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A study of substrate specificity for a CTD phosphatase, SCP1, by proteomic screening of binding partners

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ABSTRACT

RNA polymerase II carboxyl-terminal domain (RNAPII CTD) phosphatases are a newly emerging family of phosphatases. Recently a CTD-specific phosphatase, small CTD phosphatase 1 (SCP1), has shown to act as an evolutionarily conserved transcriptional corepressor for inhibiting neuronal gene transcription in non-neuronal cells. In this study, using the established NIH/3T3 and HEK293T cells, which are expressing human SCP1 proteins under the tight control of expression by doxycycline, a proteomic screening was conducted to identify the binding partners for SCP1. Although the present findings provide the possibility for new avenues to provide to a better understanding of cellular physiology of SCP1, now these proteomic and some immunological approaches for SCP1 interactome might not represent the accurate physiological relevance in vivo. In this presentation, we focus the substrate specificity to delineate an appearance of the dephosphorylation reaction catalyzed by SCP1 phosphatase. We compared the phosphorylated sequences of the immunologically confirmed binding partners with SCP1 searched in HPRD. We found the similar sequences from CdcA3 and validated the efficiency of enzymatic catalysis for synthetic phosphopeptides the recombinant SCP1. This approach led to the identification of several interacting partners with SCP1. We suggest that CdcA3 could be an enzymatic substrate for SCP1 and that SCP1 might have the relationship with cell cycle regulation through enzymatic activity against CdcA3.

1. Introduction

The evolution of eukaryotes was accompanied by the development of specialized forms of RNA polymerases [1]. Among these eukaryotic RNAPs, RNAPII is the key component in the transcription apparatus for synthesis of mRNA and its regulation [2]. In addition, eukaryotic RNAPII keeps an unusual consensus heptapeptide sequence ($^{1}Y^{2}S^{3}P^{4}T^{5}S^{6}P^{7}S$) tandem repeated at its carboxyl terminus that is not present in other RNA polymerases [3]. Also, it is well known that coordinately regulated phosphorylation and dephosphorylation of the CTD is not only essential for the recruitment and assembly of transcription complexes but also temporally controls transcription and mRNA processing during the cell cycle [4]. It is thought to act as a scaffold to coordinate the binding of proteins involved in the different phases of transcription and couples transcription with other nuclear process such as mRNA maturation. These physiological events are largely mediated through the participation of proline-directed kinases such as cyclin-dependent kinases. And several phosphatases have been implicated in the removal of phosphates from the CTD, thereby mediating transitions in the transcription cycle [5,6]. A systematic approach examining the genome-wide distribution of CTD modifications indicated considerable interplay between CTD kinases and phosphatases, suggesting that transcription operates in a uniform mode at virtually all genes [7].

In higher eukaryotes, small CTD phosphatases (SCPs) were described, which contain a catalytic FCPH domain with Mg^{+2} binding DXDX(T/V) motif but lack a BRCT domain [8]. SCPs preferentially reverse the phosphorylation of ^{5}Ser residues of the CTD and its attraction towards the specific substrate [9]. SCPs are related to the catalytic subunit of FCP1, the 1st identified CTD phosphatase, which is highly conserved, essential enzyme for dephosphorylating the CTD of RNAPII. SCPs are also transcriptional regulators that silence neuronal genes in non-neuronal tissue [10]. Whereas the catalytic mechanisms of SCPs and the basis for

Abbreviations: RNAPII, RNA polymerase II; CTD, C-terminal domain; *p*NPP, *para*-nitrophenyl phosphate; SCP, small CTD phosphatase; CTD-SPL, CTD small phosphatase like protein; TIP120A, TATA-binding protein interacting protein 120; CdcA3, Cell division cycle-associated protein 3; UTF1, undifferentiated embryonic cell transcription factor 1.

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their CTD specificity are well understood [11], the identity of substrates that could be in explanation of the biological activities of SCPs has remained elusive. We have established NIH/3T3 and HEK293T cells carrying an inducible form of SCP1 (wild-type and D96N mutant forms) and analyzed interacting proteins with SCP1 in order to identify plausible substrates of it.

Although some proteins act primarily as single monomeric units, a significant portion of all proteins function in association with partner molecules or as components of large molecular assemblies or enzyme-substrate complexes. Thus, the protein complexes are critical in fulfilling the various cellular function or enzyme reaction mechanism. Although it has become clear that no single technique would be sufficient to meet all the requirements of proteomics-based investigations, to date proteomics techniques are applied for the investigation of protein-protein interactions, protein expression profile, and also protein quantification and have become more and more accepted as promising techniques in various research [12]. Some of proteomics results can be used to confirm interactions, which have already been assumed before, but others have delivered a huge number of novel and interesting correlations that might help in the development of novel strategies for the protein-protein interactions especially enzyme-substrate interactions. Some observations indicate that phosphatases, like kinases, use protein interaction to modulate the function of their catalytic domains and to select substrates [13]. Furthermore, phosphatases select their substrates by a combination of enzymatic specificity and spatial organization, mediated by the protein interaction.

In this new study, SCP1 was found to interact with cell division cycle-associated protein 3 (CdcA3, also designated as TOME1), which is involved in ubiquitination and degradation of the mitosis-inhibitory kinase, Wee1, leading to the dephosphorylation of Cdc2 in the late G2 phase [14], using the inducible expression system and a proteomic approach that combined coimmunoprecipitation, SDS-PAGE, and identification of interacting partner protein by mass spectrometry. In addition, the kinetic analysis of SCP1 against phosphopeptides of CdcA3 supported the identification of CdcA3 as a plausible substrate of SCP1. Finally, we arrived at some new insights regarding enzymatic clues for unraveling the enzyme specificity of a CTD phosphatase, SCP1.

2. Materials and methods

2.1. Cell culture and establishment of inducible SCP1-expressing cell lines

NIH/3T3 mouse embryonic fibroblasts and HEK293T human kidney epithelial cells were brought from ATCC (Bethesda, MD, USA) maintained as recommended by supplier supplemented with antibiotics in a humidified incubator at 37 °C with 5% CO_2. Tetracycline-inducible cell lines for specific protein expression were established as previously described [15,16]. The expressed SCP1 (wild type and D96N mutant) was tagged with Flag sequence in the N-terminal region of the cDNA.

2.2. Preparation of recombinant SCP1 and its purification

The pET 21a(+)/ΔN SCP1$^{76\text{-}261}$/His vector was introduced into *Escherichia coli* Rosetta 2 (DE3) strain. After an OD_{600} value of 0.6 was achieved, the *E. coli* culture was transferred to pre-cooled incubator at 16 °C and the recombinant protein was induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside. The recombinant SCP1 was expressed and purified by using a protocol previously described [11].

2.3. Immunoblot analysis and immunoprecipitation assays

Proteomic screenings were performed using immunocomplexes which were immunoprecipitated from total cell lysates of induced and non-induced NIH/3T3/Wt SCP1 and HEK293T/Wt SCP1 cells inducibly expressing SCP1. Cells were lysed using a lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100 containing protease and phosphatase inhibitor cocktails (Roche, Manheim, Germany) or RIPA buffer containing protease and phosphatase inhibitors. The procedures for Western analysis and immunoprecipitation assays were essentially performed as previously described [17]. Primary antibodies used were as follows: mouse anti-Flag (M2 clone, Sigma-Aldrich, St. Louis, MO, USA), rat anti-DYKDDDDK (BioLegend Inc., San Diego, CA, USA), mouse anti-HSP 90αβ (Santa Cruz Biotech. Inc., Santa Cruz, CA, USA), rabbit anti-prohibitin (Santa Cruz), mouse anti-TIP120A (IgM, Santa Cruz), rabbit anti-CTD-SPL (Santa Cruz), rabbit anti-CdcA3 (Santa Cruz), and rabbit anti-α-tubulin (AbClone Inc., Seoul, Korea).

2.4. In gel trypsin digestion, mass spectrometry analysis and database searching

The adapted in-gel digestion procedure and LC–ESI-MS/MS analysis were performed as previously described [18]. Peak lists of MS/MS spectra were processed using Analyst QS (v.1.1, Applied Biosystems, Foster City, CA, USA) software and searched against the International Protein Index (IPI) protein database version 3.20 (European Bioinformatics Institute, Hinxton, UK), the NCBI-nonredundant, and EST and other databases using Mascot operating on a local server [19].

2.5. Synthesis of phosphopeptides

The phosphopeptides generated from CdcA3 and CTD based on the PTM position cluster predicted by PTMCluster tool were synthesized by FMOC solid phase peptide synthesis using ASP48S and purified by the reverse phase HPLC using a Vydac Everest C_{18} column (Peptron Inc., Daejeon, Korea). The sequences of the synthetic phosphopeptides were ^{25}ADPR^{29}S^{p}PSA^{33}G (designated as ^{29}S) and ^{64}SDPR^{68}S^{p}PTL^{72}G (^{68}S). The characters S^{p} in peptides are corresponding the phosphorylated serine residues. The peptide sequences are designed and synthesized in terms of referring to the C-terminal domain of RNAPII (YSPT^{5}S^{p}PSYS) [20]. Previously, the specificity determinants of SCP1 were demonstrated by the structural analysis for phospho-^{5}Ser-directed dephosphorylation [11].

2.6. Phosphatase assays

SCP1-catalyzed dephosphorylation of phosphorylated substrate was performed as previously described with minor modifications [21]. Assays were conducted for 10 min at 37 °C in a buffer consisting of 50 mM sodium acetate (pH 5.5), 20 mM $MgCl_2$, 5 μM–1 mM synthetic phosphopeptides and 5 ng of wild-type or D96N mutant recombinant SCP1. Phosphate release was quantitated by using a malachite green-based colorimetric assay for inorganic phosphate by measuring the absorbance at 620 nm. The malachite green solution and inorganic phosphate standards were prepared as described [22]. To derive K_M and k_{cat} values, the data were fit by nonlinear regression to the Michaelis–Menten equation.

3. Results and discussion

3.1. Establishment of NIH/3T3 and HEK293T cell lines for SCP1

After transfection of human wild type and D96N mutant SCP1, a dominant negative form, cloned into an inducible vector [15,16],

several candidates were primarily chosen based on their antibiotics selection (10 μg/ml puromycin) and on their microscopic appearance for enhanced green fluorescence protein, and were finally selected using immunoblot analysis with anti-Flag antibodies in all of NIH/3T3 (Fig. 1A) and HEK293T cells (Fig. 1B). An increase in SCP1 expression was only shown in response to doxycycline depending on the induction time. However, after treatment of doxycycline for the indicated periods of time, when the cells were replated on plastic culture dishes with fresh medium without inducer, the SCP1 reverted to no expression (Fig. 1A and B). Thus, its expression was under the tight control of inducer and is a suitable, unique, and useful system for identifying SCP1 interactome. The established wild-type and D96N mutant SCP1 inducible NIH/3T3 and HEK293T cells were designated as NIH/3T3/Wt SCP1, NIH/3T3/D96N SCP1, HEK293T/Wt SCP1 and HEK293T/D96N SCP1, respectively. In addition, the immunoprecipitation analysis of 1 mg of these total cell lysates with anti-Flag antibody were also not shown any positive signal from the non-induced cell lines (Fig. 1C). The phosphatase activities of the immunocomplexes from the induced expressed wild type SCP1 was measured by showing the enzymatic activity against the *para*-nitrophenyl phosphate (pNPP) [21], while those from the D96N mutant SCP1 inducible cell lines of NIH/3T3 and HEK293T were not shown. Together, these data demonstrate that our inducible cell lines are effective tools for dissecting the cellular and physiological functions of each SCP1 protein.

3.2. Purification of immunocomplexes and identification of co-purifying proteins in the interactome of SCP1

The identification of protein-protein interactions is a key probing the specificity and fidelity of many enzymatic and cellular

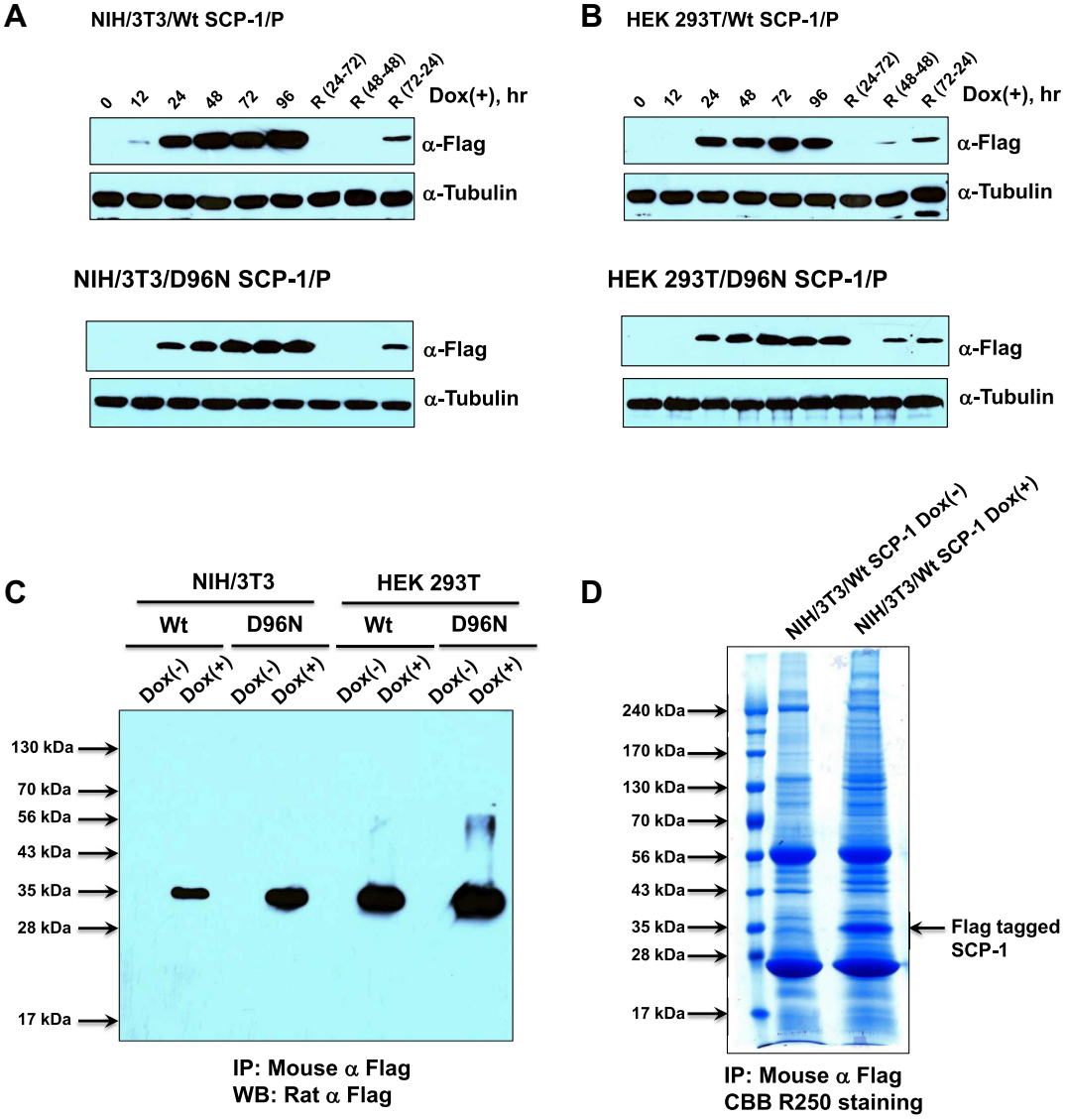

Fig. 1. Establishment of wild-type and D96N mutant SCP1 expressing inducible NIH/3T3 (A) and HEK293T cells (B). The expression levels of wild-type and D96N mutant SCP1 treated with or without 2 μg/ml of doxycycline for the indicated periods of time were monitored by immunoblot analysis. For reverted cells, the cells were treated with the inducer for the indicated periods and, after replacement with fresh medium, allowed to grow for the indicated periods of time without the inducer. The quantity of the applied protein was normalized by immunoblot analysis with anti-α-tubulin monoclonal antibody. Immunoprecipitation of wild-type and D96N SCP1 proteins was performed with 1 mg of total cell lysates from NIH/3T3/Wt SCP1, NIH/3T3/D96N SCP1, HEK293T/Wt SCP1, and HEK293T/D96N SCP1 (C). Each cell lysate was incubated with mouse Flag M2 antibody conjugated agarose beads. The affinity-purified proteins were resolved onto SDS-PAGE and were analyzed by Western blot with a specific rat antibody against the Flag sequence. Affinity-purification of SCP1-interacting proteins was carried out with Flag beads and 20 mg of total cell lysates from induced and uninduced cells. The proteins were resolved by 6–15% gradient SDS-PAGE gel (D) and visualized by colloidal Coomassie solution and subjected to trypsin digestion, and analyzed by LC–MS/MS.

physiological processes including an enzyme-substrate interaction. Recently, proteomic technology combining affinity purification of native protein complexes, separation on SDS-PAGE, and mass spectrometry is powerful for revealing interacting partners which are directly or indirectly associated with a specific protein of interest. In this study, we used four different systems to purify SCP1 in complex with interacting proteins, NIH/3T3/Wt SCP1, NIH/3T3/D96N SCP1, HEK293T/Wt SCP1, and HEK293T/D96N SCP1. The usefulness of our inducible protein expressing cell systems to validate the proteome profile changes and to elucidate the signaling network by proteomic technology has been previously [18]. Cell extracts from all two cell clones, which induced the SCP1 expression for 72 hr with doxycycline, were collected. Our use of a low ionic strength lysis buffer containing a relatively mild detergent and conventional RIPA buffer for this study allowed us to efficiently solubilize SCP1, hopefully with minimal disruption of the SCP1 interactome. The Flag tagged SCP1 proteins, as well as any associated protein components, were recovered by immunoprecipitation (Fig. 1D). Basically, among the cell clones, the SCP1 complex remained relatively invariant (Data not shown). SCP1 was immunoprecipitated from total cell lysates of four expressing systems with anti-Flag M2 antibody conjugated with Protein A agarose, and the isolated immune-complexes immobilized in protein A beads were removed using 2X Laemmli loading buffer on SDS-PAGE, and subjected to tryptic digestion followed by LC–MS/MS analysis. In parallel, as a negative control, the same procedure was undertaken but using the uninduced cell lysates (Fig. 1D). As shown in Fig. 1D, lots of proteins were specifically co-immunoprecipitated with Flag-tagged SCP1 from total cell lysates. The lanes, which are evenly cut as 10 equivalent slices, were excised from the gel in the immunoprecipitation lanes as well as the parallel negative controls for the MS analysis by destaining and in-gel digestion followed by peptide extraction. The tryptic peptides obtained from each gel slice were analyzed by LC–MS/MS. Proteins identified in the either of wild-type or D96N mutant SCP1 immunoprecipitation lanes with two or more peptides and a confidence level more than 95% (more than 40 Probability based Mowse Score), but absent in the negative control lanes of uninduced cell lysates, can be considered as a specific interactome for SCP1. Basically, four cell systems, NIH/3T3/Wt SCP1, NIH/3T3/D96N SCP1, HEK293T/Wt SCP1, and HEK293T/D96N SCP1, showed the totally same protein identities from the mass spectrometer analysis. There were 47 novel potential SCP1 interacting proteins identified in each of four cell systems (Data not shown). Based on the identities from the Databases with high probabilities, although we gave a special attention to their physiological functional categories, the commonly identified proteins from four cell lines were featureless. To further validate the proteins identified by MS/MS peptide matching to be components of the SCP1 interactome, we performed Western blot analysis on the SCP1 immunoprecipitate of the wild-type SCP1 expressing NIH/3T3 and HEK293T cells. Of the SCP1 interacting proteins identified, we selected 5 proteins that represent novel SCP1 interacting proteins, HSP 90αβ, TATA-binding protein interacting protein 120A (TIP120A), CdcA3, CTD small phosphatase-like protein (CTD-SPL), and prohibitin. As shown in Fig. 2, SCP1 protein expression was observed in SCP1 immunoprecipitate but not the negative control lysates from the uninduced cells. For further validation of any interaction and owing to the efficiency of immunoprecipitation, we performed the reciprocal immunoprecipitation with SCP1 in wild-type SCP1 expressing NIH/3T3 and HEK293T cell lysates, and the associated complexes were analyzed for the presence of expressed SCP1, HSP 90αβ, TIP120A, CdcA3, CTD-SPL (Fig. 2A–C). From these experiments, we demonstrate that four proteins were immunoprecipitated with the inducibly expressed SCP1 in two kinds of cells. Indeed, four proteins were co-immunoprecipitated with SCP1 in either two kinds of lysis buffers, a buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, protease and phosphatase inhibitor cocktails) or a conventional RIPA buffer (50 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, protease and phosphatase inhibitor cocktails). In addition, prohibitin was proven to be false positive (Fig. 2A). It is interesting that CTD-SPL, so called MYA22 from mouse origin and HYA22 from human, as a binding partner for SCP1 identified in this experiment has not been previously demonstrated. Although the present findings provide the possibility for new avenues to provide to a better understanding of cellular physiology of SCP1, these proteomic and some immunological approaches for SCP1 interactome might not represent the accurate physiological relevance in vivo. Thus, in this presentation, we focus the substrate specificity to delineate an appearance of the dephosphorylation reaction catalyzed by SCP1 phosphatase.

3.3. Determination of recombinant SCP1 substrate specificity

In this study to investigate the substrate specificity for dephosphorylation by SCP1, we expressed the recombinant SCP1 in *E. coli* and the phosphorylated sequences of the identified proteins by proteomic and some immunological approaches for SCP1 interactome searched in Human Protein Reference Database (HPRD) [23]. A truncated version of SCP1 improved the solubility as previously described [24]. Phosphatases have been promiscuous enzymes, displaying little intrinsic substrate specificity when assayed in vitro. However, there are some published evidences that they may show remarkable preference for specific substrates in vivo. In general, enzymatic specificity may be achieved by increasing the ratio of the catalytic activity of the enzyme toward physiological substrates over the background activity for similar non-physiological substrates. Such intrinsic activity can be obtained by shaping the chemical environment of the substrate binding pocket via side chain substitution. To date, no consensus identifying the preferred phosphopeptide sequence contexts for CTD phosphatases could be identified. The X-ray crystal structures of a dominant-negative form of human SCP1 (D96N mutant) bound to mono- and diphosphorylated peptides encompassing the CTD heptad repeat (^{1}Y^{2}S^{3}P^{4}T^{5}S^{6}P^{7}S) disclosed the residues in SCP1 involved in CTD binding and its preferential dephosphorylation of Phospho-^{5}Ser of the CTD heptad repeat [11]. Based upon the crystallization study of the complex of SCP1 and phosphopeptides of CTD heptad repeat in RNAPII, we selected the PX(S/T)^{p}P sequence as a specific motif of a substrate for enzymatic catalysis of SCP1. We compared the phosphorylated sequences of HSP 90αβ, TIP120A, CdcA3, and CTD-SPL reported in HPRD with the PX(S/T)^{p}P sequence, in order to find phosphopeptides, which could be dephosphorylated by SCP1. We, interestingly, found similar sequences from CdcA3 to the PX(S/T)^{p}P sequence, which are uniformly phosphorylated at ^{5}Ser position and the conserved ^{3}Pro and ^{6}Pro positions in CTD sequence, among the interacting proteins. Among 19 phosphorylated sequences of CdcA3 searched in HPRD [25], we found four plausible phosphopeptides and further selected two phosphopeptides (^{29}S (^{25}ADPR^{29}S^{p}PSA^{33}G) and ^{68}S(^{64}SDPR^{68}S^{p}PTL^{72}G)) based on the similarity with CTD heptad repeat. For comparing the specificity, we also selected two phosphopeptides (^{18}S (^{14}PAPP^{18}S^{p}PAS^{22}P) and ^{245}S (^{240}VRGPG^{245}S^{p}PPP^{250}P)) from searching result of UTF1, undifferentiated embryonic cell transcription factor 1 and a chromosome-associated protein with repressor activity expressed in pluripotent cells, in HPRD [25]. To validate the efficiency of enzymatic catalysis for synthetic phosphopeptide substrate, based on binding affinity (K_M) and turnover number (k_{cat}) were determined k_{cat}/K_M (s^{-1} mM^{-1}) (Table 1). The kinetic constants, K_M and k_{cat}, for CTD-phospho-^{5}Ser by recombinant SCP1 were 0.220 ± 0.075 mM

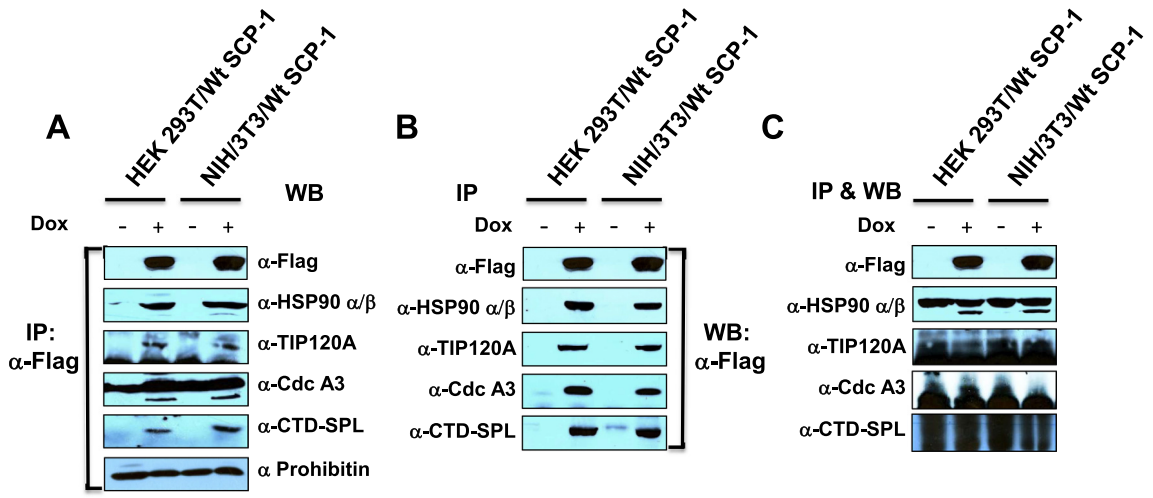

Fig. 2. Coimmunoprecipitation using anti-Flag antibody with total lysates of NIH/3T3 and HEK293T based cells (A). Total lysates from the appropriate cells were incubated with Flag beads, washed with the lysis buffer, and separated onto 10% SDS-PAGE. The gels were then immunoblotted for each protein with specific antibody: HSP 90αβ, TIP120A, CdcA3 and CTD-SPL. Prohibitin is shown as a false-negative control for immunoprecipitation. (B) Coimmunoprecipitation using specific antibodies directed against HSP 90αβ, TIP120A, CdcA3 and CTD-SPL. Total lysates (each 1 mg) from the induced and uninduced SCP1 expressing cells were mixed with the appropriate primary antibody raised against individual proteins followed by incubating with gentle rocking. Then, 50% bead slurry of Protein A agarose was added into the immunoprecipitates, incubated for 5 h, and separated onto SDS-PAGE. The gels were then immunobloted with rat anti-DYKDDDDK antibody. (C). Immunoprecipitation using specific antibodies directed against HSP 90αβ, TIP120A, CdcA3 and CTD-SPL and immunodetection (IP: immunoprecipitation and WB: Western blot analysis).

Table 1
Kinetic characterization of SCP1 against phosphopeptides. The data were the average of five independent measurements ±S.D.

	Phosphopeptide sequence	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} mM^{-1})
CTD-Phospho-^{5}Ser	YSPTSPSYSPTS^{P}PPS	0.220 ± 0.075	1.232 ± 0.189	5.600 ± 1.587
CdcA3 ^{29}S	ADPRS^{P}PSAG	0.423 ± 0.119	0.846 ± 0.109	2.000 ± 0.913
CdcA3 ^{68}S	SDPRS^{P}PTLG	0.264 ± 0.041	1.784 ± 0.110	6.752 ± 2.669
UTF1 ^{18}S	PAPPS^{P}PASP	1.044 ± 0.178	1.315 ± 0.013	1.260 ± 0.073
UTF1 ^{245}S	VRGPGS^{P}PPPPP	0.225 ± 0.018	0.671 ± 0.021	2.982 ± 1.167

and 1.232 ± 0.189 s^{-1}, respectively. Thus, k_{cat}/K_M value for CTD-phospho-^{5}Ser was 5.600 ± 1.587 s^{-1} mM^{-1}. Recombinant SCP1 exhibited a K_M of 0.423 ± 0.119 mM and an apparent k_{cat} of 0.846 ± 0.109 s^{-1} for synthetic phosphopeptide ^{29}S, but a K_M of 0.264 ± 0.041 mM and an apparent k_{cat} of 1.784 ± 0.11 s^{-1} for ^{68}S (Table 1). The specificity constant for ^{68}S is close to the range of kinetic constants observed when recombinant SCP1 was first tested for phosphatase activity against the original CTD phosphopeptide. These constants better reflect the high specificity typically associated with enzymatic activity. From these constants, we calculated the specificity constant (k_{cat}/K_M) resulting in values of 2.000 ± 0.913 s^{-1} mM^{-1} for ^{29}S synthetic phosphopeptide and 6.752 ± 2.669 s^{-1} mM^{-1} for ^{68}S. Recombinant SCP1 showed a K_M of 1.044 ± 0.178 mM and an apparent k_{cat} of 1.315 ± 0.0.013 s^{-1} for synthetic phosphopeptide ^{18}S (k_{cat}/K_M = 1.260 ± 0.073 s^{-1} mM^{-1}), and a K_M of 0.225 ± 0.018 mM and an apparent k_{cat} of 0.671 ± 0.022 s^{-1} for ^{245}S (k_{cat}/K_M = 2.982 ± 1.167 s^{-1} mM^{-1}) (Table 1). Among K_M values of four phosphopeptides except CTD-phospho-^{5}Ser, the phosphopeptides of CdcA3 ^{68}S and UTF1 ^{245}S bound strongly to SCP1, but UTF1 ^{18}S was the weakest binding phosphopeptide to SCP1. In terms of k_{cat} values of four phosphopeptides, SCP1 showed the fastest turnover rate with the phosphopeptide of CdcA3 ^{68}S, but the slowest turnover rate with the phosphopeptide of UTF1 ^{245}S. The differences of these kinetic constants could be originated from partial differences among four phosphopeptides and now we are not able to describe the partial difference. However, we speculate that there might be additional binding residues of phosphopeptides with SCP1 because the specificity of CdcA3 ^{68}S is relatively high than them of others even though all of them have PX(S/T)^{P}P from this kinetic result. Therefore, at least one of the phosphorylated sequences of CdcA3 is plausible dephosphorylated site by SCP1, although the possibility still exists that some enzymes may be designed to have poorer binding and/or catalytic efficiency as a means of regulating activity.

In conclusion, this combined study of some proteomic, immunological and kinetic approach opens new insights for enzymatic substrates of SCP1. We suggest that there are several interacting proteins with SCP1 and CdcA3 could be an enzymatic substrate of SCP1. Moreover these findings provide that SCP1 might have the relationship with cell cycle regulation through enzymatic activity against CdcA3.

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