Association of (c)AMP-Degrading Glycosylphosphatidylinositol-Anchored Proteins with Lipid Droplets Is Induced by Palmitate, H_2O_2 and the Sulfonylurea Drug, Glimepiride, in Rat Adipocytes

Günter Müller,* Sabine Over, Susanne Wied, and Wendelin Frick

Sanofi-Aventis Pharma, Therapeutic Department Metabolism, 65926 Frankfurt am Main, Germany

Received December 4, 2007

ABSTRACT: Inhibition of lipolysis in rat adipocytes by palmitate, H_2O_2 and the antidiabetic sulfonylurea drug, glimepiride, has been demonstrated to rely on the upregulated conversion of cAMP to adenosine by enzymes associated with lipid droplets (LD) rather than on cAMP degradation by the insulin-stimulated microsomal phosphodiesterase 3B (Müller, G., Wied, S., Over, S., and Frick, W. (2008) *Biochemistry 47*, 1259−1273). Here these two enzymes were identified as the glycosylphosphatidylinositol (GPI)-anchored phosphodiesterase, Gce1, and the 5′-nucleotidase, CD73, on basis of the following findings: (i) Photoaffinity labeling with 8-N_3-[^{32}P]cAMP and [^{14}C]5′-FSBA of LD from palmitate-, glucose oxidase- and glimepiride-treated, but not insulin-treated and basal, adipocytes led to the identification of 54-kDA cAMP- and 62-kDa AMP-binding proteins. (ii) The amphiphilic proteins were converted into hydrophilic versions and released from the LD by chemical or enzymic treatments specifically cleaving GPI anchors, but resistant toward carbonate extraction. (iii) The cAMP-to-adenosine conversion activity was depleted from the LD by adsorption to (c)AMP-Sepharose. (iv) cAMP-binding to LD was increased upon challenge of the adipocytes with palmitate, glimepiride or glucose oxidase and abrogated by phospholipase C digestion. (v) The 62-kDa AMP-binding protein was labeled with typical GPI anchor constituents and reacted with anti-CD73 antibodies. (vi) Inhibition of the bacterial phosphatidylinitosol-specific phospholipase C or GPI anchor biosynthesis blocked both agent-dependent upregulation and subsequent loss of cAMP-to-adenosine conversion associated with LD and inhibition of lipolysis. (vii) Gce1 and CD73 can be reconstituted into and exchanged between LD *in vitro*. These data suggest a novel insulin-independent antilipolytic mechanism engaged by palmitate, glimepiride and H_2O_2 in adipocytes which involves the upregulated expression of a GPI-anchored PDE and 5′-nucleotidase at LD. Their concerted action may ensure degradation of cAMP and inactivation of hormone-sensitive lipase in the vicinity of LD.

About 1% of all eukaryotic proteins or 10−20% of all membrane proteins that enter the secretory pathway after being targeted to the endoplasmic reticulum are posttranslationally modified at the carboxy-terminus by GPI[1] moieties, a complex glycophospholipid that serves to anchor proteins at the cell surface. The core structure of this anchor consists of ethanolamine phosphate, trimannoside, non-acetylated glucosamine and inositol phospholipid in this order (*1*, *2*). The GPI anchor is combined with the carboxyl-terminus of the protein *via* its ethanolamide amino-terminus. GPI anchoring of cell surface proteins is the most complex and metabolically expensive of the posttranslational lipid modifications. The GPI anchor is synthesized via a membrane-bound multistep pathway in the ER requiring more than 20 gene products (*3*). The pathway is initated on the cytoplasmic face of the ER and completed in the ER lumen, necessitating flipping of a glycolipid intermediate across the membrane. The completed GPI anchor is attached to proteins that have been translocated across the ER membrane and that display a GPI anchor signal sequence at the carboxy-terminus (*4*). Typically, GPI proteins transit the secretory pathway to the cell surface.

GPI proteins are ubiquitously distributed among eukaryotes from vertebrates to protozoa. Although GPI proteins are functionally diverse, many have or are predicted to have hydrolytic activity, such as alkaline phosphatase and 5′-Nuc, or serve as cell surface receptors, such as folate receptor and CD14. The importance of GPI anchoring in mammals is underscored by the observations that abrogation of GPI biosynthesis results in embryonic lethality (*5*), that impairment of GPI mannosylation leads to thromboses of the portal and hepatic veins (*6*) and that a somatic defect in the first step of GPI biosynthesis in hematopoietic human stem cells causes the acquired hemolytic disease, paroxysmal nocturnal hemoglobinuria (*7*).

* To whom correspondence should be addressed: Sanofi-Aventis Pharma Germany GmbH, TD Metabolism, Industrial Park Höchst, Bldg. H821, 65926 Frankfurt am Main, Germany; phone, +4969-305-4271; fax, +4969-305-81901; e-mail, Guenter.Mueller@sanofi-aventis.com.

[1] Abbreviations: BATC, *β*-amidotaurocholate; BSA, bovine serum albumin; (c)AMP, (cyclic) adenosine monophosphate; 5′-FSBA, 5′-*p*-fluorosulphonylbenzoyladenosine; Gce1, GPI-anchored cAMP-binding ectoprotein-1; GO, glucose oxidase; GPI, glycosylphosphatidylinositol; (G)PI-PLC, (glycosyl)phosphatidylinositol-specific phospholipase C; GPI protein, GPI-anchored protein; IBMX, isobutylmethylxanthine; LD, lipid droplets; 5′-Nuc, 5′-nucleotidase; PDE, phosphodiesterase; SDS−PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; TAG, triacylglycerol.

10.1021/bi7022915 CCC: $40.75
Published on Web 01/11/2008

So far as is known the vast majority of GPI proteins are expressed at the cell surface which represents their final functional localization. Unlike conventional hydrophobic transmembrane protein domains that span the membrane bilayer, GPI anchors only penetrate into a single membrane leaflet. This feature, as well as the saturated fatty acyl chains typically found in GPI anchors, ensures the association of GPI proteins with cholesterol-containing detergent-insoluble glyco(sphingo)lipid-enriched plasma membrane microdomains in the nanoscale that are postulated to play an important role in membrane trafficking and cell signaling (*8*−*10*). It has recently been demonstrated that GPI anchors can determine functional specificity of the protein attached by triggering the association with new protein or lipid components located in a shared plasma membrane microdomain (*11*, *12*). Moreover, on the basis of the recognized heterogeneity of plasma membrane microdomains considering their cholesterol content (*13*, *14*), the observed translocation of certain GPI proteins between microdomain of differing cholesterol content (*15*, *16*) and the well-established effects of GPI anchor removal on enzymatic activities (*17*−*19*) and ligand binding properties (*20*, *21*), it is conceivable that GPI proteins acquire distinct functions depending on the interaction of their GPI anchors with distinct lipid or protein components of the corresponding membrane microdomain.

LD are ubiquitous intracellular energy storage organelles of organisms as diverse as bacteria and humans. They are composed of a protein-coated phospholipid monolayer, which encloses a hydrophobic core of neutral storage TAG, cholesteryl esters and retinyl esters reflecting their predominant role in lipid storage for energy homeostasis and for membrane phospholipid and lipid hormone synthesis (*22*). Conversely, degradation of lipids during lipolysis induces LD fragmentation in differentiated adipocytes, underscoring the dynamic cell biology of these organelles (*23*). Moreover, LD have recently been suggested to represent target compartments for FA scavenging to protect cells from lipotoxicity (*24*, *25*). LD are thought to originate from the membrane of the ER with newly synthesized TAG being inserted between the bilayer causing the cytoplasmic leaflet to bulge and eventually bud off into the cytoplasm (*26*, *27*). However, LD are surrounded by a phospholipid monolayer that has a composition different from the ER membrane (*28*, *29*). In mammals, the *p*erilipin (in adipocytes and steroidogenic cells)−*a*dipophilin/*A*DRP (in all other mammalian cell types)−*T*IP47 (differentiating cells) PAT family of proteins, which is defined by sequence similarity and lipid-binding, has been shown to coat LD (*30*, *31*). S3−12 shares sequence similarity with PAT proteins and, like TIP47, only coats LD under certain metabolic states. The PAT proteins are thought to organize the packaging and trafficking of intracellular neutral lipids (*22*, *32*). In particular, perilipin impairs basal or facilitates upregulated lipolysis by acting either as a barrier or attachment site for HSL in phosphorylation-dependent manner (*33*).

Remarkably, extensive subproteomic analysis of LD-associated proteins from a variety of different adipose cells from mammals and *Drosophila* resulted in the identification of a multitude of proteins of distinct structure and function but so far failed to demonstrate the expression of any GPI protein at LD under basal or lipolytically stimulated conditions (*29*−*31*, *34*−*36*). However, from a theoretical point of view, it is conceivable that the GPI modification of proteins may facilitate their embedding in the LD surface phospholipid monolayer, thereby converting them into LD-associated proteins, eventually in time-dependent and regulated fashion. Interestingly, recent studies revealed considerable increases in cAMP-to-adenosine conversion activity associated with LD from primary rat adipocytes upon inhibition of lipolysis by palmitic acid, H_2O_2 and glimepiride (*83*). Since GPI-anchored versions of a cAMP-binding protein (Gce1) and AMP-hydrolyzing enzyme (CD73) have previously been identified in adipocytes (*37*−*39*), the possibility of expression of certain GPI proteins at LD of rat adipocytes under certain conditions of repressed lipolysis was tested in the present study.

MATERIALS AND METHODS

Materials. 8-N_3-[^{32}P]cAMP was bought from ICN (Eschwege, Germany). [U-^{14}C]mannose and *myo*-[U-^{14}C]inositol were purchased from Amersham-Buchler (Braunschweig, Germany); [^{14}C]5′-FSBA was synthesized by the method of Coleman and co-workers (*40*) using [U-^{14}C]adenosine and purified by thin layer chromatography on silica-gel plates developed in butan-2-one/acetone/water (12/4/3, by volume). BATC (*41*) were made available by the biotechnological production and medicinal chemistry synthesis departments of Sanofi-Aventis Pharma (Frankfurt; Germany). cAMP-Sepharose, AMP-Sepharose and protein A-Sepharose were provided by Pharmacia/LKB (Freiburg, Germany). 5′-Nuc (*Crotalus atrox*), PI-PLC (*Bacillus* cereus), adenosine 5′-[α,β-methylene]diphosphate (AMPCP) and N^6,2′-O-dibutyryladenosine 3′5′-cyclic monophosphate (dibutyryl-cAMP) were from Sigma/Aldrich (Deisenhofen, Germany). All other materials were obtained as described previously (*16*, *42*−*44*, *83*).

Primary Culture and Metabolic Labeling of Rat Adipocytes with [^{14}C]Inositol. Rat adipocytes (5 mL, 2×10^6 cells/mL) were washed twice with 50 mL of labeling medium (inositol- and glucose-free DMEM containing 25 mM Hepes/KOH, pH 7.4, 5 mM sodium pyruvate, 5% fetal calf serum and 0.5% BSA) and then incubated (2 h, 37 °C) in 20 mL of labeling medium supplemented with 50 units/mL penicillin and 50 μg/mL streptomycin sulfate in 150-mL culture flasks under 95% O_2/5% CO_2. Labeling was started by addition of *myo*-[U-^{14}C]inositol (20 μCi, 0.1 mM final concentration) and continued (5 min for short-term pulse labeling or 60 min for long-term equilibrium labeling) in the presence of various stimuli and/or inhibitors as indicated. For initiation of the chase, the cells were washed twice by flotation with 10 mL each of labeling medium containing 0.5 mM glucose and 10 mM *myo*-inositol and then suspended in 20 mL of the same medium (zero time point). After incubation (37 °C, gentle shaking) for increasing periods of time in the presence of various stimuli and/or inhibitors as indicated, the adipocytes were separated from the medium by flotation and washed with labeling medium (15 °C).

Adsorption to cAMP-Sepharose and AMP-Sepharose. 20−50 μg of protein extracted from LD and precipitated under native conditions was dissolved in 100 μL of 25 mM Mops/KOH (pH 7.0), 1 M NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 100 μM IBMX, 60 mM octyl glucoside, 0.5%

BATC and then applied in the absence or presence of 1 mM cAMP or AMP onto 1-mL columns of N^6-(2-aminoethyl)-cAMP-Sepharose (Pharmacia) or 5′-AMP-Sepharose (Sigma) which had been equilibrated with the same buffer supplemented with 0.5 mg/mL BSA. The flow-through was desalted by centrifugation through 2-mL columns of Sephadex G-25 and then precipitated under native conditions.

Photoaffinity Labeling of LD with 8-N_3-[^{32}P]cAMP and [^{14}C]5′-FSBA. Proteins extracted from LD (10−20 μL) and precipitated under native conditions were incubated (15 min, 4 °C) with 0.5 μCi of 8-N_3-[^{32}P]cAMP (5 pmol) or 20 μCi of [^{14}C]5′-FSBA (15 nmol) in 25 μL of 10 mM Tris/HCl (pH 7.4), 1 mM EDTA, 0.5 mM EGTA, 140 mM NaCl, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM IBMX, 1 mM DTT, 20 mM NaF, 25 mM glycerol-3-phosphate, 10 mM sodium pyrophosphate and protease inhibitors in the presence of 1 mM AMP (8-N_3-[^{32}P]cAMP) or 100 μM ADP ([^{14}C]5′-FSBA) in the wells of microtiter plates (96-formate) under ultrasonic treatment (bath sonicator, max. power, 3 × 15-s periods with 45-s intervals) and then irradiated with UV light (254 nm, 8000 μW/cm^2) at a distance of 0.5 cm for 1 min (*45*, *46*). The reactions were quenched by addition of 25 μL of the same buffer containing 10 mM cAMP (8-N_3-[^{32}P]-cAMP) or AMP ([^{14}C]5′-FSBA).

cAMP-Binding Activity. Binding of cAMP was measured using titration ITC using a VP-ITC calorimeter from MicroCal (Northampton,). In brief, LD proteins (5−10 μg in 100 μL of 20 mM Tris/HCl (pH 7.4), 1 mM EDTA, 150 μM IBMX, complete protease and phosphatase inhibitor tablets) were added to 1.41 mL of 40 mM Mops/KOH (pH 7.0). For each binding assay increasing amounts of cAMP (in the same buffer) were titrated (in 5-μL portions) until no more binding occurred (typcially 20 injections, with 4 min between each injection). The heat released from each injection was then directly analyzed to obtain the binding constant, and the stoichiometry of binding using the MicroCal/Origin software (MicroCal) in a method similar to that described previously (*47*). This microcalorimetric cAMP-binding assay was validated using [^{3}H]cAMP and a filtration technique as decribed previously (*48*), which resulted in qualitatively similar results.

TX-114 Partitioning. Protein samples were separated into amphiphilic and hydrophilic proteins using partitioning between TX-114-enriched and depleted phases (*49*) by mixing with the same volume (max. 0.5 mL) of ice-cold 25 mM Tris/HCl (pH 7.4), 140 mM NaCl containing 2% TX-114. After incubation (1 h, on ice), the mixture was layered onto a cushion of 0.4 mL of 0.25 M sucrose and 25 mM Tris/HCl (pH 7.4) on ice. Phase separation was induced by warming up to 37 °C and subsequent centrifugation (10000*g*, 1 min). The re-extracted lower TX-114-enriched phase and the pooled upper TX-114-depleted phase were precipitated under denaturing conditions.

Preparation of [^{14}C]Inositol-Labeled Gce1p from Yeast with Intact or Cleaved GPI Anchor. Gce1p with intact GPI anchor was purified from lactate-grown yeast cells, which had been metabolically labeled with *myo*-[^{14}C]inositol and then enzymatically converted to spheroblasts, as described previously (*45*). In brief, plasma membranes were prepared, purified by Ficoll gradient centrifugation, solubilized with 0.4% BATC and then subjected to TX-114 partitioning. Gce1p contained in the detergent-enriched phase was purified by sequential gel filtration chromatography on Sephadex S-300, affinity chromatography on N^6-(2-aminoethyl)-cAMP Sepharose and phenyl Sepharose chromatography. Elution from the columns was followed by on-line monitoring of ^{14}C-radioactivity. Partially purified Gce1p was precipitated under native conditions, then resuspended in buffer G (25 mM Tris/acetate, pH 7.4, 144 mM NaCl, 0.4% β-BATC, 20 mM octylglucoside, 0.5 mM DTT, 0.2 mM EDTA, 5% glycerol and protease inhibitor mix) at 0.2 mg protein/mL and subsequently incubated (3 h, 25 °C) in the absence or presence of 6 U/mL PI-PLC (*B. cereus*) (*20*, *37*). After addition of 10 volumes of ice-cold 2% Triton X-114, 10 mM Tris/HCl (pH 7.4), 144 mM NaCl and phase separation (incubation [2 min, 37 °C] and centrifugation [12000*g*, 1 min, 25 °C]), Gce1p with intact or cleaved GPI anchor was recovered from the lower detergent-enriched and upper detergent-depleted phases, respectively (*49*). After two re-extractions by addition of an equal volume of 10 mM Tris/HCl, 144 mM NaCl and subsequent phase separation, the combined phases were precipitated under native conditions. Radiolabeled Gce1p was suspended in buffer G at 300−1,000 dpm/μl. The enrichment and purity of Gce1p was determined during each step as described previously (*50*).

Reconstitution of Gce1p into LD. [^{14}C]Inositol-labeled Gce1p from yeast with intact or cleaved GPI anchor (5,000−10,000 dpm) or bovine brain PDE (25 mU) in 50 μL of buffer G was added to LD prepared from 1 × 10^6 adipocytes in 450 μL of buffer R (50 mM Tris/HCl, pH 7.4, 0.1 M NaCl, 0.2 mM DTT, 0.02% sodium azide, 0.7 mM $CaCl_2$, 0.7 mM $MgCl_2$, 0.7 mM $MnCl_2$) and then incubated (1 h, 25 °C). The detergent was removed by dialysis in Spectrapor 4 tubings (12−14 kDa cutoff) against 3 × 50 mL of buffer R. Following dialysis, the dialysate was mixed with 1.5 mL of 65% sucrose (w/v) and poured into a 5-mL centrifuge tube. 1.5 mL of 10% sucrose (w/v) was then layered on top of the sucrose cushion. The tube was filled to capacity with buffer R. The gradient was centrifuged (172000*g*, 60 min, 15 °C) and then allowed to coast to rest. LD floating as the upper white layer of the gradient were isolated by suction with a syringe (0.8 mL), then washed two times with buffer R by flotation and finally suspended in 50 mM Tris/HCl (pH 7.4), 150 mM NaCl, and 0.02% sodium azide.

Immunoprecipitation. Protein precipitates (10−50 μg protein) were dissolved in 1 mL of buffer IP (10 mM Tris/HCl, pH 8.0, 150 mM NaCl, 6 mM EDTA, 1% Triton X-100, protease inhibitor mix) and then precleared with protein G/A-Sepharose. After incubation (4 h, 4 °C, with rocking) with anti-CD73 antibodies (Abcam, mouse monoclonal 7G2, 1:400) preadsorbed to 20 μL of protein G/A-Sepharose (50 mg/mL) in 1 mL of buffer IP and subsequent centrifugation (5000*g*, 2 min), the collected immune complexes were washed four times with 1 mL each of buffer IP, then twice with buffer IP lacking Triton X-100 and twice with buffer IP lacking Triton X-100 and NaCl and finally dissolved (5 min, 95 °C) in 50 μL of sample buffer. Supernatant proteins were analyzed by SDS−PAGE and immunoblotting. The recovery in the amount of immunoprecipitated protein was corrected for the amount of protein actually applied to the gel as revealed by homologous immunoblotting.

Miscellaneous. Published procedures were used for the preparation and incubation of rat adipocytes (*16*, *44*), preparation of LD from rat adipocytes (*51*, *52*, *83*), extraction and precipitation of LD proteins (*83*), nitrous acid deamination, hydrogen fluoride dephosphorylation and treatment with PI-PLC (*Bacillus cereus*) of GPI proteins (*45*, *50*), SDS−PAGE and immunoblotting using chemiluminescent detection (*15*, *16*, *83*). Protein concentration was determined by using the BCA method (Pierce) with BSA as calibration standard. Phosphorimages were processed and quantified by computer-assisted video densitometry using the Storm 860 PhosphorImager system (Molecular Dynamics, Gelsenkirchen, Germany). Concentration−response curves were fitted by using the GraphPad Prism 4.03 software. Figures of phosphoimages and lumiimages were constructed using the Adobe Photoshop software (Adobe Systems, Mountain View, CA).

RESULTS

The cAMP-to-Adenosine Conversion at LD of Rat Adipocytes Is Constituted by the PDE Gce1 and the 5′-Nuc CD73. Previous studies on the molecular mechanism of the inhibition of lipolysis by palmitate, glimepiride and H_2O_2 in rat adipocytes revealed increased conversion of cAMP to adenosine by LD prepared from these cells and assayed *in vitro*, which was sensitive toward inhibitors of PDE and 5′-Nuc (*83*). To identify the putative LD-associated cAMP-/AMP-binding/degrading proteins, LD were prepared from glimepiride-, palmitate- or GO (for the generation of H_2O_2 in the incubation medium)-treated adipocytes and then photoaffinity-labeled with 8-N_3-[^{32}P]cAMP or [^{14}C]5′-FSBA in the presence of the general PDE inhibitor, IBMX, for interference with cAMP degradation. The phosphorimages of the SDS−PAGE demonstrated photoaffinity labeling of a 54-kDa protein with 8-N_3-[^{32}P]cAMP and of a 62-kDa protein with [^{14}C]5′-FSBA with LD from glimepiride-treated adipocytes (Figure 1A). The specificity of the photoaffinity labeling reactions was confirmed by (i) omission of UV irradiation, (ii) inclusion of excess of unlabeled cAMP and AMP, respectively, or (iii) depletion of cAMP- and AMP-binding proteins by adsorption to cAMP-Sepharose or AMP-Sepharose, respectively, prior to photoaffinity labeling, which in each case led to considerable quenching of photoaffinity labeling of the 54-kDa protein and 62-kDa protein. Importantly, in the absence of IBMX the two proteins resisted photoaffinity labeling (data not shown) which could be due to degradation of 8-N_3-[^{32}P]cAMP and generation of [^{32}P]-AMP efficiently competing with [^{14}C]5′-FSBA for binding by a putative PDE activity intrinsic to the 54-kDa protein cAMP-binding protein. Upon TX-114 partitioning both the 54- and 62-kDa photoaffinity-labeled proteins were recovered predominantly with the detergent rather than aqueous phases (Figure 1A) hinting to their amphiphilic nature, which is a well-known characteristic of GPI proteins (*1*, *49*). To test for modification with a GPI anchor, both proteins were treated with a bacterial (G)PI-PLC, nitrous acid or aqueous hydrogen fluoride known to cause specific cleavage of GPI anchors at distinct sites and to convert amphiphilic GPI proteins into their hydrophilic GPI anchorless versions, which are enriched in the aqueous phase upon TX-114 partitioning (*4*, *45*). Each of these treatments led to release of the two photoaffinity-labeled proteins into the aqueous phase arguing for modification of the 54-/62-kDa cAMP-/AMP-binding proteins with GPI anchors.

The putative relationship of these proteins with the upregulated cAMP-to-adenosine conversion activity at LD of the adipocytes should be reflected in correlation between the efficacies of photoaffinity labeling and lipolysis inhibition exerted by the three agents. In fact, the amounts of both the 54- and 62-kDa protein found associated with LD were significantly and concentration-dependently increased upon short-term incubation of rat adipocytes with palmitate (to up to 4-fold), glimepiride (to up to 6- to 8-fold), and GO (to up to 9- to 12-fold) compared to control cells (Figure 1B) and correlated well to the corresponding antilipolytic activities (H_2O_2 > glimepiride > palmitate). The insulin-induced elevations in the LD association of both proteins were only moderate, and did not reach statistical significance. As demonstrated above for glimepiride-treated adipocytes (Figure 1A), the amphiphilic versions of the 54-kDa cAMP- and 62-kDa AMP-binding proteins recovered with LD from palmitate- and GO-treated adipocytes were converted to their hydrophilic forms by treatment with bacterial (G)PI-PLC, nitrous acid and aqueous hydrogen fluoride (data not shown). Further evidence for involvement of the cAMP (54-kDa)- and AMP (62-kDa)-binding proteins in the conversion of cAMP to adenosine by LD was obtained by their adsorption to cAMP-Sepharose and AMP-Sepharose following extraction of the LD proteins under native conditions and subsequent assaying of the nonadsorbed proteins for cAMP-to-adenosine conversion (Figure 2). The glimepiride-, palmitate- and GO-induced pronounced increase in cAMP-to-adenosine conversion (compared to basal adipocytes) was significantly reduced after depletion of the cAMP- or AMP-binding proteins with cAMP- or AMP-Sepharose, respectively, and almost completely abrogated with a combination of both. The specificity of the depletion was demonstrated by inclusion of excess of cAMP and AMP, respectively, which left the cAMP-to-adenosine conversion unaltered compared to nonadsorbed LD proteins. Importantly, the amounts of typical LD proteins in adipocytes, such as perilipin-A, HSL, caveolin-1 and vimentin, as well as of ADRP and TIP47, which were detected as faint bands, only, in agreement with the reported low-abundance expression in mature adipocytes (*53*, *54*), did not differ considerably between glimepiride-, palmitate-, H_2O_2-treated and basal adipocytes (see ref *83*). Taken together, the upregulation of the cAMP-to-adenosine conversion by glimepiride, palmitate and H_2O_2 is correlated to pronounced and specific increases in the amounts of the GPI-modified 54-kDa cAMP- and 62-kDa AMP-binding proteins at LD of adipocytes.

Putative candiates for the LD-associated 54-kDa cAMP-binding protein represent the major cAMP-binding and cAMP-handling proteins expressed in isolated rat adipocytes, among them the RIIβ-subunit of PKA, PDE3B and Gce1. RIIβ could associate with LD on basis of its functional interaction with LD-associated caveolin-1 (*55*−*57*) in the course of cAMP-dependent phosphorylation of perilipin (*58*). Microsomal PDE3B could move to LD during their biogenesis by bulbing from the cytosolic leaflet of the endoplasmic reticulum membrane (*59*). However, blotting of LD proteins from glimepiride-, palmitate- and GO-treated adipocytes for RIIβ (data not shown) and PDE3B (see ref *83*) revealed no or only low expression, respectively, of cross-reactive

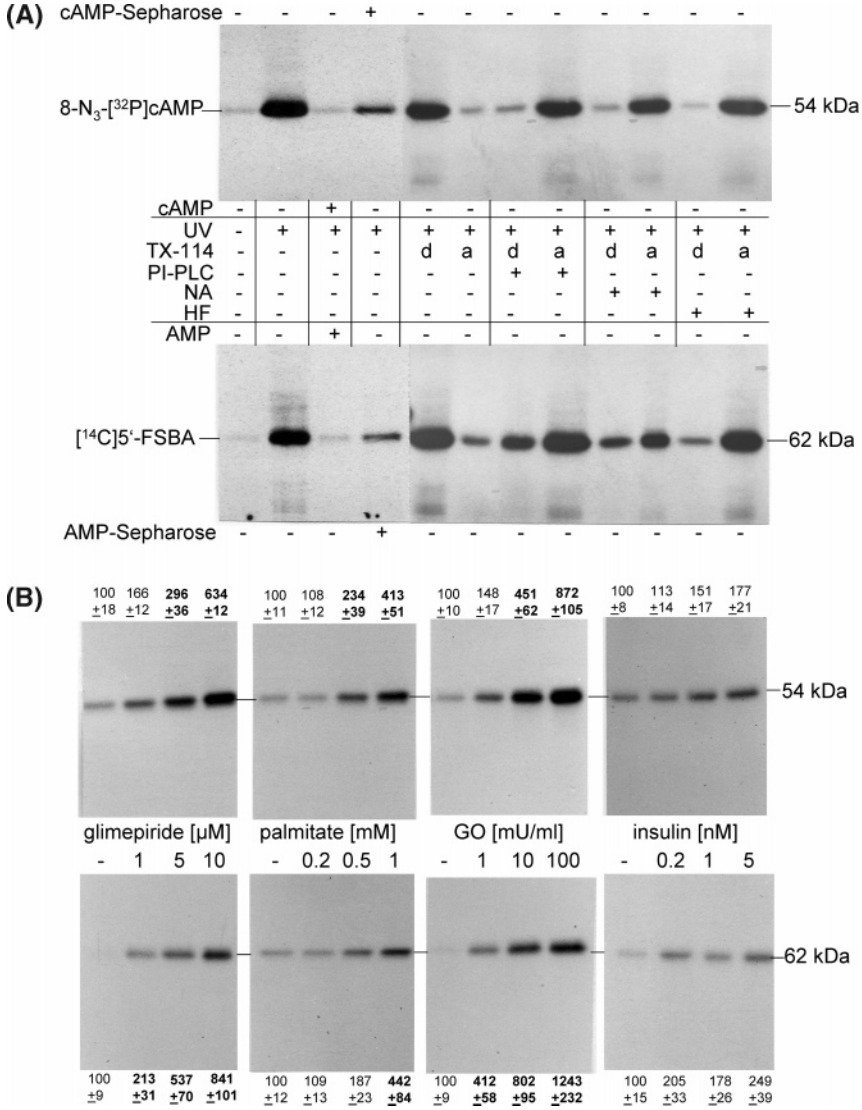

FIGURE 1: Effect of glimepiride, palmitate, GO and insulin on the expression of cAMP- and AMP-binding proteins at LD. Isolated rat adipocytes were incubated (37 °C) with glimepiride (10 μM, 60 min) (A) or in the absence or presence of increasing concentrations of glimepiride (60 min), palmitate (120 min) or GO (20 min) (B) and then separated from the medium for the preparation of LD. Proteins were extracted under native conditions, then subjected (A) or not (A, B) to adsorption to cAMP-/AMP-Sepharose, subsequently precipitated under native conditions, dissolved and finally incubated with 8-N_3-[^{32}P]cAMP or [^{14}C]5′-FSBA in the absence (A, B) or presence of cAMP or AMP (final concentration 1 mM) (A) under UV irradiation (A, B) or not (A) as indicated. Portions of the samples were subjected to lipolytic cleavage by PI-PLC (*Bacillus cereus*), deamination by nitrous acid (NA) or dephosphorylation by aqueous hydrogen fluoride (HF) or left untreated as indicated followed by TX-114 partitioning (A). Proteins in the aqueous (a) and detergent phases (d) (A) as well as of total photoaffinity labeling reactions (A, B) were precipitated under denaturing conditions and then analyzed by SDS−PAGE. Phosphorimages from a typical experiment and quantitative evaluation are given. (Means ± SD, $n = 3$; bold numbers indicate significant differences compared to control at $p \leq 0.01$.)

polypeptides. This argues against involvement of RIIβ and PDE3B in the cAMP-binding detected with LD. Previously, a *G*PI-anchored 54-kDa *c*AMP-binding *e*ctoprotein, called Gce1, has been identified at plasma membranes of isolated rat adipocytes (*37*, *38*). However so far, this protein has escaped purification and sequence determination. To test for the presence of Gce1 at LD from glimepiride-, palmitate- and GO-treated adipocytes, cAMP-binding was assayed before and after cleavage of the GPI anchor with bacterial (G)PI-PLC (Figure 3). Significant cAMP-binding was detected in LD from treated but not basal adipocytes with the relative potency of the different agents (H_2O_2 > glimepiride > palmitate) correlating well to that for the upregulation of the cAMP-to-adenosine conversion (see Figure 2). Strikingly, significant agent-induced cAMP-binding with LD was detectable only in the presence of IBMX. This argues for potent PDE activity intrinsic to LD protein(s), which is most likely due to Gce1 itself, so far recognized as 54-kDa GPI-anchored cAMP-binding protein at the plasma membranes of rodent adipocytes and yeast (*37*, *38*, *45*). Moreover, modification with GPI was also detected with the LD-associated 54-kDa cAMP-binding protein since exposure to bacterial (G)PI-PLC in the absence but not presence of the (G)PI-PLC inhibitor, GPI-2350 (*16*), led to almost complete loss of the cAMP-binding recovered with the reisolated LD.

Putative candidates for the LD-associated 62-kDa AMP-binding protein represent the major AMP-binding and AMP-handling proteins expressed in isolated rat adipocytes, among them a variety of enzymes which are allosterically regulated by AMP (e.g., glycogen phosphorylase, AMP-dependent protein kinase) or convert AMP into other nucleot(s)ides (e.g., adenylate kinase, 5′-Nuc). However, the apparent size

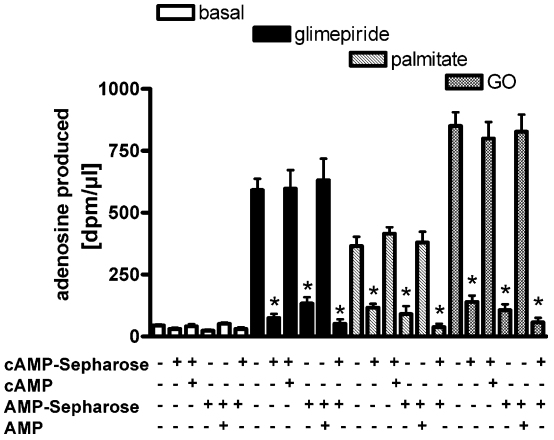

FIGURE 2: Effect of depletion of cAMP- and AMP-binding proteins from LD on the glimepiride-, palmitate- or GO-induced conversion of cAMP to adenosine. Isolated rat adipocytes were incubated (37 °C) in the absence (basal) or presence of glimepiride (10 μM, 60 min), palmitate (1 mM, 120 min) or GO (10 mU/mL, 20 min) and then separated from the medium for the preparation of LD. Subsequently, proteins were extracted from the LD and precipitated under native conditions, then dissolved, and finally adsorbed or not to cAMP-Sepharose and/or AMP-Sepharose in the absence or presence of cAMP or AMP (final concentration 1 mM) as indicated. Proteins in the flow-through or total proteins were precipitated under native conditions and then assayed for cAMP-to-adenosine conversion. (Means ± SD, $n = 4$ adipocyte preparations with determinations in duplicate; * indicates significant differences compared to treatment with glimepiride, palmitate and GO and analysis of unadsorbed proteins at $p \leq 0.01$.)

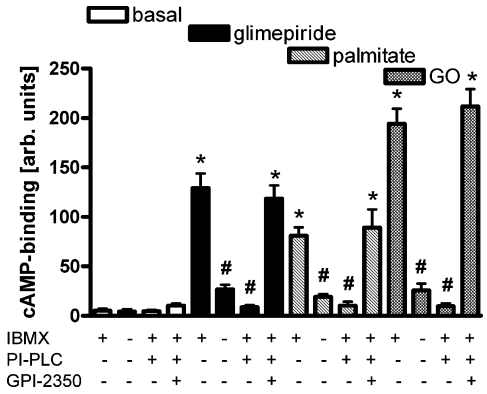

FIGURE 3: Effect of glimepiride, palmitate and GO on cAMP-binding of isolated LD. Isolated rat adipocytes were incubated (37 °C) in the absence (basal) or presence of glimepiride (10 μM, 60 min), palmitate (1 mM, 120 min) or GO (10 mU/mL, 20 min) and then separated from the medium for the preparation of LD. LD were incubated (45 min, 30 °C) without or with PI-PLC (*Bacillus cereus*) in the absence or presence of GPI-2350 (50 μM) and then reisolated by centrifugation through sucrose as described in Materials and Methods. Proteins were extracted from the LD, precipitated under native conditions and then assayed for cAMP-binding in the absence or presence of IBMX (100 μM) as indicated. (Means ± SD, $n = 3$ adipocyte preparations with determinations in triplicate; */# indicate significant differences compared to basal and to glimepiride-, palmitate- and GO-treated adipocytes, respectively, in the presence of IBMX and absence of PI-PLC at $p \leq 0.01$.)

as well as modification with a GPI moiety seemed to be most compatible with the 5′-Nuc, CD73, which has been shown to be anchored at the cell surface of a variety of cells (*60*−*62*), including cultured and primary adipocytes (*37*−*39*). To test for the presence of CD73 at LD in response to glimepiride, palmitate or GO treatment, total proteins were extracted from LD of unlabeled, photoaffinity-labeled or

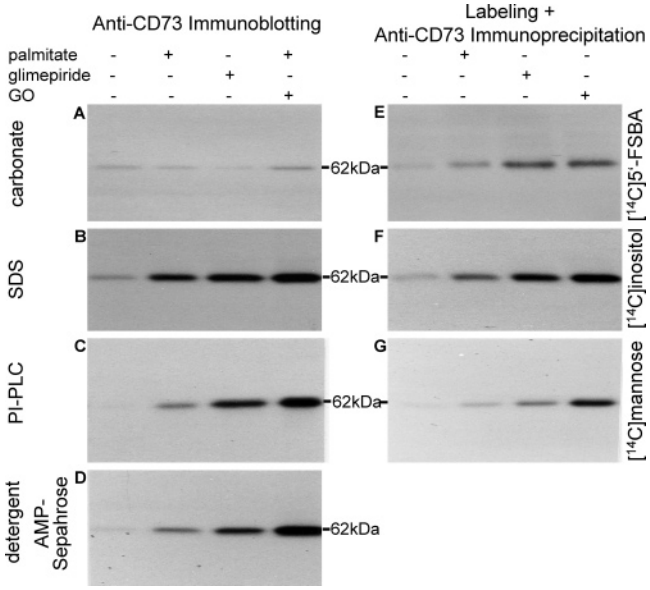

FIGURE 4: Effect of palmitate, glimepiride and GO on the expression of CD73 at LD. Isolated rat adipocytes were incubated (37 °C) with glimepiride (10 μM, 60 min), palmitate (1 mM, 120 min) or GO (100 mU/mL) and then separated from the medium for the preparation of LD. Portions of the LD were extracted for proteins under denaturing conditions (SDS). Other portions were treated with sodium carbonate (1.5 M, ph 11.5, 30 min) or PI-PLC (*B. cereus*, 1 U/mL, 2 h, 37 °C) and then subjected to sucrose gradient centrifugation. Proteins extracted or recovered from the infranatant, respectively, were precipitated under denaturing conditions and then analyzed by SDS−PAGE and immunoblotting for CD73 (BD Biosciences, mouse monoclonal 5F/B9, 0.5 μg/mL). Other portions of the LD were extracted for proteins under native conditions. After precipitation (under native conditions) and dissolution, the proteins were photoaffinity-labeled with [^{14}C]5′-FSBA and after immunoprecipitation with anti-CD73 analyzed by SDS−PAGE. Alternatively, the proteins were adsorbed to AMP-Sepharose and after elution analyzed by SDS−PAGE and immunoblotting for CD73. Chemiluminescence and phosphorimages from a typical experiment are shown repeated once with similar results.

metabolically labeled adipocytes by various methods and then immunoblotted or immunoprecipitated with anti-CD73 antibodies (Figure 4). Extraction with SDS, bacterial (G)PI-PLC or non-ionic detergent followed by adsorption to and elution from AMP-Sepharose, but not extraction with carbonate, led to the release of a 62-kDa anti-CD73 reactive polypeptide from LD of unlabeled treated, but not untreated, adipocytes as shown by the chemiluminescent images (left panels). The same was true for LD of adipocytes which had been photoaffinity labeled with [^{14}C]5′-FSBA or metabolically labeled with the GPI anchor constituents, [^{14}C]mannose or [^{14}C]inositol, as shown by the phosphorimages (right panels). The relative amounts of released anti-CD73 reactive proteins were correlated to the relative potency of the antilipolytic activity and the resulting upregulation of the LD-associated cAMP-to-adenosine conversion exerted by the three agents (H_2O_2 > glimepiride > palmitate, see also Figure 2). The observed crossreactivity of the 62-kDa polypeptide, which is subjected to massive upregulation at LD in response to palmitate, glimepiride and H_2O_2, toward two different anti-CD73 antisera in combination with its photoaffinity labeling with a 5′-AMP analogue and metabolic labeling with typical GPI anchor constituents strongly suggests its identity with the GPI protein and 5′-Nuc, CD73. The extraction behavior of CD73 argues that it binds to LD

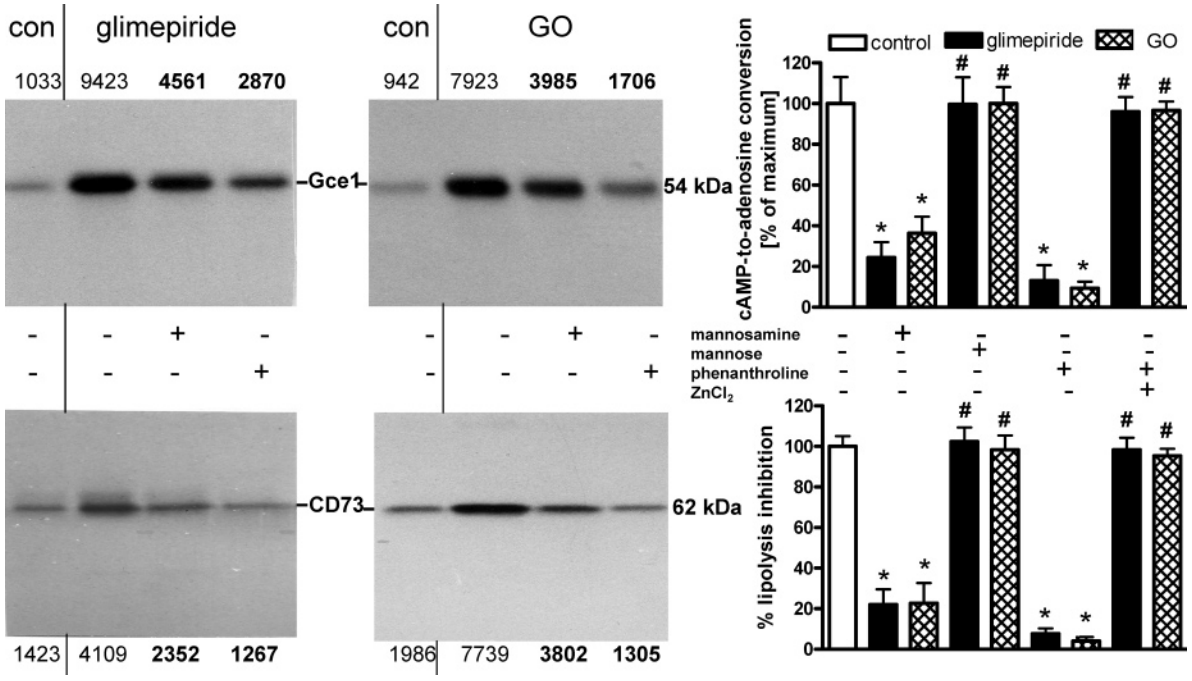

FIGURE 5: Effect of inhibition of GPI protein biosynthesis on the expression of the cAMP- and AMP-binding proteins at LD, cAMP-to-adenosine conversion and lipolysis inhibition. Isolated rat adipocytes were incubated (16 h) in primary culture in the absence or presence of mannosamine (20 mM), mannose (20 mM), 1,10-phenanthroline (250 μM) or $ZnCl_2$ (2 mM) before the addition of glimepiride (final concentration 10 μM) or GO (10 mU/mL) or buffer (control) as indicated. After further incubation (1 h), portions of the adipocytes were separated from the medium for the preparaton of LD. From portions of the LD, proteins were extracted and precipitated under native conditions, then photoaffinity-labeled with 8-N_3-[^{32}P]cAMP or [^{14}C]5′-FSBA, precipitated under denaturing conditions and finally analyzed by SDS−PAGE and phosphorimaging (left panel). Phosphorimages from a typical experiment are shown repeated once with similar results. Quantitative evaluation by phosphorimaging is given (means ± SD, $n = 3$ adipocyte preparations with single determinations; bold numbers indicate significant differences compared to glimepiride and GO treatment in the absence of inhibitor). Other portions of the LD were assayed for cAMP-to-adenosine conversion (right panel), which was set at 100% for glimepiride/GO without any addition. Other portions of the adipocytes were challenged with isoproterenol (1 μM). After further incubation (2 h) the medium was separated from the adipocytes and analyzed for glycerol. Inhibition of glycerol release by glimepiride/GO without any addition was set at 100%. (Means ± SD, $n = 4$ adipocyte preparations with determinations in duplicate; */# indicate significant differences compared to control and glimepiride/GO incubations, respectively, at $p \leq 0.01$.)

via hydrophobic (sensitivity toward SDS and non-ionic detergent) rather than electrostatic interactions (resistence toward carbonate) of its GPI anchor (sensitive toward PI-PLC).

GPI Anchorage and GPI Anchor Cleavage of Gce1 and CD73 Are Required for the Palmitate-, Glimepiride- and H_2O_2-Induced Upregulation of Their Expression at LD. The importance of GPI anchorage for the expression of the cAMP-to-adenosine conversion activity as well as of the 54-kDa cAMP-binding protein, Gce1, and the 62-kDa AMP-binding protein, CD73, at LD was further substantiated by blockade of the GPI protein biosynthesis at the level of the final transfer of the assembled GPI anchor to the protein moiety or at the level of the assembly of the GPI anchor precursor. For this, rat adipocytes were incubated in the long term with the metalloprotease inhibitor, 1,10-phenanthroline, an effective inhibitor of the GPI anchor transferase in mammalian cells (*63*) or mannosamine, a carbohydrate chain terminator during GPI anchor assembly (*64*, *65*). Analysis of the amounts of photoaffinity-labeled Gce1 and CD73 revealed significant reductions in the glimepiride-/GO-induced amounts of both proteins at LD by 60−75 and 80−100% with mannosamine and phenanthroline, respectively (Figure 5). This was paralleled by significant downregulation of the glimepiride-/GO-induced cAMP-to-adenosine conversion by isolated LD and inhibition of isoproterenol-stimulated lipolysis by 60−80 and 85−95% with mannosamine and phenanthroline, respectively. Mannose and phenanthroline in the presence of $ZnCl_2$ had no effect (Figure 5). This strongly argues for a causal relationship between GPI-anchorage, LD expression of Gce1 and CD73, cAMP-to-adenosine conversion by LD and inhibition of lipolysis.

It has been demonstrated in primary and cultured adipocytes that GPI-anchored Gce1 and CD73 located at the cell surface are susceptible to lipolytic cleavage by the activated plasma membrane GPI-PLC (*38*, *39*, *60*), which is susceptible to blockade by the inhibitor GPI-2350 (*16*). The role of GPI anchor cleavage for the expression of Gce1 and CD73 proteins as well as of the cAMP-to-adenosine conversion activity at LD was demonstrated by inhibition of the GPI-PLC in the course of addition of GPI-2350 at various time points during challenge of the rat adipcytes with glimepiride, palmitate or GO. This analysis showed that the stimulus-induced cAMP-to-adenosine conversion activity (Figure 6A) as well as photoaffinity labeling of the 54-kDa cAMP-binding protein, Gce1, (Figure 6B) observed with the LD was upregulated with half-maximal increases at 5−15 min for GO, 15−20 min for glimepiride and 30 min for palmitate. Following maximal levels of conversion activity and amount of Gce1, both declined to almost basal values (glimepiride, GO) or by 30% (palmitate) until the end of the 120-min incubation. The presence of GPI-2350 already at the start of the treatment (0 min) completely blocked upregulation of both conversion activity and amount of Gce1 in response to

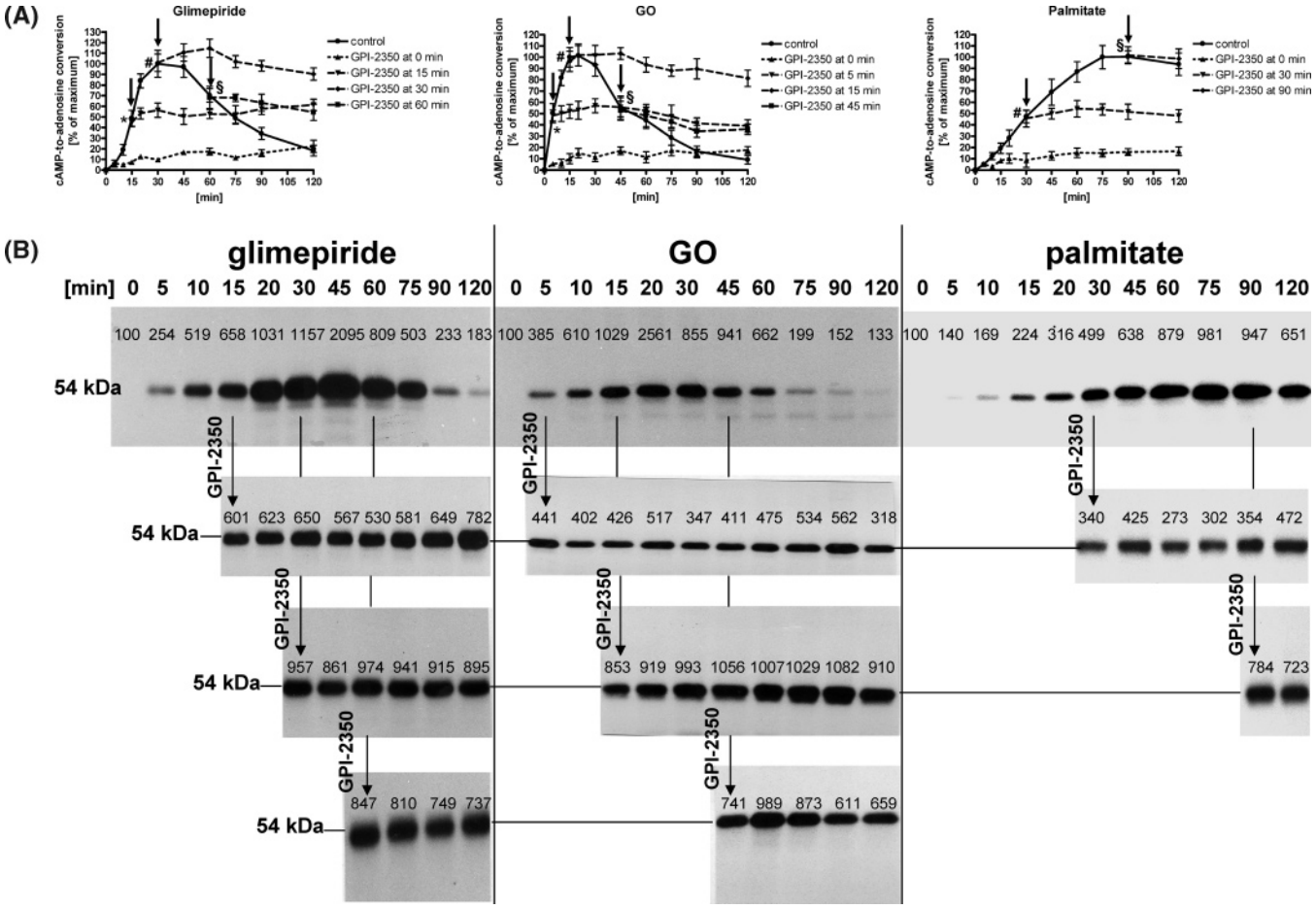

FIGURE 6: Effect of inhibition of PI-PLC on the glimepiride-, palmitate- and GO-induced conversion of cAMP to adenosine by LD. Isolated rat adipocytes were incubated (37 °C) in the absence (basal) or presence of glimepiride (10 μM), palmitate (1 mM) or GO (10 mU/mL) for increasing periods of time. At the time points indicated by arrows, GPI-2350 (final concentration 50 μM) or buffer was added and the incubation was continued for increasing periods. Thereafter, the adipocytes were separated from the medium for the preparation of LD. Portions of the LD were then assayed for the conversion of cAMP to adenosine. (A) The cAMP-to-adenosine conversion at zero time point in the absence of GPI-2350 was subtracted from each value. For each agent this difference was set at 0% for the zero time point and 100% for the maximal conversion (means ± SD, $n = 4$ adipocyte preparations with determinations in triplicate; */#/§ indicate significant differences compared to the 0-min, 15-min basal and 30-min time points, respectively, at $p \leq 0.05$). (B) From other portions of the LD, proteins were extracted and precipitated under native conditions, then photoaffinity-labeled with 8-N_3-[^{32}P]cAMP, precipitated under denaturing conditions and then analyzed by SDS−PAGE and phosphorimaging. Phosphorimages from typical experiment are shown repeated once with similar results. Quantitative evaluation by phosphorimaging is given.

the three stimuli. Addition of GPI-2350 at three different time points (corresponding to half-maximal increases, maximal increases or half-maximal decreases) during the continuous presence of glimepiride, palmitate or GO immediately terminated further upregulation and prevented further loss, respectively, of conversion activity (Figure 6A) and amount of Gce1 (Figure 6B). Thus, both upregulation and loss of the cAMP-to-adenosine conversion activity and the amount of Gce1 at LD apparently depend on functional GPI-PLC.

The GPI Protein, Gce1p, Can Be Reconstituted into LD in Vitro. The identification of GPI-anchored Gce1 and CD73 at LD of lipolytically stimulated adipocytes immediately favors a model for the binding of GPI proteins to LD driven by direct interactions between the fatty acyl chains of their GPI anchors and the phospholipids of the LD surface monolayer. Therefore, the putative reconstitution of a GPI protein into isolated LD was studied *in vitro*, as has been previously successfully performed with liposomes (*66*). For this, [^{14}C]inositol-labeled detergent-solubilized and affinity-purified Gce1p from yeast harboring the intact or (G)PI-PLC-cleaved GPI anchor was incubated with LD prepared from basal rat adipocytes. Upon dilution of the detergent and reisolation of the LD by sucrose gradient centrifugation, proteins were extracted from the LD, then subjected to TX-114 partitioning and finally analyzed by SDS−PAGE and phosphorimaging or measurement of PDE activity. The recovery of [^{14}C]inositol-labeled amphiphilic Gce1p partitioning into the detergent phase (Figure 7A) as well as PDE activity (Figure 7B) with the LD increased with the amounts of Gce1p harboring the intact (−PI-PLC), but not the cleaved GPI anchor (+PI-PLC), used in the reconstitution assay. In contrast, very little hydrophilic Gce1p partitioning into the aqueous phase (Figure 7A) was found associated with the LD after incubation with either intact or cleaved Gce1p. As expected, soluble PDE from bovine brain used as a control was not recovered with LD to any significant degree (Figure 7B). The specificity of the *in vitro* reconstitution was demonstrated by incubations of Gce1p with pure tripalmitin or triolein or of detergent-solubilized insulin receptor and glucose transporter isoform 4 with adipocyte LD. None of these proteins was recovered significantly with the top fractions upon sucrose gradient centrifugation (data not

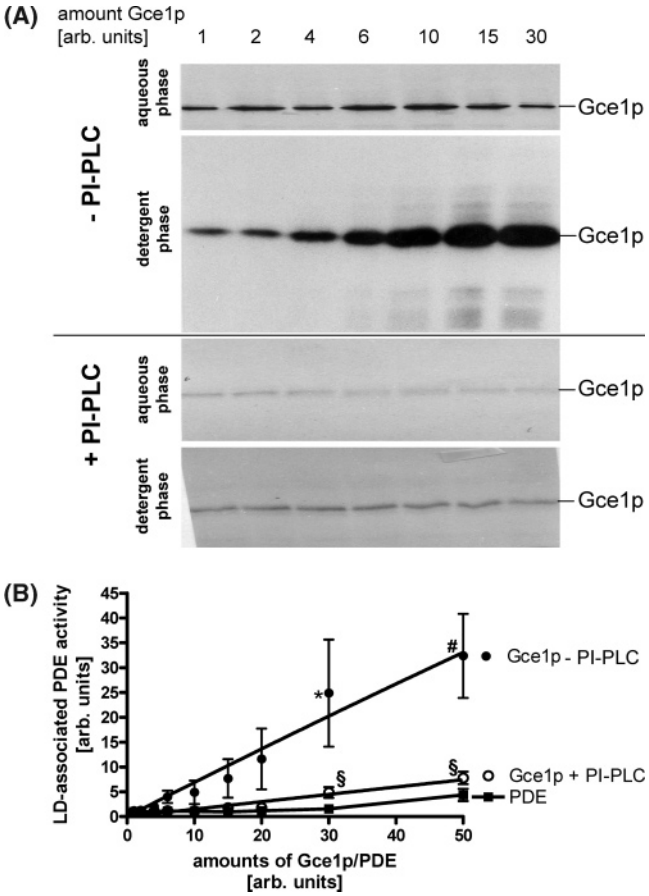

FIGURE 7: Reconstitution of Gce1 into LD. Gce1p was partially purified from yeast metabolically labeled with *myo*-[^{14}C]inositol and then treated in the absence or presence of PI-PLC (*Bacillus cereus*) as described in Materials and Methods. Subsequently, increasing amounts of detergent-solubilized Gce1p were incubated (30 min, 37 °C) with LD prepared from basal rat adipocytes. As a control, PDE (bovine brain) suspended in the same buffer (0.5 U/mL) was used instead of Gce1p. After removal of the detergent by dialysis, LD were recovered from the incubation mixtures by centrifugation through sucrose cushions and then washed by flotation. Proteins were extracted and then precipitated under native conditions. (A) Portions of the dissolved proteins were partitioned between aqueous and detergent (TX-114) phases, then precipitated under denaturing conditions and after dissolution analyzed by SDS−PAGE. The phosphorimages of a typical experiment are shown repeated once with similar results. (B) Other portions of the dissolved proteins were assayed for LD-associated PDE activity. (Means ± SD of 3 independent adipocyte preparations and PDE measurements in quadruplicate; */# indicate significant differences compared to the use of PDE at $p \leq 0.05$ and ≤ 0.01, respectively; § indicates significant differences compared to the use of Gce1p-P1-PLC at $p \leq 0.05$.)

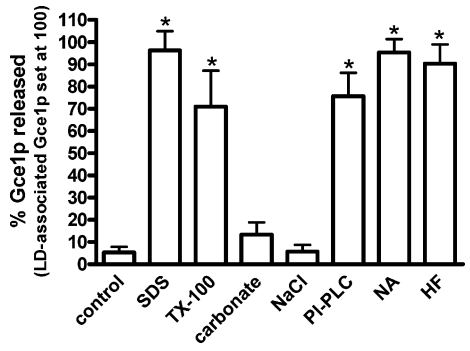

FIGURE 8: Release of Gce1 from reconstituted LD. LD reconstituted with anchor-containing Gce1p (30 arbitrary units) and recovered by centrifugation through sucrose cushions (3500 dpm, ~20 μL) were incubated with the 100 μL of SDS (10%, 10 min, 65 °C), 20% TX-100 (60 min, 37 °C), 1.5 M NaCl (60 min, 37 °C) or 1.5 M sodium carbonate (pH 11.5, 30 min, 37 °C) or subjected to treatment with PI-PLC (*B. cereus*), nitrous acid (NA) or hydrogen fluoride (HF) as described in Materials and Methods. Thereafter, 100-μL portions of the incubation mixtures were centrifuged through sucrose cushions. The infranatant below the floating upper lipids was removed by suction and then used for precipitation of proteins under denaturing conditions. After dissolution radiolabeled Gce1p was analyzed by SDS−PAGE and quantitatively evaluated by phosphorimaging with the amount remaining associated with untreated LD after the centrifugation set at 100%. (Means ± SD of 4 adipocyte preparations with determinations in duplicate; * indicates significant differences compared to control at $p \leq 0.01$.)

shown). Analysis of the type of interaction of Gce1p with the *in vitro* reconstituted adipocyte LD demonstrated that Gce1p is associated *via* hydrophobic rather than mere electrostatic interactions (with the phospholipid monolayer or other LD proteins) since it was significantly released by treatment of the LD with hot SDS or TX-100, but resisted release by incubation with carbonate or high salt (Figure 8). Compatible with the GPI anchorage of Gce1p at LD, treatment with nitrous acid, aqueous HF or bacterial (G)PI-PLC, which causes specific cleavage of GPI structures at distinct sites (*45*, *50*, *67*), resulted in almost complete liberation of radiolabeled Gce1p from LD. This extraction behavior of Gce1p from reconstituted LD, which is identical to that of CD73 from authentic LD (see Figure 4), argues for direct binding of GPI proteins to LD *via* their GPI anchor, which can be reconstituted *in vitro*.

The possibility to label LD with different GPI proteins either by metabolic labeling of adipocytes and subsequent preparation of the LD or by reconstitution of labeled and purified GPI proteins into LD *in vitro* opened the possibility to study the putative exchange of GPI proteins between distinct LD. For this, LD reconstituted with [^{14}C]inositol-labeled Gce1p from yeast and LD from [^{14}C]inositol-labeled and GO-treated adipocytes were incubated together with rat adipocyte cytosol in the presence of an ATP-regenerating system. After reisolation, the LD were adsorbed to either cAMP- or AMP-Sepharose beads and then specifically eluted for the separate recovery of Gce1p- or CD73-containing LD, respectively. SDS−PAGE and phosphorimaging of the extracted radiolabeled proteins revealed (Figure 9) that after 30 min incubation in the presence of cytosol, the Gce1p- and CD73-containing LD were quantitatively recovered with the cAMP- and AMP-Sepharose beads, respectively (as reflected in similar amounts of Gce1p and CD73 after combined compared to separate incubation of the two LD species). Remarkably, the Gce1p- or CD73-containing LD harbored small but significant amounts of CD73 and Gce1p, respectively, i.e., of that GPI protein counterpart that initially was not contained in the prepared or reconstituted LD species and therefore did not adsorb to cAMP- and AMP-Sepharose beads, respectively. In contrast, after incubation without cytosol or control incubation (zero time) or incubation of a single LD species only (Figure 9) or incubation in the absence of ATP-regenerating system (data not shown) Gce1p but not CD73 was eluted from cAMP-Sepharose and *vice versa* CD73 but not Gce1p from AMP-Sepharose. This argues for the specificity of the reaction as well as detection. Only minute amounts of Gce1p and CD73 were recovered

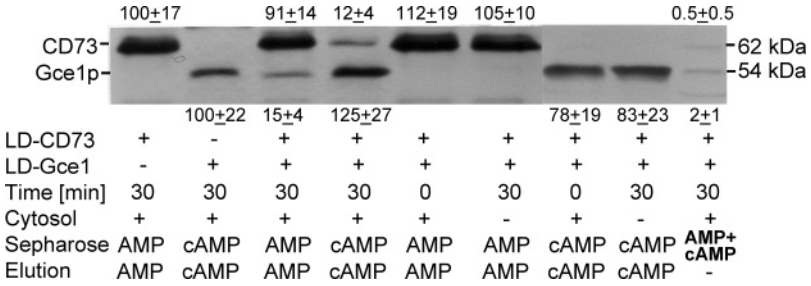

FIGURE 9: Exchange of GPI proteins between LD *in vitro*. LD prepared from untreated rat adipocytes and reconstituted with *myo*-[^{14}C]-inositol-labeled Gce1p from yeast (LD-Gce1; 1500−2000 dpm; corresponding to 2×10^6 adipocytes) were incubated (head-over rotation in 1-mL reaction vessels, 30 rpm, 37 °C) with LD prepared from *myo*-[^{14}C]inositol-labeled and GO-treated adipocytes (LD-CD73; 5000−7000 dpm; corresponding to 2×10^6 adipocytes) in the presence or absence of cytosol (125 μg of protein) from untreated adipocytes in 0.5 mL of buffer R as described for the reconstitution of LD. After 0 or 30 min, the LD were reisolated by flotation (500*g*, 2 min) and then incubated (head-over rotation in 1-mL reaction vessels, 100 rpm, 4 h, 12 °C) with cAMP-Sepharose and/or AMP-Sepharose (40 mg) in 0.5 mL of buffer R. Thereafter, the Sepharose beads were collected by centrifugation (10000*g*, 2 min), washed three times with 1 mL of buffer R each and then eluted with 1 mL of buffer R without or with 1 mM cAMP or AMP as indicated. After incubation (head-over rotation, 20 min, 12 °C), the LD released were separated from the Sepharose beads by centrifugation (10000*g*, 2 min) and then recovered from the top. Proteins were extracted from the LD and precipitated under denaturating conditions and then analyzed by SDS−PAGE. The phosphorimages of a typical experiment are shown. Quantitative evaluation (mean ± SD of three adipocyte incubations) is given with the amounts of CD73 and Gce1p recovered from incubations of CD73 and Gce1p each alone set at 100 each.

when the elution of combined cAMP/AMP-Sepharose was performed without both cAMP and AMP (Figure 9). This demonstrates specific binding of Gce1p-/CD73-containing LD to the Sepharose beads and efficient removal of unbound LD prior to elution. In conclusion, at least one of the two LD populations initially equipped with distinct GPI proteins, each, apparently has obtained a limited amount (~10%) of the GPI protein from its LD counterpart prior to its specific recovery on the basis of its initial GPI protein content. Alternatively, partial fusion between the two LD species may have occurred during the incubation prior to their recovery. Compatible with fusion are recent findings with intact NIH 3T3 cells or a cell-free system that LD can grow by fusion after they have been assembled which is independent of TAG synthesis and requires the interaction of cytosolic ATP-driven motor proteins, such as dynein, and intact microtubules (*68*). It may thus be useful to study the effect of microtubule depolymerization, e.g., by nocodazole, on the exchange of GPI proteins between distinct LD in the cell-free system. In any case, this process is time-, cytosol- and ATP-dependent and is therefore most likely physiological rather than artificial (due to, e.g., mechanical stress, fragmentation, spontaneous coagulation), but apparently independent of TAG *de novo* synthesis (absence of glucose and FA during the incubation).

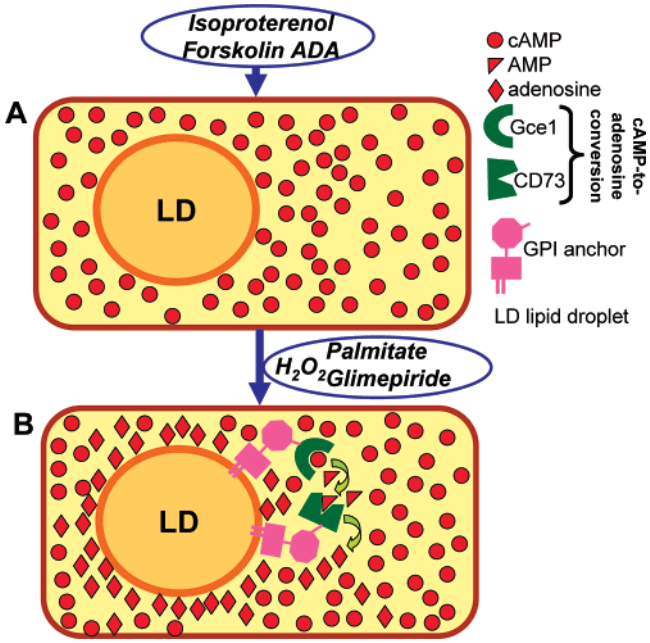

FIGURE 10: Model for the degradation of cAMP by GPI proteins at the surface of LD in rat adipocytes. (A) Short-term challenge with isoproterenol, ADA or forskolin leads to elevation of total cytosolic cAMP. (B) Palmitate, glimepiride and H_2O_2 cause conversion of cAMP *via* AMP to adenosine and thereby cAMP lowering in the immediate vicinity of the LD, only, through upregulation of the GPI-anchored PDE, Gce1, and 5′-Nuc ,CD73, at the LD surface.

DISCUSSION

Association of the GPI Proteins, Gce1 and CD73, with LD. The present study shows, for the first time, that GPI proteins can be associated with LD facing the cytoplasm rather than embedded in the outer leaflet of the plasma membrane or luminal leaflets of the endoplasmic reticulum/Golgi membranes facing the extracellular or vesicular space, respectively (Figure 10). The following findings provide strong evidence for this unexpected localization of GPI proteins at LD: (i) With the exception of the localization, the proteins newly identified at LD of rat adipocytes share all the properties previously found for the GPI proteins, Gce1 (*37*) and CD73 (*39*, *60*−*62*), i.e., apparent molecular weight (Figure 1A), photoaffinity labeling with 8-N_3-[^{32}P]cAMP and [^{14}C]5′-FSBA (Figure 1A), binding to cAMP-Sepharose and AMP-Sepharose (Figure 2), binding of cAMP (Figure 3), immunoreactivity with anti-CD73 antisera (Figure 4), amphiphilic nature and amphiphilic-to-hydrophilic conversion through cleavage by bacterial PI-PLC, nitrous acid deamination and hydrogen fluoride dephosphorylation (Figure 1A) and metabolic labeling with typical GPI anchor constituents (Figure 4). (ii) Expression of Gce1 and CD73 at LD is blocked by interference with GPI anchor biosynthesis (Figure 5). (iii) Expression of Gce1 and CD73 at LD is blocked by interference with GPI anchor cleavage by GPI-PLC (Figure 6). (iv) Gce1p prepared from yeast with intact but not lipolytically cleaved GPI anchor associates with LD *in vitro* (Figure 7). It is highly unlikely that the sucrose gradient-purified LD fractions used in the present study contained

significant amounts of plasma membranes or endoplasmic reticulum/Golgi membranes. In fact, LD differ considerably from membranous organelles with regard to both protein composition and buoyant density as revealed by immunoblotting for typical marker proteins and flotation or sedimentation, respectively, during centrifugation, which renders LD to one of the most conveniently producable and purest subcellular fraction (*83*).

Apparently, localization of GPI proteins at LD has escaped detection up to now despite the use of several subproteomic analyses of LD using different cell types (*31*, *34*−*36*), including basal and isoproterenol-induced adipocytes (*29*, *30*). This failure of the application of standard proteomics protocols may be explained as follows: (i) LD expression of GPI proteins is very low in the basal state of cells and requires induction, e.g., in rat adipocytes by antilipolytic agents, such as glimepiride, palmitate and H_2O_2 (see also discussion below). (ii) LD expression of GPI proteins seems to be transient, only, e.g., in rat adipocytes with rapid increase upon lipolysis inhibition and subsequent gradual decline to almost basal level (Figure 6). (iii) Only a subset of GPI proteins seems to be expressed at LD, e.g., in rat adipocytes Gce1 and CD73, since in preliminary studies the GPI protein, alkaline phosphatase, was not recovered with LD from basal and anti-lipolytically induced adipocytes to any significant degree (Müller, G., Schulz, A., unpublished results). (iv) GPI modification of proteins cannot be predicted with certainty from the amino acid sequence (*67*, *71*, *79*) and its experimental demonstration requires specific biochemical analyses which have not been applied to LD proteins so far. (v) The sequences of some LD-associated GPI proteins may not have been published so far, which is the case for Gce1 of rodent adipocytes, preventing their detection by mass spectroscopy. (vi) Proper separation of GPI proteins by two-dimensional gels requires cleavage of their GPI anchors by appropriate phospholipases, presumably to avoid interference of the hydrophobic phospholipid moiety of GPI anchors during isoelectric focusing. This has been demonstrated for detergent-resistant membrane fractions of BHK cells from which six GPI proteins were identified only upon PI-PLC treatment (*69*). The "shave-and-conquer" concept also led to the identification by capillary liquid chromatography and tandem mass spectroscopy of hitherto unknown GPI proteins of human and plant plasma membranes and detergent-resistant membrane fractions following their lipolytic release (*70*). The use of phospholipase D instead of PI-PLC as performed in that investigation may be of general advantage since the former activity is not affected by structural heterogeneity of the GPI anchor (*71*). Unfortunately, subproteomic 2-D-based analyses of phospholipase-treated LD have not been reported so far. There are additional examples for LD-associated proteins which were identified by rigorous biochemical analysis rather than 2-D proteomics. Recently, Fujimoto and co-workers described the production of radiolabeled TAG and cholesterylester by LD from cultured liver cells upon incubation with radiolabeled oleoyl-CoA (*72*). This implies the presence of acyl-coenzyme A:diacylglycerol/cholesterol acyltransferases in LD which both remained undetected in former proteomic studies of LD proteins of the same cells (*34*) as well as adipocytes (*30*).

Taken together, the 52-kDa cAMP-binding protein, Gce1, and the 62-kDa AMP-binding protein, CD73, apparently constitute the components of the cAMP-to-adenosine conversion activity found associated with LD (Figure 2). They presumably act as the rolipram-sensitive PDE and the 5′-Nuc, respectively, which are apparently involved in local lowering of cAMP in the vicinity of LD (Figure 10) and downregulation of lipolysis by palmitate, glimepiride and H_2O_2 (*83*). The PDE activity of adipocyte Gce1 has escaped detection so far, which is explained best with the presence of IBMX in the assays used previously for the identification of Gce1 as a cAMP-binding protein (filtration assay for [^{3}H]-cAMP binding, photoaffinity labeling with 8-N_3-[^{32}P]cAMP, affinity purification on cAMP-Sepharose; refs *37*−*39*). Gce1 shares with PDE4 the sensitivity toward rolipram (*73*, *74*, *83*), but nevertheless represents a novel type of PDE with unique properties: It is (i) modified with a GPI moiety, (ii) localized both at the cell surface and at the LD surface in adipocytes and (iii) regulated by change in its localization rather than by phosphorylation (Müller, G., and Schulz, A., unpublished data). However, final proof that Gce1 and CD73 exert the PDE and 5′-Nuc activities, which in concert underly the LD-associated cAMP-to-adenosine conversion activity, will require gene disruption and overexpression studies.

Upregulation of the LD Association of Gce1 and CD73 by Palmitate, Glimepiride and H_2O_2. The association of the two GPI proteins, Gce1 and CD73, with LD was recognized as concentration-dependent increases in their photoaffinity-labeling (Figure 1B) and in the cAMP-to-adenosine conversion activity (Figure 2) of LD prepared from glimepiride-, palmitate- and H_2O_2-induced adipocytes, but hardly of LD from insulin-induced and basal cells. A causal relationship between the upregulation of the LD expression of Gce1 and CD73, cAMP-to-adenosine conversion by LD and inhibition of isoproterenol-stimulated lipolysis in response to palmitate, glimepiride and H_2O_2 was suggested by their blockade upon inhibition of GPI anchor biosynthesis (Figure 5) and, unexpectedly, inhibition of the GPI-PLC (Figure 6). The requirement for GPI-PLC action for upregulation of LD expression is reminiscent of the previously reported translocation of Gce1 and CD73 from high cholesterol- to low cholesterol-containing plasma membrane microdomains in rat adipocytes (*13*, *15*, *16*). Future studies have to address the molecular mechanism for the upregulation of Gce1 and CD73 at LD including their site(s) of synthesis and storage and the role of GPI anchor cleavage. The results may also help understanding how GPI proteins gain access to the cytoplasmic phospholipid monolayer of the LD, since they typically use vesicular trafficking for their transport to and from the cell surface with the polypeptide as well as the glycan portion of the GPI anchor always facing the non-cytoplasmic (luminal and extracellular) spaces (*2*, *4*, *75*). Moreover, inhibition of the GPI-PLC also prevented the gradual loss of Gce1 and CD73 from LD. Thus, Gce1 and CD73 can be released from the LD surface by lipolytic cleavage as observed here following their maximal LD expression despite the continuous presence of palmitate, glimepiride and H_2O_2 (Figure 6). This downregulation of Gce1 and CD73 at LD may reflect desensitization or feedback inhibition in the course of prolonged antilipolytic challenge of adipocytes.

Reconstitution of GPI Proteins into LD in Vitro. At a first glance, GPI modification of proteins seems to fit perfectly to their anchorage at the surface phospholipid monolayer of

LD with the proteinaceous ectodomain facing entirely the cytoplasm and enabling the handling of hydrophilic ligands and substrates, such as cAMP by Gce1 and AMP by CD73. Typical LD-associated proteins of hydrophobic nature and with structural function have been found to traverse the LD from the outer shell to the inner core during their life cycle (*57*), which would impede any catalytic function at the LD surface. Furthermore, it is conceivable that GPI proteins associate with preformed LD by spontaneous or protein-mediated partitioning of the fatty acyl moieties of their GPI anchor into the LD phospholipid monolayer. This biosynthetic pathway would enable upregulation of GPI protein expression at preformed LD in the short term as observed here for Gce1 and CD73 in response to glimepiride, palmitate and H_2O_2. The underlying mechanisms may be analogous to the recently elucidated guidance of C-tail-anchored proteins with a transmembrane domain close to their carboxy-terminus to the cytoplasmic leaflet of the ER membrane (*76*). In the case of moderate hydrophobicity and a certain degree of water solubility of the transmembrane domain, the C-tail-anchored proteins apparently insert spontaneously after contacting the bilayer without assistance from proteins. C-tail-anchored proteins with more hydrophobic transmembrane domains, which tend to form aggregates, require interaction with a chaperone to remain in the insertion-competent state during cytoplasmic passage and to be released for spontaneous insertion into the bilayer after contact of the chaperone with the cytoplasmic face of the endoplasmic reticulum membrane. On the basis of the chain length and saturation of the fatty acyl moieties found in typical GPI proteins (*67*), it is conceivable that *in vivo* the GPI anchors of Gce1 and CD73 have to be masked by chaperons to facilitate their transfer from the site of their synthesis/storage to the LD surface. Interestingly, the chaperone HSP70 has recently been found to be associated with LD in 3T3-L1 adipocytes in transient and regulated fashion (*77*). The demonstrated reconstitution of purified Gce1p from yeast into LD from rat adipocytes *in vitro* (Figure 7) which mimics the extraction behavior of adipocyte Gce1 from authentic adipocyte LD (Figure 8) suggests that spontaneous or protein-mediated insertion of GPI proteins into LD may occur also *in vivo*.

The possibility of the reconstitution of any recombinant GPI protein of interest into LD of various origin (*78*, *79*) in combination with the observed exchange of the reconstituted GPI proteins between distinct LD (Figure 9) may have broad implications for future biotechnological applications which encompass "two-dimensional" binding or catalytic processes occurring at the (phospho)lipid−water interface. Lipases, for instance, are well-known to alter their kinetic properties and/or specificity upon contact with hydrophobic surfaces, such as lipidic substrates, a phenomenon called interfacial activation (*80*). It will be interesting to study the hydrolytic efficacy of a recombinant lipase with a covalently attached GPI anchor after its reconstitution into LD.

In conclusion, the presented findings of the transient expression of certain GPI proteins, such as Gce1 and CD73, at the LD of adipocytes supplement the growing list of LD-associated proteins recruited from other cellular compartments in a cell type-specific and regulated fashion (*81*, *82*). In addition to the storage of neutral lipids and the regulation of lipid metabolism, LD might therefore have a general role in managing the availability and sequestration of proteins for various purposes, such as regulated inactivation of proteins, prevention of toxic protein aggregates, and localized delivery of signaling molecules, as is apparently true for the (c)AMP-hydrolyzing enzymes, Gce1 and CD73.

REFERENCES

1. Low, M. G., and Saltiel, A. R. (1988) Structural and functional roles of glycosylphosphatidylinositol in membranes, *Science 239*, 268−275.
2. Ikezawa, H. (2002) Glycosylphosphatidylinositol (GPI)-Anchored Proteins, *Biol. Pharm. Bull. 25*, 409−417.
3. Takeda, J., and Kinoshita, T. (1995) GPI-anchor biosynthesis, *Trends Biochem. Sci. 20*, 367−371.
4. Orlean, P., and Menon, A. K. (2007) Thematic review series: lipid posttranslational modifications. GPI anchoring of protein in yeast and mammalian cells, or: how we learned to stop worrying and love glycophospholipids, *J. Lipid Res. 48*, 993−1011.
5. Nozaki, M. K., Ohishi, K., Yamada, N., Kinoshita, T., Nagy, A., and Takeda, J. (1999) Developmental abnormalities of glycosylphosphatidylinositol-anchor-deficient embryos revealed by Cre/loxP system, *Lab. Invest. 79*, 293−299.
6. Almeida, A. M., Murakami, Y., Layton, D. M., Hillmen, P., Sellick, G. S., Maeda, Y., Richards, S., Patterson, S., Kotsianidis, I., and Mollica, L. (2006) Hypomorphic promoter mutation in PIGM causes inherited glycosylphosphatidylinositol deficiency, *Nat. Med. 12*, 846−851.
7. Inoue, N. Y., Murakami, Y., and Kinoshita, T. (2003) Molecular genetics of paroxysmal nocturnal hemoglobinuria, *Int. J. Hematol. 77*, 107−112.
8. Brown, D. A., and London, E. (1998) Functions of lipid rafts in biological membranes, *Annu. Rev. Cell Dev. Biol. 14*, 111−136.
9. Simons, K., and Toomre, D. (2000) Lipid rafts and signal transduction, *Nat. Rev. Mol. Cell Biol. 1*, 31−39.
10. Helms, J. B., and Zurzolo, C. (2004) Lipids as targeting signals: lipid rafts and intracellular trafficking, *Traffic 5*, 247−254.
11. Stefanova, I., Horejsi, V., Ansotegui, I. J., Knapp, W., and Stockinger, H. (1991) GPI-anchored cell-surface molecules complexed to protein tyrosine kinases, *Science 254*, 1016−1019.
12. Chen, Y., Thelin, W. R., Yang, B., Milgram, S. L., and Jacobsen, K. (2006) Transient anchorage of cross-linked glycosyl-phosphatidylinositol-anchored proteins depends on cholesterol, Src family kinases, caveolin, and phosphoinositides, *J. Cell Biol. 175*, 169−178.
13. Müller, G., Hanekop, N., Wied, S., and Frick, W. (2002) Cholesterol depletion blocks redistribution of lipid raft components and insulin-mimetic signaling by glimepiride and phosphoinositolglycans in rat adipocytes, *Mol. Med. 8*, 120−136.
14. Babiychuk, E. B., and Draeger, A. (2006) Biochemical characterization of detergent-resistant membranes: a systematic approach, *Biochem. J. 397*, 407−416.
15. Müller, G., Jung, C., Wied, S., Welte, S., Jordan, H., and Frick, W. (2001) Redistribution of glycolipid raft domain components induces insulin-mimetic signaling in rat adipocytes, *Mol. Cell. Biol. 21*, 4553−4567.
16. Müller, G., Schulz, A., Wied, S., and Frick, W. (2005) Regulation of lipid raft proteins by glimepiride- and insulin-induced glycosylphosphatidylinositol-specific phospholipase C in rat adipocytes, *Biochem. Pharmacol. 69*, 761−780.
17. Braun-Breton, C., Rosenberry, T. L., and da Silva, L. P. (1988) Induction of the proteolytic activity of a membrane protein in *Plasmodium falciparum* by phosphatidyl inositol-specific phospholipase C, *Nature 332*, 457−459.
18. Brewis, I. A., Turner, A. J., and Hooper, N. M. (1994) Activation of the glycosylphosphatidylinositol-anchored membrane dipeptidase upon release from pig kidney membranes by phospholipase C, *Biochem. J. 303*, 633−638.
19. Lehto, M. T., and Sharom, F. J. (1998) Release of the glycosylphosphatidylinositol-anchored enzyme eco-5′-nucleotidase by phospholipase C. catalytic activation and modulation by the lipid bilayer, *Biochem. J. 332*, 101−109.
20. Müller, G., and Bandlow, W. (1994) Lipolytic membrane release of two phosphatidylinositol-anchored cAMP receptor proteins in yeast alters their ligand-binding parameters, *Arch. Biochem. Biophys. 308*, 504−514.

21. Wang, X., Jansen, G., Fan, J., Kohler, W. J., Ross, J. F., Schornagel, J., and Ratnam, M. (1996) Variant GPI structure in relation to membrane-associated functions of a murine folate receptor, *Biochemistry 35*, 16305−16312.
22. Zweytick, D., Athenstaedt, K., and Daum, G. (2000) Intracellular lipid particles of eukaryotic cells, *Biochim. Biophys. Acta 1469*, 101−120.
23. Granneman, J. G., Li, P., Lu, Y., and Tilak, J. (2004) Seeing the trees in the forest: selective electroporation of adipocytes within adipose tissue, *Am. J. Physiol. Endocrinol. Metab. 287*, E574−E582.
24. Unger, R. H. (2002) Lipotoxic diseases, *Annu. Rev. Med. 53*, 319−336.
25. DeFronzo, R. A. (2004) Dysfunctional fat cells, lipotoxicity and type 2 diabetes, *Int. J. Clin. Pract. (Suppl.)*, 9−21.
26. Murphy, D. J., and Vance, J. (1999) Mechanisms of lipid-body formation, *Trends Biochem. Sci. 24*, 109−115.
27. Brown, D. A. (2001) Lipid droplets: proteins floating on a pool of fat, *Curr. Biol. 11*, R446−R449.
28. Tauchi-Sato, K., Ozeki, S., Houjou, T., Taguchi, R., and Fujimoto, T. (2002) The surface of lipid droplets is a phospholipid monolayer with a unique fatty acid composition, *J. Biol. Chem. 277*, 44507−44512.
29. Bartz, R., Li, W-H., Venables, B., Zehmer, J. K., Roth, M. R., Welti, R., Anderson, G. W., Liu, P., and Chapman, K. D. (2007) Lipidomics reveals that adiposomes store ether lipids and mediate phospholipid traffic, *J. Lipid Res. 48*, 837−847.
30. Brasaemle, D. L., Dolios, G., Shapiro, L., and Wang, R. (2004) Proteomic analysis of proteins associated with lipid droplets of basal and lipolytically stimulated 3T3-L1 adipocytes, *J. Biol. Chem. 279*, 46835−46842.
31. Liu, P., Ying, Y., Zhao, Y., Mundy, D. I., Zhu, M., and Anderson, R. G. W. (2004) Chinese hamster ovary K2 cell lipid droplets appear to be metabolic organelles involved in membrane traffic, *J. Biol. Chem. 279*, 3787−3792.
32. Wolins, N. E., Brasaemle, D. L., and Bickel, P. E. (2006) A proposed model of fat packaging by exchangeable lipid droplet proteins, *FEBS Lett. 580*, 5484−5491.
33. Brasaemle, D. L., Rubin, B., Harten, I. A., Gruia-Gray, J., Kimmel, A. R., and Londos, C. (2000) Perilipin A increases triacylglycerol storage by decreasing the rate of triacylglycerol hydrolysis, *J. Biol. Chem. 275*, 38486−38493.
34. Fujimoto, Y., Itabe, H., Sakai, J., Makita, M., Noda, J., Mori, M., Higashi, Y., Kojima, S., and Takano, T. (2004) Identification of major proteins in the lipid droplet-enriched fraction isolated from the human hepatocyte cell line HuH7, *Biochim. Biophys. Acta 1644*, 47−59.
35. Beller, M., Riedel, D., Jänsch, L., Dieterich, G., Wehland, J., Jäckle, H., and Kühnlein, R. P. (2006) Characterization of the *Drosophila* lipid droplet subproteome, *Mol. Cell. Proteomics 5*, 1082−194.
36. Gutierrez, E., Wiggins, D., Fielding, B., and Gould, A. P. (2007) Specialized hepatocyte-like cells regulate *Drosophila* lipid metabolism, *Nature 445*, 275−280.
37. Müller, G., Wetekam, E.-M., Jung, C., and Bandlow, W. (1994) Membrane association of lipoprotein lipase and a cAMP-binding ectoprotein in rat adipocytes, *Biochemistry 33*, 12149−12159.
38. Müller, G., Dearey, E.-A., Korndörfer, A., and Bandlow, W. (1994) Stimulation of a glycosyl phosphatidylinositol-specific phospholipase by insulin and the sulfonylurea, glimepiride, in rat adipocytes depends on increased glucose transport, *J. Cell. Biol. 126*, 1267−1276.
39. Müller, G., Dearey, E. A., and Pünter, J. (1993) The sulphonylurea drug, glimepiride, stimulates release of glycosylphosphatidylinositol-anchored plasma-membrane proteins from 3T3 adipocytes, *Biochem. J. 289*, 509−521.
40. Colman, R. F., Pal, P. K., and Wyatt, J. L . (1977) Adenosine derivatives for dehydrogenases and kinases, *Methods Enzymol. 46*, 240−249.
41. Müller, G., Korndörfer, A., Saar, K., Karbe-Thonges, B., Fasold, H., and Müllner, S. (1994) 4′-Amino-benzamido-taurocholic acid selectively solubilizes glycosyl-phosphatidylinositol-anchored membrane proteins and improves lipolytic cleavage of their membrane anchors by specific phospholipases, *Arch. Biochem. Biophys. 309*, 329−340.
42. Müller, G., Hanekop, N., Kramer, W., Bandlow, W., and Frick, W. (2002) Interaction of phosphoinositolglycan(-peptides) with plasma membrane lipid rafts of rat adipocytes, *Arch. Biochem. Biophys. 408*, 17−32.
43. Müller, G., Jordan, H., Jung, C., Kleine, H., and Petry, S. (2003) Analysis of lipolysis in adipocytes using a fluorescent fatty acid derivative, *Biochimie 85*, 1245−1256.
44. Müller, G., and Wied, S. (1993) The sulfonylurea drug, glimepiride, stimulates glucose transport, glucose transporter translocation, and dephosphorylation in insulin-resistant rat adipocytes in vitro, *Diabetes 42*, 1852−1867.
45. Müller, G., Schubert, K., Fiedler, F., and Bandlow, W. (1992) The cAMP-binding ectoprotein from *Saccharomyces cerevisiae* is membrane-anchored by glycosyl-phosphatidylinositol, *J. Biol. Chem. 267*, 25337−25346.
46. Stochaj, U., and Mannherz, H. G. (1990) Affinity labelling of 5′-nucleotidases with 5′-*p*-fluorosulphonylbenzoyladenosine, *Biochem. J. 266*, 447−451.
47. Wiseman, T., Williston, S., Brandts, J. F., and Lin, L. N. (1989) Rapid measurement of binding constants and heats of binding using a new titration calorimeter, *Anal. Biochem. 179*, 131−137.
48. Müller, G., and Bandlow, W. (1989) An amphitropic cAMP-binding protein in yeast mitochondria. 1. Synergistic control of the intramitochondrial location by calcium and phospholipid, *Biochemistry 28*, 9957−9967.
49. Bordier, C. (1981) Phase separation of integral membrane proteins in Triton X-114 solution, *J. Biol. Chem. 256*, 1604−1607.
50. Müller, G., Wied, S., Crecelius, A., Kessler, A., and Eckel, J. (1997) Phosphoinositolglycan-peptides from yeast potently induce metabolic insulin actions in isolated rat adipocytes, cardiomyocytes and diaphragms, *Endocrinology 138*, 3459−3475.
51. Greenberg, A. S., Egan, J. J., Wek, S. A., Garty, N. B., Blanchette-Mackie, E. J., and Londos, C. (1991) Perilipin, a major hormonally regulated adipocyte-specific phosphoprotein associated with the periphery of lipid storage droplets, *J. Biol. Chem. 266*, 11341−11346.
52. Wolins, N. E., Quaynor, B. K., Skinner, J. R., Schoenfish, M. J., Tzekov, A., and Bickel, P. E. (2005) S3−12, Adipophilin, and TIP47 package lipid in adipocytes, *J. Biol. Chem. 280*, 19146−19155.
53. Londos, C., Sztalryd, C., Tansey, J. T., and Kimmel, A. R. (2005) Role of PAT proteins in lipid metabolism, *Biochimie 87*, 45−49.
54. Brasaemle, D. L., Barber, T., Wolins, N. E., Serrero, G., Blanchette-Mackie, E. J., and Londos, C. (1997) Adipose differentiation-releated protein is an ubiquitously expressed lipid storage droplet-associated protein, *J. Lipid. Res. 38*, 2249−2263.
55. Ostermeyer, A. G., Paci, J. M., Zeng, Y., Lublin, D. M., Munro, S., and Brown, D. A. (2001) Accumulation of caveolin in the endoplasmic reticulum redirects the protein to lipid storage droplets, *J. Cell Biol. 152*, 1071−1078.
56. Pol, A., Martin, S., Fernandez, M. A., Ferguson, C., Carozzi, A., Luetterforst, R., Enrich, C., and Parton, R. G. (2004) Dynamic and regulated association of caveolin with lipid bodies: Modulation of lipid body motility and function by a dominant negative mutant, *Mol. Biol. Cell 15*, 99−110.
57. Robenek, H., Robenek, M. J., and Troyer, D. (2005) PAT family proteins pervade lipid droplet cores, *J. Lipid. Res. 46*, 1331−1338.
58. Cohen, A. W., Razani, B., Schubert, W., Williams, T. M., Wang, X. B., Iyengar, P., Brasaemle, D. L., Scherer, P. E., and Lisanti, M. P. (2004) Role of caveolin-1 in the modulation of lipolysis and lipid droplet formation, *Diabetes 53*, 1261−1270.
59. Robenek, M. J., Severs, N. J., Schlattmann, K., Plenz, G., Zimmer, K.-P., Troyer, D., and Robenek, H. (2004) lipids partition caveolin-1 from ER membranes into lipid droplets: updating the model of lipid droplet biogenesis, *FASEB J.* DOI: 10.1096/fj.03-0782fje.
60. Klip, A., Ramlal, T., Douen, A. G., Burdett, E., Young, D., and Cartree, G. D. (1988) Insulin-induced decrease in 5-nucleotidase activity in skeletal muscle membranes, *FEBS Lett. 238*, 419−423.
61. Zimmermann, H. (1992) 5′-Nucleotidase: molecular structure and functional aspects, *Biochem. J. 285*, 345−365.
62. Martinez-Martinez, A., Flores-Flores, C., Campoy, F. J., Munoz-Delgado, E., Fini, C., and Vidal, C. J. (1998) Biochemical properties of 5′-nucleotidase from mouse skeletal muscle, *Biochim. Biophys. Acta 1386*, 16−28.
63. Mann, K. J., and Sevlever, D. (2001) 1,10-Phenanthroline inhibits glycosylphosphatidylinositol anchoring by preventing phosphoethanolamine addition to glycosylphosphatidylinositol anchor precursors, *Biochemistry 40*, 1205−1213.
64. Lisanti, M. P., Field, M. C., Caras, I. W., Menon, A. K., and Rodriguez-Boulan, E. (1991) Mannosamine, a novel inhibitor of

glycosylphosphatidylinositol incorporation into proteins, *EMBO J. 10*, 1969−1977.
65. Field, M. C., Medina-Acosta, E., and Cross, G. A. M. (1993) Inhibition of glycosylphosphatidylinositol biosynthesis in Leishmania mexicana by mannosamine, *J. Biol. Chem. 268*, 9570−9577.
66. Lehto, M. T., and Sharom, F. J. (2002) PI-specific phospholipase C cleavage of a reconstituted GPI-anchored protein: Modulation by the lipid bilayer, *Biochemistry 41*, 1398−1408.
67. Nosjean, O., Briolay, A., and Roux, B. (1997) Mammalian GPI protein: sorting, membrane residence and functions, *Biochem. Biophys. Acta 1331*, 153−186.
68. Marchesan, D., Rutberg, M., Andersson, L., Asp, L., Larsson, T., Boren, J., Johansson, B. R., and Olofsson, S.-O. (2003) A Phospholipase D-dependent process forms lipid droplets containing caveolin, adipocyte differentiation-related protein, and vimentin in a cell-free system, *J. Biol. Chem. 278*, 27293−27300.
69. Fivaz, M., Vilbois, F., Pasquali, C., and van der Goot, F. (2000) Analysis of gycosyl phosphatidylinositol-anchored proteins by two-dimensional gel electrophoresis, *Electrophoresis 21*, 3351−3356.
70. Elortza, F., Mohammed, S., Bunkenborg, J., Foster, L. J., Nühse, T. S., Brodbeck, U., Peck, S. C., and Jensen, O. N. (2006) Modification-specific proteomics of plasma membrane proteins: Identification and characterization of glycosylphosphatidylinositol-anchored proteins released upon phospholipase D treatment, *J. Proteome Res. 5*, 935−943.
71. Hooper, N. M. (2001) Determination of glycosyl-phosphatidylinositol membrane protein anchorage, *Proteomics 1*, 748−755.
72. Fujimoto, Y., Itabe, H., Kinoshita, T., Homma, K. J., Onoduka, J., and Mori, M. (2007) Involvement of ACSL in local synthesis of neutral lipids in cytoplasmic lipid droplets in human hepatocyte HuH7, *J. Lipid Res. 48*, 1280−1292.
73. Wang, H., and Edens, N. K. (2007) mRNA expression and antilipolytic role of phosphodiesterase 4 in rat adipocytes in vitro, *J. Lipid Res. 48*, 1099−1107.
74. Snyder, P. B., Esselstyn, J. M., Loughney, K., Wolda, S. L., and Florio, V. A. (2005) The role of cyclic nucleotide phosphodiesterases in the regulation of adipocyte lipolysis, *J. Lipid Res. 46*, 494−503.
75. Stevens, V. L. (1995) Biosynthesis of glycosylphosphatidylinositol membrane anchors, *Biochem. J. 310*, 361−370.
76. Borgese, N., Brambillasca, S., and Colombo, S. (2007) How tails guide tail-anchored proteins to their destinations, *Curr. Opin. Cell Biol. 19*, 368−375.
77. Jiang, H., He, J., Pu, S., Tang, C., and Xu, G. (2007) Heat shock protein 70 is translocated to lipid droplets in rat adipocytes upon heat stimulation, *Biochim. Biophys. Acta 1771*, 66−74.
78. Caras, I. W., and Weddell, G. N. (1989) Signal peptide for protein secretion directing glycophospholipid membrane anchor attachment, *Science 243*, 1196−1198.
79. Caras, I. W., Weddell, G. N., and Williams, S. R. (1989) Analysis of the signal for attachment of a glycophospholipid membrane anchor, *J. Cell Biol. 108*, 1387−1396.
80. Ben Ali, Y., Chahinian, H., Petry, S., Müller, G., Lebrun, R., Verger, R., Carriere, F., Mandrich, L., Rossi, M., Manco, G., Sarda, L., and Abousalham, A. (2006) Use of an inhibitor to identify members of the hormone-sensitive lipase family, *Biochemistry 45*, 14183−14191.
81. Cermelli, S., Guo, Y., Gross, S. P., and Welte, M. A. (2006) The lipid-droplet proteome reveals that droplets are a protein-storage depot, *Curr. Biol. 16*, 1783−1795.
82. Welte, M. A. (2007) Proteins under new management: lipid droplets deliver, *Trends Cell Biol.*, in press.
83. Müller, G., Wied, S., Over, S., and Frick, W. (2008) Inhibition of lipolysis by palmitate, H_2O_2 and the sulfonylurea drug, glimepiride, in rat adipocytes depends on cAMP degradation by lipid droplets, *Biochemistry 47*, 1259−1273.

BI7022915