Structural Integrity of Histone H2B in Vivo Requires the Activity of Protein L-Isoaspartate O-Methyltransferase, a Putative Protein Repair Enzyme

Arlene L. Young‡, Wayne G. Carter‡, Hester A. Doyle§, Mark J. Mamula§, and Dana W. Aswad¶

From the ‡Department of Molecular Biology and Biochemistry, University of California, Irvine, California 92697 and the §Section of Rheumatology, Yale University School of Medicine, New Haven, Connecticut 06510

Protein L-Isoaspartate O-Methyltransferase (PIMT) is postulated to repair β-aspartyl linkages (isoaspartyl (isoAsp)) that accumulate at certain Asp-Xaa and Asn-Xaa sites in association with protein aging and deamidation. To identify major targets of PIMT action we cultured rat PC12 cells with adenosine dialdehyde (AdOx), a methyltransferase inhibitor that promotes accumulation of isoAsp in vivo. Subcellular fractionation of AdOx-treated cells revealed marked accumulation of isoAsp in a 14-kDa nuclear protein. Gel electrophoresis and chromatography of nuclei 3H-methylated in vitro by PIMT revealed this protein to be histone H2B. The isoAsp content of H2B in AdOx-treated cells was ~18 times that in control cells, although no isoAsp was seen in other core histones, regardless of treatment. To confirm the relevance and specificity of this effect, we measured isoAsp levels in histones from brains of PIMT knockout mice. IsoAsp was found at near stoichiometric levels in H2B extracted from knockout brains and was at least 80 times greater than that in H2B from normal mice. Little or no isoAsp was detected in H2A, H3, or H4 from mice of either genotype. Accumulation of isoAsp in histone H2B may disrupt normal gene regulation and contribute to the reduced life span that characterizes PIMT knockouts. In addition to disrupting protein function, isoAsp has been shown to trigger immunity against self-proteins. The propensity of H2B to generate isoAsp in vivo may help explain why this histone in particular is found as a major antigen in autoimmune diseases such as lupus erythematosus.

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**EXPERIMENTAL PROCEDURES**

**Materials—**S-Adenosyl-[methyl-3H]methionine (15 Ci/mmol) was purchased from PerkinElmer Life Sciences. AdoMet of lower specific activity was prepared by dilution with unlabeled, purified AdoMet (19). Calf thymus histone H2B and mixed core histones were purchased from Roche Molecular Biochemicals. All other chemicals, including AdoHx, were purchased from Sigma. Synthesis and characterization of the peptide E3 (Ac-GRGPGPGRGPGPG-RG) has been described (20). PIMT from cow brain, used in early experiments, was prepared as described by Aswad and Deight (21). Recombinant rat PIMT, used in later experiments, was prepared according to David and Aswad (22). Rat PC12 cells were obtained from Dr. Gabor Tigyi at the University of California, Irvine. All culture media were obtained from Life Technologies, Inc.

**PIMT knockout (−/−) and wild-type (+/+)** littermates were bred from PIMT (+/−) founder pairs generously provided by Dr. Edward Kim, Gladstone Institute of Cardiovascular Disease, San Francisco and Dr. Stephen Clarke of the University of California, Los Angeles (13). Mice were screened for the presence of the neo gene and the absence of the PIMT gene by polymerase chain reaction analysis of tail DNA. Primer sequences were: PIMT forward, 5'-gggcaagaccgtcaga-3'; PIMT reverse, 5'-ggcagtgcagcactgctt-3'; neo forward, 5'-gggcaagaccgtcagc-3'; neo reverse, 5'-ggcagtgcagcactgctg-3'. Mice were sacrificed by cervical dislocation at ~4–6 weeks of age. Brains were rapidly removed and frozen at ~80 °C prior to analysis.

**PC12 Cell Culture and Subcellular Fractionation—**Cells were seeded at 1 × 10⁶ cells/ml in 50 ml of RPMI 1640 medium supplemented with 10% normal calf serum, 100 units/ml penicillin/streptomycin, and 10% calf serum. Plates were purchased from Sigma. Synthesis and characterization of the primer sequences were: PIMT forward, 5'-cgcatcgagcgagcacgtactcgg-3'; PIMT reverse, 5'-cggcatcgagcgagcacgtactcgg-3'. To determine how isoAsp accumulation is distributed among various cell fractions, we carried out a similar incubation of PC12 cells in the presence or absence of 10 μM AdoHx for 24 h and then fractionated the cells by differential centrifugation as described under "Experimental Procedures." Samples of each fraction were 3H-methylated in vitro with purified PIMT and then subjected to SDS-PAGE at pH 2.4. Fig. 1A shows that a substantial AdoHx-dependent increase in isoAsp occurred in all of the fractions analyzed. The smallest increase occurred in the mitochondrial fraction, whereas much of this apparent increase may be due to cross-contamination by the membrane fraction. Previous studies on the basal level of isoAsp sites in subcellular fractions of rat brain revealed significantly lower levels of isoAsp in the mitochondrial fraction than in synaptic membranes (29).

Of the AdoHx effects seen in Fig. 1A, the single most dramatic increase was observed at 14 kDa in the nuclear fraction. To confirm that this increased labeling was caused by isoAsp and not the modification of arginine residues by endogenous protein arginine methyltransferase 1 (30), we carried out methylation of a +AdoHx nuclear extract with or without added PIMT. We also included in this assay the highly effective R3 peptide substrate for protein arginine methyltransferase 1 to further reduce any endogenous protein arginine methylation (20). Fig. 1B shows that methylation of the 14-kDa protein was indeed AdoHx-dependent and therefore reflects increased isoAsp re-
The identification of histone H2B as the major source of isoAsp accumulation in nuclei of AdOx-treated PC12 cells—The apparent mass and nuclear location of the 14-kDa protein suggested to us that it might be a histone. We therefore isolated nuclei from +Adox and −Adox cells, subjected them to $^3$H-methylation in vitro, and then extracted the histone-rich fraction with 0.2 M HCl. SDS-PAGE at pH 2.4 revealed that virtually all of the 14-kDa protein was solubilized by the acid, and none was present in the acid-insoluble pellet (data not shown).

To determine which histone(s) or histone-like protein(s) carried the $^3$H-methyl label, we analyzed acid extracts of $^3$H-methylated nuclei by polyacrylamide gel electrophoresis in a UAT buffer system commonly used for histone separation. As shown in Fig. 2, most of the $^3$H-labeled metabolite comigrated with a histone H2B standard and not with standards of the other three core histones (H2A, H3, and H4). Automethylation of PIMT was seen in both lanes. Automethylation of PIMT has been reported (31, 32) and has been attributed to partial deamidation/isomerization of Asp$^{188}$–Gln$^{189}$ and Asp$^{217}$–Lys$^{218}$ (33). Adox-dependent methylation was also seen in proteins near and just below the position of histone H2A. This may reflect a small increase in isoAsp in native and partially degraded forms of H2A and/or the presence of isoAsp in other nuclear proteins that co-extract with histones.

Because isoAsp formation is a non-enzymatic intramolecular reaction observed in many (but not all) proteins during in vitro aging at physiological pH and temperature, we incubated a commercial preparation of purified calf thymus H2B (which is identical in sequence to rat H2B) for 1 or 3 days at 37 °C, pH 7.4 and then assayed the histone for $^3$H-methyl-accepting capacity. As shown in Fig. 2B, the methylation capacity of calf H2B increased progressively over this incubation period. We have also observed a selective accumulation of isoAsp in H2B when purified rat mononucleosomes were incubated under the same conditions.2 To confirm the identity of H2B as the major methyl acceptor in nuclei from AdOx-treated cells, we subjected the same type of $^3$H-methylated acid extracts used in Fig. 2 to reversed-phase HPLC. Fig. 3 shows again that Adox treatment of PC12 cells selectively and dramatically increased the methylation capacity of H2B. Separation of histones by HPLC facilitates the estimation of individual histone amounts (achieved by comparing peak sizes with those of histone standards run under the same conditions), allows for easy quantitation of $^3$H-methyl incorporation by liquid scintillation counting of collected fractions, and is carried out under solvent conditions (0.3% trifluoroacetic acid) that stabilize isoAsp methyl esters. We therefore used the results of Fig. 3 to estimate the stoichiometry of isoAsp accumulation in H2B afforded by Adox treatment. Data from panel A indicate that $\sim$0.37% of the H2B molecules from Adox-treated cells were methylated, whereas data from panel B indicate that only 0.02% of the H2B molecules from control cells were methylated. Although the methylation capacity is not high in either case, the increase is at least 18-fold.

The absolute rate of isoAsp formation in H2B in vivo is almost certainly greater than 0.37% per 24 h. This conclusion is based on the fact that incubation of PC12 cells with Adox does not completely inhibit PIMT activity (12) and the fact that the instability of protein methyl esters typically results in yields of 80% or less (24). With these considerations in mind, we estimate that the rate of isoAsp formation in histone H2B is roughly 1% per day in cultured PC12 cells.

Selective Accumulation of IsoAsp in Histone H2B of PIMT-deficient Mice—The recent availability of PIMT KO mice provided us with an independent means of assessing the specificity and physiological relevance of isoAsp formation in histones.

Brains from PIMT$^(-/-)$ or (+/+) littersmates were homogenized and used to prepare a crude nuclear fraction as described under “Experimental Procedures.” After $^3$H-methylating the nuclei in vitro with purified PIMT, histones were extracted and then separated by HPLC as in Fig. 3. As seen in Fig. 4, the results obtained with histones from PIMT$^(-/-)$ and (+/+) mice were strikingly similar to the results obtained with histones from the +Adox and −Adox PC12 cells, respectively.

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*W. G. Carter and D. W. Aswad, unpublished data.*
Isoaspartate in Histone H2B

Isoaspartate accumulation was seen exclusively in histone H2B. We calculated isoAsp stoichiometries of 8.1 and 0.09% in H2B from the [−/−] and [+/+] mice, respectively, resulting in a difference of at least 80-fold between the two genotypes. As with the data from Fig. 3, the absolute stoichiometries calculated here probably underestimate the true level and rate of isoAsp formation in the KO mice. In this case, the rate of isoAsp formation is underestimated not only because methyl esters are lost during the methylation and subsequent handling and analysis but also because of the likelihood that accumulation of damaged, isoAsp-containing H2B molecules is attenuated and ultimately limited by proteolytic degradation pathways (34). We suspect that 10–12% of H2B molecules in the [−/−] mice contain isoAsp. Regardless of the exact stoichiometry, the isoAsp content of H2B in the KO mice is considerably higher than in the normal mice, and the absolute level of isoAsp in these mice is an order of magnitude greater than that found during short-term, partial inhibition of PIMT in PC12 cells.

Biological Implications of IsoAsp Formation in Histones—Our findings suggest that histone H2B generates isoAsp sites at a rate of ∼1% per day in rodents. Accumulation of isoAsp sites under normal conditions is apparently held in check by the action of PIMT. When PIMT activity is reduced by chemical inhibition (AdOx) or by genetic means, isoAsp accumulates well beyond its normal levels in numerous proteins. The ability of PIMT to repair isoAsp sites in vitro implies that it may also function in vivo as a repair enzyme. An alternative in vivo role for PIMT is that it promotes rapid degradation of isoAsp-containing proteins.

Histones are abundant proteins that exhibit extremely slow turnover. As components of the nucleosome, core histones play a key role in stabilizing DNA structure and in mediating alterations in the accessibility of DNA to nucleic acid polymerases and regulatory proteins during transcription and cell division. Depending on the location(s) in H2B, isoAsp sites could destabilize nucleosome structure and/or disrupt interaction of the flexible N terminus of H2B with the surrounding DNA and with proteins that regulate chromatin structure. Accumulation of isoAsp in H2B may make an important contribution to the reduced life span of PIMT KO mice and to any mammalian condition in which PIMT expression is abnormally low.

In vitro aging of synthetic peptides and structured proteins indicates that isoAsp forms most readily at Asn-Gly, Asn-Ser, and Asp-Gly sites when the polypeptide flexibility encompassing such sequences is high (47). The presence of an Asp-Gly sequence in positions 25–26 at the boundary of the highly flexible N terminus of H2B may explain why isoAsp accumulation is unique to this histone. The only other occurrence of these three isoAsp-prone sequences in rat core histones is Asn63-Ser64 found in the more structured central region of histone H2B. None of the other three core histones have any Asn or Asp sites that are predicted to isomerize rapidly. Recent studies suggest that Asp25-Gly26 is in fact the major site of isopeptide bond formation in H2B. This means that PIMT-dependent conversion of the isopeptide bond to a normal peptide bond would constitute a complete repair of the damaged histone. Such a repair would not be complete if isoAsp arose via deamidation of an Asn site.

Lowenson et al. (34) have recently reported on the phenotype of PIMT KO mice in which PIMT activity was selectively restored to neurons using a transgene controlled by the neuron-specific enolase promoter. Although PIMT activity in the transgenic brains was restored to only 13% of its normal value, these mice lived 5 times longer than the KO mice, and epileptic seizures were correspondingly delayed. This suggests that neurons have a stronger need than other cell types to keep isoAsp levels in check, an idea that fits well with the observations that brain exhibits relatively high levels of PIMT in mammals and that the accumulation of isoAsp sites in PIMT KO mice is most dramatic in the brain (13, 14). Although histones are abundant in all cells that have DNA, the highly complex functions of neurons may be particularly sensitive to abnormalities of gene expression.

The selective accumulation of isoAsp in histone H2B also has interesting implications regarding molecular mechanisms that lead to autoimmunity. Recent studies showed that isoAsp sites can render some self-proteins immunogenic (17). By the proc-
ess of epitope spreading, antibodies are also made to other regions of the same protein and to other proteins that strongly associate with the isoAsp protein. This effect, combined with our discovery that histone H2B has an unusual propensity to isoAsp formation in vivo, may explain why anti-histone antibodies are often found in humans afflicted with autoimmune disease (35) and why histone H2B in particular is one of the most commonly found self-antigens in these individuals (36, 37).

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