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► **To cite this version:**

Markus A. Seeliger, Martin Spichty, Sadie E. Kelly, Mark Bycroft, Stefan M.V. Freund, et al.. Role of Conformational Heterogeneity in Domain Swapping and Adapter Function of the Cks Proteins. *Journal of Biological Chemistry*, 2005, 280 (34), pp.30448-30459. 10.1074/jbc.M501450200 . hal-03026515

HAL Id: hal-03026515

<https://hal.science/hal-03026515>

Submitted on 26 Nov 2020

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Role of conformational heterogeneity in domain swapping and adapter function of the Cks proteins.

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*Abbreviations: APC, anaphase promoting complex; Cdk2, Cyclin dependent kinase 2;
Cks, Cyclin dependent kinase subunit; HSQC, heteronuclear single quantum coherence;
MD, molecular dynamics; NOESY, nuclear Overhauser enhancement spectroscopy;
SCF, Skp-Cullin-F-box proteins; Skp2, S-phase Kinase associated protein; TOCSY, total
correlation spectroscopy; TROSY, transverse relaxation-optimised spectroscopy.*

Cks proteins are adapter molecules that coordinate the assembly of multi-protein complexes. They share the ability to domain swap by exchanging a β -strand, β 4. Here we use NMR spectroscopy and molecular dynamics simulations to investigate the dynamic properties of human Cks1 and its response on assembly with components of the SCF^{Skp2} ubiquitin ligation machinery. In the NMR experiment with the free form of Cks1, a subset of residues displayed elevated R_2 values and the cross-peaks of neighbouring residues were missing from the spectrum, indicating a substantial conformational exchange contribution on the microsecond to millisecond timescale. Strikingly the region of greatest conformational variability was the β 4-strand that domain swaps to form the dimer. Binding of the ligand common to all Cks proteins, Cdk2, suppressed the conformational heterogeneity. This response was specific to Cdk2 binding; in contrast, binding of Skp2, a ligand unique to human Cks1, did not alter the dynamic behaviour. Short-time (< 5 ns) molecular dynamics simulations indicate that those residues of Cks1 that form the binding site for phosphorylated ligands are considerably more flexible in the free form of Cks1 than they are in the Cdk2-Cks1 complex. A cooperative interaction between Cdk2 and Cks1 is suggested which reduces the configurational entropy of Cks1 and therefore facilitates phosphoprotein binding. Indications of an unusual dynamic behaviour of strand β 4 in the free form of Cks1 were obtained from longer-time (50 ns) dynamics simulations. A spontaneous reversible unzipping of hydrogen bonds between β 4 and β 2 was observed suggesting an early intermediate structure for unfolding and/or domain swapping. We propose that the dynamic properties of the β -sheet and its modification upon ligand binding underlie the domain swapping ability and the adapter function of Cks proteins.

Introduction

Cks1 is a member of the cyclin dependent kinase subunit (Cks) family of proteins. Cks proteins were first identified as functioning in the control of the cell cycle by binding to Cyclin dependent kinase 2 (Cdk2) and regulating its function in some, as yet unknown, way ¹. It is only recently, however, that their involvement in targeted protein degradation *via* the ubiquitin pathway has been discovered. First, in what is thought to be the canonical Cks function, it was found that they are required for the activating phosphorylation of the APC (anaphase promoting complex) ubiquitin ligase ^{2,3}; more recently Cks1, one of the two human Cks homologues, was shown to be essential for the activity of another ubiquitin ligase, the SCF^{Skp2} ⁴⁻⁶.

The Cks proteins have very high sequence identity. The main difference is that some of the homologues have additional sequences at the N- and C-termini and a longer loop between the α -helices. The core structure is a 4-stranded β -sheet. One face of the β -sheet forms the binding site for Cdk2, whilst on the other face there is a cluster of conserved residues that is thought to bind phosphorylated proteins (referred to as the phosphate-binding site) ⁷ (Figure 1). The less conserved α -helical region caps one end of the β -sheet and biochemical and mutagenesis studies have shown that this part of the protein binds the substrate recognition component, Skp2, of the SCF ⁶. Domain swapping has been observed for several Cks proteins with the C-terminal β -strand, β 4, exchanging with another molecule to form a dimer pair ⁸⁻¹⁰. The process is mediated by a 'hinge loop' sequence and two conserved proline residues in the hinge loop are the main determinants of whether the protein adopts the monomer or the dimer form: mutation of the first or second proline creates an obligate monomer or dimer, respectively, whereas

mutation of any other residue in the hinge loop has little effect on the monomer-dimer equilibrium ¹¹.

Although the biological pathways in which Cks proteins act have been identified, the underlying mechanistic role remains to be determined. They have been proposed to function as macromolecular adapters or docking factors: In the context of their canonical function, they are thought to facilitate or direct the binding of Cdk2 to a partly phosphorylated substrate such as the APC by binding both Cdk2 and the phosphoprotein simultaneously. One requirement is therefore the presence of multiple binding sites. A second requirement may be the interdependence of these binding sites. Support for such a model came first from our domain swapping studies which revealed allosteric behaviour whereby a change in the conformation of the Cdk2-binding site altered the ligand affinity of the phosphate-binding site ¹². Also, we have shown that human Cks1 directs the SCF^{Skp2} ubiquitination machinery to its target p27 by binding Skp2, Cdk2 and p27 in a synergistic manner ¹³. To investigate the structural requirements of an adapter molecule, we have used NMR TROSY (transverse relaxation-optimised spectroscopy) ^{14,15} to probe the dynamic behaviour of Cks1 and its response upon step-wise assembly with the components of the SCF^{Skp2} machinery.

We show by NMR experiments that uncomplexed Cks1 is a conformationally heterogeneous molecule and that the most labile region maps to β 4, the β -strand that undergoes domain swapping. Cks1 responds differently to binding different ligands. Whilst the binding of Skp2 does not change the dynamic properties, the binding of the canonical Cks ligand, Cdk2, freezes out the heterogeneity. To gain more detailed information about the effect of Cdk2 binding on the flexibility of Cks1, we performed molecular dynamics simulations for free Cks1 and Cks1 in complex with Cdk2 using

implicit (EEF1¹⁶) and explicit water solvation. The experiments and simulations both show that the binding of Cdk2 restricts the conformational flexibility of Cks1 residues that comprise the phosphate-binding site, supporting a cooperative interaction between Cdk2 and Cks1 for the binding of phosphorylated ligands. Also, long-time dynamics (50 ns) were performed to provide insights into the unusual dynamic behaviour of $\beta 4$ detected by NMR. Cross-correlations observed in the simulations were used to examine coupling among the motion of different portions of Cks1 before and after Cdk2 binding. The results suggest that a dynamic response to ligand binding, i.e., a change in overall flexibility and a redistribution of conformations, contributes to the allostery^{17,18}, rather than the more classical ‘enthalpic’ mechanism of allostery induced by discrete structural changes.

Experimental NMR results

Missing cross-peaks indicate conformational exchange.

The HSQC spectrum of the 79-residue Cks1 is shown in Figure 2A. It was immediately apparent that several cross-peaks were missing from the spectrum and that the cross-peaks observed had large intensity variation. This is indicative of conformational exchange on an intermediate NMR timescale. We screened a range of experimental conditions in order to eliminate alternative origins of line broadening. Spectra were recorded at protein concentrations from 40 μM to 1 mM and no changes were observed in the linewidths of the cross-peaks, indicating that aggregation was not involved. Furthermore, analytical ultracentrifugation and size-exclusion chromatography experiments indicate that the protein is >99% monomeric in this protein concentration range (data not shown). We attempted to recover non-observable cross-peaks by varying

field-strength (experiments were run on spectrometers with proton frequencies between 500 MHz and 800 MHz) and temperature (273 K – 303 K, well below the melting temperature of 328 K). Lowering the temperature to 278 K and increasing the field strength to 800 MHz resulted in additional very faint, broad peaks; however the spectral quality was not good enough to obtain additional assignments. Experiments optimised for minimal solvent saturation did not recover additional cross-peaks and this observation, as well as the lack of pH dependence, suggests that the absence of cross peaks for a subset of residues is not associated with rapidly solvent exchanging amide protons.

We also looked at the mutant P62A, because the equivalent mutant (P90A) in *S. pombe* Cks protein Suc1 was found previously to greatly reduce the line-broadening observed for that protein ¹⁹. In the case of Cks1, however, there was no significant difference between the spectra of P62A and wild type. Although the formation of a domain-swapped dimer is the other potential cause for the missing cross-peaks, the dissociation constant is too high for the dimer to be significantly populated (the dissociation constant for wild type Cks1 is in the ten millimolar range ²⁰ and for the mutant P62A, which showed similar line broadening, it is at least an order of magnitude higher). Moreover, the timescale of monomer-dimer interconversion is of the order of tens of minutes, much slower than the microsecond-millisecond timescale of conformational exchange that causes line broadening.

Conformational heterogeneity maps to the domain swapping β strand 4.

The HSQC spectrum of Cks1 was assigned using standard triple resonance experiments complemented by NOESY-based techniques. To investigate the

conformational exchange process further, the backbone dynamics was analysed by measuring longitudinal (R_1) and transverse (R_2) relaxation rates (Figure 3). The average ratio of R_2/R_1 of ~ 12 (corresponding to a correlation time τ_c of 10.7 ns) is in the range expected for a 9 kDa protein. This is consistent with the results above that indicate the line-broadening is not a result of Cks1 being oligomeric. A subset of residues display elevated R_2 values (Arg20 in strand β_2 , His60 and Glu61 in the hinge loop between β_3 and β_4 , these were excluded from the calculation of the R_2/R_1 ratio) and surrounding these are residues without visible cross-peaks in the HSQC spectrum. These results strongly suggest that chemical exchange on a microsecond to millisecond timescale contributes significantly to the relaxation mechanism at these sites.

There was a single structural element for which there were no cross-peaks in the HSQC spectrum of Cks1. This stretch of sequence involves the domain swapping β -strand 4. In addition to those residues adjacent to the missing cross-peaks, a number of other residues, mostly in the β -sheet, also exhibited significantly higher R_2 values than the average, notably residue Arg20 in the phosphate-binding site.

Cks1 dynamics are modified upon binding of Cdk2.

Cdk2 binds to one face of the Cks1 β -sheet (Figure 1) with an affinity of 1.5 μM ¹³. We looked at the effect of Cdk2 binding using ^{13}C , ^{15}N -labelled Cks1 in complex with unlabelled Cdk2. Due to solubility limitations, Cks1 was at a concentration of 150 μM with Cdk2 in slight excess. To improve the relaxation properties of the 44 kDa complex the side chain protons in Cks1 were perdeuterated ($>80\%$)²¹, and the spectral quality was improved by incorporation of TROSY building blocks¹⁵. The TROSY-HSQC spectrum of Cks1 in the presence of Cdk2 is shown overlaid on that of free Cks1 (Figure 2B). The

two spectra were recorded in parallel on samples that had been dialysed in the same beaker. The two spectra are very different. Most significantly, many more cross-peaks are observed from the Cks1 subunit in the complex when compared with the number of cross-peaks observed for free Cks1. These results indicate that we have recovered the majority of cross-peaks missing in the free Cks1 spectrum. There was also more uniformity in cross-peak intensity and shape in the Cdk2-bound Cks1 spectrum.

Cdk2 binding affects the whole β -sheet of Cks1.

The large change in the spectrum required a re-assignment of Cdk2-bound Cks1 using a series of triple resonance experiments and NOESY-based experiments acquired with a sample of sidechain-perdeuterated ^{15}N , ^{13}C -labelled Cks1 in complex with unlabelled Cdk2. The changes on binding Cdk2 were then mapped onto the Cks1 structure (Figure 4). Of the cross-peaks that could be assigned in the Cdk2-bound spectrum that were missing in the free Cks1 spectrum, most were one-residue or two-residue extensions of stretches of assignments that were made in the free form. The remaining additionally assigned residues mapped to a single stretch of sequence, the domain swapping β -strand 4.

Changes in the HSQC spectrum of Cks1 on binding Cdk2 map to residues throughout the β -sheet as well as to residues in the α -helical region. First, the cross-peaks that were not visible in the free Cks1 spectrum due to line-broadening were recovered upon binding Cdk2. Second, of those residues with cross-peaks that were visible in the free Cks1 spectrum many showed significant chemical shift changes (Figure 5). Of significance are residues like Arg20 located in the phosphate-binding site

on the opposite surface of the β -sheet to the Cdk2-binding site and Asn45 in helix α 2 of the Skp2-binding site (Figure 5).

Cks1 binds with high affinity to the 63 kDa heterodimer Skp1-Skp2, the substrate recognition component of the SCF^{Skp2} 13,22. A sample of 150 μ M ^2H , ^{15}N -labelled Cks1 was prepared in complex with (a slight excess of) unlabelled Skp1-Skp2 heterodimer. The 110 kDa complex of ^2H , ^{15}N -labelled Cks1 and unlabelled Cdk2 and Skp1-Skp2 was also assembled. Analytical gel filtration of the NMR samples was used to confirm the formation of the complexes. The elution profiles showed one major peak at a volume expected for the complex and minor peaks corresponding to the non-Cks1 component(s) which were always used in excess of Cks1 to ensure there was no free Cks1 in solution. SDS-PAGE subsequently confirmed that all components of the complex were present in the major peak. No peak was observed at the volume expected for free Cks1 and no peak was observed at the void volume which would correspond to high order oligomers. Integration of the peak volumes showed that no sample was lost due to aggregation. Finally the correlation times for the Cks1-Skp1-Skp2 and Cks1-Cdk2-Skp1-Skp2 complexes were estimated based on the amide relaxation behaviour and the values obtained agree well with those expected for their respective molecular weights.

Figure 2C shows the TROSY-HSQC spectrum of Skp1-Skp2-bound Cks1 overlaid on that of free Cks1. The two spectra were recorded in parallel on samples that had been dialysed in the same beaker. In contrast to Cdk2-bound Cks1 (Figure 2B), the spectrum of Skp1-Skp2-bound Cks1 is similar in appearance to that of free Cks1, and notably no additional cross-peaks were recovered. There was an increase in linewidth, as would be expected due to the high molecular weight of the complex. There were also significant chemical shift differences compared with free Cks1 and these were mostly

limited to residues in the α -helical region, Asp27, Glu36, Glu40, Glu42, Gln49, consistent with the interface mapped previously by site-directed mutagenesis (Figure 1)⁶. Surprisingly Asn45, a residue found to be critical for the interaction with Skp2, does not show a significant chemical shift difference. The TROSY-HSQC spectrum of Cks1 in the quaternary complex of Cks1-Cdk2-Skp1-Skp2 was similar in appearance to that of the binary complex of Cks1-Cdk2. Thus, the NMR spectra indicate that the binding of Skp1-Skp2, unlike Cdk2, does not induce large changes in the internal dynamics of Cks1.

Theoretical results

Flexibility of native ensemble changes upon Cdk2-binding.

The structures of Cks1 and its complex with Cdk2 have been solved by X-ray spectroscopy. There are two structures available for Cks1 (PDB entries 1DKS and 1DKT)²³. In both structures Cks1 is crystallized as dimer; the structure 1DKS has a bound phosphate and 1DKT has a metavanadate bound at the phosphate-binding site. In the X-ray structure of the Cdk2-Cks1 complex (PDB entry 1BUH)⁷ the phosphate binding site is unoccupied. The structure of Cks1 is very similar in all three X-ray structures; the rms deviation of the C $^{\alpha}$ atoms is less than 1.5 Å (residues 5 – 74). Because of the higher experimental resolution, 2.6 Å (1BUH) vs. 3.2/2.9 Å (1DKS/1DKT), we used the crystal structure of the complex as the starting point of the calculations for both the complex Cdk2-Cks1 and the uncomplexed Cks1. We simulated both systems for 5 ns with explicit solvation. The Cks1 unit was solvated in a sphere of water molecules (radius 37 Å) and the solvent molecules were confined to a sphere by using stochastic

boundary conditions ²⁵. For the simulation of complexed Cks1, the Cdk2 unit was modelled as illustrated in Figure 6.

The simulations of free and Cdk2-bound Cks1 yield stable trajectories (Figure 8A). The rms deviation curves for free and complexed Cks1 plateau after 1 ns of equilibration at about the same value of 1.3 Å. The average structures of the Cks1 unit calculated for the time interval 1 – 5 ns show C^α deviations from the complex crystal structure of 1.02 Å in the case of free Cks1 and 0.94 Å in the case of the complex, respectively. The main difference between these two structures is found in the bulge region of strand β1 (Ser9,Asp10,Lys11). In the complexed form of Cks1 the side chain oxygen of Ser9 forms a hydrogen bridge with the backbone amide proton of His21 (strand β2, see Figure 9A), as seen in all three crystal structures. This hydrogen bonded configuration is not stable in the free form of Cks1. In the absence of the Cdk2 unit, the side chain oxygen of Ser9 forms an intra-strand hydrogen bond with the backbone amide proton of Asp10 (Figure 9B) resulting in the loss of an inter-strand hydrogen bond between β1 and β2.

The isotropic rms fluctuations of the backbone nitrogens calculated from the simulations show that the flexibilities of free and complexed Cks1 differ considerably at two sites (Figure 7). The backbone nitrogen fluctuations of residues 10 – 15, and especially Lys11 (bulge in strand β1) and Glu15 (loop β1-β2), and the residues of the hinge loop are significantly higher in the free structure of Cks1 than in the complex.

The increased flexibility of the hinge region in free Cks1 can be understood by looking at the X-ray structure of the complex. There are intermolecular hydrogen bonds between the backbone of His60 (Cks1) and the side chain of Lys237 (Cdk2) and between the side chain of His60 (Cks1) and the backbone of Leu174 (Cdk2). Furthermore there is

a salt bridge between the side chains of Glu61 (Cks1) and Lys237 (Cdk2). In the free form of Cks1 these interactions are missing and the hinge becomes more flexible. For the bulge in $\beta 1$ there are no direct interactions with the Cdk2 unit that could explain the lower flexibility in the complex in comparison to free Cks1. Instead, residues Tyr12 and His21 that interact with both the bulge and the Cdk2 unit seem to constrict the fluctuations of Asp10, Lys11.

Atomic fluctuations can also be estimated from the experimental B -factors of crystal structures,

$$B = \frac{8 \cdot \pi^2}{3} \langle \Delta r_i^2 \rangle$$

The correlation between the calculated fluctuations and those obtained from B -factors is reasonable, the correlation coefficient amounts to 0.61 for the complex and 0.54 for free Cks1, respectively. The smaller correlation for free Cks1 is the result of large deviations for residues of the bulge region and the coil between $\alpha 1$ and $\alpha 2$. The disagreement for the bulge region can be understood if one considers that in the crystal structures of uncomplexed Cks1 (1DKS, 1DKT) the protein is present as a dimer and the two Cks1 units interact with each other at strand $\beta 1$ (Figure 10) which most likely suppresses the high flexibility of the bulge region and of those residues located across on strand $\beta 2$ (Arg20, His21).

The change in flexibility of the entire stretch Ser9 – Val22 and the alteration of the inter-strand hydrogen bond configuration at the bulge region is a possible explanation for the chemical shift differences observed upon complex formation for a large number of residues (e.g. Tyr12, Asp14, Glu15, Arg20, His21, and Val22). Furthermore, the dramatic decrease of flexibility for residue Lys11 upon complex formation is in accord

with the re-appearance of the corresponding peak in the TROSY spectra. The immobilization of Lys11 upon complex formation may play a role in the adapter function of Cks1. Lys11 together with Arg20, Trp54 and Arg71 form the phosphate-binding site as seen in the crystal structure 1DKS (Figure 11A). All four residues exhibit a higher flexibility in the free form than in the complex (Figure 11B,C), and, therefore, the entropy cost of binding phosphorylated ligands should be smaller in the complex.

Cdk2-binding decreases configurational entropy of Cks1.

The higher flexibility of free Cks1 in comparison to Cdk2-bound Cks1 can be quantified in terms of the vibrational entropy. We performed a quasiharmonic analysis²⁶ for the MD simulations of free and complexed Cks1 using only the C α atoms of the Cks1 unit. The mass of each C α atom was set to the total mass of the corresponding residue. With the resulting normal modes, the vibrational entropy is²⁷:

$$S_{vib} = \sum_{k=1}^{3N-6} \left[-N_A k \ln(1 - e^{-h\nu_k/kT}) + \frac{N_A h \nu_k}{T} \frac{e^{-h\nu_k/kT}}{1 - e^{-h\nu_k/kT}} \right]$$

where N_A is Avogadro's number, k is Boltzmann's constant, h is Planck's constant and ν_k is the frequency of normal mode k . The summation is over all vibrational modes. The calculated vibrational entropy amounts to 917.3 cal mol⁻¹ K⁻¹ for complexed Cks1 and 943.1 cal mol⁻¹ K⁻¹ for free Cks1, respectively. Accordingly, the vibrational entropy differs by 25.8 cal mol⁻¹ K⁻¹ which corresponds to an energy difference of more than 7.5 kcal mol⁻¹ at room temperature. If the decreased flexibility of Cks1 as seen in the complex with Cdk2 is necessary for the binding of a phosphorylated protein, the free form of Cks1 would need to overcome an additional entropic cost that could be as large as 7.5 kcal mol⁻¹ for Cks1 to bind phosphorylated proteins, relative to that required in the

Cdk2-Cks1 complex. The affinities of free and Cdk2-bound Cks1 for phosphopeptides have been measured by fluorescence anisotropy titrations. A small phosphopeptide, however, would be more flexible than an intact phosphoprotein and would probably not rigidify Cks1 to the same extent; the effect of Cdk2 complex formation on the phosphopeptide binding ability of Cks1 may therefore be small. The presence of Cdk2 slightly increased the binding affinity of Cks1 for phosphopeptides derived from the APC (SEK, F. Herzog, J.M. Peters & LSI, unpublished results). Cdk2 also increased the affinity of Cks1 for a p27 phosphopeptide, if Skp2 was also present¹³; Skp2 may reduce the flexibility of the p27 phosphopeptide. There are no experimental data available regarding the effect of Cdk2 on the affinity of Cks1 for intact phosphoproteins.

Correlated motions in Cks1 are suppressed upon complex formation.

Further insights into how complex formation changes the internal motions of the Cks1 unit are obtained from the covariance matrices of the spatial MD atom displacements. In the free form of Cks1 (Figure 12A) there are strong positive backbone N cross-correlations between stretches of residues 6 – 14 (β 1) and 18 – 25 (β 2), 18 – 25 (β 2) and 65 – 74 (β 4), 54 – 60 (β 3) and 66 – 70 (β 4), and a strong negative correlation between 40 – 45 (α 2) and 70 – 74 (β 4, C-terminus). We also calculated the residue correlations by averaging the covariance elements over all atoms of each residue. The resulting correlation pattern is similar to that obtained from the backbone N correlations, but considerable less intense. The correlations between the β -strands are not surprising since the β -strands interact with each other and together form the β -sheet. Remarkable is the strong negative correlation between helix α 2 and the end of strand β 4 (the C-terminus), two structural motifs that are separated by more than 15 Å. This correlated

motion between residues of the binding sites for Skp2 ($\alpha 2$) and p27 (Arg71 of $\beta 4$) could play a role in the adapter function of Cks1 in that it provides a communication mechanism between the two binding sites. Upon complex formation with Cdk2 the correlations in Cks1 become weaker in general (Figure 12B), in accord with the smaller amplitude of the fluctuations. Complex formation suppresses the correlated motions of the β -sheet and between $\alpha 2$ and the C-terminus.

Reversible separation of strands $\beta 2$ and $\beta 4$ in free Cks1.

Strand $\beta 4$ exhibits very low fluctuations in the MD simulations not only in the complex but in the free form of Cks1 as well. Referring to the NMR measurements, the cross-peaks of residues 65 – 71 are missing in the TROSY-HSQC spectrum of free Cks1 and re-appear upon complex formation, which points to an increased flexibility of $\beta 4$ in the free form of Cks1. Strand $\beta 4$ shows very low fluctuations in both of the 5 ns MD simulations (free and complexed Cks1), just as it does in the normal mode analysis. However, indications about the unusual dynamics behavior of $\beta 4$ as seen in the NMR experiments are obtained from longer time-scale dynamics. We simulated free Cks1 for 50 ns using the implicit solvation model EEF1. The C^α rms deviation from the crystal structure is illustrated in Figure 7B. The rmsd plateaus at about 7 ns to a value of 2.3 Å. There is a slight but distinct increase of the all-atom rms deviation at 20 ns. It drops down again at about 30 ns. Inspection of the MD trajectory reveals that a conformational change occurs at the C-terminus (end of strand $\beta 4$) in the time period between 22 and 32 ns. Two hydrogen bonds between $\beta 2$ and $\beta 4$ are broken and the side chain of Arg70 bridges now the two strands (Figure 13A). Arg70 thereby acts as a "scissor" by "cutting"

the inter-strand hydrogen bonds. In the time-interval 35 – 50 ns the Arg70 side chain moves out again and the two hydrogen bonds between $\beta 2$ and $\beta 4$ are reformed, as in the crystal structure (Figure 13B). The "unzipping" of the terminal hydrogen bonds between $\beta 4$ and $\beta 2$ is certainly crucial for the domain swapping of $\beta 4$ or the unfolding of $\beta 4$ to an extended (open) form. Molecular dynamics simulation of the unfolding pathway of Suc1²⁸ showed that the interaction between $\beta 2$ and $\beta 4$ is stronger than any other interaction between structural motifs; it is retained even at 500 K. Whereas Suc1 has to unfold almost completely in order to exchange strand $\beta 4$ ²⁹, Cks1 follows a different pathway with the interaction between $\beta 2$ and $\beta 4$ broken at the beginning followed by the separation of $\beta 4$ from $\beta 3$ ³⁰. The conformation with the inserted Arg70 side chain may be regarded as an early intermediate structure on the pathway of unfolding for $\beta 4$ -strand exchange. The complete separation of $\beta 4$ is, of course, not observed in the limited time of the molecular dynamics simulation, but the calculations have demonstrated two factors that might play an important role in the domain swapping process: a) the terminal hydrogen bonds of $\beta 4$ with $\beta 2$ can be broken by the insertion of the Arg70 side chain, and b) the hinge loop exhibits a very high flexibility in the free form that should facilitate the separation of $\beta 4$ from $\beta 3$.

Weak interaction between Cdk2 and Ck1 in the complex.

We also studied the complex Cdk2-Cks1 with the implicit solvation model EEF1 by performing a normal mode analysis. An extensive structure minimization was necessary (see Methods) to determine a minimum energy structure with only positive eigenvalues in the normal mode analysis. The rms displacement of the C^α atoms from the

crystal structure after minimization is 1.31 Å. As a result of the minimization a slight movement of the two units, Cdk2 and Cks1, relative to each other is observed. The normal mode analysis shows that the complex structure features a very flat potential energy surface, e.g. the frequency of the three lowest modes (after the overall translational & rotational modes have been removed) is lower than 1.7 cm⁻¹. The eigenvectors of these modes describe global motions of the Cks1 unit on the surface of Cdk2. This suggests that the interaction between Cdk2 and Cks1 is weak and that, therefore, the translational and rotational modes of the Cks1 unit relative to Cdk2 are low frequency modes of the complex. Indeed, the experimentally measured affinity of Cks1 for Cdk2 is modest (micromolar range) ¹³.

Discussion

A model for the adapter function of Cks proteins.

There is increasing evidence for the key role played by Cks proteins in the ubiquitin-proteasome pathway ^{2-5,31,32}. Their importance was most recently highlighted by a study linking the Cks proteins to the function of the proteasome ³³. The results of this study suggest that Cks proteins are required to recruit the proteasome for a non-proteolytic (and possibly also proteolytic) role in the transcription of *CDC20* whose gene product activates the APC ubiquitin ligase. A common function of Cks proteins is therefore emerging in directing the assembly of a variety of protein complexes. However, the underlying mechanism remains unresolved. In this study we used the human protein Cks1 which assembles together SCF^{Skp2}, Cdk2 and p27 and thereby drives p27 ubiquitination and subsequent degradation by the proteasome. There are two conserved surfaces on the Cks β-sheet that are involved in molecular recognition (Figure 1): One

surface binds Cdk2 and although the identities of the substrates for the other site (referred to here as the phosphate-binding site) are unknown to date, the evidence points to phosphorylated proteins (such as p27) located within larger assemblies involved in ubiquitination pathways.

The NMR experiments show an exchange phenomenon for some residues of the β -sheet in free Cks1 on a microsecond to millisecond timescale, indicating the presence of more than one conformation, and this dynamic property is modified upon binding of the canonical Cks partner, Cdk2 (Figure 4). The β -sheet of the Cks proteins is unusual as neither faces pack against any other structural element. Instead, it is very solvent-exposed with a conserved binding site occupying each of its faces (Figure 1). The solvent accessible surface (probe radius = 1.4 Å) for the 32 residues of the β -sheet is about 1900 Å² for free Cks1 and 1500 Å² for Cdk2-bound Cks1, respectively. Interestingly, the fraction hydrophobic/hydrophilic residues for this solvent exposed β -sheet (1.6) is much higher than for the entire protein (1.0). The 'naked' β -sheet may have evolved to allow the Cks adapter to bind multiple substrates simultaneously and the high degree of solvent exposure could be a factor contributing to the conformational heterogeneity.

The crystal structure of Cks1 is well defined with a relatively uniform distribution of *B*-factors over the structure and the different crystal derivatives (1DKS and 1DKT) overlay with small rms. Thus, there is no indication of the heterogeneity that is observed in solution. Further, the crystal structures of uncomplexed Cks1 and Cdk2-bound Cks1 are highly superimposable (C^α rmsd is < 1.1 Å for residues 5 – 74) and have similar *B*-factors (see Figure 7), in contrast to their very different solution properties. This strongly suggests that the internal dynamics of Cks1 are frozen out upon binding Cdk2; the effect is mimicked in the crystal of uncomplexed Cks1 where the protein forms a dimer (see

Figure 10). Molecular dynamics simulations performed on a short time-scale ($< 5\text{ns}$) show that the internal dynamics of Cks1 does not alter dramatically upon complex formation in terms of the cross-correlation for the fluctuations of different residues (Figure 12), but a subset of residues features significantly lower fluctuations in the complex (see below). Moreover longer time molecular dynamics shows the existence of an inherent flexibility in the uncomplexed Cks1.

Conformational heterogeneity has been observed in response regulators such as Spo0F and nitrogen regulatory protein C³⁴⁻³⁷. The data point to a mechanism of allosteric activation^{38,39} whereby the unphosphorylated protein interconverts between two conformers, populating predominantly the inactive one, and phosphorylation activates the protein by shifting this pre-existing ensemble to the active conformer. We can extend this mechanism of activation to a different class of proteins, macromolecular adapters like Cks1, where it is ligand binding rather than phosphorylation that induces the population shift⁴⁰⁻⁴². In view of the proposed biological role of Cks proteins as coordinators of multi-protein assembly, we suggest the following model for Cks action: The exposed β -sheet of the Cks protein samples more than one conformation in solution and as such binds phosphorylated substrates with low affinity. Cdk2 binding to the β -sheet locks it in a single relatively rigid conformation, one that is assembly-competent with a high affinity for its target proteins. Experiment and simulation both point to this model. Residues that display conformational heterogeneity in free Cks1 and that have an altered chemical environment upon binding Cdk2 include those in the phosphate-binding site (Figure 4): Arg20 has an elevated R_2 value and its cross-peak is significantly shifted on binding Cdk2; the cross-peak for Lys11 is not visible in the free Cks1 spectrum and is recovered in the Cdk2-bound spectrum. In the simulations, the bulge region of strand $\beta 1$

(Asp10, Lys11) exhibits a significantly higher flexibility in the free form of Cks1 than in the complex. The binding of Cdk2 stabilizes the bulge region and prevents the switching between different hydrogen bond configurations seen in the free form of Cks1. Furthermore, Arg20 in strand β 2, located opposite of the bulge of strand β 1, displays an increased flexibility in the free form of Cks1, as do neighbouring residues Tyr19 and His21. These observations support the recently proposed mechanism of crosstalk between the Cdk2- and phosphoprotein-binding sites¹³; the modulation of the flexibility of the β 1 bulge region – a structural motif common to Cks proteins – seems to play a key role in the communication. Moreover, once Cks1 is "locked" in one conformation, the calculations suggest that the entropy loss on binding a phosphorylated protein could be decreased by as much as 7.5 kcal mol⁻¹ upon complex formation with Cdk2. The model is in accord with the observed enhancement in Cks1 affinity for a p27 phosphopeptide¹³ and for APC-derived phosphopeptides (SEK, F. Herzog, J.M. Peters, unpublished results) on binding Cdk2. An activation mechanism such as the one proposed may help to ensure that the assembly processes coordinated by Cks adapters take place in the correct order.

Domain swapping as a side-effect of function.

The ability to adopt different oligomeric states by domain swapping is common to all five members of the Cks family that have been studied in solution to date^{8-10,20,43,44}. The finding that the domain-swapping β -strand in both Cks1 and Suc1 has conformational heterogeneity suggests that this facilitates the swapping process. The microsecond fluctuations that are detected by NMR are not sufficient to release the β -strand to domain swap but they may reflect a larger scale motion occurring in the partly

unfolded state from which domain swapping occurs¹¹. A 50 ns-molecular dynamics simulation for the free form of Cks1 suggests that one contribution to the unbinding of the β 4 strand arises from the insertion of the side chain of Arg70, which separates β 4 from β 2.

It is important to note that neither the domain swapping ability nor the conformational heterogeneity arise from an intrinsic instability of the Cks structure: the stability of different family members varies widely and can be relatively high (from 3 kcal mol⁻¹ at 25 °C for Cks1²⁰ to 6.5 kcal mol⁻¹ for Cks1 from *S. cerevisiae* (MAS & LSI, unpublished results) and 7.5 kcal mol⁻¹ for Suc1⁴⁴). It is also not the case that the Cks proteins are partially unfolded molecules, as kinetic and equilibrium unfolding studies show that they behave as cooperatively folded structural units^{20,44}.

We do not know whether Cks proteins domain swap *in vivo* and/or whether this process is functionally relevant, but in view of the high dissociation constant of the dimer, this seems unlikely. Rather, the ability to domain swap may be a side effect of the conformational heterogeneity that is required for its adapter function. Do other proteins that domain swap also display such behaviour in solution? Of the few domain swapping proteins that have been studied by NMR, YopH and Cyanovirin-N display regions of internal mobility on the micro/millisecond timescale^{45,46}. And two other proteins that domain swap, ribonuclease A and Spo0A, can adopt multiple conformations in crystals^{47,48}. For some of these proteins, the structural heterogeneity may be relevant to the function.

Controlling assembly and activity of protein complexes.

The canonical Cks function is thought to be that of an adapter between Cdk2 and phosphorylated substrates, and the solution properties of Cks1 reflect this role: First, the relatively modest, micromolar, affinity for Cdk2 may be important to prevent Cks1 from being permanently attached to Cdk2 and to allow competition for Cdk2 between Cks1 and the other human Cks protein, Cks2. As Cks2 does not bind to Skp2, this commits Cdk2 complexes to different signalling pathway. Second, the dynamic properties observed here by experiment and simulation show how the binding of Cdk2 to one face of the β -sheet could enable the tight binding of phosphorylated substrates such as p27 to the other face. Such a mechanism would explain the absolute requirement of Cks1 to bind to Cdk2 in order for the ubiquitination of p27 to occur ⁶.

We showed previously that the affinity of Cks1 for p27 is enhanced by assembly of Cks1 with Skp1-Skp2 ¹³. The interaction between Cks1 and Skp1-Skp2 contrasts with that between Cks1 and Cdk2: The affinity for Skp1-Skp2 is very high ¹³ and this is consistent with Cks1 and Skp1-Skp2 being permanently assembled, as is suggested by their parallel expression levels during the cell cycle ⁶. The mode of binding of Skp2 to Cks1 revealed by NMR is also different from that of Cdk2 to Cks1. Skp2 binds to the α -helical region; i.e., chemical shift differences are localised to these residues, and there is not the dramatic change in internal dynamics that occurs when Cdk2 binds. In the absence of a crystal structure it remains unresolved how Cks1 and Skp2 work together to bind p27 with high affinity. Possible models include: (1) Skp2 binding induces structural changes in Cks1, (2) Cks1 binding induces structural changes in Skp2, (3) Cks1 and Skp2 form a bimolecular interface for binding p27. The NMR results showing no global

change in Cks1 structure or dynamics upon binding Skp1-Skp2 now allows us to rule out model (1).

The evidence presented here strongly suggests that the dynamic properties of Cks1, as revealed by NMR experiments and molecular dynamics simulations, are functionally relevant. First, conformational heterogeneity has now been seen for two Cks proteins, Cks1 and Suc1; second, this property is altered by binding of the canonical ligand. Interestingly, Elongin C, a protein that acts as an adapter in another ubiquitin ligase, CBC^{VHL} , behaves similarly⁴⁹. On complex formation with Cdk2, the flexibility is significantly decreased for those residues of Cks1 that form the binding site for the phosphorylated ligand. This suggests a cooperative interaction between Cdk2 and Cks1 which reduces the configurational entropy of Cks1 and therefore facilitates substrate binding. Our results also have wider implications for dynamic mechanisms underlying signalling pathways and for the organisation of protein networks^{17,41,50-54}. The dynamic behaviour of Cks1 and the signalling between binding events add weight to the idea of an adapter molecule binding several proteins onto its surface in a cooperative manner. The proposed mechanism of allosteric activation of an adapter in response to binding one of its ligands could be a way to ensure the correct order of assembly of multi-protein complexes in general and, in particular, the timely recruitment of target proteins for ubiquitination and subsequent degradation.

Experimental procedures

Protein purification

E. coli expression plasmid for GST-Cdk2 was a generous gift of J.A. Endicott, University of Oxford, UK and GST-Skp1/Skp2 was a generous gift of A. Hershko, Technion Haifa, Israel. Isotopes were obtained from Goss Scientific Instrument Limited, UK and other chemicals were obtained Sigma.

C41 or BL21DE3 cells were transformed with the previously described expression vector for Cks1²⁰ and grown in 2xTY media containing ampicillin at 37 °C to an OD_{600nm} of 0.6. They were then harvested in sterile centrifuge bottles by centrifugation for 10 min at 5000 rpm at 4 °C. The cell pellet was re-suspended in M9 media in D₂O with the appropriate carbon (glucose or ¹³C-glucose) and nitrogen sources (¹⁵NH₄Cl), using twice the initial rich media volume. Cells were grown for a further 2 hours at 25 °C and induced with 0.1 mM IPTG overnight at 25 °C. Expression of Cdk2 and Skp1-Skp2 were carried out as described previously¹³. Proteins were purified by nickel affinity (Cks1) or glutathione affinity methods (Cdk2, Skp1-Skp2) followed by gel filtration (Superdex G75, Pharmacia). Proteins were judged to be >95% pure by SDS-PAGE. Mass spectrometry showed that Cks1 samples were >90% labelled with the appropriate isotopes. Samples were prepared for NMR in 100 mM NaPO₄ buffer pH 6.8, 100 mM NaCl, 5 mM DTT, 5% D₂O and Complete protease inhibitor cocktail (Roche). The protein complexes were assembled with Cdk2 and/or Skp1-Skp2 in slight excess relative to Cks1. The complex was then dialysed in the same beaker as a control sample of free Cks1, and NMR spectra were subsequently recorded in parallel. Samples were concentrated to ~150 μM using VivaSpin concentrators (VivaScience), then filtered and extensively degassed. To confirm that the Cks1 complexes were formed and remained

assembled during NMR data acquisition, an aliquot of the NMR sample was loaded on a gel filtration column. An elution peak was observed at the volume expected for the respective complex. There were also minor peaks at volumes expected for Cdk2 and/or Skp1-Skp2 corresponding to the slight excess relative to Cks1 (used to ensure there was no free Cks1 in solution).

NMR spectroscopy

Spectra were recorded at 298 K on Bruker DRX 600 and DRX 800 NMR spectrometers. Sequence-specific resonance assignments for ^2H , ^{15}N , ^{13}C -labeled Cks1 wild type, both free and in complex with Cdk2, were made based on the following triple-resonance TROSY experiments: HN(CO)CA, HNCA, CBCA(CO)NH, HNCACB (as reviewed in ⁵⁵). ^{15}N -edited 3D NOEST experiments were acquired using ^{15}N -labelled Cks1 to confirm and extend sequential assignments. For the resonance assignment of Cks1 in complex with Cdk2, the 1:1 complex of Cks1 was performed with unlabelled Cdk2. Data were processed and analysed with Felix 97 (MSI).

TROSY-HSQC spectra of ^2H , ^{15}N -labelled Cks1 in complex with (unlabelled) Skp1-Skp2, with Cdk2 and with Skp1-Skp2 and Cdk2 were also recorded in 50 mM Tris-HCl pH 7.5, 200 mM NaCl, 2 mM DTT, 5% D_2O and Complete protease inhibitor cocktail (a buffer required for optimal stability of the Skp1-Skp2 component). The spectrum of free Cks1 was always recorded at the same time for comparison, using a sample that had been dialysed in the same beaker as the sample of the complex.

Chemical shift changes upon Cdk2 binding were calculated as the root mean square deviation of the chemical shifts of corresponding peaks in free Cks1 compared with those in Cdk2-bound Cks1. The chemical shifts in the ^{15}N dimension were divided

by 10 to correct for the gyromagnetic ratio between ^1H and ^{15}N nuclei. Thus,

$$rmsdt = \sqrt{\left(H_i^{Cks1} - H_i^{Cks1/Cdk2}\right)^2 + \left(\frac{N_i^{Cks1} - N_i^{Cks1/Cdk2}}{10}\right)^2},$$
 where H_i^{Cks1} and N_i^{Cks1} are the

chemical shift of residue i in free Cks1 in the proton / nitrogen dimension and $H_i^{Cks1/Cdk2}$

and $N_i^{Cks1/Cdk2}$ are the respective chemical shifts of residue i in Cks1 in complex with

Cdk2.

Relaxation measurements

^{15}N T_1 , T_2 experiments were recorded at a ^{15}N frequency of 60.13 MHz on a Bruker DRX600 spectrometer (as reviewed in ⁵⁶). T_1 relaxation delays were set to 16, 80, 160, 240, 320, 400, 480, 600, 720, 840, 1000, 1200ms. T_2 relaxation delays were set to 10, 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220, 240 ms. Peak volumes were fitted to a single exponential decay function using the program Kaleidagraph (Abelbeck Software). In all the experiments a relaxation delay of 3 s was used. The 2D experiments were acquired with 1024 x 128 complex points.

Computational methods

General

All calculations were carried out with the program CHARMM ⁵⁷. For the the calculations with implicit solvation the polar-hydrogen topology (PARAM19) with the EEF1 model was used ¹⁶, and for the simulations with explicit water the all-hydrogen

topology (PARAM22) was employed⁵⁸. The crystal structure of the complex Cdk2-Cks1 (PDB entry 1BUH) served as starting point for the setup of all structural models.

Calculations with implicit solvation model

Setup of structures. The terminal residues 1 – 4 & 74 – 79 of Cks1 and 295 – 298 of Cdk2 are missing in the crystal structure and were neglected in the calculations, i.e., residue 5 of Cks1 was treated as the N-terminus, and residues 75 of Cks1 and 294 of Cdk2 as the C-termini, respectively. Missing intermediate residues 40 – 46 of Cdk2 were constructed in the following way: initially placed coordinates were first minimized with 2000 steepest descent (SD) and 10000 adopted basis Newton-Raphson (ABNR) steps. Subsequently, simulated annealing for these residues was performed by molecular dynamics, i.e., the residues were heated up within 20 ps to 2000 K and kept at this temperature for 500 ps and then cooled down to 50 K within 2 ns. The resulting structure was again minimized with 2000 SD and 5000 ABNR steps. The rest of the complex was kept fixed during this procedure. For the calculations of free Cks1 the Cdk2 unit was not present.

Minimizations and normal mode analysis. The structures were minimized with 5000 SD and 10000 ABNR steps. During the first 10000 minimization steps harmonic restraints were applied to the backbone atoms and the strength of the restraints was slowly decreased to zero. The second derivatives for the normal mode analysis were calculated analytically. The normal mode calculations yielded no negative eigenvalues. The absolute values of the rotation and translation modes are $< 0.04 \text{ cm}^{-1}$.

Molecular dynamics simulation. A trajectory of 50 ns was started from the minimized structure of free Cks1 using standard heating and early equilibration protocols⁵⁷ for the first 20 ps. The system was then coupled to a heat bath (300 K)

applying the Berendsen algorithm. The leap-frog integrator was used with a time-step of 1 fs (SHAKE constraints for all bonds of hydrogens to heavy atoms).

Calculations with explicit water solvation

Setup of the structural models. In order to determine the protonation state of the ionizable groups, the corresponding pKa values for free Cks1 and the complex Cdk2-Cks1 were estimated from standard continuum electrostatics calculations [Donald Bashford & Martin Karplus, pKa's of Ionizable Groups in Proteins: Atomic Detail from Continuum Electrostatic Model]. Residues His21, His36, His60 of the Cks1 unit feature pKa values higher than pH 7 in free Cks1 and in the complex as well. Those histidines were protonated; for all other ionizable residues their standard protonation state at pH 7 was used.

The N-terminal residue 4 of Cks1 was capped by an acetyl group and the C-terminal residue 74 by an amino methyl group. For the simulation of Cdk2-bound Cks1, residues of Cdk2 farther than 44 Å from the center of mass of the Cks1 unit were deleted; and for the simulation of free Cks1 the entire Cdk2 unit was deleted. A sphere of preequilibrated modified TIP3 water molecules (radius 37 Å) centered on the center of mass of the Cks1 unit was overlaid and water molecules overlapping with crystal structure elements were deleted. Crystal waters outside the overlaid sphere were deleted, too. This resulted in an extensive solvation of the Cks1 unit with about 6 - 8 water shells surrounding the protein. Bad contacts between the overlaid waters and the crystal structure were removed by performing 100 step of steepest descent minimization keeping protein atoms fixed. The water molecules were relaxed by a short dynamics run of 50 ps at 300 K (see below for details on MD). During this relaxation process, all protein atoms

were kept fixed. This overlay and relaxation procedure was repeated five times with different orientations of the overlaying water sphere. This resulted in a total of 6905 TIP3 water molecules for free Cks1 and 6249 TIP3 waters for the Cdk2-bound Cks1, respectively. The outermost water molecules of the water sphere were deleted to obtain a density closest to that of pure water at 300 K and 1 atm (0.0334 molecules/ Å³) for the volume occupied by the solvent. This resulted in 6769 waters for free Cks1 and 6147 waters for Cdk2-bound, respectively.

Minimizations and molecular dynamics. Structures with equilibrated water molecules were minimized by 2000 SD and 5000 ABNR steps. For the minimization of the complex, Cdk2 residues more than 35 Å away from the center of mass of the Cks1 unit were kept fixed, and residues within 28 to 35 Å were confined to their original positions with harmonic restraints. The harmonic force constant for each residue was chosen proportional to the inverse of the average *B*-factor of this residue with a proportionality factor $3kT/8\pi^2$ (= 7.9 kcal mol⁻¹ for T = 300 K), so that the fluctuations of the harmonic oscillations resembled the experimental fluctuations obtained from the *B*-factors of the X-ray structures. All other atoms were allowed to move freely. For the minimization of free Cks1, no restraints were used. MD trajectories of 5 ns were started from the minimized structures using stochastic boundary conditions with radius 37 Å²⁵. Water molecules within radius 30 to 37 Å were treated by Langevin dynamics with a friction coefficient of 62 ps⁻¹ for the water oxygen atoms and a heat bath temperature of 300 K. All other atoms were treated by Newtonian dynamics. The equation of motion was integrated with the leap-verlet integrator using a time-step of 2 fs (holonomic SHAKE constraints for all bonds of hydrogens to heavy atoms). All restraints of the minimizations were maintained. To keep the protein centered in the water sphere, the

center of mass of the Cks1 unit was harmonically restrained using a force constant 50 kcal mol⁻¹ Å⁻².

Quasiharmonic analysis. The C^α coordinates of the Cks1 unit were extracted from snapshots taken every 0.1 ps of the equilibrated MD trajectory (1 – 5 ns). The mass of each C^α atom was set to the total mass of the corresponding residue. The C^α structures were reoriented in order to remove translational and rotational modes. Then the mass-weighted fluctuation covariance matrix was diagonalized to obtain the quasiharmonic eigenvectors and frequencies ²⁷.

Cross-correlation analysis of atomic fluctuations. The Cks1 snapshot structures of the MD trajectory (1 – 5 ns) were first reoriented in order to remove translational and rotational modes before the normalized covariance matrix of atomic fluctuations was calculated. The backbone N plots in Figure 12 only include cross-correlations between backbone N atoms. For the residue cross-correlation, the diagonal elements correspond to averages over all covariance elements involving atoms of the same residue; and the off-diagonal elements are obtained by averaging over all covariance elements involving atoms of two different residues.

Acknowledgements

This work was supported by the Medical Research Council of the UK (MRC). MAS was supported by an External Research Studentship from Trinity College, Cambridge. MS was supported by the Swiss National Science Foundation, the Roche Research Foundation, and the CHARMM Development Project at Harvard University. The work done at Harvard was supported in part by the National Institute of Health (USA). Computational resources were provided by the National Energy Research Scientific Computing Center (USA).

Legends to Figures

Figure 1. Structure of Cks1 showing the binding sites for Cdk2, Skp2 and phosphorylated substrate. The side chains of residues in the binding sites are shown, with residues in the Cdk2-binding site in green, in the phosphate-binding site in blue and the Skp2-binding site in red.

Figure 2. NMR Data for assembly of Cks1 with Cdk2 and Skp1-Skp2. (A) HSQC of ^{15}N -labelled free Cks1. (B) TROSY-HSQC of ^2H , ^{15}N -labelled Cks1 in complex with Cdk2 (red); ^2H , ^{15}N -labelled free Cks1 (black) is shown for comparison. (C) TROSY-HSQC of ^2H , ^{15}N -labelled Cks1 in complex with Skp1-Skp2 (red); ^2H , ^{15}N -labelled free Cks1 (black) is shown for comparison. Samples were prepared in 100 mM NaPO_4 buffer pH 6.8, 100 mM NaCl, 5 mM DTT, 5% D_2O . The concentration of Cks1 was 150 μM , with Cdk2 or Skp-Skp2 in slight excess.

Figure 3. R_1 and R_2 values of free Cks1. The secondary structure elements are shown above the plot.

Figure 4. Changes in Cks1 dynamics on binding Cdk2. Three views are shown for clarity: (A) The Cks1-Cdk2 complex. (B) Structure of the Cks1 subunit in the same orientation as in (A). (C) Structure of the Cks1 subunit as viewed from Cdk2. Residues in dark blue are those that could be assigned in free Cks1, residues in orange and red are those that could be assigned only in the Cdk2-bound Cks1. Residues in orange are short extensions of sequence that could be assigned in the free form, whereas the residues in red, comprising β strand 4, are the only continuous stretch of sequence that is missing in

the free Cks1 spectrum and that is recovered in the Cdk2-bound spectrum. Residues in yellow are those with elevated R_2 values.

Figure 5. Chemical shift changes in Cks1 on binding Cdk2. Chemical shift changes upon Cdk2 binding were calculated as the root mean square deviation of the chemical shifts of cross-peaks in free Cks1 compared with corresponding peaks in Cdk2-bound Cks1. The chemical shifts in the ^{15}N dimension were divided by 10 to correct for the gyromagnetic ratio between ^1H and ^{15}N nuclei. Thus,

$$rmsdt = \sqrt{\left(H_i^{Cks1} - H_i^{Cks1/Cdk2}\right)^2 + \left(\frac{N_i^{Cks1} - N_i^{Cks1/Cdk2}}{10}\right)^2}, \text{ where } H_i^{Cks1} \text{ and } N_i^{Cks1} \text{ are the}$$

chemical shift of residue i in free Cks1 in the proton / nitrogen dimension and $H_i^{Cks1/Cdk2}$ and $N_i^{Cks1/Cdk2}$ are the respective chemical shifts of residue i in Cks1 in complex with Cdk2.

Residues with a chemical shift change > 0.2 ppm are coloured red on the structure of Cks1 and their side chains are shown. Residues with a change < 0.2 ppm are coloured blue. The greatest chemical shift changes are (i) Cdk2-binding interface: residues D14 and E15 (in the turn between $\beta 1$ and $\beta 2$) form ionic interactions with Cdk2 and are hydrogen bonded to Cdk2 backbone atoms; residues Y12 ($\beta 1$) and H21 ($\beta 2$) are hydrogen bonded in the complex with Cdk2. (ii) Distant from the Cdk2 binding interface: residues R20 and N45. These are key residues in the phosphate-binding site (R20) and the Skp2-binding site (N45). Different views of the protein are shown in (A)-(C).

Figure 6. Structural model for the molecular dynamics simulation of Cdk2-bound Cks1 in explicit water. Residues of Cks1 (purple) and residues of Cdk2 within 28 Å of the center of mass of the Cks1 unit (red) are allowed to move freely, and Cdk2 residues between 28 and 35 Å (blue) were confined to their original positions with harmonic restraints. All other residues of Cdk2 were either fixed (cyan surface) or removed from the model completely if they had no atoms within 7 Å of the solvent boundary (37 Å, indicated as a blue cloud).

Figure 7. Calculated fluctuations of backbone nitrogen atoms for free and Cdk2-bound Cks1. Isotropic root mean-square fluctuations determined from *B*-factors (X-ray) and from molecular dynamics (MD) trajectories using explicit TIP3P water solvation (1–5 ns). The X-ray fluctuation curve of uncomplexed Cks1 is obtained by averaging over the fluctuations of the four monomers found in the two dimer crystal structures 1DKS and 1DKT.

Figure 8. RMSD plots for MD simulations. The rms deviation of the C^α atoms from the complex crystal structure (PDB entry 1BUH) is plotted as a function of the simulation time. (A) Trajectories of free and Cdk2-bound Cks1 using explicit TIP3P water solvation (only unconstrained C^α atoms of Cdk2 are included). (B) Trajectory of free Cks1 using the implicit solvation model EEF1. The rms deviation of all non-hydrogen atoms is shown in addition. The arrows mark the insertion into, and displacement from, strands β4 and β2 of the Arg70 side chain.

Figure 9. The bulge region of strand β 1. The figure shows the backbone of the average structures of Cdk2-bound Cks1 (A) and free Cks1 (B) determined from molecular dynamics simulations with explicit water. For reasons of clarity residues of the Cdk2 unit and side chains of Cks1 are not shown except for Ser9.

Figure 10. Interactions between monomers A and B in the dimer crystal structure of Cks1 (PDB entry 1DKS). (A) Overview of the crystal structure with monomers A (blue) and B (red) shown as cartoon model. (B) Enlarged cutout region as indicated in (A). Residues that show intermolecular hydrogen bonds are shown as colored stick model and are labeled with residue type & number and monomer identifier.

Figure 11. The phosphate binding site of Cks1 and flexibility of Lys11. (A) The figure illustrates how phosphate binds to Cks1 as seen in the dimer crystal structure of Cks1 (PDB entry 1DKS1). (B) C^α rms overlay of 20 snapshots from the molecular dynamics of free Cks1 with explicit water (1 – 5 ns) taken every 0.2 ns. Only a cutout is shown as indicated by the frame in (A). The structures were reoriented for the overlay to remove translational and rotational motions. The backbone is shown as light grey C^α trace and the side chains of Lys11, Arg20, Trp54, and Arg71 as colored stick models. (C) The same as (B) but for the simulation of the complex Cdk2-Cks1. The Cdk2 unit is not shown for clarity.

Figure 12. Contour plot for the covariance matrices of the spatial atom displacements of Cks1. The covariance elements were determined from the molecular dynamics simulations with explicit water for free Cks1 (A) and Cdk2-bound Cks1 (B). In

the lower right half of each plot, the normalized covariance elements of only the backbone nitrogen atoms are illustrated, whereas in the upper left half the averages over all atoms of each residue are shown.

Figure 13. Insertion of Arg70 side chain into strands $\beta 2$ and $\beta 4$ of free Cks1. The end of strand $\beta 4$ (C-term) and the loop $\beta 1$ - $\beta 2$ is shown for two average structures of time-intervals 22 – 32 ns (A) and 35 – 50 ns (B) of the molecular dynamics simulation with implicit solvation (EEF1). Side chains are not shown except for Arg70.

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