Binding analyses of the refolded recombinant protein of human p21(waf1/cip1) expressed in *E.coli* – an evaluation on its use in exploring the novel p21 interacting proteins by immunoprecipitation

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Abbreviation: PCNA, proliferating cell nuclear antigen, CDK, cyclin dependent kinase, IP, immunoprecipitation

Abstract

The p21 $^{(Waf1/Cip1)}$, the general inhibitor of cyclin dependent kinase (CDK), is known for its role for cell cycle arrest for DNA repair when genomic lesions occur. However, recent findings on the novel p21 $^{(Waf1/Cip1)}$ interacting proteins have suggested that p21 $^{(Waf1/Cip1)}$ may have different functions besides CDK inhibitors. To explore the novel p21 $^{(Waf1/Cip1)}$ interacting proteins, we have cloned the human p21 $^{(Waf1/Cip1)}$ cDNA into *E. coli* expression vectors to obtain the recombinant proteins from the transformed bacteria. The recombinant proteins were overexpressed and were found to be present in inclusion bodies in bacteria. However, we used a previously described scheme to denature and refold the recombinant p21 proteins and the refolded p21 was found to show the binding activities as expected from the native protein, i.e. the interactions with its well-known targets such as PCNA, CDK and cyclin D1. By using immunoprecipitation, we found that this refolded p21 protein specifically recognized more than a dozen proteins in UV-irradiated Chinese hamster (CHO-K1) cells which is p21 deficient. One of the proteins identified by the subsequent MALDI-TOF procedures was PCNA. These analyses suggest that the refolded, recombinant p21 protein is suitable for exploring the novel p21 interacting proteins with immunoprecipitation method.

Keywords: p21 $^{(Waf1/Cip1)}$, recombinant protein, CDK, PCNA, cyclin D1, interacting proteins, immunoprecipitation, MALDI-TOF.

1. Introduction

Eucaryotic cell cycle progression is regulated via the balance between cyclin-dependent kinase (CDK) activation and inhibition [1]. $p21^{(Waf1/Cip1)}$ is a universal inhibitor of CDKs in mammals. It inhibits the G1 cyclin CDK activity by interacting with cyclin-CDK complexes and the DNA replication by interacting with proliferating cell nuclear antigen (PCNA, the sliding clamp of DNA polymerase δ/ε), through its N-terminal and C-terminal domains, respectively [2-8]. p21 has been known for its role in the p53 checkpoint control which monitors the genomic integrity of cells [9]. The expression of p21 can be induced by p53 to arrest cell cycle for DNA repair when cells are inflicted with DNA lesions by either internal or external factors.

Interestingly, recent discoveries found that p21 has new activities that are unrelated to its function as CDK inhibitors. For instance, p21 can interact with procaspase-3 to inhibit caspase-3 activation and to resist Fas-mediated cell death [10]. A study using a yeast-two hybrid system has found that p21 interacts with a zinc finger, nuclear protein, Ciz1which may regulate the cellular localization of p21[11]. Besides, p21 can interact with the transcription activator STAT3 (signal transducers and activators of transcription) and the interaction blocks the STAT induction of gene expression in response to cytokine receptor stimulation [12]. STAT proteins are in cytoplasm before the receptor is stimulated. Following stimulation, STAT proteins dimerize, translocate into the nucleus, and activate specific target genes. In addition, the protooncogene product c-Myc has been found to bind to p21 via the PCNA-binding region and hence relieves PCNA from p21 for DNA replication, meanwhile the E-box-dependent transcription by Myc-Max is blocked [13]. Moreover, p21 forms a complex with Rho-kinase and inhibits its activity in vitro and in

vivo [14]. Rho-kinase plays important roles in stress fiber, focal adhesion formation, smooth muscle contraction, cytokinesis, and neurite retraction.

Therefore, the p21 protein seems to participate in complicated and unexpected regulatory circuits and it is likely that more novel p21 interacting proteins may remain to be discovered.

In this report, we describe the binding activities of the refolded recombinant human $p21^{(Waf1/Cip1)}$ protein which was obtained as inclusion bodies from the transformed bacteria and subsequently undergone the denaturing and refolding scheme. Our purpose was to evaluate if the recombinant human p21 protein is suitable for identifying novel p21 interacting proteins. Although the bacterially expressed recombinant p21 proteins have been described before [15], its use of this kind has not been reported. Our study indicates that the refolded recombinant human p21 protein could recognize the cellular PCNA, cyclin D1 and CDK2 in the cell extracts prepared from Chinese hamster cells. More interestingly, the recombinant p21 protein appeared to specifically recognize more than a dozen proteins in UV-irradiated hamster cells. Our previous studies have indicated that UV induces substantial apoptosis in these cells and the cells are p21 deficient.

2. Materials and methods

2.1 Construction of the bacterial expression vectors of human $p21^{(Waf1/Cip1)}$ and the recombinant protein expression and purification

The human $p21^{(Waf1/Cip1)}$ cDNA clone [16] was used as the template and a pair primers (forward 5’-cccggatccATGTCAGAACCGGCT-3’ and reverse 5’-cccgtcgacTTAGGGCTTCCTCTT-3’) for polymerase chain reaction to amplify the

DNA fragment for cloning the gene in full-length into the bacterial expression vector pET-30a or pET-32a (Novagen) to give pET-30a-p21(or pET-32a-p21). In the above-mentioned primer sequences, the regions of p21 were in capital letters and the cloning sites, i.e. Bam H1 and SalI were underlined. The fidelity of the cloned p21 sequences in expression vectors was proved by DNA sequencing (not shown). *E. coli* strain (BL21 DE3) was transformed with the p21 expression vectors and the transformed bacteria were used for expressing the recombinant p21 proteins. The recombinant p21 proteins were induced in the transformed bacteria with IPTG at 37 ^{o}C for 3 h or under the specified conditions. The induced-culture was centrifuged at 6000 rpm for 15 min, and the pellet was resuspended in 5 ml ice-cold lysis buffer (10 mM Tris-HCl, pH 8.8 and 0.1 mM lysozyme) for 1 h on ice. Aliquots of 10 mM Tris-HCl , pH 8.8 solution were added to give a final volume of 25 ml. The French-Press was then performed twice to breakdown the cell walls and the total cell lysate was centrifuged at 6000 rpm for 15 min. The soluble and pellet fractions were separated and tested for the presence of p21 protein with 12 % SDS- polyacrylamide gel electrophoresis and Western blotting analysis using anti-p21 antibody (Santa Cruz, sc-6264). As most of the recombinant p21 proteins were in pellet fraction, the pellets were collected and dissolved in 4.5 M urea with 10 mM Tris base and 0.1 M β- mercaptoethanol (β-ME) and the His-tag containing recombinant proteins were further purified with nickel-column affinity chromatography.

2.2 Denaturation and renaturation of recombinant human p21

The procedures were performed according to those described previously [17, 18,]. The protein solution obtained from nickel-column affinity chromatography was dialyzed

against buffer 1 (10 mM Tris base, pH 11, 1 M urea, 0.1 mM β-ME and 5% glycerol) for 72 h at 4 ^{o}C. The urea was then removed by dialysis against buffer 2 (10 mM Tris base, pH 11 , 0.1 mM β-ME and 5% glycerol) for 24 h at 4 ^{o}C, To neutralize the pH, the refolding mixture was dialyzed against buffer 3 (10 mM Tris base, pH 8.8, 0.1 mM β-ME and 5% glycerol) for 24 h at 4 ^{o}C. Finally, glycerol was removed by dialysis against buffer 4 (10 mM Tris base, pH 8.8, 0.1 mM β-ME) for 4 h at 4 ^{o}C.

2.3 Immunoprecipitation

The immunoprecipitation procedures described previously [19], were adopted. For a typical assay, 1-2 μg of the recombinant p21 protein was added with 300 μl of total cell lysate (about 500- 700 μg proteins), 200 μl of dH_2O and 500 μl of 2 x immunoprecipitation (IP) buffer (2 X IP buffer : 2% Triton X-100, 300 mM NaCl, 20 mM Tris-HCl pH 7.4, 2 mM EDTA pH 8.0, 2mM EGTA, 0.4mM PMSF, 1% NP-40). The mixtures were incubated at 4°C for 1.5 h. Then, 2 μg of primary antibody was added to the mixtures and incubated at 4 ^{o}C for 1.5 h with constant rotation. After that, 50 μl of 10 % protein A-agarose (Amersham) was added to the mixture and incubated with agitation for 1.5 h at 4°C. The mixture was then centrifuged with 5000 g for 5 min, and the supernatant was discarded. The remaining pellets were washed with 1 ml of 1 ×IP buffer for 3 times. The pellets were then resuspended with 20 μl of 2 × SDS sample buffer (8% SDS, 40% glycerol, 0.2% bromophenol blue, 20% β-ME). Finally, the pellets were boiled for 15 min, and mixture was then centrifuged to separate the insoluble particles from the solution which was saved for subsequent analysis by 12 % SDS-PAGE and Western blotting analysis. For Western analyses the antibodies: anti-PCNA (Oncogene,

NA03-200UG), anti-p21 (Santa Cruz, sc-6264), anti-cdk2 (Santa Cruz, sc-6248), anti-cyclin D1 (Santa Cruz, sc-8396) and anti-actin (Santa Cruz, sc-1616) were used.

3. Results

3.1 The recombinant human p21 $^{(Waf1/Cip1)}$ protein from E. coli.

The recombinant protein of human p21 cloned in pET30a vector was induced by IPTG in transformed *E. coli* as illustrated in Fig 1A (top panel). The induced-protein was confirmed to be p21 by the Western blotting using anti-p21 antibody (Fig 1A, bottom panel). It was noted that besides the major protein a slightly smaller protein in less abundance was also recognized by the p21 antibody. This minor species was also seen even when different vectors (e.g. pET32a) was used, and it was co-purified with the major band during affinity chromatography purification with nickel column (see Fig 1C), suggesting that the minor protein contained the His_6-tag. And since the His_6-tag was (in theory) fused with the recombinant p21 protein at the N-terminal end, the minor protein was likely a truncated version of p21 with a deletion at C-terminal (see more in discussion).

While to isolate the recombinant p21 protein, we noted that most of the proteins of interest were in the form of inclusion bodies. Only few of the proteins were in the soluble fraction even if the induction was performed at conditions thought to be in favor of the soluble form, for instance, induction with low concentrations of IPTG and/or at low temperatures (see Fig 1B). We also had tried different vectors, for instance, pET-32a but we found that the including of thioredoxin to the recombinant protein did not improve the solubility of the recombinant proteins. Since the soluble recombinant p21 proteins were

too few in quantity for our further biochemical analyses, we decided to harvest the inclusion bodies and applied the denaturing-refolding procedures described previously [17] to obtain the soluble, refolded recombinant p21 proteins. For this end, the pellets of recombinant protein were first solubilized into urea containing buffer and purified with nickel column as illustrated in Fig 1C, then the purified proteins were subjected to a sequential series of denaturation and refolding as described in the materials and methods. To examine if the refolded protein had any secondary structures, the circular dichroism spectra of the recombinant p21 was collected (Fig 2A) and analyzed by the SELCON3 program with the CA reference (Fig 2B). This analysis gave 9.9% of the α-helix, 35.5% of β-sheet, 22.6% of turn and 32.1% of unordered structure, which is not similar to those described in the previous report [15]. Nevertheless, both studies agree that the recombinant p21 protein had low portion of α-helix.

3.2 The recombinant human p21 $^{(Waf1/Cip1)}$ protein bound PCNA, CDK2 and Cyclin D1

It has been known that p21 $^{(Waf1/Cip1)}$ binds PCNA at its C-terminal domain, and interacts with cyclin and CDK at N-terminal domain (see Fig 3A). To examine if the refolded recombinant p21 protein could bind PCNA, we performed the immunoprecipitation (IP) experiments using the crude lysate of hamster cells (CHO-K1). Previously, we have found that p21 is absent in CHO-K1 cells at either normal or UV-irradiated conditions [20,21]. Hence, there would be no endogenous p21 to compete with the recombinant p21 for PCNA. In the IP experiments, cell lysate was mixed with the recombinant p21 protein and the proteins interacting with recombinant p21 protein were pulled-down by anti-p21 antibody and the subsequent binding of protein-A

conjugated with agarose. The presence of PCNA in the proteins pulled-down was analyzed in Western-blotting using anti-PCNA antibody. The results shown in Fig 3B (top panel) indicate that the hamster PCNA was recognized by the recombinant p21. As compared lanes 1 and 4, PCNA was not detected in the pellets unless the anti-p21 antibody was included. The amount of PCNA pulled-down was apparently dependent on the cell lysate as compared the lanes 1 and 2. Interestingly, the heat treated-p21 lost the binding of PCNA significantly (see lanes 1 and 3). As a positive control, lane 5 shows the direct blotting of PCNA in the lysate. The interaction between PCNA and the recombinant p21 was also shown by the reciprocal IP, i.e. immunoprecipitation of PCNA first with anti-PCNA antibody and then Western blotting for the recombinant p21 (see lane 1, bottom panel of Fig 3B). As a negative control, IP with anti-actin antibody did not co-precipitate p21 (see lane 2).

To see if the recombinant p21could bind cyclin D1/CDK2 complex, a similar experiment was performed with CHO-K1 cell lysate , i.e. the IP first with anti-p21 antibody then Western blotting analysis for the presence of CDK2. As expected, the recombinant p21 bound CDK2 (see Fig 3C, top panel). Furthermore, this binding was verified with the reciprocal IP, i.e. IP with anti-CDK2 or anti-Cyclin D1 first, then Western analysis for the presence of recombinant p21 protein (Fig 3C, bottom panel). When antibodies against CDK2 or cyclin D1 were used to immunoprecipitate CDK2 or cyclin D1, p21 was detected in the subsequent Western analysis (lanes 1 and 2, Fig 3C bottom). It was noted that although the recombinant p21 proteins in the reaction contained major and minor bands (see lane 4), the minor band was preferentially bound

by the cellular CDK2 or cyclin D1 (lanes 1 and 2). As mentioned earlier, the minor band was likely the truncated version of recombinant p21 at C-terminal. .

3.3 The use of recombinant human p21 (Waf1/Cip1) protein to identify its interacting proteins in mammalian cells

The results described above suggest that the recombinant human p21 protein seemed to be suitable for exploring the p21 interacting proteins. Since we have found, in our previous study that UV irradiation fails to induce p21 in CHO-K1 cells and the absence of p21 plays a critical role in the UV-induced apoptosis, we suspected that the p21 target proteins but not bound by the p21 due to the absence of p21 might be crucial to the UV-induced apoptosis. Thus, we performed the immunoprecipitation assay with the recombinant p21 and the cell lysates prepared from the UV-irradiated CHO-K1 cells to search for the proteins. Typical results were shown in Fig 4, where the proteins co-precipitated with p21 were separated by SDS-PAGE and revealed by the silver staining. By comparing the patterns between lanes 4 and 5-7 (Fig 4), we could see many specific bands presumably the proteins interacting directly or indirectly with the recombinant p21. For identification, we arbitrarily divided these proteins into 3 groups (see the vertical lines between lanes 7 and 8). Although some of these proteins seemed to be present in the cells even those without UV treatment, some distinctive proteins among the groups were only seen in UV-irradiated cells, especially at later hours after irradiation (lane 7). We estimated total about 20 proteins were recognized by the recombinant p21 in cells at this condition.

Several p21 (Waf1/Cip1) interaction proteins were undergone the identification procedures with MALDI TOF MS. One of these proteins (encircled by block, Fig 5) has been undergone all the preparative procedures including the in-gel digestion and mass spectrometry before further analyzed by the Bio Tools program (from BRUKER DALTONIOS) according to the rat database of NCBI reference and was shown to be very similar to rat PCNA (Fig 5). Though the score was not high, it may be due to variation between rat and hamster PCNA. At the moment, database of hamster proteins remains incomplete.

4. Discussion

The general CDK inhibitor, $p21^{(Waf1/Cip1)}$ has been known for its role in cell cycle arrest for repairing the genomic damages. Although CDK and PCNA are well known targets of p21, other cellular proteins might also interact directly or indirectly with p21 to modulate the cell cycle arrest, DNA repair, cell death or others such as cell differentiation. In this study, we have tried to explore these novel p21 interacting proteins by using the recombinant protein of p21 obtained from bacterial and the cell lysate from Chinese hamster ovary cells (CHO-K1) which are p21 deficient even under UV irradiation. Although in this report, we did not identify novel p21 target protein, we showed the evidence that the system we used had great potential in identifying these target proteins.

Although we found the recombinant p21 proteins expressed in E. coli with pET expressing vectors were in inclusion bodies, we have adopted a denature-refolding scheme described previously by one of the authors and have obtained in good quantities the soluble recombinant p21 protein. The method of denature-refolding scheme has been

successfully applied to a number of studies with a variety of system including mammals [22-24]. Although we are not sure how similar this refolded protein was similar to the native protein, the refolded protein appeared to posses the binding activities expected for native protein. We also noted that there were two forms of the recombinant p21 produced in the bacteria. One was the full-length p21, the other, we reckoned, as the C-terminal truncated version of p21. At this moment, we do not have a sound theory to interpret the genesis of the truncated p21 protein, nor do we know where exactly the truncation took place, nevertheless, we found this truncated p21 was preferentially recognized by CDK2 or cyclin D1 (bottom, Fig 3C). This preferential binding may be due to the absence of C-terminal, which hindered the binding of PCNA and yet facilitated the access of CDK2 or cyclin D1, which bind to the p21 at the N-terminal. This explanation is consistent with the observation that PCNA only bound with the full-length p21 but not the C-terminal truncated protein (bottom, Fig 3B). The PCNA binding domain is located at C-terminal of p21. These observations gave us a lesson to explore the novel p21 target proteins in a more effective way. Since one of the most common obstacles of identifying novel proteins by MALDI TOF MS method is the problem to collect enough protein for analysis, one could enrich the proteins of interest by immunodepletion or other suitable methods to reduce PCNA or CDK to avoid the competition of p21 (bat) by these abundant targets.

It is also worth mentioning the CHO-K1 cells we used for the source of cell lysate in immunoprecipitation (IP). As we mentioned earlier, this cell line does not express p21 when the cell is UV-irradiated. For doing IP with the recombinant p21, the endogenous p21 is undesirable. Thus, the cell lysate from this hamster cell line is superior as the

endogenous p21 is concerned. The only drawback is the current deficiency of hamster protein database as compared to others such as human, mouse or rat. This was illustrated by our experience: one of the hamster proteins picked by the recombinant p21 protein was identified by MALDI TOF MS to be similar to rat PCNA; although PCNA was listed to be the most likely candidate, the score similar to rat PCNA was not impressive. We reckon that the deficiency of hamster protein database may be improved as more studies involve the hamster cells. Nonetheless, we found in our experiment that a great numbers of proteins in the UV-irradiated or non-irradiated hamster cells were picked by the recombinant p21. There are about 20 different proteins recognized by p21 in cells 15 h after UV radiation. About 50% of UV-irradiated cells enter apoptosis at 24 h following UV. Thus, potentially these proteins are involved in determining the cell survival or death in either direct or indirect interaction with p21.

In summary, we have found that the refolded, recombinant human p21 protein expressed in *E. coli*, seems to be suitable for proteomic search for the cellular novel p21 interacting proteins by immunoprecipitation method with lysate obtained from p21 deficient cells such as Chinese hamster ovary (CHO-K1) cells.

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Figure legends

Fig 1. Expression of the recombinant protein of human p21 (Waf1/Cip1). (A) SDS-PAGE analysis of the crude extracts from E. coli cells with pET-30a (lanes 1 and 2) or pET-30a-p21 plasmid (lanes 3 and 4). The recombinant protein of human p21 (marked with **) was induced with 2 mM IPTG. (*bottom panel*) Western blotting analysis of the recombinant protein of human p21 with anti-p21 antibody(Santa Cruz, sc-6264). (B) The recombinant protein of human p21 was expressed mainly as insoluble bodies in E coli. (*top*) Induction of the recombinant protein of p21 was done at 16 ^{o}C with 0.1 mM IPTG for 0, 3, 6, 9, 12 or 15 h, and the total cell lysates (lanes 2-7) or their soluble fractions (lanes 8-13) were examined for the presence of the recombinant protein. Lane M, protein marker. Lane 1, BSA. (*bottom*) Western blotting of recombinant p21. (C) Affinity column chromatography of the recombinant p21 with nickel column. The crude proteins were applied to the column and proteins eluted with imidazole of gradient concentrations after thorough wash. The proteins eluted at each fraction (1-17) of chromatography were reveal by SDS-PAGE and stained with commasie blue. It was noted the major band was co-purified with the major one.

Fig 2. The circular dichroism spectroscopy of the refolded, recombinant p21. CD spectra in the UV region (260–200 nm) were recorded on a Jasco J 720C spectropolarimeter at 20 °C. A 0.1 cm-light-path cuvette was used to reduce the light scattering of the solution. Data are expressed in molar ellipticity ( )(deg cm_2 $dmol^{-1}$), calculated based on the mean residue weight 110.55 dalton for the recombinant

$p21^{(Waf1/Cip1)}$. The solvent contribution on CD was subtracted from each spectrum. The component secondary structures—i.e., α-helix, distorted α-helix, β-sheet, distorted β-sheet, turns, and unordered—were analyzed by the singular value decomposition algorithm provided by the SELCON3 program as described in [18].

Fig 3. (A) the p21 domain structure. (B) The recombinant p21 (Waf1/Cip1) could bind the recombinant PCNA. *Top*: about 1 μg of the recombinant p21 (Waf1/Cip1) was mixed with the different amount of the cell extracts, and the mixtures were incubated at 4°C for 1.5 h. The mixtures were IP with anti-p21 mAb, and the precipitates were analyzed by Western blotting with the anti-PCNA mAb. Lane 1, IP mixture contained 600 μg of cell extracts; lane 2, IP mixture contained 200 μg of cell extracts; lane 3, similar to lane 1 except the recombinant p21 was heat denatured at 100 °C for 15 min; lane 4, similar to lane 1 except the anti-p21 mAb was omitted; lane 5, direct Western blotting of PCNA, with 50 μg of cell extracts. *Bottom*: The mixtures were IP with anti-PCNA mAb. And the precipitates were analyzed by Western blotting with the anti-p21mAb .Lane 1, the anti-PCNA mAB was added to the mixtures; lane 2, similar to lane 1 except the anti-PCNA mAb was omitted; lane 3, direct western blotting with 0.5 μg of the recombinant p21. (C) *top*: similar to that described in (B, *top* panel) except the immunoprecipitates were analyzed by Western blotting with the anti-CDK2 mAb (lane 1). Lane 2, the anti-p21 antibody was omitted; lane 3, direct Western blotting of CDK2. *Bottom*: similar to that described in (B, *bottom* panel) except the anti-CDK2 (lane1), anti-cyclin D1(lane 2) or anti-actin (lane 3) was used first before western

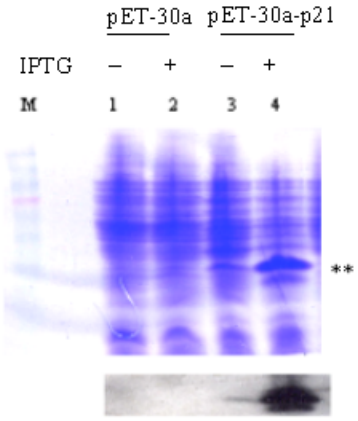
blotting of p21. Lane 4: direct Western blotting of the recombinant p21.

Fig 4. Proteins of CHO-K1 cells co-immunoprecipitated with the recombinant p21. The proteins co-precipitated with the recombinant p21 were separated by SDS-PAGE and revealed by silver staining. Lane 1, direct loading of an aliquot of cell lysate (about 5 μg) from log-phased cells; lane 2, direct loading of anti-p21 antibody (about 0.2 μg); lane 3, direct loading 1 μg recombinant p21; lane 4, IP assay, the mixture contained no cell lysate only recombinant p21 protein (1 μg)and anti-p21 antibody (2 μg); lane 5, similar to lane 4 except the IP mixture contained 1 mg of cell lysate from log-phased cells; lane 6, similar to lane 5 except the IP mixture contained 1 mg of cell lysate from UV-irradiated cells (8 h after 25 J/m^2 UVC); lane 7, similar to lane 6 except the IP mixture contained 1 mg of cell lysate from UV-irradiated cells (15 h after 25 UV);. lane 8, similar to lane 5 except anti-p21 antibody was omitted. The specific proteins co-precipitated with the recombinant p21 were marked with the vertical bars. The protein band encircled was identified to be PCNA by use of MALDI-TOF described in Fig 5.

Fig 5. Identification of the protein band encircled (Fig 4) by use of MALDI-TOF. The protein bands of interest were excised and undergone in gel digestion before analyzed with a Bruker ultraflex MALDI-TOF mass spectrometer (Bruker Daltonics) in positive ion mode. (A) MALDI-TOF spectra. (B) similarity search for the protein of interest according to rat database of NCBI.

Fig 1

(A)


pET-30a
pET-30a-p21
IPTG
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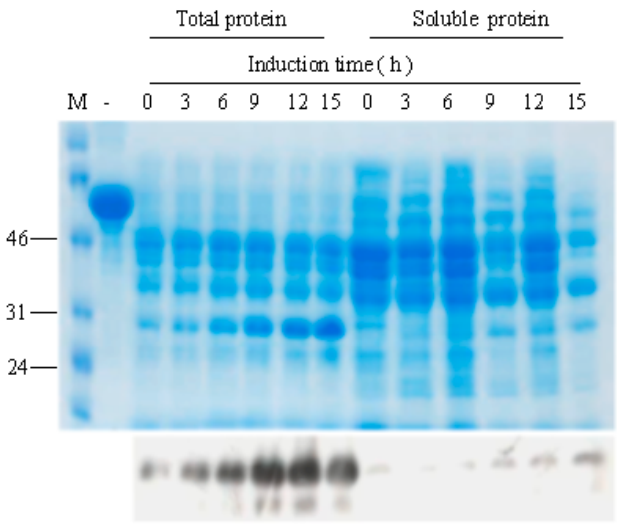
**

(B)


Total protein
Soluble protein
Induction time (h)
M
-
0
3
6
9
12
15
0
3
6
9
12
15
46
31
24

(C)

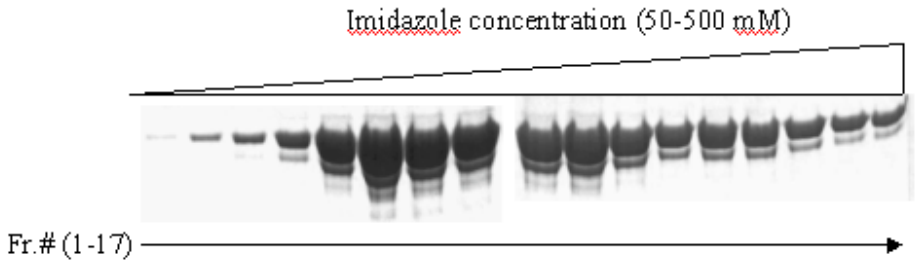
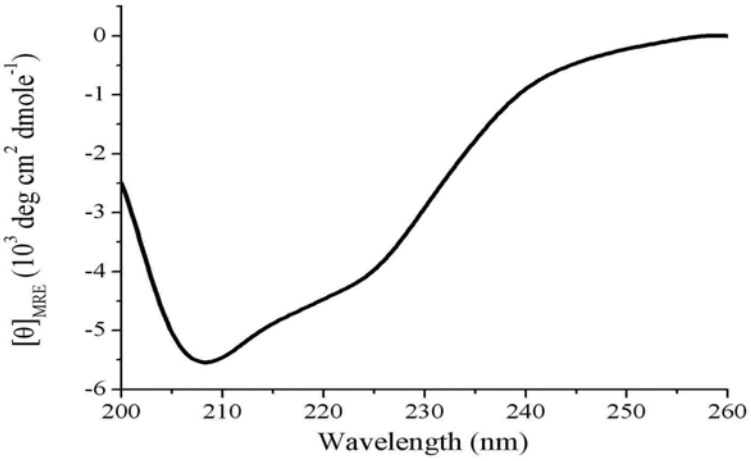

Imidazole concentration (50-500 mM)
Fr.# (1-17)

Fig 2

(A)


0
-1
-2
-3
-4
-5
-6
200
210
220
230
240
250
260
Wavelength (nm)
$[\theta]_{MRE}$ (10^3 deg cm^2 dmole^{-1})

(B)

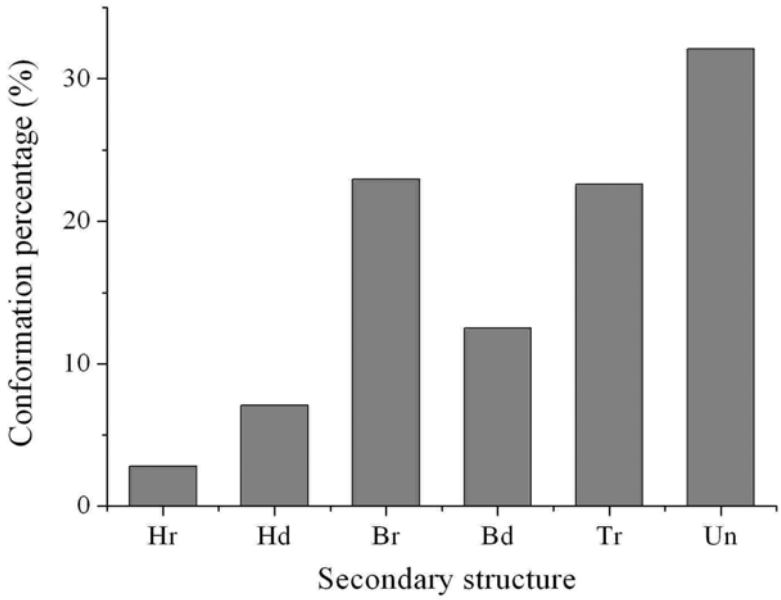
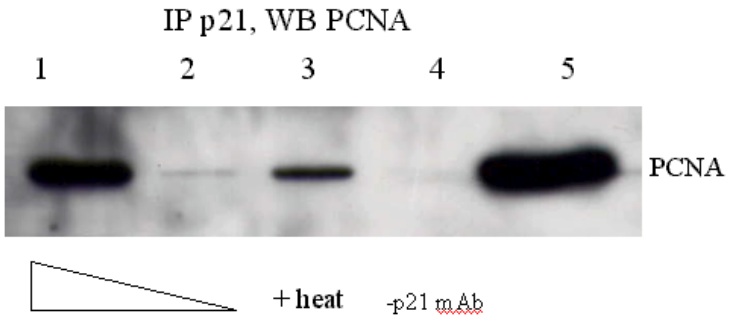
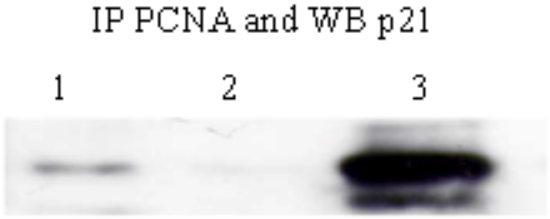

30
20
10
0
Hr
Hd
Br
Bd
Tr
Un
Secondary structure
Conformation percentage (%)

Fig 3

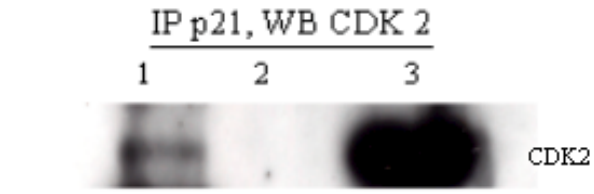
(A) p21 binding domains

(B)





(C)



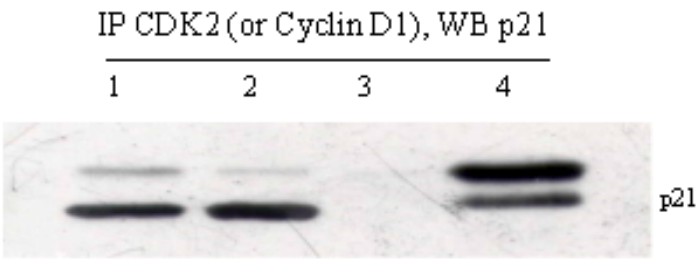


Fig 4

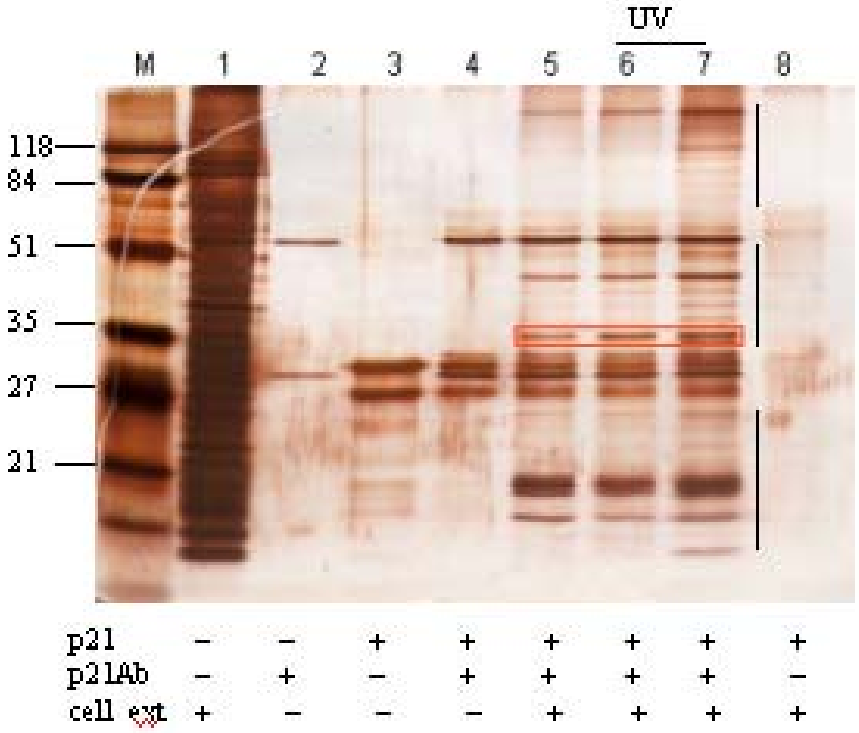
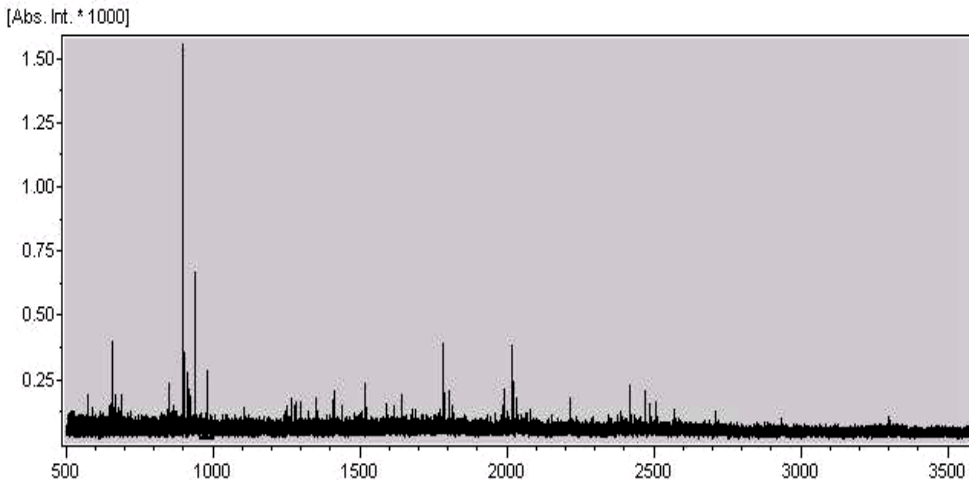


Fig 5

(A)



(B)

Index

	Accession	Mass	Score	Description
1.	gi|11693142	28730	49	proliferating cell nuclear antigen [Rattus norvegicus
2.	gi|27703480	15459	38	similar to glyceraldehyde 3-phosphate dehydrogenase |
3.	gi|27665532	21886	34	hypothetical protein XP_243713 [Rattus norvegicus]
4.	gi|27721583	19743	34	similar to cathelin-related protein precursor - mouse
5.	gi|477866	10191	33	MHC beta chain - rat (fragment)
6.	gi|27730721	22125	33	similar to hypothetical protein FLJ14345 [Homo sapier
7.	gi|27658904	12995	33	similar to ATP-binding cassette, sub-family E (OABP),
8.	gi|71829	49488	33	fibrinogen gamma-A chain precursor - rat
9.	gi|27686907	13226	32	similar to ribosomal protein S13, cytosolic [validate
10.	gi|27705160	22217	32	similar to chromosome 20 open reading frame 86 [Homo