BIO DESIGN

pISSN 2288-6982 | eISSN 2288-7105

CRYSTALLIZATION P 141-144

Crystallization and preliminary diffraction analysis of DUSP28 through identification of a pseudo-thrombin cleavage site

Won Hong[1], Bonsu Ku[2], Myungbin Kim[1], Seung Jun Kim[2] and Seong Eon Ryu[1]*

[1]Department of Bioengineering, College of Engineering, Hanyang University, Seoul 04763, Korea, [2]Disease Target Structure Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon 34141, Korea

*Correspondence: ryuse@hanyang.ac.kr

Dual specificity protein phosphatases (DUSPs) belong to the protein tyrosine phosphatase (PTP) family. DUSPs dephosphorylate both phospho-serine/threonine and phospho-tyrosine of mitogen activated protein kinases (MAPKs) and play important roles in cell growth, regulation and signaling. DUSP28, a member of the atypical DUSPs, has dephosphorylation activity towards proteins involved in cellular signaling processes. DUSP28 is also implicated in the development of pancreatic cancer and liver cancer. The atomic resolution structure of DUSP28 should help the structure-based design of specific and potent therapeutics. However, the structure and detailed function of DUSP28 have not been elucidated yet. Here, we prepared a large quantity of DUSP28 protein and crystallized the protein. During the protein preparation, we encountered an unexpected proteolytic cleavage in the middle of the protein domain and overcame the problem by identifying and mutating the pseudo-thrombin cleavage site. By using the purified protein, we were able to grow diffraction quality crystals and collected a 2.1 Å resolution diffraction data. The preliminary diffraction analysis revealed that the crystal is in the space group $P3_121$ with unit cell parameters of a = 78.85 Å, b = 78.85 Å, c = 90.26 Å, α = 90.00°, β = 90.00° and γ = 120.00°

INTRODUCTION

Protein tyrosine phosphatases (PTPs) dephosphorylate phosphor-tyrosine/threonine and phosphor-tyrosine of target proteins that are involved in cellular signal transduction and regulation (Alonso and Pulido, 2016; Tonks, 2006). The human genome contains approximately 110 of PTP family proteins that are largely divided into the classic PTP family and the dual specificity phosphatases (DUSP) family (Alonso et al., 2004; Bermudez et al., 2010). The catalytic domain structures and sequences of both classical PTP and DUSP family proteins are highly similar (Jeong et al., 2014). The active site of PTPs is conserved with the P-loop and the WPD-loop (the D-loop in DUSPs). DUSPs dephosphorylate both phospho-serine/threonine and phosphor-tyrosine, while classical PTPs are specific to phosphor-tyrosine. The active site pocket of DUSPs is shallower than that of classical PTPs to fit the short side chains of phospho-serine/threonine (Jeong et al., 2014; Yuvaniyama et al., 1996).

Due to their functions in critical cellular processes, DUSPs are targets for drug development against cancers, diabetes, immune diseases, and neuronal diseases (Bermudez et al., 2010; Keyse, 2008; Patterson et al., 2009). In particular, DUSPs play an important role in cell growth and survival by regulating map kinase (MAPK) signaling pathway. DUSPs can dephosphorylate MAPK proteins including ERK, JNK and p38. DUSPs are divided into typical and atypical subgroups. Typical DUSPs contain MAPK binding domain (MKB) as a regulatory domain. Atypical DUSPs lack the regulatory MKB domain. Atypical DUSPs, like typical DUSPs, can dephosphorylate MAPK and play roles in cell growth, apoptosis and cell signaling.

DUSP28, which is an atypical DUSP, has a conserved P-loop in the active site. Of note is that, unlike other human DUSPs containing the P-loop with the HCXXXXXR motif, DUSP28 has tyrosine instead of the conserved histidine resulting in the P-loop of the YCXXXXXR motif (Jeong et al., 2011; Lee et al., 2017). However, the function of the replaced tyrosine has not been identified. The enzyme activity of DUSP28 appears to be affected by physiological concentration of zinc ion (Jeong et al., 2011). In the cell, metal ion is maintained in a specific range of concentrations and involved in the function of the cell (Maret, 2010). Especially, divalent metal ions are combined with proteins to regulate their activity (Maret et al., 1999).

DUSP28 is associated with liver and pancreatic cancers and implicated as a potential anti-cancer drug target (Lee et al., 2017; Lee et al., 2016). The atomic resolution structure of DUSP28 can greatly aid efforts to develop specific and potent therapeutics through structure-based designs. However, the structure of DUSP28 has not been determined. In the current study, we

crystallized human DUSP28 for the atomic resolution structure determination. During purification of the protein samples for crystallization, we encountered unexpected proteolytic cleavage, which was solved by introducing a mutation in the pseudo-thrombin recognition site that was found in the wild type DUSP28 sequence. From the resulting mutation, we were able to prepare stable proteins and to obtain diffraction-quality crystals and collect 2.1 Å diffraction data.

RESULTS AND DISCUSSION

The full-length human DUSP28 (residues 1-167) was cloned and expressed by using an *E.coli* over-expression system (Table 1). Our initial expression and purification efforts encountered a problem in the purification step involving the removal of histidine tag (His-tag) by thrombin digestion. Although the His-tag affinity-purification yielded a single band, the subsequent thrombin cleavage for the His-tag removal resulted in two extra bands at around 12 kDal and 7 kDal positions) in addition to the His-tag cleaved full-length DUSP28 band at around 20 kDal position indicating that there was a nonspecific cleavage (Figure 1a). A close examination of the DUSP28 sequence revealed a pseudo-thrombin cleavage site in the middle of the expressed protein.

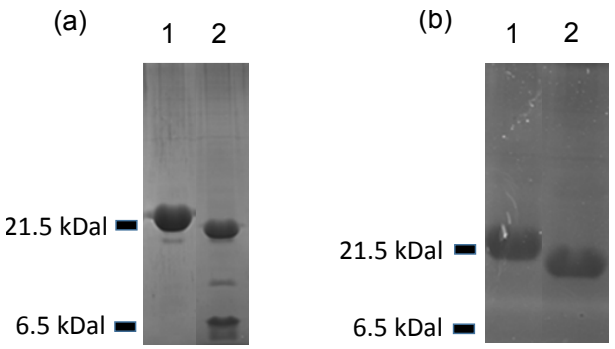

FIGURE 1 | The pseudo-thrombin cleavage site. The purified DUSP28 proteins before and after thrombin cleavage were shown with SDS-PAGE. (a) Thrombin digestion of the His-tag purified wild type proteins. Lane 1, before thrombin cleavage; lane 2, after thrombin cleavage. (b) Thrombin digestion of the His-tag purified R59Q proteins. Lane 1, before thrombin cleavage; lane 2, after thrombin cleavage.

Although the regular thrombin cleavage sequence is Leu-Val-Pro-Arg-Gly-Ser, thrombin can cleavage A-B-Pro-Arg-X-Y sequence where A and B are hydrophobic amino acids and X and Y are non-acidic amino acids (Guan and Dixon, 1991; Hefti et al., 2001; Jornvall et al., 1979). DUSP28 has the pseudo-thrombin cleavage site in residues 56-61 (Pro-Gly-Pro-Arg-Ala-Pro). The cleavage in the pseudo site would result in two fragments of about 60 and 110 amino acids whose sizes correspond to the two bands observed during thrombin cleavage (Figure 1a). To solve this problem, we mutated the region of DUSP28 sequence from Pro-Gly-Pro-Arg-Ala-Pro to Pro-Gly-Pro-Gln-Ala-Pro (the R59Q mutation). The mutation from Arg to Gln was to conserve the size of residue and minimize the impact on the whole protein structure. In addition to the mutation in the pseudo-thrombin cleavage site, we mutated the active site cysteine (Cys103) to serine (Ser103) to minimize non-specific aggregation and oxidation. Thrombin digestion of the resulting DUSP28 construct with the R59Q and C103S mutations (DUSP28_R59Q/C103S) resulted in one band (Figure 1b)

The first crystal was obtained from the sitting drop method with a condition (0.1 M Tris-Cl, pH 8.5, 0.2 M NaCl, 25% PEG 3350) from the Hampton Research index 73, but the crystal had cracks. So, for the high quality crystals, we refined the crystallization condition around the initial condition. We tried various precipitant and salt concentrations and found that optimal crystallization conditions include NaCl concentrations of minimum 0.2 M to maximum 0.4 M and PEG 3350 concentrations of minimum 21% in pH 8.5. Crystals suitable for x-ray diffraction and data collection were obtained in the drop of the mixture of 0.2 µl DUSP28 protein solution (20 mg/ ml concentration in 20 mM Tris-Cl, pH 8.0, 0.2 M NaCl and 4 mM DTT) with 0.2 µl reservoir buffer (0.1 M Tris-Cl, pH 8.5, 0.3 M NaCl and 26% PEG 3350)

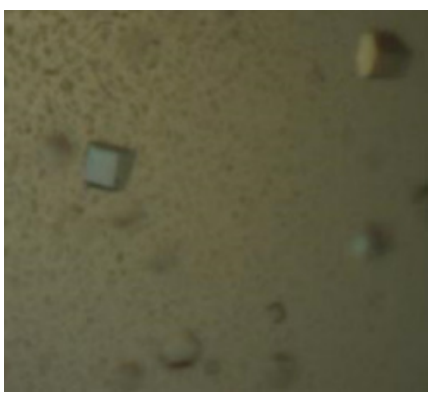

FIGURE 2 | Crystals of human DUSP28. The crystals were obtained with a reservoir solution containing 0.1 M Tris-Cl, pH 8.5, 0.3 M NaCl and 26% PEG 3350 at 291 K.

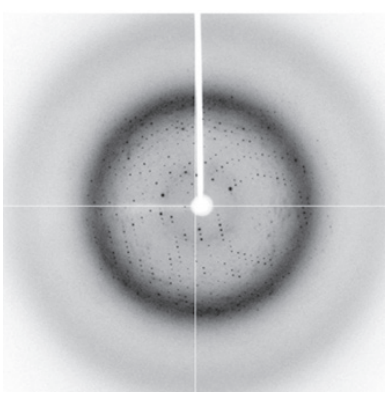

FIGURE 3 | Diffraction pattern. An 1.0 ° oscillation photograph of the DUSP28_R59Q/C103S crystal.

TABLE 1 | Human DUSP28 production information

Source organism	Human
DNA source	cDNA
Forward primer	GGCCCATATGGGACCGGCAGAAGCTGGG
Reverse primer	GGCCGAATTCTCAAGCCTCAGGGCCCAACCC
Mutation primer (R59Q)	CCCGGCCCGCAAGCGCCCGGC GCCGGGCGCTTGCGGGCCGGG
Mutation primer (C103S)	TGCCTAGTCTACTCCAAGAACGGCCGC GCGGCCGTTCTTGGAGTAGACTAGGCA
Cloning vector	pET28a
Expression host	*E. coli* BL21 (DE3) pLysS
Complete amino acid sequence of the construct produced	MGPAEAGRRG AASPVPPPLV RVAPSLFLGS ARAAGAEEQL ARAGVTLCVN VSRQQPGP**Q***A PGVAELRVPV FDDPAEDLLA HLEPTCAAME AAVRAGGACL VY**S***KNGRSRS AAVCTAYLMR HRGLSLAKAF QMVKSARPVA EPNPGFWSQL QKYEEALQAQ SCLQGEPPAL GLGPEA

* Mutated residues (R59Q and C103S) are highlighted with stars.

TABLE 2 | Crystallization

Method	Sitting drop vapor diffusion
Plate type	96-Well Sitting-Drop Crystallography Plate
Temperature (K)	291
Protein concentration (mg ml^{-1})	20
Buffer composition of protein solution	20 mM Tris-Cl, pH 8.0, 0.2 M NaCl and 4 mM DTT
Composition of reservoir solution	0.1 M Tris-Cl, pH 8.5, 0.3 M NaCl and 26% PEG 3350
Volume of drop (μl)	0.4 (protein: reservoir = 1:1)
Volume of reservoir (μl)	70

TABLE 3 | Data collection and processing statistics

Diffraction source	beamline 7A, Pohang Light Source (PLS)
Wavelength (Å)	1.0000
Temperature (K)	100
Space group	$P3_121$
a, b, c (Å)	78.85, 78.85, 90.26
α, β, γ (o)	90.00, 90.00, 120.00
Resolution range (Å)	50.0 - 2.1 (2.14-2.10)*
Completeness (%)	99.3 (28.8)
R_{merge} (%)	5.1 (4.4)
Average I/σ(I)	47.2 (99.1)
Redendency	8.0

* Statistics in parenthesis are for the highest resolution bin.

(Table 2). The crystal of DUSP28 diffracts to 2.1 Å resolution (Figure 2). Initial analysis revealed that space group of the crystal was P3121 with unit cell parameters of a = 78.85 Å, b = 78.85 Å, c = 90.26 Å, α = 90.00°, β = 90.00° and γ = 120.00°. The data collection statistics are shown in Table 3.

METHODS

Cloning and purification of the recombinant proteins

The full length gene of DUSP28 was PCR-amplified with forward and backward primers (Table 1). Primers contained restriction sites for NdeI and EcoRI. The PCR product was digested with NdeI and EcoRI, and ligated with pET28a vector (Invitrogen). To prevent aggregation and thrombin non-specific cutting (see Results and Discussion), another DNA construct was prepared by site-directed mutagenesis (DUSP28 R59Q C103S). The cloned DUSP28 DNA was introduced into *E.coli* strain BL21 (DE3) competent cell. For the induction of protein over-expression the incubation temperature was lowered to 18℃ when the OD600 reached 0.7 with 37℃ incubation. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to 0.1 mM concentration and incubated in 18℃ for 16 hours. Cells were harvested and re-suspended in lysis buffer (50 mM Tris-Cl, pH 7.5, 500 mM NaCl, 1 mM PMSF, and 0.2% 2-mercaptoehanol), and sonicated in ice. After centrifugation of cell lysate, affinity column (GE healthcare hitrap TALON crude) was equilibrated in the equilibration buffer (50 mM Tris-Cl, pH 7.5, 0.5 M NaCl and 0.5 mM PMSF). After that, the supernatant of cell lysate was loaded into the affinity column. Next, the column was washed by the wash buffer (50 mM Tris-Cl, pH 7.5, 0.5 M NaCl and 0.5 mM PMSF). The protein was eluted by the elution buffer (50 mM Tris-Cl, pH 7.5, 0.5 M NaCl, 10 mM 2-mercaptoethanol and 0.5 M imidazole). After finishing affinity chromatography, protein buffer was changed to a buffer containing 20 mM Tris-Cl, pH 8.0, 0.2 M NaCl and 40 mM 2-mercaptoethanol by using a stirred cell. His-tag was removed by cleavage with thrombin for 15 hours. Finally, gel filtration chromatography column (GE healthcare Sephacryl S-100) was equilibrated by the

equilibration buffer (20 mM Tris-Cl. pH 8.0, 0.2 M NaCl and 4 mM DTT). After that, protein was loaded into the gel filtration column and protein concentration was determined by the Bradford assay method (Bradford, 1976). Fractions containing the protein were collected and concentrated to 20 mg/ml.

Crystallization and X-ray data collection

Initial crystallization conditions were searched by using commercial screening solutions by using the sitting drop vapor diffusion method. The first crystal was obtained at 18℃ with the condition from the Hampton Research index 73 (0.1 M Tris-Cl, pH 8.5, 0.2M NaCl, 25% PEG 3350). Crystals suitable for x-ray diffraction and data collection were obtained in the drop of the mixture of 0.2μl DUSP28 protein solution (20 mg/ml concentration in 20 mM Tris-Cl, pH 8.0, 0.2 M NaCl and 4 mM DTT) with 0.2 μl reservoir buffer (0.1 M Tris-Cl, pH 8.5, 0.3 M NaCl, 26% PEG 3350). The drops were sealed with 70 μl of reservoir buffer in crystallization well and incubated at 18℃. Crystals appeared in approximately two weeks. X-ray diffraction data were collected on the ADSC Q270 detector at the Pohang Light Source (PLS) beamline 7A. Crystals were soaked with cryo-protective buffer containing 20% glycerol in the reservoir buffer and frozen with nitrogen gas. The collected data was processed using the program HKL2000

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ACKNOWLEDGMENTS

We are grateful for the use of beamline 7A at the Pohang Accelerator Laboratory in Korea.

Original Submission: Dec 1, 2017
Revised Version Received: Dec 18, 2017
Accepted: Dec 18, 2017

REFERENCES

Alonso, A., and Pulido, R. (2016). The extended human PTPome: a growing tyrosine phosphatase family. *FEBS J* **283**, 2197-2201.

Alonso, A., Sasin, J., Bottini, N., Friedberg, I., Osterman, A., Godzik, A., Hunter, T., Dixon, J., and Mustelin, T. (2004). Protein tyrosine phosphatases in the human genome. *Cell* **117**, 699-711.

Bermudez, O., Pages, G., and Gimond, C. (2010). The dual-specificity MAP kinase phosphatases: critical roles in development and cancer. *Am J Physiol Cell Physiol* **299**, C189-202.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248-254.

Guan, K.L., and Dixon, J.E. (1991). Eukaryotic proteins expressed in Escherichia coli: an improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. *Anal Biochem* **192**, 262-267.

Hefti, M.H., Van Vugt-Van der Toorn, C.J., Dixon, R., and Vervoort, J. (2001). A novel purification method for histidine-tagged proteins containing a thrombin cleavage site. *Anal Biochem* **295**, 180-185.

Jeong, D.G., Kim, S.Y., Yun, J.H., and Kim, J.H. (2011). Characterization of a Dual-Specificity Protein Phosphatase, Human DUSP28. *J Life Sci* **21**, 31-35.

Jeong, D.G., Wei, C.H., Ku, B., Jeon, T.J., Chien, P.N., Kim, J.K., Park, S.Y., Hwang, H.S., Ryu, S.Y., Park, H., Kim, D.S., Kim, S.J., and Ryu, S.E. (2014). The family-wide structure and function of human dual-specificity protein phosphatases. *Acta Crystallogr D Biol Crystallogr* **70**, 421-435.

Jornvall, H., Fish, W.W., and Bjork, I. (1979). The thrombin cleavage site in bovine antithrombin. *FEBS Lett* **106**, 358-362.

Keyse, S.M. (2008). Dual-specificity MAP kinase phosphatases (MKPs) and cancer. *Cancer Metastasis Rev* **27**, 253-261.

Lee, J., Lee, J., Yun, J.H., Choi, C., Cho, S., Kim, S.J., and Kim, J.H. (2017). Autocrine DUSP28 signaling mediates pancreatic cancer malignancy via regulation of PDGF-A. *Sci Rep* **7**, 12760.

Lee, J., Lee, J., Yun, J.H., Jeong, D.G., and Kim, J.H. (2016). DUSP28 links regulation of Mucin 5B and Mucin 16 to migration and survival of AsPC-1 human pancreatic cancer cells. *Tumour Biol* **37**, 12193-12202.

Maret, W. (2010). Metalloproteomics, metalloproteomes, and the annotation of metalloproteins. *Metallomics* **2**, 117-125.

Maret, W., Jacob, C., Vallee, B.L., and Fischer, E.H. (1999). Inhibitory sites in enzymes: zinc removal and reactivation by thionein. *Proc Natl Acad Sci U S A* **96**, 1936-1940.

Patterson, K.I., Brummer, T., O'Brien, P.M., and Daly, R.J. (2009). Dual-specificity phosphatases: critical regulators with diverse cellular targets. *Biochem J* **418**, 475-489.

Tonks, N.K. (2006). Protein tyrosine phosphatases: from genes, to function, to disease. *Nat Rev Mol Cell Biol* **7**, 833-846.

Yuvaniyama, J., Denu, J.M., Dixon, J.E., and Saper, M.A. (1996). Crystal structure of the dual specificity protein phosphatase VHR. *Science* **272**, 1328-1331.