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Phylogenetic evidence of a role for 5-hydroxymethyluracil-DNA glycosylase in the maintenance of 5-methylcytosine in DNA

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ABSTRACT

5-Hydroxymethyluracil (HmUra) is formed in DNA as a product of oxidative attack on the methyl group of thymine. It is also the product of the deamination of 5-hydroxymethylcytosine (HmCyt) which may be formed via oxidation of 5-methylcytosine (MeCyt). HmUra is removed from DNA by a DNA glycosylase which, together with HmCyt-DNA glycosylase, is unique among DNA repair enzymes in being present in mammalian cells but absent from bacteria and yeast. We found HmUra-DNA glycosylase activity in a wide variety of vertebrate and invertebrate animals (except *Drosophila*) and in protozoans. In most vertebrate organisms the highest specific activity was in nervous and immune system tissue. The phylogenetic distribution of HmUra-DNA glycosylase correlates with the presence of 5-methylcytosine (MeCyt) as a regulator of gene expression. This distribution of activity supports the contention that HmUra-DNA glycosylase aids in the maintenance of methylated sites in DNA.

INTRODUCTION

Reactive oxygen species generated by ionizing radiation and endogenous oxidative metabolic processes interact with DNA, damaging both the sugar-phosphate backbone and the bases (1−3). Several distinct repair enzymes have been identified in bacteria, yeast and mammalian tissues which effect removal of oxidized sugar residues and bases (4−8) The phylogenetic conservation of such activities has been taken as evidence that most species are subject to oxidative stress and that the resultant DNA modifications are sufficiently deleterious to promote the evolutionary development of repair enzymes (9).

A notable exception to the phylogenetic conservation of oxidative damage DNA repair enzyme activities is 5-hydroxymethyluracil-DNA glycosylase (HmUra-DNA glycosylase). Its substrate, the 5-hydroxymethyluracil (HmUra) residue in DNA, may be formed as a result of oxidative attack on the methyl group of Thy (1,10−13). HmUra-DNA glycosylase activity was present in extracts of murine and rodent cells and tissues and calf thymus but was not detectable in extracts of bacteria and yeast (14−18). In murine tissue extracts, specific activity of the enzyme was highest in brain and thymus (16). This contrasted with the distribution of activity of the ubiquitous repair enzyme Uracil-DNA glycosylase (Ura-DNA glycosylase) which correlated with the proliferative activity of the tissue and was lowest in brain (19).

In our previous reports we suggested that the reason for the limited phylogenetic distribution of the enzyme was that the HmUra residue in DNA might be only weakly mutagenic (16,18,20). Its mutagenicity could result from the lower energy of hydrogen bonding of Ade:HmUra pairs as compared to Ade:Thy pairs and/or conformational differences between the nucleosides of the two pyrimidines (21−23). In a unicellular

organism a rare mutational event might not be of sufficient consequence to promote development of a repair enzyme. However, in tissue such as brain and thymus, both of which contain cell populations which are markedly diverse at the molecular level (24), a rare mutational event or an error in transcription resulting from the oxidation of Thy to HmUra might be sufficiently deleterious to promote development of a pathway for enzymatic removal.

To further investigate this hypothesis, we undertook a survey of the phylogenetic distribution of HmUra-DNA glycosylase together with an examination of the specific activity of the enzyme in tissues of different species.

MATERIALS AND METHODS

Species examined: Species examined in this study are listed in Table 1. Wistar rats were a gift of Dr. M. Davitz, Department of Pathology, N.Y.U. Medical Center. Lobsters, chickens and turtles were purchased from local food suppliers. Frogs were obtained from the Marine Biological Laboratory, Woods Hole, MA. Carp were provided by Dr. C. Chun, New York Aquarium, Brooklyn, NY. *Aplysia* were a gift from Dr. J. Schwartz, Howard Hughes Institute, Center for Neurobiology and Behavior, Columbia University. Earthworms, flatworms, brown hydra, green hydra, *Amoeba* and *Chilomonas* were purchased from the Carolina Biological Supply Company. *Schizosacchoromyces pombe* were a gift of Dr. H. Klein, Department of Biochemistry, N.Y.U. Medical Center.

All animals were sacrificed immediately upon receipt in our laboratory, except for the protozoans. *Amoeba* and *Chilomonas* were cultured on wheat medium. Hot pasteurized spring water was added to a sterile 100ml culture dish containing 2 wheat grains (Carolina Biological). The cultures were cooled to room temperature and inoculated. The cultures were maintained at 20°C to 22°C under conditions of dim to moderate light and a neutral or slightly alkaline pH. The cultures were covered to exclude dust but not air. In 3 weeks, there were harvests of about a thousand protozoa per dish.

Preparation of tissue extracts: Larger organisms (rat, chicken, frog, turtle, carp, lobster, and sea hare) were sacrificed, and the organs were dissected, pooled, and washed in Hanks Balanced Salt Solution (GIBCO). Pooled organs from at least two specimens were used for each determination. Adult females were used for all studies. Samples of each tissue were fixed in a 10% formaldehyde solution for confirmatory histological examination. The remaining tissues were then suspended in three volumes of 0.01 mM Tris, 0.001 mM EDTA, pH 8.0 and homogenized with a Potter-Elvejheim apparatus. The mixture was filtered through gauze and then sonicated, as described previously (16,17). After sonication, the solution was centrifuged and decanted, and dithiothreitol was added to a final concentration of 1.0 mM. The protein concentration was measured using the Bradford method (BIO-RAD protein assay) and then adjusted to 5 mg/ml.

The smaller organisms (*Drosophila*, earthworm, flatworm, hydra species, protozoans) were prepared in the same manner except that the whole organisms were pooled, washed and homogenized. For *Schizosacchoromyces pombe*, the cells were first converted to spheroplasts (25) prior to homogenization.

Assay of DNA glycosylase activities: DNA glycosylase activities were determined using assay conditions developed and described previously (16−18). Serial dilutions of sonicated cellular extracts were incubated with [^{3}H] PBS phage DNA (50,000−98,000 cpm/μg) to measure Ura-DNA glycosylase activity and with [^{3}H]SPO1 phage DNA (Strain IP4 from

TABLE 1 SPECIES EXAMINED IN THIS STUDY

Classification	Species (Common name)
Kingdom Protista	*Amoeba proteus*
	Chilomonas sp.
Kingdom Fungi	*Schizosaccharomyces pombe*
Kingdom Animalia	
Phylum	
Coelenterata	*Chlorohydra viridissima* (green hydra)
	Hydra littoralis (brown hydra)
Platyhelminthes	*Dugesia tigrina* (flatworm)
Mollusca	*Aplysia californica* (sea hare)
Annelida	*Lemlumbricus sp.* (earthworm)
Arthropoda	*Homarus americanus* (lobster)
	Drosophila melanogaster (fruit fly)
Chordata	
sub Vertebrata	
class Osteichthyes	*Cyprinus* (carp)
class Reptilia	*Pseudemys* (turtle)
class Amphibia	*Rana pipiens* (frog)
class Aves	*Gallus gallus* (chicken)
class Mammalia	*Rattus* (rat)

The Bacillus Genetic Stock Center, Ohio State University, growth and DNA extraction as for PBS) (10,000−50,000 cpm/μg) to determine HmUra-DNA glycosylase activity. The final NaCl concentration was adjusted to 25 mM. The reaction was terminated after 15 minutes for Ura-DNA glycosylase activity and after 6 hours for HmUra-DNA glycosylase activity by adding an equal volume of 1% bovine serum albumin and five volumes of acetone at 4°C. We have previously determined that release of HmUra and Ura was linear over these time intervals for extracts of calf thymus (18) and of hamster V79 cells (16). The mixture was refrigerated at 4°C for 30 minutes and then centrifuged for 10 minutes at 8000 rpm in an SS-34 rotor in a Sorvall RC-2 Centrifuge. The supernatant fluid was decanted and the pellet washed with an additional 5 volumes of acetone and recentrifuged. The acetone fractions were combined and dried under reduced pressure. The residue was then redissolved in water and filtered. HPLC analysis was performed on a 5 μm Ultrasphere analytical ODS C-18 column using water as eluant (1 ml/min) and non-radioactive HmUra or Ura as the appropriate UV marker. The presence of radioactive material coeluting with authentic base was determined using a Radiomatic Beta 1C radioactivity flow detector. The specific activity of each enzyme was determined from the linear portion of the v *vs* $[E]_t$ plot. Coefficients of variation for sequentially performed determinations typically ranged from 25−40%. Ura-DNA glycosylase activity was generally present in amounts 10−100 times greater than HmUra-DNA glycosylase, and its presence confirmed that the tissue extracts were suitably prepared for assays of HmUra-DNA glycosylase activity.

TABLE 2 SPECIFIC ACTIVITY OF URA-DNA GLYCOSYLASE AND HMURA-DNA GLYCOSYLASE IN VERTERBRATE AND LARGE INVERTEBRATE ORGANISMS
*p*moles/min/mg

Rat	Thymus	Brain	Liver	Spleen	Kidney	
HmUra	1.9	1.8	0.69	1.7	0.85	
Ura	120	9.5	130	37	110	
Frog	Thymus	Brain	Liver	Spleen	Kidney	
HmUra	2.2	3.6	0.21	0.26	0.24	
Ura	67	35	36	14	27	
Carp	Thymus	Brain	Liver	Spleen	Post. Kidney	Ant. Kidney
HmUra	1.7	1.2	0.09	0.20	0.79	ND
Ura	87	9.3	8.1	15	32	22
Chicken		Brain	Liver	Spleen	Kidney	Bursa
HmUra		0.70	0.10	0.37	0.64	0.71
Ura		41	1.2	1.6	32	40
Turtle		Brain	Liver	Spleen	Kidney	
HmUra		0.54	0.95	0.20	0.78	
Ura		2.1	6.3	1.2	ND	
Aplysia	Digestive Organ	Muscle	Ganglion	Reproductive System		
HmUra	6.2	0.72	0.09	0.89		
Ura	120	110	27	19		
Lobster	Digestive Organ	Testis	Brain			
HmUra	0.96	0.33	ND			
Ura	4.1	2.9	0.56			

Note: ND indicates that activity was not detected at a level of less than 0.02 pmoles/min/mg.

RESULTS

HmUra-DNA glycosylase activity in vertebrates. HmUra-DNA glycosylase activity was found in all vertebrate organisms examined (Table 2), indicating that HmUra-DNA glycosylase is not limited to mammals. The highest levels of activity were generally found in the brain and immune system tissue (thymus, spleen, bursa). Similar results in mice were previously reported (16). However, such a distribution of specific activity was not found in turtle tissue.

HmUra-DNA glycosylase activity in invertebrates. HmUra-DNA glycosylase activity was detected in all invertebrate organisms examined (Table 2 and Table 2) except *Drosophila*, indicating that HmUra-DNA glycosylase is not limited to vertebrate species. In the two invertebrate organisms in which tissue specific activity could be measured, the nervous system did not show the high levels of activity found in most vertebrates.

HmUra-DNA glycosylase activity in unicellular organisms. We previously reported that the unicellular yeast *Saccharomyces cerevesiae* lacked HmUra-DNA glycosylase, indicating that HmUra-DNA glycosylase was not essential to eukaryotic survival (16). An additional distantly related fungal species, *Schizosaccharomyces pombe*, was also found here to lack

TABLE 3 SPECIFIC ACTIVITY OF URA-DNA GLYCOSYLASE AND HMURA-DNA GLYCOSYLASE IN SMALL INVERTEBRATES AND PROTISTS
*p*moles/min/mg

Organism	HmUra	Ura
D. Melanogaster	ND	ND
Earthworm	3.0	16
Flatworm	0.55	420
Brown hydra	5.1	250
Green hydra	2.9	270
Amoeba	21	650
Chilomonas	30	640

Note: ND indicates that activity was not detected at a level of less than 0.02 pmoles/min/mg.

HmUra-DNA glycosylase, although it contained measurable amounts of Ura-DNA glycosylase (120 pmoles/min/mg). However, two species of protists had high levels of HmUra-DNA glycosylase activity (Table 3) indicating that HmUra-DNA glycosylase is not obligatorily absent from unicellular organisms. These results suggest that a fundamental difference between the Kingdom Protista and the Kingdom Fungi is the ability to repair HmUra in DNA.

Phylogenic and tissue distribution of Ura-DNA glycosylase. A byproduct of these studies was the accumulation of a large body of data on the tissue distribution of Ura-DNA glycosylase (Tables 2 and 3). The tissue distribution of Ura-DNA glycosylase was generally different from that of HmUra-DNA glycosylase and was consistent with the hypothesis that there is a close link between the proliferative capacity of organs and levels of Ura-DNA glycosylase (19,26−28). Our studies confirm that Ura-DNA glycosylase is present in tissues of adults of all species (4) except *Drosophila* (29). However, it should be noted that a recent report described the presence of Ura-DNA glycosylase activity in embryos and larval forms of *Drosophila* (30).

DISCUSSION

This survey indicates that HmUra-DNA glycosylase activity is widely distributed throughout the animal kingdom (Table 4). Its absence from yeast indicates it is not essential for the survival of all eukaryotic organisms, but its presence in protists indicate that it is not simply related to the multicellularity of the organism.

Lindahl has posited that repair enzymes have evolved in response to deleterious modifications of DNA resulting from endogenous stresses such as deamination, aberrant methylation and oxidation (4). Since bacteria, yeast and mammalian cells all contain repair enzyme activities directed against 5,6-dihydroxy-5,6-dihydrothymine (thymine glycol) and related base modifications (31−35), it is likely that oxidative modification of bases occurs in all phyla. Therefore, the absence of HmUra-DNA glycosylase activity from bacteria and yeast led us to propose that the oxidation of Thy to HmUra in a pre-existing AT pair must be more deleterious to complex eukaryotic organisms, which contain HmUra-DNA glycosylase, than to bacteria or yeast, which do not (16).

Based upon the results of this survey and upon our discovery of a second distinct repair enzyme in mammalian tissue, 5-hydroxymethylcytosine-DNA glycosylase (HmCyt-DNA

TABLE 4 SUMMARY OF THE PHYLOGENIC DISTRIBUTION OF HmUra-DNA GLYCOSYLASE

A. Organisms containing HmUra-DNA glycosylase activity

Vertebrates
- Human (16)
- Calf (17)
- Mouse (15)
- Rat
- Chicken
- Frog
- Turtle
- Carp

Invertebrates
- Lobster
- Sea hare
- Earthworm
- Flatorm
- Brown hydra
- Green hydra
- *Amoeba*
- *Chilomonas*

B. Organisms without detectable HmUra-DNA-glycosylase activity

Invertebrates
- *D.Melanogaster*(fruit fly)

Fungi
- *Saccharomyces cerevesiae* (16)
- *Schizosaccharomyces pombe*

Bacteria
- *E.Coli* (WP2, KS391) (16)
- *E.Coli* (BP325, B2375, BP2457) (16)
- *B. subtilis* (14)
- *Dienococcus radiodurans* (16)

Note: Data is from this study unless otherwise noted.

glycosylase) (17,18), we have proposed a second hypothesis to account for the unique phylogenetic distribution of HmUra-DNA glycosylase. HmCyt-DNA glycosylase, like HmUra DNA glycosylase, was also not detectable in bacterial extracts (17). The existence of this enzyme suggests that the methyl groups of 5-methylcytosine (MeCyt) residues in DNA are subject to oxidative attack, resulting in formation of 5-hydroxymethylcytosine (HmCyt), in the same way that the oxidation of Thy residues yields HmUra. HmCyt residues, a normal constituent of the DNA of T-even phages, readily undergo deamination, yielding HmUra (36). Thus, HmUra may be formed by this second mechanism as well as via oxidation of Thy. The major difference between these pathways of HmUra formation is that deamination of HmCyt results in a Gua:HmUra mismatch while the direct oxidation of Thy in a preexisting AT pair produces an Ade:HmUra pair (18,20).

The phylogenetic distribution of HmUra-DNA glycosylase described in this report suggests that the enzyme is found in organisms which use MeCyt in their DNA for the

control of gene expression. MeCyt is generally found as a minor component of DNA in higher eukaryotes and in most lower eukaryotes (37,38) and is believed to function in the control of eukaryotic gene expression (39–41). However, the yeast species we examined do not contain methylated cytosine residues (42). While the DNA of some *E. coli* strains contains MeCyt, the function of this base in *E. coli* seems to be related to preventing restriction and to directing the strand specificity of mismatch repair (43,44). *Drosophila* DNA is also free of MeCyt residues (45). Therefore, the absence of HmUra-DNA glycosylase activity from this organism is consistent with the hypothesis that it is the formation of HmUra from MeCyt which promoted the evolutionary development of this repair enzyme.

In contrast to higher invertebrate and vertebrate species, which generally contain MeCyt, and with fungi, which do not, protozoans show marked interspecies variation in the extent of methylation of cytosine. Several species of ciliates including *Paramecium* (46), *Tetrahymena* (47,48), *Stylonicha* (49) and *Oxytricha* (50) contain no MeCyt while MeCyt is present in the ciliate *Blepharism japonicum* (51) and in several species of dinoflagellates, phytoflagellates (52) and slime molds (53). If our hypothesis is correct, then our finding that *Amoeba* and *Chilomonas* contain HmUra-DNA glycosylase would suggest that these organisms contain MeCyt in their DNA. However, the presence or absence of MeCyt in these species has not yet been determined.

The absence of HmUra-DNA glycosylase in yeast, bacteria and *Drosophila* further suggests that HmUra-DNA glycosylase is one of a group of enzymes involved in maintaining the integrity of MeCyt residues in genomic DNA. If MeCyt is oxidized to HmCyt, it can be repaired by HmCyt-DNA glycosylase (17). If MeCyt is first oxidized and is then subsequently deaminated, or vice versa, the resultant HmUra would be subject to repair by HmUra-DNA glycosylase (16,18). If MeCyt deaminates to form Thy, the resultant GT mismatch could be repaired by a mismatch repair system (54–56). In support of this scheme is the recent observation that a 200 kDa protein factor which binds to GT mismatches is also absent from *Drosophila* and *Saccharomyces cerevesiae* (56). Furthermore, it has recently been shown that the process of GT mismatch repair in human cells involves a DNA glycosylase (57). It is possible that these repair enzymes appeared evolutionarily only when the maintenance of MeCyt residues in DNA became critical for gene regulation.

Our results suggest that there are at least two determinants for the levels of HmUra-DNA glycosylase activity in animal tissues. The first determine whether the organism does or does not contain the gene for this enzyme as reflected by the presence or absence of DNA glycosylase activity. We now believe that the use of MeCyt in DNA as a regulatory element in gene expression constitutes one such determinant. The second type of determinant may be tissue specific; i.e., species which contain the gene express different amounts of enzyme activity in different tissues. The finding of high HmUra-DNA glycosylase levels in organs of the nervous or immune system in most of the vertebrates studied (rat, mouse, frog, chicken and carp) suggests that the activity is necessary for maintenance of normal immune and nervous function. Support for this idea can be drawn from the fact that organisms which contain high levels of HmUra-DNA glycosylase activity in nervous and immune tissue also show allograft rejection and mixed lymphocyte reactivity (aspects of major histocompatibility complex function) (58). However, since turtle tissue demonstrated high specific activity of HmUra-DNA glycosylase in liver and kidney as well, additional determinants of tissue specific activity must also be operative in vertebrates.

The lack of detectable levels of enzyme activity in lobster brain and the low levels of

enzyme activity in the ganglion of *Aplysia* suggests that different factors determine expression of enzyme activity in invertebrates. However, it is also possible that the differences in specific activity are, in part, artifactual since we expressed enzyme specific activity as a function of protein content. Since *Aplysia* has very large neurons, it may be that were we able to quantitate HmUra-DNA glycosylase activity as a function of cell number, HmUra-DNA glycosylase activity might prove to be higher in *Aplysia* and Lobster ganglia as compared to other invertebrate organs.

Our comparisons of HmUra-DNA glycosylase activity between tissues must be interpreted cautiously. Since the enzyme incubation lasted six hours, it is possible that variations in specific activities among tissues represent differences in levels of proteases, nucleases, inhibitors or other factors in the tissue extracts and not differences in HmUra-DNA glycosylase activity. It will not be possible to rigorously confirm the tissue specific differences in enzyme activity until antibodies and molecular probes become available.

Our laboratory is currently undertaking the purification of HmUra-DNA glycosylase and HmCyt-DNA glycosylase to permit us to study the molecular genetics of these enzymes and thereby further elucidate their contribution to the repair of oxidative damage to DNA.

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