Molecular Characterization of Peptidylarginine Deiminase in HL-60 Cells Induced by Retinoic Acid and 1α,25-Dihydroxyvitamin D₃ *

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Three types of peptidylarginine deiminase (PAD), which converts a protein arginine residue to a citrulline residue, are widely distributed in animal tissues. Little is known about PAD of hemopoietic cells. We found that PAD activity in human myeloid leukemia HL-60 cells was induced with the granulocyte-inducing agents retinoic acid and dimethyl sulfoxide and with the monocyte-inducing agent 1α,25-dihydroxyvitamin D₃. We cloned and characterized a PAD cDNA from retinoic acid-induced cells. The cDNA was 2,238 base pairs long and encoded a 663-amino acid polypeptide. The HL-60 PAD had 50–55% amino acid sequence identities with the three known enzymes and 73% identity with the recently cloned keratinocyte PAD. The recombinant enzyme differs in kinetic properties from the known enzymes. Immunoblotting and Northern blotting with an antiserum against the enzyme and the cDNA, respectively, showed that the enzyme in cells and tissues is not yet known (20, 21). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby advertised solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBankTM/EMBL Data Bank with accession number(s) AB017919.

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¶ The abbreviations used are: PAD, peptidylarginine deiminase; BAEK, N-benzoyl-N-arginine ethyl ester; Bz-l-Arg, N-benzoyl-l-arginine; DTT, dithiothreitol; GST, glutathione S-transferase; MPO, myeloperoxidase; PAGE, polyacrylamide gel electrophoresis; PBS(–), Mg²⁺- and Ca²⁺-free phosphate-buffered saline; PCR, polymerase chain reaction; RA, all-trans-retinoic acid; RACE, rapid amplification of cDNA ends; TPA, 12-O-tetradecanoylphorbol-13-acetate; 1α,25-(OH)₂D₃, 1α,25-dihydroxyvitamin D₃; bp, base pairs; kb, kilobases; family of post-translational modification enzymes which convert arginine residues to citrulline residues in the presence of calcium ion. Enzymatic deimination in vitro changes the functional properties of various proteins and alters their secondary and tertiary structures (1–4). Deimination of keratins, filagrin, and trichohyalin is involved in the process of keratinization of skin and hair (4–9). Deiminated keratins and filaggrin are found in the cornified layer of the epidermis and deiminated trichohyalin is localized in the medulla of hair and the inner root sheath of hair follicles and these modifications are tightly linked to cell-specific stages of epitherm differentiation and hair follicle development (5–9). Extensively deiminated forms of myelin basic protein are also found in normal infant brain and in demyelinated areas of brain with multiple sclerosis, and this deimination is thought to be associated with immature myelination (10, 11). We reported a correlation between deimination of vimentin in mouse peritoneal macrophages and ionomycin-induced apoptosis (12). Deimination of a 70-kDa nuclear protein in cultured keratinocytes associated with apoptosis was also reported recently (13). All these findings suggest involvements of PAD in biological as well as pathological processes. There are at least three types of PAD in various rodent tissues which seem to be cell type specific (3, 14–16). Their substrate specificities for BAEK and Bz-l-Arg and their antigenic properties are different. PAD type II purified from rat muscle has been well characterized. It is also present in the brain, spinal cord, and some secretory tissues. PAD types I and III are mainly present in the epidermis and uterus and in hair follicles, respectively. PAD cDNAs for types I, II, and III have been isolated from rat, mouse, and sheep, but not from humans (9, 17–19). Their amino acid sequences constituting 662 to 673 amino acid residues have been deduced. Recently, a novel PAD cDNA named type IV was isolated from a keratinocyte cell line from a newborn rat and rat epidermis, but the distribution of the enzyme in cells and tissues is not yet known (20, 21).

PAD activities in rat granulocytes and mouse peritoneal macrophages have been reported, but nothing is known about the enzyme properties or structures of the enzymes (22). We studied PAD in human myeloid leukemia HL-60 cells, which can be induced to differentiate into granulocytes by retinoic acid and into monocyte/macrophages by 1α,25-(OH)₂D₃ or TPA (23). We report here the molecular characterization of HL-60 cell PAD induced by retinoic acid and regulation of its expression in myeloid differentiation.

EXPERIMENTAL PROCEDURES

Chemicals—Gigapack III Gold packaging extract and λZAP II/EcoRI/ calf intestine alkaline phosphatase-treated vector were from Stratagene, La Jolla, CA; Me₂SO, dimethyl sulfoxide; nt, nucleotides; PAGE, polyacrylamide gel electrophoresis.
Induction of Peptidylarginine Deiminase in HL-60 Cells

Northern Blotting—Total RNAs were isolated from HL-60 cells by the acid guanidinium thiocyanate method (32) and poly(A)⁺ RNA was isolated using an oligo(dt)-cellulose column (29). The poly(A)⁺ RNA was separated by electrophoresis in denaturing 0.8% agarose gel containing 2.5 mM formaldehyde, transferred to a Hybond-N nylon membrane, and UV cross-linked. The membrane was hybridized with a 32P-labeled full-length hPAD cDNA probe. (5.0 x 10⁶ cpm/3.5 ng/ml) in a solution of 50 mM formamide, 6 x SSPE, 0.1% SDS, 0.1% sonicated heat-denatured salmon sperm DNA, 5 x Denhardt's solution, and 0.5% dextran sulfate at 42°C for 24 h. The membranes were finally washed in 0.1 x SSC, 0.1% SDS at 65°C, and autoradiographed as described above.

Preparation of a Recombinant GST-hPAD—The entire coding sequence of PAD cDNA was constructed from a 5'-RACE cDNA and 7-2 cDNA by overhang extension by PCR. Briefly, a 5'-RACE cDNA was amplified using an M13 p8 primer (TOYOBO) and the antisense primer (nt 329-310) described above and then treated with T4 DNA polymerase to excise the 3' extruded portion. The PCR product, whose 3' end overlaps the 5' end (nt 246-329) of the 7-2 cDNA sequence, was annealed with KpnI-cut 7-2 DNA and cloned at 68°C. The elongated product was amplified with a sense primer (hPAD-ex1: 27-mer) consisting of a 5' EcoRI site (underlined) and a 19-nt sequence (nt 27-45: 5'-CGAAATTCATGGCCAGGACATG-3'), an antisense primer (hPAD-ex2: 35-mer) consisting of a 5' EcoRI-Ncol site (underlined) and a 19-nt sequence (nt 2098-2075: 5'-CCGAATTCGCGCGCGCGACGGCGCATGACAC-3') of the PAD cDNA. The PCR product was digested with EcoRI and subcloned into an EcoRI site of pGEX 4T1 containing a thrombin site and named pGEX-hPAD. The hPAD cDNA was also subcloned into an EcoRI site of pGEX 6P-1 containing a 3C protease site. BL21 cells transformed with pGEX-hPAD were grown in 2 x YT medium at 25°C to a cell density of 1.0 x 10⁶/ml for 600 min and then with addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for a further 5 h. The cells were collected by centrifugation, resuspended in 20 mM Tris-HCl (pH 7.6), 1 mM EDTA, and 0.1% Triton X-100 and disrupted by 2–3 passages through a French press. The cell lysate was brought to a concentration of 1 mM NaCl and centrifuged at 15,000 x g for 30 min. The supernatant was loaded on a glutathione-Sepharose 4B column and the column was thoroughly washed with lysis buffer containing 0.1 mM NaCl. The recombinant fusion protein was eluted with a solution of 10 mM glutathione in 50 mM Tris-HCl (pH 8.0), 0.1 mM NaCl, and 0.1% Triton X-100. The yield of enzyme activity was about 26%.

Preparation of Antiserum against PAD—Purified GST-hPAD (360 μg) in complete Freund's adjuvant was injected into rabbits and then they were given a booster injection of the same antigen in incomplete Freund's adjuvant. Anti-PAD serum was applied to a GST-Sepharose column. The unabsorbed fraction contained anti-PAD activity. An aliquot was diluted 100-fold with PBS (−) and then incubated with 280 μg/ml recombinant GST at room temperature for 20 min before use for immunoblotting. This preincubation was necessary for bleaching a non-specific band of about 70 kD.

SDS-PAGE and Immunoblotting—Sample proteins were subjected to SDS-10% PAGE by the method of Laemmli (33) and then transferred to a nitrocellulose membrane. For immunostaining of deiminated proteins, the membrane was treated at 37°C for 3 h with the medium for chemically modifying citrulline residues and then modified citrulline residues were detected by coupled immunoreactions with rabbit anti-modified citrulline IgG (0.125 μg/ml) for 1 h and horseradish peroxidase conjugate of goat anti-rabbit IgG (1:5,000) for 1 h by a reported method (34). Immunoblotting of PAD was performed using anti-GST-hPAD serum (1:3,000) or anti-rabbit type II PAD and bound IgG was detected with a horseradish peroxidase conjugate of goat anti-rabbit IgG (1:5,000) (Bio-Rad) using a chemiluminescence reagent kit, Renaissance (NEN Life Science Products). The blot was reprobed with anti-MPO serum (1:1,000) after depoing with a solution of 2% SDS, 62.5 mM Tris-HCl (pH 6.5), 0.1 M 2-mercaptoethanol as described before (34).

RESULTS

Expressions of PAD Activity in HL-60 Cells Induced to Differentiate into Granulocytes and Monocytes—When HL-60 cells were grown in the presence of RA, a granulocyte inducing agent, their PAD activity increased in the exponential phase of cell growth and reached a plateau in the stationary phase. No activity was detected in the absence of RA throughout the 3-day culture period (Fig. 1, A and B). During cell growth in the

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presence of RA, the MPO activity of the cells rapidly decreased to about 10% of that of control cells, indicating differentiation of the HL-60 cells into granulocytes (Fig. 1C), as reported previously (25). Various compounds are known to induce differentiation of HL-60 cells into granulocytes, monocytes, or macrophages (23). After additions of these compounds, the cells were examined for expression of PAD. Table I summarizes the ratios of the HL-60 cells into granulocytes (Fig. 1). The cells were harvested and disrupted by freeze-thawing. A, protein deiminating activity. Cell lysates equivalent to 2 × 10⁶ cells were incubated in the absence and presence of 10 mM CaCl₂ for 1 h with or without 10 μM CaCl₂ and then subjected to SDS-PAGE. The protein blots were probed with anti-modified citrulline IgG as horseradish peroxidase-goat anti-rabbit IgG as a secondary antibody as described under “Experimental Procedures.” Lanes 1–3, untreated and RA- and 1α,25-(OH)₂D₃-treated cell lysates, respectively, incubated in the absence of Ca²⁺; lanes 4–6, the same as lanes 1–3, respectively, except that the lysates were incubated with Ca²⁺. B, protein blots stained with Amido Black 10B. Lanes 1–3, untreated cell lysate, and RA- and 1α,25-(OH)₂D₃-treated lysates, respectively. C, immunoblotting of HL-60 PAD. Protein blots containing 7 milliunits of PAD activity of RA- and 1α,25-(OH)₂D₃-treated cell lysates were probed with anti-rat type II PAD IgG as described above. Lanes 1 and 2, 2.8 and 14 milliunits of rat PAD II, respectively; lanes 3 and 4, 7 milliunits of PAD of RA-cell lysates and 7 milliunits of PAD of 1α,25-(OH)₂D₃-cell lysates, respectively.

FIG. 1. Time courses of expressions of PAD activity in HL-60 cells without or with treatment with RA. HL-60 cells were seeded at 3 × 10⁵ cells/ml and grown in the absence (●) or presence of 1 μM RA (○) for the indicated times. Cell numbers of the cultures and enzymatic activities of PAD and MPO were determined in three separate cultures, as described under “Experimental Procedures.” Bars indicate standard deviations. A, cell numbers of the cultures. B, PAD activities with BAEE as a substrate of the cells. C, MPO activities as a decreasing marker during differentiation of the cells.

FIG. 2. Protein deiminating activity of HL-60 cells grown in the absence and presence of RA and 1α,25-(OH)₂D₃. HL-60 cells were cultured in the absence or presence of 1 μM RA and 0.1 μM 1α,25-(OH)₂D₃ for 3 days as described in the legend to Fig. 1. The cells were harvested and disrupted by freeze-thawing. A, protein deiminating activity. Cell lysates equivalent to 2 × 10⁶ cells were incubated in the absence and presence of 10 mM CaCl₂ at 37 °C for 1 h and then subjected to SDS-PAGE. The protein blots were probed with anti-modified citrulline IgG using horseradish peroxidase-goat anti-rabbit IgG as a secondary antibody as described under “Experimental Procedures.” Lanes 1–3, untreated and RA- and 1α,25-(OH)₂D₃-treated cell lysates, respectively, incubated in the absence of Ca²⁺; lanes 4–6, the same as lanes 1–3, respectively, except that the lysates were incubated with Ca²⁺. B, protein blots stained with Amido Black 10B. Lanes 1–3, untreated cell lysate, and RA- and 1α,25-(OH)₂D₃-treated lysates, respectively. C, immunoblotting of HL-60 PAD. Protein blots containing 7 milliunits of PAD activity of RA- and 1α,25-(OH)₂D₃-treated cell lysates were probed with anti-rat type II PAD IgG as described above. Lanes 1 and 2, 2.8 and 14 milliunits of rat PAD II, respectively; lanes 3 and 4, 7 milliunits of PAD of RA-cell lysates and 7 milliunits of PAD of 1α,25-(OH)₂D₃-cell lysates, respectively.

TABLE I

| Added inducers         | Activity* |
|------------------------|-----------|
|                        | units/mg  |
| None                   | NDb       |
| RA                     | 0.091 ± 0.012 |
| Me₂SO                  | 0.041 ± 0.012 |
| 1α,25-(OH)₂D₃         | 0.052 ± 0.005 |
| TPA                    | ND        |

* Values are mean ± SD for three separate cell cultures. ND, not detectable.

6), but on incubation without Ca²⁺ no deiminated proteins were detected (lanes 2 and 3). Untreated cell lysates did not show any deiminated proteins, regardless of the presence or absence of Ca²⁺ (Fig. 2A, lanes 1 and 4). These results indicate that PADs in the RA cell lysates and 1α,25-(OH)₂D₃ cell lysates can deiminate various cellular proteins in the presence of Ca²⁺.

In addition, the absence of detectable deiminated proteins in the intact cells suggested that a few proteins might be targeted slightly under in vivo conditions. Immunostaining of similar protein blots loaded with RA- and 1α,25-(OH)₂D₃ cell lysates containing 7 milliunits of PAD with anti-rat PAD type II IgG did not give any positive signals, although 2.8 and 14 milliunits of PAD of rat muscle PAD type II gave bands of about 72 kDa (Fig. 2C). This also suggested that the HL-60 PADs produced in cells cultured with RA or 1α,25-(OH)₂D₃ differ from the type II enzyme.

**Cloning and Characterization of a Human PAD cDNA**—To isolate and characterize the HL-60 PAD, we used a cDNA cloning strategy. We constructed a cDNA library in λZAP II
from HL-60 cells treated with RA for 3 days, and then screened the library by plaque hybridization with rat PAD type II cDNA as a probe. Two positive cDNA clones, 7-2 and 13-2, were selected and sequenced. Their sequences overlapped, but a sequence for a 5'-portion of PAD mRNA was missing. Thus, a 5'-portion of PAD cDNA was prepared by the 5'-RACE method. Several 5'-RACE cDNAs were obtained and sequenced. They had the same sequence. Three overlapping cDNAs were 5'-RACE cDNA (nt 1 to 329), 7-2 cDNA (nt 246 to 2,286), and 13-2 cDNA (nt 1,374 to 2,286). Alignment of the 5'-RACE cDNA and the 7-2 and 13-2 cDNAs gave a full-length cDNA named human PAD V cDNA (hPAD V cDNA). The cDNA was 2,286 bp long, and consisted of a 5'-untranslated region of 26 bp, a coding region of 1,992 bp, a 3'-untranslated region of 268 bp including a polyadenylation signal, AATAAA (nt 2,236 to 2,241), and a poly(A) tail (nt 2,264 to 2,286). The coding sequence encoded a polypeptide of 663 amino acid residues with a calculated molecular mass of 74,100 Da. The calculated pI of the protein was 6.12. The deduced amino acid sequence showed 55, 50, and 55% identities with those of rat PAD types I, II, and III, respectively, and 73% identity with rat keratinocyte PAD type IV, whose distribution in cells and tissues is not yet known. The carboxyl two-thirds of the sequences were relatively conserved, while the sequences of their amino-terminal one-thirds were more divergent (data not shown).

Expression and Characterization of a Recombinant HL-60 PAD—To express the above cloned PAD cDNA as a GST fusion protein in E. coli, we constructed the entire coding sequence (nt 1,374 to 2,286) of PAD from a 5'-RACE cDNA and inserted it into the pGEX 4T-1 vector. An isolated construct of pGEX-hPAD contained a 5'-untranslated region of 26 bp, a 3'-untranslated region of 268 bp including a polyadenylation signal, AATAAA (nt 2,236 to 2,241), and a poly(A) tail (nt 2,264 to 2,286). The coding sequence encoded a polypeptide of 663 amino acid residues with a calculated molecular mass of 74,100 Da. The calculated pI of the protein was 6.12. The deduced amino acid sequence showed 55, 50, and 55% identities with those of rat PAD types I, II, and III, respectively, and 73% identity with rat keratinocyte PAD type IV, whose distribution in cells and tissues is not yet known. The carboxyl two-thirds of the sequences were relatively conserved, while the sequences of their amino-terminal one-thirds were more divergent (data not shown).

Induction of Peptidylarginine Deiminase in HL-60 Cells

**TABLE II**

| Substrate | V_{max} | K_{m} | K_{cat} | K_{cat}/K_{m} |
|-----------|---------|------|--------|--------------|
| BAEE      | 17.3    | 1.4  | 2.7    | 1.9          |
| Bz-L-Arg  | 17.3    | 0.9  | 2.7    | 2.9          |

K_{cat}/K_{m} for these substrates were estimated from Lineweaver-Burk plots (Table II). The K_{m} value for BAEE was the same as that for Bz-L-Arg. The K_{m} for BAEE was larger than that for Bz-L-Arg. The K_{cat}/K_{m} ratio for Bz-L-Arg was 1.5 times that for BAEE. The K_{m} value for Bz-L-Arg with lysates of cells cultured with 1a,25-(OH)_{2}D_{3} was estimated to be 0.7 mM and was similar to the value of 0.9 mM of that with the recombinant enzyme. The other kinetic properties of the recombinant enzyme also appeared to reflect the properties of PADs in cells cultured with RA and 1a,25-(OH)_{2}D_{3}, which had relatively higher activity for Bz-L-Arg than for BAEE, as mentioned above.

The action of the recombinant PAD on cellular proteins in uninduced HL-60 cell lysates containing no endogenous PAD was also examined. The recombinant enzyme was incubated with an uninduced cell lysate with various concentrations of CaCl$_2$ of up to 10 mM and the resulting deiminated proteins were analyzed by immunoblotting with anti-modified citrulline IgG (Fig. 4). The deiminated proteins with a large range of molecular weights increased in a CaCl$_2$ concentration-dependent manner (lanes 1–7). Without CaCl$_2$, no deiminated protein was detected (lane 1). With 10 mM CaCl$_2$, a faint signal at the dye front was detected (lane 2) and its intensity increased with increase in CaCl$_2$ concentration. With 0.5 mM CaCl$_2$, four strong signal bands of 33, 34, 40, and 50 kDa besides the front band were seen (lane 4). With over 1 mM CaCl$_2$, numerous cellular proteins were deiminated (lanes 5–10). These results indicated the absolute requirement of Ca$^{2+}$ for PAD action and the preference of PAD for some cellular proteins at a limited concentration of CaCl$_2$. The different patterns of proteins deiminated by endogenous PAD in cell lysates (Fig. 2A) and by recombinant enzyme added to cell lysates (Fig. 4) might be due to different subcellular localizations of the endogenous cellular PAD and recombinant enzyme.$^2$

Regulation of PAD Gene Expression in HL-60 Cells during Granulocyte and Monocyte/Macrophage Differentiations—We studied the dynamic nature of PAD expression in HL-60 cells by immunoblotting and Northern blotting. First, antiserum to a purified GST-hPAD protein was raised and its specificity for the recombinant enzyme added to cell lysates (Fig. 4) was examined. The antiserum is specific for cellular 67-kDa PAD. We also examined the tempo-

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$^2$ K. Nakashima, T. Hagiwara, A. Ishigami, S. Nagata, H. Asaga, M. Kuramoto, T. Senuhu, and M. Yamada, unpublished data.
ral changes of PAD expression in HL-60 cells cultured for 5 days with or without granulocyte- and monocyte/macrophage-inducing agents in the same way (Fig. 5B, upper panel). During granulocyte differentiation induced by RA or Me₂SO, a 67-kDa band became detectable on day 2 of culture and its signal intensity gradually increased during culture for 5 days (lanes 4–11). Similarly, during monocyte differentiation induced by 1α,25-(OH)₂D₃, a band of 67 kDa became detectable on day 2 of culture and its intensity increased until the end of the culture period (lanes 12–15). Untreated cultures gave no bands (lanes 1–3). Moreover, surprisingly, differentiated macrophages induced by TPA gave no bands (lanes 16–19). Rapid decrease in the amount of precursor MPO and progressive decrease in the amount of MPO were observed on the same blot, confirming the differentiation of HL-60 cells into granulocytes, monocytes, and macrophages under these culture conditions reported previously (23, 25, 26) (Fig. 5B, lower panel). These results indicated that the same 67-kDa PAD is produced in RA- and Me₂SO-induced granulocytes and also in 1α,25-(OH)₂D₃-induced monocytes, but is not produced in TPA-induced macrophages.

Next, we examined the amount of PAD mRNA in similarly induced HL-60 cells using the above cloned cDNA as a probe for Northern blotting (Fig. 6, upper panel). The cells cultured with RA, Me₂SO, and 1α,25-(OH)₂D₃ for 2 days gave a major band (about 2.6 kb) and a minor band (about 3.2 kb) of mRNA (lanes 2–4). Untreated cells gave no band (lane 1). Rehybridization of the blot with a 5′ portion-specific probe (a 1.2-kbp sequence upstream of the XhoI site), the portion of which diverges in PADs, also gave bands of 3.2- and 2.6-kb mRNA (data not shown), suggesting that the 3.2-kb species was closely related to 2.6-kb mRNA. Decreasing intensities of MPO mRNA signals and similar intensities of glyceraldehyde-3-phosphate dehydrogenase mRNA signals during these treatments served as a cell differentiation marker and internal control, respectively. These results of Northern blotting and immunoblotting suggested that expression of the PAD gene is linked with granulocyte and monocyte differentiations of HL-60 cells and is regulated at the transcriptional level.

**DISCUSSION**

In this work we characterized a novel PAD induced in HL-60 cells during myeloid differentiation. This is the first characterization of PAD from cells of hematopoietic origin and human origin. The PAD activity in HL-60 cells was induced during RA- and Me₂SO-induced granulocyte differentiation and also during 1α,25-(OH)₂D₃-induced monocyte differentiation, but not in TPA-induced macrophage differentiation. Expression of the PAD appears to be closely linked to cell-specific stages of myeloid differentiation of HL-60 cells. Mouse peritoneal macrophages and mouse macrophage-like cell lines show PAD activities (12, 22). Although HL-60 cells can differentiate during normal myeloid differentiation in vivo and express many differentiation-associated markers, not all the phenotypes expressed in normal mature cells are always induced during HL-60 cell differentiations (23). Studies are needed on the dynamic expression of PAD during maturation of HL-60 monocytes into macrophages and in normal human monocytes and macrophages. The PAD of HL-60 monocytes appears to be identical with that of HL-60 granulocytes: both have similar activity on Bz-L-Arg and BAEE. The sizes of PADs detected by
Induction of Peptidylarginine Deiminase in HL-60 Cells

immunoblotting in HL-60 granulocytes and monocytes were the same and the sizes of the mRNAs in the two types of cells were also the same. Several of the same phenotypes are known often to be induced in both granulocyte and monocyte differentiation of HL-60 cells (23). Since both PAD mRNAs and proteins were simultaneously detectable during HL-60 cell differentiation, the expression of PAD is regulated at a transcriptional level. HL-60 cells have RA and D3 receptors (36, 37), and its amino acid sequence. Type IV cDNA has been cloned judging from its mobility on SDS-PAGE, its kinetic properties, and its amino acid sequence. Type IV occurs in cells and tissues has not yet been demonstrated. The amino acid sequence of HL-60 PADs was compared with those of four known rat enzymes (20). HL-60 PAD showed 73% amino acid sequence homology with that of rat keratinocyte PAD IV and 50–55% homologies with those of rat PAD types I, II, and III (17-21). The carboxyl two-thirds of the sequences of PADs are relatively well conserved, but their amino-terminal one-third portions are divergent. Interestingly, seven highly conserved sequences each consisting of 6 to 9 amino acid residues are located in PAD sequences and of these some are thought to be a Ca2+-binding site, a substrate recognition site or a catalytic site. However, by comparison of its sequences, it is hard to determine these sites. Homology search also revealed no Ca2+ binding motif such as an EF-hand and C2 motif in PAD. For understanding these functional sites, studies are required on the relationship of enzyme structures and their functions.

The biological role(s) of PAD in myeloid differentiation and in mature granulocytes, monocytes, and macrophages is entirely unknown. No deiminated cellular proteins were found in intact RA- and 1α,25-(OH)2D3-induced HL-60 cells as shown in Fig. 2, although their cell lysates could deiminate cellular proteins on addition of Ca2+. These results suggest that PADs in intact cells are activated by external signals. When HL-60 granulocytes and monocytes are stimulated by the chemotactic factors fMet-Leu-Phe and leukotriene B4, the cytosolic free Ca2+ concentration of a few micromolar is transiently elevated through calcium influx, and Ca2+ ionophore stimulation also elevates the cytosolic concentration to 10 µM (38-40). Recently we reported that mouse peritoneal macrophages selectively deiminate vimentin when stimulated by Ca2+ ionophore and that deiminated vimentin is accumulated in the periphery of nuclei. These events are considered to cause early changes in nuclear morphology with simultaneous apoptosis. PAD in cells is considered to be involved in a degenerative process through deimination of intermediate filaments such as vimentin and keratins. Interestingly, citrulline residues of deiminated filagrin are constituents of epitopes recognized by autoantibodies in patients with rheumatoid arthritis (41-42). The function of polymorphonuclear leukocytes and macrophages infiltrating into the synovial cavity of patients with rheumatoid arthritis is considered to be associated with inflammation and immune responses elicited by autoantibodies. The role of PAD in granulocytes/polymorphonuclear leukocytes and macrophages may be induced by external Ca2+ stimuli generated in host defense responses of inflammation and immune responses. The PAD cDNA from hemopoietic cells and antiserum reported in this work should aid in studies on PAD in various stages of cells during granulocyte and monocyte/macrophase development.

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