The Alpha Helix of Ubiquitin Interacts with Yeast Cyclin-Dependent Kinase Subunit CKS1†

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ABSTRACT: Ubiquitin serves as a molecular zipcode to direct and sort ubiquitinylated proteins into distinct biological pathways. Although novel modes of ubiquitin interaction have recently been characterized, conventional ubiquitin-binding domains (UBDs) recognize ubiquitin through a hydrophobic pocket centered around isoleucine 44 and lined by residues in beta sheets 3 and 4. In this study, we report a novel mode of interaction between ubiquitin and the cyclin-dependent kinase subunit of *Saccharomyces cerevisiae*, Cks1p, an adaptor protein involved in transcriptional regulation through recruitment of proteasomal subunits to gene promoters. Cks1p interacts specifically with monoubiquitin and tetraubiquitin with an affinity several orders of magnitude greater than that of other ubiquitin-binding domains and in an unconventional fashion, which differs from interactions documented so far between ubiquitin and conventional UBDs. The loop between helices alpha 1 and alpha 2, and to a minor extent the *N*-terminal α-helix of Cks1p, are involved in the interaction with the α-helix of ubiquitin, instead of its I44-centered hydrophobic pocket. Not only is this the first time the α-helix of ubiquitin is implicated in a protein/protein interaction, thereby shedding new light on the mechanisms of ubiquitin recognition, but also the first report of a direct physical interaction between ubiquitin and Cks1p, inferring a role for ubiquitin binding in the transcriptional function of Cks1p.

Post-translational modification of proteins by ubiquitinylation is a tightly regulated mechanism that modifies the biochemical properties of target proteins and seals their fate by affecting their subcellular localization, activity, interactions with partners and substrates, or their overall stability (*1*). Similar modifications may occur through conjugation of ubiquitin-like proteins (UBLs[1]), such as Sumo (small ubiquitin-like modifier) and Nedd8 (also called RUB: related to ubiquitin), which, as their names indicate, share the same structural fold with ubiquitin but possess different biochemical properties, which are conferred to the proteins they modify (*2*). The ubiquitin or UBL tag can, therefore, be thought of as a molecular zipcode that addresses and sorts proteins into distinct cellular pathways, including transcriptional regulation, endocytosis, DNA repair, and signal transduction in addition to proteasome-mediated degradation (*3*−*5*). Moreover, the length and linkage specificity of ubiquitination is intimately related to the fate of the ubiquitinylated target (*6*−*9*), polyubiquitinylation with lysine 48 (K48) chains (polymers of ubiquitin linked through their K48) generally leading to proteasome-mediated degradation in contrast to monoubiquitinylation, which is preferentially associated with endocytosis, lysosomal degradation, and transcriptional regulation (*1*, *2*).

The mechanistic basis underlying recognition of ubiquitinylated proteins is still poorly understood but is believed to involve both structural determinants within the ubiquitin moiety and the target protein itself. The interaction between ubiquitin and ubiquitin-binding domains (UBDs) is much better documented, and the structural features of ubiquitin and several ubiquitin chains have been well characterized (*6*−*12*). Ubiquitin is a small (8.5 kDa), compact protein composed of four beta sheets interconnected by a single α-helix and a short 3_{10} helix (*10*). Archetypal UBDs bind ubiquitin through a hydrophobic pocket centered around isoleucine 44 (I44), lined by residues in beta sheets 3 and 4, particularly leucine 8, histidine 68, lysine 48, and valine 70 (L8, K48, H68, and V70) (*6*). This mode of interaction is of modest affinity, in the micromolar range, which is thought to be compatible with the dynamic and reversible physiological requirements of this type of interaction. Nine different classes of conventional UBDs have been described (*5*, *6*), the most notorious of which are the UBA (ubiquitin-associated), the CUE (coupling ubiquitin to endoplasmic

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[1] Abbreviations: APC, anaphase promoting complex; cdk, cyclin-dependent kinase; Cks, cyclin-dependent kinase subunit; CUE, coupling of ubiquitin conjugation to the endoplasmic reticulum; FITC, fluorescein isothiocyanate; RUB, related to ubiquitin; RUZ, Rabex-5 ubiquitin-binding zinc finger; Sumo, small ubiquitin-like protein modifier; Ub, ubiquitin; UBA, ubiquitin-associated; UBD, ubiquitin-binding domain, UBL, ubiquitin like; UIM, ubiquitin-interacting motif; UBM, ubiquitin binding motif; UBP, ubiquitin-specific processing protease; UBZ, ubiquitin-binding zinc finger; UPS, ubiquitin/proteasome system; ZnF, zinc finger.

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reticulum degradation), and the UIM (ubiquitin-interacting motif) (*13*−*22*). More recently, four novel sequence motifs or domains have been described, which recognize ubiquitin through residues other than those engaged in its hydrophobic pocket (*23*−*26*). The so-called RUZ (Rabex-5 ubiquitin-binding zinc finger) motif of Rabex-5, contacts ubiquitin through aspartate 58 (*23*, *24*). The ZnF UBP of isopeptidase T recognizes the C-terminal G75G76 dipeptide of ubiquitin (*25*). The UBM (ubiquitin-binding motif) and the UBZ (ubiquitin-binding zinc finger) domains identified in Y family DNA polymerases interact with a surface adjacent to the I44 patch of ubiquitin, displaced toward L8 (*26*). The discovery of new families of UBDs interacting with residues other than those strictly centered at the I44 pocket, together with the extreme sequence conservation of ubiquitin throughout evolution (*27*), suggests that all accessible surface residues of ubiquitin are potential candidates for recognition by UBD-containing proteins.

The ubiquitin/proteasome system (UPS) determines the fate of a number of cell cycle regulators including cyclins, the regulatory subunits of cyclin-dependent kinases (*28*, *29*). Cks proteins (for cyclin-dependent-kinase subunit) play a central role in cell cycle progression through physical association with cyclin-dependent kinases (cdks) and stabilization of cdk−cyclin complexes (*30*−*34*). These small (9−18 kDa), highly conserved proteins are also known to function as adaptors by facilitating the recruitment of phosphoprotein regulators, such as Cdc25, Myt1, and Wee1, to cyclin-dependent kinases, thanks to an anion-binding pocket (*35*). Moreover, human CksHs1 is involved in the recruitment of the SCF ubiquitin ligase Fbox component Skp2 to the cdk inhibitor p27, thereby promoting ubiquitinylation and degradation of the latter (*36*, *37*). In budding yeast, Cks1p modulates the proteolysis of M phase targets, including the anaphase inhibitor Pds1 and the mitotic cyclin Clb2 through physical interaction with the proteasome (*38*). In addition, we recently uncovered a role for Cks1p in the transcriptional regulation of the APC activator *CDC20* in particular, and in transcriptional regulation more generally, through the recruitment of proteasomal subunits to the promoters of target genes (*39*). These and other findings have lead us and others to propose that Cks1p and proteasomal subunits might be recruited to actively transcribed chromatin to participate in transcriptional remodeling activities (*39*−*41*).

Given the relationship between Cks proteins and several components of UPS (*36*−*40*) as well as the growing body of evidence for the implication of ubiquitin and ubiquitin-like proteins in transcriptional regulation and chromatin remodeling (*42*−*47*), we asked whether ubiquitin might serve as an adaptor in this process through physical interaction with Cks1p. In this study, we report a novel, high affinity interaction between ubiquitin and the cyclin-dependent kinase subunit Cks1p and demonstrate that it differs from conventional interactions described between archetypal UBDs and ubiquitin. Instead, it involves the α-helix of ubiquitin on the one hand and specific sequences present in only budding yeast Cks1p and fission yeast p13suc1 on the other. This is the first time the α-helix of ubiquitin is implicated in a protein/protein interaction, and it is also the first report of a direct physical interaction between Cks proteins and ubiquitin. We discuss the implications of this interaction with respect to the mechanisms of ubiquitin recognition and the transcriptional function of yeast Cks1p.

EXPERIMENTAL PROCEDURES

Materials. Ubiquitin of bovine origin was purchased from Sigma-Aldrich. K48- and K63-linked tetraubiquitin chains were purchased from BioMol International LP. Yeast Rad23 was generously provided by G. Divita and G. Lledo. Oligonucleotide primers were purchased from Eurogentec. Glutathione Sepharose, Chelating Sepharose, and CNBr-activated Sepharose were all purchased from GE Healthcare. Anti-Cks1 and anti-ubiquitin were purchased from Tebubio, Santa-Cruz and used for Western blotting according to the manufacturer's instructions.

Plasmid Constructs and Deletion Mutants of Cks1. The pRK171-CKS1 construct was from S. I. Reed. The cDNA encoding Cks1 was subcloned into the pGex6P1 vector. Deletion mutants of CKS1 were generated either by PCR−deletion/subcloning strategy or by site-directed PCR mutagenesis using the QuickChange Mutagenesis Kit (Stratagene). The ΔQ and the ΔI mutants were generated by PCR−mutagenesis by deleting base pairs corresponding to the polyQ sequence of Cks1p (residues 118−133) and amino acids 57−66 (DYFNSEVGTL), respectively. In the pRK171-CKS1 construct, the ΔN mutant was generated by PCR−mutagenesis by deleting residues 7−24 (AFQGRKLTDQER-ARVLLEF) from the wild-type sequence of Cks1p. The ΔNΔI mutant was generated through dual deletion of residues 7−24 and 57−66 in both the pRK171-CKS1 and the pGex6P1-CKS1 constructs. In addition the GST-ΔN24-Cks1 construct was generated directly by subcloning the corresponding PCR product into the pGex6P1 vector. Deletion mutants ΔIA (SDYFNSEV deleted) and ΔIB (GTLRILT deleted) as well as alanine mutants of each of the residues from D57 to R67 were generated using the QuickChange Mutagenesis Kit (Stratagene). pET6His-tagged wheat ubiquitin was from O. Coux. pET6His-tagged yeast Smt3 was provided by E. Johnson. Chimeric 6His-Smt3 α-helix ubiquitin (Smt3p-αUb) was generated by replacing the sequence encoding the α-helix of Smt3p (residues 45−56, LR-RLMEAFAKRQ) with that encoding the α-helix of wheat ubiquitin (residues 23−34, IDNVKSKIQDKE) according to the Quick Change Mutagenesis protocol (Stratagene). Expression vectors for recombinant ubiquitin mutants I44A and H68A as well as GST-Dsk2-UBA were provided by M. Shirakawa. The pGex-Rad23-UBA plasmid was provided by C. Dargemont.

Protein Expression and Purification. All proteins were expressed in *E. coli* by induction with 0.5 mM IPTG for 4 h at 37 °C. Cks proteins expressed without a tag were purified essentially as described previously (*33*, *48*). Alternatively GST-Cks proteins were purified on Glutathione Sepharose, and the GST-tag was removed by treatment with prescission protease and gel filtration chromatography for titration experiments. 6His-tagged wheat ubiquitin and 6His-tagged Smt3 were purified by affinity chromatography on Chelating Sepharose. Ubiquitin mutants were purified by heat treatment, and GST-Dsk2-UBA was purified on Glutathione Sepharose, as described previously (*20*). All proteins were finally applied onto a Superdex 75 16/60 FPLC gel filtration column (GE Healthcare) for their final purification, and pure

proteins were concentrated in 50 mM phosphate buffer at pH 7.5 and 150 mM NaCl on an Amicon ultrafiltration unit.

Peptide Synthesis. The peptides used in this study were synthesized and purified to 90% by GL Biochem, Ltd. (Shanghai, China). The 20-mer peptide derived from the major α-helix of ubiquitin (Alpha Ub), VESSDTIDNVKSKIQDKEG-C, corresponds to residues 17−35 of *S. cerevisiae* ubiquitin (helix I23-E34). The 33-mer peptide encompassing residues involved in α-helices 1 and 2 as well as the insertion of *S. cerevisiae* Cks1p (Cks1pep) MLPKAMLKVIPSDYFNSEVGTLRILTEDEWRG-C corresponds to residues 45−76. The 25-mer peptide encompassing residues involved in α-helices 1 and 2 of human CksHs1 (CksHs1pep) MLPKDIAKLVPKTHLMSESEWRNL-C corresponds to amino acids 23−36. As indicated in their sequence, an additional C-terminal cysteine was incorporated into these peptides upon synthesis for labeling purposes. The control peptide used in this study corresponds to a peptide derived from a 14−3−3 binding sequence of the Cdc25C phosphatase, GLYRSPSMPE.

Fluorescent Labeling of Proteins and Peptides. All proteins and peptides except ubiquitin were labeled on a single cysteine with a 10-fold molar excess of fluorescein isothiocyanate (FITC) in phosphate buffer at pH 7.5 and 150 mM NaCl as recommended by the manufacturer (Molecular Probes). Cks1p was labeled on its unique cysteine, C90. Ubiquitin was labeled with FITC on primary amines (N-terminal labeling) in essentially the same fashion, except that the pH of the phosphate buffer was greater than 8.5. Following labeling, the excess fluorophore was removed by purification of proteins and peptides on NAP-10 columns (GE Healthcare).

Fluorescence Spectroscopy Titration Experiments. Fluorescence spectroscopy titration experiments were performed as described previously in potassium phosphate buffer at pH 7.2 and 150 mM NaCl at 25 °C (*49*). Extrinsic fluorescence of fluorescein-labeled proteins was monitored between 500 and 560 nm following excitation at 495 nm. Data were analyzed and fitted according to a standard quadratic equation, using the Grafit 4.0 software (Erathicus, Ltd.), as described previously (*49*). Dissociation constant values (*K*d) reported in this study correspond to the average of three or four independent representative experiments.

Pull-Down Experiments. Untagged Cks proteins or ubiquitin were cross-linked to CNBr-activated Sepharose according to the manufacturer's instructions (GE Healthcare). GST-tagged Cks proteins were coupled to Glutathione Sepharose. 6His-ubiquitin and 6His-Smt3 were coupled to Chelating Sepharose activated with $NiCl_2$. Incubations were performed in 50 mM TrisHCl buffer at pH 7.5−8.0 containing 50−100 mM NaCl for 1 h at room temperature. Samples were then washed three times with the same buffer, boiled in Laemmli buffer, and analyzed by SDS−PAGE followed by Western blotting.

Models of Cks1p and Ubiquitin. Ribbon diagrams of Cks1p and ubiquitin structures were generated from the coordinates of Cks1p crystal structure (1QB3) (*50*), human ubiquitin crystal structure (1UBI) (*10*), and *S. cerevisiae* ubiquitin NMR structure (1WR1) (*17*) using PyMOL software, version 0.93 (Delano Scientific, LLC, San Carlos, USA) and Swiss-PDB Viewer software, version 3.7 (*51*) (http://www.expasy-.org/spdbv/).

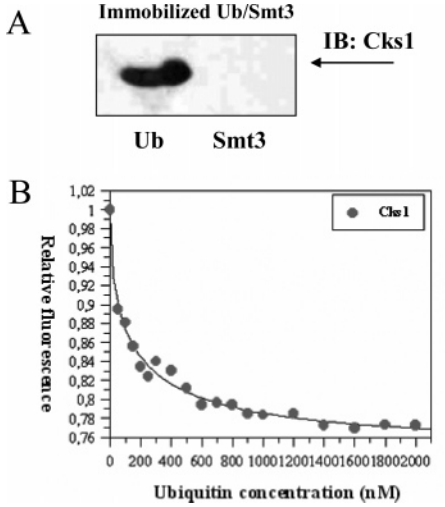

FIGURE 1: Ubiquitin interacts specifically with Cks1p. (Panel A) Equal amounts of 6His-ubiquitin and 6-HisSmt3 were immobilized on CNBr Sepharose beads and incubated with purified recombinant Cks1p. Specific retention of Cks1p was detected by Western blotting with anti-Cks1 antibodies. (Panel B) Titration of fluorescein-labeled Cks1p with ubiquitin. Curve fitting and the calculation of dissociation constants were performed as described previously (*25*). A representative example is shown with a *K*d value of 74 ± 6 nM.

RESULTS

S. cerevisiae Cks1p Interacts Specifically and with High Affinity with Ubiquitin in Vitro. To address whether ubiquitin might serve as an adaptor in the recruitment of Cks1p to transcriptional loci, we asked whether Cks1p might interact physically with ubiquitin. We incubated purified recombinant Cks1p with recombinant ubiquitin, which had been immobilized on CNBr-activated Sepharose in physiological conditions (pH 7.2, 100 mM NaCl) and found that it was efficiently retained by ubiquitin (Figure 1A). The converse experiment, in which ubiquitin was incubated with Cks1p immobilized on CNBr-activated Sepharose resin yielded the same result (data not shown), revealing that immobilization of either partner does not prevent their interaction. In contrast to ubiquitin, however, immobilized recombinant Smt3p, the *S. cerevisiae* representative of Sumo proteins (*52*, *53*), did not retain Cks1p (Figure 1A), indicating that Cks1p does not generally bind all ubiquitin-like proteins in spite of their conserved structural fold.

To further characterize the interaction between Cks1p and ubiquitin, we performed titration experiments by fluorescence spectroscopy using fluorescein-labeled Cks1p (Cks1p-FITC). The addition of increasing concentrations of unlabeled monoubiquitin produced 25% quenching of Cks1p fluorescence, and curve fitting yielded a dissociation constant of 91 ± 21 nM, indicative of a high affinity interaction between Cks1p and ubiquitin (Figure 1B, Table 1). In contrast, titration of Cks1p with Smt3p did not produce any significant changes in the extrinsic fluorescence of Cks1p-FITC, in agreement with the pull-down experiments, indicating that Cks1p and Smt3p do not interact *in vitro* (Table 1). The finding that Cks1p bound monoubiquitin with such high affinity, whereas conventional UBDs interact with ubiquitin with micromolar affinity, prompted us to characterize this interaction further. In particular, UBDs interact with ubiquitin chains in which the ubiquitin moieties are linked through K48 with greater affinity than with monoubiquitin because

Table 1: Dissociation Constants Determined for the Interactions between Cks1p and Ubiquitin Variants[a]

fluorescent substrate	ligand	*K*d (μM)
Cks1p-FITC	ubiquitin	0.091 ± 0.021
Cks1p-FITC	tetraubiquitin (K48-linked)	0.125 ± 0.025
Cks1p-FITC	tetraubiquitin (K63-linked)	0.094 ± 0.034
ubiquitin-FITC	Cks1p	0.099 ± 0.008
ubiquitin-FITC	GST-Dsk2-UBA	6.6 ± 1.93
Cks1p-FITC	ubiquitin I44A mutant	0.123 ± 0.022
Cks1p-FITC	ubiquitin H68A mutant	0.115 ± 0.02
Cks1p-FITC	α-helix ubiquitin	0.141 ± 0.025
Cks1p-FITC	Smt3	no binding
Cks1p-FITC	Smt3-α-helix ubiquitin	0.350 ± 0.100

[a]Fluorescence spectroscopy titration experiments were performed by titrating 100 nM fluorescently labeled Cks1p or ubiquitin with unlabeled ligand in potassium phosphate buffer at pH 7.2 and 150 mM NaCl at 25 °C. Spectra were analyzed and corrected for dilution, as described previously (*49*). Data plotting, curve fitting, and dissociation constant calculation, according to a standard quadratic equation, were performed using the Grafit software (Erathicus, Ltd.). The *K*d values shown correspond to the average of three to four independent representative experiments.

of their closed conformation, which enables the formation of a diubiquitin sandwich around the UBD (*11*). Hence, we examined the ability of Cks1p-FITC to interact with K48-tetraubiquitin chains by fluorescence spectroscopy. Interestingly, we found that Cks1p bound K48-tetraubiquitin with an affinity similar to that for monoubiquitin, with a dissociation constant of 125 ± 25 nM (Table 1). Moreover, experiments performed with tetraubiquitin K63-linked chains reveal that Cks1p exhibits similar, only slightly higher affinity for K63-linked chains, with a dissociation constant of 94 ± 34 nM (Table 1). Taken together, these data reveal that Cks1p interacts specifically with ubiquitin, which by definition indicates that Cks1p harbors a UBD. However, the mode of interaction between Cks1p, ubiquitin, and K48- or K63-linked ubiquitin chains does not resemble that of conventional UBDs, whose propensity to interact with tetraubiquitin chains is favored over monoubiquitin.

Fluorescently Labeled Ubiquitin Interacts with Cks1p in an Unconventional Fashion. The finding that Cks1p bound ubiquitin with submicromolar affinity, whereas the dissociation constant values for all other UBDs described to date are in the micromolar range (*K*d 10−500 μM) (*6*, *7*, and references therein, *23*−*26*), together with the lack of significant differences in the affinity of Cks1p for tetraubiquitin chains, suggested that Cks1p does not recognize ubiquitin in the same fashion as other UBDs. However, in all of the studies reported for UBDs, dissociation constants were determined by surface plasmon resonance in which the substrate is fixed, whereas our values were obtained by fluorescence titration experiments performed in solution. Hence, to avoid any discrepancies due to differences in experimental approaches, we performed fluorescence titration experiments to compare the affinity of Cks1p with that of an archetypal UBD, the UBA of *S. cerevisiae* Dsk2p (*19*, *20*) for FITC-labeled ubiquitin. The titration of FITC-ubiquitin with Cks1p lead to significant quenching of its fluorescence (20%) (Figure 2A), and curve fitting yielded a dissociation constant of 99 ± 8 nM, quite similar to the value obtained in the converse experiment (Table 1). In contrast, the titration of FITC-ubiquitin with GST-UBA-Dsk2 yielded a dissociation constant several orders of magnitude greater, with a value of 6.6 ± 1.93 μM (Table 1), which is comparable to the value determined by surface plasmon resonance in previous studies (*20*). Similar results were obtained for the UBA of Rad23p, another archetypal UBD (data not shown, *15*). These data indicate that the ubiquitin-binding moiety of Cks1p does not resemble conventional UBDs such as the UBAs of Dsk2p or Rad23p, in that it interacts with ubiquitin with much higher affinity and, therefore, presumably in a different fashion.

Ubiquitin Interacts with Cks1p through Its α*-Helix.* In spite of their structural diversity, most UBDs interact with the hydrophobic pocket centered around I44, buried on one face of ubiquitin (*6*). In particular, the UBAs of Rad23p and Dsk2p have been shown to contact residues in β3 and β4 beta sheets of the ubiquitin moiety, which line the hydrophobic pocket through residues in their α1 and α3 helices. Ubiquitin residues I44 and H68 are critical for this interaction, and their mutation impairs the robust binding of ubiquitin to an UBA (*20*). To determine whether Cks1p behaved like conventional UBDs, and in particular like UBAs, we examined its ability to interact with ubiquitin mutants I44A and H68A. The titration of Cks1p-FITC with either of these mutants revealed that the mutation of I44 or H68 to alanine barely affected ubiquitin binding to Cks1p, with *K*d values of 123 ± 22 and 115 ± 20 nM, respectively (Table 1). These results contrast with the complete disruption and the significant impairment of the interaction described between Dsk2p-UBA and the I44A and H68A mutants, respectively, arguing that the ubiquitin/Cks1p interaction is mediated through a contact surface other than the I44-centered hydrophobic pocket of ubiquitin (Table 1, Figure 2A, *20*).

The overall structural conservation of ubiquitin throughout evolution, together with the recent reports of novel recognition patches distinct from the I44 pocket on the surface of ubiquitin, suggests that practically any accessible surface residues are candidates for contacts with novel, unconventional UBDs. In particular, an examination of the molecular surface of ubiquitin points to the α-helix of ubiquitin, which is fully exposed at the surface of the ubiquitin molecule and lies on the opposite face of ubiquitin with respect to the I44 patch, as a potential candidate (Figure 2B). To verify whether this helix, or some of its residues, might indeed interact with Cks1p, we designed a peptide derived from the α-helix of *S. cerevisiae* ubiquitin and examined its ability to bind Cks1p in pull-down and fluorescence spectroscopy experiments (Table 1, Figure 2A and C). We found that this peptide readily interacted with Cks1p-FITC with a dissociation constant of 141 ± 25 nM and that it retained recombinant Cks1p in pull-down assays, thereby identifying it as an entirely novel recognition site at the surface of ubiquitin and establishing its implication in the Cks1p−ubiquitin interaction. As shown in Figure 2D, the sequence of the α-helix of ubiquitin from different species (yeast, wheat, or bovine) is highly conserved, whereas the α-helix of Smt3p displays no homology whatsoever. Therefore, to further confirm the critical role of the α-helix of ubiquitin in the interaction with Cks1p, we generated a His-tagged chimera, Smt3p-αUb, in which the α-helix of Smt3p was replaced by that of ubiquitin, and assessed its ability to interact with Cks1p-FITC by fluorescence spectroscopy. In contrast to Smt3p, which did not interact with Cks1p, we found that the Smt3p-αUb

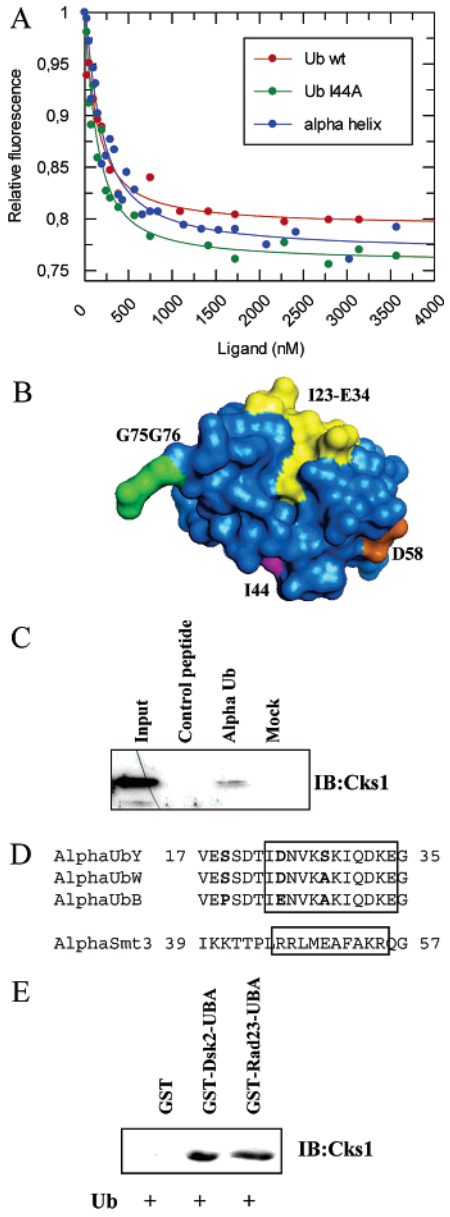

FIGURE 2: Ubiquitin binds Cks1p through its α-helix. (Panel A) Titration of fluorescein-labeled Cks1p with wild-type ubiquitin, its I44A mutant, or a peptide derived from ubiquitin. Curve fitting and the calculation of dissociation constants were performed as described previously (*25*). Representative examples are shown for wild-type ubiquitin (red circles), the I44A ubiquitin mutant (green circles), and α-helix ubiquitin peptide (blue circles) with *K*d values of 103 ± 26, 92 ± 23, and 135 ± 25 nM, respectively. (Panel B) Surface representation of ubiquitin generated from coordinates of human ubiquitin crystal structure (1UBI) (*10*) with PyMOL software, version 0.93 (Delano L. W. Scientific, San Carlos, USA). Residues 23−34, forming the α-helix shown in yellow, are located at the surface of ubiquitin, far from the other residues involved in interactions with UBDs: Ile44 in pink, Asp58 in orange, and diglycine 75 and 76 in green. (Panel C) A peptide derived from the α-helix of ubiquitin immobilized on CNBr Sepharose beads and incubated with recombinant Cks1p. Specific retention of Cks1p was probed by Western blotting as in Figure 1A. Equal amounts of a peptide with no biological relevance to this study (control peptide) and resin alone (mock) were used as controls. (Panel D) Sequence of the peptide derived from the α-helix of yeast ubiquitin (AlphaUbY) and Smt3 (AlphaSmt3) shown together with corresponding sequences in wheat (AlphaUbW) and bovine ubiquitin (AlphaUbB). (Panel E) Cks1p incubated with GST (lane 1), GST-Dsk2-UBA (lane 2), or GST-Rad23-UBA (lane 3) immobilized on GST-Sepharose and preincubated with ubiquitin. The Cks1p retained on the resins was detected by Western blotting.

chimera bound Cks1p with an affinity 3-fold lower than that of full-length, wild-type ubiquitin with an average *K*d value of 350 ± 100 nM (Table 1). Taken together, these data indicate that the α-helix of ubiquitin is necessary and sufficient for the interaction between Cks1p and ubiquitin, even in the context of an entirely foreign environment (Smt3p). The weaker affinity of the Smt3p-αUb chimera for Cks1p compared to that of wild-type ubiquitin and the α-helix peptide is most likely due to charge or steric repulsion from residues in the Smt3 entity.

We finally asked whether Cks1p was capable of interacting with ubiquitin while the latter bound a conventional UBD or whether their binding was mutually exclusive, the binding of one partner somehow preventing access or inducing a conformational change that might preclude interaction of the third partner. To address this issue, Cks1p was incubated with GST, GST-Dsk2-UBA, or GST-Rad23-UBA, previously immobilized on GST Sepharose and preincubated with ubiquitin. As shown in Figure 2E, we found that Cks1p was indeed retained by the UBA of Dsk2p and of Rad23p preincubated with His-tagged ubiquitin. In the absence of ubiquitin, Cks1p is not retained by either of these GST-tagged UBAs (data not shown). These data not only confirm our finding that Cks1p binds a patch on ubiquitin, which is distinct from the conventional Ile44-centered pocket, but also indicate that Cks1p can form a ternary complex with ubiquitin and a conventional UBD.

Selectivity of Ubiquitin for Cks1p. Identification of Cks1p Interaction Determinants. S. cerevisiae Cks1p bears three features that distinguish it from other eukaryotic Cks orthologs: an N-terminal extension, part of which adopts a helicoidal fold in the crystal structure of Cks1p (α0) (*50*) and which is absent in other Cks orthologs except in those of *S. pombe* and *Leishmania.* sp.; a 10-mer insertion between α1 and α2 helices, which is also found in the *S. pombe* ortholog p13suc1; and a C-terminal extension of 30−35 amino acids, which is absent in all other orthologs and harbors a 16-mer polyglutamine tail (Figure 3A and B). To identify Cks1p determinants implicated in the interaction with ubiquitin, we first asked whether the human orthologs CksHs1 and CksHs2 were equally capable of binding ubiquitin. As shown in Figure 4A, unlike Cks1p, human CksHs1 and CksHs2 immobilized on CNBr-Sepharose were unable to retain ubiquitin, indicating that one or several of the additional extensions specific to *S. cerevisiae* Cks1p were responsible for the interaction with ubiquitin. Furthermore, the titration of fluorescent ubiquitin with recombinant CksHs1 and CksHs2 confirmed that these proteins were altogether unable to interact with ubiquitin in solution (Table 2). In contrast, we found that fission yeast p13suc1 bound fluorescent ubiquitin with high affinity very similar to that of Cks1p, with a dissociation constant of 92 ± 28 nM (Table 2), hinting at a role for the N-terminal extension and the 10-mer insertion of these yeast proteins in the interaction with ubiquitin.

To further identify which extensions were responsible for the ubiquitin/Cks1p interaction, we generated several deletion mutants of Cks1p and characterized their ability to bind ubiquitin *in vitro* (Figure 4B and C). Pull-down experiments indicated that deletion of the C-terminal polyglutamine extension had no major impact, in agreement with the high affinity interaction measured between ubiquitin and p13suc1,

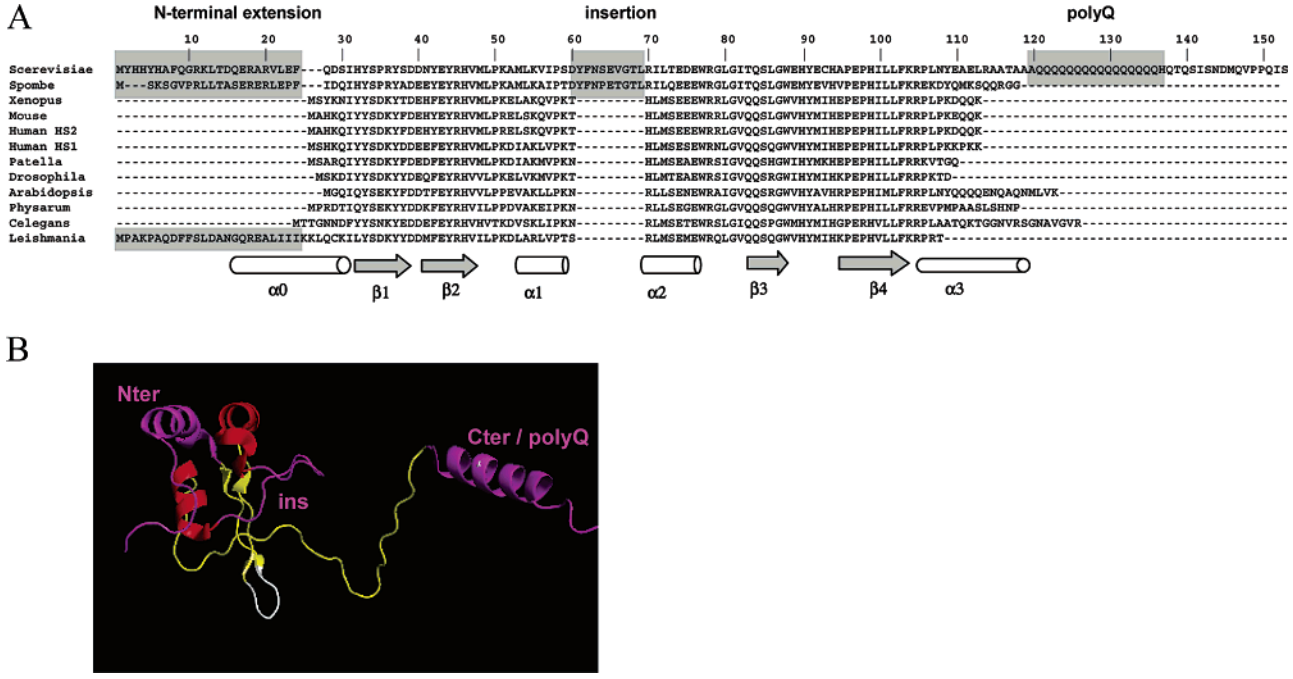

FIGURE 3: Features specific to *S. cerevisiae* Cks1p. (Panel A) Homology alignment of Cks proteins from different species using MULTALIN (http:// npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_multalin.html). Distinctive features of Cks1p are highlighted. Secondary structure elements of Cks1p determined by X-ray crystallography (*18*) are shown below the alignment. (Panel B) Model of Cks1p. Ribbon diagram of Cks1p generated using the coordinates of Cks1p crystal structure (1QB3) (*50*) with PyMOL software, version 0.93 (Delano L.W. Scientific, San Carlos, USA). The N-terminal extension, the insertion, and the C-terminal extension harboring the polyglutamine stretch are highlighted in magenta.

which lacks the C-terminal extension of Cks1p (Figure 3A). However, the deletion of the first 24 amino acids corresponding to the N-terminal extension (ΔN24) modestly affected ubiquitin binding, and the deletion of the insertion (ΔI) alone or together with the deletion of the N-terminal extension (ΔN24ΔI) significantly impaired this interaction. On the basis of these results, we further characterized the interactions between ubiquitin and Cks1p mutants lacking the N-terminal extension or the insertion. The ΔN24 mutant presented a small but reproducible decrease in affinity compared to wild-type Cks1p, with a *K*d value of 152 ± 23 nM, whereas the ΔI mutant lost all ability to bind ubiquitin (Table 2). As expected, the ΔN24ΔI mutant did not bind ubiquitin either. To clearly establish the role of the insertion of Cks1p in the interaction with ubiquitin, we examined the affinity of two fluorescently labeled peptides for ubiquitin: Cks1pep, corresponding to the α1/insertion/α2 sequence of Cks1p, and Hs1pep, spanning the sequence corresponding to α1 and α2 of human CksHs1. As shown in Figure 4D and Table 2, Cks1pep bound ubiquitin with high affinity with a *K*d value of 174 ± 40 nM, whereas Hs1pep did not interact with ubiquitin at all. Taken together, these results argue that although the N-terminal extension of Cks1p contributes to the interaction with ubiquitin, the 10-mer insertion between α-helices 1 and 2 is the primary element responsible for this interaction.

In order to characterize the novel ubiquitin-binding motif of Cks1p in more detail, we generated two deletion mutants, ΔIA (SDYFNSEV) and ΔIB (GTLRILT), and compared their ability to bind immobilized ubiquitin in pull-down experiments. As shown in Figure 4E, we found that the first moiety of the insertion was required for the interaction between Cks1p and ubiquitin. In an attempt to define a minimal ubiquitin-binding motif within this sequence, we generated a series of individual mutants by alanine scanning (from D57 to V63) and examined their ability to interact with ubiquitin. However, none of these individual mutants was capable of disrupting the Cks1p−ubiquitin interaction (data not shown), indicating that several residues are likely to cooperate in this interaction. This being said, we found this interaction to be independent of salt concentration, suggesting that it does not involve any charged residues or salt bridges (data not shown).

Model of the Ubiquitin−Cks1p Interaction. On the basis of our studies, we generated a model to schematize this novel mode of interaction between the α-helix of ubiquitin, the N-terminal extension, and the insertion of Cks1p (Figure 5). In this model, the structures of Cks1p and of ubiquitin are oriented such that the N-terminal extension and the insertion of Cks1p point toward the major α-helix of ubiquitin. This representation clearly shows that Cks1p residues in the beta sheets responsible for interactions with cdks on the one hand and those involved in the anion-binding pocket on the other (*48*, *50*) are far from those involved in the interaction with ubiquitin. Likewise the α-helix of ubiquitin and the I44-centered hydrophobic pocket of ubiquitin lie on opposite faces of ubiquitin. In this model, it, therefore, appears that while interacting with one another, both Cks1p and ubiquitin may remain capable of binding other partners, such as a cyclin-dependent kinase, a phosphoprotein regulator for Cks1p, or a conventional I44-binding UBD for ubiquitin. As such, this model allows for a more global representation of both Cks1p and ubiquitin as adaptors, each of which may bring together additional partners in the context of a larger multimeric complex.

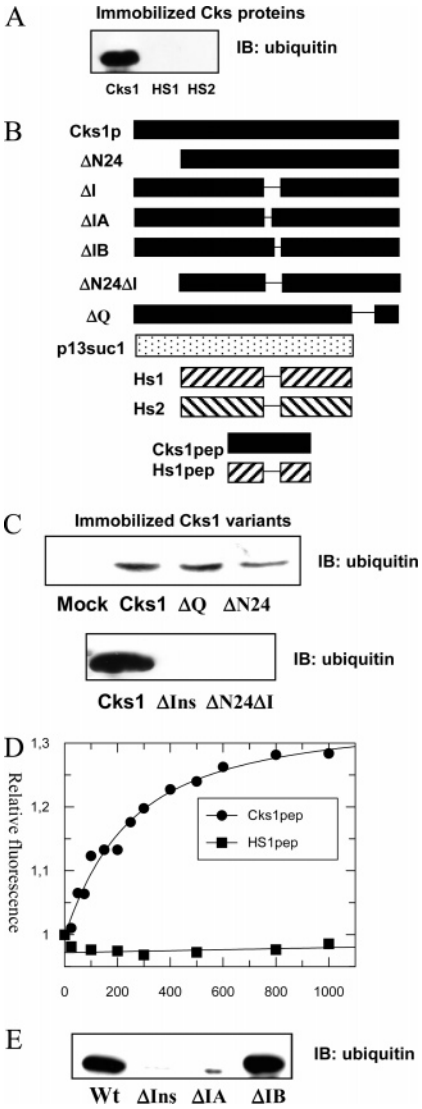

FIGURE 4: Ubiquitin interaction determinants of Cks1p. (Panel A) Cks1p, CksHs1, and CksHs2 were immobilized on CNBr Sepharose beads and incubated with recombinant ubiquitin. Specific retention of ubiquitin was detected by Western blotting with anti-ubiquitin antibodies. (Panel B) Schematic representation of wild-type Cks1p and its deletion mutants compared to human CksHs1, CksHs2, and p13suc1. (ΔN24), Cks1p deleted for its 24 first N-terminal residues; (ΔI), for the insertion between α-helices α1 and α2; (ΔIA) and (ΔIB), for the first and second half of the insertion, respectively; (ΔN24ΔI), for both the N-terminal extension and the insertion; and (ΔQ), for the polyglutamine tail. (Nterpep), peptides spanning the N-terminal extension of Cks1p; (Cks1pep), the alpha1-insertion-alpha2 of Cks1p; and (Hs1pep), alpha1-alpha2 of CksHs1. (Panel C) Equal amounts of the deletion mutants of Cks1p described in panel B immobilized on CNBr Sepharose beads and incubated with recombinant ubiquitin. Differential retention of ubiquitin by these mutants was detected by Western blotting with anti-ubiquitin antibodies. The lower part of the Figure was overexposed with the aim of detecting any traces of ubiquitin binding to the ΔI or ΔN24ΔI mutants. (Panel D) Titration of FITC-labeled Cks1pep and Hs1pep peptides with ubiquitin by fluorescence spectroscopy. (Panel E) His-tagged ubiquitin immobilized on Chelating Sepharose incubated with equal amounts of GST-tagged Cks1, ΔI, ΔIA (SDYFNSEV deleted), or ΔIB (GTLRILT deleted). Retention of these mutants by ubiquitin was probed by Western blotting with anti-Cks1 antibodies.

DISCUSSION

Cks proteins entertain a very close relationship with components of the ubiquitin/proteasome system. Human CksHs1 has been shown to interact with the E3 ubiquitin ligase component Skp2 (*36*, *37*, *54*, *55*), and *S. pombe* p13suc1 was found to bind activated APC/cyclosome (*56*). We have shown that budding yeast Cks1p recruits proteasomal subunits to transcriptional loci to promote transcriptional activation (*39*). As such, given the implication of both the UPS and Cks1p in gene transcription (*39*, *42*−*47*), we asked whether ubiquitin might be involved in a physical interaction with Cks1p. In this study, we have demonstrated that ubiquitin indeed interacts with Cks1p *in vitro*, revealing that by definition, Cks1p harbors a ubiquitin-binding domain. However, the affinity of this interaction determined by fluorescence titration experiments is several orders of magnitude greater than that of the UBDs characterized to date (*6*−*8*, *23*−*26*), inferring that Cks1p harbors a novel type of UBD that does not bind ubiquitin in a conventional fashion. In agreement with this statement, we have shown that Cks1p interacts with the α-helix of ubiquitin, in contrast to conventional UBDs that bind ubiquitin through their I44-centered hydrophobic pocket (*23*−*26*). Moreover, we have provided evidence that the α-helix of ubiquitin is both sufficient and necessary for this interaction.

Table 2: Dissociation Constants Determined for the Interactions between Ubiquitin and Cks1p Variants[a]

fluorescent substrate	ligand	*K*d (μM)
ubiquitin-FITC	Cks1p	0.099 ± 0.008
ubiquitin-FITC	p13Suc1	0.092 ± 0.028
ubiquitin-FITC	CksHs1	no binding
ubiquitin-FITC	CksHs2	no binding
Cks1p-FITC	ubiquitin	0.091 ± 0.021
Cks1p-ΔNter24-FITC	ubiquitin	0.152 ± 0.023
Cks1p-ΔI−FITC	ubiquitin	no binding
Cks1pep-FITC	ubiquitin	0.174 ± 0.04
HS1pep-FITC	ubiquitin	no binding

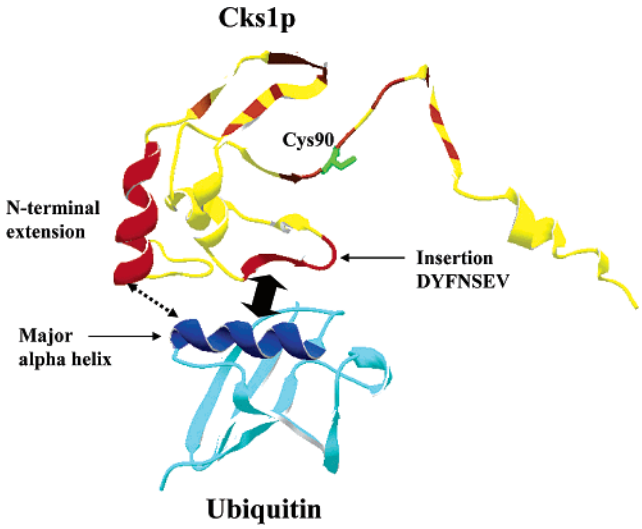

FIGURE 5: Model of the ubiquitin-Cks1p interaction. Ribbon diagram of Cks1p (yellow and red) and ubiquitin (light and dark blue) structures. The coordinates of the Cks1p crystal structure (1QB3) (*50*) and of the *S. cerevisiae* ubiquitin NMR structure (1WR1) (*17*) were used to generate the Figure using the SwissPdb Viewer software, version 3.7 (*51*, http://www.expasy.org/spdbv/). Cks1p Cys90, which was labeled in this study, is shown in green. The residues involved in the cdk-binding site of Cks1p are in brown. The N-terminal extension and insertion residues DYFNSEV (in red) of Cks1p are positioned pointing toward the major α-helix (dark blue) of ubiquitin to illustrate how they are likely to interact.

The α-helix of ubiquitin sticks out at the surface of ubiquitin, much like the handle of a mug, and this accessibility makes it a very good candidate for protein/protein interactions. Moreover, the sequence of ubiquitin is extremely conserved across species overall with only three amino acid substitutions from human to yeast, two of which are essentially conserved substitutions within the major α-helix of ubiquitin (E24 to D and A28 to S) and the third immediately preceding the helix itself (S19 to P in human ubiquitin), which may introduce a kink further exposing the helix for interactions (Figure 2D). The finding that this α-helix is implicated in protein-partner binding sheds new light on the mechanisms of ubiquitin recognition. Indeed, together with other recent studies that have identified UBDs making surface contacts with residues other than those involved in ubiquitin's notorious I44-centered pocket (*23*−*26*), our work suggests there are more modes of ubiquitin binding to partners than were previously thought to exist. Interestingly, a recent report has mapped a common Sumo-interacting motif (SIM) on Sumo-1 and Sumo-2, which involves two residues in the α-helix (*57*). This report further hints at a function of the α-helix of ubiquitin and UBLs in interactions with partners.

The specificity of Cks1p for ubiquitin versus Smt3p is easily understood considering that their surface charge distribution is completely different and when comparing the biochemical properties of their helices. The isoelectric point of *S. cerevisiae* ubiquitin's α-helix, which spans residues 23−34 (IDNVKSKIQDKE) is 6.27, and the conserved substitutions between yeast, plant, and human ubiquitin within this helix do not significantly modify its biochemical properties. In contrast, the isoelectric point of Smt3p's major α-helix, which spans residues 46−55 (RRLMEAFAKR) is 11.71. Conversely, we have shown that the specificity of ubiquitin for Cks1p and p13suc1 is primarily mediated by a conserved insertion between α-helices α1 and α2: DYFNSEVGTL, amino acids 57−66 in Cks1p. More specifically, we have found that the DYFNSEV moiety of the insertion loop is required for the interaction between Cks1p and ubiquitin and that several residues cooperate in this interaction but that charged residues are unlikely to be involved. The insertion is a short sequence, which loops out from the Cks1p protein, and is, thus, accessible to interact with the α-helix of ubiquitin. Alignment of the two yeast insertions reveals that they are almost identical with only two substitutions: Cks1p V63 is replaced by T in p13suc1 and S61 by P. The latter substitution is likely to introduce a kink in p13suc1, which may further help orient residues immediately preceding it (DYFN) for their interaction with ubiquitin. The N-terminal extension of Cks1p contributes to ubiquitin binding but is insufficient, in the absence of the insertion loop, to sustain a stable interaction between Cks1p and ubiquitin. Further structural characterization by NMR or X-ray crystallography should provide a more complete understanding of the specific residues involved in the Cks1p/ubiquitin interface.

The systematic mutation of all of ubiquitin's residues (*27*) previously revealed that only a specific subset of residues was essential for viability, most notably Phe4 and Ile44, and these do not include any residues from the α-helix of ubiquitin (except for perhaps K27A, which confers a temperature-sensitive phenotype at 37 °C). However, it should be noted that only yeast viability and endocytosis were assayed in the study of the ubiquitin mutants (*27*). Given the function of Cks1p in transcriptional regulation, it seems reasonable to surmise that certain residues in the α-helix of ubiquitin may be required for certain aspects of gene transcription. Indeed, histone monoubiquitylation and deubiquitylation processes are a *sine qua none* for transcriptional activation and have been inferred to regulate chromatin structure and dynamics, similar to acetylation, methylation, and phosphorylation (*44*, *45*). In a recent report concerned with histone ubiquitinylation, a model of ubiquitinylated histone H2A was proposed, in which the ubiquitin moiety is located at the surface of the nucleosome with its α-helix clearly exposed toward the solvent and accessible for protein/protein interactions (*58*). This structural model reconciles the Cks1p/ubiquitin interaction with the function of Cks1p in gene transcription, allowing us to speculate that recruitment of Cks1p to chromatin may be dependent on histone ubiquitinylation. As such, Cks1p might play a role in protecting histones from deubiquitinylation upon activation of gene transcription. Alternatively, it might interact with ubiquitinylated transcription factors bound to chromatin prior to or concomitant to transcription complex assembly. Moreover, because Cks1p recruits proteasomal subunits to actively transcribed genes, we cannot exclude that its binding to a ubiquitinylated chromatin-associated partner might protect it from polyubiquitinylation and subsequent proteasome-mediated degradation.

An unanswered query in the biology of ubiquitin interactions is how ubiquitin dissociates from conventional UBDs. It has been suggested that a UBD itself could somehow be modified and that this would create an energetic, electrostatic, or conformational change responsible for dissociation. Alternatively, the interaction of a protein-like Cks1p with the opposite face of the ubiquitin molecule, the handle of the mug, could introduce an allosteric change that would liberate the hydrophobic pocket of ubiquitin from a third parner. Cooperation and competition between different partners that bind different faces/patches of ubiquitin as well as insights into the combinatorial assembly of different ubiquitin-binding domains are clearly important issues to be addressed in the future. Likewise, the regions within Cks1p primarily involved in the interaction with ubiquitin are distinct from the anion-binding pocket and the surfaces that mediate interactions with cdks and with Skp2. As such, Cks1p may be able to recognize and bring together ubiquitinylated partners, a phosphorylated transcription factor, and a cyclin-dependent kinase in one complex. In the context of transcriptional regulation, Cks1p might facilitate the interaction between multiple partners, in line with its general adapter function, or simply interact cooperatively with a single factor that is both monoubiquitinylated and phosphorylated. Moreover, the binding of Cks1p to a ubiquitinylated partner may promote the formation of a larger macromolecular complex, together with a UBD-containing protein that binds the hydrophobic pocket on the opposite face of the ubiquitin moiety. Our finding that Cks1p, the UBA of Dsk2p or Rad23p, and ubiquitin indeed form a ternary complex suggests that there is no steric hindrance precluding the assembly of such a macromolecular complex.

In conclusion, not only is this the first report of a direct physical interaction between Cks1p and ubiquitin, it is also

the first time the α-helix of ubiquitin has been implicated in a protein/protein interaction, thereby inferring novel functions for Cks proteins and providing new insights into the mechanisms of ubiquitin recognition. This finding offers exciting perspectives for the future, and work is in progress to identify proteins other than Cks1p that interact with the major α-helix of ubiquitin as well as ubiquitinated proteins that interact with Cks1p. Moreover, the finding that *S. cerevisiae* Cks1p but not the human orthologs binds ubiquitin suggests that this interaction is related to functions that are unique to Cks1p, more specifically transcriptional regulation. Future efforts will aim to address how the Cks1p−ubiquitin interaction affects Cks1p function and whether it contributes to transcriptional activation *in vivo*.

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