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Comparison of enzymatic properties between hPADI2 and hPADI4☆

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Abstract

In the sera of rheumatoid arthritis (RA) patients, autoantibodies directed to citrullinated proteins are found with high specificity for RA. Peptidylarginine deiminases (PADIs) are enzymes responsible for protein citrullination. Among many isoforms of PADIs, only PADI4 has been identified as an RA-susceptibility gene. To understand the mechanisms of the initiation and progression of RA, we compared the properties of two PADIs, human PADI2 and human PADI4, which are present in the synovial tissues of RA patients. We confirmed their precise distribution in the RA synovium and compared the stability, Ca^{2+} dependency, optimal pH range, and substrate specificity. Small but significant differences were found in the above-mentioned properties between hPADI2 and hPADI4. Using LC/MS/MS analysis, we identified the sequences in human fibrinogen indicating that hPADI2 and hPADI4 citrullinate in different manners. Our results indicate that hPADI2 and hPADI4 have different roles under physiological and pathological conditions. Further studies are needed for the better understanding of the role of hPADIs in the initiation and progression of RA.

Keywords: Citrullination; PADI2; PADI4; Fibrinogen

Peptidylarginine deiminase (PADI) catalyzes the conversion of peptidylarginine to peptidylcitrulline in the presence of Ca^{2+}. Citrulline is a non-coding native amino acid and peptidylcitrulline is produced by post-translational modification of arginine residues by PADI. This conversion causes a very small change in the molecular mass (less than 1 Da) and loss of one positive charge. Such changes could affect the conformation of the protein and the interaction with other molecules [1]. In humans, five PADI isoforms have been identified and they have different tissue distributions [2–8]. As known citrullinated proteins in vivo, for example, there are keratins [9,10] and filaggrin [11–13], vimentin [14], myelin basic protein (MBP) [15], histone [16,17], nucleophosmin/B23 [16], and fibrinogen or fibrins [18]. But, not much is known about the specificity of PADI toward protein substrates.

PADI and citrullination of proteins are associated with human diseases [18–21]. In RA, various antibodies including anti-citrullinated peptide antibodies are generated in the patients as autoantibodies. It is reported that the family of autoantibodies directed to proteins

☆ *Abbreviations:* BAEE, benzoyl-L-arginine ethyl ester; BCEE, benzoyl-L-citrulline ethyl ester; Bz, benzoyl; DTT, dithiothreitol; OEt, *O*-ethylester; OMe, *O*-methylester; Tos, tosyl.

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containing modified citrulline [12,13,18] has higher specificity and sensitivity for diagnosis of RA than the widely used rheumatoid factor (RF) [22]. Anti-citrullinated protein autoantibodies can be detected in early stage RA and could be useful for diagnosis of early stage RA in clinical practice [23–25]. Further, a strong association is reported between RA and the functional variants of the gene encoding PADI4 in the Japanese population [2]. These findings suggest that citrullination in self-peptides and the subsequent break of tolerance to citrullinated peptides occurs at very early stage RA or is associated with the onset of RA.

In RA synovial tissues, PADI2 and PADI4 have been detected [2,26,27]. Therefore, either or both types can participate in the citrullination of proteins of RA synovial tissues, but their respective roles remain obscure. It is considered that various factors regulate their roles under physiological conditions. Factors that regulate the respective roles include tissue distribution and cellular localization, chronological regulation of expression, biochemical conditions that activate PADI enzymes, substrate specificity, and residual preference. In this study, we examined the localization of human PADI2 (hPADI2) and human PADI4 (hPADI4) in RA synovium. And we characterized the properties of hPADI2 and hPADI4 by comparing stability, Ca^{2+} dependency, optimal pH range, and activities towards 4 arginine derivatives and 2 proteins, human fibrinogen and human filaggrin, which are known to be citrullinated in vivo and recognized by autoantibodies in RA sera [9,18]. We compared the citrullinated sites in fibrinogen by hPADI2 and hPADI4 in vitro using liquid chromatography with tandem mass spectrometry (LC/MS/MS) analysis.

Materials and methods

Chemicals. L-Citrulline, Bz-Arg-OEt, and Bz-Arg-OMe were from Sigma. Bz-Arg-NH_2 was from Tokyo Kasei Kogyo. Tos-Arg-OMe was from Wako Pure Chemicals Industries.

Cloning of human PADI2 and PADI4, and human filaggrin. We obtained full-length hPADI2 cDNA from ResGen (Invitrogen), and we obtained hPADI4 and filaggrin cDNA by PCR using human bone marrow cDNA [2] and human skin cDNA [28] as the templates, respectively. The hPADI2 cDNA was cloned into a pDEST10 vector (Invitrogen) for expression in SF9 cells. The hPADI4 cDNA was cloned into a pDEST17 vector (Invitrogen) and the filaggrin cDNA was cloned into a pET22b (+) vector (Novagen) for expression in *Escherichia coli*.

Expression and purification of human PADI2, PADI4, and filaggrin. Recombinant hPADI2 protein expressed in *E. coli* was purified as previously described [7]. A recombinant baculovirus that encoded his-tagged hPADI2 was generated using a BAC-TO-BAC Baculovirus Expression System (Gibco-BRL). The expression of the recombinant hPADI2 protein was confirmed by Western blotting and then purified using Q Sepharose, phenyl Sepharose, and HiPrep26/60 Sephacryl S-300 High Resolution (Amersham) according to the manufacturer's instructions. SDS–PAGE was performed to analyze the purified protein from the infected cells. Purified recombinant PADI2 was dialyzed with 20 mM Tris–HCl (pH 7.6), 500 mM NaCl, 2 mM DTT, 1 mM EDTA, and 20% glycerol. We used purified hPADI2 expressed by *E. coli* and the baculovirus system for assays of the activities on arginine derivatives and used purified hPADI2 expressed by the baculovirus system for assays of the activities on protein substrates because hPADI2 expressed by the baculovirus system showed higher activity than hPADI2 expressed by *E. coli* (Table 1) and almost the same activity as hPADI2 expressed in mammalian cells (data not shown). His-tagged hPADI4 was expressed in bacteria BL21-SI (Invitrogen) by 0.3 M NaCl induction for 3.5 h at 30 °C and his-tagged filaggrin was expressed in BL21 (DE3) codon plus (Stratagene) by 1 mM IPTG induction for 3 h at 37 °C. Fusion proteins were purified by HiTrap column (Amersham) or Ni–NTA (Qiagen) according to the manufacturer's instructions. hPADI4 and filaggrin were dialyzed extensively against hPADI4 dialysis buffer (10 mM Tris–HCl [pH 7.6], 150 mM NaCl, 200 μM DTT, and 1 mM EDTA) and the filaggrin dialysis buffer (10 mM Tris–HCl [pH 7.6], 20 mM $CaCl_2$, and 5 mM DTT), respectively.

Preparation and purification of antiserum against hPADI2 and hPADI4. We synthesized hPADI2 derived peptide (CDGVVEKNNP KKASWT), purified and immunized in rabbits, and then purified antiserum by affinity chromatography on the peptide column (Immuno-Biological Laboratorie, Gunma, Japan). Anti-hPADI4 antibody was prepared as previously described [2].

Immunohistochemistry. Paraffin sections of synovial tissues were incubated at 4 °C for 12 h with rabbit polyclonal antibody to hPADI4 or hPADI2 at 100 ng/section and visualized by VECTASTAIN ABC Universal kit using 3,3′-diaminobenzidine as a substrate. All of the sections were counterstained with hematoxylin.

Assays of hPADI2 and hPADI4 activities on arginine derivatives. The activities of hPADI2 and hPADI4 on arginine derivatives were measured as described previously [5]. Briefly, in each enzymatic assay, purified enzyme was mixed with reaction buffer containing 25 mM Tris–HCl (pH 7.6), 20 mM $CaCl_2$, 5 mM DTT, and various concentrations of arginine derivatives. Then the reaction mixtures were incubated at 37 °C for 10 min. After incubation, reactions were stopped by 33 mM EDTA. The concentration of citrulline was determined by the colorimetric method [29]. All the activity measurements were performed in triplicate.

One unit (U) of the enzyme was defined as the amount of the enzyme that catalyzed the production of 1 μmol BCEE at 37 °C for 1 h

Table 1
Kinetic parameters of hPADI2 and hPADI4 for arginine derivatives

Substrate	K_m (mM)	V_{max} (μmol/mg/h)
hPADI2 expressed in E. coli		
Bz-Arg-NH_2	1.01 (± 0.18)	10.8 (±0.4)
Bz-Arg-OEt	0.73 (±0.10)	37.6 (±0.8)
Bz-Arg-OMe	1.97 (±0.15)	23.8 (±0.4)
Tos-Arg-OMe	5.61 (±1.27)	23.9 (±1.8)
hPADI2 expressed by baculovirus		
Bz-Arg-NH_2	1.23 (±0.09)	31.7 (±0.4)
Bz-Arg-OEt	0.15 (±0.01)	88.3 (±0.1)
Bz-Arg-OMe	0.77 (±0.02)	73.0 (±0.5)
Tos-Arg-OMe	1.63 (±0.10)	83.4 (±1.3)
hPADI4 expressed in E. coli		
Bz-Arg-NH_2	0.16 (±0.01)	115.9 (±1.1)
Bz-Arg-OEt	0.91 (±0.09)	108.0 (±1.9)
Bz-Arg-OMe	0.63 (±0.04)	77.0 (±0.8)
Tos-Arg-OMe	—	—

The incubation mixtures were composed of 25 mM Tris–HCl (pH 7.6), 20 mM $CaCl_2$, 5 mM DTT, various amounts of the arginine derivatives, and 0.03 U hPADI2 or hPADI4, and were incubated at 37 °C.

under the conditions described above. The kinetic assay for BAEE was estimated from the data of the enzymatic activity assay at 37 °C for 20 min using the direct linear plot and Hanes–Woolf plot. Protein concentration was determined by the method of Bradford [30] with bovine serum albumin as a standard.

Assays of hPADI2 and hPADI4 activities on protein substrates. Human fibrinogen (American Diagnostics) and human filaggrin were each mixed with hPADI2 or hPADI4 and incubated for 5 and 20 min, respectively, at 37 °C. The concentration of citrulline was measured as described above. The kinetic assay for the protein substrates was performed using the Lineweaver–Burk plot.

SDS–PAGE and Western blotting. Protein samples were resolved by 10% SDS–PAGE and then transferred to a PVDF membrane (Bio-Rad). Blotted membranes used for the detection of citrullinated proteins by hPADI2 or hPADI4 were chemically modified prior to immunostaining, as described in the instructions of the anti-citrulline detection kit (Upstate).

Detection of citrullinated sites of human fibrinogen by LC/MS/MS. hPADI2 or hPADI4 was mixed with the reaction buffer containing 25 mM Tris–HCl (pH 7.6), 20 mM $CaCl_2$, 5 mM DTT, and 6 μM human fibrinogen (American Diagnostics), and incubated for 3 h at 37 °C. An aliquot of 25 μl of the fibrinogen treated with hPADI2, hPADI4 or no enzyme was mixed with 50 μl of 0.1 M NH_4HCO_3, pH 7.8, 6 M guanidine–HCl, and 10 mM EDTA. The denatured fibrinogen was reduced and alkylated with 10 mM DTT and 55 mM iodoacetamide. The buffer of the proteins was changed to 20 mM NH_4HCO_3, pH 8.0, and was deglycosylated by peptide:*N*-glycanase F (New England Biolabs). Digestion of deglycosylated proteins with trypsin (Promega), chymotrypsin, and Glu-C (Roche Applied Science) was performed separately for 12 h. Adding formic acid stopped the enzymatic reaction and the samples were stored at −20 °C until use.

The mixture of the digested peptides was analyzed by LC/MS/MS. The Q-TOF2 mass spectrometer (Micromass) was equipped with a CapLC chromatography system (Waters) using a homemade electrospray ionization tip packed with Develosil ODS media (Nomura Chemical). The MS/MS data were searched against the SWISS-PROT database with protease specificities under consideration of a fixed modification (carbamoylmethylation of Cys) and variable modifications (citrullination of Arg, deamidation of Asn and Gln, oxidation of Met, and pyroglutamination of N-terminal Gln) using the Mascot program (Matrix Sciences). The criteria for the citrullinated sites were: (i) appearance of a peptide of a mass shift of +1 Da in the sample treated with PADIs; (ii) disappearance of the peptide in the sample without PADIs; and (iii) good assignment of a mass shift of +1 Da on Arg in the MS/MS spectra of the peptide by automated or manual inspection. When a non-citrullinated peptide observed in the sample without hPADIs was not detected in the sample with hPADIs, we considered this site as highly citrullinated. When a non-citrullinated peptide observed in the sample without hPADIs was also detected in the sample with hPADIs, we considered this site as partially citrullinated. When a non-citrullinated peptide was not observed in the sample without hPADIs, we designated this site just as observed.

Results

Immunohistochemical localization of hPADI2 and hPADI4 in rheumatoid arthritis synovium

To determine whether hPADI2 and hPADI4 were distributed differentially in the synovial tissue of patients with RA synovitis, fresh-frozen synovial tissues were obtained and immunohistochemical analysis on cryostat tissue sections was performed with an antibody to

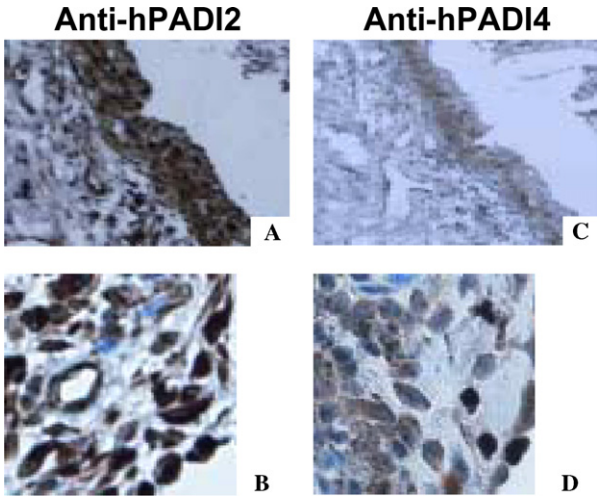

Fig. 1. Immunohistochemical localization of hPADI2 and hPADI4 in human RA synovium. Cryosections of RA synovium were analyzed by immunostaining with anti-hPADI2 (A,B) and hPADI4 (C,D), respectively. All sections were counterstained with hematoxylin. Original magnification: A and C, 4×; B and D, 40×.

hPADI2 and hPADI4 (Fig. 1). hPADI2 was expressed in both the lining layer and sublining region (Fig. 1A), while hPADI4 was expressed mainly in the sublining region (Fig. 1C). hPADI2 was expressed in the cytoplasm (Fig. 1B), while hPADI4 was expressed in both the cytoplasm and nuclei (Fig. 1D). In the deep lining layer, hPADI2 protein was detected more than hPADI4 protein (Figs. 1A and C). These results indicate that hPADI2 and hPADI4 proteins are localized in the RA synovium and the expression patterns are somewhat different.

Catalytic activities of hPADI2 and hPADI4 on arginine derivatives

To characterize the properties of hPADI2 and hPADI4, each of them was expressed recombinantly as a His-tagged protein and purified. The purified proteins were subjected to SDS–PAGE analysis. These preparations gave single major bands of about 76 and 70 kDa, respectively. With 4 arginine derivatives, kinetic properties were observed for recombinant hPADI2 and hPADI4 (Table 1). Compared with the hPADI2 activity toward the α-amino substituents, hPADI2 showed higher activity toward the benzoyl group than the tosyl group. Toward the α-carboxyl substituents, hPADI2 showed high activity in the order of *o*-ethyl group > *o*-methyl group > *o*-carboxyl free radical. The properties of hPADI2 toward arginine derivatives were similar to those of rabbit skeletal muscle PADI [31]. On the other hand, compared with the hPADI4 activity toward the α-amino substituents, hPADI4 showed high activity toward the benzoyl group but did not show any activity toward the tosyl group, although hPADI2 did. Toward the α-carboxyl substituents, hPADI4 showed high

activity in the order of *o*-carboxyl free radical > *o*-ethyl group > *o*-methyl group. Thus, the patterns of the kinetic properties of recombinant hPADI2 and hPADI4 for arginine derivatives were clearly different.

Calcium dependency, stability, and optimal pH range of activities of hPADI2 and hPADI4 on BAEE

We next assessed the effect of calcium and pH on the activities of hPAID2 and hPADI4 using BAEE as a substrate. Activities of both hPADI2 and hPADI4 were increased by calcium in a concentration-dependent manner, but the dependency was different (Fig. 2A). The activity of hPADI2 reached 50% of the maximum at about 0.62 mM, while the activity of hPADI4 reached 50% of the maximum at about 0.75 mM (Fig. 2A). Thus, the Ca^{2+} concentration for half-maximal activity of hPADI2 was somewhat lower than that of hPADI4. Concerning the optimal pH range, hPADI2 was most active in the pH range from pH 6.0 to 10.0, while

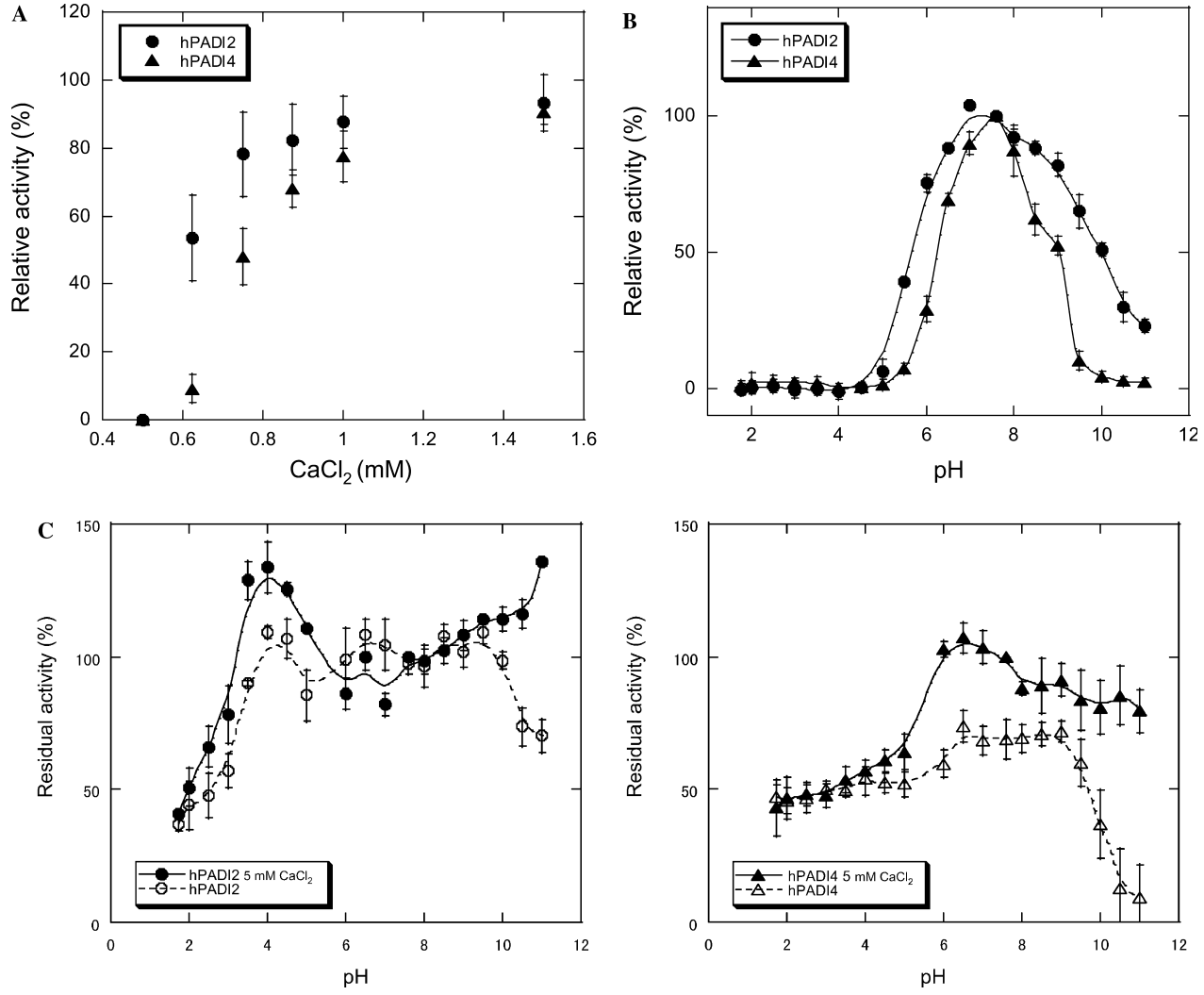

Fig. 2. Characterization of hPADI2 and hPADI4. (A) Effect of calcium. The incubation mixtures were composed of 10 mM Tris–HCl (pH 7.6), 5 mM DTT, 10 mM BAEE, 150 mM NaCl, 0.02 U hPADI2 or hPADI4, and various concentration of $CaCl_2$, and were incubated at 37 °C for 40 min. The concentration of citrulline was determined by the colorimetric method [29]. Symbols: ●, hPADI2; ▲, hPADI4. The data of relative activity were calculated as the percent of activity obtained at 20 mM $CaCl_2$. Values represent means ± SD of data from triplicate experiments. (B) pH dependence. The incubation mixtures were composed of 5 mM DTT, 10 mM BAEE, 150 mM NaCl, 0.02 U hPADI2 or hPADI4, and various buffers as follows: 10 mM acetate buffer (pH 1.75–6.0), 10 mM Mes buffer (pH 6.5), 10 mM Tris–HCl buffer (pH 7.0–8.0), and 10 mM glycine–NaOH buffer (pH 8.5–11.0). The mixtures were incubated at 37 °C for 40 min. The concentration of citrulline was determined by the colorimetric method [29]. Symbols: ●, hPADI2; ▲, hPADI4. The data of relative activity were calculated, as the percent of activity obtained at 10 mM Tris–HCl (pH 7.6). Values represent means ± SD of data from triplicate experiments. (C) Effect of pH on stability. A quantity of 0.02 U of hPADI2 (left panel) or hPADI4 (right panel) was dissolved in 10 mM buffer to a final concentration of 10% glycerol in the absence (○: hPADI2, △: hPADI4) and in the presence (●: hPADI2, ▲: hPADI4) of 5 mM $CaCl_2$, and allowed to stand at 25 °C for 60 min. The concentration of citrulline was determined by the colorimetric method [29]. Buffers used were acetate buffer (pH 1.75–6.0), Mes buffer (pH 6.5), Tris–HCl buffer (pH 7.0–8.0), glycine–NaOH buffer (pH 8.5–11.0). The data of residual activity were calculated, as the percent of activity obtained at 10 mM Tris–HCl (pH 7.6), 5 mM $CaCl_2$. Values represent means ± SD of data from triplicate experiments.

hPADI4 was from pH 6.5 to 9.0 (Fig. 2B). Compared with hPADI2, hPADI4 a showed narrow optimal pH range. We also estimated the pH stability of the two in the presence and absence of Ca^{2+} (Fig. 2C). In the absence of Ca^{2+}, the activity of hPADI2 was reduced at pH 5.0, below pH 3.5, and above pH 10.5. In the presence of Ca^{2+}, the activity was reduced below pH 3.0. Enhancements of the activity were observed at around pH 4.0 and 10.0 (left panel of Fig. 2C). In the case of hPADI4, the activity was reduced at below pH 5.0 and above pH 10.0 in the absence of Ca^{2+}. Also, the activity was reduced totally compared with the activity in the presence of Ca^{2+}. In the presence of Ca^{2+}, the activity was reduced below pH 5.0 (right panel of Fig. 2C). Thus, hPADI2 and hPADI4 had somewhat different stability, Ca^{2+} dependency, and optimal pH range.

Activities of hPADI2 and hPADI4 for protein substrates

We investigated the activities of hPADI2 and hPADI4 on human fibrinogen and human filaggrin. We detected the citrullination of them by anti-modified citrulline antibody (anti-MC) (data only for hPADI4 shown in Fig. 3). Citrullination of human fibrinogen by hPADI2 or hPADI4 was clearly detected by anti-MC. The Aα-chain was highly citrullinated compared with either the Bβ-chain or the γ-chain by both enzymes. In the Aα-chain, we detected a similar change in SDS–PAGE mobility of citrullinated substrates by both enzymes (Fig. 3A). In the case of human filaggrin, dramatic change in mobility on SDS–PAGE by citrullination was detected for assays with either enzyme, which was similar to the report on the change in mobility by citrullination of mouse filaggrin by rabbit skeletal muscle PADI [1] (Fig. 3B). Comparison of activity on human fibrinogen between hPADI2 and hPADI4 under the same reaction conditions using the same unit quantity indicated activity on fibrinogen relative to BAEE of hPADI2 was higher than that of hPADI4 (Fig. 4A). We determined the kinetic parameters of the activities of hPADI2 and hPADI4 toward human fibrinogen using the Lineweaver–Burk plot (Fig. 4B and Table 2). Also we determined the kinetic parameters of the activities of hPADI2 and hPADI4 for human filaggrin (Table 2). hPADI2 showed almost the same activity toward human fibrinogen and human filaggrin. On the other hand, hPADI4 showed higher activity toward human fibrinogen than toward human filaggrin. We confirmed the difference in activities of recombinant hPADI2 and hPADI4 on protein substrates as well as arginine derivatives.

Identification of citrullinated sites in human fibrinogen Aα-chain and Bβ-chain by hPADI2 and hPADI4

After we confirmed that hPADI2 and hPADI4 exhibited different enzyme activity in citrullination of human

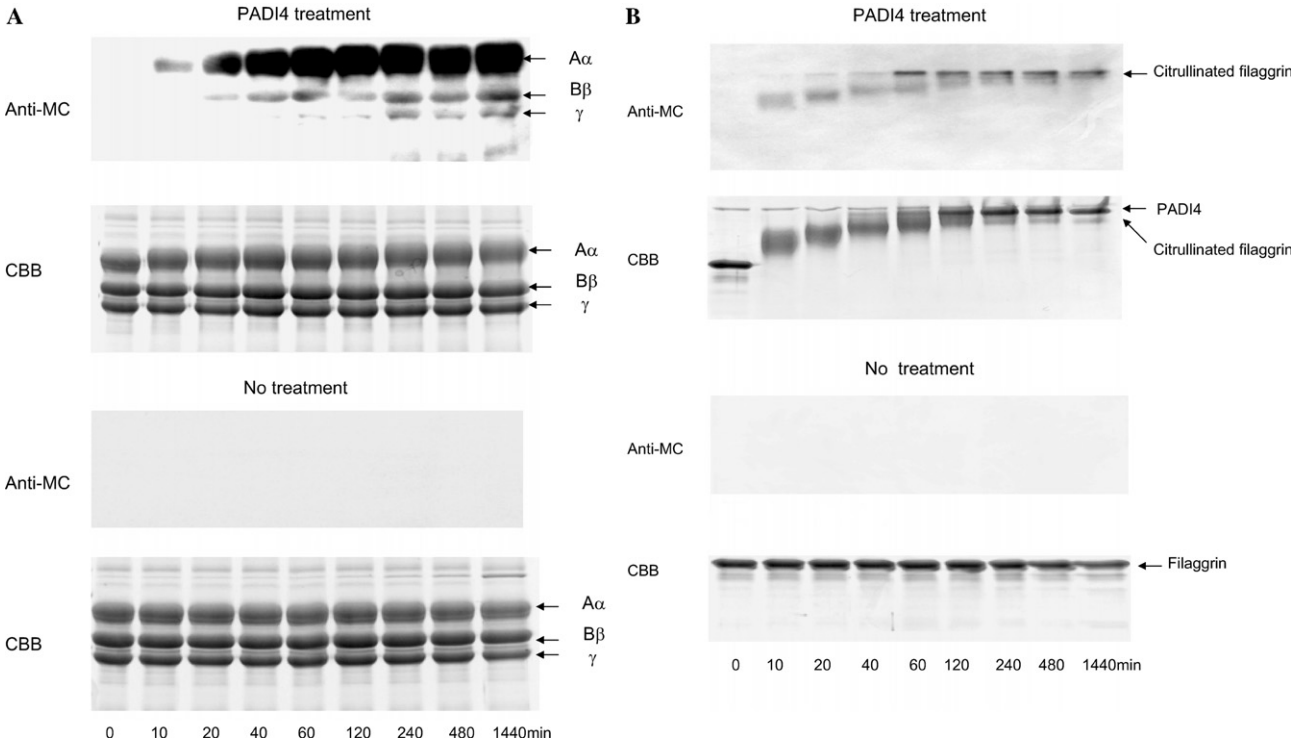

Fig. 3. Citrullination of human filaggrin and fibrinogen by hPADI4. Detection of citrullination of human fibrinogen (A) or human filaggrin (B) by hPADI4. Samples catalyzing hPADI4 for 0, 10, 20, 40, 60, 120, 240, 480, and 1440 min, respectively, were resolved by 10% SDS–PAGE, stained with CBB or transferred to a PVDF membrane, and developed with anti-MC.

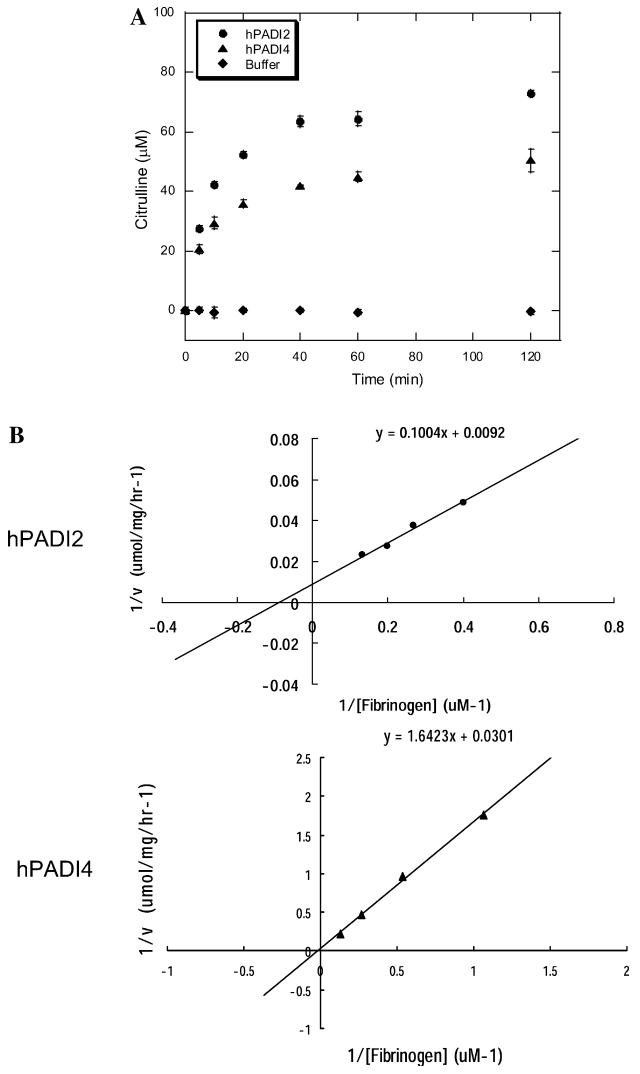

Fig. 4. (A) Time courses of the citrullination of human fibrinogen by hPADI2 and hPADI4. The incubation mixtures were composed of 25 mM Tris–HCl (pH 7.6), 20 mM $CaCl_2$, 5 mM DTT, 6 μM human fibrinogen, and 0.03 U hPADI2 or hPADI4, and were incubated at 37 °C. Symbols: ●, hPADI2; ▲, hPADI4; and ■, buffer for control. Values represent means ± SD of data from triplicate experiments. (B) Determination of Michaelis–Menten kinetic parameters of hPADI2 and hPADI4 for human fibrinogen.

Table 2
Kinetic parameters of hPADI2 and hPADI4 for human fibrinogen and filaggrin

Substrate	K_m (mM)	V_{max} (μmol/mg/h)
hPADI2		
Human fibrinogen	0.013	111.8
Human filaggrin	0.001	96.5
hPADI4		
Human fibrinogen	0.055	33.2
Human filaggrin	0.064	8.0

The incubation mixtures were composed of 25 mM Tris–HCl (pH 7.6), 20 mM $CaCl_2$, 5 mM DTT, various amounts of the native proteins, and 0.015–0.06 U hPADI2 or hPADI4, and were incubated at 37 °C.

fibrinogen, we next examined whether there were any differences in the citrullinated sites in human fibrinogen by hPADI2 and hPADI4. We identified the citrullinated sites of the Aα-chain and Bβ-chain of human fibrinogen by hPADI2 and hPADI4 using LC/MS/MS (Fig. 5). In both the Aα-chain and Bβ-chain, multiple sites were detected to be citrullinated. Some of the sites were catalyzed by both isozymes and others by either isozymes. In the γ-chain, only one site was identified that was citrullinated by both enzymes (data not shown). We detected that the number of catalyzed sites was more in the Aα-chain than in the Bβ-chain (Fig. 5). This result was consistent with the result of a higher signal in the citrullinated Aα-chain than in the citrullinated Bβ-chain detected with anti-MC (Fig. 3A). We identified 16 citrullinated sites that were citrullinated by both hPADI2 and hPADI4 in the Aα-chain and 4 sites in the Bβ-chain, though the degree of citrullination in these sites varied between the two isozymes. In the Aα-chain, we identified 6 sites that were citrullinated by hPADI2 but not by hPADI4, while we did not identify any sites citrullinated by hPADI4 but not by hPADI2. In the Bβ-chain, we identified 3 sites citrullinated only by hPADI2 and one site citrullinated only by hPADI4. In total, 21 sites were citrullinated by both hPADI2 and hPADI4, 9 were by hPADI2 but not by hPADI4, and 1 site was catalyzed only by hAPDI4.

Discussion

Citrullination of peptidylariginine to peptidylcitrulline by PADI is considered to play an important pathogenic role in RA because anti-citrullinated peptide antibody is highly specific in RA [18] and also because the PADI4 gene has an RA-susceptible variant [2]. Among the 5 known human PADI isozymes, hPADI2 and/or hPADI4 are considered to be responsible for the pathogenesis of RA because of their tissue distribution [2,26,27]. Although both PADI2 and PADI4 are detected in RA synovial tissues, their substrates and roles may be different. First of all, PADI2 and PADI4 are expressed by different cells in the synovial tissue and their intracellular localization seems to be different as anticipated from the fact that PADI4 has a nuclear localization signal unique among the 5 isozymes. Besides the localization, expression and activation of PADI2 and PADI4 were reported to be separately regulated at multiple steps, such as transcription, translation, and enzyme activation in monocytes and macrophages [27]. In this study, we showed that PADI2 and PADI4 had different properties in terms of substrate specificity, intermolecular residual preference, Ca^{2+} dependency, and optimal pH range as well as synovial distribution in RA synovium.

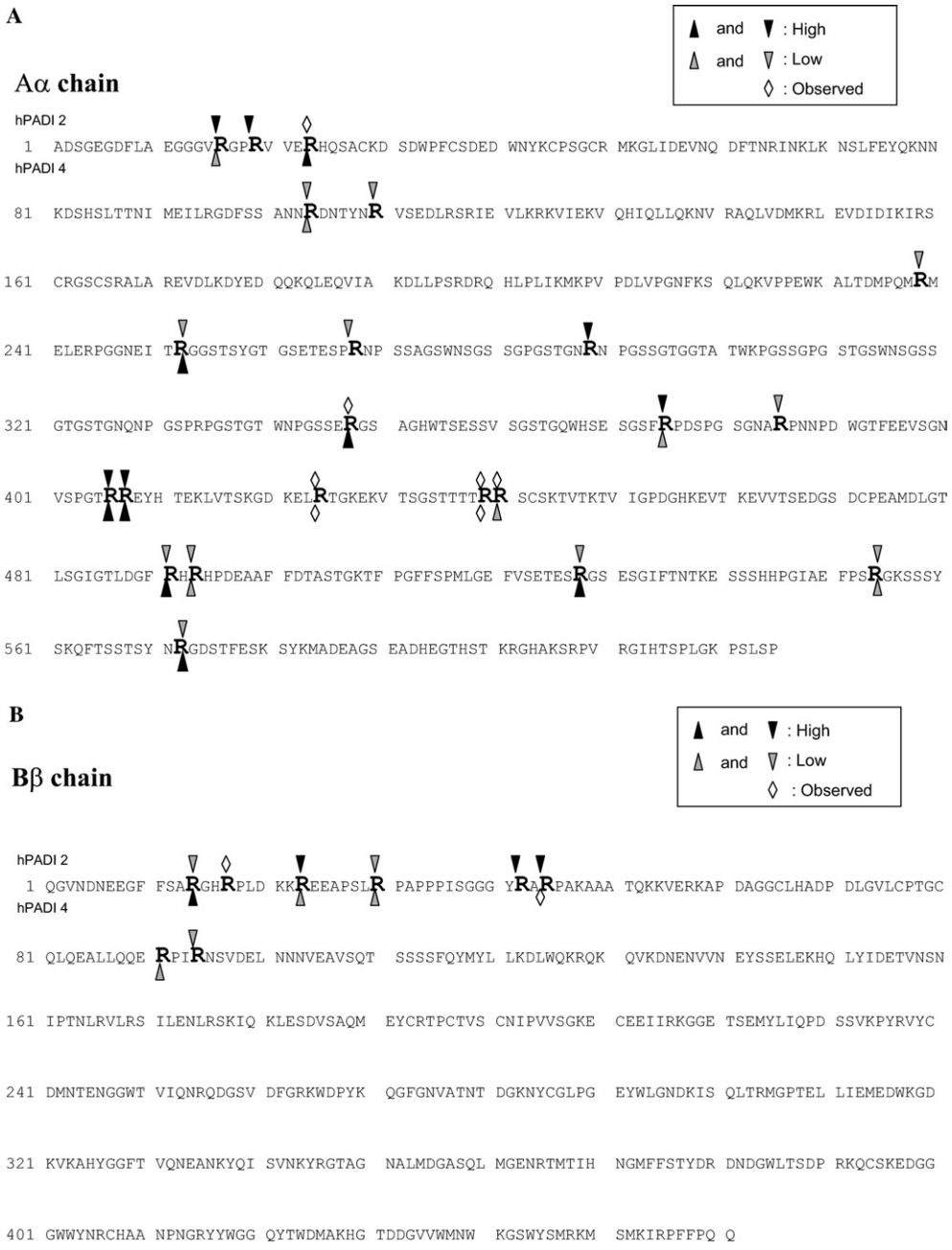

Fig. 5. Identification of citrullinated sites in human fibrinogen by hPADI2 and hPADI4. (A) Identification sites in Aα-chain of human fibrinogen by hPADI2 and hPADI4. (B) Identification sites in Bβ-chain of human fibrinogen by hPADI2 and hPADI4. The symbols for the sites citrullinated by hPADI2 are indicated above the line, and those for hPADI4 are indicated below the line. Highly, partially, and observed citrullinated sites of R are marked with black closed triangles, gray closed triangles, and open diamond shapes, respectively.

From the results of the difference in activities between hPADI2 and hPADI4 on arginine derivatives (Table 1), we confirmed that the catalysis-active site of hPADI2 and hPADI4 interacts with the imino-group differently depending on the side chain structure in the arginine derivatives. It seems reasonable to extrapolate from this that citrullination of peptidylarginine is also influenced by neighboring amino acid residues and other intramolecular chemical conditions in a subtype-specific manner, as seen in arginine derivatives. Therefore, we anticipated that hPADI2 and hPADI4 should have different substrate specificity and arginine residue preference. We selected human fibrinogen and human filaggrin for physiologic substrates because they have been reported to be recognized by anti-citrullinated protein antibody in RA sera [9,18]. Our in vitro experiments revealed that hPADI2 and hPADI4 catalyzed fibrinogen and filaggrin differently and citrullinated arginines in fibrinogens were also different between the two isozymes (Fig. 5).

Besides specificity of substrate and residue specificity in vitro, co-localization of the hPADI isozyme and its substrates is an important factor to determine in vivo enzyme–substrate relationships. It is not known precisely where self-peptides are citrullinated by hPADIs. Because all the hPADIs require high concentration of Ca^{2+}, citrullination by hPADIs must be carried out either intracellularly under tight control of the cation concentration or extracellularly with leakage of enzymes on apoptosis or necrosis of cells. Only hPADI4 is localized in the nucleus (identified by possession of a nuclear localization signal), but both hPADI2 and hPADI4 are localized in the cytosol (Fig. 1). Such differences in localization between hPADI2 and hPADI4 would affect the physiologic substrate repertoire of each.

Expressions of PADIs are regulated at the transcription and translation levels, and also translated enzymes are inactivated in steady state [27]. As our data indicated, hPADI2 and hPADI4 require high concentration of Ca^{2+} and regulation of pH within the optimal range for their activity. Therefore, it is considered that intracellular activation of hPADIs is controlled by transient increase in Ca^{2+} concentration (Fig. 2A). Though not much is known regarding the relationship between the rise in Ca^{2+} and physiologic hPADI activities such as citrullination of histones by hPADI4 [32,33] or citrullination associated with terminal differentiation of epidermal keratinocytes by hPADI2 [7], Ca^{2+} must be involved in their control. Because Ca^{2+} dependency was different between hPADI2 and hPADI4, their regulation by Ca^{2+} might be different in the Ca^{2+} signaling mechanism and/or Ca^{2+} concentration threshold. On the other hand, under extracellular conditions, Ca^{2+} concentration is sufficient for PADI activity. Therefore, once PADIs leak out of the cells by apoptosis or necrosis or some other mechanism(s), they could become activated and citrullinate extracellular proteins. In RA synovial tissue extracellular citrullinated fibrin is present [18]. Fibrin is located in RA synovium as a consequence of extravasation, therefore fibrin(ogen) is anticipated to be citrullinated extracelluarly [18]. Self proteins citrullinated intracellularly or extracellularly under some conditions as mentioned above could lead to autoimmune reaction in RA and the induction of autoimmunity is considered to be related to chemical changes of self molecules and HLA-restricted recognition of citrulline-containing peptides [34,35]

In summary, hPADI2 and hPADI4 show differential tissue distribution and pH- and calcium-dependent properties, and have substrate specificity for arginine derivatives, protein substrates and residue specificity in protein substrates. These differential properties and specificities suggest different roles under physiological and pathological conditions. Because autoimmunity towards citrullinated proteins is one of pertinent phenomena in RA, further investigations of the physiological and pathological roles of hPADIs would make significant contribution to the fields of molecular biology and immunology.

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