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Interaction studies between the p21Cip1/Waf1 cyclin-dependent kinase inhibitor and proliferating cell nuclear antigen (PCNA) by surface plasmon resonance

Martine Knibiehler[a], Françoise Goubin[a], Nathalie Escalas[a], Zophonias O. Jónsson[b], Honoré Mazarguil[a], Ulrich Hübscher[b], Bernard Ducommun[a,*]

[a]*Institut de Pharmacologie et de Biologie Structurale, Université P. Sabatier, 205 Route de Narbonne, 31077 Toulouse, France*
[b]*Universität Zürich-Irchel, Institut für Veterinärbiochemie, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland*

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Abstract The cyclin-dependent kinase (CDK) inhibitor p21Cip1 consists of two domains that interact with CDKs and proliferating cell nuclear antigen (PCNA), respectively. We have investigated the interaction between p21Cip1 and PCNA using surface plasmon resonance (SPR) technology and compared the results with those obtained from other sources such as the yeast two-hybrid system. Whilst other methods are only semi-quantitative, the SPR technique allowed us to determine the kinetic parameters of the interaction. The apparent equilibrium constant K_D calculated for these kinetic parameters was 3.2×10^{-7} M. We further demonstrate the use of SPR to study the interaction between mutant proteins and to determine their actual K_D. The interaction between p21Cip1/PCNA is shown to be dependent upon the trimeric conformation of PCNA since a point mutant that abolishes PCNA-PCNA interaction also abolishes PCNA's interaction with p21Cip1. Finally, we demonstrate that SPR can be used to characterise the interaction of p21Cip1 and PCNA in the presence of short competitive peptides.

Key words: PCNA; Cyclin-dependent kinase inhibitor; p21Cip1/Waf1; Cell cycle

1. Introduction

Cyclin-dependent kinases (CDKs) regulate the key transitions of the eukaryotic cell cycle. Their activities are regulated at different levels [1] including specific inhibitors called cyclin-dependent kinase inhibitors (CKIs), which have been identified by virtue of their physical interaction with the CDKs. The inhibitor p21Cip1/WAF1 plays an important growth control function by arresting the cell cycle in G1-phase in response to extra- or intracellular signals such as DNA damage [2]. p21Cip1 has been shown to interact both with the CDK2 kinase and with proliferating cell nuclear antigen (PCNA) an auxiliary protein of DNA polymerase δ [3].

In order to identify the p21Cip1 inhibitor domains that are involved in the interaction with CDK2 and with PCNA, we performed extensive mutagenesis using the alanine-scanning mutagenesis strategy [4]. A large number of p21 mutants were produced as fusion proteins with GST (glutathione *S*-transferase), purified and used in an in vitro interaction assay with either in vitro translated CDK2 or purified PCNA. This work led to the identification of several mutants that were deficient for either of these interactions and suggested that binding domains for both partners of p21Cip1 were localised to the amino- and carboxy-terminal parts of the protein, respectively [4]. Other groups used different approaches to reach similar conclusions [5–7].

Recent advances in our understanding of the molecular basis of growth control suggest that cyclin-dependent kinases and their regulators, i.e. inhibitors such as p21Cip1, might represent potential targets for new pharmacological strategies. However, molecular interactions between cell cycle regulators are generally analysed at a superficial level. In particular, the kinetics and thermodynamics of the interactions are rarely characterised, information that is essential for the development of screening strategies designed to identify modulators and ultimately to aid rational drug design.

The BIAcore (Pharmacia Biosensor) is a surface plasmon resonance (SPR)-based system for real time analysis of biospecific interactions [8]. To study the interactions between biomolecules, the BIAcore system uses an optical technique that measures the refractive index at the sensor chip surface. A ligand is immobilised onto the dextran-coated gold surface of a sensor chip and its interaction with a soluble analyte flowing through the dextran matrix is monitored by changes in refractive index which are proportional to mass increases resulting from the interaction. Collected sensorgrams can subsequently be used to estimate the association and dissociation rate constants, by either linearised transformation [9] or non-linear curve-fitting methods [10] using the BIAevaluation 2.1 software (Pharmacia Biosensor).

Here we report the use of SPR technology to investigate the interaction between p21Cip1 and PCNA. We compare the results obtained by this approach with previous in vitro interaction data and with in vivo interactions measured using the two-hybrid system in yeast. We report here the kinetic parameters of the p21Cip1-PCNA interaction and demonstrate the use of this strategy to assay competitive peptides. Furthermore, we show that a point mutation of PCNA that abolishes trimer formation [11] also totally impairs its interaction with p21Cip1, indicating that this complex formation is dependent on the oligomerisation state of PCNA.

2. Materials and methods

2.1. Materials

The BIAcore system, CM5 research grade sensor chips, coupling reagents and HBS buffer were purchased from Pharmacia Biosensor (Uppsala, Sweden). *E. coli* and budding yeast growth media were from Bio101 Inc. (La Jolla, CA). Glutathione Sepharose was from Pharmacia (Uppsala, Sweden) and electrophoresis reagents from BioRad (Hercules, CA).

*Corresponding author. Fax: (33) 05 61 17 59 94.
E-mail: ducommun@IPBS.fr

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2.2. Protein purification

Constructs expressing glutathione *S*-transferase fusion proteins were made as previously described [4] in the pGEX-KG vector. Production of recombinant proteins was achieved after induction with 0.4 mM IPTG (isopropyl thiogalactoside) for 4 h at 37°C in Luria broth. GST-p21Cip1 fusion proteins wild-type and ASM mutants were produced in *E. coli* BL21(DE3)pLysE and purified from inclusion bodies as previously described [12]. GST-CTE, encoding the carboxy-terminal half of p21Cip1 (residues 75–164), was soluble and was therefore purified as follows: harvested cells were sonicated in PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) containing protease inhibitors (0.1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin and 10 μg/ml TPCK (tosyl phenylalanine chloromethyl ketone)) and centrifuged at 27 000×*g* for 30 min. The supernatant was adjusted to 0.1% Triton X-100 and incubated with glutathione Sepharose for 3 h at 4°C. After three washes with 5-fold bed volume (50 mM Tris-HCl pH 8.0, 50 mM NaCl), GST-CTE was eluted with 50 mM Tris-HCl (pH 8.0) containing 10 mM glutathione. All purified proteins were stored at −80°C until use. Wild-type PCNA and the Y114A mutant were purified exactly as described [11].

Protein concentrations were determined using the Bradford [13] assay (Bio-Rad). Purified proteins were resolved by SDS-polyacrylamide gel electrophoresis according to Laemmli [14].

The two peptides used in the BIAcore assays were in a 5 mM solution in DMSO. Working solutions were made by dilution in HBS buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% Surfactant p-20) and filtered through 0.22 μm Millex GV filters (Millipore).

2.3. Two-hybrid assay

Plasmids pGBT9-CIP1 and pGBT9-ASMx (x=the number of the respective mutant) were constructed by cloning the entire coding sequences of wild-type or mutant p21CIP1 downstream and in frame with the GAL4 DNA-binding domain in the *Eco*RI and *Bam*HI sites of pGBT9 cloning vector (Clontech); the plasmid pACT-PCNA, expressing the human PCNA protein fused to the GAL4-activation domain in the pACT2 vector (Clontech), was kindly provided by I. Salles (Grenoble, France). For interaction assays, the yeast strain SFY526 (*MATa*, *ura3-52*, *his3-200*, *ade2-101*, *lys2-801*, *trp1-901*, *leu2-3,112*, *can*^{r}, *gal4-542*, *gal80-538*, *URA3::GAL1-lacZ*) [15] was transformed with equal quantities of pACT-PCNA and pGBT9-CIP1 or pGBT9-ASMx plasmids. Transformants were selected on SD synthetic medium minus Leu-Trp plates at 30°C and grown for 2–3 days. Independent transformants were grown overnight (OD_{600} = 1) in 5 ml of SD minus Leu-Trp medium at 30°C and β-galactosidase activity was then measured on crude cellular extracts. Cells were collected by centrifugation, washed once in 1 ml of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 40 mM β-mercaptoethanol, 1 mM PMSF, pH 7.0) and disrupted in 300 μl of Z buffer by vortexing with glass beads at 4°C for 2 min. A 10 μl aliquot of this extract was used for the determination of protein concentration; assay for β-galactosidase activity was initiated by the addition of 150 μl of *o*-nitrophenyl β-D-galactopyranoside solution (4 mg/ml in 0.1 M phosphate buffer pH 7.0) and subsequent incubation at 30°C until yellow color developed, and for 3 h maximal duration. The reaction was then stopped by the addition of 500 μl of 1 M Na_2CO_3 and tubes were stored on ice. Reaction mixtures were centrifuged briefly, and the OD_{420} of the supernatant was measured; β-galactosidase activity in Miller units was calculated according to the formula: units=$OD_{420}\times 380/t\times P$, where t=time of incubation (in min) and P=proteins added to Z buffer in mg.

Table 1
List of the p21Cip1 mutants used in this study and in vitro interaction with PCNA[a]

Name	Sequence	% PCNA[a]
p21	Wild-type	100
ASM7	D52A	81
ASM19	H152A, K154A, R155A, R156A	0
p21CTE	Amino acids 75–164	25
GST	Glutathione *S*-transferase	0

The extent of interaction of these GST fusion proteins with PCNA was determined in an in vitro assay (see [4]), and is expressed as a percentage of binding relative to the interaction measured with wild-type p21Cip1.
[a]Our unpublished data and see also [4].

Table 2
Determination of the interaction between PCNA and p21Cip1 mutant proteins using the two-hybrid assay in *Saccharomyces cerevisiae*

Partners[a]	β-Galactosidase activity[b]	% of control
PCNA-bd	2.5	0
PCNA-p21Cip1	425	100
PCNA-ASM7	590	139
PCNA-ASM19	156	36
ad-ASM19	1.7	0

[a]PCNA was in fusion with GAL4-activating domain (ad) and p21Cip was in fusion with GAL4 DNA-binding domain (bd).
[b]β-Galactosidase activity was determined as described in [19].

2.4. SPR analysis: Immobilisation procedure and buffers

PCNA or GST-p21Cip1 wild-type proteins were covalently coupled to the dextran of sensor chip CM5 research grade. The carboxymethylated dextran was first activated during 3 to 5 min. (5 μl min^{-1} flow rate) with 0.1 M *N*-hydroxysuccinimide (NHS) and 0.4 M *N*-ethyl-*N'*-(dimethylaminopropyl)carbodiimide (EDC) in water. The ligand was then injected through the reactive surface (30 μl 0.14 μM for PCNA, 20 μl 0.5 μM for GST-p21Cip1) in 10 mM acetate buffer (pH 4.5) with a continuous flow of HBS (5 μl/min). The residual NHS esters on the sensorchip surface were then deactivated over 6 min with 1 M ethanolamine, pH 8.5, in water. Final regeneration of the matrix was performed with 0.1 N NaOH.

2.5. Study of the interaction and calculation of constants

Purified recombinant p21Cip1 wild-type and mutant proteins, PCNA wild-type and the Y114A mutant, and peptides were diluted in HBS buffer. A 40 μl sample was injected over the PCNA or GST-p21Cip1 coupled surface for 8 min at a 5 μl/min flow rate. Multiple injections at various concentrations were performed for each ligand. For each concentration a control injection was performed on an activated-deactivated sensor chip. The sensorgrams were recorded and, after subtraction of the control, the data were fitted to a single site binding model by non-linear least-squares curve fitting using the BIAevaluation 2.1 software (Pharmacia Biosensor). The apparent dissociation rate constant k_{off} was calculated by iterative fitting to first order model. For the association phase, we calculate k_s (k_s is the slope of the plot of instant rate dR/dt vs. R), at various concentrations (C) of analyte and the association rate constant k_{on} was calculated by linear curve fitting of a plot of k_s vs. C. In control experiments performed with the GST moiety alone, no interaction with PCNA was found.

3. Results

3.1. p21Cip1-PCNA interaction in the yeast two-hybrid system

The list of the p21Cip1 mutant proteins used in this study is shown in Table 1. The level of interaction with PCNA was determined in vitro as already described [4] and was normalised to 100% with wild-type p21Cip1. This set of p21 recombinant proteins includes wild-type and mutant sequences which are able to interact with PCNA and those which are not. Under the experimental conditions of the in vitro assay that was used, mutant ASM19 was totally unable to bind PCNA. The carboxy-terminal part of the protein (residues 75–164) that comprises the PCNA-binding domain [4,5] only partially interacted with PCNA (25%). The ASM7 mutant that does not interact with CDK2 [4] was not significantly affected in its interaction with PCNA.

To investigate further the interaction between the wild-type and mutant p21Cip1 and PCNA, we first used a two-hybrid assay in the budding yeast *Saccharomyces cerevisiae*. Wild-type or relevant mutants of p21Cip1, and PCNA, were fused

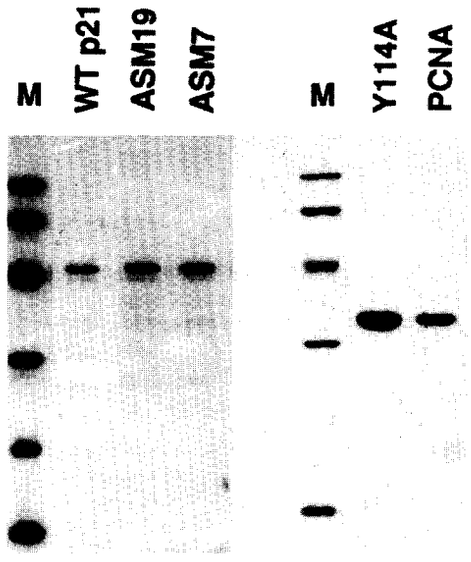

Fig. 1. SDS-PAGE of the proteins used in this study. Purified proteins were subjected to SDS-PAGE (12.5% acrylamide) and Coomassie stained. Wild-type p21Cip1 (WT) (1 μg), mutants p21Cip1 (ASM19 (1 μg) and ASM7 (1 μg)), proliferating cell nuclear antigen (PCNA) (1 μg) and PCNA mutant Y114A (2 μg), are shown. Molecular mass markers (M) are 97, 67, 43, 30, 20 and 14 kDa.

to the DNA-binding domain and to the activation domain of the yeast transcriptional activator GAL4, respectively. Plasmids encoding these fusion proteins were cotransformed into the yeast strain SFY526, carrying the lacZ reporter gene under the control of GAL4-binding sites. A quantitative determination of the interaction between the two proteins was performed by measuring β-galactosidase activity on total cell lysates. The results of a typical experiment are reported in Table 2. No *trans*-activation was observed when the fusion between the GAL4-activating domain and PCNA was co-expressed with GAL4 DNA-binding domain (bd) alone. Similarly, fusion of any of the p21Cip1 mutants with GAL4 DNA-binding domain was unable to activate β-galactosidase transcription when co-expressed with GAL4-activating domain alone (last line of Table 2 and data not shown). Interaction between PCNA and wild-type p21Cip1 was very strong, resulting in high β-galactosidase activity. As expected, mutant ASM7 which we previously identified in vitro as unable to bind CDK2 interacted normally with PCNA. The latter interaction was moreover slightly stronger than that found with the wild-type p21Cip1 (139%). Mutant ASM19 which in vitro was totally unable to bind PCNA was found in two-hybrid assay to be still able to interact although more weakly than the wild-type (36%). These results provide valuable information (see Section 4), however, the two-hybrid assay does not allow an exclusive and quantitative determination of the interaction between two proteins.

3.2. *p21Cip1-PCNA interaction analyzed with SPR*

To evaluate the kinetic parameters of interaction, we used the SPR technology. Purified wild-type PCNA (see Section 2) was immobilised onto CM5 BIAcore sensor chip. The p21Cip1 protein, the mutant forms (ASM7, ASM19) and the truncated protein (CTE) were purified as described in Section 2 to approx. 90–95% homogeneity (Fig. 1). These proteins were injected at different concentrations onto the immobilised PCNA ligand (2000 RU) and the sensorgrams were recorded (data not shown). Dissociation (k_{off}) and association (k_{on}) constants were derived from the data collected with concentration ranging from 5 to 20 μM using a single site binding model with the BIAevaluation 2.1 software. The apparent dissociation and association constants are reported in Table 3. The calculated apparent equilibrium constant K_D for wild-type p21Cip1 was 3.2×10^{-7} M. For the mutant ASM7, which binds to PCNA in an in vitro assay and in the two-hybrid system, the K_D value was similar to that of the wild-type (2.4×10^{-7} M). A considerable decrease in the interaction between mutant ASM19 and PCNA was found, with a K_D about 100-fold lower than that of wild-type p21Cip1 (3.9×10^{-5} M). Surprisingly, the carboxy-terminal domain of p21Cip1 that is known to interact with PCNA also displayed a lowered interaction constant for PCNA (K_D: 4.1×10^{-6} M), suggesting that the two functional domains of p21Cip1 are not fully independent.

Thus, the SPR technology allows a fine discrimination between the binding properties of several mutants and furthermore gives access to essential kinetic data.

3.3. *Interaction between p21 and PCNA is dependent on the trimerisation of PCNA*

The importance of the trimerisation of PCNA for its interaction with other proteins has recently been shown using a tyrosine to alanine mutation that is unable to oligomerise [11]. We have comparatively investigated the interaction properties of PCNA and Y114A-PCNA with p21Cip1 using BIAcore.

Wild-type p21Cip1 was covalently coupled on a sensor chip and both forms of PCNA were injected. As described above and also shown in Fig. 2, wild-type PCNA interacted with p21Cip1 that was coated on the sensorchip matrix with a calculated K_D of 3.0×10^{-7} M. Mutant Y114A-PCNA did not interact at all with p21Cip1, and consequently no association constant could be determined.

3.4. *Competing peptide*

We next asked whether the BIAcore system might be used in order to screen molecules that may inhibit the interaction between p21Cip1 and PCNA. To address this question, we used a synthetic p21-like 20mer peptide that has been shown to interact with PCNA [5]. A 19-mer p21Cip1 unrelated peptide (YIVPEDKREMWMACIKEAA) was used as a control. In the first experiment, a 20 μM concentration of either peptide was continuously passed in the flow on the PCNA sensor chip, then 40 μl of 20 μM wild-type p21Cip1 were injected (5 μl/min). The sensorgrams obtained in that experiment are

Table 3
Interaction between PCNA and p21Cip1: Determination of the association and dissociation constants[a]

	k_{off} (s^{-1})	k_{on} (M^{-1} s^{-1})	K_D (M)[b]
Wild-type p21	3.7×10^{-4}	1.1×10^{3}	3.2×10^{-7}
ASM7	2.7×10^{-4}	1.1×10^{3}	2.4×10^{-7}
ASM19	3.9×10^{-3}	1.0×10^{2}	3.9×10^{-5}
CTE	8.7×10^{-3}	2.1×10^{3}	4.1×10^{-6}

[a]Dissociation (k_{off}) and association (k_{on}) rate constants were calculated using the BIAevaluation 2.1 software (Pharmacia) from data collected from multiple reproducible experiments (see text).
[b]The equilibrium constant K_D is calculated from the ratio k_{off}/k_{on}

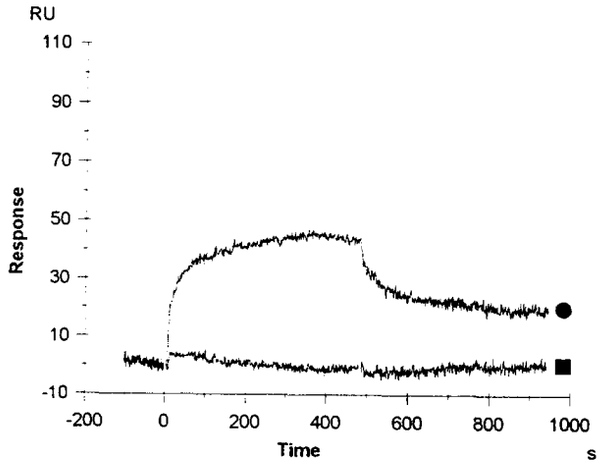

Fig. 2. SPR experiments indicate that p21Cip1 interacts with wild-type PCNA but not with the Y114A-PCNA mutant. Sensorgrams recorded after the injection of 40 μl of a 1 μM solution of wild-type PCNA (●) or Y114A PCNA mutant (■) on a sensor chip on which GST-p21Cip1 was immobilised. A control sensorgram recorded after injection of the same analytes on a blank sensor chip has been subtracted for each curve.

shown in Fig. 3A. The control peptide injected into the flow buffer had no effect on the association and dissociation constant (calculated K_D was 2.9×10^{-7} M). However, when the p21-like 20-mer peptide was used, the interaction with PCNA was completely abolished. The upward shift of the baseline is due to the difference in refringence, and is independent of the actual binding between the analyte and the ligand.

We next performed the converse experiment where p21Cip1 was immobilised on the sensor chip and PCNA was used as the analyte. A pre-incubation between PCNA and a 5 M excess of either the control or the p21-like 20-mer peptide was performed before the injection. In a control experiment, we demonstrated that the p21-like 20-mer peptide did not interact at all with immobilised p21Cip1 (not shown). As shown in Fig. 3B, the p21-like 20-mer peptide completely abolished interaction between PCNA and p21Cip1, while the control peptide had no effect.

4. Discussion

We have investigated the interaction between the cyclin-dependent kinase inhibitor p21Cip1 and proliferating cell nuclear antigen (PCNA). A recent study reported a good correlation between the strength of the interactions as predicted by the two-hybrid approach and the in vitro determination of the affinity, permitting discrimination between high-, intermediate-, and low-affinity interactions [16]. Therefore, we decided first to compare the interaction of mutant proteins with PCNA using the two-hybrid assay in *S. cerevisiae*. These results were of particular interest, since mutant ASM19, that does not bind PCNA in an in vitro assay, was found to interact efficiently in this system (36% of the wild-type). This strongly suggests that, in this type of in vivo assay, unknown stabilising partners may cooperate to allow an interaction to occur. This point should be investigated further. Mutant ASM7 was found to bind PCNA more strongly than the wild-type. As demonstrated by the determination of the K_D using SPR, this result is not due to an increase in affinity, but rather to a peculiar property of that mutant when expressed in yeast. A similar observation was made when ASM7 was overexpressed in the fission yeast *Schizosaccharomyces pombe* [17]. In that system, ASM7 mutant was shown to be more efficient at binding PCNA and consequently more active in stopping cell proliferation. The molecular basis of that result remains to be investigated.

We conclude that the two-hybrid assay does not provide a quantitative characterisation of the interaction parameters between two proteins and that it is clearly influenced by other in vivo molecular events. We therefore decided to make use of SPR technology to investigate these interactions further.

Three important conclusions can be drawn from the SPR data. (i) First, the interaction constant between PCNA and p21Cip1 could be calculated and the effects of mutations of p21Cip1 on these parameters quantitated. For instance, mutant ASM19 displayed a 100-fold lowering of the calculated

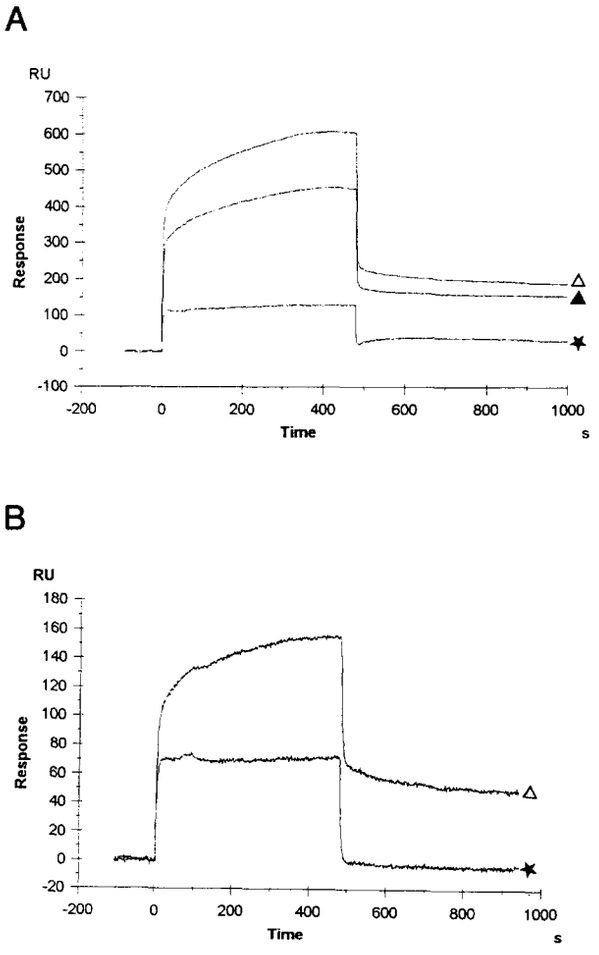

Fig. 3. SPR experiments indicate that peptides can compete in the p21Cip1-PCNA interaction. (A) The p21Cip1-like 20-mer peptide (KRRQTSMTDFYHSKRRLIFS, ★) or the unrelated peptide (YIVPEDKREMWMACIKEAA, ▲) were added (20 μM) to the buffer flow passing over a sensor chip on which PCNA was immobilised. 40 μl of a 20 μM wild-type GST-p21Cip1 solution containing 20 μM p21-like 20-mer peptide, control peptide or buffer (△) was injected. (B) A mixture of a 5-fold molar excess of peptide and PCNA (0.3 μM) was incubated for 10 min and 40 μl were injected onto a sensor chip on which GST-p21Cip1 was immobilised. (△) No peptide, (★) peptide p21Cip1-like 20-mer.

equilibrium constant as compared to wild-type p21Cip1. Both the association, k_{on}, and the dissociation, k_{off}, rate constants were affected. This result is in full agreement with the lack of interaction in the in vitro assay that we initially used to screen the mutants. However, this result demonstrates that in fact the interaction is not completely abolished, indicating that other residues of p21Cip1, such as Met-147 and Phe-150, are also important for this interaction [5]. The carboxy-terminal domain of p21Cip1, that is sufficient to bind PCNA, also displayed a lowered affinity for PCNA. This result strongly suggests that the two domains of p21Cip1 that allow separate interaction with CDK2 and PCNA are not fully independent.

(ii) Second, we clearly demonstrated that a mutant of PCNA (Y114A), that is unable to trimerise [11], does not interact at all with p21Cip1. The complete lack of interaction strongly suggests that binding of p21Cip1 to PCNA is strongly dependent upon trimerisation of PCNA.

(iii) Third, we confirm here that a 20-mer peptide that mimicks the carboxy-terminal domain of p21 is able to bind PCNA [5]. Using this peptide we clearly demonstrated that the BIAcore system can be used to perform a screen for molecules that might inhibit interaction between PCNA and p21Cip1. A similar approach could also be used to search for molecules that enhance this interaction.

In conclusion, SPR technology offers the possibility of studying the interaction parameters between biomolecules in great detail. As shown here for p21Cip1 and PCNA, it allows the calculation of association and dissociation constants that characterise the interaction, information that is not usually provided by the most commonly used assays. There are several methods for analysing the complex data collected by SPR, the most commonly used being available in BIAcore's software package. Discussions on their validity have appeared in the literature ([18] and references therein). In this report, we focused on the specific advantages of SPR as compared to other approaches used when studying interactions between cell cycle regulatory proteins. Our results clearly demonstrate that this technology provides invaluable and novel information on these interactions.

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