Protein–protein interactions involved in the recognition of p27 by E3 ubiquitin ligase

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The $p27^{Kip1}$ protein is a potent cyclin-dependent kinase inhibitor, the level of which is decreased in many common human cancers as a result of enhanced ubiquitin-dependent degradation. The multiprotein complex SCF^{Skp2} has been identified as the ubiquitin ligase that targets p27, but the functional interactions within this complex are not well understood. One component, the F-box protein Skp2, binds p27 when the latter is phosphorylated on Thr^{187}, thus providing substrate specificity for the ligase. Recently, we and others have shown that the small cell cycle regulatory protein Cks1 plays a critical role in p27 ubiquitination by increasing the binding affinity of Skp2 for p27. Here we report the development of a homogeneous time-resolved fluorescence assay that allows the quantification of the molecular interactions between human recombinant Skp2, Cks1 and a p27-derived peptide phosphorylated on Thr^{187}. Using this assay, we have determined the dissociation constant of the Skp2–Cks1 complex (K_d 140 ± 14 nM) and have shown that Skp2 binds phosphorylated p27 peptide with high affinity only in the presence of Cks1 (K_d 37 ± 2 nM). Cks1 does not bind directly to the p27 phosphopeptide or to Skp1, which confirms its suggested role as an allosteric effector of Skp2.

Key words: cancer, Cks1, p27, proteasome, Skp2, ubiquitin.

INTRODUCTION

The progression of eukaryotic cells through the cell cycle is tightly controlled by the temporal activation of cyclin-dependent kinases (CDKs) and the accumulation and turnover of their inhibitors [1]. $p27^{Kip1}$ is a natural inhibitor of the G1 cyclin–CDK complexes that functions as a negative regulator of the G1→S phase transition [2,3]. As such, it is frequently targeted for inactivation through enhanced ubiquitin-dependent proteolysis in many common human malignancies [4–6]. p27 is primed for degradation by phosphorylation on Thr^{187} by CDK2–cyclin E, which is followed by ubiquitination [7–10]. Several recent studies have established SCF^{Skp2} as the ubiquitin ligase (or E3) specific for p27 [11–13]. SCF^{Skp2} is a multiprotein complex that consists of Skp1, Cul1, Rbx1 and the F-box protein Skp2 [14,15]. The specificity of SCF^{Skp2} for phosphorylated p27 depends on the ability of Skp2 to bind p27 only when the latter is phosphorylated specifically on Thr^{187} [11,13]. The role of Skp2 as the p27-recognition component of SCF^{Skp2} is supported by the observations that addition of recombinant Skp2 to Skp2-depleted cell extracts restores the *in vitro* ubiquitination of phoshorylated p27 [13], and that cells from $Skp2^{-/-}$ mice accumulate phoshorylated p27 [16]. Although SCF^{Skp2} reconstituted from purified recombinant components is capable of carrying out the ubiquitination of p27 *in vitro*, the reaction is extremely inefficient [11]. However, addition of the small cell cycle regulatory protein Cks1 renders the ubiquitination of p27 by this reconstituted system highly efficient, indicating that Cks1 is an essential activator of SCF^{Skp2} [17,18].

Cks1 belongs to a class of essential cell cycle regulatory proteins (Cks1/Suc1) identified initially through their function as genetic suppressors of defective CDK genes in yeast [19,20]. Human Cks1 and its close orthologue Cks2 are both capable of binding CDK–cyclin complexes, but the functional significance of this binding in the regulation of the cell cycle is still not clear [21,22]. Using two different experimental approaches, we and others have shown that Cks1 plays an important role in the regulation of ubiquitin-dependent degradation of the CDK inhibitor p27 [17,18]. Mice nullizygous for Cks1 had smaller than normal body size and accumulated high cellular levels of phosphorylated p27 [18]. Additionally, Cks1 bound Skp2 *in vitro* and dramatically increased the affinity of Skp2 for phosphorylated (on Thr^{187}) p27 and the efficiency of p27 ubiquitination by recombinant SCF^{Skp2} *in vitro* [17,18].

Although the main components of the p27 ubiquitin ligase complex have now been identified, their functional interactions in the process of ubiquitination of p27 are still poorly understood. Here we report the study of the binding between purified human recombinant Cks1, Skp2 and a p27-derived peptide using a homogeneous time-resolved fluorescence (HTRF) assay. This assay format allows the measurement of protein–protein interactions free in solution under physiological conditions. We found that Cks1 binds Skp2 with high affinity, and that this binding increases the affinity of Skp2 for a p27-derived peptide phosphorylated on Thr^{187}. Cks1 did not bind the phosphorylated p27 peptide directly, which suggests that its effect on the Skp2–p27 interaction is mediated via allosteric modification of Skp2.

EXPERIMENTAL

Materials

Europium (Eu)-labelled antibodies against glutathione S-transferase (GST), the FLAG epitope and the His_6 tag were purchased from PerkinElmer Wallac (Gaithersburg, MD, U.S.A.). Allophycocyanin (APC)-labelled streptavidin, anti-GST

Abbreviations used: APC, allophycocyanin; CDK, cyclin-dependent kinase; DTT, dithiothreitol; GST, glutathione S-transferase; HBS, Hanks buffered saline; HTRF, homogeneous time-resolved fluorescence.

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and anti-FLAG antibodies were from Prozyme (San Leandro, CA, U.S.A.), and GST was from Sigma (St. Louis, MO, U.S.A.). Biotin–(glycogen synthase kinase-3β peptide) was obtained from Bachem (King of Prussia, PA, U.S.A.). Biotinylated p27 peptide phosphorylated on Thr^{187} [biotin–*P*-p27; biotin–Aca-SDGSPNAGSVEQT(p)PKKPGLRRRQT-$CONH_2$, where Aca is aminocaproic acid] and biotinylated unphosphorylated p27 peptide (biotin–p27; biotin-Aca-SDGSPNAGSVEQTPKKPGLRRRQT-$CONH_2$) [13] were synthesized at Hoffmann-La Roche Inc. using standard methods. They were purified by reverse-phase HPLC and confirmed by amino acid analysis and MS. All other chemicals were purchased from Sigma unless indicated otherwise in the text.

Plasmid construction

An expression plasmid for GST–Skp2 and GST–Skp1 (pET/GST-Skp2-Skp1) was constructed by PCR amplification of the coding regions for Skp1 and Skp2 from pcDNA1/hSkp1 and pcDNA3/Skp2 respectively. Skp2 was fused in-frame at the N-terminus with GST followed by a ribosomal binding site. These two DNA fragments were cloned into the pET11 vector (Novagen, Madison, WI, U.S.A.) in tandem with GST–Skp-2 upstream of the Skp1 coding sequence as a dicistronic expression unit. Plasmid DNA was transformed into BL-21(DE3) cells for protein expression. A baculovirus expression plasmid for His_6-tagged Skp1 [pFastBac/$(His)_6$-Skp1] was constructed by inserting a PCR-amplified Skp1 coding sequence with an N-terminal hexahistidine tag into *Bam*HI/*Xba*I-predigested pFastBac vector (Invitrogen, Carlsbad, CA, U.S.A.). All plasmid constructs were sequenced for insert orientation and DNA sequence confirmation.

Protein expression and purification

For purification of GST–Skp2/Skp1, cells from a 5-litre bacterial culture were collected by centrifugation and resuspended in 200 ml of lysis buffer [20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM dithiothreitol (DTT) and 0.5 % Nonidet P40] containing 200 mg/ml lysozyme. The cell suspension was sonicated five times (1 min each), with a 2 min rest on ice between bursts. The homogenate was centrifuged at 10 000 ***g*** for 20 min, and the supernatant was combined with 30 ml of glutathione–Sepharose equilibrated with lysis buffer. The slurry was tumbled at 4 °C for 1 h. The resin was then washed with 10 vol. of lysis buffer, followed by 20 vol. of lysis buffer minus Nonidet P40. The protein complex was eluted by incubation of the resin with 5 vol. of 20 mM Tris/HCl, 150 mM NaCl, 1 mM DTT and 20 mM glutathione (pH 7.8), followed by vacuum filtration through a 0.2 μm polyether sulphone membrane. The protein was concentrated using an Amicon Stir Cell (Amicon, Beverly, MA, U.S.A.) with a 30 000 Da cut-off membrane. The samples were then dialysed against 2×1 litre of 20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM DTT and 20 % (v/v) glycerol.

Cks1 and FLAG–Cks1 were purified as described previously [23]. The identity of all purified proteins was confirmed by MS and sequencing of N-terminal peptides.

HTRF ASSAYS

All HTRF binding assays were performed in black flat-bottomed 384-well plates (Costar # 3710) using assay buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 0.05 % (w/v) BSA and 1 mM DTT. Time-resolved fluorescence was measured by a Victor-5 plate reader (PerkinElmer Wallac) using excitation at 340 nm and emission at 665 nm (50 μs delay and 100 μs acquisition window) and 615 nm (400 μs delay and 400 μs acquisition window).

Saturation binding between GST–Skp2/Skp1 and FLAG–Cks1

Samples of 20 μl of FLAG–Cks1 (final 62.5 nM) and 20 μl of GST–Skp2/Skp1 (final 2.8–541 nM; 1.5-fold serial dilutions) in assay buffer were mixed and incubated at 37 °C with shaking for 1 h. Then 10 μl of Eu-labelled anti-FLAG antibody (final 0.32 μg/ml; 2 nM) and 10 μl of APC-labelled anti-GST antibody (final 1.25–244 nM; 1.5-fold serial dilutions) were added in assay buffer. The mixture was shaken at room temperature for another 30 min and read as described above. Each data point was performed in quadruplicate.

Competition assay with Cks1

Samples of 10 μl of FLAG–Cks1 (final 78 nM) and 10 μl of Cks1 (final 0.69 nM–1.5 μM; 3-fold serial dilutions) were mixed in assay buffer, followed by the addition of 20 μl of GST–Skp2/Skp1 (final 44 nM). The mixture was incubated at 37 °C with shaking for 1 h. Then 20 μl of premixed Eu-labelled anti-FLAG antibody (final 0.32 μg/ml; 2 nM) and APC-labelled anti-GST antibody (final 6.9 μg/ml; 43 nM) was added and the samples were shaken at room temperature for another 30 min and read as above. Each data point was performed in quadruplicate.

GST–Skp2/Skp1 and biotin–*P*-p27 binding in the absence or presence of Cks1

Samples of 10 μl of Cks1 (final 33.3 μg/ml; 3.45 μM) or buffer and 10 μl of GST–Skp2/Skp1 (final 1.75–1094 nM; 5-fold serial dilutions) were mixed in assay buffer and preincubated for 10 min at room temperature. Then 20 μl of biotin–*P*-p27 (final 9.38–5863 nM; 5-fold serial dilutions) was added, and the mixture was incubated at 37 °C with shaking for 1 h. This was followed by the addition of 20 μl of premixed Eu-labelled anti-GST antibody (final 0.32 μg/ml; 2 nM) and APC-labelled streptavidin (final 6 μg/ml; 15 nM). The mixture was shaken at room temperature for 30 min and read as above.

FLAG–Cks1 and biotin–*P*-p27 binding in the absence or presence of GST–Skp2/Skp1

Samples of 10 μl of GST–Skp2/Skp1 (final 66.6 μg/ml; 0.7 μM) or buffer and 10 μl of FLAG–Cks1 (final 11.6–940 nM; 3-fold serial dilutions) were mixed in assay buffer and preincubated for 10 min at room temperature. Then 20 μl of biotin–*P*-p27 peptide (final 9.28–1160 nM; 5-fold serial dilutions) was added, and the mixture was incubated at 37 °C with shaking for 1 h. This was followed by the addition of 20 μl of premixed Eu-labelled anti-FLAG antibody (final 3.2 μg/ml; 20 nM) and APC-labelled streptavidin (final 6 μg/ml; 15 nM). The mixture was shaken at room temperature for 30 min and read as above.

Saturation binding between GST–Skp2/Skp1 and biotin–*P*-p27 and biotin–p27 peptides

Samples of 10 μl of GST–Skp2/Skp1 (final 8.8 nM) and 10 μl of Cks1 (final 1.7 μM) were mixed in assay buffer and preincubated for 10 min at room temperature. Then 20 μl of biotin–***P***-p27 peptide (final 0.013–0.75 μM; 1.5-fold serial dilutions) or biotin–p27 peptide (final 0.013–1.15 μM; 1.5-fold serial dilutions) were added, and the samples were incubated at 37 °C with shaking for

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1 h. This was followed by the addition of 20 μl of premixed Eu-labelled anti-GST antibody (final 0.16 μg/ml; 1 nM) and APC-labelled streptavidin (final 0.17–10 μg/ml for biotin–*P*-p27 and 0.17–15 μg/ml for biotin–p27 peptide; 1.5-fold serial dilutions). The samples were mixed by shaking at room temperature for 30 min and read as above. Each data point was performed in quadruplicate.

Biacore studies

Binding interactions were performed on a Biacore 1000 instrument using BIAevaluation software for data evaluation. A Biacore Sensor Chip SA was used for capture of biotinylated target pepides. The system running buffer was standard BIA-certified Hanks buffered saline containing 0.01 M Hepes, pH 7.4, 0.15 M NaCl, 3 mM EDTA and 0.005 % (v/v) polyoxyethylenesorbitan. Lyophilized peptides were solubilized in 100 % (v/v) DMSO to a concentration of 10 mM and then diluted in Hanks buffered saline to 1 μM for capture. The chip was conditioned with 3×1 min pulses of 1 M NaCl/50 mM NaOH, and sample peptides were injected manually to a level of 200–300 RU (1000 RU correspond to 1 ng of protein/mm^2). The first of four flow cells (Fc1) on the SA chip was used as a reference surface with a captured biotinylated control peptide. To determine the kinetic constants for the binding interaction, a full concentration series of GST–Skp2/Skp1 was prepared by serial dilution in running buffer at concentrations from 1 nM to 2 μM. The Cks1 concentration was kept constant at 250 nM. A continuous flow rate of 40 μl/min was used for all experiments. Each cycle for each concentration began with an injection of sample for an association phase of 3 min. At the end of the injection, the switch back to running buffer began the dissociation phase (3 min). The chip was then regenerated by injection of 20 μl of 20 mM NaOH. A single method for automatic operation was written and run for GST–Skp2/Skp1 in the presence of Cks1 and then again in the absence of Cks1. Sensorgrams from the biotin–p27 peptide (Fc2) after subtracting the reference surface (Fc1) confirmed no binding with or without Cks1. The level of non-specific binding due to Cks1 protein alone was subtracted from each cycle. Sensorgrams from each concentration series were fit into a simple one-to-one binding model to obtain kinetic constants.

RESULTS

Expression and purification of recombinant human Skp2, Skp1 and Cks1

Our previous attempts to express and purify Skp2 in bacterial cells produced very low yields, most probably due to rapid *in vivo* degradation of the protein. It has been shown that this problem can be overcome by co-expression of Skp2 and Skp1 from a dicistronic vector [24]. We cloned Skp2 and Skp1 into a dicistronic expression vector (GST–Skp2/Skp1) and affinity purified the complex using its GST tag. This procedure yielded a high level of almost homogeneous GST–Skp2 and Skp1, as revealed by SDS/PAGE, showing two bands with molecular masses corresponding to the two proteins (Figure 1B, lane 2). A small amount of His_6-tagged Skp1 was purified for control binding experiments (Figure 1B, lane 1). Cks1 and its FLAG-tagged version were expressed at high levels in bacterial cells (Figure 1A, lanes 1 and 2), which allowed their purification using conventional methods [23]. All proteins used in the binding experiments were at least 90 % pure.

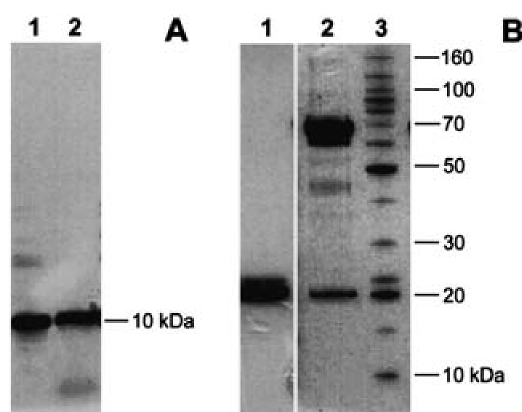

Figure 1 PAGE analysis of purified recombinant proteins

Protein fractions containing 5 μg of total protein were dissolved in Laemmli buffer and run on 4–12 % (w/v) gradient NuPAGE gels. The protein fractions were visualized by staining with Coomassie Blue. (**A**) Cks1 fractions: lane 1, Cks1; lane 2, FLAG–Cks1. (**B**) Skp1 and Skp2 fractions: lane 1, His_6–Skp1; lane 2, GST–Skp2 and Skp1; lane 3, BenchMark (Invitrogen) protein ladder.

HTRF assay for protein–protein binding

HTRF energy transfer is a recently developed technology that has been applied successfully to protein–protein and ligand–receptor binding assays [25]. While traditional ELISA or radioisotopic assays involve multiple washing steps to separate free and bound label, which may cause denaturation of biological reagents and distortion of assay results, HTRF is a homogeneous assay format that allows the measurement of interactions between free molecules in solution under physiological conditions. It utilizes europium chelate (Eu), a source of long-lived fluorescence, and cross-linked APC as a donor–acceptor pair for fluorescence energy transfer. The long-lived nature of acceptor emission (665 nm) helps to eliminate short-lived background fluorescence and substantially improves the signal-to-noise ratio. The donor Eu emission at 615 nm only decreases slightly upon energy transfer, so the ratio of emission at 665 nm to that at 615 nm is used in HTRF assays to correct for fluorescence quenching. In the present study, we have used the HTRF assay as a principal tool to quantify protein–protein interactions among some purified components of the p27 ubiquitin ligase.

To study the interactions between Skp2 and Cks1, we developed an HTRF assay in which APC-labelled anti-GST antibody and Eu-labelled anti-FLAG antibody were used as capture molecules for GST–Skp2/Skp1 and Flag-Cks1 respectively (Figure 2A). A different version of the assay was developed to follow the interaction between Skp2 and a p27-derived peptide (24-mer) shown previously to interact with Skp2 *in vitro* [13]. In this assay, Eu-labelled anti-GST antibody and APC-labelled streptavidin were used as the donor–acceptor pair to capture GST–Skp2 and biotin–*P*-p27 peptide respectively (Figure 2B).

Binding affinity between Cks1 and Skp2

It has been shown previously that labelled Cks1 can bind the F-box protein Skp2 when immobilized on Sepharose beads through a GST tag [17,18]. However, these experiments were carried out in the presence of other protein components from the reticulocyte lysate and under conditions that did not allow quantitative measurements of the interaction. We have used the HTRF binding assay (Figure 2A) to, first, find out if binding occurs between purified human recombinant Cks1 and Skp2 and,

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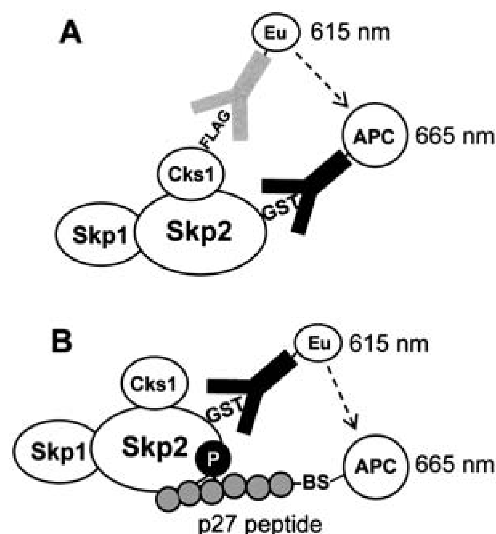

Figure 2 Principal scheme of the HTRF assay

(A) Assay for binding between Skp2 and Cks1; **(B)** assay for binding between Skp2 and biotin–*P*-p27 peptide. Fluorescence energy transfer (arrows) is achieved between Eu cryptate and APC conjugated to antibodies specific for the interacting macromolecules upon complex formation. BS designates biotin–streptavidin complex.

secondly, to determine the binding constant, should an interaction occur. When GST–Skp2/Skp1 and FLAG–Cks1 were incubated at 37 °C in buffer for 1 h, strong emission at 665 nm was measured (Figure 3), while no signal was detected in the absence of GST–Skp2/Skp1 or FLAG–Cks1 (results not shown), indicating that the energy transfer resulted from the association of GST–Skp2/Skp1 and FLAG–Cks1. In order to determine if Skp1 plays a direct role in the binding to Cks1, we replaced GST–Skp2/Skp1 in the assay with His_6–Skp1 and used Eu-labelled anti-His_6 antibody and APC-labelled anti-FLAG antibody. No detectable binding between His_6–Skp1 and Cks1 was observed (results not shown). Since the small His_6 tag is unlikely to alter the properties of Skp1, we concluded that the molecular association between GST–Skp2/Skp1 and Cks1 is mediated by GST–Skp2. The GST tag is a relatively large protein domain. To rule out an interaction between FLAG–Cks1 and the GST portion of GST–Skp2, we replaced GST–Skp2/Skp1 with purified GST protein in the HTRF assay. This assay did not register any signal over the background at the same GST concentrations as those used when GST–Skp2/Skp1 gave a strong signal (results not shown). These results indicate that, under the conditions used in the Skp2–Cks1 binding assay, GST cannot bind Cks1. Therefore the observed binding between FLAG–Cks1 and GST–Skp2/Skp1 is due to a direct interaction between FLAG–Cks1 and Skp2.

To rule out a contribution of the FLAG tag on Cks1 to the binding between Skp2 and Cks1, and to quantify the interaction, we determined the dissociation constant (K_d) using a saturation binding study. In this assay, FLAG–Cks1 was titrated with increasing concentrations of GST–Skp2/Skp1, but the molar ratio of APC-labelled anti-GST antibody and GST–Skp2/Skp1 was kept constant, so that GST–Skp2/Skp1 was labelled uniformly across the titration. An increasing background signal was detected with increasing concentrations of APC-labelled anti-GST antibody owing to non-specific energy transfer. This background was measured by conducting the assay in the absence of FLAG–Cks1 and subtracted from the signal obtained with the full reaction mixture. The background-corrected data were then analysed with the one-site binding equation using GraphPad Prism software, and the K_d was calculated to be 140 ± 14 nM (Figure 3A). Next, we tested untagged Cks1 as a competitor in the HTRF binding assay. An IC_{50} of 160 ± 1 nM was obtained using sigmoidal dose–response curve fitting (Figure 3B), and the K_i was calculated to be 103 ± 1 nM, implying that the FLAG tag has no significant effect on the affinity of Cks1 for GST–Skp2/Skp1. Therefore the binding constants derived from both saturation and competitive binding assays reflect the true interaction between Skp2 and Cks1. This binding was also found to be ionic-strength-dependent and pH-sensitive, with optimal binding observed in 150 mM NaCl at pH 7.5, coinciding with physiological salt and pH conditions (Table 1).

Skp2 binds a p27 phosphopeptide only in the presence of Cks1

Previous experiments have shown that a 24-mer peptide derived from the p27 sequence (amino acids 175–198) can bind Skp2 immobilized on Sepharose beads *in vitro* only when the peptide is phosphorylated on Thr^{187} [13]. This phosphorylation can be

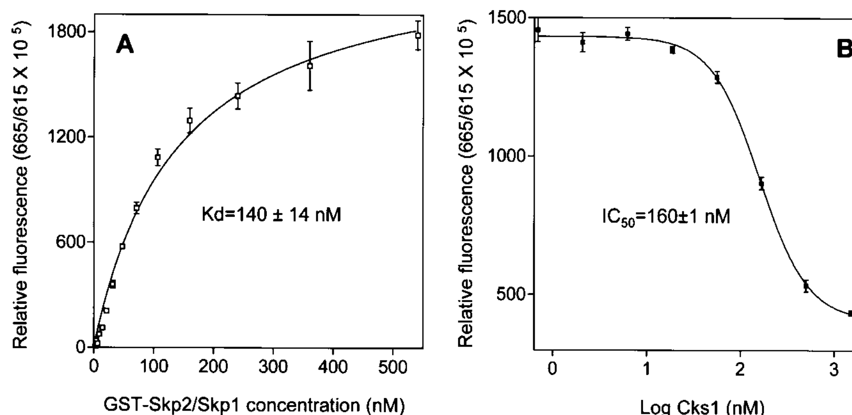

Figure 3 Binding affinity between GST–Skp2/Skp1 and Cks1

(**A**) Saturation binding assay. FLAG–Cks1 (62.5 nM) was titrated with increasing concentrations of GST–Skp2, and the K_d was determined using a saturation binding plot in GraphPad. (**B**) Competitive inhibition of the binding between GST–Skp2/Skp1 and FLAG–Cks1 by Cks1. The binding of GST–Skp2/Skp1 and FLAG–Cks1 was measured in the presence of increasing concentrations of non-tagged Cks1. Each data point represents the mean ± S.E.M. of quadruplicate samples.

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Table 1 pH- and ionic-strength-dependence of GST–Skp2/Skp1 and FLAG–Cks1 binding

(a) To test the effect of pH, the binding between GST–Skp2/Skp1 (140 nM) and FLAG–Cks1 (176 nM) was measured in 20 mM Tris/HCl, containing 140 mM NaCl. The pH was adjusted by adding concentrated HCl or NaOH. (b) The effect of ionic strength was measured in 10 mM Tris/HCl, pH 7.5, at various NaCl concentrations. Binding was calculated as a percentage of the maximum HTRF signal.

(a)

pH	Binding (%)
6.0	24.7
6.5	31.8
7.0	82.4
7.5	100
8.0	72.8

(b)

NaCl (mM)	Binding (%)
20	51.0
40	74.9
80	86.1
160	100
320	71.8

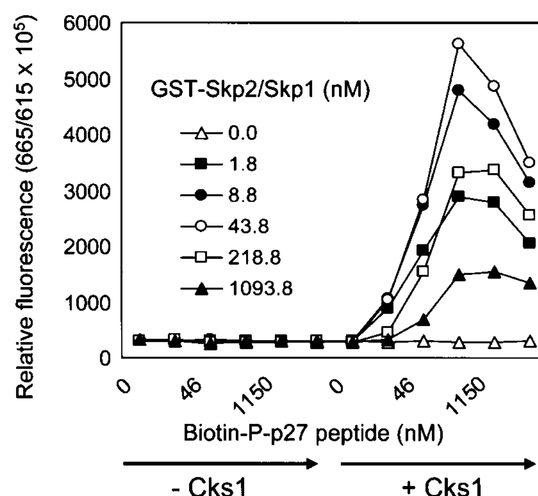

Figure 4 Skp2 binds biotin–*P*-p27 peptide only in the presence of Cks1

GST–Skp2/Skp1 and biotin–*P*-p27 peptide were cross-titrated in the absence or presence of a fixed amount of Cks1 (3.45 μM), and the fluorescence signal was plotted against concentration. The fluorescence signal decreases at high concentrations of GST–Skp2/Skp1 or biotin–*P*-p27 peptide due to saturation of the Eu-labelled antibody (2 nM) or APC-labelled streptavidin (15 nM) respectively, that are used at fixed concentrations.

carried out by CDK2–cyclin E both *in vitro* and *in vivo*, and appears to be critical for the targeting of p27 for ubiquitination [8–10]. Therefore this peptide can be used as a p27 surrogate in studying its functional interactions with Skp2. It has been shown that Cks1 can significantly enhance the binding of Skp2 to the phosphorylated, but not to the unphosphorylated, form of the p27 peptide immobilized on Sepharose beads [17,18]. Those experiments used unfractionated *in vitro* translation reactions containing Skp2 labelled with ^{35}S. Because of the crude nature of the assay, the results were only semi-quantitative, at best. To address quantitatively the role of Cks1 in the interaction between purified Skp2 and p27, we used a phosphorylated (on Thr^{187}) and an unphosphorylated version of the p27-derived peptide described above, both containing N-terminal biotin tags (biotin–*P*-p27 peptide and biotin–p27 peptide respectively).

The binding between biotin–*P*-p27 peptide and GST–Skp2/Skp1 was investigated using a version of the HTRF assay in which energy transfer was achieved by APC-labelled streptavidin and Eu-labelled anti-GST antibody, as shown in Figure 2(B). To determine the ratios of the two interacting partners that generate optimal energy transfer, we cross-titrated both GST–Skp2/Skp1 and biotin–*P*-p27 peptide in the absence or presence of excess Cks1 (3.45 μM). The data showed that phosphorylated p27 bound GST–Skp2/Skp1 only in the presence of Cks1, and the maximal signal/background ratio was achieved at peptide concentration of approx. 1 μM (Figure 4). This result confirmed previous observations that Cks1 can dramatically increase the binding affinity between Skp2 and phospho-p27. To ensure that the GST tag on Skp2 does not contribute to the observed binding between biotin–*P*-p27 peptide and GST–Skp2/Skp1, we tested the ability of GST alone to bind biotin–*P*-p27 peptide. When purified GST was mixed with biotin–*P*-p27 peptide in the presence of Cks1, both within concentration ranges equivalent to those shown in Figure 4, no signal over the background was registered, indicating that GST alone cannot bind the peptide (results not shown). In an alternative control experiment designed to measure the effect of the biotin tag on the interaction, we replaced biotin–*P*-p27 peptide with an irrelevant biotinylated peptide [biotin–(glycogen synthase kinase-3β)]. Again, we were not able to register any measurable signal (results not shown). These control experiments confirmed that the tags did not contribute to the observed binding between Skp2 and biotin–*P*-p27 peptide.

To quantify the effect of Cks1 on the affinity of Skp2 for the biotin–*P*-p27 peptide, we determined the dissociation constant of the interaction between GST–Skp2/Skp1 and the biotin–*P*-p27 peptide in the presence of Cks1. A K_d of 37 ± 2 nM was calculated from the saturation binding plot (Figure 5A), indicating a strong molecular interaction. Next, we measured the binding between Skp2 and the biotin–p27 peptide. GST–Skp2/Skp1 bound the biotin–p27 peptide with much lower affinity, and this affinity did not change with the addition of Cks1. The K_d was 435 ± 20 nM in the absence and 468 ± 23 nM in the presence of Cks1 (Figure 5B). As an independent means of validation of our data, we used a Biacore instrument, which allows the measurement of macromolecular interactions by an alternative technology not involving the use of antibodies. In the presence of Cks1, the binding constant for the binding of GST–Skp2/Skp1 to the biotin–*P*-p27 peptide (K_d=42 ± 5 nM) was found to be nearly identical to that determined by the HTRF assay (results not shown).

Cks1 acts as an allosteric effector of the Skp2–p27 interaction

Although it has been shown that Cks1 can dramatically increase the affinity between Skp2 and p27, the mechanism underlying this effect is not well understood [17,18,26]. According to one model, the affinity of Skp2 for p27 is increased allosterically by the binding of Cks1 to Skp2 [18]. Another model suggests that Cks1 may serve as a molecular adapter in the interaction between Skp2 and p27 [26]. This latter model is based on the observation that Cks1 itself can bind phosphorylated p27 [17]. To determine if purified Cks1 is capable of binding p27 phosphopeptide in solution, we used FLAG–tagged Cks1 and biotin–*P*-p27 peptide in a HTRF assay similar to the one depicted in Figure 2(B), but replacing the anti-GST antibody with Eu-labelled anti-FLAG antibody. Cross-titration of FLAG–Cks1 (11.6–940 nM) and biotin–*P*-p27 peptide (9.28–1160 nM) did not reveal any detectable binding in the absence of GST–Skp2/Skp1.

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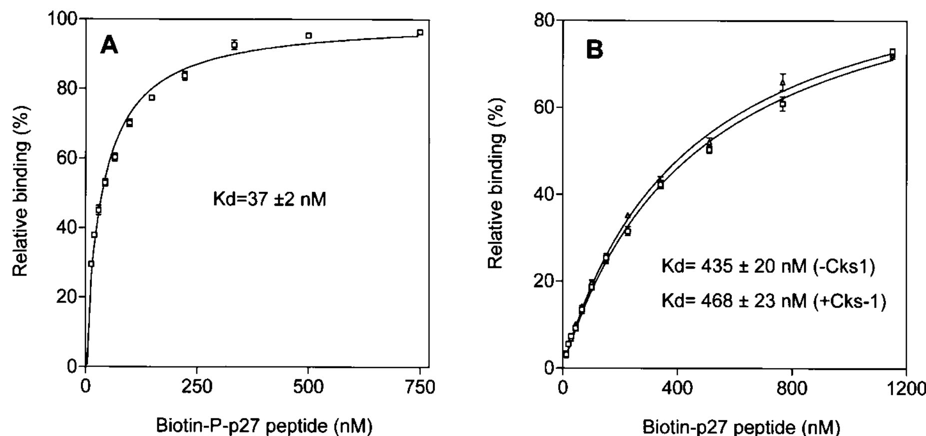

Figure 5 Binding affinities between Skp2 and p27 peptides

(**A**) Saturation binding plot of the interaction between GST–Skp2/Skp1 and biotin–*P*-p27 peptide in the presence of Cks1. GST–Skp2/Skp1 was mixed with a saturating concentration of Cks1 (1.7 μM) and titrated with increasing concentrations of biotin–*P*-p27 peptide. The K_d was determined based on a one-site binding model using GraphPad. (**B**) Saturation binding plot of the interaction between GST–Skp2/Skp1 and biotin–p27 peptide in the presence or absence of Cks1. GST–Skp2/Skp1 was premixed with or without Cks1 as in (**A**), and titrated with biotin–p27 peptide. The K_d was determined as in (**A**).

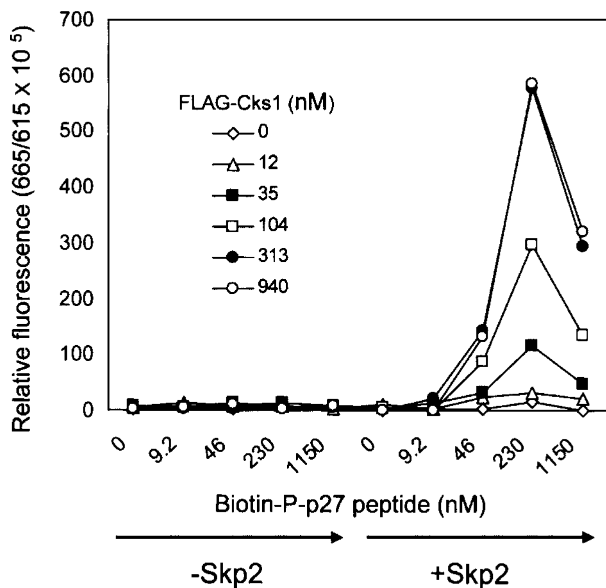

Figure 6 Cks1 alone does not bind biotin–*P*-p27 peptide

FLAG–Cks1 and biotin–*P*-p27 peptide were cross-titrated in the absence or presence of a fixed amount of GST–Skp2/Skp1 (0.7 μM), and the fluorescence signal was plotted against the concentration of each of the components. The decrease in the signal at high concentrations of biotin–*P*-p27 peptide is due to saturation of the APC-labelled streptavidin used at a fixed concentration (15 nM).

However, a significant increase in the fluorescence signal was observed when GST–Skp2/Skp1 was added to the reaction mixture at 0.7 μM (Figure 6). This signal increase most probably reflects the binding of Skp2 to biotin–*P*-p27 peptide that occurs in the presence of Cks1 (Figure 4), rather than a Skp2-induced direct interaction between Cks1 and the peptide. If the observed binding is via Skp2, the distance between the FLAG tag on Cks1 and biotin on the peptide will be greater than the distance between two directly interacting macromolecules, and this would result in less efficient energy transfer. In agreement with this expectation, the fluorescence signal in the binding reaction of FLAG–Cks1 and biotin–*P*-p27 peptide in the presence of GST–Skp2/Skp1 (Figure 6) was significantly lower than the signal generated by the binding of GST–Skp2/Skp1 to biotin–*P*-p27 peptide (Figure 4). Thus, under conditions in which FLAG–Cks1 binds strongly to GST–Skp2, we cannot detect any interaction between FLAG–Cks1 and biotin–*P*-p27 peptide, suggesting that the effect of Cks1 is exerted allosterically through its interaction with Skp2.

DISCUSSION

Recently it has been found that the efficiency of p27 ubiquitination can be increased dramatically by adding the small protein Cks1 to SCF^{Skp2} [17,18]. ^{35}S-labelled Cks1 has been shown to be capable of binding GST–Skp2 immobilized on glutathione–Sepharose beads *in vitro* and to increase significantly the binding between Skp2 and p27 *in vitro* and *in vivo* [17,18]. These studies have measured the interaction between Skp2 and Cks1 under conditions where one protein partner originated from unpurified fractions labelled by *in vitro* translation and the other one was immobilized on a solid support.

Here we report a quantitative study of protein–protein interactions between purified human recombinant Skp2 and Cks1. We developed a HTRF assay that allowed quantification of the binding between Skp2 and Cks1, and between Skp2 and a p27-derived phosphopeptide free in solution. It is based on the energy transfer between Eu and APC that can occur upon physical binding between two macromolecular components. This binding is detected through the use of specific antibodies conjugated with Eu or APC and reactive against tags on the interacting molecules. In a series of control experiments, we demonstrated that none of the tags on the molecules studied (GST on Skp2, FLAG on Cks1, or biotin on p27 peptide) can interact non-specifically with other components of the assay and thus contribute to the observed interactions. Therefore the data obtained using this binding assay should reflect the true affinities of the recombinant proteins. The data, however, may or may not represent the true affinities between the native proteins in the cell, due to the fact that they are expressed in bacterial cells and may not have correct folding and post-translational modifications. On the other hand, X-ray crystallographic studies of bacterially expressed Skp1/Skp2 complexes suggest that the Skp2 leucine-rich repeat pocket, thought to be the site of substrate binding, is folded similarly to other leucine-rich repeat structures [24].

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Using this assay, we confirmed that Cks1 can bind Skp2, and determined the K_d of the interaction to be 140 ± 14 nM (Figure 3). Untagged Cks1 can compete effectively with FLAG–Cks1 for binding to Skp2 (IC_{50} 160 ± 1 nM), which shows that the small tag did not significantly affect the binding properties of Cks1. We also examined the ability of Skp2 to bind a p27-derived peptide that carries the phosphorylation site for CDK2–cyclin E (Thr^{187}) and has been shown previously to interact only when the peptide is phosphorylated [13]. In our system, using purified proteins, we were unable to detect reliable binding between GST–Skp2 and biotin–*P*-p27 peptide in the absence of Cks1 (Figure 4). However, strong binding was registered in the presence of Cks1, with a K_d of 37 ± 2 nM (Figure 5). Interestingly, Skp2 was able to bind the non-phosphorylated p27 peptide with much lower affinity in either the presence or the absence of Cks1 (Figure 5B). The fact that the K_d was not affected by Cks1 suggests that biotin–p27 peptide binds to a different site on the Skp2 molecule, where the conformation is not sensitive to Cks1 binding. Although p27-derived peptides have been used as a surrogate for p27 in binding studies with Skp2 [13,17], their affinity may differ from that of the native protein, and the binding constant between Skp2 and p27 *in vivo* may deviate significantly from the value determined in the present study.

The observations that Cks1 can bind Skp2 and enhance its affinity for p27, and hence the efficiency of p27 ubiquitination, *in vitro* have led to the conclusion that Cks1 mediates the interaction between Skp2 and p27 [17,18]. However, alternative models have been proposed to explain the mechanism of this effect [26]. According to one model, Cks1 serves as a bridge between p27 and Skp2. This model is based on the observation that ^{35}S-labelled Cks1 can bind phosphorylated (Thr^{187}) p27 peptide in the absence of Skp2 [17]. In the second model, Cks1 plays the role of an allosteric factor that binds Skp2 and increases its affinity for p27 without interacting directly with p27 [18]. In our assay system, purified FLAG–Cks1 did not show detectable binding to biotin–*P*-p27 peptide even at the highest concentration tested of 1 μM. This result supports the model in which Cks1 is an allosteric effector that increases the affinity of Skp2 for p27.

It is difficult to identify the reasons for the discrepancy between the present study and the previous observation of Cks1–p27 peptide binding [17], as the experimental design and conditions employed were quite different. First, the peptides used in the studies were not identical. Both peptides are C-terminal and contain the critical phosphorylation site, Thr^{187}, but the peptide used in our study was five amino acids longer. Secondly, in our assay the interaction was measured free in solution, while in the earlier study it was based on immobilized peptides. Thirdly, the sensitivity of detection of radioactively labelled protein by autoradiography is greater, and can potentially pick up minute quantities of ^{35}S-labelled Cks1 retained on the *P*-p27 peptide beads. Finally, traces of Skp2 from the reticulocyte lysate may bind ^{35}S-labelled Cks1, thus mediating the interaction with *P*-p27 on the beads indirectly. Since the ratio of Cks1 to *P*-p27 peptide and the affinity of the interaction have not been determined, the functional significance of the observed binding between Cks1 and p27 is not clear. It is reasonable to speculate that, if Cks1 plays the role of a bridging factor between Skp2 and p27, its binding affinities for both Skp2 and the p27 peptide should be similar. In our hands, purified Skp2 bound the biotin–*P*-p27 peptide with high affinity in the presence of Cks1, while the binding of purified Cks1 alone to the peptide was below the detection level of the assay. These results lend support to the model in which Cks1 functions as an allosteric regulator of the interaction between Skp2 and p27, and thus of E3 ligase activity [18,26]. Although it is unlikely, we cannot exclude the other possibility that Skp2 can itself alter the conformation of Cks1 so that it can bind p27 and therefore serve as a bridging molecule, as suggested previously [17,26]. Determination of the crystal structure of the p27–Skp2–Cks1 complex should resolve these issues in the future.

It has been well documented that a substantial fraction of the cellular CDK2 is associated with Cks1, but the functional significance of this is not well understood [21,22]. In the light of the newly discovered role of Cks1 as an activator of SCF^{Skp2}, this interaction may play an important role in the function of the E3 ligase–p27 complex. In the proposed alternative bridging model [26], Cks1 can bind and allosterically affect Skp2, while at the same time it can interact with the CDK2–cyclin–phospho-p27 complex via CDK2. A study published during the preparation of our present manuscript [28] addressing the roles of different Cks1 sequence motifs in its interactions with Skp2 and p27 has shown that efficient binding of Skp2 to the p27–CDK2–cyclin E complex under non-saturating conditions requires the CDK2-binding site on Cks1. Therefore it appears plausible that the activating function of Cks1 may not be limited to its effect on the conformation of Skp2, but may be exerted through a more complex mechanism, with the participation of CDK2–cyclin E as an integral part of the p27 ubiquitination machinery.

Loss or a low level of p27 has been associated with poor prognosis in a wide variety of human cancers [6,29]. The restoration of high p27 levels might offer a novel approach to cancer therapy. It has been shown that the low cellular levels of p27 in tumours are due mainly to an elevated rate of proteolytic degradation by the ubiquitin/proteasome system [4,5]. Understanding the mechanism of p27 ubiquitination might help to identify novel molecular targets for therapeutic intervention. The binding affinities between recombinant Cks1 and Skp2 (140 nM) and Skp2–p27 phosphopeptide (40 nM) *in vitro* suggest that these protein–protein interactions might fall in the tractable range for development of chemical inhibitors.

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