Deimination of Histone H2A and H4 at Arginine 3 in HL-60 Granulocytes†

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ABSTRACT: Interplay of various covalent modifications of histone tails has an essential role in regulation of chromatin function. Peptidylarginine deiminase (PADI) 4 deiminates protein arginine to citrulline in a Ca^{2+}-dependent manner and is present in the nucleus of granulocyte-differentiated HL-60 cells. When these cells are treated with the calcium ionophore A23187, core histone deimination occurs. To determine the deimination sites of histones, histone species were purified by reverse-phase high-performance liquid chromatography (RP-HPLC) from the cells. Immunoblotting using antimodified citrulline antibody indicated that histones H2A, H3, and H4 but not H2B were deiminated. H2A and H4 were digested with *Staphylococcus aureus* V8 protease, and the digests were separated by RP-HPLC. Immuno dot-blotting and mass spectrometry showed that the deiminated residues were present in H2A (1−56) and H4 (1−52) regions but not in other regions. The H2A peptide (1−56) was digested with α-chymotrypsin, and the deiminated peptide was separated from the corresponding nondeiminated peptide by RP-HPLC. The deiminated residue was found to be limited to residues 1−23. Similarly, digestion of the H4 peptide (1−52) with endoproteinase Asp-N and separation of the deiminated peptide from the nondeiminated peptide indicated that the deiminated residue was limited to residues 1−23. Mass spectrometry of lysylendopeptidase digests of the H2A (1−23) and H4 (1−23) peptides showed that deimination occurred at arginine 3 of the N-terminal sequence Ac-SGRGK common to H2A and H4. These results suggest that PADI4 deiminates only a restricted site of target proteins in cells. Deimination of histones is discussed in relation to chromatin structure and function.

The nucleosome core particle consists of a 146-bp DNA wrapped around a histone octamer (H2A, H2B, H3, and H4) whose tails protrude from it and are involved in nucleosome−nucleosome interactions and nonhistone protein−histone interactions to form the higher-order chromatin structure (*1*−*4*). The histone tails are subject to a series of enzyme-catalyzed modifications including acetylation, methylation, and phosphorylation, and ubiquitinatation that may influence, singly or in combination, short- and long-term chromatin template activity and chromosome condensation (*4*−*9*). Besides these well-known modifications, deimination was recently found to have a repressive effect on transcriptional activation of the estrogen-regulated gene (*10*, *11*).

The conversion of protein arginine residues to citrulline is called deimination and is catalyzed by peptidylarginine deiminase (PADI,[1] EC 3.5.3.15) in the presence of Ca^{2+} (*12*, *13*). This conversion changes the positively charged guanidinium group of arginine to the uncharged ureido group of citrulline and is thought to cause profound alterations in protein structure, function, distribution, and metabolism (*14*−*19*). At least four types of PADI are widely distributed in tissues (*20*−*24*). They deiminate various tissue-specific target proteins. The extent of myelin basic protein deimination is modulated in the central nervous system of infants during early development and is involved in maturation of myelin sheaths (*15*). Various deiminated forms of keratin K1 and filaggrins are found in the cornified layer of the epidermis and function in the keratinization that occurs in the last stage of keratinocyte differentiation (*25*−*27*). Among the four enzymes, only PADI4 (previously called PAD V) is present in the nucleus, with the other PADIs being present in the cytoplasm (*28*). PADI4 was, originally, found in bloodstream granulocytes and cultured HL-60 granulocytes, which are produced from undifferentiated HL-60 cells with retinoic acid (*23*, *28*, *29*). When cells are stimulated with the calcium ionophore A23187, preferential core histone deimination occurs in the nucleus (*28*, *30*). The physiological function of this deimination is not yet known. Identification of the deimination site is fundamental for understanding the significance of the resulting modification in cell function. We purified and characterized deiminated histones from HL-60 granulocytes by successive digestions with various enzymes and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI−TOF MS). This paper

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[1] Abbreviations: PADI, peptidylarginine deiminase; MALDI−TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; AMCA, antimodified citrulline antibody; RP-HPLC, reverse-phase high-performance liquid chromatography; TFA, trifluoroacetic acid; Lep, lysylendopeptidase; MS, mass spectrometry; Ac, acetyl group; Me, methyl group; Cit, citrulline.

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reports that histones H4, H2A, and H3 but not H2B are deiminated on stimulation of cells with A23187 and that the deiminated sites of H2A and H4 are at arginine 3 in the N-terminal sequence Ac-SGRGK, which is present in both H4 and H2A.

MATERIALS AND METHODS

HL-60 Granulocytes and Calcium Ionophore Treatment. HL-60 granulocytes were prepared by cultivating HL-60 cells with 1 μM *all-trans* retinoic acid (Sigma) for 3 days as described (*23*). The cells (3×10^8) were suspended in 150 mL of Locke's solution (0.15 M NaCl, 5 mM KCl, 2 mM $CaCl_2$, 10 mM Hepes-HCl at pH 7.3, and 0.1% glucose) and incubated at 37 °C with or without 5 μM A23187 (Merck) for 15 min as described (*30*).

Extraction of Histones. Cells were treated directly with 0.2 M H_2SO_4 overnight at 4 °C, and the extracted protein was precipitated with 25% trichloroacetic acid. The precipitate was dissolved in 1 mM HCl, and core histones were precipitated with 0.5 M $HClO_4$ to remove H1 histones. The resultant precipitate was washed with acetone-acidified 0.05 M HCl and with acetone. Protein concentrations were determined by the method of Bradford with calf thymus whole histone as a standard (*31*).

Immunoblotting. Histones were resolved by 15% SDS−PAGE and blotted onto polyvinylidene fluoride membranes (Millipore). For detection of deiminated proteins, the blots were treated with citrulline modification medium and then with 1% glutaraldehyde in Mg^{2+}- and Ca^{2+}-free phosphate-buffered saline for 10 s at room temperature (*30*). The blots were probed with antimodified citrulline antibody (AMCA) (0.125 μg/mL) and then with a horseradish peroxidase conjugate of goat anti-rabbit IgG as described (*32*, *33*). The bound peroxidase was detected with an enhanced chemiluminescence kit (Perkin−Elmer Life Sciences). Peptide samples were bound covalently by amino coupling to a Biodyne C membrane (Pall) activated with equal volumes of a mixture of 0.1 M *N*-hydroxysuccinimide and 0.48 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, and the remaining reactive group was deactivated with 0.2 M Tris-HCl at pH 8.3. Citrulline residues were detected as described above.

Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC). RP-HPLC was performed with a TOSOH HPLC-gradient system using a model 8010. Histone separation was performed on a Develosil 300 C4 HG-5 column (200 × 4.6-mm inner diameter; 5-μm particle size; Nomura Chemicals, Aichi, Japan). Samples of ~800 μg were injected onto the column equilibrated with 33% acetonitrile in 0.05% trifluoroacetic acid (TFA) at 45 °C at a flow rate of 1 mL/min. Histones were eluted with a linear gradient of acetonitrile of 33−58% in 0.05% TFA over 50 min. Histone fractions were collected, lyophilized, and stored at −20 °C. Digests of H4 and H2A histones were digested with *Staphylococcus aureus* V8 protease, and the digests were separated using a Hydrosphere C18 column (150 × 4.6-mm inner diameter; 3.5-μm particle size; YMC Co., Kyoto, Japan). The digests were injected onto the column equilibrated with 5% acetonitrile in 0.05% TFA at 40 °C at a flow rate of 1 mL/min. Peptides were eluted with a linear gradient of acetonitrile of 5−50% in 0.05% TFA over 45 min. H2A and H4 N-terminal peptides were digested with α-chymotrypsin and endoproteinase Asp-N, respectively, and the digests were injected onto the same C18 column equilibrated with 5% acetonitrile in 0.05% TFA at 40 °C at a flow rate of 1 mL/min. Peptides were eluted with a linear gradient of 5−30% acetonitrile in 0.05% TFA over 50 min. Fractions were collected, lyophilized, and stored at −20 °C.

Proteolytic Digestion. Histone H2A (100 μg) and H4 (100 μg) were digested at 37 °C for 2 and 5 h, respectively, with *S. aureus* V8 protease (Sigma) at an enzyme/substrate molar ratio of 1:40 in 20 mM ammonium bicarbonate at pH 4.0. Histone H2A peptides (residues 1−56) were digested at 37 °C for 1.5 h with TLCK-treated α-chymotrypsin (Sigma) at an enzyme/substrate molar ratio of 1:500 in 20 mM sodium phosphate at pH 7.8. Histone H4 peptides (residues 1−52) were digested at 37 °C for 16 h with endoproteinase Asp-N (Roche Diagnostics) at an enzyme/substrate molar ratio of 1:1700 in 50 mM sodium phosphate at pH 7.8. N-Terminal peptides (residues 1−23) of histones H2A and H4 were digested at 37 °C for 3 h with lysylendopeptidase (Lep, Wako Pure Chemical Industries) at an enzyme/substrate molar ratio of 1:300 in 0.1 M *N*-ethylmorpholine-acetic acid at pH 8.4. The digests were lyophilized and dissolved in 0.1% TFA for mass spectrometry.

MALDI−TOF MS. MALDI−TOF MS was performed on a Voyager DE (Applied Biosystems) using a reflector-mode measurement or a linear-mode measurement. Peptides (~100 pmol/μL) in 0.1% TFA were mixed with 10 volumes of the matrix solution, 2,5-dihydroxybenzonic acid (10 mg/mL in 50% acetonitrile−0.1% TFA) containing standard peptides for internal calibration. The ProteoMass peptide and protein MALDI−MS calibration kit (Sigma) were bradykinin fragment 1−7 (757.3997 Da), P14R (1533.8582 Da), ACTH fragment 18−39 (2465.1989 Da), insulin-oxidized B chain (3494.6513 Da), and insulin (5730.6087 Da). Expected masses to compare with the observed MALDI−TOF MS results were calculated using Protein Prospector (*34*).

RESULTS

RP-HPLC of Deiminated Histones. HL-60 granulocytes were incubated with or without calcium ionophore A23187 for 15 min, and their histones were extracted with 0.2 M H_2SO_4 and recovered as core histones by 0.5 M $HClO_4$ precipitation. When the fraction was chromatographed on a C4 column in a 33−55% linear gradient of acetonitrile at 45 °C, several protein fractions were separated and numbered 1−6 in order of their elution (Figure 1A). To identify histone molecular species, the fractions were subjected to 15% SDS−PAGE and the resolved proteins were blotted onto a membrane and stained for proteins and deiminated proteins. On the basis of protein mobility, fractions 1 and 2 were identified as histones H2B and H4, respectively, and fractions 3 and 4 and fractions 5 and 6 were identified as histones H2As and H3s, respectively (left panel of Figure 1B). Immunoblotting using AMCA showed that fractions 2−6 had deiminated proteins (right panel of Figure 1B). Thus, H4, H2A, and H3 but not H2B were deiminated. Also, no citrulline residue in H2B was detected by colorimetry (data not shown). In addition, histone deimination occurred in the cells with but not without A23187 (Figure 1B). RP-HPLC of nondeiminated histones prepared without A23187 treat-

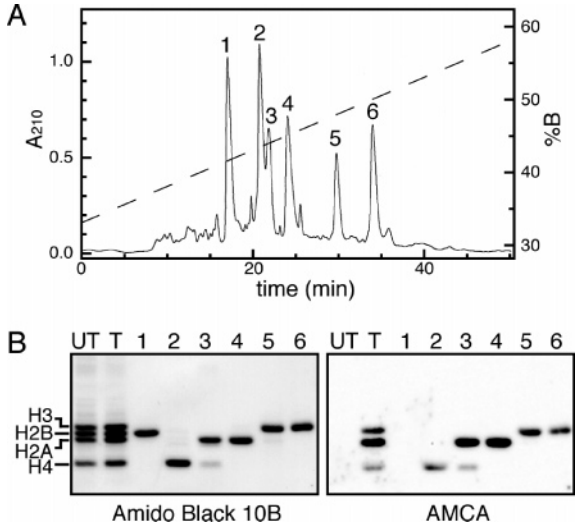

FIGURE 1: Separation of HL-60 granulocyte deiminated histones. (A) RP-HPLC of deiminated histones. A23187-treated granulocyte histones were chromatographed on a Develosil C4-HG 5 column in a linear gradient of 33−58% solvent B in solvent A at a flow rate of 1 mL/min over 50 min. Solvent A was 0.05% TFA, and solvent B was acetonitrile containing 0.05% TFA. (B) SDS−PAGE of histone fractions. Fractions 1−6 from the chromatograms were subjected to 15% SDS−PAGE and blotted on a membrane. The membrane was stained with Amido Black 10B, and the same membrane was stained for deiminated proteins using AMCA. Lanes UT and T, histones (2 μg) extracted from HL-60 granulocytes incubated without or with A23187, respectively, for 15 min. Lanes 1−6, 0.5 μg of protein from fractions 1−6, respectively.

ment gave virtually the same protein elution profile as that of deiminated histone (data not shown), indicating that deiminated histones were not separated from nondeiminated histones under these conditions.

Determination of the Deimination Site in Histone H2A. Fraction 4 H2A described above was digested with V8 protease, and the digest was separated into 8 peptide fractions numbered 1−8 by RP-HPLC on a C18 column with a 5−50% linear gradient of acetonitrile (Figure 2A). The fractions obtained were analyzed by MALDI−TOF MS. All of the peptides in the fractions were assigned to positions in H2A (residues 1−129) and found to cover all of the expected peptides generated by V8 (data not shown). Among them, fractions 3, 4, 5, and 6 were all N-terminal peptides (1−56) modified at least with an acetyl group, and fractions 7 and 8 were C-terminal peptides (65−129 and 62−129), respectively. The MS could not distinguish deiminated peptides from the corresponding nondeiminated peptides. The distribution of several isotopic species of the same size as these peptides made it difficult to detect the calculated mass difference of 0.98 between the arginine and citrulline residues. Fractions of peptides were covalently linked to a carboxyl membrane and examined for deiminated peptide using AMCA. Immuno dot-blots, Figure 2A, showed that fractions 3, 4, 5, and 6 contained deiminated peptides but that other fractions contained no deiminated peptides, indicating that H2A deimination occurs in the N-terminal 56-residue sequence but not in other regions including the C-terminal tail.

N-Terminal peptides pooled from fractions 3−6 (shown in Figure 2A) were digested with α-chymotrypsin and separated into fractions numbered 1−17 and 4′ in the same way as described above, except for the use of a shallower

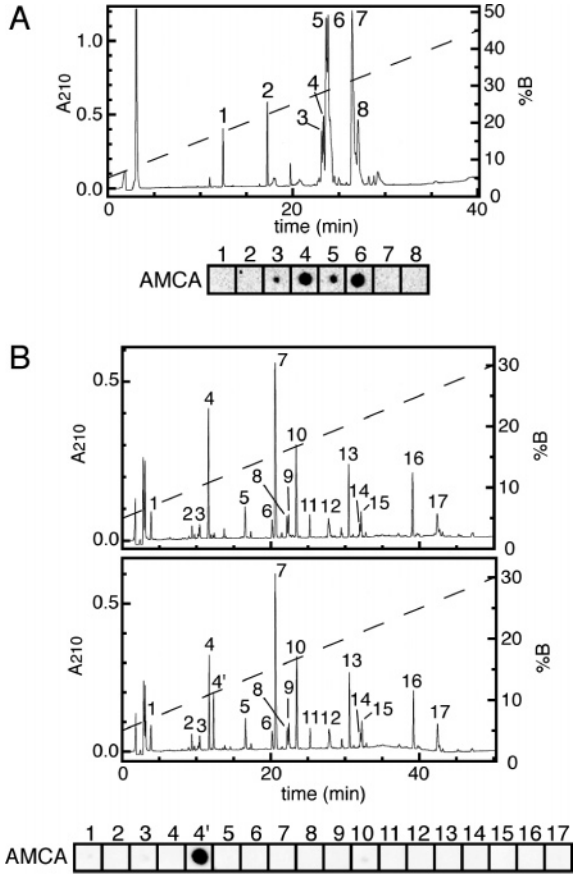

FIGURE 2: RP-HPLC of deiminated H2A peptides. (A) RP-HPLC of V8 digest of H2A. A V8 digest of H2A (fraction 4 in Figure 1A) was chromatographed on a YMC hydrosphere C18 column in a linear gradient of 5−45% solvent B in solvent A at a flow rate of 1 mL/min over 40 min. The fractions (0.05 μg) numbered on the chromatogram were spotted on a carboxyl-activated membrane, and the blots were probed using AMCA as described for Figure 1. (B) RP-HPLC of chymotryptic digest of H2A N-terminal peptide (1−56). Fractions 3−6 shown in A containing deiminated N-terminal peptides (1−56) were pooled and digested with chymotrypsin, and the digests were separated in the same way as above, except that elution was performed with a linear gradient of 5−30% solvent B in solvent A over 50 min. Upper and lower chromatograms show elution profiles of a nondeiminated and deiminated histone sample, respectively. Note that the latter has one additional fraction numbered 4′. The fractions (0.05 μg) numbered on the lower chromatogram were bound to a membrane, and then the blots were probed for deiminated peptides as described above.

linear gradient of acetonitrile (lower panel of Figure 2B). H2A N-terminal peptides (1−56) prepared from a nondeiminated histone sample were also digested and run in the same way (upper panel of Figure 2B). The upper and lower chromatograms have the same elution profiles, except that the latter has an additional fraction 4′. MALDI−TOF MS of the fractions from the latter indicated that the fractions covered all of the fragments generated with α-chymotrypsin, and most of them were assigned to positions in H2A (1−56) (data not shown). Fractions 4 and 4′ gave mass-to-charge ratios (*m*/*z*) of 2370.35 and 2371.38, respectively (parts a and b of Figure 3). The values of *m*/*z* and mass difference of 1.03 between peptides 4 and 4′ indicated that these peptides were a nondeiminated and deiminated N-terminal peptide (1−23), respectively. Furthermore, immuno dot-blotting of the latter fractions indicated that fraction 4′ but not 4 was a deiminated peptide, while other fractions were

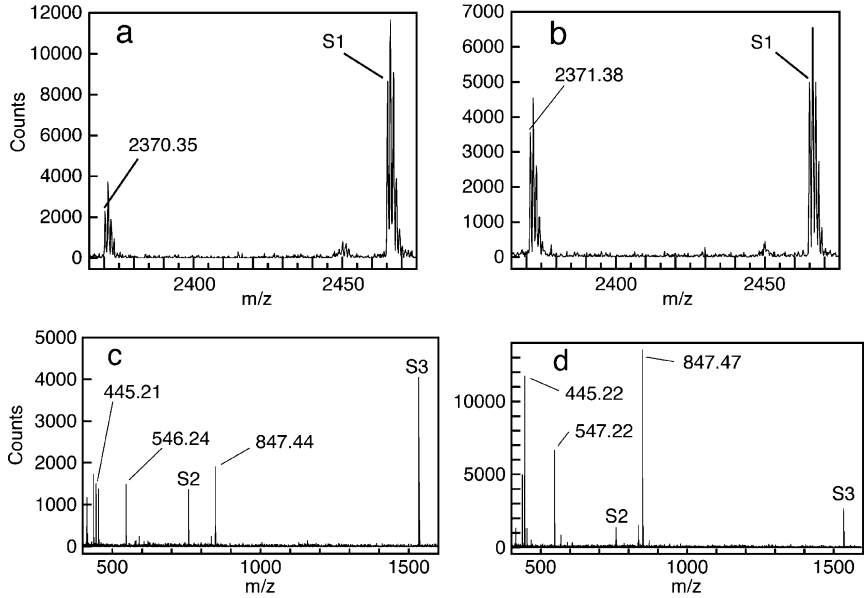

FIGURE 3: MS spectra of nondeiminated and deiminated H2A peptides. Peptides 4 and 4′ (a and b) shown in Figure 2B, lower chromatogram, and Lep digests of peptides 4 and 4′ (c and d) were subjected to MALDI−TOF MS with peptides S1, S2, and S3 as standards. S1, 2465.20 Da; S2, 757.39 Da; and S3, 1533.85 Da.

Table 1: Sequence and Modification of Histone H2A Peptides Deduced by MALDl−TOF MS

peptides[a]	*m/z* (observed)	calculated mass $[M + H]^+$ monoisotopic	peptide sequence and modifications	residues
			3 11 17 20	
4	2370.35	2328.34	Ac-SGRGKQGGKARAKAKTRSSRAGL	1-23
Lep digest	546.24	504.28	Ac-SGRGK	1-5
	445.21	445.29	ARAK	10-13
	847.44	847.48	TRSSRAGL	16-23
4'	2371.38	2328.34	Ac-SGRGKQGGKARAKAKTRSSRAGL + 1Di[b]	1-23
Lep digest	547.22	504.28	Ac-SGRGK + 1Di	1-5
	445.22	445.29	ARAK	10-13
	847.47	847.48	TRSSRAGL	16-23

[a] Peptides 4 and 4′ isolated from a digest of deiminated H2A (lower panel of Figure 2B) were digested with Lep. [b] Deimination of arginine residue to citrulline residue.

nondeiminated peptides (Figure 2B). Deiminated H2A was estimated to constitute 38% of H2A by densitometry of fractions 4 and 4′ with absorbency at 210 nm.

To narrow down the deimination site, peptides (1−23) 4 and 4′ were digested with Lep and the digests were analyzed by MALDI−TOF MS (parts c and d of Figure 3). Peptide 4 gave three signals with values in *m*/*z* of 445.21, 546.24, and 847.44, which were assigned to peptides (residues 10−13, 1−5, and 16−23), respectively. Peptide 4′ also gave three signals with values in *m*/*z* of 445.22, 547.22, and 847.47 (Table 1). Among them, only one peptide (residues 1−5) had a mass difference of 0.98 in the 4 and 4′ digests. Thus, the nondeiminated and deiminated peptides were identified as Ac-SGRGK and Ac-SGCitGK, respectively. This indicated that H2A deimination occurs in R3.

Determination of the Deimination Site in H4. The isolated H4 described above was digested with V8 protease, and the digest was separated into 12 peptide fractions numbered 1−12 by RP-HPLC with a linear gradient of 5−50% acetonitrile in the same way as described above (Figure 4A). All of the peptides were assigned to positions in H4 (1−102) and covered all of the expected peptides generated by V8 (data not shown). Fractions 4 and 5 were N-terminal peptides (1−52) that contained either one or two acetyl groups, respectively, on the basis of the analysis described below, while fraction 10 was found to be the C-terminal peptide (75−102) and fraction 12 was found to be the C-terminal peptide (53−102). Fraction 6, like fraction 5, contained an N-terminal peptide (1−52) modified by two acetyl groups and additionally an unidentified peptide. The MS could not distinguish deiminated and nondeiminated peptides in these fractions. Immuno dot-blots, Figure 4A, revealed that fractions 4, 5, and 6 contained deiminated peptides but that other fractions had no deiminated peptides. The percentage of fraction 6 deiminated peptide of the total amount of deiminated peptides from fractions 4, 5, and 6 was 1.8%. Therefore, the fraction 6 immuno dot-blot signal was accounted for by the presence of the N-terminal peptide (1−52) in fraction 6, which spilled over from fraction 5. These results together indicated that the H4 N-terminal region (1−52) was deiminated, but other regions, including the C-terminal tail, were not.

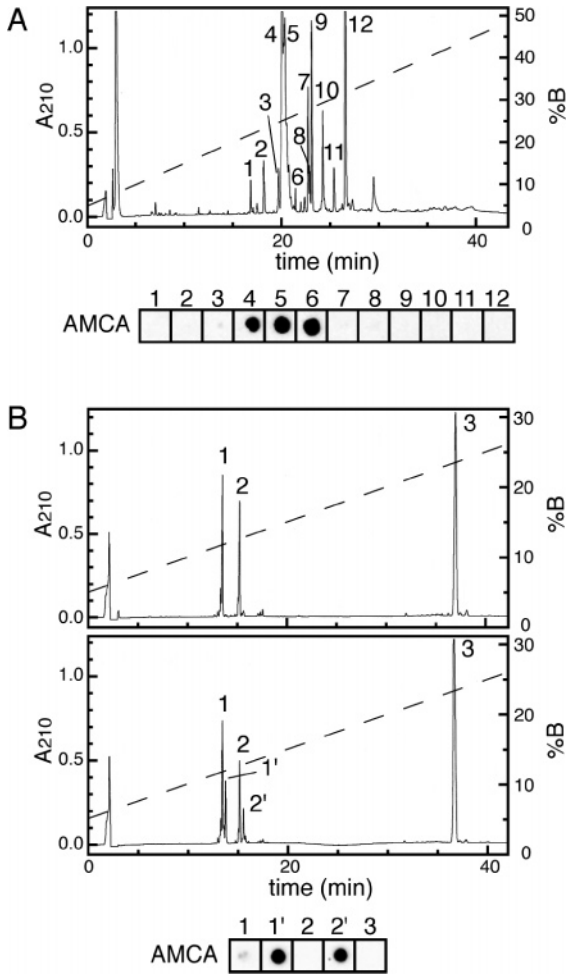

FIGURE 4: RP-HPLC of deiminated H4 peptides. (A) RP-HPLC of V8 digest of H4. A V8 digest of H4 (fraction 2 of Figure 1A) was separated by RP-HPLC on a C18 column as described for Figure 2A. The fractions (0.05 μg) numbered on the chromatogram were spotted on a membrane, and the blots were probed using AMCA as described for Figure 2. (B) RP-HPLC of Asp-N digest of H4 N-terminal peptide (1−52). Fractions 4 and 5 shown in A were pooled and digested with endoproteinase Asp-N, and the digest was chromatographed on the above same column in the same way as for Figure 2. Upper and lower chromatograms show elution profiles from a nondeiminated and deiminated histone-derived sample, respectively. Note that the latter has two additional fractions numbered 1′ and 2′. The fractions (0.01 μg) numbered on the lower chromatogram were spotted on a membrane, and the blots were stained for deiminated peptides as described for Figure 2.

Next, fractions 4 and 5 were pooled and digested with endoproteinase Asp-N, and the digest was separated into four peptide fractions named 1 and 2 and 1′ and 2′ by RP-HPLC (lower panel of Figure 4B). N-Terminal peptides prepared from a nondeiminated H4 sample were also digested and then run in the same way (upper panel of Figure 4B). A comparison of the upper and lower chromatograms shows that the latter contained two additional peaks, designated 1′ and 2′ (Figure 4B). Fractions of the latter were analyzed by MALDI−TOF MS. Fractions 1 and 1′ gave *m*/*z* values of 2430.48 and 2431.56, respectively (parts a and b of Figure 5), indicating a mass difference of 1.08 between the two peptides. These results indicated that peptides 1 and 1′ each contain one acetyl modification and two methyl modifications (1−23) without and with one deiminated site, respectively. Fractions 2 and 2′ gave *m*/*z* values of 2472.53 and 2473.47, respectively, indicating a mass difference of 0.94 between peptides 2 and 2′ (parts c and d of Figure 5). Peptides 2 and 2′ each contain two acetyl modifications and two methyl modifications (1−23) without and with one deiminated site, respectively. Fraction 3 was assigned to position 24−52 and was not deiminated. The amounts of deiminated peptides 1′ and 2′ were both estimated to be 30% of the total amounts of the corresponding peptides by densitometry of the chromatogram (lower chromatogram of Figure 4B). Furthermore, immuno dot-blotting of fractions 1, 1′, 2, and 2′ confirmed that fractions 1′ and 2′ were deiminated peptides, while fractions 1 and 2 were not deiminated (Figure 4B, immuno dot-blots).

To narrow down the deiminated site, peptides 1 and 1′ and peptides 2 and 2′were digested with Lep and the digests were analyzed by MALDI−TOF MS (parts e−h of Figure 5). Peptides 1 and 1′ gave two signals with *m*/*z* of values of 546.21 and 992.64 and those with *m*/*z* of 547.23 and 992.67, respectively, indicating a mass difference of 1.02 between the two smaller peptides and no mass difference between the two larger peptides (parts e and f). The smaller and larger peptides were assigned to positions 1−5 and 17−23, respectively (Table 2). Peptides 2 and 2′ gave two signals with *m*/*z* of 546.31 and 1347.85 and those with *m*/*z* of 547.25 and 1347.85, respectively, indicating a mass difference of 0.94 between the smaller peptides and no mass difference between the larger peptides (parts g and h). The smaller and larger peptides were assigned to positions 1−5 and 13−23, respectively (Table 2). The difference between peptides 1 and 2 was accounted for by the absence and presence of one acetylated site in sequence 13−23, respectively. The sequences of the nondeiminated and deiminated peptides (1−5) identified are Ac-SGRGK and Ac-SGCitGK, respectively. These results indicate unequivocally that H4 deimination occurs in R3.

DISCUSSION

This work indicated that brief exposure of HL-60 granulocytes to calcium ionophore induces deiminations of histone H2A, H3, and H4 but not H2B and that H2A and H4 deiminations occur preferentially at Arg 3 in the N-terminal tails.

Clear separation of histones into four molecular species by RP-HPLC at 45 °C showed H2A, H3, and H4 deiminations that are consistent with the previous observations on HL-60 granulocytes and neutrophils by immunoblotting (*28*, *30*) and facilitated the complete mapping of deimination sites on H2A and H4. HPLC separated H2A and H3 into two molecular species that were both deiminated. However, whether they are variant or modified forms of each other remains unknown. To identify deiminated sites, isolated H2A and H4 were digested successively with V8 protease and chymotrypsin and with V8 protease and endoproteinase Asp-N, respectively. The digests were separated by RP-HPLC, and the isolated peptides were identified by MALDI−TOF MS. In this procedure, immuno dot-blotting of the peptide covalently bound to a blotting membrane was effective to detect amounts in picomoles of as small as 10 amino acid deiminated peptides and also amino acid citrulline. The separation of deiminated peptides from the corresponding nondeiminated peptides by RP-HPLC was also crucial for

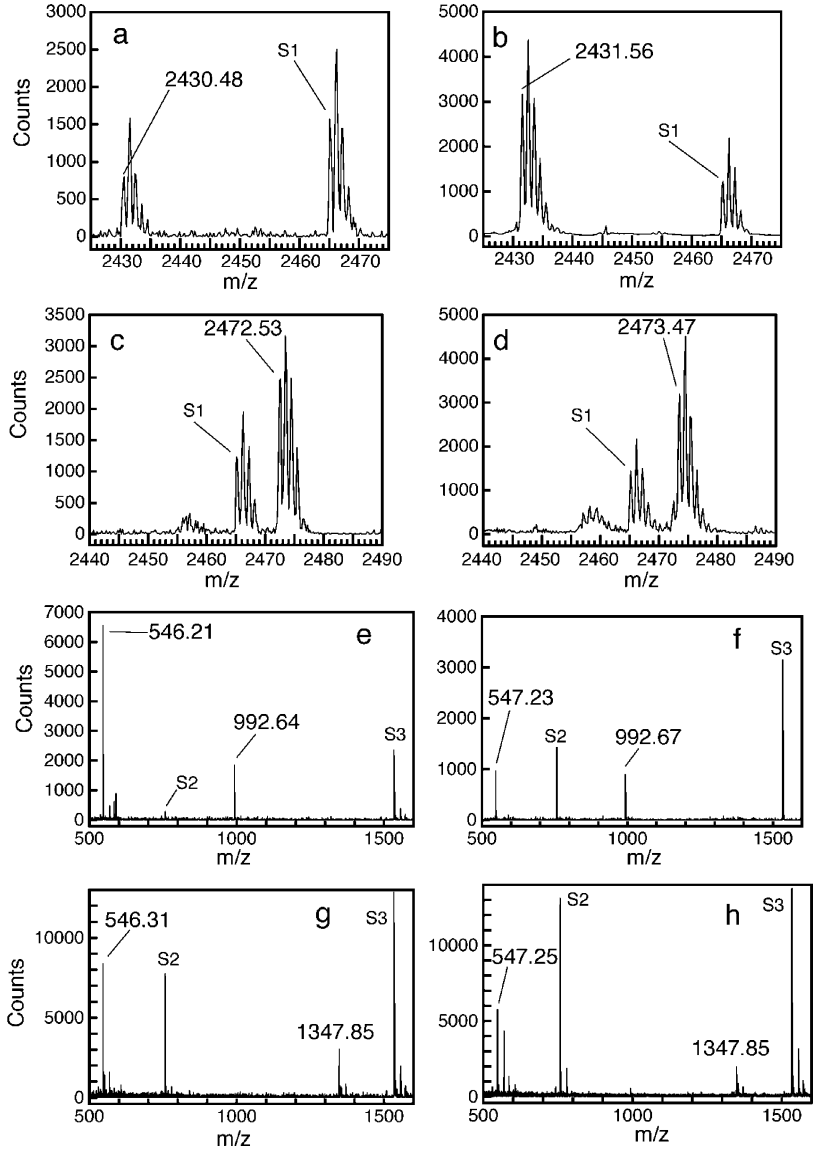

FIGURE 5: MS spectra of nondeiminated and deiminated H4 peptides. Peptides 1 and 1′ (a and b) and peptides 2 and 2′ (c and d) shown in Figure 4B, lower chromatogram, and Lep digests of peptides 1 and 1′ (e and f) and peptides 2 and 2′ (g and h) were subjected to MALDI−TOF MS with peptides S1, S2, and S3 as standards. S1, S2, and S3 were the same as described in Figure 3.

distinction between them by MS, because the abundance of isotopic molecules of peptides longer than 15 amino acids disturbed distinction of the arginine peptide from corresponding citrulline peptides (*35*). The procedure used in this work will aid in identifying deiminated sites in other proteins.

Approximately one-third of H2A and H4 and probably a similar amount of H3 was deiminated under the present cell-culture conditions. However, H2A and H4 deiminations appeared to be restricted to only Arg 3 of the N-terminal sequence Ac-SGRGK. No deimination of the other two Arg sites and four other Arg sites, respectively, in the H2A and H4 N-terminal tails was found. In addition, no H4 C-terminal Arg residue was deiminated. H3 deimination was also found in the N-terminal sequence (1−50) but not in other regions including the C-terminal tail, although site identification remained elusive (unpublished data). During this work, Wang et al. found that histone H3 Arg 8 and Arg 17 are deiminated 27 and 7%, respectively, following treatment of HL-60 granulocytes with A23187 under conditions similar to those used in this work (*11*). However, whether two deiminated sites are present in a single or different molecules is not known. H3 deimination sites do not have an apparent sequence similarity to the H2A or H4 deimination site. These results suggest that PADI4 may have some site preference within the unstructured histone tails as a target site. The crystal structure of the PADI4 benzoylarginine amide (a synthetic substrate) complex revealed that the substrate binds to a catalytic cleft that is formed by Ca^{2+} binding to the enzyme (*13*). The deimination site sequence determined will be helpful for understanding molecular recognition by the enzyme of natural target proteins.

This work also indicated that no obvious modification by methylation or acetylation besides deimination in the bulk of histone was generated on stimulation of HL-60 granulocytes with A23187, at least in the N-terminal 23-residue tails of H2A and H4. MS of the N-terminal tails indicated that the H2A N-terminal tail of the bulk of H2A was not modified at all with a methyl or acetyl group. Arg 3 deimination is the first known Arg 3 modification of the H2A N-terminal tail. In the bulk of H4 from HL-60 granulocytes, about 60% of the N-terminal 23-residue tail was modified with one dimethyl group that is probably ascribed to Lys 20 dimethy-

Table 2: Sequence and Modification of Histone H4 Peptides Deduced by MALDI−TOF MS

peptides[a]	*m/z* (observed)	calculated mass $[M + H]^+$ monoisotopic	peptide sequence and modifications	residues
			3 17 19 23	
1	2430.48	2360.43	Ac-SGRGKGGKGLGKGGAKRHRKVLR + 2Me	1-23
Lep digest	546.21	504.29	Ac-SGRGK	1-5
	992.64	964.63	RHRKVLR + 2Me	17-23
1′	2431.56	2360.43	Ac-SGRGKGGKGLGKGGAKRHRKVLR + 2Me + 1Di	1-23
Lep digest	547.23	504.29	Ac-SGRGK + 1Di	1-5
	992.67	964.63	RHRKVLR + 2Me	17-23
2	2472.53	2360.43	Ac-SGRGKGGKGLGKGGAKRHRKVLR + 1Ac + 2Me	1-23
Lep digest	546.31	504.29	Ac-SGRGK	1-5
	1347.85	1277.80	GGAKRHRKVLR + 1Ac + 2Me	13-23
2′	2473.47	2360.43	Ac-SGRGKGGKGLGKGGAKRHRKVLR + 1Ac + 2Me + 1Di	1-23
Lep digest	547.25	504.29	Ac-SGRGK + 1Di	1-5
	1347.85	1277.80	GGAKRHRKVLR + 1Ac + 2Me	13-23

[a] Peptides 1 and 1′ and Peptides 2 and 2′ isolated from a digest of deiminated H4 (lower panel of Figure 4B) were digested with Lep.

lation based on the insusceptibility of Lys 20 to Lep digestion and the rest of the N-terminal tail was further modified with one acetyl group. This modification was assigned to Lys 16 acetylation based on the insusceptibility of Lys 16 to Lep digestion. No other site, including Arg3, was modified. This suggests that Arg 3 deimination is not affected by the pre-existing dimethyl group of Lys 20 or the acetyl group of Lys 16. Lys 16 and 20 are usually a mutually exclusive pair for modifications (*8*, *36*), but this is not the case in the chromatin studied in this work, suggesting that the modifications are static in HL-60 cell granulocytes. However, this work has not excluded that the small amount of Arg 3 methylation occurs at specific genes such as estrogen-regulated genes and that the methylated Arg 3 is deiminated into a citrulline residue under the conditions (*11*).

The functional meaning of histone deimination in cells on stimulation with calcium ionophore remains unknown. The deimination level of histones is estimated to be as high as one per two nucleosome particles. Possibly, such massive histone deimination in a short time may influence the higher-order chromatin structure and release the histone from chromatin in HL-60 granulocytes and neutrophils, which are known to be susceptible to apoptosis (*37*). Calcium ionophore eventually causes apoptosis of cells (*18*, *38*). Whether the release of histones from the nucleus is associated with histone deimination and apoptosis is under investigation. In contrast, two recent reports by Cuthbert et al. and Wang et al. describe estrogen-dependent histone H3 and H4 deimination (*10*, *11*). The deimination by PADI4 antagonizes histone Arg methylation, which is essential for estrogen responsive gene activation (*10*). The deimination of methyl arginine in histones by PADI4 has also been shown to repress nuclear hormone receptor-dependent gene activation (*10*, *11*). The fate of deiminated histones remains unknown. At present, it is not clear if citrulline residues on histones can be converted back to arginine or if deiminated histones are replaced by unmodified histones. It is possible that deiminated H2A or H3 is substituted with variants of H2A or H3, which is known to be a DNA synthesis-independent process (*4*, *39*). Protein deiminations in the nucleus are possibly associated with a variety of normally occurring short- and long-term nuclear functions regulated by Ca^{2+} signaling (*37*, *40*).

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