

Purification of enzymatically inactive peptidylarginine deiminase type 6 from mouse ovary that reveals hexameric structure different from other dimeric isoforms

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ABSTRACT

The murine peptidylarginine deiminase (PAD) has five isoforms encoded by different genes and participates in a variety of cellular functions through the citrullination of target proteins. The crystal structure of human PAD4 with a dimeric form was previously solved because of the enzyme's relevance to rheumatoid arthritis. PAD6, abundant in mouse oocytes and eggs, is believed to take part in early events of embryogenesis, but its biochemical properties are little understood. Here we have purified and characterized a recombinant PAD6. A PAD6 cDNA was cloned from mouse ovary RNA and expressed in *Escherichia coli* through pET29 and pGEX vectors. When benzoyl-L-arginine ethyl ester was used as a substrate, no appreciable activity was detected with a cell homogenate under conditions where a human PAD4 cDNA caused significant activity. Both proteins were affinity-purified to near homogeneity. The circular dichroism spectra of PAD6 and human PAD4 were similar in the far ultraviolet region. On molecular sieving, PAD6 was eluted faster than human PAD4. The cross-linking of PAD6 with dimethyl suberimidate clearly showed six bands on an sodium dodecyl sulfate-polyacrylamide gel. These results indicate that PAD6 can constitute a hexameric structure. The purified PAD6 still showed no enzy-

matic activity. This unique structure and loss in enzymatic activity is strongly suggested to favor the formation of egg cytoplasmic sheets as the architectural protein.

Keywords: Peptidylarginine Deiminase; Isoform; Dimer; Hexamer; Mouse Oocytes; Cytoplasmic Sheets

1. INTRODUCTION

Peptidylarginine deiminases (PAD) (protein-arginine deiminase; E.C. 3.5.3.15) are an enzyme that catalyzes the removal of the imino-moiety of peptidylarginine to yield peptidylcitrulline and ammonia in the presence of Ca²⁺. PADs have been demonstrated to play a variety of roles in cell signaling pathways, apoptosis, differentiation and transcriptional regulation. Dysregulation of PAD activity is considered to be responsible for various diseases such as rheumatoid arthritis (RA), cancer and colitis. Therefore, development of agents regulating the PAD activity is required for reducing the disease severity [1].

In humans and rodents, the enzyme has five isoforms differing in distribution: PAD1, epidermis and uterus; PAD2, various tissues; PAD3, hair follicles; PAD4, blood cells; PAD6, egg and ovary (for reviews see, e.g., [2-4]). The genes for these PADs are clustered on chromosome 4 at D3 in mice and on chromosome 1 at p36.13 in humans [5,6], and PADs share 40% - 55% sequence homology. PAD4 has been studied extensively in rela-

tion to the pathogenesis of RA, because PAD4 gene expression is prominent in the synovial tissue of RA patients, and levels of an anti-cyclic citrullinated peptide antibody (anti-CCP antibody) are significantly augmented in their sera [7]. Therefore, the anti-CCP antibody has recently become a diagnostic marker of RA. Moreover, Suzuki *et al.* [8] found that mutations of the PAD4 gene are closely linked to RA in an SNP analysis. For these reasons, the crystal structure of human PAD4 (hPAD4) has been solved [9]. hPAD4 has a dimeric structure with a head-to-tail shape whose N- and C-terminal regions function in Ca^{2+} -binding and catalysis, respectively. The binding of Ca^{2+} to an apo-hPAD4 induces a gross conformational change resulting in a catalytically active form. Cys⁶⁴⁵ near the C-terminal is necessary for catalysis. However, only one PAD occurs in prokaryotes [10]; the *Porphyromonas gingivalis* PAD also carries a reactive Cys residue that acts as a nucleophile essential for catalysis despite no requirement of Ca^{2+} [11,12].

In contrast to PAD1 to 4, the biochemical properties of PAD6 are poorly understood. Interestingly, the crystallographic data on PAD4 along with a sequence alignment among five PADs imply that the acidic residues involved in Ca^{2+} -binding and a reactive Cys in the C-terminal region are not conserved in PAD6 [9]. These findings provide the possibility that PAD6 lacks enzymatic activity. To learn more about structure-function relationships among PADs, it is necessary to purify and characterize PAD6. Since the crystal structure of hPAD4 is available, and hPAD4 and mouse PAD4 have high amino acid sequence homology, we have compared the properties of PAD6 and hPAD4, and present data for a unique structure of PAD6.

2. MATERIALS AND METHODS

2.1. Materials

The biochemical reagents and materials used were obtained as follows: murine reverse transcriptase, Takara Bio (Kyoto, Japan); KOD⁺ DNA polymerase, Toyobo (Shiga, Japan); Sephacryl S300, Ni²⁺ Sepharose, PreScissionTM endoprotease, GE Health care; pET28 and pET29, Novagen/Merck; Luria-Bertani (LB) broth, Terrific broth (TB), dimethyl suberimidate, Nacalai tesque (Kyoto, Japan); DE52, Whatman (NJ, USA) and the Amicon UltraTM spin column (cut-off $M_r \sim 10,000$), Millipore (MA, USA). Other materials were also obtained commercially and used without further treatment.

2.2. cDNA Cloning

cDNA cloning and the construction of expression vectors were performed using general PCR-based procedures as described previously [13]. Total RNA was isolated from mouse ovaries. cDNA to PAD6 was synthe-

sized with random hexamer/oligo(dT) primers by reverse transcriptase. An aliquot of the cDNA library was amplified by KOD⁺ DNA polymerase with custom-made primers in a thermal cycler (ABI 2720) (**Figure 1(a)**). The reaction profile was 30 cycles of denaturing at 94°C for 30 sec, annealing at 55°C for 30 sec, and elongation at 68°C for 2 min. The product, once treated with phenol/chloroform, was digested with restriction endonucleases and ligated to a pET29 or pGEX6 vector precut with the corresponding restriction enzymes. These DNAs were used to transform *E. coli* BL21 (DE3) or BL21, and antibiotic-resistant colonies were subjected to DNA sequencing in a Hitachi-ABI sequencer 3100.

2.3. Purification of PAD6

Proteins were purified at 0°C - 4°C. Herein, 10 mM Tris-HCl (pH 8.0) is referred to as "Tris", unless otherwise stated. A one-fiftieth volume of overnight culture of *E. coli* harboring pET29/PAD6 cDNA (**Figure 1(b)**) was inoculated into a 2L-flask containing 500 ml of TB/kanamycin, and shaken at 37°C (95 shuttles/min) until the cell density reached 0.7 (absorbance at 600 nm). Then, isopropyl 1-thio- β -D-galactoside (IPTG) was added to make a concentration of 0.15 mM, and the culture was continued at 25°C overnight. The cells collected by centrifugation were stored in four 50-ml tubes at -80°C until used. The thawed cells were suspended in 20 ml of Tris, 1 mM EDTA, lysozyme (5 mg), 10 mM 2-mercaptoethanol and 1mM phenylmethylsulfonyl fluoride, and frozen again at -80°C for 30 min. The thawed sample was sonicated at 200-W at three intervals over a pe-

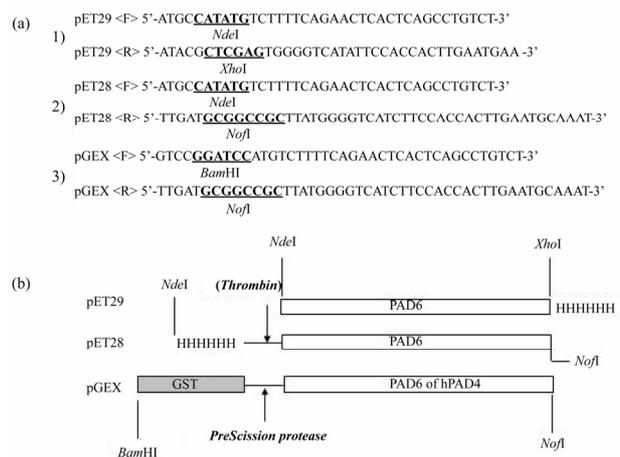


Figure 1. Primers and vectors used. (A) Forward (F) and reverse (R) primer sets, a, b, c, were used for the amplification of PAD6 cDNA from a mouse ovary cDNA library. The PCR products were cleaved with NdeI + XhoI, NdeI + NotI, or BamHI + NotI restriction endonucleases, and introduced into a pET29, pET28, or pGEX vector previously cut with the corresponding endonucleases.

riod of 1 min. After centrifugation at $12,000\times g$ for 15 min, the supernatant was diluted 2-fold with Tris, and applied to a DE52 column (2-cm diameter X 4-cm height) prewashed with Tris containing 1 mM EDTA and 10 mM 2-mercaptoethanol (Buffer A). After a wash with 60 ml of Buffer A, PAD6 was eluted with 90 ml of Tris containing 5 mM mercaptoethanol and 0.2 M NaCl (Buffer B). The eluent was adjusted to make 0.5 M NaCl and 20 mM imidazole. To this solution in 50-ml tubes was added 5 ml of Ni^{2+} Sepharose (50% v/v). The mixture was added was gently rotated for 1 h, and centrifuged at $1000\times g$ for 2 min. The precipitate was washed five times with Tris containing 0.5 M NaCl and 20 mM imidazole. Finally, PAD6 was released by adding triturated imidazole to make 0.3 M. The eluent concentrated on a spin column was applied to a Sephacryl S300 column (1.5 cm \times 98 cm) prewashed with Buffer B, and 5-ml portions were collected. The purity of each fraction was checked by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE), concentrated by a spin column again and kept in aliquots at -80°C . Alternatively, Ni^{2+} Sepharose chromatography was preceded by gel chromatography; a DE52-eluted preparation was concentrated with 70% ammonium sulfate and applied to the Sephacryl column. Each fraction (5 ml) was purified by the Ni^{2+} Sepharose treatment as above.

2.4. Purification of hPAD4 and PAD6 Derived from pGEX6

hPAD4 and PAD6 were obtained through the vector pGEX6 (**Figure 1(b)**). *E. coli* cells harboring pGEX6/hPAD4 or PAD6 were cultured overnight in TB supplemented with ampicillin and IPTG as described above. The cell homogenate was spun, and the supernatant was applied to a DE52 column equilibrated with Tris. The column was extensively washed with Tris containing 0.15 M NaCl and 10 mM 2-mercaptoethanol. The wash-out solution was brought to a concentration of 0.5 M with respect to NaCl. This preparation received 5 ml of GST-Sepharose (50% v/v), and was gently mixed by rotation for 2 h. The gels were washed 5 times with Tris containing 5 mM 2-mercaptoethanol and 0.5 M NaCl, and then the 10-ml suspension received 30 μl of Pre-Scission protease. The digestion was continued for 24 h with slow rotation. The supernatant after centrifugation was further concentrated on a spin column and applied to the Sephacryl S300 column. Aliquots of the 5-ml fraction were subjected to SDS-PAGE and active fractions were used for experiments.

2.5. Chemical Cross-Linking Experiment

The chemical cross-linking experiment was carried out as described by Davies and Stark [14]. The reaction mixture (20 μl) consisted of 0.1 M triethanolamine-HCl (pH

8.5), 2 - 5 μg of protein, and a 10- to 30-fold excess of dimethyl suberimidate-HCl. The reaction was allowed to proceed for 2 h at 20°C , and stopped by adding an equal volume of 2% SDS, 20 mM sodium phosphate (pH 7.2), 5% 2-mercaptoethanol, 0.05% bromophenol blue and 50% glycerol. After being denatured at 95°C for 2 min, the samples were subjected to 3.5% SDS-polyacrylamide disc gel electrophoresis at 8 mA/disc, and stained with Coomassie Brilliant Blue.

2.6. Enzyme Assay

PAD activity was measured in a 700- μl volume with benzoyl-L-arginine ethyl ester (BAEE) as the substrate as described previously [15], in which 10 mM dithiothreitol was included. After incubation at 37°C for an appropriate period, the reaction was terminated by adding 100 μl of 8 N perchloric acid. Citrulline in the 333- μl supernatant was reacted with 417 μl of 2.25 M $\text{H}_3\text{PO}_4/4.5$ M H_2SO_4 , 167 μl of 20 mM diacetyl monoximine, and 43 μl each of 30 mM thiosemicarbazide and $\text{NH}_4\text{Fe}(\text{SO}_4)_2$ at 99°C for 22 min. The absorbance of the reactant was recorded at 540 nm. The molar extinction coefficient for citrulline was 5×10^4 . One unit of activity was defined as the amount of enzyme catalyzing the formation of 1 μmol of product per min.

2.7. Other Methods

Circular dichroism (CD) spectra of purified proteins were recorded in a JASCO J-805 as described previously [13]. Prior to analysis, the samples were dialyzed against 10 mM potassium phosphate (pH 7.2) and 0.2 M NaCl for 15 h. Protein was measured by the Lowry method using bovine serum albumin (BSA) as a standard [16]. Site-directed mutagenesis of PAD4 was performed as described [13].

3. RESULTS

3.1. Expression of PAD6 cDNA

A PAD6 cDNA was cloned from mouse ovary RNA, ligated to a pET29 expression vector, and expressed in *E. coli* in the presence of IPTG. For the PAD enzyme assay, BAEE was used as a substrate throughout the experiment. As shown in **Figure 2**, the cell homogenate of hPAD4 showed time- and dose-dependent activity, whereas that of PAD6 did not. The latter result verifies a prediction by X-ray analysis that PAD6 may not bear enzymatic activity [9].

3.2. Purification of PAD6

PAD6 protein was purified to near homogeneity from the supernatant of the *E. coli* homogenate, because an intense band of PAD6 with a M_r of 76,777 was not seen in the insoluble fraction upon SDS-PAGE. A single band of

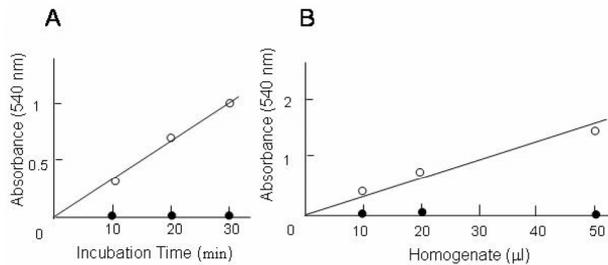


Figure 2. hPAD4 and PAD6 activity in cell homogenates. (A) The time course of PAD activity was determined colorimetrically with 50 ml of cell homogenate (~20 mg/ml) as described in Materials and Methods. Open and closed circles denote the activity of the hPAD4 and PAD6 homogenates, respectively. (B) Concentration-dependence of the hPAD4 (○) and PAD6 (●) activity of the cell homogenates. The reaction time was 30 min. The acid soluble fraction (333-ml) of the reaction mixture was subjected to color development and measured at 540 nm (the ordinate absorbance).

protein was recovered from the SDS gel and assigned as PAD6 by peptide sequencing (**Figure 3**). Ser² was found to be the N-terminal residue instead of the translation initiator Met. The yield of PAD6 was extremely low (below 0.5 mg from 1L-cultured cells), whereas that of hPAD4 exceeded 3 mg. Since hPAD4 was isolated from a GST-fused polypeptide by cleavage with PreScission protease, the N-terminal GST segment might be useful for translation efficiency, and/or protein stability. To test this possibility, PAD6 was expressed through pGEX (**Figure 1(b)**). However, the yield of PAD6 was still comparable to, or less than, that through pET29. A pET28 vector having a long N-terminal tag (20 residues) was

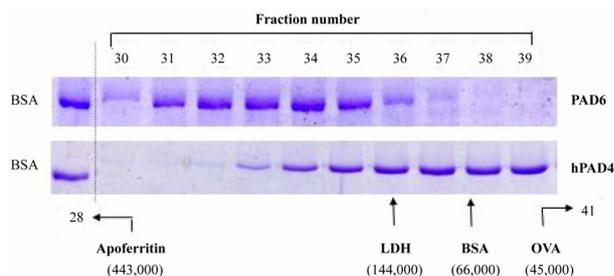


Figure 3. Elution profiles of PAD6 and hPAD4 obtained by Sephacryl S300 gel chromatography. (*Upper panel*) An ammonium sulfate-enriched preparation (~50 mg) of an eluent from a DE52 column was applied to a Sephacryl S300 column, and fraction numbers 30 to 39 were subjected to Ni²⁺ Sepharose chromatography as described in Materials and Methods. Aliquots of the imidazole-eluted samples were analyzed by 8% SDS-PAGE. (*Lower panel*) An hPAD4 preparation (~5 mg) released through *PreScission* protease digestion was applied to a Sephacryl S300 column, and aliquots of the eluent were analyzed by 8% SDS-PAGE. Separately, authentic standards were run to locate the elution position as monitored at 280 nm: LDH, lactate dehydrogenase; BSA, bovine serum albumin; OVA, ovalbumin.

also without effect (**Figure 1**), suggesting that the C-terminal His tag does not interfere with the expression. Therefore, the secondary structure of a local nucleotide sequence in the PAD6 mRNA may impede translation, or the codon usage of the PAD6 mRNA may be inadequate in prokaryotic cells.

3.3. Elution Profile of PAD6 on Sephacryl S300

PAD6 and hPAD4 were subjected to molecular sieving, and each fraction was visualized by staining after SDS-PAGE (**Figure 3**). PAD6 appeared with a broad peak at $M_r \sim 200,000$, while hPAD4 was recovered much slower than PAD6. A major band of hPAD4 was seen around the position of BSA, which differed from the expected elution position of about $M_r 150,000$ (dimer) [9].

3.4. Chemical Cross-Linking

The results of size-exclusion chromatography were slightly ambiguous. Thus, we estimated the quaternary structure based on results of chemical cross-linking experiments. RNase A, creatine kinase, lactate dehydrogenase and glutamate dehydrogenase were used as a monomer or oligomeric proteins. The protein bands were visualized by staining after disc SDS-PAGE. Unexpectedly, PAD6 revealed six bands (**Figure 4**). hPAD4 produced a dimer although its population was slightly small compared with a parent band. PAD6 via pGEX also showed a hexamer (data not shown). These results, together with those of the molecular sieving experiment, imply that hPAD4 constitutes in part a monomeric

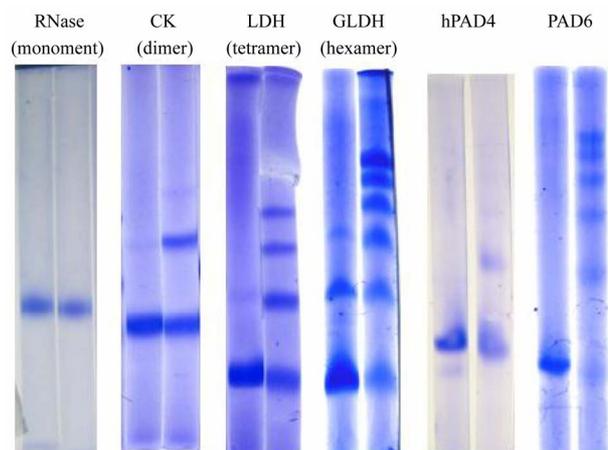


Figure 4. Chemical cross-linking of PAD6 and hPAD4 with dimethyl suberimidate. Chemical cross-linking experiments were carried out as described in Materials and Methods. RNase A (M_r 14,000), creatine kinase (CK, subunit M_r 41,000), lactate dehydrogenase (LDH, subunit M_r 35,000), and bovine glutamate dehydrogenase (GLDH, subunit M_r 52,000) were used as standards. The gel concentration was 3.5% except for RNase A (8%) and CK (6%).

population under the present conditions, and PAD6 can form a hexamer (calculated $M_r \sim 456,000$) despite that little PAD6 was seen at the elution position of apoferritin (calculated M_r 443,000). The shape of PAD6 is considered to be different from that of apoferritin.

3.5. CD Spectra

CD spectra of PAD6 and hPAD4 were measured in the far ultraviolet region (**Figure 5**). Their patterns seemed to be similar; the α -helix content was calculated to be 26.0% and 27.2% for hPAD4 and PAD6, respectively, from the $[\theta]_{\text{obs}}$ value based on the equation of Chen and Yang [17]. The CD spectrum of mouse PAD2 indicated an α -helix content of 29.1% [18]. An X-ray analysis revealed that hPAD4 is comprised of 22.4% α -helix and 39.1% β -strand [9]. It is conceivable that PAD6 and hPAD4 have a similar secondary structure.

3.6. Enzymatic Activity of Purified Preparations

The specific activity of purified hPAD4 was 1.2 $\mu\text{mol}/\text{mg}/\text{min}$, whereas purified PAD6 exhibited no detectable activity even with 50 μg and 15-h incubation. hPAD4 Cys⁶⁴⁵ is essential for catalysis because a Cys⁶⁴⁵Ala mutant did not show activity [9]. We also confirmed the Cys⁶⁴⁵Ala mutant to be enzymatically inactive. In PAD6, the corresponding residue is Ala⁶⁶⁴. It is of interest whether an Ala⁶⁶⁴Cys protein has activity. The mutant, however, did not exhibit enzymatic activity, indicating that there exist critical residues other than Ala⁶⁶⁴.

4. DISCUSSION

The structure of mammalian eggs is fortified with cytoplasmic sheets. An $M_r \sim 69,000$ protein, occurring in relatively high abundance in eggs, was found to associate with the fibrous network of intermediate filaments [19]. Wright *et al.* separated this soluble protein by two-

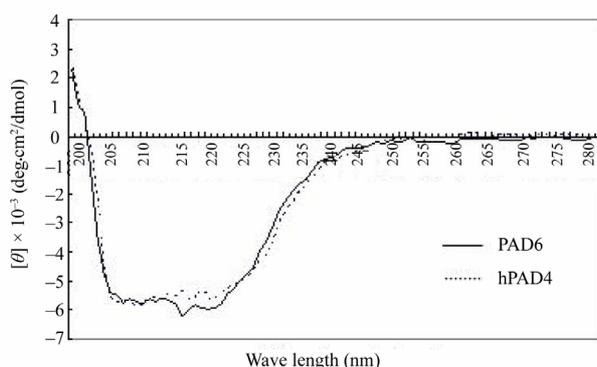


Figure 5. CD spectral profiles of PAD6 and hPAD4. About 0.3 mg/ml each of PAD6 and hPAD4 were recorded in the far ultraviolet region. The measurement conditions were: cell, 10-mm light path; response, 2 sec; band width, 1 nm, scan speed, 20 nm/min.

dimensional gel electrophoresis, identified it as an isoform of PAD by mass spectrometry, and named it ePAD [20]. An ortholog was found in humans, and ePAD was renamed PAD6 [6]. Esposito *et al.* [21] created PAD6-null mice lacking cytoplasmic sheets and female fertility, and showed an absence of positive signals in these animals when ovarian sections were stained with an anti-H4Cit3 antibody. They concluded that this was due to the lack of PAD6 activity. On the other hand, an X-ray analysis of hPAD4 along with a sequence alignment of PADs suggested that enzymatic activity may not be inherent to PAD6 [9]. In the present study, we first showed that the purified PAD6 does not exhibit enzymatic activity when BAEE is used as a substrate. A microbial PAD occurs in *P. gingivalis* whose primary sequence differs greatly from that of PAD6 [22]. Rodriguez *et al.* [11,12] showed with purified a monomeric *P. gingivalis* PAD that Cys³⁵¹ acts as a nucleophile in catalysis and no Ca²⁺ cofactor is required. These results suggest that a particular Cys residue in the active site is a prerequisite for deimination, but Ca²⁺ is not necessarily required in the microbial enzyme. Our preliminary finding that an Ala⁶⁶⁴Cys mutant did not exhibit enzymatic activity shows the necessity of acidic residues for Ca²⁺ binding. Among 19 residues involved in the binding of Ca²⁺, 13 are acidic. In human PAD6, 7 Glu and Asp residues are not conserved [9]. Human and mouse PADs are similar in primary structure. Accordingly, the PAD6 Ala⁶⁶⁴Cys mutant is expected to be incapable of binding to Ca²⁺. Whether bound Ca²⁺ exists in PAD6 remains to be determined. It is tempting to examine whether the replacement of these non-acidic residues with acidic residues could restore the enzymatic activity. The creation of a chimera between hPAD4 and PAD6 may be another approach.

Studies *in vitro* and *in vivo* have evoked a great discrepancy as to why an inactive PAD6 can function as an enzyme in the egg and ovary. Several possibilities exist: 1) a true proteinous substrate of PAD6 occurs *in vivo*; 2) an unknown protein such as a chaperone is needed for the activity; 3) Ca²⁺ is not the correct cofactor; and 4) the recombinant PAD6 differs from the naturally occurring form due to a lack of post-translational modification. Concerning the second possibility, Snow *et al.* [23] reported the necessity of tyrosine/tryptophan monooxygenase activation protein (YWHA or 14-3-3 protein) for interaction with PAD6 via phosphorylation at the stage encompassing oocytes to early embryos.

The principle finding of this study is that PAD6 is a hexamer unlike hPAD4, although the two resemble each other in secondary structure. This was not noted previously. PAD6 may be in a state of equilibrium regarding the association-dissociation of the protomer depending

on the protein concentration or the solvent. Under chemical cross-linking conditions, the PAD6 concentration was relatively high (about 0.5 mg/ml), whereas about 0.1 mg/ml of PAD6 was used for a Sephacryl S300 chromatography, and so the protein would be gradually diluted during development; this may explain why PAD6 migrated so far. Arita *et al.* [9] in their X-ray analysis queried whether the dimeric form is specific to PAD4 or common to all PADs although the residues involved in the dimerization are partially conserved. Unfortunately, we could not determine which residues play a role in the subunit-subunit interaction of hPAD4. The residues responsible for the hexamer of PAD6 are expected to be different from those in the case of hPAD4. Further study is needed to clarify whether the unique structure of PAD6 favors the formation of cytoplasmic sheets in eggs.

In conclusion, we have first demonstrated that PAD6 has no enzymatic activity when BAEE is used as a substrate, and is a hexameric protein unlike other dimeric PADs. This unique structure is suggested to favor the formation of egg cytoplasmic sheets.

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