Characterization of the cullin and F-box protein partner Skp1

Raymond W.M. Ng, Talha Arooz, Cain H. Yam, Iris W.Y. Chan, Anita W.S. Lau, Randy Y.C. Poon*

Department of Biochemistry, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong, China

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Abstract Skp1 interacts with cullins, F-box containing proteins, and forms a complex with cyclin A-Cdk2 in mammalian cells. Skp1 is also involved in diverse biological processes like degradation of key cell cycle regulators, glucose sensing, and kinetochore function. However, little is known about the structure and exact function of Skp1. Here we characterized the interaction between Skp1 and the F-box protein Skp2. We show that Skp1 can bind to Skp2 in vitro using recombinant proteins, and in vivo using the yeast two-hybrid system. Deletion analysis of Skp1 indicated that most of the Skp1 protein is required for binding to Skp2. In mammalian cell extracts, a large portion of Skp1 appears to associate with proteins other than Skp2. Biochemical analysis indicated that Skp1 is likely to be a flexible, non-spherical protein, and is capable of forming dimers.

Key words: Cell cycle; Cyclin; Cyclin-dependent kinase; Degradation; Ubiquitination

1. Introduction

The cell cycle is driven by a family of protein kinases called cyclin-dependent kinases (CDKs) [1]. The activity of CDK is tightly regulated by an intricate system of protein-protein interaction and phosphorylation [2]. Association with a cyclin subunit is required for CDK activation. CDKs are activated by phosphorylation of Thr^{161} and inhibited by phosphorylation of Thr^{14} and Tyr^{15}. Furthermore, the activity of CDKs is negatively regulated by binding to protein inhibitors of the $p21^{Cip1/WAF1}$ and $p16^{INK4A}$ family. In addition, the activities of some CDKs are also regulated by binding to folding factors [3]. Many of these CDK regulators are known to be regulated by degradation via the ubiquitin-proteasome system [4].

Comparison of the composition of the proteins that associate with cyclin-CDK complexes in normal and cancer cells reveals proteins that are important for the deregulation of cyclin-CDK in cancer cells [5]. Examples of these CDK regulators are the CDK inhibitors $p21^{Cip1/WAF1}$ and $p16^{INK4A}$, which are found in cyclin-CDK complexes in normal, but not in many transformed cells. Lack of these CDK inhibitors in transformed cells may contribute to the loss of normal inhibition of CDK activity after DNA damage and increase genomic instability. In normal human fibroblasts, cyclin A-Cdk2 exists in a quaternary complex that contains $p21^{Cip1/WAF1}$ and PCNA (proliferating cell nuclear antigen) [6]. But in many transformed cells, $p21^{Cip1/WAF1}$ and PCNA disappear from the cyclin A-Cdk2 complexes, and instead a substantial fraction of cyclin A-Cdk2 complexes are associated with a 19-kDa protein and a 45-kDa protein [5]. The 19-kDa and 45-kDa proteins were subsequently purified, cloned, and were renamed Skp1 and Skp2, respectively [7].

The mRNA level of Skp2 is most abundant during the S phase [7]. Furthermore, microinjection of anti-Skp2 antibodies or Skp2 antisense oligonucleotides into normal human fibroblasts or HeLa cells inhibits entry into S phase (but not S phase progression) [7]. These data suggest that Skp2 is an important regulator of the S phase. Interestingly, the level of Skp2 is higher in transformed cells than in normal cells, which may explain the increased abundance of Skp2 binding to cyclin A-Cdk2 in transformed cells. Consistent with this, the *SKP2* gene has been mapped to chromosome position 5p13, the *SKP1* gene to 7q11.2 [8], and close homolog or a pseudogene of *SKP1* (*SKP1B*) was mapped to 12p12 [8]. All three of these loci are associated with karyotypic alterations, known amplifications, or suspected tumor suppressor genes. The increased abundance of Skp2-containing cyclin A-Cdk2 complex in tumor cells could imply a partial deregulation of entry into S phase, and may contribute to the process of cellular transformation. The Skp1 found in cyclin A-Cdk2 complexes does not bind cyclin A-Cdk2 directly, but indirectly through Skp2 [7,9]. Skp1 binds to Skp2 via a novel structural motif in Skp2 called the F-box [9], which is found in a large number of diverse proteins including cyclin F and Cdc4p.

Identification of Skp1 homologs from *C. elegans*, *A. thaliana*, and yeast indicates that Skp1 is evolutionarily highly conserved and may play an important role in the cell [10]. This is reinforced by the findings that Skp1 is involved in diverse functions, including roles in ubiquitin-dependent protein degradation of cell cycle control and glucose sensing proteins, kinetochore function, and tumorigenesis.

Skp1 was identified as a subunit of CBF3, a multiprotein complex that binds centromere DNA in vitro [10]. Skp1 therefore represents an intrinsic kinetochore protein conserved throughout eukaryotic evolution, and may be directly involved in linking kinetochore function with the cell cycle.

Skp1 was also identified as a suppressor of cdc4 mutants and as a cyclin F-binding protein [9]. In *S. cerevisiae*, mutants of cdc4 and cdc34 are phenotypically indistinguishable, and the genes are critical for S phase progression [11]. Cdc4p, acting in concert with Cdc34p and Cdc53p, is involved in the ubiquitin-dependent degradation of multiple cell cycle regulators, including Cln2p, Cln3p, Far1p, and Sic1p [12–14]. Cyclin F was isolated as a suppressor of the G1/S deficiency of a cdc4 mutant [15], suggesting cyclin F may also be involved in the destruction of other cell cycle regulators. The

*Corresponding author. Fax: (852) (2358) 1552.
E-mail: bcrandy@usthk.ust.hk

Abbreviations: BSA, bovine serum albumin; CDK, cyclin-dependent kinase; GSH, glutathione; GST, glutathione-*S*-transferase; H6, hexahistidine; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; TCA, trichloroacetic acid

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fact that Skp1 is associated with cyclin F and Cdc4p suggests that Skp1 may also be involved in the ubiquitin-dependent destruction of cell cycle regulators. Indeed Skp1 was found to be required for ubiquitin-mediated proteolysis of Cln2p, Clb5p, and Sic1p [9]. Skp1p, Cdc4p, and Cdc53p assemble into an ubiquitin ligase complex named SCF^{Cdc4p}, that can reconstitute ubiquitination of phosphorylated Sic1p in the presence of E1 enzyme, the E2 enzyme Cdc34p, and ubiquitin [16–18]. Cdc53p is homologous to the cullins family of proteins, which are involved in cell cycle exit in *C. elegans* [19] and degradation of cell cycle regulators [19]. It is believed that various SCF complexes exist in the cell, with Cdc53 (cullins) and Skp1 associating with different F-box-containing proteins [9,20,21]. Different cellular processes are regulated by SCF complexes containing different F-box proteins: Cdc4p for the degradation of Sic1p, Skp2 for the regulation of cyclin A, Grr1p for degradation of G1 cyclin Cln2 and glucose induction of HXT expression [21], and Met30p for methionine biosynthesis [22]. The nature of the interactions between Skp1 and F-box proteins will shed light on the specific degradation as a regulatory mechanism. Towards this goal, here we describe the biochemical analysis of Skp1 and the investigation of the interaction between Skp1 and Skp2.

2. Materials and methods

2.1. Constructs

Human Skp1 was amplified from a human cDNA library (from peripheral lymphocytes transformed with EBV, a gift from K. Yamashita, Kanazawa University, Japan) by PCR with primers 5′-CACCATGGCTTCAATTAAGTTGCA-3′ (Skp1 FOR) and 5′-ACCTCGAGCTTCTCTTCACACCACTGGTT-3′; the PCR product was cut with *Nco*I-*Xho*I and put into pGEX-KG (Pharmacia, Uppsala, Sweden) (GST-Skp1 in pGEX-KG) and pET21d (Novagen, Madison, WI, USA) (Skp1-H6 in pET21d). The second residue of Skp1 was changed from a Pro to an Ala due to the subcloning. C-terminal truncation mutants of Skp1 were constructed by PCR using 5′-GACCCAATGTGCCTGGATGCG-3′ and 5′-TTGCTCGAGGAACTGGCTAATCATCTC-3′ (CΔ41), 5′-GGCTCGAGCTAGTGGTGGGTGCACCA-3′ (CΔ65), or 5′-ACCTCGAGCTAACCTTTGATGTCTA-3′ (CΔ114); the PCR products were cut with *Nco*I-*Xho*I, and ligated into pGEX-KG. N-terminal truncation mutants of Skp1 were constructed by PCR using 5′-CATCACCGAAACGCGCGAGGC-3′ and 5′-GGCCATGGATGATGAAGGAGATG-3′ (NΔ36), 5′-CCCCATGGATGACCCTCCTCCTCCTG-3′ (NΔ67), or 5′-CACCATGGGTTTGCTTGATGTTACAT-3′ (NΔ114); the PCR products were cut with *Nco*I-*Xho*I, and ligated into pGEX-KG. The sequences of the constructs were confirmed by double-stranded DNA sequencing. Human Skp2 clone (Skp2 in pBluescript SK^-) was a gift from David Beach (Cold Spring Harbor Laboratory) [7]. Skp1 in pACT2 was constructed by PCR with primers Skp1 FOR and 5′-TTTCCATGGTCATCACCGAAACGCGCGAG-3′, cleaved with *Nco*I and ligated into pACT2. To construct Skp2 in pAS2, Skp2 was amplified by PCR from Skp2 in pBluescript SK^- with primers 5′-TTCCATGGACGTATTTAAAACTCCCGGGC-3′ and 5′-ATCTCGAGTAGACAACTGGGCTTTTGCAGTGT-3′, cut with *Nco*I and *Xho*I, and ligated into pAS2. GST-Skp2 in pGEX-KG was constructed by ligation of the same PCR fragment into *Nco*I and *Xho*I cut pGEX-KG.

2.2. Expression and purification of recombinant proteins

Expression of GST-tagged and histidine-tagged proteins in bacteria and purification with GSH-agarose and nickel-NTA agarose chromatography, respectively, were as described [23]. Protein concentrations were estimated by SDS-PAGE gel and Coomassie blue staining using BSA as standard. Thrombin digestion of GST-fusion proteins and inactivation of thrombin were as described [24]. Coupled transcription-translation reactions in the presence of [^{35}S]methionine in rabbit reticulocyte lysate were performed according to the manufacturer's instructions (Promega, Madison, WI, USA).

2.3. Cell extracts

HeLa cells (human cervical carcinoma cells) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) calf serum in a humidified incubator at 37°C, in 5% CO_2. Cell-free extracts were prepared as described [23]. The protein concentration of cell lysates was measured with bicinchoninic acid protein assay system (Pierce, Rockford, IL, USA) using BSA as a standard.

2.4. Circular dichroism spectroscopy

Equilibrium circular dichroism (CD) spectra were measured on a Jasco J-720 spectropolarimeter, using a 0.1-mm path length quartz cuvette at 20°C. Samples were dissolved in buffer as specified, and protein concentrations were determined by SDS-PAGE gel using BSA as a standard. Secondary structure analysis was performed using the Secondary Structure Estimation 38 program according to the manufacturer, using the reference CD spectra as described [25].

2.5. Nano-differential scanning calorimetry

Calorimetry was performed using a Nano-Differential Scanning Calorimeter (NDSC) from Calorimetry Sciences Corporation (CSC). Purified Skp1-H6 was dissolved in buffer A to a concentration of approximately 0.5 mg/ml (estimated by SDS-PAGE gel using BSA as a standard), and was degassed prior to the run. A heating scan from 0°C to 100°C at a scan rate of 1°C/s was used, followed by a cooling scan from 100°C to 0°C at the same rate. Analysis was performed using DSC analysis software from Microcal Corp., to calculate the molar enthalpy and transition temperatures of the folding and refolding reactions after subtraction of the baseline.

2.6. Gel filtration chromatography

Samples (500 μl) were applied onto a Superose 12 HR10/30 column (Pharmacia) and subjected to FPLC chromatography with buffer A (20 mM Tris-Cl, pH 7.5, 100 mM NaCl, 1 mM EDTA) with a flow rate of 0.4 ml/min. Fractions were collected at 1 fraction/min. The fractions were lyophilized in a Speedvac and dissolved in 100 μl of SDS-sample buffer. Molecular size standards for the gel filtration column were: BSA (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (28.8 kDa), RNaseA (13.7 kDa). Stokes' radii of the standards used were BSA (35.5 Å), ovalbumin (30.5 Å), carbonic anhydrase (24.3 Å), RNaseA (16.4 Å). The Stokes' radius of Skp1 was estimated by applying the Porath and the Laurent and Killander relationships [26]. Around 20 μg of each standard protein or Skp1-H6 was loaded. For cell extracts, 200 μg of extracts were applied onto the column and fractionated with the conditions as described above.

2.7. Glycerol gradient centrifugation

Isokinetic 5–25% glycerol gradients (in buffer A) were poured with a two chamber gradient maker connected to a peristaltic pump (Gilson Miniplus3, 0.4 ml/min). Gradients (9 ml) were poured by displacement method with a glass capillary tube at the bottom of the centrifugation tube (open ended PP ultracentrifuge tube of 11 ml capacity). The glass tube was carefully removed vertically afterwards without disturbing the gradient. About 50 μg of each standard and Skp1-H6 in 200 μl were loaded onto the gradients. Alternatively, standards and 150 μl of 1 mg/ml cell extracts were loaded onto the gradients. Centrifugation was performed in Sorvall Combiplus Ultracentrifuge with a Sorvall TH641 swinging bucket rotor at 40 000 rpm for 23 h at 4°C. Fractions were collected at 0.4 ml/fraction from the bottom of the tube using a fine capillary tube. The fractions were TCA-precipitated, and dissolved in 150 μl SDS-sample buffer (for Skp1-H6) or 100 μl SDS-sample buffer (for cell extracts). The sedimentation coefficient of the sample protein was estimated by comparison with the standards [27]. The sedimentation coefficients of the standards used were: BSA (4.31×10^{-13} s), ovalbumin (3.55×10^{-13} s), carbonic anhydrase (3.23×10^{-13} s), RNaseA (1.78×10^{-13} s). From the sedimentation data alone, the molecular weight can be approximated by:

$$\frac{s_1}{s_2} = \left(\frac{M_1}{M_2}\right)^{2/3}$$

where s is sedimentation coefficient and M is molecular size in kDa [27].

2.8. Combination of gel filtration chromatography and glycerol gradient centrifugation data

Calculation of true molecular size by combining the Stokes' radius

from gel filtration chromatography, and the sedimentation coefficient from glycerol gradient centrifugation was performed as described [28].

2.9. Binding assays

Bacterially expressed GST-fusion proteins (~100 ng) were incubated with rabbit reticulocyte lysate programmed to contain ^{35}S-labeled proteins (~5 μl) at 30°C for 30 min. GST-fusion proteins were then recovered with 15 μl GSH-agarose in 250 μl bead buffer (50 mM Tris-Cl, pH 7.4, 5 mM NaF, 250 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.1% Nonidet P-40, 2 μg/ml aprotinin, 15 μg/ml benzamidine, 1 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor). After incubation at 4°C with end-to-end rotation for 45 min, the beads were washed five times with 250 μl of bead buffer. The samples were then dissolved in 30 μl of SDS-sample buffer and the bound ^{35}S-labeled proteins were detected by SDS-PAGE followed by PhosphorImager analysis (Molecular Dynamics).

2.10. Cross-linking of proteins

Purified proteins (5 μl) were incubated with 2 μl of 750 mM sodium phosphate buffer (pH 7.0), 5 μl of 0.004% glutaraldehyde, and 8 μl of water at 23°C. At the indicated time points, 4-μl samples were taken and dissolved in 36 μl of SDS-sample buffer. Part of the samples (10 μl) was applied onto SDS-PAGE and stained with Coomassie blue stain.

2.11. Yeast two-hybrid interactions

Yeast two-hybrid interaction assays were performed as described [29]. Yeast strain Y190 was co-transformed with a combination of Skp1 in pACT2, Skp2 in pAS2, and the corresponding vectors as indicated. Colonies were grown on Leu-, Tyr-selection plates containing X-gal. Interacting clones produced a dark blue color and non-interacting clones remained white in color.

2.12. Antibodies and immunological methods

Goat anti-Skp1 antibodies were raised against a peptide corresponding to the C-terminal 20 amino acids of human Skp1 (sc-1568, Santa Cruz Biotechnology). Goat anti-Skp2 antibodies were raised against a peptide corresponding to the N-terminal 19 amino acids of human Skp2 (sc-1567, Santa Cruz Biotechnology). Rabbit antibodies against Cdk2 and E72 anti-cyclin A monoclonal antibody [23] were as previously described. Immunoblots were performed as described [30].

3. Results

3.1. Interaction between Skp1 and the F-box-containing protein Skp2

Skp1 and Skp2 were isolated as interactors of cyclin A-Cdk2 complexes from mammalian cells. Fig. 1 shows that our recombinant Skp1 and Skp2 can also interact in a yeast two-hybrid system. Reporter activity was detected when Skp1 and Skp2 were co-transformed, but not when Skp1 or Skp2 were transformed individually into yeasts. To see whether Skp1 and Skp2 can also interact in vitro, ^{35}S-labeled Skp2 translated in rabbit reticulocyte lysate was incubated with GST-Skp1 or GST, and the proteins then recovered with

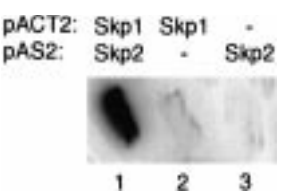

Fig. 1. Interaction between Skp1 and Skp2. Yeast cells were co-transformed with Skp1 in pACT2 and Skp2 in pAS2 (lane 1), Skp1 in pACT2 and pAS2 vector (lane 2), or pACT2 vector and Skp2 in pAS2 (lane 3). Colonies were put onto a selection plate containing X-gal. Interacting clones produced a dark blue color and non-interacting clones remained white in color (rendered to gray scale here).

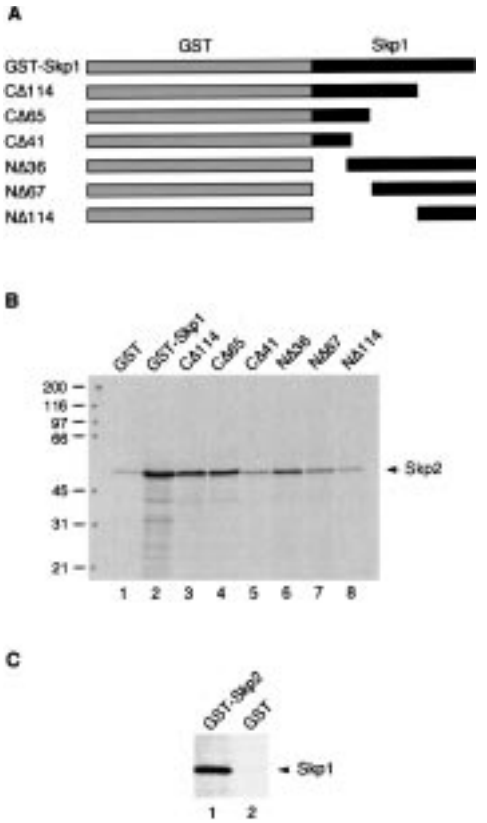

Fig. 2. Binding of deletion mutants of Skp1 to Skp2. A: Summary of GST-Skp1 deletion mutants. GST-Skp1 was deleted from the N-terminus or the C-terminus as described in Section 2. NΔ or CΔ denoted deletions from the N-terminus and C-terminus, respectively, and the numbers indicate the amino acid residues in Skp1 that the mutants were deleted to (full length Skp1 contains 163 amino acid residues). B: Binding of Skp2 to GST-Skp1 and deletion mutants. Skp2 translated in reticulocyte lysate in the presence of [^{35}S]methionine (5 μl) was incubated with 100 ng of GST (lane 1), GST-Skp1 (lane 2); C-terminal deletion mutants of GST-Skp1: CΔ114 (lane 3), CΔ65 (lane 4), CΔ41 (lane 5); or N-terminal deletion mutants of GST-Skp1: NΔ36 (lane 6), NΔ67 (lane 7), NΔ114 (lane 8) at 23°C for 30 min. The GST-fusion proteins were then recovered with GSH-agarose as described in Section 2. The samples were dissolved in SDS-sample buffer and associated Skp2 was detected by SDS-PAGE followed by analysis with a PhosphorImager. The positions of the molecular size markers (in kDa) are shown on the left. C: Binding of Skp1 to GST-Skp2. Skp1-H6 was translated in a coupled transcription-translation rabbit reticulocyte lysate system in the presence of [^{35}S]methionine. The lysates were incubated with bacterially expressed GST-Skp2 (lane 1) or GST (lane 2). GST-Skp2 and bound proteins were then precipitated with GSH-agarose, and the Skp1-H6 was detected by SDS-PAGE followed by analysis with a PhosphorImager.

GSH agarose. Fig. 2B shows that Skp2 was retained by GST-Skp1 but not by the GST control (lanes 1 and 2).

To have an idea of the region of Skp1 that is involved in binding to Skp2, we constructed deletion mutants of GST-Skp1 that were truncated from either the N-terminus or the C-terminus. The deletion mutants are summarized schematically in Fig. 2A. NΔ or CΔ denoted deletions from the N-terminus and C-terminus of Skp1, respectively, and the numbers indicate the amino acid residues in Skp1 that the mutants were deleted to. These GST-Skp1 deletion mutants were expressed in bacteria, and their ability to bind rabbit reticulocyte lysate translated Skp2 was compared. Fig. 2B shows that the smallest deletion of Skp1 from the N-terminus (NΔ36)

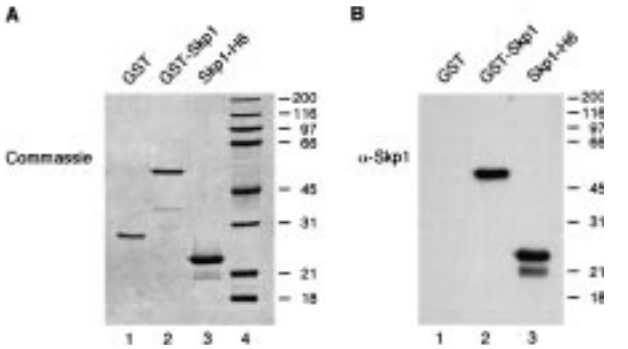

Fig. 3. Expression and purification of recombinant Skp1. A: GST (lane 1), GST-Skp1 (lane 2), and Skp1-H6 (lane 3) were expressed in bacteria and purified as described in Section 2. The purified proteins were applied onto SDS-PAGE and stained with Coomassie blue. Molecular size markers were loaded in lane 4. B: Purified GST (lane 1), GST-Skp1 (lane 2), and Skp1-H6 (lane 3) (~10 ng) were applied onto SDS-PAGE and immunoblotted with antibodies raised against the C-terminal 20 amino acids peptide of Skp1.

already disrupted most of its binding to Skp2 (15% of wild-type GST-Skp1 from quantitation of the PhosphorImager). Further deletions from the N-terminus (NΔ67 and NΔ114) reduced the binding to Skp2 close to background level. The ability of Skp1 to bind Skp2 could be tolerated slightly more by deletion of Skp1 from the C-terminus; CΔ114 and CΔ65 still retained more than 20% binding to Skp2 in comparison to wild-type GST-Skp1. Further deletion of Skp1 from the C-terminus (CΔ41) abolished all the binding to Skp2. Hence it is likely that most of the Skp1 is required for binding to Skp2, with the N-terminal region playing a slightly more prominent part.

To eliminate the possibility that Skp1 was just a very 'sticky' protein that bound non-specifically to any protein in in vitro assays, we performed the inverse experiment by translating Skp1 in rabbit reticulocyte lysate in the presence of [^{35}S]methionine, and assaying whether the Skp1 could bind to GST-Skp2. Fig. 2C shows that Skp1 translated in rabbit reticulocyte lysate was recruited by GST-Skp2 fusion protein, but not by GST alone. This indicates that Skp1 at least binds specifically to Skp2 and not to GST.

3.2. Expression and purification of recombinant Skp1

To further characterize the biochemical properties of Skp1, human Skp1 was subcloned into vectors that expressed C-terminal hexa-histidine-tagged Skp1 (Skp1-H6) and GST-fusion Skp1 (GST-Skp1), respectively, in bacteria. Skp1-H6 and GST-Skp1 were purified with nickel-NTA agarose chromatography and GSH-agarose chromatography, respectively. Fig. 3A shows the purity of the purified recombinant proteins by SDS-PAGE and Coomassie blue staining. The identity of the GST-Skp1 and Skp1-H6 were confirmed by immunoblotting with an antibody raised against the C-terminal peptide of Skp1 (Fig. 3B, lanes 2 and 3). In comparison, GST alone was not detected by the anti-Skp1 antibody (lane 1). In the Skp1-H6 preparation, the two minor faster migrating bands were unlikely to be degradation products of Skp1-H6 because they were also recognized by the anti-C-terminal peptide antibodies (Fig. 3B). These faster migrating bands are likely to be oxidative forms of Skp1-H6 (J. Endicott and R.Y.C.P., unpublished data).

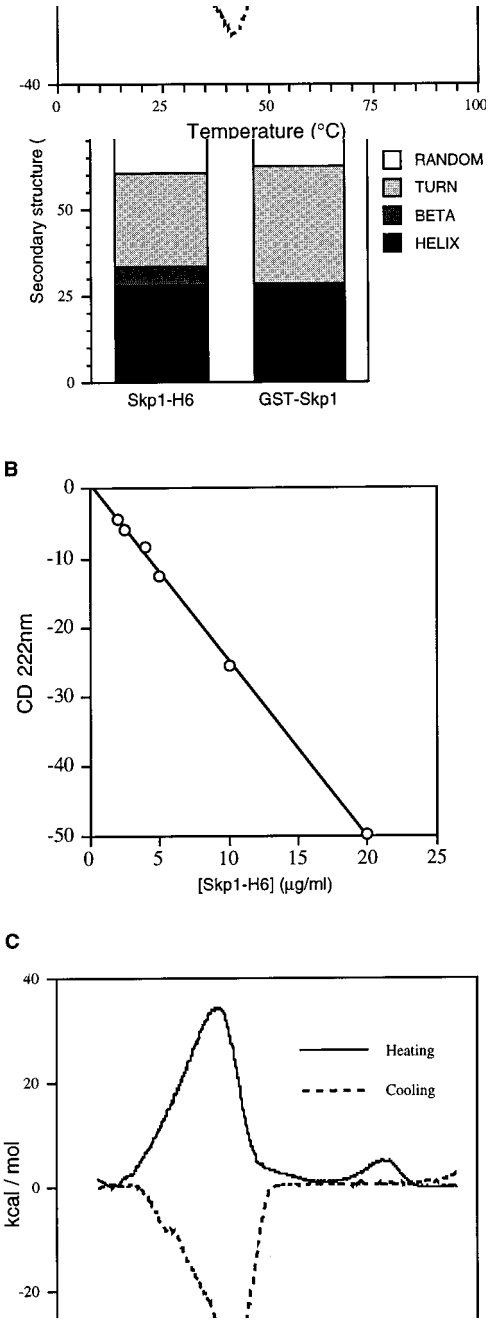

Fig. 4. Circular dichroism spectroscopy and nano-differential scanning calorimetry analysis of Skp1. A: Purified Skp1-H6 and GST-Skp1 (5 μg/ml) were analyzed by circular dichroism as described in Section 2. The percentages of the calculated α-helix, β-sheet, turn, and random coil structures of the proteins are shown. B: Different concentrations of purified Skp1-H6 (2.0, 2.5, 4.0, 5.0, 10.0, 20.0 μg/ml) were subjected to circular dichroism spectroscopy analysis. The measurement of helical structure (CD 222 nm) is plotted against the protein concentration. C: The thermal stability of Skp1-H6 was analyzed by nano-differential scanning calorimetry as described in Section 2. The energy per mol was measured in one heating scan (0–100°C) (upper solid line) followed by one cooling scan (100–0°C) (lower dotted line).

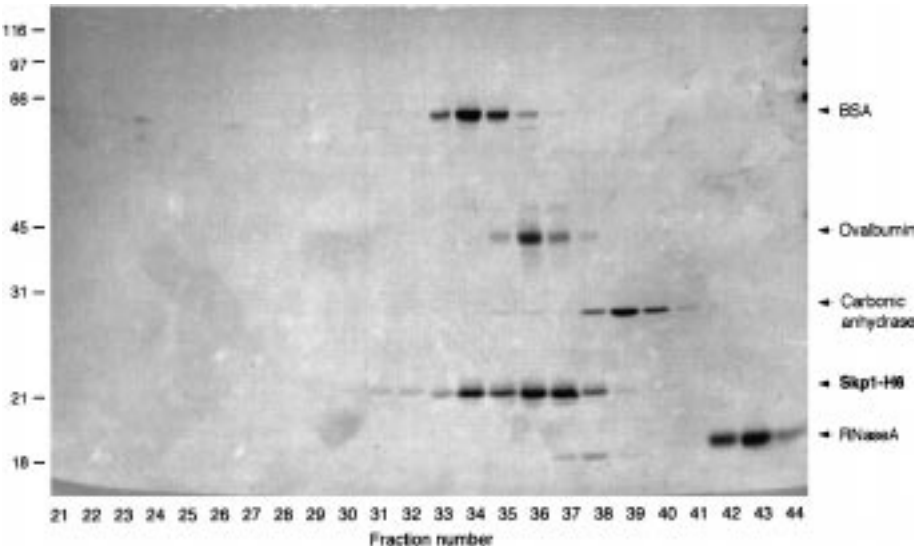

Fig. 5. Gel filtration chromatography of recombinant Skp1. A mixture of Skp1-H6 and molecular size marker proteins was fractionated by gel filtration chromatography as described in Section 2. The standard proteins used were BSA (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (28.8 kDa), RNaseA (13.7 kDa). The fractions were lyophilized, dissolved in SDS-sample buffer, and loaded onto a 17.5% SDS-PAGE gel. The proteins were visualized by Coomassie blue staining. The positions of the molecular size standards (in kDa) for the SDS-PAGE are shown on the left.

To see whether the recombinant Skp1 proteins have folded into any secondary structure, purified Skp1-H6 and GST-Skp1 were analyzed by circular dichroism spectroscopy. Fig. 4A shows the estimated percentage of secondary structures in Skp1-H6 and GST-Skp1. From these rough estimations, Skp1-H6 contains about 28% α-helix, 6% β-sheets, 26% turns, and 40% random coils. More importantly, the percentage of helical estimation in the Skp1-H6 did not change significantly by varying the concentration of Skp1-H6 (Fig. 4B). These results indicate that Skp1-H6 contained secondary structure, and the overall secondary structure did not change significantly at different concentrations of the protein. The concentration of recombinant Skp1 used for the rest of this study was in the range of 1–5 μg/ml.

To have an idea of the stability of Skp1-H6, nano-differential scanning calorimetry was used to analyze the endothermic and exothermic processes associated with purified Skp1-H6. Fig. 4C shows that Skp1-H6 appeared to start to denature at a fairly low temperature (~25°C), and peaked at ~40°C. The apparent denaturation of Skp1-H6 was not completed in a narrow temperature range, suggesting that there could be subdomains within Skp1-H6 that have different unfolding temperatures, or both intermolecular and intramolecular interactions existed for Skp1-H6. Interestingly, there was a minor peak of unfolding at around 75°C when heating was applied to Skp1-H6. After the heating scan, the Skp1-H6 sample was cooled at the same rate as the previous heating scan. The shape of the energy-temperature curve for cooling is nearly a mirror image of the heating curve. This suggests that most of the Skp1-H6 appeared to have refolded. However, the cooling curve is slightly shifted to a higher temperature, and the minor peak at ~75°C seen during denaturation was not observed during renaturation. These data show that the purified Skp1-H6 proteins may be thermal stable, which is in agreement with the observation that Skp1-H6 was not precipitated by boiling (unpublished data).

3.3. The native molecular size of Skp1

Given the possibility that bacterially expressed Skp1-H6 may not be a monomer, we next investigated the size of Skp1-H6 by size fractionation chromatography. Skp1-H6, together with several molecular size standards, was applied onto gel filtration chromatography; the fractions were analyzed by SDS-PAGE and Coomassie blue staining (Fig. 5). Significantly, we found that Skp1-H6 migrated at a size much larger than expected for a monomer (predicted size 20 kDa). The major peak of Skp1-H6 (fractions 36–37) appeared to have a size larger than carbonic anhydrase (28.8 kDa) and similar to ovalbumin (43 kDa). Skp1-H6 behaved as a fairly uniform population on gel filtration chromatography, without any Skp1-H6 migrating at the front of the column (suggesting that there was little protein aggregation). However, a minor peak of Skp1-H6 migrating at a slightly larger size (fraction 34) was reproducibly observed, which may represent Skp1-H6 dimers (see below). From these gel filtration chromatography data alone, Skp1-H6 seemed to have a size much larger (~43 kDa and ~66 kDa, respectively, for the two populations) than its predicted monomeric size.

To see whether Skp1-H6 were monomers or dimers, we measured the native molecular size of Skp1-H6 by combining the Stokes' radius (from gel filtration chromatography) and the sedimentation coefficient (from glycerol gradient centrifugation) according to the method of Siegel and Monty [28]. The Stokes' radius of Skp1-H6 was estimated by comparing with the standards from the gel filtration chromatography (Fig. 5) and applying the Porath and the Laurent and Killander relationships [26]. We estimated the Stokes' radius of the major peak of Skp1-H6 to be ~29.7 Å. To measure the sedimentation coefficient of Skp1-H6, Skp1-H6 and standard proteins were loaded onto 15–25% glycerol gradient and subjected to centrifugation. Fractions were collected after centrifugation and analyzed by SDS-PAGE and Coomassie blue staining. The fact that the standards were applied together

with Skp1-H6 onto the same gradients ensured that they served as internal standards irrespective of the variation of the gradient preparations (data not shown). The sedimentation coefficient of Skp1-H6 was estimated to be 1.97×10^{-13} s ($\pm0.02\times10^{-13}$ s). Unlike the gel filtration results, Skp1-H6 did not behave as a larger protein than its predicted size by gradient centrifugation. Using the sedimentation data alone, Skp1-H6 has an estimated size of $\sim$17 kDa.

When the data from the gel filtration chromatography and glycerol gradient centrifugation were combined, we calculated the size of Skp1-H6 to be about 24 ± 1 kDa. The minor population of Skp1-H6 with an apparent larger size on gel filtration chromatography was estimated to have a size of approximately 50 kDa (this is a rough approximation because the minor peak was not consistently well resolved by gradient centrifugation). These results indicate that Skp1-H6 was mainly a monomer, and it migrated anomalously on gel filtration chromatography. However, there is also a population that is of larger size, possibly representing the dimeric form of Skp1-H6.

Dimeric form of Skp1 was further demonstrated by covalently cross-linking of purified Skp1-H6 by glutaraldehyde (Fig. 6). Skp1-H6 was incubated with either buffer or the cross-linking agent glutaraldehyde. The reactions were stopped at different time points by mixing with SDS-sample buffer; the samples were then analyzed with SDS-PAGE and Coomassie blue staining. A portion of Skp1-H6 was seen to migrate at approximately double the size of monomeric Skp1-H6 after incubation with glutaraldehyde (lanes 4–7), but not after incubation with buffer alone (lanes 2–3). This larger peptide after cross-linking was also recognized by anti-Skp1 antibodies (data not shown).

3.4. *Skp1 associates with Skp2 as well as other proteins in mammalian cell extracts*

To see whether the size of the endogenous Skp1 in cell lysates was any different from that of recombinant Skp1, extracts from human cervical carcinoma HeLa cells were fractionated by gel filtration chromatography as before. The fractions were applied onto SDS-PAGE, transferred to

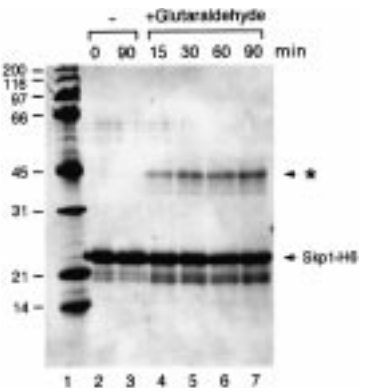

Fig. 6. Cross-linking of Skp1. Purified Skp1-H6 (about 2 μg per lane) was incubated with buffer (lanes 2-3) or with buffer containing glutaraldehyde (lanes 4–7). At the indicated time points, the reactions were terminated by addition of SDS-sample buffer and analyzed with SDS-PAGE followed by Coomassie blue staining. Molecular size markers are loaded in lane 1 (indicated in kDa on the left). The positions of the monomeric Skp1-H6 are indicated on the right and the position of the dimeric Skp1-H6 is indicated by the asterisk.

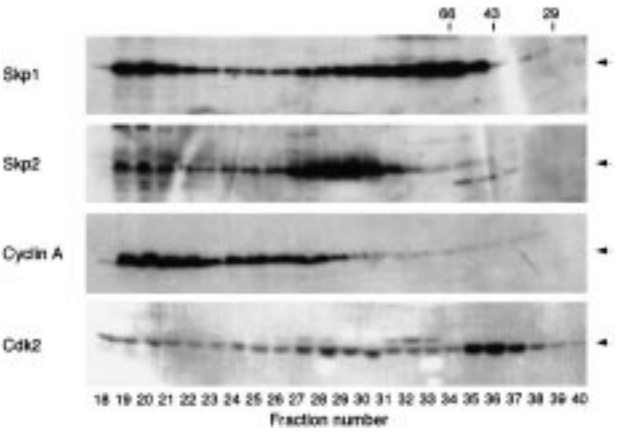

Fig. 7. Endogenous Skp1 in mammalian cell extracts. HeLa cell extracts were subjected to gel filtration chromatography as described in Section 2. The fractions were lyophilized, dissolved in SDS-sample buffer, and loaded onto a 17.5% SDS-PAGE gel. Proteins were transferred onto Immobilon membranes and immunoblotted with antibodies against Skp1 and Skp2 as indicated. Immunoblottings for cyclin A and Cdk2 are also shown for comparison.

membranes and immunoblotted with antibodies against Skp1 (Fig. 7). In comparison to recombinant Skp1, the endogenous Skp1 in the cell extracts was widely distributed on gel filtration chromatography. This reflects the fact that Skp1 was associated with other proteins in the cell, including some in very large complexes. When we immunoblotted the same column fractions with antibodies against Skp2, Skp2 was found to be restricted to a single major peak (and some in very large complexes). Although some portion of Skp1 co-migrated with Skp2 on gel filtration chromatography, the majority of Skp1 did not, indicating that only a portion of Skp1 may be complexed to Skp2 in the cell.

4. Discussion

Skp1 is a small protein that binds to kinetochore components, as well as to cullins and F-box proteins for the ubiquitination-dependent degradation of other proteins. Understanding the biochemical properties of Skp1 is important for understanding how Skp1 functions in these processes. Here we describe the production of GST- and histidine-fusion proteins of human Skp1 from bacteria. Circular dichroism spectroscopy of recombinant Skp1 revealed that it contains secondary structures, and the secondary structures were independent of Skp1 concentration. Nano-differential scanning calorimetry revealed that Skp1 denatured at low temperature, but apparently could refold when the temperature was lowered. The low denaturation temperature of Skp1 could reflect the ability to undergo large conformational changes after binding to other proteins.

On gel filtration chromatography, the main population of Skp1-H6 co-migrated with the 43-kDa ovalbumin. But these Skp1-H6 were likely to represent monomeric Skp1-H6 (predicted size 20 kDa), which we think migrated anomalously on gel filtration columns. A second peak of Skp1-H6 on gel filtration columns was likely to represent dimers. Combination of gel filtration chromatography and gradient centrifugation analysis indicated that the size of the main population of Skp1-H6 is around 24 kDa, and that of the minor population is around 50 kDa. The existence of Skp1 dimers was sup-

ported by the fact that after treatment of Skp1-H6 with a cross-linking agent, some Skp1-H6 can be detected to migrate at approximately double the size of Skp1-H6 on denatured SDS-PAGE. All these biophysical data suggest that Skp1-H6 could be a fairly flexible, non-spherical protein, and is capable of forming dimers.

We found that Skp1 could interact with Skp2 in vitro using bacterially expressed and rabbit reticulocyte expressed proteins, and in vivo using the yeast two-hybrid system. Skp1 also binds to Skp2 in mammalian cell extracts [7]. But based on the gel filtration chromatography analysis of cell extracts (Fig. 7), a substantial portion of Skp1 appeared to associate with proteins other than Skp2 in mammalian cells. These proteins probably include other F-box proteins like cyclin F, and the E3 ubiquitin ligase component cullin, the kinetochore protein complex, as well as other yet unidentified partners of Skp1. We are currently characterizing potential Skp1-interacting clones identified by cDNA library screening using the yeast two-hybrid system (unpublished data).

Deletion analysis of Skp1 suggests that most of Skp1 is necessary for binding to Skp2, but the N-terminal region seems to be more important than the C-terminal region. Interesting, cullin (CUL1) was reported to bind to the N-terminal half of Skp1 [20]. It would also be interesting to investigate which part of Skp1 is involved in binding to the kinetochore protein complex. We found that similar regions in Skp1 were involved in binding to Skp2 and cyclin F (unpublished data). Skp1 is a relatively small protein, and it is possible that it can tolerate only a limited amount of deletion before the ability to bind other proteins is lost. Like all deletion analyses, it is important to note that truncation of part of Skp1 may have completely disrupted the whole structure of the remaining protein. In comparison to Skp1, the region of Skp2 that binds Skp1 is more well defined, partly because Skp2 is a larger protein than Skp1. The F-box region of Skp2 is sufficient to bind to Skp1, and mutations of conserved residues in the F-box in Cdc4p [18] and Skp2 (unpublished data) abolished their binding to Skp1.

The structure of Skp1 would be very revealing for the understanding of the functions of Skp1 in different biological processes. As shown here, the yield and purity of bacterially expressed Skp1-H6 after one column purification were sufficiently high for us to pursue further crystal structure analysis (J. Endicott and R.Y.C.P., unpublished data). We showed that Skp1-H6 contained secondary structures (by circular dichroism spectroscopy), and the majority of Skp1-H6 behaved as a uniform population on gel filtration chromatography. The population of Skp1-H6 that dimerized and the small population that was oxidized may present problems for structural studies. Furthermore, the low temperature of denaturation of Skp1-H6 may also render the crystallization process more demanding. We found that Skp1-H6 translated in rabbit reticulocyte lysate was able to bind to bacterially expressed GST-Skp2, suggesting the recombinant Skp1-H6 is functional in terms of binding to Skp2. Based on the truncation analysis described here, it is probably not useful to study the structure of a truncated version of Skp1, because the binding to its physiological partners is also compromised. Eventually, the structures of Skp1 in a complex with F-box proteins and cullins will provide more understanding on how these proteins target substrates for ubiquitination.

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