Biochemical and Biophysical Research Communications **267,** 296–299 (2000)
doi:10.1006/bbrc.1999.1900, available online at http://www.idealibrary.com on IDEAL®

A Water-Soluble Analogue of Glucosaminylphosphatidylinositol Distinguishes Two Activities That Palmitoylate Inositol on GPI Anchors

William T. Doerrler*,[1] and Mark A. Lehrman†,[2]
**Cell Regulation Graduate Program and †Department of Pharmacology, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, Texas 75390-9041*

Received November 19, 1999

2-Palmitoylation of the inositol residue occurs during biosynthesis of glycosylphosphatidylinositol (GPI) anchors, but the enzymology of this step has been enigmatic. With endogenously synthesized glucosamine-PI (GlcN-PI; a GPI intermediate), a CoA-dependent palmitoyl-CoA-independent acyltransfer activity (AT-1) has been reported in rodent preparations. In contrast, a palmitoyl-CoA-dependent GlcN-PI acyltransferase activity (AT-2) was reported in both rodent and yeast preparations with a novel water-soluble dioctanoyl GlcN-PI analogue, GlcN-PI(C8). We report that AT-1, as well as AT-2, can be detected in rodent microsomes with GlcN-PI(C8), thus demonstrating the coexistence of these activities in a single membrane preparation and the general utility of GlcN-PI(C8) for studying the GPI pathway. Unexpectedly, AT-2 was peripherally associated with microsomes, a property atypical for GPI biosynthetic enzymes.

Many eukaryotic proteins are anchored to the extracellular face of the plasma membrane by glycosylphosphatidylinositols (GPI) anchors (1). GPI anchors have core structures widely conserved throughout evolution, consisting of phosphatidylinositol (PI), glucosamine (GlcN), three residues of mannose (Man), and phosphoethanolamine (P-EthN) on the third mannose that serves as the attachment point for protein (PI-GlcN-Man_3-P-EthN). Starting with PI the individual units are added enzymatically in the endoplasmic reticulum (ER) to yield a free GPI anchor precursor. By transamidation C-terminal fragments of proteins destined to acquire GPI-anchors are removed, with concomitant attachment of the newly exposed carboxy-terminal residues to amino groups on the terminal ethanolamines of free GPIs (2). Additional anchor modifications occur in a species, cell, and tissue specific manner (1).

The GPI anchor pathway is similar, though not identical, among mammals, yeasts, and parasites (3). An important difference is acylation of the 2-position of inositol with palmitate. Inositol 2-acylation follows addition of GlcNAc to PI and deacetylation of GlcNAc-α-PI to form GlcN-α-PI. Such acylation renders GPIs resistant to PI-specific phospholipase C (PI-PLC). In essentially all mammals free GPIs have 2-acyl inositol, yet most mammalian GPI anchored proteins are PI-PLC sensitive. Thus, the acyl groups are probably removed following transfer to protein. While the function of the acyl group on inositol is unclear, in rodents (4, 5) and yeast (6) it has been suggested that acylation precedes, and is required for, subsequent mannosylation of the GlcN residue. In contrast, inositol 2-acylation follows mannosylation in the parasite *T. brucei* (7).

In rodents two types of acyl-transferring activities have been reported to act upon GlcN-PI. As first reported by Stevens and Zhang (8) and independently confirmed by Franzot and Doering (9), endogenous GlcN-α-PI in mouse and hamster microsomes, respectively, can be acylated by an activity (possibly a transacylase) that requires free CoA and uses an unidentified endogenous acyl donor. In contrast, Doerrler and Lehrman (5) used a water soluble dioctanoyl analogue of GlcN-α-PI (GlcN-α-PI(C8)) to detect an activity in hamster (as well as yeast) microsomes that trans-

Abbreviations used: CHO, Chinese hamster ovary; GPI, glycosylphosphatidylinositol; GlcN-PI, glucosaminyl phosphatidylinositol; GlcN-PI(C8), dioctanoyl glucosaminyl phosphatidylinositol; MPD, mannosylphosphoryl-dolichol; TLC, thin layer chromatography; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; HAP, hydroxyapatite; PI-PLC, bacterial PI-specific phospholipase C.

[1] Present address: Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710.
[2] To whom correspondence should be addressed. Fax: 214-648-8626. E-mail: mlehrm@mednet.swmed.edu.

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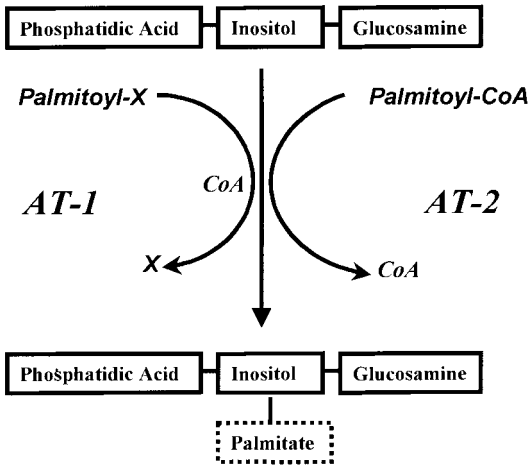

FIG. 1. Acylation of the inositol residue of GlcN-PI by AT-1 and AT-2. GlcN-PI is shown schematically with its phosphatidic acid, inositol, and glucosamine components indicated. After reaction with AT-1 or AT-2, mannosylation of the glucosamine by GPI mannosyltransferase-I can occur.

ferred palmitate from palmitoyl-CoA to GlcN-PI(C8). An acyl-CoA dependent activity in yeast preparations was also reported with endogenous GlcN-PI by Costello and Orlean (6). For simplicity, we term these two acyltransferring activities AT-1 and AT-2, and distinguish them by their requirements for free CoA or acyl-CoA, respectively (Fig. 1). Although the enzymatic donor is unknown and therefore cannot be radiolabeled, AT-1 can be detected if the endogenous GlcN-PI is made radioactive biosynthetically. In contrast, radiolabeled GlcN-PI(C8) is unavailable but AT-2 can be detected with [^{3}H]palmitoyl-CoA. Further, both AT-1 and AT-2 can be followed by subsequent extension of the acylated product with dolichol-P-[2-^{3}H]mannose, produced by addition of GDP-[2-^{3}H]mannose to the microsomes.

Rodent cells therefore contain two seemingly redundant activities that palmitoylate the inositol residues of GPI precursors (Fig. 1). As a first step toward a complete evaluation of these activities we report conditions by which AT-1, in addition to AT-2, is detected with GlcN-PI(C8). GlcN-PI(C8) was then used to partially characterize AT-2. Unexpectedly AT-2 was peripherally associated with microsomes, atypical for GPI biosynthetic enzymes. Thus, in addition to establishing to co-existence of AT-1 and AT-2 in the same membrane preparation, these results demonstrate the general usefulness of GlcN-PI(C8) for studying inositol palmitoylation and subsequent reactions in the GPI pathway.

EXPERIMENTAL PROCEDURES

Materials. GDP-[^{3}H]mannose (15 Ci/mmol) was from American Radiolabeled Chemicals. [^{3}H]palmitate (43 Ci/mmol) was from DuPont. Cell culture supplies were from Life Technologies, except serum (Atlanta Biologicals). Heparin-agarose and SP-Sepharose were purchased from Amersham-Pharmacia Biotech and hydroxyapaptite was purchased from Bio-Rad. CHAPS was purchased from Roche Molecular Biochemicals.

Preparation of [^{3}H]palmitoyl-CoA and GlcN-α-PI(C8). [^{3}H]Palmitoyl-CoA was prepared by ligation of [^{3}H]palmitate to free CoA as described (10). GlcN-α-PI(C8) was a gift of Dr. J. R. Falck of this institution and synthesized as described (11).

Cell culture. CHO-K1 cells (12) were maintained at 37°C in F-12 medium buffered at pH 7.4 with 15 mM Na-HEPES and supplemented with 2% FCS and 8% calf serum, in the absence of antibiotics.

Preparation of microsomal membranes. Cultured cells were subjected to hypotonic swelling, and microsomal membranes were prepared from either swollen cultured cells or tissues by Dounce homogenization in a solution containing 0.5 M NaCl, 20 mM Tris-Cl (pH 7.4) and 100 μg/ml DNase I (12). As described in the text AT-2 was displaced from microsomes by NaCl, and an alternate homogenization solution without NaCl containing 20 mM Tris-Cl (pH 7.4), 10% sucrose and 1 mM EDTA was found to give improved yields of microsomally associated AT-2 (data not shown).

In vitro assay for acylation and mannosylation of GlcN-PI(C8). Labeling of GlcN-PI(C8) with GDP-[^{3}H]mannose was carried out as previously described (5). Labeling with [^{3}H]palmitoyl-CoA was carried out in a 0.1 ml volume containing 100 ng GlcN-PI(C8), 1 μCi (0.25 μM) [^{3}H]palmitoyl-CoA, 0.1 M Tris-Cl (pH 8.0), 1 mM Na_3EDTA and a source of enzyme. Detection of activity required that detergent be present at concentrations below its critical micellar concentration. Therefore, samples containing CHAPS were diluted into reaction buffer to 0.2% or less. Following incubation at 37°C for 20 min, the reaction was partitioned between butanol and water. The butanol phase was backwashed once with butanol saturated water, dried under a stream of nitrogen, and lipids were dissolved in 50 μl chloroform:methanol (C:M) (2:1) and applied to a preactivated Whatman Silica Gel 60 TLC plate. The plate was developed once in C:M:H_2O (65:25:4), sprayed with fluor (13), and exposed to X-ray film (Kodak) overnight.

RESULTS

Both AT-1 and AT-2 Can Be Detected with GlcN-PI(C8)

As discussed above, AT-1 and AT-2 are distinguished by their requirements for free CoA and acyl-CoA, respectively. Since microsomes are capable of synthesizing acyl-CoA in the presence of ATP and free CoA, in some experiments apyrase was added to destroy ATP. In order to compare the two activities directly acylated products were identified by transfer of [2-^{3}H]mannose. As shown in Fig. 2 Man-GlcN-(acyl)PI(C8) (lipid 1) is formed in the presence of ATP by CHO-K1 microsomes without added CoA (lane 2), presumably due to trace amounts of endogenous CoA. Lipid 1 was formed more efficiently when CoA was added, up to 10 μM CoA (lanes 5 and 8). Apyrase blocked formation of lipid 1 with concentrations of CoA up to 10 μM (lanes 3, 6, 9), showing that AT-2 was being detected. However, with 100–1000 μM CoA lipid 1 was formed even in the presence of apyrase (lanes 12 and 15), a characteristic of AT-1. By comparison, with endogenous GlcN-PI as the acceptor Stevens and Zhang (8) determined that 1

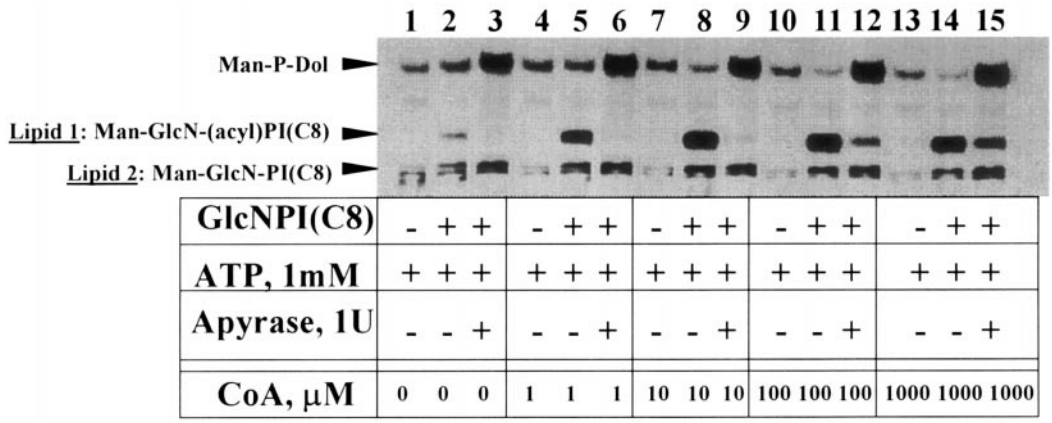

FIG. 2. Detection of AT-1 and AT-2 with GlcN-α-PI(C8). 50 μg CHO-K1 microsomes were incubated in the presence of 50 mM Tris-Cl (pH 7.4), 5 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM 5′NaAMP, 1 mM Na_2ATP and 0.26 μM GDP-[^{3}H]mannose in the presence or absence of 100 ng GlcN-PI(C8) and apyrase (1 U, Sigma) for 5 min at 37°C. Coenzyme A (Sigma) was then added at the indicated final concentrations and the incubation was continued for an additional 20 min at 37°C. Lipids were subsequently extracted and analyzed by thin layer chromatography and fluorography as described (5). Identification of [^{3}H]MPD is based upon earlier work (16). The basis for the greater abundance of [^{3}H]MPD in the presence of apyrase is unknown. The structures of lipid 1 and 2 are based upon earlier determinations (5) and are [^{3}H]Man-GlcN-(acyl)PI(C8) and [^{3}H]Man-GlcN-PI(C8), respectively. An unknown [^{3}H]mannose-containing lipid comigrates with lipid 2 (lanes 1, 4, 7, 10, 13) but does not require the presence of GlcN-PI(C8). The R_f values are: MPD, 0.82; Lipid 1, 0.65; and Lipid 2, 0.55/0.57 (doublet).

μM CoA was maximal for AT-1. The basis for this difference is unclear. The unacylated product Man-GlcN-PI(C8) (lipid 2) was evident at all concentrations of CoA and apyrase (lanes 3, 6, 9, 12, 15), demonstrating that the GPI mannosyltransferase-I was unaffected. GlcN-PI(C8) can therefore be used as an acceptor for AT-1 with 100–1000 μM free CoA in the presence of apyrase. By comparison, AT-2 can be detected with submicromolar concentrations of palmitoyl-CoA (5) or with 10 μM CoA plus ATP.

AT-2 Is Peripherally Associated with Microsomes

As described in the introduction, AT-2 in CHO-K1 cell microsomes is conveniently assayed by directly monitoring the transfer of [^{3}H]palmitate from [^{3}H]palmitoyl-CoA onto the inositol residue of GlcN-PI(C8). AT-2 was not stimulated by 5 mM $MgCl_2$, 5 mM $MnCl_2$, 5 mM $CaCl_2$, 1 mM ATP, or 1mM GTP, and activity was maximal between pH 6.5 and 8.0 (data not shown). In a preliminary survey of conditions, AT-2 was partially released from microsomes by 2% CHAPS (33 mM). Surprisingly, 1.0 M NaCl also released AT-2 from microsomes. Together, the two treatments acted somewhat synergistically (Fig. 3). The effects of NaCl demonstrate that AT-2 requires ionic interactions to associate with microsomes. The effects of CHAPS may be more complex, as they could be attributed ionic and/or non-ionic interactions.

After extraction of microsomes with a solution containing 1 M NaCl, 2% CHAPS, 10% glycerol, 50 mM Tris-Cl (pH 7.4), 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 5 μg/ml aprotinin, and 0.1 mM TLCK, and dialysis against 50 mM Tris-Cl (pH 7.4), 1 mM Na_3EDTA, 0.5% CHAPS, and 0.1 M NaCl, AT-2 remained soluble and active for several weeks at 4 degrees. In preliminary experiments the extracted AT-2 bound to, and was eluted from, hydroxyapatite, SP-sepharose, and heparin-agarose. In addition to CHO-K1 cells, AT-2 activity was found in microsomes from HeLa cells, YH16.33 cells, and mouse brain, kidney, liver, and lung. When normalized to microsomal protein, CHO-K1 AT-2 activities were approximately 20-fold higher than in the tissues (data not shown).

DISCUSSION

Since AT-1, like AT-2, can be detected with the water-soluble acceptor substrate GlcN-PI(C8), future studies of these enzymes and the role(s) of inositol acylation will be greatly facilitated. An endogenously synthesized water-insoluble acceptor is not required to assay AT-1, and both activities can be detected and compared in the same preparation of microsomes. The

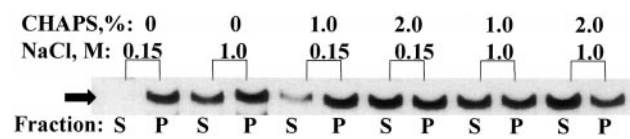

FIG. 3. Displacement of AT-2 from microsomes. 1 mg of CHO microsomes was treated at 0°C in a volume of 0.2ml with a combination of NaCl and/or the detergent CHAPS at the indicated concentrations. The tubes were centrifuged 60 min at 4°C at 60,000 rpm (128,000 × g) in a Beckman tabletop ultracentrifuge. 10 μl of the supernatant (S) and the pellet (P; rehomogenized in 0.2 ml of the original extraction solution) were assayed for AT-2 as described under Experimental Procedures.

properties of AT-2 reported here also suggest strategies for its purification. The displacement of AT-2 from microsomes with NaCl was particularly surprising since all previously characterized enzymes in the GPI pathway have been thought to be firmly membrane-associated. Recently, Kinoshita's group (14) reacted GlcN-PI(C8) with AT-2 and GPI mannosyltransferase-I to create an acceptor for EthN-P addition, extending the utility of GlcN-PI(C8) to at least 3 sequential reactions of the GPI pathway.

The results reported here for AT-1 and AT-2 may be particularly useful since no mutant defective in GlcN-PI inositol acylation has yet been identified in any cell type, precluding the use of genetic and molecular cloning methods successfully employed for many other GPI biosynthetic steps where mutant cells were isolated. There is no obvious reason for the absence of inositol acylation mutants, but perhaps AT-1 and AT-2 serve redundant functions. This would make isolation of recessive mutants highly difficult. Alternatively, mutants in inositol acylation may be leaky, and still display sufficient GPI anchored proteins to escape selection methods for GPI anchor deficiencies.

It has been suggested that acylation of the 2-position of inositol with palmitate may enhance transverse flipping of GPI intermediates by increasing net hydrophobicity. Acylation also appears to precede and enhance the addition of the first mannose to the growing GPI anchor precursor by GPI mannosyltransferase-I in *S. cerevisiae* and mammals (4–6). As discussed earlier (5), this may be due to stereochemical constraint. We are not aware of any other situations in eukaryotes where a prior lipid modification is needed for a subsequent glycosyltransferase reaction. Thus, the yeast/mammalian GPI mannosyltransferase-I may represent a novel class of glycosyltransferase. In contrast, the corresponding trypanosomal GPI mannosyltransferase-I acts prior to acylation (7), and both the mannosyltransferase and acyltransferase from *T. brucei* have recently been shown to be promising drug targets (15).

ACKNOWLEDGMENTS

We gratefully acknowledge Biswanath Praminik, who provided invaluable assistance with tissue culture, and thank Dr. Taroh Kinoshita for sharing unpublished results. This work was supported by NIH Grant GM38545 and Welch Grant I-1168.

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