The Sir2 family of NAD+‐ dependent histone/protein deacetylases has been implicated in a wide range of biological activities, including gene silencing, life span extension, and chromosomal stability. Recent evidence has indicated that these proteins produce a novel metabolite O‐acyt‐ADP‐ribose (OAADPr) during deacetylation. Cellular studies have demonstrated that this metabolite exhibits biological effects when microinjected in living cells. However, the molecular targets of OAADPr remain to be identified. Here we have analyzed the ADP‐ribose‐specific Nudix family of hydrolases as potential in vivo metabolizing enzymes of OAADPr. In vitro, we found that the ADP‐ribose hydrolases (yeast YSA1, mouse NudT5, and human NUDT9) cleaved OAADPr to the products AMP and acetylated ribose 5′‐phosphate. Steady‐state kinetic analyses revealed that YSA1 and NudT5 hydrolyzed OAADPr with similar kinetic constants to those obtained with ADP‐ribose as substrate. In dramatic contrast, human NUDT9 was 500‐fold less efficient (kcat/Km values) at hydrolyzing OAADPr compared with ADP‐ribose. The inability of OAADPr to inhibit the reaction of NUDT9 with ADP‐ribose suggests that NUDT9 binds OAADPr with low affinity, likely due to steric considerations of the additional acetylated‐ribose moiety. We next explored whether Nudix hydrolytic activities against OAADPr could be observed in cell extracts from yeast and human. Using a detailed analysis of the products generated during the consumption of OAADPr in extracts, we identified two robust enzymatic activities that were not consistent with the known Nudix hydrolases. Instead, we identified cytoplasmic esterase activities that hydrolyze OAADPr to acetate and ADP‐ribose, whereas a distinct activity residing in the nucleus is consistent with an OAADPr‐specific acetyltransferase. These findings establish for the first time that select members of the ADP‐ribose hydrolases are potential targets of OAADPr metabolism. However, the predominate endogenous activities observed from diverse cell extracts represent novel enzymes.
GX, EX, REUEEXEGU, where U represents a bulky, hydrophobic amino acid, usually Ile, Leu, or Val and X represents any amino acid (27). Based on predictive (29) and structural studies (30), the Nudix box designates a unique loop-helix-loop motif, involved in the binding of the substrate (31) and in catalysis (32, 33). The nucleoside diphosphate linkage is the common feature of the wide range of substrates for the family, which include NADH, nucleoside polyphosphates, nucleotide sugars, and (deoxy)ribonucleoside triphosphates. These substrates are thought to be either potentially deleterious compounds, cell signaling molecules, regulators, or metabolic intermediates whose concentrations require modulation during fluctuations of the cellular environment (34). Hence, proposed physiological functions of these enzymes are to eliminate potentially toxic nucleotide metabolites from the cell (35, 36) and to regulate the concentrations of nucleotide cofactors and signaling molecules for optimal cell growth and survival (37).

The purpose of the current study is to investigate the Nudix hydrolase family of enzymes as viable targets of OADPr metabolism. To explore this idea, we examined the ability of Nudix enzymes, specifically the ADP-ribose (ADPr) hydrolases, to catalyze the hydrolysis of OADPr. We found that select members of the ADP-ribose hydrolases are capable of efficient hydrolysis of OADPr, suggesting that certain members may be crucial in regulating in vivo levels of OADPr. To examine this possibility, we analyzed endogenous enzymatic activities toward OADPr from a variety of cellular sources. We show that human HeLa, mouse 3T3 fibroblast, and yeast cell extracts harbor robust enzymatic activities toward OADPr. However, we demonstrate that the products generated from these cellular activities are not consistent with known Nudix hydrolase activities. This finding raises important biological questions concerning the physiological functions of OADPr metabolizing enzymes detected in cellular extracts as well as the observed diverse catalytic efficiency of ADP hydrolases on OADPr.

EXPERIMENTAL PROCEDURES

Cell Culture—HeLa S3 cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum, 30 μg/ml L-glutamine, 10 units/ml penicillin, and 10 μg/ml streptomycin at 37 °C and 5% CO2. 3T3 fibroblast cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum, 30 μg/ml L-glutamine, 10 units/ml penicillin, and 10 μg/ml streptomycin at 37 °C and 5% CO2.

Preparation of Nuclear Extracts—Cell monolayers were washed twice with ice-cold phosphate-buffered saline, pH 7.4, and 4 °C and removed from the culture dish by scraping. Cells were pelleted by centrifugation using a clinical centrifuge for 10 min at 4 °C. The cell pellet was resuspended in 4 volumes of ice-cold water and recentrifuged, and the supernatant containing the cytoplasmic fraction was collected and stored and at −80 °C. The cell pellet was then resuspended in 4 volumes of ice-cold water and recentrifuged for 10 min at 4 °C. The cell pellet was resuspended in 3 volumes of glass bead disruption buffer (200 mM Tris-Cl, pH 7.9, 10 mM MgCl2, 1 mM EDTA, 5% glycerol, 1 mM DTT, 0.5 mM ammonium sulfate, 1 mM PMSF), and a cell paste was created. Four volumes of chilled acid-washed beads (Sigma) were added to the cell paste, the suspension was vortexed at maximum speed for 5 min, and the tubes were placed on ice for 4 min. Vortexing was repeated three times. The glass beads were allowed to settle, and the supernatant was decanted. To remove insoluble protein, the supernatant was centrifuged for 60 min at 12,000 rpm (15,800 × g). The supernatant, which represents the crude extract, was collected and stored at −80 °C.

Conversion Assay Using HeLa Cell Nuclear Extract—The presence of ADP-ribose activity in HeLa cell monolayers was determined. This weight was said to be equal to the packed cell volume (in milliliters), and for all subsequent steps was considered as 1 volume. Cells were resuspended in 4 volumes if ice-cold water and immediately centrifuged for 5 min at 3,500 rpm (2,170 × g) at 4 °C. The cell pellet was resuspended in 3 volumes of glass bead disruption buffer (20 mg Tris-Cl, pH 7.9, 10 mM MgCl2, 1 mM EDTA, 5% glycerol, 1 mM DTT, 0.5 mM ammonium sulfate, 1 mM PMSF), and a cell paste was created. Four volumes of chilled acid-washed beads (Sigma) were added to the cell paste, the suspension was vortexed at maximum speed for 5 min, and the tubes were placed on ice for 4 min. Vortexing was repeated three times. The glass beads were allowed to settle, and the supernatant was decanted. To remove insoluble protein, the supernatant was centrifuged for 60 min at 12,000 rpm (15,800 × g). The supernatant, which represents the crude extract, was collected and stored at −80 °C.
preparation of O-acetyl-ADP-ribose and the products were analyzed by reverse-phase HPLC and a colorimetric phosphatase assay. 

Matrix-assisted Laser Desorption Ionization Mass Spectrometry Analyses—MALDI mass spectrometry was performed at the Environmental Health Sciences Center, Oregon State University, as described previously (22).

RESULTS 

Certain Members of the ADP-Ribose Hydrolase Family Efficiently Hydrolyze OAc-ADPR—We performed initial literature searches to identify enzymes capable of metabolizing the closest known metabolite of OAADPr, ADPr. The most promising finding was the NuDT family of enzymes, which consists of a group of phosphoanhydrases present in organisms ranging in complexity from viruses to humans (34). The substrates of this enzyme family include nucleoside triphosphates, co-enzymes, sugar nucleotides, and dinucleoside polyphosphates (34). Interestingly, ADPr is a favored substrate for several members of the NuDT family (Refs. 34, 39–43; reviewed in Ref. 27). We have demonstrated that eichinoderm oocytes (26), human (Fig. 1) and S. cerevisiae cell extracts (data not shown) possess robust enzymatic activities that metabolize OAADPr, although the products of these reactions had not been elucidated. It was therefore logical to screen a number of NuDT hydrolases as potential enzymes responsible for the robust metabolism of OAADPr observed in these cellular extracts. To explore the idea that OAADPr may be a substrate for these hydrolases, we assayed the ADP-sugar pyrophosphatase, YSA1, from S. cerevisiae (YSA1) (40), the murine ADP-sugar diphosphatase mNuDT5 (42), and the human ADP-diphosphatase NUDT9 (44). These enzymes were chosen because their preferred substrates are ADP-sugars with preference for ADPr (44, 45). A common mechanism of virtually all members of the NuDT hydrolase superfAMILY is a nucleophilic attack by water on a pyrophosphate linkage in the substrate (43). This nucleophilic attack occurs on the alpha or beta phosphorus producing a reaction product corresponding to the nucleoside 5'-monophosphate (43). Because the products generated from ADPr hydrolysis are AMP and ribose 5'-phosphate (39, 45, 46), the reaction products predicted from the conversion of OAADPr would be AMP and acetylated ribose 5'-phosphate. To provide evidence for this prediction, YSA1 was incubated with either OAADPr or ADPr and the products were analyzed by reverse-phase HPLC and a colorimetric phosphatase assay.

Reverse-phase HPLC of this reaction indicated that the disappearance of substrate ADPr or OAADPr was coincident with the appearance of AMP (Fig. 2, A and B, respectively). To confirm the elution position of AMP, 100 μM of an authentic AMP standard was co-injected onto the HPLC, and the elution profile was monitored (Fig. 2C). As expected, UV absorption at 260 nm demonstrated that AMP co-eluted with the A260 peak seen with YSA1 enzymatic turnover of OAADPr (Fig. 2B). To detect the cleavage of OAADPr, we generated radiolabeled O-[3H]OAADPr using the Sir2 homolog HST2 and the synthetic,

![](https://example.com/figure1.png)

**Fig. 1.** Enzymatic conversion of OAADPr in HeLa cell lysates. O-[3H]OAADPr, 1.7 mM, was added to HeLa cell whole cell lysate (circles), heat-denatured whole cell lysate (diamonds), and to a buffer control (25 mM Tris, pH 7.2) (squares). At 0, 30, 60, and 180 min, samples were injected onto the HPLC; fractions were collected, and radioactivity of fractions was determined by scintillation counting. Percent hydrolysis of O-[3H]OAADPr was determined by comparing the radioactivity of fractions to a t = 0 time point.

![](https://example.com/figure2.png)

**Fig. 2.** HPLC analysis of the YSA1 reaction with ADPr and O-[3H]OAADPr. A, elution of products from the YSA1 enzymatic hydrolysis of ADPr, detected by UV absorbance at 260 nm. Conditions: 50 mM Tris-Cl, pH 7.5, 7 mM MgCl2, 300 μM ADPr, 37 ng of YSA1 enzyme for 3 min 37 °C before quenching the reaction with trifluoroacetic acid to a final concentration of 1%. B, elution of products from the YSA1 enzymatic hydrolysis of O-[3H]OAADPr, detected by UV absorbance at 260 nm. Conditions: 50 mM Tris-Cl, pH 7.5, 7 mM MgCl2, 300 μM OADPr, 37 ng of YSA1 enzyme for 3 min 37 °C before quenching the reaction with trifluoroacetic acid to a final concentration of 1%. C, reverse-phase HPLC elution of AMP standard co-elutes with the A260 products from YSA1 enzymatic conversion of OAADPr. Conditions: AMP was added to a final concentration of 100 μM to the quenched reaction described in B. D, elution of tryptinated acetyl products generated from the YSA1 hydrolysis of O-[3H]OAADPr. Samples were injected onto the HPLC; fractions were collected and detection of the tritiated product was determined by scintillation counting.

5'-monophosphate (43). Because the products generated from ADPr hydrolysis are AMP and ribose 5'-phosphate (39, 45, 46), the reaction products predicted from the conversion of OAADPr would be AMP and acetylated ribose 5'-phosphate. To provide evidence for this prediction, YSA1 was incubated with either OAADPr or ADPr and the products were analyzed by reverse-phase HPLC and a colorimetric phosphatase assay.

Reverse-phase HPLC of this reaction indicated that the disappearance of substrate ADPr or OAADPr was coincident with the appearance of AMP (Fig. 2, A and B, respectively). To confirm the elution position of AMP, 100 μM of an authentic AMP standard was co-injected onto the HPLC, and the elution profile was monitored (Fig. 2C). As expected, UV absorption at 260 nm demonstrated that AMP co-eluted with the A260 peak seen with YSA1 enzymatic turnover of OAADPr (Fig. 2B). To detect the cleavage of OAADPr, we generated radiolabeled O-[3H]OAADPr using the Sir2 homolog HST2 and the synthetic,
acetylated histone H3 peptide, [3H]AcH3, where the radioactivity resides on the methyl position of the acetyl group. Cleavage of OADPr by the ADPr hydrolase YSA1 between the α and β phosphates would be expected to generate acetylated ribose 5'-phosphate as the other reaction product. Upon O-[3H]AADPr conversion by YSA1, we found a single tritium-labeled product eluting at fraction 6 in reverse-phase HPLC analysis (Fig. 2D). Next, we attempted to verify this tritiated product as acetylated ribose 5'-phosphate. To accomplish this, YSA1 was incubated with O-[3H]AADPr, and the reaction products were collected after separation by HPLC. If the product is acetylated ribose 5'-phosphate then we would expect to retrieve equal moles of acetyl-ribose to phosphate. The acetyl-containing species was quantitated by using the specific activity of the AADPr hydrolase reaction contains approximately equivalent molar amounts of phosphate, indicating cleavage of OADPr between the α and β phosphates, producing reaction products in accordance with AMP and acetylated ribose 5'-phosphate.

These findings reveal the capacity of the ADPr hydrolase YSA1 to metabolize OADPr to the reaction products AMP and acetylated ribose 5'-phosphate. To further characterize OADPr as a substrate for the ADPr hydrolase family of enzymes, we next determined the steady-state kinetic constants of YSA1, mNudT5, and NUDT9 for OADPr. For these assays, enzyme was incubated with varying concentrations of substrate, and the initial velocities were measured using a colorimetric phosphate assay described under “Experimental Procedures.” Saturation curves were constructed and kinetic constants calculated from fits using the Michaelis-Menten equation. The activities toward OADPr were compared with ADPr (Table I). Our results with ADPr were similar to those reported previously (Table I) (40, 42, 44).

OADPr is hydrolyzed by YSA1 at a maximal rate ($k_{cat} = 37 s^{-1}$) 2-fold less than that measured with ADPr (Table I) (Fig. 3A) and with a $k_{cat}/K_m$ value 3.5-fold less than with ADPr as substrate. The mNudT5 hydrolyzes each substrate at a similar maximal rate (0.8–0.9 s$^{-1}$), and the ratio of $k_{cat}/K_m$ values is nearly 1:1 (Table I) (Fig. 3B). These data demonstrate the capacity of YSA1 and mNudT5 to bind and hydrolyze OADPr with similar kinetic parameters as those for ADPr. Strikingly, this is not the case with NUDT9, which exhibits a $k_{cat}/K_m$ value that is 500-fold less than with ADPr (Fig. 3C). Because the saturation curve for OADPr (Fig. 3C) is linear through as high as 800 μM substrate, accurate $V_{max}$ or $K_m$ values could not be obtained. The $k_{cat}/K_m$ value (448 ± 24 s$^{-1}$) for NUDT9 with OADPr was determined from the slope of the line fitted by linear-least squares regression (Table I). If it is assumed that $V_{max}$ is unchanged or 2-fold slower with OADPr as substrate compared with ADPr (a reasonable assumption based on the results with NudT5 and YSA1, see above) and fitting the data to the Michaelis-Menten equation.

Human Cell Extracts Harbor Two Non-Nudix OADPr Metabolizing Activities—As previously indicated, we found that
human and yeast cells possess robust enzymatic activities that metabolize OAAADPr (Fig. 1). To explore the hypothesis that the metabolism of OAAADPr observed in cell extracts resulted from a Nudix hydrolase activity, we set out to identify the products of this reaction. If the reaction products were indeed AMP and acetylated ribose 5'-phosphate, we could conclude that a Nudix-like enzyme may be responsible for the observed turnover.

Initially we analyzed whether these enzyme activities could be localized to either the cytoplasm or nucleus. To accomplish this, HeLa nuclear and cytoplasmic pools were fractionated and assayed for OAAADPr metabolizing enzymes. Both nuclear and cytoplasmic extracts were incubated with O-[3H]AADPr for various times, and the loss of radioactivity in the reverse-phase HPLC peak corresponding to O-[3H]AADPr was monitored (Fig. 4). Interestingly, we observed activity in both the nuclear and cytoplasmic fractions, however, the labeled acetyl-containing product of OAAADPr turnover was different between the two cellular compartments (Fig. 4). Upon O-[3H]AADPr conversion by the cytoplasmic compartment, the tritium-labeled product elutes at fraction 5 in the reverse-phase HPLC analysis (Fig. 4, left panel). The nuclear OAAADPr metabolizing activity generates a tritium-labeled product that elutes at fraction number 10 (Fig. 4, right panel). Following the adenine moiety, we observed, by HPLC and UV-detection at $A_{260}$, that both the cytoplasmic and nuclear enzymes appear to generate the same reaction product eluting at fraction 6 (Figs. 5 and 6). Also, we have demonstrated that nuclear and cytoplasmic extracts from 3T3 fibroblasts and S. cerevisiae yeast whole cell extracts are also capable of metabolizing OAAADPr, producing analogous reaction products to those seen with HeLa cytoplasmic and nuclear extracts. These data suggest the existence of different cellular enzymes in both the nucleus and cytoplasm of HeLa, 3T3 fibroblast cells, and yeast extracts that utilize OAAADPr.

2 L. A. Rafty, M. T. Schmidt, A.-L. Perraud, A. M. Scharenberg, and J. M. Denu, unpublished observations.
Characterization of a Cytoplasmic OAADPr Metabolizing Activity—Next, we attempted to characterize the products of these reactions and establish if they were consistent with Nudix hydrolase activity. Analysis of the HeLa cell cytoplasmic enzymatic activity toward OAADPr revealed that the reaction products are not consistent with generating AMP and acetylated ribose 5'-phosphate. Several biochemical criteria indicate that one of the cytoplasmic enzymes is an esterase, hydrolyzing OAADPr to ADPr and acetate. First, we verified acetate as one of the reaction products. HeLa cell cytoplasmic extract was incubated with O-[3H]AADPr, the products were analyzed by reverse-phase HPLC, and the fractions were analyzed by liquid scintillation counting. We detected radioactivity resulting from OAADPr conversion to AMP and ADPr by one or more esterases located in the cytoplasm.

Characterization of a Nuclear OAADPr Metabolizing Activity—As previously discussed, we observed that the nuclear OAADPr metabolizing activity generates a tritium-labeled product that elutes at fraction 10 (Fig. 4, right panel). This elution position is far more hydrophobic than the elution profile for acetate alone, which elutes at fraction 5 (Fig. 5E), or for acetylated ribose 5'-phosphate, which elutes at fraction 6 (Fig. 2D). These findings suggest that the nuclear OAADPr metabolizing activity is not consistent with either an esterase- or Nudix-like activity. To shed light on the nature of this nuclear OAADPr metabolizing enzyme, the products generated from the nuclear enzymatic turnover of OAADPr were extensively studied. HeLa cell nuclear extract was incubated with O-[3H]AADPr, and the products were analyzed by reverse-phase HPLC, the colorimetric phosphatase assay, and MALDI mass spectral analysis. When nuclear extract was incubated with OAADPr, reverse-phase HPLC demonstrated that the disappearance of substrate O-[3H]AADPr was coincident with the appearance of an A_{260} absorbing species eluting at fraction 6 (Fig. 6A). To explore the possibility that this product was ADPr, 50 μM authentic ADPr was co-injected with the nuclear extract conversion assay described in Fig. 6A, and reverse-phase HPLC was performed. ADPr co-eluted with the A_{260} peak generated from the enzymatic turnover O-[3H]AADPr (Fig. 6B). Because acetate was clearly not a reaction product, we examined the possibility that the enzyme was cleaving O-[3H]AADPr between the β-phosphate and ribose 5'-OH moiety to yield ADP and acetylated ribose. To explore this, co-
Fig. 5. HPLC analysis of the O-[³H]AADPr metabolizing activity in HeLa cytoplasmic extract. A, control reaction. Conditions: 1.5 mM O-[³H]AADPr, 100 µg of HeLa cytoplasmic extract for 0 min at RT before quenching with trifluoroacetic acid to 1%. B, elution of products from HeLa cytoplasmic extract enzymatic hydrolysis of O-[³H]AADPr, detected by UV absorbance at 260 nm. Conditions: 1.5 mM O-[³H]AADPr, 100 µg of HeLa cytoplasmic extract for 60 min at RT before quenching with trifluoroacetic acid to 1%. C, elution of ADPr standard correlates exactly with the A_{250} reaction product from HeLa cytoplasmic extract enzymatic hydrolysis of O-[³H]AADPr. ADPr was added to a final concentration of 10 µM to the quenched reaction described in B prior to HPLC analysis. D, HPLC product analysis of YSA1 incubated with the products generated in B. Conditions: 40 ng of YSA1 and 7 mM MgCl₂ were added to the reaction described in B for 10 min, 37 °C prior to HPLC analysis. E, HPLC analysis of acetate as a product generated in B. The reaction described in B was injected onto the HPLC; fractions were collected and radioactivity of fractions was determined by scintillation counting. The elution position of acetate was then determined by using 1.0 nmol of sodium [³H]acetate (100,000 cpm/nmol) and detection by liquid scintillation counting (data not shown).

injections with ADP were performed. Interestingly, HPLC analysis revealed that ADP eluted at the same position as ADPr (Fig. 6C). Because ADP and ADPr elute together on the HPLC, colorimetric phosphate assays were used to distinguish between these two possibilities. Either alkaline phosphatase or alkaline phosphatase and a ADPr hydrolase (NUDT9) was incubated with the unknown A_{250} absorbing species, and the amount of phosphate liberated in each case was quantitated. ADP would be hydrolyzed to adenosine by alkaline phosphatase whether NUDT9 was present or absent (1 mol of ADP producing 2 mol of P₃ and 1 mol of adenosine), whereas ADPr would only yield free phosphate in the presence of a Nudix-specific pyrophosphatase activity to generate products AMP and ribose 5'-phosphate, which are then sensitive to complete hydrolysis by alkaline phosphatase. Only in the presence of both alkaline phosphatase and NUDT9 enzyme was significant phosphate released. Of the total phosphate detected, only 13% could be liberated by alkaline phosphatase only, whereas 87% of the total required NUDT9 and alkaline phosphatase for detection. This suggests that the major product represented by the HPLC fraction 6 is indeed ADPr and not ADP.

To provide further evidence that ADPr is the product generated from the nuclear enzymatic metabolism of OAADPr, HPLC fractions containing this product were subjected to MALDI mass spectral analysis for mass determination. The adduct yielded a mass (positive molecular ion in MALDI) of 560 m/z (Fig. 7), consistent with the enzymatic formation of ADPr. Together these findings provide strong evidence that ADPr is a reaction product generated from the HeLa nuclear conversion of OAADPr. This finding, combined with the observation that the tritium-labeled product is distinct from either acetate or acetylated phospho-5'-ribose, suggests that the nuclear enzymatic activity is an OAADPr-specific acetyltransferase and is not consistent with an esterase or traditional Nudix hydrolase activity. Currently the properties of this acetyl-containing product in fraction 10 and the identity of the enzyme responsible for the transferase activity are under investigation.
FIG. 7. Mass spectral analysis of the A_{260} product generated by the HeLa nuclear enzymatic activity. MALDI mass spectrometry was used to identify a mass of 560 m/z, consistent with the formation of ADPr.

DISCUSSION

This study demonstrates in vitro that certain members of the ADPr hydrolase family are potential targets of OAADPr metabolism, whereas others display a clear preference for ADPr turnover. Moreover, we demonstrate that HeLa, 3T3 fibroblast, and S. cerevisiae cell extracts harbor robust enzymatic activities toward OAADPr. However, our data suggest that the products generated from these endogenous enzymes are not consistent with known Nudix hydrolase reactions. Instead, we show that OAADPr is utilized by one or more cytoplasmic enzymes that catalyze OAADPr hydrolysis to acetate and ADPr, and by one or more nuclear enzymes that catalyze the transfer of the acetyl moiety to an acceptor protein or small molecule. Although no clear endogenous Nudix-like activity was observed in our studies, these observations do not rule out the possibility that such activities do exist and are important physiologically. The two observed activities may be present at much higher levels than specific Nudix hydrolases and may have greater catalytic efficiencies toward OAADPr compared with Nudix hydrolases, thereby competing with endogenous Nudix hydrolases for OAADPr turnover. Consequently, the detection of endogenous Nudix activity may be masked by these robust cellular activities. The findings, that ADPr hydrolases display unique specificity for OAADPr and that there exists other cellular enzymes capable of hydrolyzing OAADPr, provide strong circumstantial evidence for the importance of OAADPr in signaling and other cellular processes.

Although the function(s) of OAADPr have not been established, it has been proposed to act as a second messenger, a regulator of other enzymatic processes, or as a substrate for other enzymes (22, 23). Our data are fully consistent with these possibilities. First, our finding of an esterase-like activity (or activities) located in the cytoplasmic compartment of cells suggests this activity may play an integral role in regulating the levels of OAADPr by catalyzing OAADPr to acetate and ADPr. Thus, in this instance OAADPr could initiate a signaling cascade as an important second messenger. Our identification of an OAADPr-specific acetyltransferase activity in the nuclear compartment of cells indicates that OAADPr can function as a co-enzyme substrate for other enzymatic reactions. The function of these specific transferases as well as the identity of the acceptor protein or small molecule remains to be uncovered. Our finding that select members of the ADPr hydrolase family of enzymes (for example mNudT5 and YSA1) are capable of efficient hydrolysis of OAADPr, whereas NUDT9 is essentially inactive toward OAADPr (as reflected in the $k_{cat}/K_m$ values, Table 1), further supports the notion that OAADPr is a potential second messenger as well as an in vivo substrate for other enzymes. Lack of activity between OAADPr and NUDT9 raises a number of intriguing questions pertaining to OAADPr function.

Interestingly, NUDT9 has a mitochondrial leader sequence that gives rise to a mature 34.2-kDa mitochondrial protein (45). Recently, it has been reported that mitochondria from mammalian cells contain intrinsic NAD$^+$-dependent deacetylase activity (50). This activity was shown to be inhibited by nicotinamide but not by Trichostatin A, making it consistent with an Sir2 type of deacetylase activity. Furthermore, this deacetylase activity was identified as the nuclear-encoded human Sir2 homolog hSirT3 (50). This finding raises interesting questions for the biological roles of OAADPr in the mitochondria and elsewhere. SIRT3-dependent generation of OAADPr in the mitochondria would be resistant to hydrolysis by NUDT9, implying an important role in maintaining OAADPr levels within the mitochondria. It has recently been proposed that OAADPr may be an important signaling molecule that mediates Sir2-like enzyme function on metabolic networks (51). Moreover, it has been speculated that the conserved family of Sir2 proteins are involved in sensing cellular energy and redox states (20). Supporting a link between Sir2-like enzymes and metabolism, Lin et al. (52) have demonstrated that yeast lifespan extension under limiting glucose requires Sir2 and increased respiration. These observations, combined with the results from this study, suggest that the Sir2 family of enzymes may control a variety of metabolic or signaling pathways through the formation of OAADPr. Identification of the enzymes and the reactions they catalyze will be a critical step in uncovering the functions of this unique metabolite.

Acknowledgments—We are thankful to Dr. Maurice Bessman (Dept. of Biology and McCollum-Pratt Institute, The John Hopkins University) for YSA1 and Drs. Jeffrey Miller and Hanjing Yang (Dept. of Microbiology and Molecular Genetics and the Molecular Biology Institute, University of California-Los Angeles) for mNudT5, Brian Arbogast (Oregon State University) for the MALDI mass spectral analysis, and Dr. Michael D. Jackson (Oregon Health & Sciences University) for helpful discussions and technical assistance.

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Analysis of O-Acetyl-ADP-ribose as a Target for Nudix ADP-ribose Hydrolases
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J. Biol. Chem. 2002, 277:47114-47122.
doi: 10.1074/jbc.M208997200 originally published online October 4, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M208997200

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