Glycosylphosphatidylinositol-anchor intermediates associate with Triton-insoluble membranes in subcellular compartments that include the endoplasmic reticulum

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Glycosylphosphatidylinositol (GPI)-anchored proteins are resistant to solubilization with Triton X-100 at 4 °C, and they can be recovered in Triton-insoluble membranes (TIMs) that float to a characteristic buoyant density. Because the GPI structure itself has been shown to target GPI-anchored proteins to TIMs, we investigated the association of GPI-anchor intermediates with TIMs. GPI-anchor biosynthesis involves a pathway of some 10 steps that take place in the endoplasmic reticulum (ER). These intermediates include glucosaminyl-acylphosphatidylinositol [GlcN-(acyl)PI] and later mannosylated GPIs, denoted H6, H7 and H8, that are present not only in the ER but also in other cell compartments, including the plasma membrane. At least two-thirds of the GlcN-(acyl)PI in HeLa D cells and mannosylated

GPIs in K562 cells were found in TIMs. Although previous reports have considered TIMs to be derived primarily from the plasma membrane, we recovered TIMs from subcellular fractions enriched in ER membranes. The ER marker calnexin and GPI-anchored proteins as well as *N*-acetylglucosaminyl-phosphatidylinositol and mannosylated GPIs were present in ER-TIMs. Interestingly, GlcN-PI and H7 were less enriched in ER-TIM than the other GPIs, suggesting that ER-TIMs might reflect a compartmentalization of the GPI-anchor biosynthetic pathway in the ER.

Key words: ER, GPIs, subcellular-membrane microdomains.

INTRODUCTION

Glycosylphosphatidylinositol (GPI) addition is a lipid modification responsible for anchoring many cell-surface proteins in diverse organisms from yeast to mammals [1]. The GPI-anchor structure includes a conserved core glycan consisting of EthN-P-6Man α 1-2Man α 1-6Man α 1-4GlcN α 1-6-inositol-P- (where EthN is ethanolamine) that in mammals is attached to a glycerolipid. Proteins destined to be GPI-anchored are synthesized with a C-terminal GPI signal sequence that is cleaved soon after translocation into the endoplasmic reticulum (ER) lumen and replaced by a pre-synthesized GPI anchor [2].

GPI-anchored proteins have been proposed to become associated with clustered sphingolipids and cholesterol in the *trans*-Golgi network while *en route* to the cell surface [3,4]. Presumably as a consequence of this clustering, GPI-anchored proteins become resistant to solubilization with Triton X-100 at 4 °C and are recovered in Triton-insoluble membranes (TIMs) that float to a characteristic buoyant density during sucrose-gradient centrifugation [3]. Other lipid-modified proteins like non-receptor-type tyrosine kinases, heterotrimeric G-proteins and caveolin are also recovered in TIMs (reviewed in [5,6]). These proteins are attached to the inner leaflet of the plasma membrane (PM), whereas GPI-anchored proteins are anchored

to the outer leaflet. A few transmembrane proteins apparently without lipid modifications are also present in TIMs [7,8].

The presence in TIMs of caveolin, a protein that coats flask-shaped invaginations of the PM named caveolae [9], led to the assumption that TIMs were enriched preparations of caveolae and to the interchangeable use of both terms. However, GPI-anchored proteins in TIMs were detected in the absence of caveolin or caveolae in neuroblastoma cells [10] and lymphocytes [11]. Furthermore, even in cells expressing caveolin, immunoprecipitation of some components of TIMs did not bring down caveolin, indicating the heterogeneity of TIMs [12,13]. Based on this and other evidence [14] it appears that TIMs are probably not restricted to caveolae.

Electron microscopy of TIMs revealed a heterogeneous population of vesicles and membrane fragments that seem to contain a lipid bilayer [3,15]. TIMs do not appear to be an artifact that arises from Triton X-100 extraction for the following reasons. (i) A lag time is required to incorporate endogenous [3] and exogenous [16] GPI-anchored proteins into TIMs. This delay was also reported for the tyrosine kinase p59^{lun} after translocation to the PM [17]. (ii) TIMs can be generated *in vitro* from artificial membranes with lipid compositions similar to those in TIMs [18]. (iii) TIMs can be obtained using detergent-free protocols [19,20]. (iv) A correlation between localization in TIMs and enzymic

Abbreviations used: APP, amyloid precursor protein; ER, endoplasmic reticulum; PI, phosphatidylinositol; GlcN-PI, glucosaminyl-PI; GlcNAc, *N*-acetylglucosamine; GlcNAc-PI, *N*-acetylglucosaminyl-PI; GPI, glycosylphosphatidylinositol; HBSS, Hanks balanced salt solution; PIP₂, phosphatidylinositol 4,5-bisphosphate; PLAP, placental alkaline phosphatase; PM, plasma membrane; SM, sphingomyelin; TIMs, Triton-insoluble membranes; DMEM, Dulbecco's modified Eagle's medium; CHO, Chinese hamster ovary; Tos-Lys-CH₂Cl, tosyl-lysylchloromethane; CMW, chloroform/methanol/water; DAF, decay-accelerating factor.

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activity was reported for p56^{lck} [21]. In this study the pool of p56^{lck} associated with TIMs was found to be hyperphosphorylated and of lower kinase activity than the p56^{lck} pool outside TIMs, prompting the proposal that Lck in TIMs constitute a reservoir of enzyme that can be readily activated [21].

Since many components of TIMs, including GPI-anchored proteins, reside primarily in the PM, these membrane domains were assumed to be present mainly at the cell surface. Nevertheless, it is noteworthy that molecules thought to reside preferentially in the ER, such as the presenilins involved in the metabolism of amyloid precursor protein (APP), are present in TIMs [22].

The importance of establishing the contribution of membranes other than the PM to TIMs has been acknowledged in recent reviews [5,23]. Simons and Ikonen [5] emphasized that the origin of TIMs could be determined only by isolating specific organelles before detergent extraction. It has been assumed that TIMs do not exist in the ER because of the low cholesterol and sphingolipid content of this organelle. Although the importance of cholesterol and sphingomyelin (SM) in the organization of TIMs has been established, it is still not clear whether these lipids are required absolutely for their formation. The following observations suggest that they are not: (i) TIMs can be isolated from a cell mutant that lacks glycosphingolipids [6]; (ii) metabolic inhibition of sphingolipid synthesis by fumonisin B₁ did not alter the distribution of the GPI-anchored folate receptor in TIMs [24]; and (iii) cholesterol depletion by cyclodextrin did not affect the association of Thy-1 or GM₁ with TIMs [25].

The GPI anchor appears to be responsible for the Triton X-100 resistance of GPI-anchored proteins, because hybrid proteins carrying the same polypeptide but with a transmembrane anchor are absent from TIMs [26]. Such reports have prompted us to ask whether free GPIs (i.e. those not linked to proteins) are associated with TIMs and whether this association affects GPI metabolism. In mammalian cells free GPIs are synthesized in the ER in excess of the amounts needed to anchor proteins [27]. Some of this remnant pool of free GPIs escapes the ER and can be found in cell compartments outside the ER, including the PM [28–30]. These observations raise the possibility that, in addition to serving as precursors for GPI anchoring in the ER, other roles for GPIs may depend on their membrane localization. In this report we show an enrichment of free GPIs in TIMs from both whole-cell extracts and ER membranes. Interestingly, the extent of their association depends on the particular GPI-anchor intermediate and on its subcellular localization.

EXPERIMENTAL

Materials

Dulbecco's modified Eagle's medium (DMEM), RPMI 1640, Ham's F-12, inositol free-DMEM and glucose free-RPMI 1640 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. [2-³H]Mannose (20 Ci/mmol), *myo*-[2-³H]inositol (20 Ci/mmol), [methyl-³H]choline (85 Ci/mmol), and [³H]acetate (7.6 Ci/mmol) were from American Radiolabeled Chemicals. Tran³⁵S-labelTM (70 % [³⁵S]methionine and ~15 % [³⁵S]cysteine, 1175 Ci/mmol) was from ICN. Anti-calnexin and anti-caveolin monoclonal antibodies were from Transduction Laboratories. Polyclonal and monoclonal antibodies against placental alkaline phosphatase (PLAP) were from Dako and Accurate respectively. Polyclonal antibodies against the APP were raised using a peptide corresponding to the last 20 C-

terminal residues. Glucosaminyl-acylphosphatidylinositol {GlcN-(acyl)[³H]PI} was extracted from HeLa D cells labelled with [³H]inositol and purified by chromatography on octyl-Sepharose as described previously [31].

Cell-culture conditions

The HeLa D cell line isolated in our laboratory from HeLa S3, as described in Sevillever et al. [32], was maintained in DMEM supplemented with 10 % newborn calf serum, 100 i.u./ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine in an atmosphere of 5 % CO₂. Erythroleukaemia K562 cells and the derived mutant K cells [33] were cultured in RPMI 1640 supplemented with DMEM. Chinese hamster ovary (CHO)-K1 cells transfected with human placental alkaline phosphatase (G9PLAP) were a kind gift of Dr. Victoria Stevens (Emory University, Atlanta, GA, U.S.A.) [34] and were maintained in Hams's F-12 medium supplemented with 10 % fetal calf serum, 100 i.u./ml penicillin, 100 µg/ml streptomycin and 100 µg/ml geneticin.

Isolation of TIMs

Cell lysis and sucrose-gradient centrifugation for the isolation of TIMs were conducted basically as described elsewhere [3]. Briefly, 10⁸ cells were washed with Hanks balanced salt solution (HBSS) and resuspended in 1.5 ml of 50 mM Hepes (pH 7.4) containing 0.15 M NaCl, 5 mM EDTA and 1 % Triton X-100 supplemented with 1 µg/ml leupeptin, 1 µg/ml pepstatin and 0.1 mM Tos-Lys-CH₂Cl (tosyl-lysylchloromethane, or 'TLCK'; buffer A) on ice. All subsequent steps were carried out at 4 °C. The cells were lysed with 10 strokes in a manual tissue homogenizer over the course of 1 h, and the homogenate was centrifuged for 3 min at 2500 *g* to pellet unbroken cells and large insoluble material. The supernatant of the low-speed centrifugation (1 ml) was mixed with an equal volume of 80 % sucrose and placed at the bottom of a 12-ml centrifuge tube, and a discontinuous sucrose gradient was formed by overlaying 6 ml of 38 % sucrose and 3 ml of 5 % sucrose. After 19 h of centrifugation in a Beckman SW41 rotor at 230 000 *g*, 1-ml fractions were collected from the top. TIMs floated to the interface between 5 and 38 % sucrose and appeared as an opalescent band. In some experiments TIMs were pelleted by dilution of the sucrose with 50 mM Hepes supplemented with proteinase inhibitors and centrifugation for 2 h at 230 000 *g* in an SW41 rotor. The pelleted membranes were extracted again in 1 ml of buffer A and collected following centrifugation.

Metabolic labelling of GPIs and TLC analysis

K562 or mutant K cells (2–5 × 10⁸) were preincubated at 37 °C for 1 h in 20–40 ml of glucose-free RPMI 1640 supplemented as above but with dialysed 10 % newborn calf serum and 10 µg/ml tunicamycin. [³H]Mannose (1–2 mCi) was then added and the cells were incubated for another 2 h. HeLa D cells were plated in 10 175-cm² flasks and incubated with 50–100 µCi of [³H]inositol for 3 days. Approximately 0.1 % of [³H]mannose was incorporated in Man₅GPIs and 9 % of [³H]inositol in GlcN-(acyl)PI. Radiolabel in the media was removed with three washes in HBSS buffer, and labelled cell pellets were extracted in buffer A and subjected to sucrose-gradient centrifugation as described in the previous section. Gradient fractions were dialysed against 20 mM sodium phosphate (pH 7.0) to eliminate the sucrose, dried and extracted twice with 1 ml of chloroform/methanol/water (CMW, 10:10:3, by vol.). Lipid extracts were dried and partitioned between water-saturated n-butanol and n-butanol-saturated water, and the n-butanol phases were run on TLC with CMW

(10:10:3) as the developing solvent, as described elsewhere [35]. Labelled lipids were identified as outlined previously [35,36] and quantified either by radioscanning the TLC plate with a BioScan System 200 Imaging Scanner and/or by spraying the plate with EN³HANCE (NEN) to locate labelled bands by fluorography and then quantifying the radioactivity by scintillation counting of the scraped silica. Means $\pm$ S. D. were from duplicate experiments.

Radiolabelling of choline-containing lipids

K562 cells (2×10^7) were labelled for 48 h with 300 μ Ci of [³H]choline. The radiolabelled cells were combined with 5×10^8 unlabelled cells before Triton X-100 extraction. Sucrose gradient fractions were obtained as described above. The [³H]choline-labelled lipids from the sucrose fractions were dialysed, dried and extracted in 600 μ l of water, 720 μ l of methanol containing 2% acetic acid and 720 μ l chloroform. The organic phase was collected, dried and resuspended in chloroform/methanol (2:1, by vol.) before running on TLC. The lipids were resolved on TLC developed in chloroform/acetone/methanol/acetic acid/water (60:24:18:12:6, by vol.) and the positions of SM and phosphatidylcholine were established using commercial standards.

Cholesterol determination

K562 cells (2×10^8) were labelled with 600 μ Ci of [³H]acetate in RPMI 1640 supplemented with 10% lipoprotein-deficient newborn calf serum for 20 h. TIMs from the subcellular fractions were obtained as described previously. Non-dialysed aliquots (125 μ l) of the sucrose-gradient fractions were combined with 125 μ l of 20% taurodeoxycholate and 500 μ l of Dole reagent (2-propanol/heptane/water, 78:20:2, by vol.) and extracted with 250 μ l of heptane. The heptane phase was collected, dried and run on TLC (light petroleum/diethyl ether/acetic acid, 80:20:1, by vol.) together with cholesterol standards. The migration of cholesterol standards was established by spraying the plate with ferric chloride reagent (50 mg FeCl₃·6H₂O, 90 ml of water, 5 ml of glacial acetic acid and 5 ml of concentrated H₂SO₄). After heating the plate, cholesterol appeared as a purple spot with this reagent.

Subcellular fractionation

Membrane organelles were isolated in fractions as described by Vidugiriene and Menon [37], with minor modifications. Cells [$(2-5) \times 10^8$] were collected by centrifugation, and were washed twice with HBSS and once with 10 ml of 10 mM Hepes (pH 7.5) containing 0.25 M sucrose, 1 mM dithiothreitol, 1 mM PMSF, 1 μ g/ml leupeptin and 0.1 mM Tos-Lys-CH₂Cl. The cell pellet was resuspended in 10 ml of this buffer, transferred to a nitrogen cavitation bomb (Parr Instruments), and held at a pressure of 450 p.s.i. ($\approx$ 3000 kPa) for 30 min. *Staphylococcus aureus* nuclease (1.5 units/ml) was added to the cavitated cell lysate, and the mixture was further disrupted with 10 strokes in a tissue homogenizer over the course of 1 h at 4 °C and clarified by centrifugation at 10000 *g* for 15 min at 4 °C. The postnuclear supernatant (5 ml, fraction 1) was layered on top of a step sucrose gradient composed of 2 ml each of 38, 30 and 20% sucrose in 10 mM Hepes, pH 7.5, and centrifuged for 2 h at 140000 *g* in an SW41 rotor. Three opalescent bands at the sucrose interfaces were collected from the top of the tube and designated fractions 2–4 and, together with the pellet at the bottom of the tube, were pelleted by dilution of the sucrose with

10 mM Hepes and centrifugation for 2 h at 230000 *g* in the same rotor. The organelle-specific marker enzymes used to determine the purity of the fractions were: alkaline phosphodiesterase I for PM, α -mannosidase II for Golgi and dolichol-P-mannose synthase for ER, and the activities of these three enzymes were measured as described elsewhere [37]. When lipids (GPIs, SM, phosphatidylcholine and cholesterol) were analysed, radio-labelled and unlabelled cells were combined to obtain a total of $2-5 \times 10^8$ cells before subjecting them to the subcellular-fractionation protocol described above. In some experiments pelleted membranes from subcellular fractions were extracted with 1 ml of buffer A by periodic vortexing for 1 h at 4 °C, and the extract was mixed directly with 80% sucrose for flotation and collection of TIMs.

In vitro labelling of GPIs in ER membranes

ER membranes (1–2 mg, fraction 4) obtained as described above were resuspended in 300 μ l of 10 mM Hepes (pH 7.5) supplemented with 5 mM MgCl₂, 0.5 mM dithiothreitol, 1 μ g/ml leupeptin, 0.1 mM Tos-Lys-CH₂Cl, 0.2 μ g/ml tunicamycin, 1 mM ATP, 1 mM GTP and 1 mM dimercaptopropanol. The reaction was initiated by the addition of 15 μ Ci of [³H]UDP-*N*-acetylglucosamine (GlcNAc) and the membranes were incubated at 37 °C for 60 min. Unincorporated radiolabel was removed by washing the membranes with 10 mM Hepes (pH 7.5) supplemented with proteinase inhibitors and pelleting them at 230000 *g* for 1 h in an SW41 rotor. The washed membrane pellet was extracted in buffer A and subjected to sucrose-gradient centrifugation as described above, and 1-ml fractions were dialysed, dried and extracted by the method of Bligh and Dyer [38]. The final chloroform-enriched phase was dried, and glycolipids were resolved on TLC with chloroform/methanol/1 M NH₄OH (10:10:3, by vol.) as the solvent. Approximately 1% of the radioactivity was incorporated into *N*-acetylglucosaminyl-phosphatidylinositol (GlcNAc-PI) and glucosaminyl-phosphatidylinositol (GlcN-PI).

³⁵S-Labeling and immunoprecipitation

CHO and HeLa D cells [$(1-5) \times 10^6$] were labelled for 15 min at 37 °C in 5 ml of methionine- and cysteine-free MEM containing 3 mCi of Trans³⁵S-label. In chase experiments the initial labelling medium was removed, replaced by 10 ml of regular medium, and the cells were incubated for another 1 h. After the incubations all subsequent manipulations of the cells were carried out at 4 °C. The washed ³⁵S-labelled cells were mixed with unlabelled cells from 10 confluent dishes and TIM and non-TIM fractions were obtained as described above. TIM (500 μ l, a pool of fractions 2–5 of the gradient) and non-TIM (a pool of fractions 8–11 of the gradient) fractions were combined and brought to 5 ml in RIPA buffer (50 mM Tris/HCl, pH 8.0, supplemented with 1% Nonidet P-40, 0.1% SDS and 0.5% deoxycholate), incubated overnight at 4 °C with 1 μ g/ml antibody and 20 μ l of Protein A-agarose beads. The beads were washed three times in RIPA buffer and the bound proteins were eluted by boiling in loading buffer and were resolved by SDS/PAGE (10% gel). After the run, the gels were either soaked in Amplify (Amersham) and subjected to fluorography or exposed to a PhosphorImager screen. Quantification of the signal was achieved using ImageQuant after scanning the films or the PhosphorImager screens in a Molecular Dynamics PhosphorImager. Duplicate measurements from independent experiments were averaged and these values ($\pm$ S. D.) are indicated in the text.

RESULTS

Free GPIs associate with TIMs from whole-cell lysates

A pathway for GPI biosynthesis has been proposed in mammalian cells (Scheme 1). This pathway is based on earlier studies of GPI biosynthesis in trypanosomes and on analyses of mammalian free GPIs, some of which accumulate in mutant cell lines that do not express GPI-anchored proteins (reviewed in [39]).

Scheme 1 Proposed pathway for GPI biosynthesis in mammalian cells

The sequential transfer of components to PI is shown, where inositol is represented by a hexagon; phosphate by P; fatty acyl or alkyl groups by wavy lines; mannose by M; and ethanolamine by EthN. Identified cofactors are indicated in some steps (e.g. dolichol-phosphomannose, DPM, and phosphatidylethanolamine, PE), and boxed letters under the arrows indicate known complementation-class mutations. The last three structures are denoted H6, H7 and H8.

GlcN-(acyl)PI, the fourth intermediate in the GPI pathway in Scheme 1 and the substrate for the first mannosyltransferase, accumulates to unusually high levels in HeLa D cells. When these cells were labelled with [3 H]inositol to the steady state, the TLC profile of the labelled lipids showed a pattern typical for HeLa D cells with five species identified previously as phosphatidylinositol 4,5-bisphosphate (PIP_2), phosphatidylinositol 4-phosphate, phosphatidylinositol (PI), *lyso*-PI and GlcN-(acyl)PI (Figure 1, lane C) and with PI accounting for about 85% and GlcN-(acyl)PI for about 10% of the total radiolabel [31]. To analyse the association of these lipids with TIMs, the labelled cells were lysed with Triton X-100 at 4 °C. The lysate was clarified by low-speed centrifugation to eliminate unbroken cells and large fragments, and the supernatant, which retained about 85% of the cell-associated radiolabel, was subjected to a standard sucrose-gradient centrifugation protocol. In this protocol, TIMs are defined as membrane fragments that float to the 5/38% sucrose interface [3]. The opacity at this interface (gradient fractions 3 and 4) was confirmed as TIMs by analysis of previously reported TIM markers like GPI-anchored proteins and SM. Labelled lipids from each fraction of the gradient were separated by TLC and found to differ in their distribution (Figure 1). Fractions corresponding to TIM (fractions 2–5) contained roughly equal amounts of PI and GlcN-(acyl)PI but almost no *lyso*-PI. TIM fractions were enriched not only in GlcN-(acyl)PI but also in PIP_2 , in agreement with a recent report [40]. Quantitative analysis of the distribution of labelled GlcN-(acyl)PI from this gradient (Figure 2A) revealed that $69 \pm 3\%$ was associated with TIMs. In contrast, only $12 \pm 6\%$ of the labelled PI was associated with TIMs (Figure 2B), suggesting that PI is a useful indicator of a cellular phospholipid that largely resides outside TIMs. The flotation of GlcN-(acyl)PI in TIM fractions was not an intrinsic feature of this glycolipid itself. A negligible amount of purified GlcN-(acyl)PI was recovered in TIMs when subjected to centrifugation in the absence of a cell lysate (Figure 2C).

The GPIs with the highest steady-state levels in most cell lines appear to be the last three GPIs in the pathway (H6, H7 and H8

Figure 1 Distribution of [3 H]inositol-labelled lipids in Triton X-100 extracts from HeLa D cells

HeLa D cells (5×10^7) were incubated with 50 μCi of [3 H]inositol for 3 days at 37 °C. After extraction in buffer A containing 1% Triton X-100 for 1 h at 4 °C and sucrose-gradient centrifugation, 1-ml dialysed fractions were dried, extracted with CMW (10:10:3), partitioned into *n*-butanol and analysed by TLC and fluorography as described in the Experimental Procedures section. Fractions 2–5 were assumed to contain TIMs, and fractions 8–11 were denoted non-TIM fractions. Lane C contains a sample of total labelled lipids extracted directly from HeLa D cells with CMW (10:10:3). PIP, phosphatidylinositol 4-phosphate.

Figure 2 Association of GlcN-(acyl)PI with TIMs in HeLa D cell extracts

(A, B) HeLa D cells labelled with [³H]inositol were processed as in Figure 1, and the TLC bands corresponding to radiolabelled GlcN-(acyl)PI (A) or PI (B) from sucrose-gradient fractions were quantified by scintillation counting as outlined in the Experimental Procedures section. (C) Purified GlcN-(acyl)[³H]PI (1×10^5 c.p.m.), dried and resuspended in buffer A alone, was fractionated by sucrose-gradient centrifugation as in Figure 1. Radioactivity in each fraction was measured by scintillation counting.

in Scheme 1). These GPIs, denoted Man₃GPIs because they contain the three-mannose core, can be detected following metabolic labelling with [³H]mannose or [³H]inositol of a number of wild-type cell lines including lymphomas, T-cell hybridomas, HeLa and the erythroleukaemia line K562. The mutant K cell line accumulates about three times as much of each Man₃GPI as its parental K562 line, apparently because the transamidase that attaches the GPI anchor to proteins is missing or defective [33,41]. To determine whether other free GPIs in addition to GlcN-(acyl)PI associate with TIMs, K562 and mutant K cells were labelled with [³H]mannose and the distribution of Man₃GPIs in TIMs was examined. As shown in Figure 3, about 85–95% of all three Man₃GPIs were found in TIM fractions from both K562 and mutant K cells, except for H7 in K562 cells, where 65% was recovered in TIMs. Thus GPIs are associated with TIMs, not only in cell lines like HeLa D and mutant K that show unusually high levels of particular GPIs, but also in wild-type K562 cells. Furthermore, the interaction of Man₃GPIs with

Figure 3 Association of Man₃GPIs with TIMs in K562 and mutant K cells

K562 and mutant K cells (1×10^6) were incubated with 1 mCi of [³H]mannose for 2 h and extracted with buffered Triton X-100 as described in the Experimental Procedures section. The extract supernatants, which contained almost all the cell-associated radiolabel, were fractionated by sucrose-gradient centrifugation, and the dialysed fractions were analysed as in Figure 2. In K562 cells $82 \pm 2\%$ of H6, $67 \pm 3\%$ of H7 and $85 \pm 5\%$ of H8 were associated with TIMs, whereas in mutant K cells $85 \pm 1\%$, $82 \pm 4\%$ and $91 \pm 4\%$ of H6, H7 and H8, respectively, were associated with TIMs.

TIMs was stable, since treatment of isolated TIMs with a second cycle of Triton X-100 extraction and sucrose-gradient centrifugation (see the Experimental Procedures section) failed to release any Man₃GPIs from the TIM fraction (results not shown).

Man₃GPI association with TIMs depends on their intracellular localization

Having demonstrated that GPIs are components of TIMs, we used them as reporters to investigate whether TIMs exist in the ER. Subcellular fractions from [³H]mannose-labelled K562 and mutant K cells were prepared by a protocol [37] that gave excellent fraction resolution in extracts of BW5147.3 thymoma cells. Subcellular fractions from K562 cells, particularly the PM and Golgi complex, were less well resolved (Table 1). Fraction 2 contained most of the PM and Golgi markers (alkaline phosphodiesterase and α -mannosidase II, respectively) in the post-nuclear supernatant, while fraction 4 and the pellet accounted for more than 70% of the ER markers with only a small contamination by α -mannosidase II and alkaline phosphodiesterase I (Table 1). A similar distribution of organelle markers was obtained when this subcellular fractionation protocol was applied to HeLa D cells (results not shown). Our subcellular fraction resolution is comparable with that obtained with this protocol in other cell lines [42,43]. Furthermore, in our and in all other reports, the ER marker predominated in fraction 4 and the pellet, and we have designated these fractions the ER and ER pellet, respectively. In this study we compared the compositions of these two ER fractions only with that of fraction 2, and our resolution was sufficient for this purpose. The subcellular distribution of the

Table 1 Subcellular distribution of protein, enzyme markers, choline-containing lipids and Man₃GPIs from K562 cells

Cells (5×10^8) were radiolabelled and subcellular fractions were prepared and assayed as described in the Experimental Procedures section. Fractions were numbered as in the original protocol [37], with fraction 1 corresponding to the 5-ml input at the top of the gradient. Calculations of the percentages were based only on fractions 2, 3 and 4, and the pellet. Membrane- and organelle-specific enzyme markers were alkaline phosphodiesterase (PM), mannosidase II (Golgi) and dolicholphosphomannose (DPM) synthase, GlcNAc-PI synthase, GlcNAc-PI deacetylase and calnexin (ER). Lipids were quantified after TLC separation and scintillation counting of the scraped silica as described in the Experimental Procedures section, and calnexin was determined as described in Figure 5. Values are given as the means $\pm$ S.D. of duplicate experiments. The total protein recovery in fractions 2–4 and the pellet, determined using a BCA kit (Pierce), was 8.3 mg.

Subcellular fraction ...	Cell component (%)			
	2	3	4	Pellet
Total protein	47 $\pm$ 1	30 $\pm$ 1	17 $\pm$ 1	6 $\pm$ 2
Enzymic marker				
Plasma membrane	47 $\pm$ 8	26 $\pm$ 0	16 $\pm$ 0	5 $\pm$ 4
Golgi	62 $\pm$ 12	18 $\pm$ 5	13 $\pm$ 4	7 $\pm$ 3
ER				
DPM synthase	5 $\pm$ 3	26 $\pm$ 7	43 $\pm$ 7	26 $\pm$ 9
GlcNAc-PI synthase	3 $\pm$ 0	17 $\pm$ 3	47 $\pm$ 10	33 $\pm$ 13
GlcNAc-PI deacetylase	2 $\pm$ 1	13 $\pm$ 2	53 $\pm$ 10	32 $\pm$ 10
Calnexin	11 $\pm$ 2	28 $\pm$ 1	35 $\pm$ 6	25 $\pm$ 5
Man ₃ GPIs				
H6	33 $\pm$ 5	26 $\pm$ 8	16 $\pm$ 5	25 $\pm$ 0
H7	11 $\pm$ 1	18 $\pm$ 1	25 $\pm$ 4	46 $\pm$ 5
H8	26 $\pm$ 4	33 $\pm$ 7	15 $\pm$ 2	27 $\pm$ 2
SM	26 $\pm$ 1	38 $\pm$ 5	32 $\pm$ 1	4 $\pm$ 2
Cholesterol	20 $\pm$ 2	38 $\pm$ 1	29 $\pm$ 1	12 $\pm$ 1
Phosphatidylcholine	22 $\pm$ 1	35 $\pm$ 1	30 $\pm$ 6	13 $\pm$ 4

Table 2 Compositional characterization of ER-TIMs from K562 cells

Subcellular fractions were isolated from radiolabelled cells as in Table 1 and extracted with buffered Triton X-100. The extract supernatants were fractionated by sucrose-gradient centrifugation and the fractions were analysed by TLC as described in the Experimental Procedures section. The means ($\pm$ S.D.) of duplicate experiments are shown. The percentage of total protein in TIMs was 2% in each of the three subcellular fractions. n.d., not determined.

Subcellular fraction ...	Cell component (% in TIMs)		
	2	4	Pellet
Man ₃ GPIs in K562			
H6	78 $\pm$ 4	71 $\pm$ 6	36 $\pm$ 8
H7	59 $\pm$ 5	47 $\pm$ 4	25 $\pm$ 11
H8	76 $\pm$ 5	70 $\pm$ 1	44 $\pm$ 14
SM	93 $\pm$ 3	85 $\pm$ 4	70 $\pm$ 5
Cholesterol	76 $\pm$ 4	73 $\pm$ 1	52 $\pm$ 8
Phosphatidylcholine	64 $\pm$ 7	45 $\pm$ 5	16 $\pm$ 7
Calnexin	n.d.	64 $\pm$ 7	25 $\pm$ 3

Man₃GPIs in K562 cells is also shown in Table 1, and about half of the total Man₃GPIs were found in the ER fractions (ER and ER pellet). In mutant K cells a smaller percentage of the Man₃GPIs (about 30%) was recovered in these fractions (results not shown). The individual distributions for H6 and H8 were similar to that of the total Man₃GPIs in both cell lines, but a higher percentage of H7 was found in ER fractions from both K562 and mutant K cells (70 and 50%, respectively). Thus in both cell lines H7 was enriched in ER fractions relative to H6 and H8. From these data two conclusions can be drawn. (i) In the

absence of GPI anchoring in mutant K cells, more GPI-anchor precursors escape the ER; and (ii) H7, the most likely GPI-anchor precursor candidate [28], escapes the ER to a lesser extent than either H6 or H8 in both cell lines.

When the isolated subcellular fractions were extracted with buffered Triton X-100 and subjected to sucrose-gradient flotation, an opalescent band of TIMs at the 5/38% sucrose interface was obtained from fraction 2 (PM/Golgi) and fraction 4 (ER), and occasionally from the pellet (ER pellet). The extent of association of each Man₃GPI with TIMs was determined by TLC analysis of gradient fractions (Table 2). Several points are noteworthy. At least 50% of all three Man₃GPIs were found in TIMs from subcellular fractions enriched in PM and Golgi markers. Substantial percentages of the Man₃GPIs in ER fractions also were found in TIMs, although for each Man₃GPI the percentage associated with TIMs in the ER was lower than that in the PM/Golgi. A smaller percentage of H7 than of H6 and H8 was found in TIMs from all subcellular fractions. Finally, Man₃GPIs in the ER pellet were far less associated with TIMs than Man₃GPIs in any other subcellular fraction. When equal amounts of subcellular membranes (based on protein determination) were extracted with Triton X-100, the percentages of GPIs in TIMs were similar to those in Table 2 (results not shown). This result indicates that variations in the protein-to-detergent ratio did not bias the extent of GPI association with TIMs in different subcellular fractions.

Choline-containing lipids and cholesterol were also analysed. Surprisingly, we recovered significant amounts of cholesterol and SM in ER fraction 4, contrasting with the assumption that these lipids are not present to a great extent in the ER (Table 1). When these lipids were analysed in TIMs from subcellular fractions, higher percentages of SM and cholesterol than of phosphatidylcholine were recovered in TIMs from fraction 2 (PM/Golgi), in agreement with previous reports [3]. Similarly, TIMs from ER fractions were more enriched in SM and cholesterol than in phosphatidylcholine, especially in the ER pellet (Table 2).

ER components are present in TIMs

Although unlikely, it is possible that contamination of the ER fractions with other organelles accounts for the opalescent floating bands and the high amounts of GPIs and SM recovered at the TIM position in subcellular fraction 4 and the pellet. To overcome this potential problem we looked for ER markers in TIMs. The early intermediates of the GPI biosynthetic pathway, GlcNAc-PI and GlcN-PI GPIs were shown to be synthesized mainly in ER fractions by *in vitro* labelling of purified membranes with [³H]UDP-GlcNAc ([37], and see also Table 1). Thus GlcNAc-PI and GlcN-PI can be viewed as ER markers. To establish their distribution in ER-TIMs, membranes from fraction 4 were labelled with [³H]UDP-GlcNAc and then TIMs were isolated. Substantial amounts of GlcNAc-PI ($\approx$ 30%) but much lower quantities of GlcN-PI ($\approx$ 10%) were found in TIMs (Figure 4). Changing the ratio of GlcNAc-PI to GlcN-PI by leaving GTP out of the reaction mixtures [44] did not alter the extent of the association of these lipids with TIMs (results not shown).

We also looked at ER-resident proteins. Calnexin, an ER chaperone transmembrane protein, was present mainly in ER fractions (fraction 4 and pellet) from K562 (Table 1 and Figure 5, top panel) and HeLa D (Figure 5, bottom panel) cells. Flotation analyses showed that 64 and 24% of the calnexin in fraction 4 (Table 2), and 25 and 36% of the calnexin in the pellet from K562 and HeLa D cells (Table 2 and Figure 5), respectively, were recovered in TIMs. As expected, a very weak signal was

Figure 4 GlcNAc-PI but not GlcN-PI is present in ER-TIMs

ER membranes from K562 cells were incubated with 15 μ Ci of [3 H]UDP-GlcNAc for 1 h at 37 $^{\circ}$ C, and TIM fractions were obtained and extracted for TLC analysis as described in the Experimental Procedures section. GlcNAc-PI and GlcN-PI were measured by radioscanning of the plate and scintillation counting of the scraped silica bands.

Figure 5 Calnexin in TIMs from subcellular fractions

Subcellular fractions 2 (f2) and 4 (f4), and the pellet were obtained, extracted in 1% Triton X-100 and subjected to flotation in a sucrose gradient as described in the Experimental Procedures section. Dialysed fractions 2–5 and 8–11 were combined to obtain TIMs (T) and non-TIMs (nT) respectively, incubated in 60 mM octylglucoside for 1 h on ice, and precipitated with trichloroacetic acid. The precipitates were run on SDS/PAGE (10% gel) together with HeLa cell lysate as a positive control (+). Calnexin in K562 and HeLa D cells was detected after Western blotting using an enhanced chemiluminescence (ECL) kit according to the manufacturer's specifications. The films were preflashed before exposure, and bands on the developed films were quantified with ImageQuant software.

present in fraction 2 (PM/Golgi) from both cell lines (Table 1 and Figure 5).

In order to demonstrate the presence of TIMs in the ER independently of subcellular fractionation, we took a different approach. We analysed the processing of N-linked glycans on GPI-anchored glycoproteins, because the ER localization of these proteins can be established quite precisely from changes either in their molecular mass and/or in their susceptibility to endoglycosidases as they leave this compartment. After a 15-min pulse with 35 S-labelled amino acids, proteins synthesized in the ER should still remain in this compartment. We labelled HeLa cells for 15 min with 35 S, and TIM and non-TIM pools were isolated. DAF (decay-accelerating factor) is probably the most abundant GPI-anchored glycoprotein in HeLa cells. Its immature form in the ER has a molecular mass of 43 kDa [45] and its mature form is around 60 kDa. Even before immunoprecipitation, TIM fractions were enriched (based on the protein contents of the TIM and non-TIM fractions) in a protein(s) of about 40 kDa (probably the ER form of DAF, Figure 6A, lane 1). Immunoprecipitation of TIM and non-TIM fractions with a monoclonal antibody against DAF confirmed this protein to be ER-DAF and revealed that $35 \pm 11\%$ of it was in TIMs (Figure 6A, lane 3).

Finally we followed PLAP, a 60-kDa GPI-anchored glycoprotein, in transfected CHO cells. PLAP was easily detectable in TIM fractions, both after a 15-min pulse with 35 S-labelled amino acids and following a subsequent 1-h chase in the absence of label (Figure 6B, band with the asterisk). Furthermore, a slight increase in the apparent molecular mass of PLAP was noticeable as glycan maturation occurred. To confirm that PLAP was still in the ER after the 15-min pulse, it was immunoprecipitated and treated with endoglycosidase H. The complete susceptibility of pulse-labelled PLAP to endoglycosidase H (Figure 6C) indicated that the protein remained in the ER during the labelling period. TIMs obtained following the 15-min pulse were characterized by immunoprecipitation of three membrane proteins: PLAP, APP and caveolin (Figure 6D). Detectable amounts of PLAP ($18 \pm 2\%$) were present in the TIM fraction after the pulse. In contrast APP, despite having a stronger signal than PLAP, was virtually absent from these TIMs. This result showed that the association of proteins (i.e. DAF and PLAP) with ER-TIMs is not dictated by their level of expression. Caveolin on the other hand was recovered almost exclusively in ER-TIMs (Figure 6D). Finally we followed the PLAP and APP distribution in TIMs after glycoprotein maturation. PLAP was recovered almost exclusively in TIMs after a 1-h chase (Figure 6E), in agreement with previous reports [3,4]. On the other hand, APP after the 1-h chase ran as a doublet containing an upper mature band and a lower band corresponding to the ER form, and both were absent from TIMs (Figure 6E).

DISCUSSION

Previous studies have demonstrated that TIMs are enriched in cholesterol, glycosphingolipids, SM [3] and PIP₂ [40]. We report here that TIMs are also enriched in another class of lipids, free GPIs. We also show that TIMs are not restricted to the PM and probably to the Golgi apparatus but that they also can be isolated from ER membranes.

To study the association of free GPIs with TIMs, we initially focused on GlcN-(acyl)PI because this early intermediate in the GPI pathway is easily detected following steady-state labelling of HeLa D cells with [3 H]inositol [32]. TIM fractions from these cells appeared typical in their enrichment in GPI-anchored proteins and depletion of PI ([3,40], and see Figure 2B), and they

Figure 6 ER-resident proteins are present in TIMs

Cells were pulse-labelled with ^{35}S -labelled amino acids and TIM (T) and non-TIM (nT) fractions were prepared as described in the legend of Figure 5. TIM fractions usually accounted for approximately 3% of the counts in both pulse and pulse and chase experiments. Aliquots of the T and nT pools were run on SDS/PAGE before and after immunoprecipitation of the indicated proteins with antibodies and radiolabelled bands were detected by fluorography. (A) ^{35}S -Labelled protein (lanes 1 and 2) and DAF profiles (lanes 3 and 4) in T and nT fractions from HeLa D cells after a 15-min pulse. (B) ^{35}S -Labelled proteins in TIMs after a 15-min pulse and a pulse followed by a chase of CHO cells. The band corresponding to PLAP is labelled with an asterisk. (C) Endo H treatment [4 mU of endoglycosidase H (Boehringer) in 60 mM P_i buffer, pH 5.5, supplemented with 200 μg of PMSF] for 7 h at 37 $^{\circ}\text{C}$ of ^{35}S pulse-labelled PLAP after immunoprecipitation and elution from Protein A-agarose beads with 1% SDS. (D) T and nT fractions from a 15-min pulse of CHO cells subjected to immunoprecipitation with antibodies against PLAP, APP and caveolin. Note that caveolin immunoprecipitates were run in a different gel and that the immunoreactive band had the molecular mass expected for caveolin (≈ 20 kDa). (E) T and nT fractions from the pulse-chase of CHO cells were subjected to immunoprecipitation with antibodies against PLAP and APP.

also contained 66% of the cellular GlcN-(acyl)PI. Man_3GPIs , later intermediates in the GPI-anchor pathway, were associated with TIMs in even greater percentages in K562 and mutant K cells. Studies with wild-type K562 cells were informative because they showed that the association of GPIs with TIMs does not depend on their unusual accumulation in mutant or atypical cell lines.

To our knowledge, the subcellular distribution of TIMs has not been previously investigated. Establishing the subcellular origin of TIMs simply from their content of known organelle markers is difficult because these markers may be excluded from or concentrated in TIMs [15,23]. Therefore, we addressed this issue by isolating subcellular fractions and extracting TIMs from them. TIMs derived from ER fractions of K562 cells were clearly indicated by a faint opalescent band with significant protein content at the 5/38% sucrose interface following gradient centrifugation. To establish firmly the existence of TIMs in the ER and to ensure that the presence of small amounts of PM and Golgi markers in the ER fractions does not lead to misinterpretation, we focused on ER-resident proteins. We determined that significant amounts of calnexin, an ER chaperone protein, and the immature ER forms of two GPI-anchored proteins, DAF and PLAP, were recovered in TIMs, confirming the presence of these membrane domains in the ER.

Characterization of the subcellular fractions revealed that some 50–75% of the Man_3GPIs from fraction 2 enriched in PM and Golgi markers were found in TIMs. These percentages were similar to those from intact cells. Thus the subcellular fractionation process itself did not appear to distort GPI association with TIMs. In addition, substantial amounts of the Man_3GPIs in the ER were associated with TIMs. This suggested that TIMs in the ER served to concentrate at least these glycolipids in a fashion resembling that of TIMs in Golgi and PM.

Our data showing the association of some GPI-anchored proteins in ER-TIMs appear to contrast with a previous interpretation that GPI-anchored proteins acquire detergent insolubility when they reach the Golgi apparatus [3]. However, in those studies TIMs were isolated by high-speed centrifugation, and recent reports [46,47] as well as our own unpublished work (D. Sevillever) with free GPIs as markers have demonstrated that under certain conditions and/or in some cell lines TIMs may fail to sediment at high speed but still float in a sucrose gradient. Furthermore, Brown and Rose acknowledged that a small fraction of the GPI-anchored protein becomes insoluble even before reaching the Golgi (see Figure 2 in [3]).

Whereas Man_3GPIs were associated with TIMs in the ER, quantitative differences in the extent of TIM association were apparent. For each Man_3GPI the percentage associated with

TIMs in the ER fractions was lower than that in the PM/Golgi fraction. This trend was also observed when SM, cholesterol and GPI-anchored proteins in the ER were analysed. This could arise simply because the lower content of cholesterol and glycosphingolipids in ER membranes results in weaker interactions among ER-TIM components that cannot withstand the 1% Triton treatment. There are precedents for the importance of a lower detergent concentration (i.e. below 0.2%) to establish the presence of certain proteins (e.g. GAP-43 and the IgE receptor) in TIMs [48,49]. Therefore, extraction in 1% Triton may result in underestimation of the association of some components with TIMs, particularly in the ER.

Interestingly, the association of Man₃GPIs with TIMs was selective: a smaller percentage of H7 was associated with TIMs than of either H6 or H8. Analysis of early intermediates of the GPI-anchor biosynthetic pathway, GlcNAc-PI and GlcN-PI, also demonstrated that the interaction of GPIs with TIMs is selective. GlcNAc-PI was shown to be present in ER-TIMs whereas GlcN-PI was almost absent from this fraction. It may be argued that GlcNAc-PI synthesized in a subcellular ER fraction is transferred during the reaction to contaminant PM/Golgi membranes where it is recovered in TIMs. However, we rejected this possibility because no change in the percentage of GlcNAc-PI present in TIMs was observed when the ER membrane preparations were supplemented with the same amounts of PM/Golgi membranes (results not shown).

It is very unlikely that structural differences between H7 and H6/H8 (i.e. a single ethanolamine-P group) and between GlcNAc-PI and GlcN-PI (i.e. acetylation of the GlcN) are responsible for their different enrichment in TIMs. It is possible that GPI association with TIMs is correlated with low rates of GPI turnover. Pulse-chase kinetic studies showed that turnover of H7 is considerably faster than that of H8 and H6 [28]. Differences in TIMs association also might reflect the compartmentalization of the GPI biosynthetic pathway. In support of this hypothesis, we (A. Wongkajornsilp, D. Sevillever and T. L. Rosenberry, unpublished work) and others [50] have data suggesting that GPI intermediates exist in more than one pool in the ER.

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REFERENCES

- McConville, M. J. and Ferguson, M. A. J. (1993) *Biochem. J.* **294**, 305–324
- Udenfriend, S. and Kodokula, K. (1995) *Annu. Rev. Biochem.* **64**, 563–591
- Brown, D. A. and Rose, J. K. (1992) *Cell* **68**, 533–544
- Cerneus, D. P., Ueffing, E., Posthuma, G., Strous, G. J. and van der Ende, A. (1993) *J. Biol. Chem.* **268**, 3150–3155
- Simons, K. and Ikonen, E. (1997) *Nature (London)* **387**, 569–572
- Brown, D. A. and London, E. (1998) *Annu. Rev. Cell Dev. Biol.* **14**, 11–136
- Millán, J., Puertollano, R., Fan, L., Rancano, C. and Alonso, M. A. (1997) *Biochem. J.* **321**, 247–252
- Deans, J. P., Robbins, S. M., Polyak, M. J. and Savage, J. A. (1998) *J. Biol. Chem.* **273**, 344–348
- Rothberg, K. G., Heuser, J. E., Donzell, W. C., Ying, Y.-S., Glenney, J. R. and Anderson, R. G. W. (1992) *Cell* **68**, 673–682
- Gorodinsky, A. and Harris, D. A. (1995) *J. Cell Biol.* **129**, 619–627
- Fra, A. M., Williamson, E., Simons, K. and Parton, R. (1994) *J. Biol. Chem.* **269**, 30745–30748
- Stan, R.-V., Roberts, W. G., Predescu, D., Ihida, K., Saucan, L., Ghitescu, L. and Palade, G. E. (1997) *Mol. Biol. Cell* **8**, 595–605
- Waugh, M. G., Lawson, D. and Hsuan, J. J. (1999) *Biochem. J.* **337**, 591–597
- Schnitzer, J. E., McIntosh, D. P., Dvorak, A. M., Liu, J. and Oh, P. (1995) *Science* **269**, 1435–1439
- Lisanti, M. P., Scherer, P. E., Vidugiriene, J., Tang, Z., Hermanowski-Vosatka, A., Tu, Y., Cook, R. F. and Sargiacomo, M. (1994) *J. Cell. Biol.* **126**, 111–126
- van den Berg, C. W., Cinek, T., Hallet, M. B., Horejsi, V. and Morgan, B. P. (1995) *J. Cell Biol.* **131**, 669–677
- van't Hoff, W. and Resh, M. D. (1997) *J. Cell Biol.* **136**, 1023–1035
- Schroeder, R., London, E. and Brown, D. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 12130–12134
- Smart, E. J., Ying, Y.-S., Mineo, C. and Anderson, R. G. W. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 10104–10108
- Song, K. S., Li, S., Okamoto, T., Quilliam, L. A., Sargiacomo, M. and Lisanti, M. P. (1996) *J. Biol. Chem.* **271**, 9690–9697
- Rodgers, W. and Rose, J. K. (1996) *J. Cell Biol.* **135**, 1515–1523
- Lee, S.-J., Liyanage, U., Bickel, P. E., Xia, W., Lansbury, P. T. and Kosik, K. S. (1998) *Nat. Med.* **4**, 730–734
- Kurzchalia, T. V., Hartmann, E. and Dupree, P. (1995) *Trends Cell Biol.* **5**, 187–189
- Stevens, V. L. and Tang, J. (1997) *J. Biol. Chem.* **272**, 18020–18025
- Ilangumaran, S. and Hoessli, D. C. (1998) *Biochem. J.* **335**, 433–440
- Arreaza, G., Melkonian, K. A., LaFevre-Bernt, M. and Brown, D. A. (1994) *J. Biol. Chem.* **269**, 19123–19127
- Puotinen, A. and Conzelmann, A. (1992) *J. Biol. Chem.* **267**, 22673–22680
- Rosenberry, T. L., Sevillever, D. and Medof, M. E. (1994) *Brazilian J. Med. Biol. Res.* **27**, 151–159
- van't Hoff, W., Rodriguez-Boulant, E. and Menon, A. K. (1995) *J. Biol. Chem.* **270**, 24150–24155
- Singh, N., Liang, L.-N., Tykocinski, M. L. and Tartakoff, A. M. (1996) *J. Biol. Chem.* **271**, 12879–12884
- Sevillever, D., Humphrey, D. R. and Rosenberry, T. L. (1995) *Eur. J. Biochem.* **233**, 384–394
- Sevillever, D., Schiemann, D., Guidubaldi, J., Medof, M. E. and Rosenberry, T. L. (1997) *Biochem. J.* **321**, 837–844
- Mohney, R. P., Knez, J. J., Ravi, L., Sevillever, D., Rosenberry, T. L., Hirose, S. and Medof, M. E. (1994) *J. Biol. Chem.* **269**, 6536–6542
- Stevens, V. L., Zhang, H. and Harreman, M. (1996) *Biochem. J.* **313**, 253–258
- Sevillever, D. and Rosenberry, T. L. (1993) *J. Biol. Chem.* **268**, 10938–10945
- Hirose, S., Prince, G. M., Sevillever, D., Ravi, L., Rosenberry, T. L., Ueda, E. and Medof, M. E. (1992) *J. Biol. Chem.* **267**, 16968–16974
- Vidugiriene, J. and Menon, A. K. (1993) *J. Cell. Biol.* **121**, 987–996
- Bligh, E. G. and Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37**, 911–917
- Stevens, V. (1995) *Biochem. J.* **310**, 361–370
- Pike, L. J. and Casey, L. (1996) *J. Biol. Chem.* **271**, 26453–26456
- Chen, R., Udenfriend, S., Prince, G. M., Maxwell, S. E., Ramalingam, S., Gerber, L. D., Knez, J. and Medof, M. E. (1996) *Proc. Natl. Acad. Sci. U.S.A.* **93**, 2280–2284
- Watanabe, R., Kinoshita, T., Masaki, R., Yamamoto, A., Takeda, J. and Inoue, N. (1996) *J. Biol. Chem.* **271**, 26868–26875
- Inoue, N., Watanabe, R., Takeda, J. and Kinoshita, T. (1996) *Biochem. Biophys. Res. Commun.* **226**, 193–199
- Stevens, V. L. (1993) *J. Biol. Chem.* **268**, 9718–9724
- Medof, M. E., Walter, E. I., Roberts, W. L., Haas, R. and Rosenberry, T. L. (1986) *Biochemistry* **25**, 6740–6747
- Ilangumaran, S., Briol, A. and Hoessli, D. C. (1997) *Biochim. Biophys. Acta* **1328**, 227–236
- Naslavsky, N., Stein, R., Yanai, A., Friedlander, G. and Taraboulos, A. (1997) *J. Biol. Chem.* **272**, 6324–6331
- Arni, S., Keilbaugh, S. A., Ostermeyer, A. G. and Brown, D. A. (1998) *J. Biol. Chem.* **273**, 28478–28484
- Field, K. A., Holowka, D. and Baird, B. (1997) *J. Biol. Chem.* **272**, 4276–4280
- Menon, A. K., Baumann, N. A., van't Hoff, W. and Vidugiriene, J. (1997) *Biochem. Soc. Trans.* **25**, 861–865

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