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Membrane-associated NAD$^+$ glycohydrolase from rabbit erythrocytes is solubilized by phosphatidylinositol-specific phospholipase C

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NAD$^+$ glycohydrolase (NADase) present on the surface of rabbit erythrocytes is a membrane-bound ectoenzyme that can be solubilized by phosphatidylinositol-specific phospholipase C (PIPLC). As much as 70% of the cell-associated NADase was made soluble by treatment with PIPLC. The portion of NADase that remained cell-associated after an initial PIPLC treatment proved to be resistant to subsequent solubilization attempts. Further analysis showed that release of NADase from erythrocytes could not be attributed to the action of proteinases or phospholipase C. Erythrocytes obtained from other mammals were analyzed and found to have variable amounts of PIPLC-susceptible NADase. Practically, this finding can be used to easily solubilize membrane-bound NADase as a first step in its purification.

Introduction

NAD$^+$ glycohydrolase (NADase, EC 3.2.2.5) is an enzyme capable of catalyzing the hydrolysis of NAD to form nicotinamide and adenosine diphosphoribose. NADases are widely distributed in nature, being found in microorganisms, plants, and all animal tissues [1–6]. In mammals, NADases are generally found in association with the plasma membranes of most cell types, including erythrocytes [4,5]. Rabbit erythrocytes possess relatively high NADase activity when compared to erythrocytes from other species [6], with the NADase activity being exclusively located on the outer plasma membrane surface [6,7]. Many attempts have been made to solubilize and purify erythrocyte NADase using conventional methods [8]. To date these have met with only limited success.

In this report, we describe the results of experiments which establish that the majority of rabbit erythrocyte NADase is bound to the plasma membrane via a phosphatidylinositol linkage. An effective solubilization of NADase from intact rabbit erythrocytes (> 70%) was easily accomplished by treatment with phosphatidylinositol-specific phospholipase C (PIPLC) obtained from *Bacillus cereus*.

Materials and Methods

Preparation of PIPLC. The PIPLC was prepared from *B. cereus* essentially as described by Ikezawa and Taguchi [9]. Following growth in routine culture medium (10 g polypeptone, 10 g

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yeast extract, 5 g NaCl and 0.4 g Na_2HPO_4 per liter, adjusted to pH 7.0) the supernatant was collected by centrifugation and ammonium sulfate was added to 80% saturation. After an overnight incubation (4° C), the precipitate was collected by centrifugation (25 000 × *g*), redissolved in a small volume of distilled water and dialyzed against 5 mM Tris-maleate buffer (pH 6.5). The dialyzed material was then loaded onto a CM-Sephadex column preequilibrated in Tris-maleate buffer. Effluent fractions from this column contained the PIPLC activity. The fractions with PIPLC activity were then pooled, concentrated, and dialyzed against 20 mM Tris-HCl (pH 8.5). The sample was then loaded onto a DEAE-cellulose column preequilibrated with the Tris-HCl buffer, and eluted with a linear NaCl gradient (0–0.3 M). Fractions containing PIPLC were pooled and concentrated.

Enzymatic activity was quantitated by measurement of the alkaline phosphatase-releasing activity of PIPLC from HeLa cell membranes. A small aliquot of sample being tested for PIPLC activity was added to HeLa cells ($2 \cdot 10^5$) in 0.29 M sucrose/ 10 mM NaCl/10 mM Hepes buffer (pH 7.0). After a 30 min incubation at 37° C the supernatant was withdrawn and tested for soluble alkaline phosphatase activity according to the method described by Low and Finean [10]. One unit of PIPLC was defined as the amount of enzyme necessary to release 1 unit of alkaline phosphatase from HeLa cell membranes per h. The PIPLC-containing preparation used in this study contained 10 units/μg of protein. We further evaluated our PIPLC preparation by examining its ability to solubilize other molecules purported to be attached to the plasma membrane by a phosphotidylinositol-type linkage. Utilizing the assay described by Ellman et al. [11] it was found that acetylcholinesterase could be released from rabbit erythrocytes by treatment with PIPLC. Our PIPLC preparation was also capable of solubilizing the Thy-1 molecule from murine thymocytes without affecting the surface density of major histocompatibility complex class I molecules (data not shown).

Erythrocyte preparations and PIPLC treatment protocol. Fresh heparinized blood was centrifuged at 400 × *g* for 10 min at 4° C. The plasma and buffy coat were discarded and the erythrocytes were centrifuged and resuspended in 0.173 M Tris-HCl buffer (pH 7.6) to a final cell concentration of approximately $5 \cdot 10^6$ cells/ml. A specified amount (see Results) of the PIPLC preparation was then added and incubated at 37° C. Following incubation the cells were pelleted by centrifugation, the supernatant removed, and the cell pellet lyzed by adding 1 vol. of 0.05% Triton X-100 to solubilize all remaining cell-associated NADase activity.

NADase assay. NADase activity was quantitated by measurement of the formation of [*carbonyl*-^{3}H]nicotinamide from [*carbonyl*-^{3}H]-NAD following elution from a Dowex-1-chloride column [12]. Unless otherwise stated, the assay was run in 0.1 M potassium phosphate buffer (pH 7.0), containing 0.2 mM [*carbonyl*-^{3}H]NAD (0.1 μCi) in a total volume of 200 μl. After 1 h incubation at 37° C the reaction was terminated by adding 1 ml of ice-cold 20 mM Tris-HCl (pH 7.6) and the mixture was then applied to the Dowex-1 column. The [*carbonyl*-^{3}H]nicotinamide was recovered by elution with 4 ml of 20 mM Tris-HCl (pH 7.6). A unit of NADase was defined as the amount of enzyme required to hydrolyze 1 pmol of [*carbonyl*-^{3}H]NAD per h. All assays were run in duplicate.

Results

Treatment of rabbit erythrocytes with PIPLC causes a release of membrane-associated NADase

Rabbit erythrocytes ($5 \cdot 10^6$ cells/ml) were incubated at 37° C in 0.173 M Tris-HCl buffer (pH 7.6) with various concentrations of PIPLC (0–4 μg/ml). After 60 min the samples were centrifuged (9000 × *g*) for 5 min and the supernatants were collected and quantitatively assayed for soluble NADase activity. The NADase activity found in a Triton X-100 lysate of an equivalent number of rabbit erythrocytes served as a positive control. The results (Fig. 1) demonstrate that PIPLC was capable of releasing approximately 70% of the cell-associated NADase in a dosage-dependent manner, with maximal release occurring PIPLC concentrations of approximately 0.5 μg/ml. Spectrophotometric analysis of the supernatants at 412 nm established that the solubilization of rabbit erythrocyte NADase activity by PIPLC was not

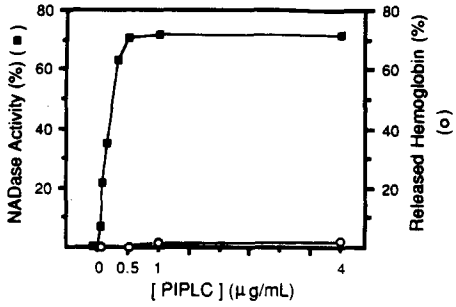

Fig. 1. Solubilization of NADase from rabbit erythrocytes by PIPLC. Erythrocytes ($5 \cdot 10^6$ cells/ml), suspended in 0.173 M Tris-HCl buffer (pH 7.6), were incubated with increasing concentrations of PIPLC at 37 °C (total reaction volume: 1.2 ml). After 1 h the sample was centrifuged ($9000 \times g$) for 5 min. NADase activity was measured in the supernatants. The PIPLC-soluble NADase activity was taken as the percentage of the total NADase extracted by treatment of the erythrocytes with 0.05% Triton X-100.

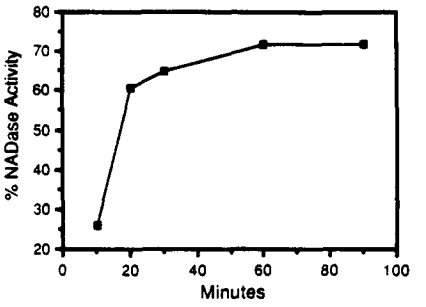

Fig. 2. Solubilization of NADase from erythrocytes by PIPLC. Erythrocytes ($5 \cdot 10^6$ cells/ml) were incubated with PIPLC (1 μg/ml) at 37 °C. At the designated times, duplicate samples were centrifuged and soluble NADase activity was measured as described for Fig. 1. The results are presented as the percentage of total NADase activity.

due to cell lysis, as in four separate experiments maximal hemoglobin release was 0.3–0.6% of the total. The incubation of rabbit erythrocytes with PIPLC (1 μg/ml) for various periods of time (0–90 min) determined that the enzymatic release of NADase was also time-dependent, reaching a maximum by approximately 30 min under the conditions employed (Fig. 2). Once again, maximal release was approximately 70% of the total erythrocyte NADase activity.

We wished to examine the possibility that the NADase was being solubilized by phospholipase C (PLC), since this enzyme copurifies with PIPLC [9]. Divalent cations inhibit PIPLC activity while stabilizing that of PLC, providing a method by which these two lipase activities can be distinguished [9]. Erythrocytes were treated with PIPLC in the presence of varying concentrations of $ZnCl_2$ (0–10 mM) and the resulting supernatants were analyzed for NADase activity (Fig. 3). It was found that $ZnCl_2$ inhibits the release of NADase in a concentration-dependent fashion. The possibility existed that $ZnCl_2$ did not inhibit PIPLC, but rather interfered with the NADase activity in some fashion. To rule this out, supernatants were dialyzed (M_r cutoff: 2000) against 0.1 M potassium phosphate buffer (pH 7.0) for 24 h and their NADase activity was determined. Similarly diminished levels of NADase activity were observed even when the $ZnCl_2$ was removed. We next examined the efficacy of our PIPLC preparation in the presence of 1,10-phenanthroline (Sigma, St. Louis), a chelating agent that reportedly inhibits PLC [9]. We found that 1,10-phenanthroline had no influence on NADase release at a concentration of 0.1 mM. The ability to inhibit release with $ZnCl_2$ together with the inability to do the same with 1,10-phenanthroline indicated to us that NADase was not being solubilized by PLC. To gain further evidence of this, commercially available PLC (from *B. cereus*, Sigma) which nonexclusively utilizes L-α-phosphatidylcholine as a substrate, was examined for its ability to release

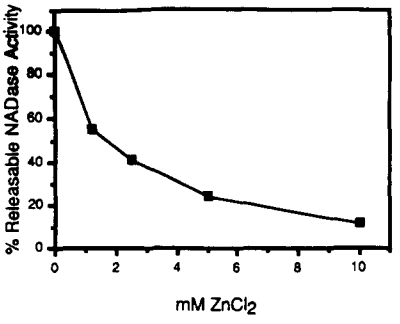

Fig. 3. The effect of $ZnCl_2$ on the solubilization of NADase. Erythrocytes ($1 \cdot 10^7$ cells/ml), suspended in 0.173 M Tris-HCl buffer (pH 7.6), were incubated with PIPLC (4 μg/ml) and varying concentrations of $ZnCl_2$ at 37 °C. After 1 h, duplicate samples were centrifuged ($9000 \times g$) for 5 min. NADase activity was measured in the supernatants. The results are presented as a percentage of the total releasable NADase activity.

NADase from erythrocytes. Thus far, there have been no reports PLC being able to solubilize confirmed phosphatidylinositol-linked molecules. In accordance with this, we observed that equivalent concentrations of commercially available PLC could solubilize minimal amounts of NADase from erythrocytes, but only when extensive hemolysis occurred (data not shown).

A similar concern is that bacterial proteinases are present within our PIPLC preparation and that the release of NADase from erythrocytes can be attributed to a proteolytic mechanism. To address this, erythrocytes were incubated with PIPLC at 1 μg/ml and 4 μg/ml in the presence of a proteinase inhibitor mixture containing 50 μM each of amastatin, antipain, chymostatin, elastinal, leupeptin and phosphoramidon (Sigma). It was found that for both concentrations of PIPLC the presence of proteinase inhibitors did not diminish the amount of NADase activity released into the supernatants.

A fraction of the rabbit erythrocyte NADase is PIPLC-resistant

Finding that PIPLC treatment of rabbit erythrocytes resulted in the release of approximately 70% of the cell-associated NADase activity led us to question whether the remaining NADase activity could be liberated by an additional PIPLC treatment. Aliquots of rabbit erythrocytes were treated with PIPLC as described previously and the supernatants and cell pellets were analyzed for NADase activity (Table I). This procedure resulted in approximately 72% of the total activity being found in the supernatant fraction. Exposure of parallel erythrocyte samples to a second 1 h PIPLC treatment, followed by the supernatant and cell pellet being analyzed for NADase activity, determined that 25–30% of rabbit erythrocyte NADase activity is not susceptible to cleavage by PIPLC.

Evaluation of various mammalian species for phosphatidylinositol-linked erythrocyte NADase

NADase is an ectoenzyme that can be found to be associated with erythrocyte membranes of all mammalian species [6]. Rabbits represent the species of animal having the highest per cell erythrocyte NADase activity, with variable amounts being reported to exist associated with erythrocyte membranes from other species [6]. Having determined that most NADase on rabbit erythrocyte membranes was linked via phosphatidylinositol led us to question whether erythrocyte NADase from other species was membrane-associated via a similar type of linkage. The results of this study (Table II) determined that the rabbit was somewhat unique, the majority of its

TABLE I

RABBIT ERYTHROCYTE NADase CAN BE SOLUBILIZED BY EXPOSURE TO PIPLC

Erythrocytes were incubated for 1 h at 37°C with a final PIPLC concentration of μg/ml. The cells were then either Triton X-100-solubilized and the NADase activity determined (none) or retreated with PIPLC employing the same conditions. Duplicate samples of pelleted cells and supernatants were assayed for NADase activity (see Materials and Methods) and the enzyme activities presented as units/10^6 cells. The numbers in parentheses represent percentages of total activity found in the tested fraction.

Erythrocyte treatment	NADase activity (units/10^6 cells)	
	cell-associated	soluble
None	181.3 ± 1.18	0
Primary PIPLC	51.3 ± 0.72 (28.2)	130.0 ± 0.94 (71.70)
Secondary PIPLC	48.1 ± 0.85 (26.5)	3.5 ± 0.14 (1.92)

TABLE II

THE PRESENCE OF PIPLC-RELEASABLE NADase ON ERYTHROCYTES FROM VARIOUS SPECIES

The control represents the total NADase activity present in 10^6 Triton X-100-solubilized cells from each species. Experimental conditions were essentially as described in the legend of Table I, except that the incubation time was reduced to 30 min since human and horse erythrocytes were prone to hemolysis following longer incubation periods. The numbers in parentheses are the percentages of the total NADase activities.

Species tested	Erythrocyte NADase activity (units/10^6 cells)		
	control	PIPLC-treated	
		cell-associated	soluble
Rabbit	182.00	53.00 (30)	127.00 (70)
Sheep	118.00	104.00 (88)	14.00 (12)
Cow	90.00	75.00 (83)	15.00 (17)
Mouse	1.60	1.60 (100)	0.00 (0)
Human	0.62	0.53 (86)	0.09 (14)
Horse	0.24	0.15 (64)	0.09 (36)

NADase being susceptible to PIPLC cleavage. All of the other species tested, with the mouse being the single exception, did exhibit a small percentage (12–36%) of PIPLC-cleavable NADase activity. No correlation existed between the absolute amount of NADase and the percentage of PIPLC-susceptible enzyme activity.

Discussion

Most of the glycoproteins present on cell surfaces are anchored to the membrane via hydrophobic peptide domains that penetrate the lipid bilayer. It is now appreciated, however, that numerous exceptions to this generalization exist. Recent evidence indicates that several types of membrane proteins are associated with the plasma membrane via a phosphatidylinositol linkage. These include: acetylcholinesterase [13], alkaline phosphatase [14], 5′-nucleotidase [15], the murine Thy-1 antigen [16], decay-accelerating factor [17], and the variant surface glycoprotein of African trypanosomes [18].

We present evidence here that the majority of rabbit erythrocyte NADase is anchored to the plasma membrane via a phosphatidylinositol linkage. NADase was quantitatively solubilized from the surface of rabbit erythrocytes by their treatment with PIPLC purified from *B. cereus* under conditions where there was negligible hemolysis. Due to the difficulties associated with the purification and characterization of NADases from plasma membranes using conventional procedures, the use of PIPLC solubilization may provide a useful initial step in the purification of this enzyme.

The results of our studies indicate that the NADase molecules residing on the surface of rabbit erythrocytes possess a dichotomous nature with respect to their susceptibility to PIPLC. Approximately 30% of the erythrocyte NADase activity proved to be resistant to cleavage by PIPLC. Our analysis of the PIPLC-mediated solubility of NADase from erythrocytes obtained from a number of mammalian sources indicates that the rabbit is somewhat unique with regard to the percentage and total amount of PIPLC-releasable NADase. However, all of the species tested, with the mouse being the single exception, demonstrated the presence of some PIPLC-releasable erythrocyte NADase activity. Our observation that not all of the erythrocyte NADase is susceptible to PIPLC is consistent with the observations made by others who have investigated the PIPLC solubility of Thy-1 [16], acetylcholinesterase [13], alkaline phosphatase, and 5′-nucleotidase [15]. In each of these studies, there appeared to be a portion of the protein or ectoenzyme that remained cell-associated following PIPLC treatment. The reason for this differential susceptibility is not clear, although several possibilities exist. It appears that neural cell adhesion molecules (N-CAM) can be attached to the plasma membrane by a hydrophobic transmembrane region or, alternatively, by a phosphatidylinositol linkage and that these two different forms occur as a result of differential mRNA splicing [19]. Others have suggested that not all phosphatidylinositol-linked molecules are equally exposed and that those resistant to release are less accessible to PIPLC [17]. Another possibility is that release-resistant molecules are modified in such a way that they are not suitable substrates for PIPLC [20].

From the data presented here it is not possible to determine the reason for the differential susceptibility of NADase to PIPLC. We do, however, feel confident that the PIPLC in our enzyme preparation was responsible for the solubilization of most of the NADase activity from the erythrocyte membranes. The experiments in this report indicate that NADase can be included among the growing number [20] of membrane-associated molecules which can employ a phosphatidylinositol anchorage. Future studies in this area will be required before the biochemistry and regulatory aspects of this novel linkage can be fully appreciated.

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