1,10-Phenanthroline Inhibits Glycosylphosphatidylinositol Anchoring by Preventing Phosphoethanolamine Addition to Glycosylphosphatidylinositol Anchor Precursors†

Karl J. Mann and Daniel Sevlever*

Department of Pharmacology, Mayo Foundation for Medical Education and Research, and Department of Research, Mayo Clinic Jacksonville, Jacksonville, Florida 32224

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ABSTRACT: The glycosylphosphatidylinositol (GPI) moiety is widely used to anchor a functionally diverse group of proteins to the plasma membrane of eukaryotes. In mammals, the predominant glycan structure of the GPI anchor consists of EthN-P-Man-Man-(EthN-P)Man-GlcN attached to an inositol phospholipid. In a smaller percentage of anchors analyzed to date, a third P-EthN group linked to the middle mannosyl residue was found. The transfer of the three P-EthN groups present in the GPI glycan core is likely to be carried out by three different GPI-phosphoethanolamine transferases (GPI-PETs). Here we report that 1,10-phenanthroline (PNT), a commonly used inhibitor of metalloproteases, is a novel inhibitor of GPI anchor synthesis. Addition of PNT to cells caused the accumulation of GPI anchor intermediates that are substrates for GPI-PETs, suggesting that these enzymes are the targets of PNT. $ZnCl_2$ blocked the effect of PNT, a known Zn chelator, and Zn itself was able to stimulate the GPI anchor synthesis, indicating that this cation is likely to be required for GPI-PET activity. PNT acutely inhibited the synthesis of GPI-anchored proteins, but the synthesis was rapidly restored once the inhibitor was washed out. Therefore, PNT will be a useful tool to study the metabolism and trafficking of GPI anchor intermediates by providing a switch to turn the pathway on and off.

Glycosylphosphatidylinositols (GPIs)[1] have been recognized as alternative membrane anchors for cell surface proteins in eukaryotes ranging from simple unicellular organisms, such as yeasts and protozoan parasites, to more complex organisms, such as plants (*1*) and mammals (*2*, *3*). Although no physiological role of the GPI structure itself has been identified, the properties that a GPI anchor confers to the attached protein are in many cases of importance for the function and/or fate of that protein in the cell (*4*−*7*).

In mammals, GPI anchor synthesis consists basically of the sequential addition of GPI anchor components (i.e., sugars, palmitic acid, and phosphoethanolamine groups) to PI (*8*, *9*) (see Figure 1 for a view of the pathway). The biosynthetic pathway is initiated in the cytoplasmic face of the endoplasmic reticulum (ER) with the addition of *N*-acetylglucosamine to PI and subsequent deacetylation of *N*-acetylglucosaminyl-PI (GlcNAc-PI) to produce GlcN-PI (*10*−*12*). The orientation on the ER membranes of the following step, addition of palmitic acid to GlcN-PI, has not been established. However, topology studies of the gene products involved in the addition of the second mannosyl residue (Man2) to GlcN-(acyl)PI (*13*), as well as in downstream steps, indicated that the rest of the biosynthetic reactions including transfer of the GPI anchor to proteins take place in the ER lumen (*14*). During the synthesis of the mammalian GPI anchor, three different GPI-phosphoethanolamine transferases (GPI-PETs) add a phosphoethanolamine (P-EthN) group to each one of the three Man residues of the GPI glycan core. The first P-EthN group is added to Man_1-GPI (H2) by GPI-PET-1 to generate H5 (Figure 1). This step is carried out in yeast by the product of the MCD4 gene (*15*) and in mammals by its recently cloned homologue, PIG-N (*16*). H6, the substrate for the second GPI-PET, is generated by the addition of two Man residues to H5. Subsequent transfer of a P-EthN to the third Man residue (Man3) in H6 produces H7 (*17*). This step is key for GPI anchoring because this P-EthN group provides the linkage between the glycolipid and the protein. The product of the PIG-F gene alone can carry out this reaction (*18*), but another recently cloned gene, PIG-O, has been reported to also be involved in the transfer of this P-EthN into H6 (*19*). The last intermediate of the pathway (H8) is generated by the addition of a third P-EthN to H7 (*20*). The mammalian gene responsible for this step has not yet been cloned, but the yeast homologue Gpi-7 has been identified (*21*). Based on structural similarities between the last two GPI anchor intermediates and the anchors attached to proteins and on kinetic studies, H7 and to a lesser extent H8 are the most likely GPI anchor donors to polypeptides (*22*). The preassembled GPI anchor is then transferred en bloc by a transamidase into newly synthesized proteins in the ER (*14*,

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* To whom correspondence should be addressed at the Department of Research, Mayo Clinic Jacksonville, 4500 San Pablo Rd., Jacksonville, FL 32224. Phone: (904) 953-7354, FAX: (904) 953-7370, E-mail: sevlever.daniel@mayo.edu.

[1] Abbreviations: GPI, glycosylphosphatidylinositol; PNT, 1,10-phenathroline; P-EthN, phosphoethanolamine; PET, phosphoethanolamine transferase; ER, endoplasmic reticulum; PLD, phospholipase D; PBS, phosphate-buffered saline; PLAP, placental alkaline phosphatase; ManN, mannosamine; DP, detergent phase; AP, aqueous phase.

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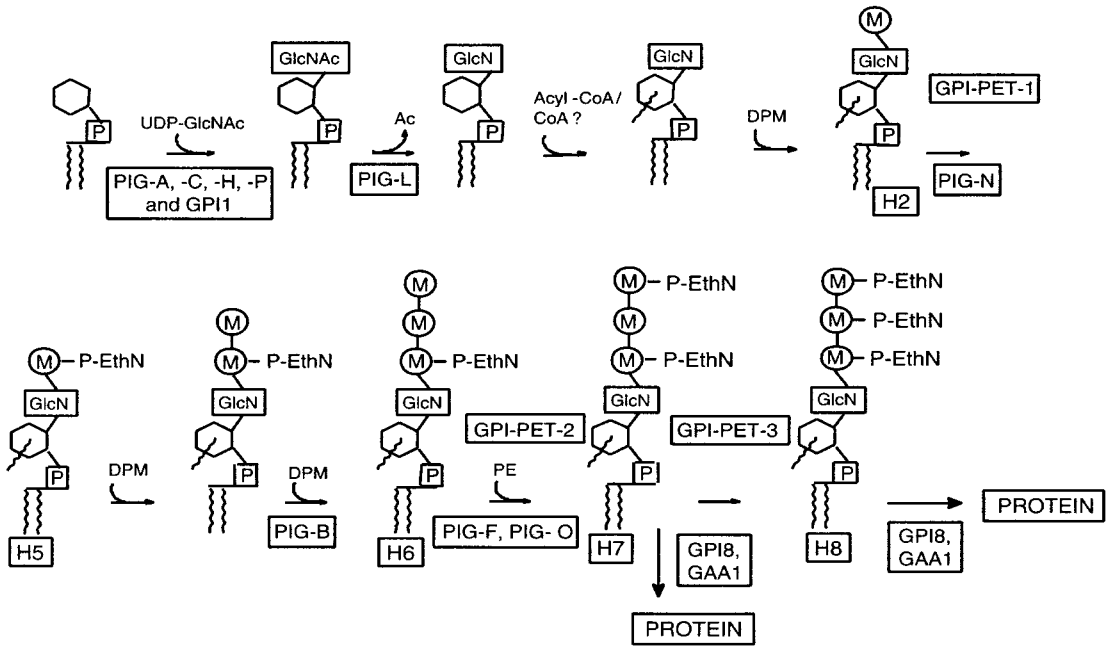

FIGURE 1: Mammalian GPI anchor biosynthetic pathway. Known steps of the main pathway leading to the assembly of the GPI anchor are depicted. Genes responsible for some of the reactions are shown below the arrows, while identified cofactors and steps in which GPI-PETs are involved are indicated above. Included in the first reaction with the other four genes is the recently cloned gene PIG-P (*46*). The donor(s) of P-EthN to Man1 and Man2 has (have) not been established. Mannoses are indicated with an M inside a circle, inositol with a hexagon, and palmitic acid and acyl and alky groups with a wavy line.

23). Soon after, while GPI-anchored proteins are still in the ER, palmitic acid is removed from the inositol ring of the glycolipid anchor (*24*).

GPI anchor intermediates (also referred as GPIs later in the text) are synthesized in excess of the cellular needs to anchor proteins. Furthermore, in addition to their presence in the ER, GPIs have been found in intracellular compartments and at the plasma membrane (*25*−*28*). Study of the mechanisms and routes involved in the intracellular trafficking of GPIs has been hampered by the lack of specific probes. One such potential probe is GPI-PLD, the only enzyme that specifically recognizes GPI structures whether attached to proteins or not (*29*). This enzyme is expressed in many tissues and cell lines, and endogenous GPI-PLD has been shown to intracellularly cleave and release a small but detectable pool of GPI-anchored proteins into the medium (*30*). That release was prevented by the addition of PNT, an inhibitor of GPI-PLD, to the cell culture medium (*30*). In the present study, we attempted to determine the role of endogenous GPI-PLD in the metabolism of GPI anchor intermediates by using PNT as a tool to inhibit the enzyme, and we discovered that PNT blocks the synthesis of the GPI anchor.

EXPERIMENTAL PROCEDURES

Reagents. Dulbecco's modified Eagle's medium (DMEM), RPMI 1640, Ham's F-12, glucose-free RPMI 1640, glucose-free DMEM, and phosphate-buffered saline (PBS) were from Gibco. Methionine- and cystine-free MEM was from ICN. Heat-inactivated newborn calf serum and fetal calf serum were from Sigma and Hyclone, respectively. Silica gel 60 TLC plates were from Merck. [2-^{3}H]Mannose (20 Ci/mmol) and Tran^{35}S-label (70% [^{35}S]methionine and ∼15% [^{35}S]cysteine, 1175 Ci/mmol) were from ARC and ICN, respectively. Anti-caveolin monoclonal antibodies were from Transduction Laboratories. Polyclonal antibodies against placental alkaline phosphatase (PLAP) were from Accurate.

Cell Culture Conditions. HeLa S3 cells were maintained in DMEM supplemented with 10% fetal calf serum, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamine in an atmosphere of 5% CO_2. K562-derived mutant K cells (*31*) and EL4-derived lymphoma mutant F cells were cultured in RPMI 1640 supplemented as with DMEM except that 10% horse serum was used with the mutant F cells. Chinese hamster ovary (CHO)-K1 cells transfected with human PLAP (G9PLAP) were a kind gift of Dr. Victoria Stevens (Emory University) (*32*) and were maintained in Ham's F-12 medium supplemented with 10% fetal calf serum, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 100 μg/mL Geneticin.

[^{3}H]Mannose Labeling. Cells at ∼1 × 10^6 cells/mL were preincubated for 1 h at 37 °C in glucose-free medium supplemented with 10 μg/mL tunicamycin, 100 IU/mL penicillin, 100 μg/mL streptomycin, 2 mM glutamine, 100 μg/mL glucose, and 10% dialyzed newborn calf serum. [^{3}H]-Man (50−100 μCi/mL) was then added for a pulse of 30 min or 1 h. When PNT was included in the pulse, it was added 30 min before starting the labeling. After the pulse, the labeled cells were washed 3 times with PBS (30 mM sodium phosphate, 155 mM NaCl, pH 7.4) before extraction of the lipids with chloroform/methanol/water (CMW, 10:10:3) or in preparation for a chase. Chase medium consisted of PBS supplemented with 5 mM Man and PNT. When chloride salts of transition metals were tested, they were also added 30 min before [^{3}H]Man. CMW extracts were dried, and labeled lipids were partitioned between 500 μL of butanol-saturated water and 500 μL of water-saturated butanol. Butanol phases were run on TLC using CMW (10:10:3) as developing solvent, and labeled GPIs were identified by comigration with previously characterized GPIs.

Quantification of the species was done by radioscanning the TLC using a BioScan System 200 Imaging Scanner. After being scanned, the plates were sprayed with EN^{3}HANCE and subjected to fluorography.

^{35}S Labeling and Immunoprecipitation. G9PLAP CHO cells in 6-well plates were preincubated for 1 h at 37 °C in 1 mL of medium supplemented with 500 μM PNT or 5 mM mannosamine (ManN). The cells were then washed and incubated for 15 min with 500 μCi of Tran^{35}S-label (70% [^{35}S]methionine and ~15% [^{35}S]cysteine) in 0.5 mL of methionine- and cysteine-free DMEM supplemented with 1 mM proline, 100 IU/mL penicillin, 100 μg/mL streptomycin, 2 mM glutamine, and 10% dialyzed newborn calf serum in the continued presence of PNT or ManN. After washing the cells to remove the unincorporated radiolabel and in recovery experiments of PNT, the cells were chased in Ham's-F12 medium with and without PNT or ManN for the periods of time noted in the figure legends. After collecting and washing the cells, they were extracted on ice with 700 μL of extraction buffer consisting of 20 mM sodium phosphate buffer (pH 6.9) containing 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 2% (w/w) Tx-114 and supplemented with 1 μg/mL leupeptin, 1 μg/mL pepstatin. The cells were incubated for 30 min on ice with intermittent vortexing, and then the lysates were centrifuged for 10 min at 14000*g*. The cell pellets were reextracted with 300 μL of extraction buffer, and both extracts were combined. Phase separation was achieved by warming the samples to 37 °C followed by a brief centrifugation at 2500 rpm. The upper aqueous phase (AP) was removed and replaced with a preequilibrated AP for a second phase partition. Both the AP and the detergent phase (DP) were brought to 1 mL with phosphate buffer, and PLAP was immunoprecipitated by addition of 1 μg/mL antibody and 20 μL of Protein A-agarose beads (Life Technologies). The samples were incubated overnight in a rocker at 4 °C, and the beads were collected by centrifugation and washed 3 times before PLAP was eluted by boiling in loading buffer. The eluted proteins were resolved in 10% SDS−PAGE (Bio-Rad Laboratories). After the run, the gels were either soaked in Amplify (Amersham) and subjected to fluorography or exposed to a PhosphoImager screen.

RESULTS

PNT Prevents the Metabolism of H6. GPI-PLD is useful as a topological probe for GPI-anchored proteins based on the following evidence. (1) Endogenous GPI-PLD cleaved GPI-anchored proteins while in transit from the ER to the plasma membrane (*30*). (2) Cytoplamically targeted GPI-PLD had no access to the luminally oriented GPI-anchored proteins in transport vesicles (*33*). HeLa cells are among the cell lines where endogenous GPI-PLD activity has been demonstrated (*30*), and GPIs can easily be detected in these cells upon [^{3}H]Man labeling (*17*). Therefore, we employed HeLa cells to determine whether GPIs are a substrate for endogenous GPI-PLD. The cells were labeled with [^{3}H]Man, and lysates generated with Tx-100 were incubated overnight with and without the addition of PNT. TLC analysis of the labeled lipids in the control sample showed H6 and H8, as well as GPI-PLD products running close to the origin of the plate (*25*) (Figure 2, upper panel). PNT treatment eliminated these fragments, resulting in a concomitant increase of H6 and H8 and in detectable H7 levels (Figure 2, upper panel). Thus, GPIs could be cleaved by endogenous GPI-PLD, and this activity was inhibited by PNT. To investigate whether GPI-PLD cleaves GPIs in intact cells, we pulse-labeled HeLa cells with [^{3}H]Man for 1 h and followed it with a 3 h chase in the absence or presence of PNT in the medium. H6 and H8 were the main GPI species labeled during the pulse, while H8 became the predominant species in the chase performed in the absence of PNT (Figure 2, middle panel). In contrast, the profile was very similar to that of the pulse (i.e., H6 and H8 were the predominant species) when the chase medium

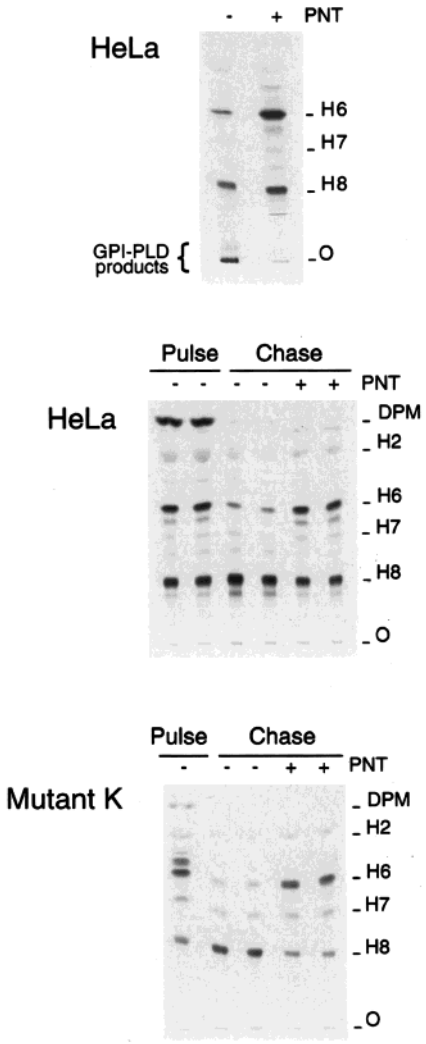

FIGURE 2: PNT affects GPI synthesis. Upper panel: HeLa cells (~2 × 10^6) were labeled with 50 μCi of [^{3}H]Man for 1 h. The washed cells were lysed, and the lysates were incubated overnight at 37 °C in 50 mM Hepes/HCl (pH 6.5) containing 0.1 mM $CaCl_2$ and 0.05% Triton X-100 with or without the addition 500 μM PNT. The lipids were extracted as described under Experimental Procedures before TLC analysis. Middle panel: HeLa cells (7 × 10^6) were labeled with 500 μCi of [^{3}H]Man for 1 h. At the end of the pulse, one-third of the cells was immediately extracted, and the remaining two-thirds of the cells was resuspended in 5 mL of PBS containing 5 mM Man supplemented with or without PNT and incubated for an additional 3 h at 37 °C. Duplicate samples of the pulse and the chase for the two conditions from two independent experiments were run on TLC. Lower panel: Mutant K cells (2 × 10^7) were pulse-labeled and chased as in the middle panel. One-fifth of the cells was analyzed after the pulse while the remaining cells were extracted after the chase. TLC profiles of pulse and duplicate chase samples with and without PNT are shown.

included 500 μM PNT (Figure 2, middle panel). Quantitation of these GPIs indicated that the ratio H6/H8 in the chase plus PNT was close to the pulse ratio (1.6 vs 1.5, respectively) and was ~6 times lower in the chase without PNT. Radiolabel lost from H6 during the chase was not quantitatively recovered in H7 and H8, perhaps because H7 and to a lesser extent H8 were transferred to proteins (*22*). Therefore, to simplify the analysis, we repeated the above experiments using mutant K cells, a K562-derived line defective in the transfer of GPI anchors to proteins but able to synthesize all the GPI anchor intermediates (*31*). After a 1 h pulse with [^{3}H]Man, H6 was the predominant labeled species among GPIs in mutant K cells, and that was also the case when a 3 h chase was performed in the presence of PNT (Figure 2, lower panel). However, in cells chased without PNT, most of the label in H6 was lost, and H8 became the predominant labeled species (Figure 2, lower panel). The H6/H8 ratio following the chase in the presence of PNT was 5.9, very similar to that of the pulse (6.3), but this ratio was ~20 times lower after the chase in the absence of PNT. These results suggested that PNT was preventing the metabolism and/or catabolism of H6, but we did not detect GPI-PLD products in the chase without PNT (compare the upper panel with the middle and lower panels in Figure 2). Therefore, it seemed unlikely that the GPI pattern obtained in the presence of PNT was caused by inhibition of GPI-PLD. If that were the case, one would have to explain first why the enzyme cleaved only H6, and second why GPI-PLD products were not detected. Both questions could be answered by proposing that H6 resides in a GPI-PLD-rich compartment separated from H7 and H8, and that the GPI-PLD cleavage products are rapidly degraded. However, an alternative explanation for the lack of conversion of H6 into H7 and H8 in the presence of PNT was that PNT inhibited GPI-PETs, since in these last two steps of the pathway only the addition of P-EthN groups by GPI-PET-2 and -3 is involved. If the second explanation were correct, GPI-PET-1 also should be sensitive to PNT, resulting in inhibition of the synthesis of H6 because this GPI contains one P-EthN group in the first Man residue.

PNT Inhibits H6 Synthesis while Causing Accumulation of H2. We used mutant F cells to analyze the effect of PNT on GPI intermediates upstream of H6. These cells are defective in the conversion of H6 to H7 due to a mutation on the PIG-F gene (*18*). After a 1 h pulse with [^{3}H]Man in the presence of 250 μM PNT, H6 was reduced by 50%, and a parallel accumulation of H2 was observed. Doubling the PNT concentration to 500 μM reduced the synthesis of H6 even further, and more H2 was accumulated (Figure 3, upper panel). PNT treatment also caused the accumulation of H2 in mutant K cells during continuous labeling with [^{3}H]Man (Figure 3, lower panel). At 10 min, the level of H2 was already well above the control, and it continued to grow during the 3 h of pulse. Neither H7 nor H8 was detected at any time point in the PNT-treated samples. These results reinforced the notion that PNT is inhibiting the synthesis of H7 and H8 rather than preventing the catabolism of H6 by GPI-PD.

So far the assignments of the species accumulated in the presence of PNT (i.e., H2 and H6) were based on their comigration on TLC with standards. To verify these assignments, we took advantage of the inability of jack bean

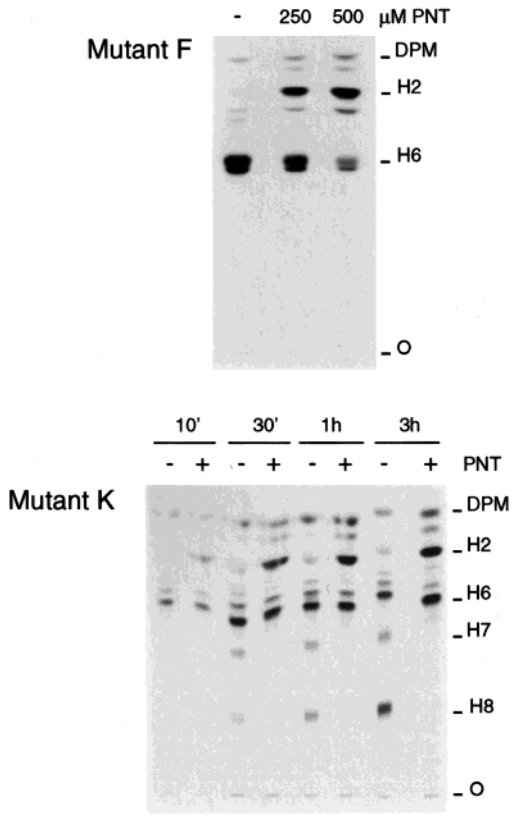

FIGURE 3: PNT inhibits GPI-PETs. Upper panel: Mutant F cells (6×10^6) in three 1 mL aliquots of labeling medium were preincubated for 30 min at 37 °C. PNT was added to 250 and 500 μM to two of the samples, and incubation was continued for an additional 30 min. The cells were then labeled by addition of 100 μCi of [^{3}H]Man for 1 h at 37 °C before lipid extraction. Lower panel: Mutant K cells (1×10^7) in two 4 mL aliquots of labeling medium were preincubated for 30 min without or with 250 μM PNT. The cells were then labeled with 500 μCi of [^{3}H]Man, and 1 mL aliquots were taken at the indicated time points and immediately processed for TLC analysis.

α-mannosidase to cleave Man residues substituted with P-EthN groups. Three HeLa samples were treated with α-mannosidase: two had been incubated with 50 and 500 μM PNT and the third without PNT. H8 served as negative control for α-mannosidase cleavage because this GPI was resistant to the enzyme due to the presence of a P-EthN group in each one of its Man residues (Figure 4). H6, on the other hand, contains a P-EthN group only in Man1. The other two unsubstituted Man residues thus are susceptible to α-mannosidase, and upon cleavage H6 is converted to H5, as seen with both PNT-treated and untreated samples in Figure 4. H2, the substrate of GPI-PET-1, has only an unmodified Man residue and therefore is susceptible to α-mannosidase cleavage. When the putative H2 accumulated in the presence of 500 μM PNT was treated with α-mannosidase, it was converted into an unlabeled GPI species (Figure 4). This experiment confirmed our initial characterization of H6 and H2 as the species that accumulated in the presence of PNT.

Titration of the PNT effect on GPI synthesis in HeLa cells showed that with increasing concentrations of the inhibitor the level of H6 reached a maximum at 100 μM PNT, at which point H2 became detectable (Figure 5). Higher PNT concentrations caused more H2 accumulation and a parallel reduction of H6. H7 and H8 levels began to decrease at 10

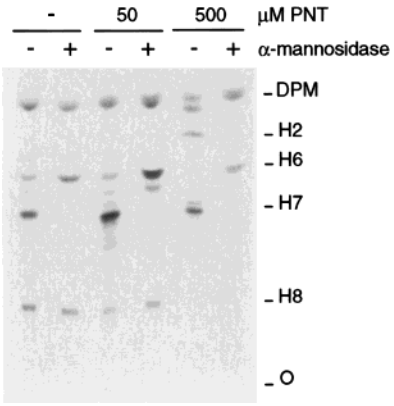

FIGURE 4: Identification of PNT-accumulated GPIs by α-mannosidase. Butanol phases (~20 000 cpm) of extracts from HeLa cells labeled as in Figure 3 with the indicated concentration of PNT were dried and resuspended in 25 μL of 0.2 M NaOAc buffer (pH 5.0), 0.2% taurocholate containing 38 units/mL jack bean α-mannosidase. After overnight incubation at 37 °C, another unit of enzyme was added, and incubations were continued for an additional 5 h. Control samples without the enzyme were treated in parallel. After the incubation, samples were partitioned in butanol for TLC analysis.

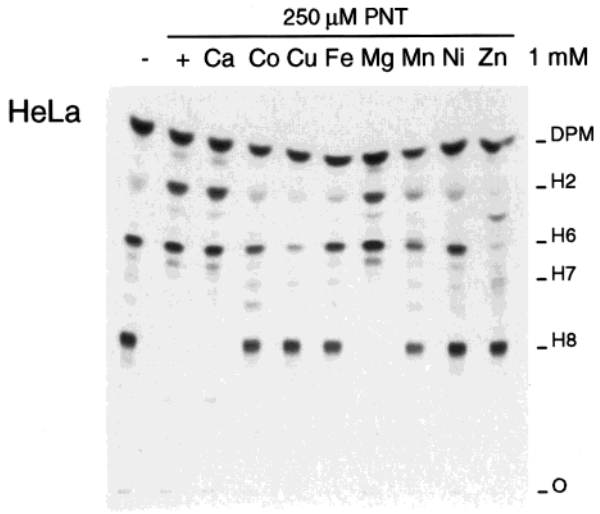

FIGURE 6: Transition metals reverse PNT inhibition. HeLa cells (7×10^5) were incubated for 30 min in 1 mL of labeling medium containing 250 μM PNT and 1 mM chloride salts of the indicated transition metals. The cells were then labeled for 1 h with 50 μCi of [^{3}H]Man before extraction and subsequent TLC analysis.

μM PNT, and at 250 μM PNT they were below detectable levels. GPIs in mutant K cells showed a similar profile with increasing concentrations of PNT, and 250 μM PNT was again the lowest concentration sufficient to completely block the synthesis of H7 and H8 (data not shown).

PNT Chelation of Zn^{2+} Is Responsible for Inhibition of GPI Synthesis. PNT is known to chelate transition metal cations, and it is often referred to as a Zn chelator. To determine whether metal cations can override the PNT effect, we preincubated the cells with 500 μM PNT together with 1 mM concentrations of different divalent cations. Since Ca^{2+} and Mg^{2+} are not transition metals, they were also included as negative controls, and as expected, neither of them was able to overcome the PNT inhibition (Figure 6). In contrast, H7 and H8 were detected with all the transition metals tested, suggesting that they reversed the effect of PNT. However, not all the metals showed the same potency, as Zn^{2+} was the most efficient followed by Cu^{2+} (Figure 6). Although this approach did not directly indicate that a transition metal is actually required for GPI synthesis, it strongly pointed to such a requirement. To directly address the role of Zn^{2+}, we supplemented the labeling medium with $ZnCl_2$. After pulsing the cells for only 30 min with [^{3}H]Man in the presence of 1 mM $ZnCl_2$, a chase-like profile was obtained with H8 as the only GPI species labeled (Figure 7, upper panel). While 0.1 mM ZnCl had no effect, 0.5 mM ZnCl was sufficient to convert all newly synthesized GPI to H8. A similar though less dramatic effect was obtained with mutant K cells, where 0.5 mM $ZnCl_2$ produced a 3-fold and a 2-fold increase in H7 and H8 levels, respectively, whereas no stimulation was observed with 0.5 mM $CuCl_2$ (Figure 7, lower panel).

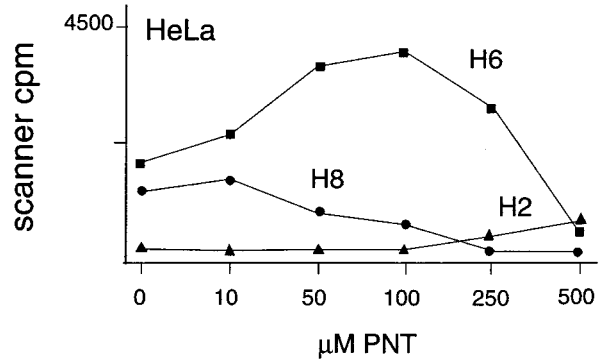

FIGURE 5: Dose-dependent inhibition of GPI synthesis by PNT. HeLa (1.5×10^6) cells were preincubated in 1 mL of labeling medium for 30 min with the indicated concentrations of PNT at 37 °C. The cells were then incubated for 1 h with 80 μCi of [^{3}H]Man, and the lipids were extracted and run on TLC. GPIs were quantitated using the integration software provided by the BioScan System Scanner. For clarity, H7 was not plotted. Representative data from one out of three independent experiments were graphed.

PNT Inhibits the Synthesis of GPI-PLAP. If PNT blocks the synthesis of H7 and H8, the only GPIs that can be transferred to proteins, then PNT should also prevent GPI anchoring of proteins. To demonstrate this point, we examined the synthesis of GPI-anchored proteins in the presence of PNT in CHO cells transfected with GPI-PLAP (G9PLAP). The cells were treated with PNT or ManN, pulse-labeled with a [^{35}S]methionine and [^{35}S]cysteine mixture for 15 min, and were chased for 20 min or 2 h in the presence of PNT or ManN. ManN inhibits GPI anchoring by blocking the addition of the third Man residue to GPIs (*34*, *35*), and was added as a positive control. Incorporation of ^{35}S into proteins during the pulse was decreased by 60−70% in the PNT-treated cells. Following the chase, the ^{35}S-labeled proteins were extracted in Tx-114 and partitioned in detergent (DP) and aqueous (AP) phases before immunoprecipitation with anti-PLAP antibodies. After 20 min of chase in untreated cells, mature as well as immature PLAP (unglycosylated) were detected in the DP. In contrast, in treated cells, a signal corresponding to immature PLAP was barely detectable in the presence of PNT and greatly reduced with ManN (Figure 8A, upper panel). Maturation was completed in the untreated cells after a 2 h chase and further incorporation of radiolabel was observed, whereas in the PNT sample the signal was completely gone. In the ManN-treated cells, the amount of newly synthesized PLAP was substantially reduced compared to the untreated sample, and full matura-

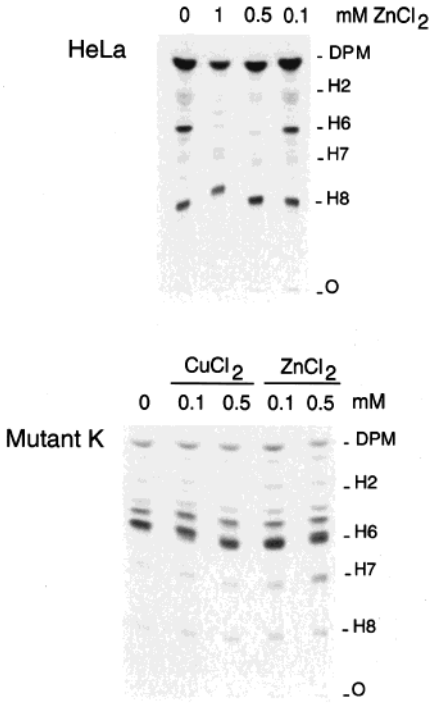

FIGURE 7: Zn^{2+} stimulates the synthesis of GPIs. Upper panel: HeLa cells (1×10^6) were labeled for 30 min with 70 μCi of [^{3}H]Man in 1 mL of media after a 30 min preincubation with the indicated concentrations of $ZnCl_2$ and 250 μM PNT. Lower panel: Mutant K cells (4×10^6) were preincubated for 30 min in labeling medium supplemented with 0.1 or 0.5 mM $CuCl_2$ and $ZnCl_2$ and then with 100 μCi of [^{3}H]Man for 30 min. The incubations were stopped by CMW, and the butanol phases were run on TLC for analysis.

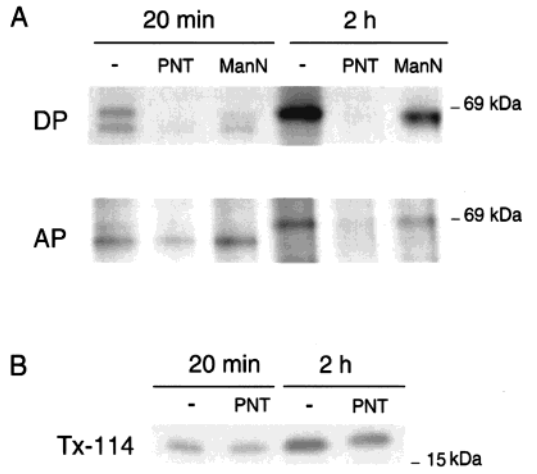

FIGURE 8: PNT inhibits the synthesis of GPI-PLAP. Panel A: G9PLAP cells in a 6-well plate were preincubated for 1 h in the presence of 500 μM PNT or 5 mM ManN, and the washed cells were then labeled with 500 μCi of Tran^{35}S-label for 15 min as described under Experimental Procedures. Where indicated, the inhibitors were added to the pulse as well as the chase media. After washing the unincorporated radiolabel, the cells were incubated in Ham's-F12 medium for 20 min and 2 h and extracted with Tx-114-containing buffer. PLAP was immunoprecipitated from the detergent (DP) and aqueous phases (AP) as detailed under Experimental Procedures, and the samples were analyzed by SDS−PAGE. Panel B: G9PLAP cells were pretreated with PNT and labeled as indicated in panel A, caveolin was immunoprecipitated directly from the Tx-114 extracts without phase separation, and the eluted proteins were resolved in SDS−PAGE.

tion was not achieved because ManN also affects N-glycosylation (Figure 8A, upper panel). GPI-anchored proteins are preferentially recovered in the DP, but they shift to the AP upon PI-PLC treatment. PLAP in the DP of control and PNT-treated samples was susceptible to PI-PLC and therefore GPI-anchored (data not shown). The AP contains proteins without the hydrophobic GPI signal sequence, which is cleaved by the GPI anchor transferase regardless of whether there is GPI anchor to be transferred. The anchorless peptide is then susceptible to a rapid degradation (*36*). We were able to detect PLAP in the AP even in the PNT-treated sample (Figure 8A, lower panel), but it was necessary to expose the film 3 times longer than in the analysis of the DP. The specificity of the PNT effect on GPI-anchored proteins was checked by analyzing caveolin, a non-GPI-anchored protein expressed in G9PLAP CHO cells. These cells were pulse-labeled and chased as described above, and caveolin was immunoprecipitated directly from the Tx-114 extracts without phase separation. Although some reduction in the caveolin signal in samples treated with PNT was observed (Figure 8B), probably reflecting the minor inhibitory effect of PNT on protein synthesis, it was not near the profound effect observed on the synthesis of GPI-PLAP.

GPI Anchor Synthesis Is Restored after PNT Removal. To assess whether PNT inhibition of GPI synthesis was reversible, cells were pulse-labeled with [^{3}H]Man for 1 h in the presence of PNT and chased in the absence of the inhibitor. In preliminary experiments with HeLa cells, we observed that normal amounts of H7 and H8 were synthesized following a 3 h chase after a pulse with PNT (data not shown). Therefore, to establish how fast GPI anchor synthesis is recovered in HeLa cells after PNT treatment, we chased for shorter periods of time, 10 and 30 min. After only 10 min, the recovery was already 50% (the ratio H6/H8 was ~1 in the controls and 1.9 in the samples treated with PNT). No difference was observed between the treated and untreated samples after 30 min of chase (Figure 9A, upper panel). Interestingly, in mutant K cells, where no transfer of GPI anchor to proteins takes place, the recovery was much slower. In these cells, even after 3 h the recovery was only 10% (Figure 9A, lower panel).

Since at least in HeLa cells GPI anchor synthesis recovered very fast (~30 min) after removal of PNT, we investigated whether a similar rapid recovery occurred with GPI-anchored proteins. G9PLAP cells were ^{35}S labeled with a short pulse in the presence of or absence of PNT, and the cells were chased for 1 h without the inhibitor. When the DPs of treated and untreated cells were compared, no difference in the GPI-PLAP was observed (Figure 9B). Therefore, GPI anchoring was restored as soon as GPI anchor synthesis resumed.

DISCUSSION

In this study, we report that PNT is a novel inhibitor of GPI anchor synthesis. We provide evidence suggesting that PNT targets GPI-PETs, the enzymes responsible for the addition of P-EthN groups to Man residues of GPIs. The PNT effect on GPI anchor synthesis is reversible, and it appears to be mediated through chelation of Zn^{2+}. Furthermore, we showed a direct stimulatory effect of Zn^{2+} on GPI synthesis, raising the possibility that GPI-PETs are Zn

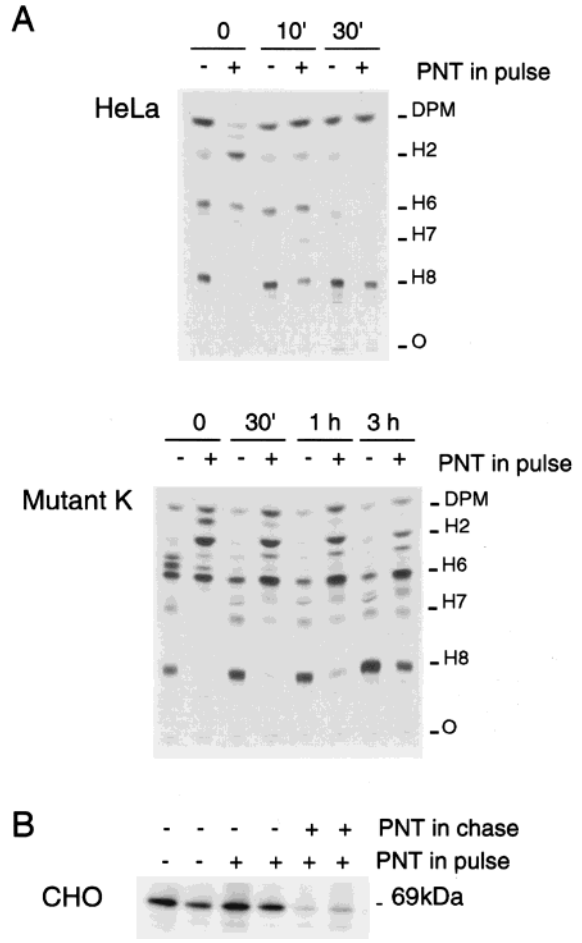

FIGURE 9: PNT inhibition of GPI anchor synthesis is reversible. Panel A: HeLa cells (5×10^6) and mutant K cells (1×10^7) were preincubated for 30 min in medium supplemented without or with 250 μM PNT and pulsed for 1 h with 250 μCi of [^{3}H]Man. One-third of the HeLa cells and one-fourth of the mutant K cells were extracted after the pulse. The remaining treated and untreated cells were chased in medium without PNT as described previously. One-third and one-fourth aliquots from HeLa and mutant K cells, respectively, were extracted at the indicated time points. TLC profiles of labeled lipids are shown. Panel B: G9PLAP cells incubated without or with 500 μM PNT were labeled with 500 μCi of Tran^{35}S-label for 15 min and chased for 1 h in the presence or absence of PNT as described in the legend of Figure 8. Labeled GPI-PLAP from the DP was immunoprecipitated and analyzed as detailed under Experimental Procedures. Duplicates samples from independent experiments are shown in the flurogram.

enzymes. This notion is supported by sequence analysis of the recently identified mammalian and yeast genes involved in the transfer of P-EthN groups into the Man residues of GPIs (*16*, *19*, *21*, *37*, *38*) that revealed homology with phosphodiesterases and nucleases, some of which are Zn enzymes (*39*, *40*).

Our studies were prompted by a report by Metz and colleagues showing that release of decay accelerating factor (DAF), a GPI-anchored protein expressed in HeLa cells, into the medium was inhibited by addition of PNT to cells (*30*). Since endogenous GPI-PLD was shown in that study to be responsible for DAF secretion and PNT was reported to inhibit GPI-PLD, it was concluded that inhibition of the enzyme prevented the release of DAF into the medium (*30*). In light of our findings, it is possible that the reported reduction of DAF secretion was a consequence of inhibition of GPI anchoring of DAF by PNT rather than of inhibition of GPI-PLD or, alternatively, the compounded effect of PNT on both.

We confirmed the presence of GPI-PLD activity in HeLa cells, and we demonstrated that endogenous GPI anchor intermediates are cleaved by the enzyme in the detergent cell lysates. Furthermore, as previously reported, PNT inhibited the hydrolysis of GPI anchor intermediates by GPI-PLD (Figure 2, upper panel). However, in intact cells we failed to detect GPI-PLD cleavage of GPIs (Figure 2, middle and lower panels) as it was reported for GPI-anchored proteins (*30*). GPI-PLD is likely to travel through the secretory pathway together with GPI-anchored proteins before being secreted. Recent reports indicated that H8 in the plasma membrane is accessible with cell-impermeant probes and that its arrival at the cell surface can be prevented by blocking vesicular transport (*25*, *28*). Therefore, the lack of detectable cleavage of H8 by GPI-PLD may be an indication that the enzyme and GPIs are transported in different secretory vesicles.

PNT-induced accumulation of GPI anchor intermediates that are substrates for GPI-PET-1 (H2) and GPI-PET-2 (H6) strongly suggests that these enzymes are inhibited by PNT. H7 is generated as a result of the transfer of a P-EthN group into H6, and H7 is converted into H8 by the addition of a third P-EthN group. Therefore, H7 and H8 should not accumulate in a chase with PNT, and in fact their levels should remain constant if no significant amounts are transferred to proteins. This was observed in cells pulsed with [^{3}H]Man in the absence of PNT (to allow the synthesis of H7 and H8) and chased in the presence of the inhibitor (Figure 2, middle and lower panels). Interestingly, while the three GPI-PETs were affected by PNT, GPI-PET-1 was less susceptible because it allowed a reduced synthesis of H6 (Figure 3). Even when higher PNT concentrations were tested (up to 2 mM), we were unable to completely block the synthesis of H6 (data not shown). PNT had a similar effect in in vitro experiments, where it blocked the conversion of H2 to H5 using either exogenous or endogenous GPI acceptors[2] as well as the synthesis of H7 and H8 (our data not shown). GPI-PET-2 and GPI-PET-3 transfer a P-EthN group to the 6-OH of Man3 and Man2, respectively, while GPI-PET-1 transfers P-EthN to the 2-OH of Man1. This different specificity is reflected in the yeast sequences of the putative GPI-PETs, where GPI-PET-2 and GPI-PET-3 show higher homology between them than either of them does with GPI-PET-1 (*19*). Therefore, it is likely that these sequence differences are responsible for the lower susceptibility of GPI-PET-1 to PNT. Consistent with these differences, an inhibitor of GPI anchor synthesis (YW3548) was recently found to inhibit GPI-PET-1, apparently without affecting GPI-PET-2 or GPI-PET-3 and only slighting reducing the expression of GPI-anchored proteins in mammalian cells (*16*). Interestingly, the profile of GPI anchor intermediates generated by YW3548 is quite different from the one produced by PNT. Upon a 24 h incubation with YW3548, cells accumulated H3 (a GPI species containing two Man residues without P-EthN) and synthesized GPI species with three Man residues and P-EthN groups in Man2 and Man3 but not in Man1(*16*). These differences may be

[2] H. Yeongjin and T. Kinoshita, personal communication.

due to the different cell types used in both studies, because exposure of mutant F cells to YW3548 resulted in the accumulation of H2 (*16*), as was the case in our studies with PNT (Figure 3, upper panel).

PNT inhibition of GPI-PLAP synthesis in G9PLAP CHO cells after only 1 h of incubation was puzzling since the levels of H7 and H8 in HeLa cells remained steady even after 3 h in the presence of PNT (Figure 2, middle panel). This may indicate that the pool of GPIs labeled with [^{3}H]Man is quite inert and/or that de novo synthesis of GPIs is needed for anchoring. Unfortunately, we were unsuccessful in labeling GPIs with [^{3}H]Man in G9PLAP cells because the efficiency was very low, and thus H8 was barely detected. This may be due to a smaller inert GPI pool in G9PLAP than in HeLa cells and hence to a higher proportion of GPIs that turn over rapidly in G9PLAP cells. Therefore, it is possible that the difference in the size of the GPI pools is responsible for the rapid inhibition of GPI anchoring in G9PLAP cells. Against this argument, we observed that DAF synthesis in HeLa cells was also inhibited after 1 h incubation with PNT (data not shown) despite the presence of a large pool of GPI anchor precursors in these cells.

Although at high concentrations PNT can be toxic to cells, we did not detect gross morphological changes in the treated cells under microscopic observation, and most of them remained attached to the plates during the incubations. PNT did not affect the uptake of [^{3}H]Man by the cells, and in fact there was a slight but consistent increase (10−15%) in the incorporation in all the cell lines studied. Furthermore, the data showing the rapid restoration of GPI anchor synthesis and protein anchoring capabilities (Figure 9) indicated no serious deleterious effects on cells exposed to PNT.

PNT is often used to inhibit metalloproteases, some of which were recently identified as GPI-anchored (*41*, *42*). Therefore, it will be important in the future to also take in account its effect on GPI anchor synthesis, particularly in cases such as the processing of the amyloid precursor protein where metalloproteases as well as GPI-anchored proteins are involved (*43*).

In spite of the fact that the mammalian GPI anchor biosynthetic pathway has been elucidated for quite some time, little is known of its regulation and only a few inhibitors of the pathway have been described. PNT is among a very small group of inhibitors of GPI synthesis, including 2-fluoro-2-deoxyglucose (*44*), ManN (*36*), and a fungus metabolite, YW3548 (*45*), that work on intact cells. PNT will be a powerful tool to manipulate the GPI anchor pathway, and it should be a useful reagent to study the intracellular trafficking of GPIs. In future studies, we plan to investigate the effect of PNT on GPI anchor intermediates of other organisms such as yeast and *Trypanosoma brucei* where no information on the GPI-PETs is currently available.

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