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Membrane Association of Lipoprotein Lipase and a cAMP-Binding Ectoprotein in Rat Adipocytes

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ABSTRACT: cAMP-binding ectoprotein (Gce1) and lipoprotein lipase (LPL) are anchored to plasma membranes of rat adipocytes by glycosylphosphatidylinositol (GPI) moieties as demonstrated by cleavage by bacterial phosphatidylinositol-specific phospholipase C (PI-PLC), reactivity with anti-crossreacting determinant antibodies (anti-CRD), and metabolic labeling with radiolabeled palmitic acid and myo-inositol. Quantitative release from the membrane of LPL and Gce1 requires both lipolytic removal of their GPI anchors and the presence of either 2 M NaCl or 1 mM inositol 1,2-cyclic monophosphate or inositol 1-monophosphate. PI-PLC-cleaved and released LPL or Gce1 reassociates with isolated plasma membranes of rat adipocytes and, less efficiently, with membranes of 3T3 fibroblasts. The specificity of the reassociation is demonstrated (i) by its inhibition after pretreatment of the membranes with trypsin, (ii) by its competition with inositol 1,2-cyclic monophosphate and inositol 1-monophosphate in a concentration-dependent manner, and (iii) by the limited number of binding sites. Enzymic or chemical removal as well as masking with anti-CRD antibodies of the terminal inositol (cyclic) monophosphate moiety of hydrophilic Gce1 and LPL significantly impairs the reassociation. These data suggest that in rat adipocytes GPI-proteins are not readily released from the cell surface upon lipolytic cleavage, but remain associated through a receptor which specifically recognizes the terminal inositol (cyclic) monophosphate epitope of the (G)PI-PLC-cleaved GPI moiety. This interaction may have implications for the regulated membrane release of GPI-proteins and for their possible internalization.

GPI-proteins,¹ a subclass of ectoproteins in both lower and higher eucaryotes, are anchored to the outer leaflet of the plasma membrane by covalently attached GPI glycolipids which are characterized by a high degree of structural conservation from yeast to man. Their linear core structure is built from phosphatidylinositol bound *via* nonacetylated glucosamine to three mannose residues. The nonreducing end is linked to ethanolamine *via* a phosphodiester bridge. From this conserved core glycan various carbohydrate and/or phosphoethanolamine residues may branch off [for a review see Ferguson and Williams (1988), Low (1989), and McConville and Ferguson (1993)]. During the biosynthesis of GPI-proteins, the amino group of ethanolamine of the complete preformed GPI structure is coupled in a pseudo transpeptidation reaction to the carboxy terminus of the mature fully translated polypeptide chain, thereby replacing a transmembrane domain present in the protein precursor [for a review see Doering et al. (1990) and Thomas et al. (1990)].

Despite the lack of a cytoplasmic domain some GPI-proteins seem to have important roles in transmembrane signaling

processes, as has been suggested recently by two lines of evidence: First, cross-linking with antibodies of the T-cell antigens, Thy-1, Ly-6, Qa-2, and human decay-accelerating factor, leads to Ca^{2+} influx, altered tyrosine phosphorylation patterns, and cell proliferation, all of which are strictly dependent on the membrane anchorage of these proteins by GPI moieties (Robinson et al., 1989; Shenoy-Scaria et al., 1992; Su et al., 1991). Second, cleavage of the GPI anchor of heparan sulfate proteoglycan by a PLC in response to serum factors and insulin in a rat hepatocyte cell line has been correlated with the effects on contact inhibition and growth (Ishihara et al., 1987). To study in greater detail the molecular mechanisms of signal transfer across plasma membranes involving GPI-proteins, we are using isolated rat adipocytes. These cells are characterized by an exquisitely high sensitivity to stimulation of both short-term regulatory and metabolic processes as well as long-term growth effects by certain hormones like insulin which might use GPI-proteins as components in their signal transduction cascades.

In a first series of experiments, we studied the mode of membrane association of LPL and cAMP-binding ectoprotein, Gce1, which have been demonstrated previously to be anchored in plasma membranes of cultured 3T3 adipocytes and the yeast *Saccharomyces cerevisiae*, respectively, by covalently attached GPI moieties (Chan et al., 1988; Müller et al., 1992, 1993). Here we show that Gce1 and LPL are covalently modified by GPI in isolated rat adipocytes, too. However, after cleavage of the GPI anchors with PI-PLC, both Gce1 and LPL remain associated with the adipocyte surface *via* bipolar interactions between the residual GPI structure and a plasma membrane receptor protein. Possible functional implications for binding of lipolytically cleaved GPI-proteins to plasma membranes will be discussed.

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¹ Abbreviations: GPI, glycosylphosphatidylinositol; GPI-protein, proteins which have been synthesized with a GPI membrane anchor irrespective of whether this moiety is preserved or cleaved; (G)PI-PLC-(D), (glycosyl)phosphatidylinositol-specific phospholipase C(D); BATC, 4'-aminobenzamidothiobutyric acid; CRD, crossreacting determinant of the soluble form of trypanosomal variant surface glycoprotein; LPL, lipoprotein lipase; Gce1, glycolipid-anchored cAMP-binding ectoprotein; TLC, thin-layer chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TX-114, Triton X-114.

MATERIALS AND METHODS

Materials. 8-N₃-[³²P]cAMP (20 Ci/mmol) was bought from ICN, Eschwege, FRG; myo-[U-¹⁴C]inositol (245 mCi/mmol), myo-[U-¹⁴C]inositol 1-monophosphate (55 mCi/mmol), phosphatidyl[U-¹⁴C]inositol (220 mCi/mmol), [U-¹⁴C]-palmitic acid (650 mCi/mmol), and scintillation cocktail ACSII were provided by Amersham-Buchler, Braunschweig, FRG; EN³HANCE was purchased from New England Nuclear, Dreieich, FRG; type 1 crude collagenase (240 units/mg) was bought from CooperBiochemical, Freehold, NJ; protein A-Sepharose was provided by LKB/Pharmacia, Freiburg, FRG; glucosamine hydrochloride, mannose, mannose 6-phosphate, inositol 1,2-cyclic monophosphate, and inositol 1-monophosphate were provided by Sigma, Deisenhofen, FRG; recombinant N-glycanase F and protease inhibitors were delivered from Boehringer Mannheim, FRG; partially purified PI-PLC (*Bacillus thuringiensis*) was bought from Biotrend, Köln, FRG; other materials were obtained as described in previous publications (Müller & Bandlow, 1989, 1991, 1992, 1993); male Wistar rats were bred and delivered by Hoechst AG, Tierzucht Kastengrund, FRG; polyclonal anti-CRD antibodies raised against purified soluble VSG (sVSG) from MITat 1.2 in New Zealand White rabbits were kind gifts from Dr. P. Overath, Tübingen, FRG; polyclonal rabbit antiserum against purified human milk LPL was donated by Dr. C.-S. Wang, Oklahoma, OK.

Isolation of Rat Adipocytes. Adipocytes were isolated from epididymal fat pads of 140- to 160-g male Wistar rats under sterile conditions as described previously (Rodbell, 1964) with the following modifications: The fat tissue (0.5–1 g) was minced in 50-mL polyethylene vials containing 3 mL of KRH buffer (10 mM HEPES/KOH, pH 7.4, 125 mM NaCl, 5.5 mM KCl, 2 mM CaCl₂, 1.5 mM MgSO₄, 2.5 mM NaH₂PO₄, 2.5% BSA, and 2 mM D-glucose) and incubated (10–20 min, 37 °C) with collagenase (0.1 mg/mL routinely, or as indicated in the figure legends) under gentle stirring with a Teflon-coated bar. The suspension was passed through a 150-μm nylon filter, washed twice with 30 mL of KRH buffer containing 5% BSA by flotation, collected from the top of the medium after centrifugation (1000g, 30 s, 25 °C), and then washed once with 30 mL of KRH buffer containing 1% BSA and finally twice with 20 mL of Dulbecco's minimal essential medium (DMEM), 0.1% BSA, 0.5 mM glucose, and 25 mM HEPES/KOH (pH 7.4). After the final wash, the packed adipocyte suspension was diluted in 2 mL of the same medium/g of fat tissue (2–2.5 × 10⁵ cells/mL) for subsequent photoaffinity or metabolic labeling.

Metabolic Labeling of Adipocytes. Freshly isolated or primary cultured adipocytes (5 mL; 2 × 10⁵ cells/mL) were washed twice by flotation with 50 mL of inositol- and glucose-free DMEM containing 5% fetal calf serum, 0.5% BSA, 2 mM sodium pyruvate, and 25 mM HEPES/KOH (pH 7.4) and then suspended in 10 mL of the same medium containing 50 units/mL penicillin, 25 μg/mL streptomycin, and 20 μCi of myo-[U-¹⁴C]inositol or 100 μCi of [U-¹⁴C]palmitic acid. After incubation (12 h, 37 °C) in 50-mL polystyrene tubes under gentle agitation, the adipocytes were separated from the medium by flotation two times with 40 mL of DMEM containing 2.5% BSA, 0.5 mM glucose, 10 mM unlabeled myo-inositol (for inositol labeling only), and 25 mM HEPES/KOH (pH 7.4) and finally concentrated to 2 × 10⁵ cells/mL of DMEM containing 0.1% BSA, 0.5 mM glucose, and 25 mM HEPES/KOH (pH 7.4).

Photoaffinity Labeling of Adipocytes and Plasma Membranes with 8-N₃-[³²P]cAMP. Freshly isolated adipocytes, 20-mL portions of cells (2 × 10⁵ cells/mL), were washed by flotation once with 20 mL of DMEM containing 0.1% BSA, 25 mM HEPES/KOH (pH 7.4), 1 mM EDTA, 1 mM DTT, and 0.1 mM PMSF concentrated to 2 × 10⁶ cells/mL in the same buffer (1.9 mL) and then transferred to the wells of 12-well culture plates. Photoaffinity labeling was performed as described (Pomerantz et al., 1975; Müller et al., 1993) with the following modifications: To each well was added 100 μL of 8-N₃-[³²P]cAMP (100 μCi/mL; 5 μM), 50 mM KCl, 40 mM MgCl₂, 10 mM MnCl₂, and 2 mM 5'-AMP. The suspension was incubated (30 min, 20 °C) in the dark in the absence or presence of 0.25 mM unlabeled cAMP (as a control) and then irradiated at 312 nm with a UV handlamp (8000 μW/cm²) at a distance of 2 cm for 2 min. After addition of 20 μL of 10 mM cAMP, the suspension was transferred to 30-mL polystyrene tubes, diluted with 18 mL of DMEM containing 0.1% BSA, 0.5 mM glucose, and 25 mM HEPES (pH 7.4), and washed by flotation two times with the same buffer. For photoaffinity labeling of plasma membranes, the plasma membrane pellet was suspended in 1 mL of 20 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 50 mM KCl, 2 mM MgCl₂, 0.5 mM MnCl₂, 0.1 mM 5'-AMP, 1 mM DTT, 0.5 mM EDTA, and 0.1 mM PMSF at 0.2 mg of protein/mL, transferred to the wells of 12-well culture plates, and supplemented with 2 μCi of 8-N₃-[³²P]cAMP (0.1 nmol). The suspension was incubated (30 min, 4 °C) in the dark in the absence or presence of 0.1 mM unlabeled cAMP (as a control) and then irradiated as described above. The labeling was terminated by the addition of 10 μL of 10 mM unlabeled cAMP and 5-fold dilution with 20 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 1 mM EDTA, and 100 μM PMSF. The photolabeled plasma membranes were centrifuged through a sucrose cushion as described for salt-washed membranes.

Preparation of Plasma Membranes. Adipocytes from 10 g of tissue (approximately 5 × 10⁶ cells) were washed twice by flotation with homogenization buffer (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.25 M sucrose) containing 200 μM PMSF and 0.5 μg/mL leupeptin, pepstatin, and aprotinin each, resuspended in 50 mL of the same buffer, homogenized with 10 strokes in a motor-driven (500 rpm) Teflon-in-glass homogenizer, and centrifuged (1500g, 5 min, 15 °C). The infranatant below the fat cake was removed from the pelleted cell debris with a syringe. The homogenate was centrifuged (12000g, 15 min, 4 °C). Plasma membranes were prepared according to published procedures (McKeel & Jarett, 1970; Karnieli et al., 1981; Müller & Wied, 1993) with the following modifications: The pellet was suspended in 15 mL of homogenization buffer and re-centrifuged (1000g, 10 min). Aliquots (7.5 mL) of the supernatant were supplemented with 32 mL of Percoll medium containing 0.25 M sucrose and 20 mM Tris-HCl (pH 7.4) and centrifuged (48000g, 20 min, 4 °C). Plasma membranes were recovered from the upper third of the gradient (white band, 2 mL), diluted with 18 mL of homogenization buffer containing 200 μM PMSF, and centrifuged (20000g, 30 min, 4 °C). The pellet was resuspended in 10 mL of the same buffer and layered onto a 20-mL cushion of 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 1.12 M sucrose. After centrifugation (100000g, 70 min, 4 °C), the plasma membranes were recovered at the interface (2 mL), diluted with 18 mL of homogenization buffer containing 200 μM PMSF, and collected by centrifugation (48000g, 15 min, 4 °C). The pellet was washed once with the same buffer

and then resuspended in the desired buffer for further processing.

Salt Wash and Centrifugation of Plasma Membranes through a Cushion of Sucrose. The plasma membrane pellet (see above) was suspended in 2 M NaCl, 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 100 μ M PMSF at 0.25 mg of protein/mL and incubated (60 min, 4 °C). After 3-fold dilution with 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 100 μ M PMSF, 6 mL of the suspension was layered onto a 3-mL cushion of 0.55 M sucrose, 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 100 μ M PMSF and centrifuged (150000g, 60 min, 4 °C, swing-out rotor). The supernatant above the sucrose layer was removed with a syringe and precipitated with 8% PEG 6000 in the presence of 100 μ g/mL BSA (see below) prior to purification of Gce1 and LPL. The membrane pellet was rinsed once with 10 mL of homogenization buffer and then used for solubilization of membranes and purification of the GPI-proteins.

Solubilization of Plasma Membranes. The plasma membrane pellet was suspended in 0.15% BATC (Müller et al., 1994), 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 100 μ M PMSF, 50 μ g/mL antipain hydrochloride, 0.5 μ g/mL leupeptin, and pepstatin and aprotinin each at 0.1 mg of protein/mL and incubated (30 min, 4 °C). Samples (2.5 mL) were layered on top of a 1-mL cushion of 0.55 M sucrose, 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 100 μ M PMSF and centrifuged (150000g, 60 min, 4 °C, swing-out rotor). The supernatant above the sucrose layer was removed with a syringe and used for purification of Gce1 and LPL.

N-Glycanase Digestion of Plasma Membranes. Photolabeled plasma membranes were suspended in 0.1 M sodium phosphate buffer (pH 8.6), 5 mM DTT, 1% SDS, and 0.1 mM PMSF at 2 mg of protein/mL. After heating (5 min, 95 °C) and centrifugation (15000g, 5 min), the supernatant was diluted 10-fold with the same buffer lacking SDS and a 0.5-mL portion incubated (16 h, 30 °C) with 1.5 units of N-glycosidase F. The digestion was terminated by TCA precipitation. The acetone-washed and dried protein pellet was dissolved in sample buffer and analyzed by SDS-PAGE.

PI-PLC Digestion. For treatment of adipocytes, photoaffinity or metabolically labeled cells (2×10^5 cells/mL) were concentrated 10-fold by flotation in DMEM containing 0.5 mM glucose, 0.1% BSA, and 25 mM HEPES/KOH (pH 7.2). Portions (2.5-mL) were incubated (30 min, 37 °C) with 2 units of partially purified PI-PLC (*B. thuringiensis*). After digestion, the adipocytes were washed by flotation with the initial buffer and adjusted to 2×10^5 cells/mL. For treatment of plasma membranes, the collected plasma membranes (50–100 μ g of protein) were suspended in 500 μ L of 25 mM HEPES/KOH (pH 7.2), 1 mM EDTA, 1 mM DTT, and 200 μ M PMSF and then incubated (2 h, 30 °C) with 0.2 units of PI-PLC.

Purification of Gce1. Affinity chromatography was performed as described (Müller & Bandlow, 1992; Müller et al., 1993) using the following modifications: 1.8 mL of solubilized plasma membranes or PEG-precipitated proteins from salt-washed membranes (approximately 140–180 μ g of protein isolated from 10^6 cells) was supplemented with 200 μ L of 1.5 M NaCl and then mixed with 8 mL of *N*⁶-(2-aminoethyl)-cAMP-Sepharose (10 mg/mL in 20 mM Tris-HCl, pH 7.4, 1 mM EDTA). After incubation (30 min, 4 °C, gentle stirring), the mixture was loaded on top of 20-mL spin columns, which were washed twice with 10 mL of 20 mM Tris-HCl (pH 7.4), 100 mM sodium citrate, 150 mM NaCl, 2 mM DTT, 1 mM MgCl₂, 1% BSA, 10% glycerol, and 0.1 mM

PMSF using centrifugation (1000g, 30 s, 4 °C) and then eluted with 10 mL of the same buffer containing 0.1 mM cAMP. The initial 1.5 mL of the eluate was desalting by centrifugation through a 5-mL column of Sephadex G-25 and then precipitated with 8% PEG 6000. The precipitate was washed once with 0.8% PEG 6000 and then dissolved in 20 mM Tris-HCl (pH 7.4) and 1 mM EDTA.

Immunoprecipitation. Immunoprecipitation with polyclonal anti-LPL antibodies was performed according to Müller et al. (1994) by suspending the collected plasma membrane or PEG-precipitated proteins from salt-washed membranes in sample buffer, subsequent heating (5 min, 95 °C), and centrifugation followed by addition of anti-LPL antiserum to the diluted supernatant (0.1% final SDS concentration). Immunoprecipitation with anti-CRD antiserum was performed either with SDS (2%)-denatured membranes (0.1% final SDS concentration) according to Müller et al. (1993) or with BATC (0.1% or 2%)-solubilized membranes (0.1% final BATC concentration). For washing of the immunoprecipitates immunoprecipitation buffer containing 2% NP-40, 0.1% NP-40, and no detergent was used in this order. For sequential immunoprecipitation with anti-LPL and anti-CRD antibodies the washed anti-LPL immunoprecipitates were suspended in sample buffer, heated (5 min, 95 °C), and centrifuged (12000g, 5 min). The supernatant was diluted 20-fold with immunoprecipitation buffer lacking detergent and then incubated with anti-CRD antibodies.

Nitrous Acid Deamination. Samples of metabolically labeled and purified LPL or Gce1 were dried and resuspended in 0.1 mL of 50 mM sodium acetate (pH 3.5). To one half of the sample was added 0.1 mL of 0.33 M NaNO₃; the other half was supplemented with 0.1 mL of 0.33 M NaCl (control) as described (Ferguson et al., 1988). The samples were then incubated (6 h, 25 °C), dried again, resuspended in 10 mL of H₂O, desalting on YC05 ultrafiltration membranes (Amicon Corp., Freehold, NJ), and finally lyophilized prior to SDS-PAGE or TLC analysis.

Miscellaneous Procedures. Published procedures were used for TX-114 partitioning (Bordier, 1981; Müller & Bandlow, 1993), digestion with partially purified GPI-PLD from rabbit serum (Müller et al., 1992), PI-PLC/GPI-PLD digestion and affinity purification of Gce1p from *S. cerevisiae* (Müller & Bandlow, 1994), TLC analysis (Müller & Bandlow, 1993) followed by a second development using 1-butanol/ethanol/NH₄OH/H₂O (2:2:1:2 v/v), protein determination (Popov et al., 1975), SDS-PAGE, and fluorography using EN³HANCE (Müller & Zimmermann, 1987).

RESULTS

Identification, Topology, and Mode of Anchorage to Adipocyte Plasma Membranes of Gce1 and LPL. Since to our knowledge no GPI-proteins have been described and characterized so far in isolated rat adipocytes, the first series of experiments was designed for their identification. Possible candidates were the cAMP-binding ectoprotein, Gce1 and LPL, both of which have been found to be anchored in plasma membranes of mouse 3T3 adipocytes by covalently attached GPI (Chan et al., 1988; Müller et al., 1993).

To identify Gce1, intact rat adipocytes were isolated by very mild collagenase digestion (0.1 mg/mL, 20 min, 37 °C) and photoaffinity-labeled with membrane-impermeable 8-N₃-[³²P]cAMP. A single protein ($M_r = 62\,000$) was photolabeled with high efficiency (Figure 1). The specificity of the photoaffinity labeling was demonstrated by inclusion of a 1000-

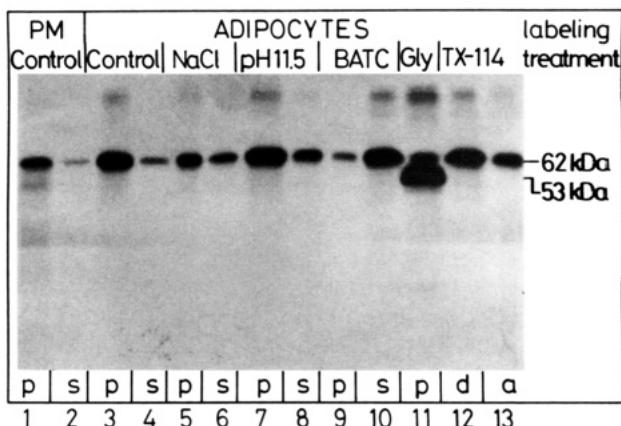


FIGURE 1: Photoaffinity labeling and membrane association of Gce1. Isolated adipocyte plasma membranes (PM; lanes 1 and 2) or adipocytes (lanes 3–13) were photoaffinity-labeled with 8-N₃-[³²P]cAMP (labeling). Plasma membranes were prepared from the adipocytes and aliquots (125 µg of protein in 0.5-mL final volume) incubated (60 min, 30 °C; treatment) in the absence (Control, lanes 1–4) or presence of 2 M NaCl (lanes 5 and 6), 0.5 M (NH₄)₂CO₃ at pH 11.5 (lanes 7 and 8), 0.15% BATC (lanes 9 and 10), N-glycanase (Gly, lane 11), or 2% (final concn) TX-114 (lanes 12 and 13). After centrifugation through a sucrose cushion (lanes 1–11) or phase separation (lanes 12 and 13), the pellet (p) and supernatant (s) fractions or the detergent (d) and aqueous (a) phases, respectively, were analyzed by SDS-PAGE and autoradiography. Molecular masses indicated in the right margin were derived from marker proteins run in parallel.

fold molar excess of unlabeled cAMP (see Figure 2). Less than 2% of radiolabel was incorporated into the 62-kDa protein compared to the absence of competitor (compare lanes 1 and 2). Most of the labeled protein was found associated with the pellet fraction of untreated plasma membranes after centrifugation through a sucrose cushion (Figure 1, lane 3), and only a minor portion was recovered from the soluble fraction (lane 4). To characterize the nature of the membrane association of Gce1, photoaffinity-labeled plasma membranes were extracted with high salt or at alkaline pH followed by centrifugation. Densitometric scanning of the fluorogram of the SDS-PAGE analysis of the pellet and supernatant fractions revealed that high-salt and alkaline pH treatments caused membrane release of only 25–30% (lanes 5 and 6) and 10–15% (lanes 7 and 8), respectively, of total photoaffinity-labeled Gce1. The limited extraction of Gce1 under these conditions may be due to the basal (constitutive) activity of an endogenous GPI-PLC which has been identified in rat adipocytes (Müller et al., in press; see below and Discussion). The major portion of total Gce1 remained associated with the membrane pellet (lanes 5 and 7) which was in agreement with the 70–80% recovered from the detergent-enriched phase after TX-114 partitioning (lanes 12 and 13). In contrast, more than 95% of the radiolabeled protein was solubilized by 0.15% BATC (lanes 9 and 10). According to this extraction and solubilization behavior Gce1 is considered as membrane protein interacting with the membrane *via* either a proteinaceous transmembrane region or a covalently attached GPI moiety.

N-Glycosylation of this protein was suggested by digestion of the photoaffinity-labeled plasma membranes with N-glycanase under denaturing conditions. The fluorogram of the SDS-PAGE revealed a reduction in apparent molecular mass of the major portion of Gce1 from 62 to 55–50 kDa (lane 11). The glycoprotein nature of the photoaffinity-labeled protein and its labeling with the membrane-impermeable photoprobe using intact adipocytes provided strong evidence that at least the cAMP-binding domain of Gce1 is located at the outer face of the plasma membrane. This view is in

agreement with the high efficiency of photoaffinity labeling of sealed plasma membrane vesicles with 8-N₃-[³²P]cAMP (lanes 1 and 2). The portion of right-side-out vesicles [about 65–75% as determined by trypsin digestion (0.5 mg/mL for 60 min at 4 °C) of isolated plasma membrane vesicles and subsequent detection of the glucose transporter isoform Glut4 by western blotting with antibodies against the carboxy-terminal 16 amino acids which are located at the cytoplasmic face of the plasma membrane; thus the ratio of the anti-Glut4 immunoreactivity of intact trypsin-treated and solubilized undigested membranes will provide an estimate of the portion of right-side-out vesicles which have shielded the cytoplasmic face of the membrane (Glut4 epitope) against tryptic degradation (G. Müller, unpublished results)] correlated well with the 55–70% efficiency of photoaffinity labeling of plasma membrane vesicles compared to identical amounts of total plasma membrane protein from photoaffinity-labeled adipocytes (compare 1 and 3). The shift in molecular mass after treatment with N-glycanase demonstrates N-glycosylation and, in addition, confirms the plasma membrane location of Gce1.

To substantiate these findings, the accessibility to proteolysis from the outer face of the plasma membrane was studied. For this, adipocytes were isolated with increasing concentrations of collagenase (Figure 2). The efficiency of the subsequent photoaffinity labeling of the extensively washed adipocytes with 8-N₃-[³²P]cAMP declined dramatically with increasing collagenase concentrations (lanes 1, 3, and 4). The highest concentration (1 mg/mL) usually used for isolation of insulin-sensitive rat adipocytes resulted in photoaffinity labeling of only 0.5% of the amount of Gce1 detected after very mild collagenase treatment (0.1 mg/mL). Consequently, 0.1 mg/mL was used for adipocyte isolation in all subsequent experiments. Interestingly, adipocytes isolated after incubation with 0.1–1 mg/mL collagenase did not differ significantly in their sensitivity and responsiveness of glucose transport stimulation by insulin (data not shown). Furthermore, no photoaffinity labeling of the regulatory subunit of protein kinase A with 8-N₃-[³²P]cAMP using rat adipocytes isolated under these conditions was detectable after SDS-PAGE and fluorography of total cellular homogenate proteins (data not shown). This provides strong evidence for integrity of the major portion of these cells (including their insulin receptors). The pronounced sensitivity of Gce1 to degradation by collagenase in intact cells confirms its ectoprotein nature. In contrast to the failure of photolabeling Gce1 after harsh treatment with collagenase, incubation of plasma membranes prepared from photoaffinity-labeled adipocytes which have been isolated by mild collagenase treatment (0.1 mg/mL) with a second portion of collagenase (0.66 mg/mL; lane 5) revealed a broad area of photoaffinity-labeled proteins ranging from 62 kDa (less than 3% of total radiolabel) to 51 kDa (>40% of total radiolabel). This confirms that Gce1 contains a cAMP-binding ectodomain which can be efficiently cleaved off by proteolytic activities contained in crude collagenase preparation. Obviously, the generated fragments fail to become photoaffinity-labeled.

To elucidate whether Gce1 is modified by a covalently attached GPI moiety, metabolic labeling experiments were performed with typical constituents of GPI lipids. Adipocytes were labeled to equilibrium with *myo*-[¹⁴C]inositol or [¹⁴C]-palmitic acid. Plasma membranes were prepared, and the solubilized Gce1 protein was enriched by cAMP-Sepharose affinity chromatography. Eluted proteins were analyzed by SDS-PAGE and fluorography. Figure 2 shows that a single

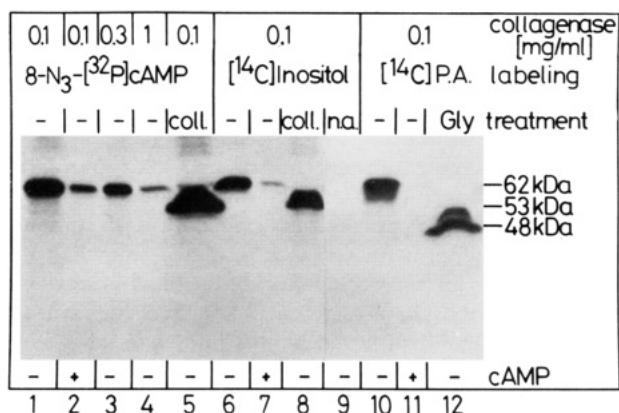


FIGURE 2: Metabolic labeling of Gce1. Adipocytes were prepared by digestion with 0.1, 0.3, or 1 mg/mL collagenase and then photoaffinity-labeled with 8-N₃-[³²P]cAMP (lanes 1–5) or metabolically labeled with myo-[¹⁴C]inositol (lanes 6–9) or [¹⁴C]palmitic acid (P.A.; lanes 10–12) (labeling). The metabolically labeled Gce1 was affinity-purified by cAMP-Sepharose chromatography from the solubilized plasma membranes (lanes 6–12). As controls, excess of cAMP was included during photoaffinity labeling (lane 2) or during affinity purification (lanes 7 and 11). Aliquots of total photoaffinity-labeled plasma membrane protein (75 µg of protein) and of affinity-purified Gce1 (3500 dpm) were incubated (30 min, 37 °C) in 300-µL final volume with 0.2 mg (lane 5) or 0.02 mg (lane 8) collagenase (coll.), respectively, or N-glycanase (Gly) or subjected to nitrous acid deamination (n.a.) (treatment). Total photoaffinity-labeled plasma membrane proteins (lanes 1–5) and affinity-purified proteins (lanes 6–12) were analyzed by SDS-PAGE and fluorography. The molecular masses indicated in the right margin were derived from marker proteins run in parallel.

protein was recovered metabolically labeled with both myo-[¹⁴C]inositol (lane 6) and [¹⁴C]palmitic acid (lane 10). This protein comigrated with 62-kDa photoaffinity-labeled Gce1 (lane 1). The identity of the metabolically labeled and affinity-purified protein with photoaffinity-labeled Gce1 was further substantiated by the sensitivity of the affinity-purified material to collagenase or N-glycanase digestion. Subsequent SDS-PAGE and fluorography shows the characteristic degradation patterns of the [¹⁴C]inositol-labeled protein by collagenase (0.066 mg/mL) (lane 8) and of the [¹⁴C]palmitic acid-labeled protein by N-glycanase (lane 12) as observed for the photoaffinity-labeled protein (lane 5 and Figure 1, lane 11). The specificity of the affinity purification procedure was confirmed by addition of an excess of cAMP to a plasma membrane sample prior to incubation with the cAMP-Sepharose beads. Almost no [¹⁴C]inositol-labeled Gce1 was recovered under these conditions (lanes 7 and 11). These data demonstrate that Gce1 contains myo-inositol and palmitic acid. Incorporation of myo-[¹⁴C]inositol into the GPI structure but not into the polypeptide portion of Gce1 under the labeling conditions used was suggested by the complete loss of the ¹⁴C-label from the affinity-purified protein after nitrous acid deamination (lane 9), which causes removal of the phosphatidylinositol moiety from the GPI structure (Ferguson et al., 1988).

In order to examine whether LPL is also anchored by GPI in rat adipocytes, plasma membranes were prepared from myo-[¹⁴C]inositol-labeled cells and treated with high salt followed by centrifugation or subjected to TX-114 partitioning. LPL was assayed in the pellet/supernatant fractions or detergent/aqueous phases, respectively, by immunoprecipitation with specific anti-LPL antibodies. SDS-PAGE and fluorography of the immunoprecipitates (Figure 3, upper panel) revealed a single radiolabeled protein in the membrane pellet (lane 1) and detergent phase (not shown) with an apparent molecular mass of 60 kDa identical to that

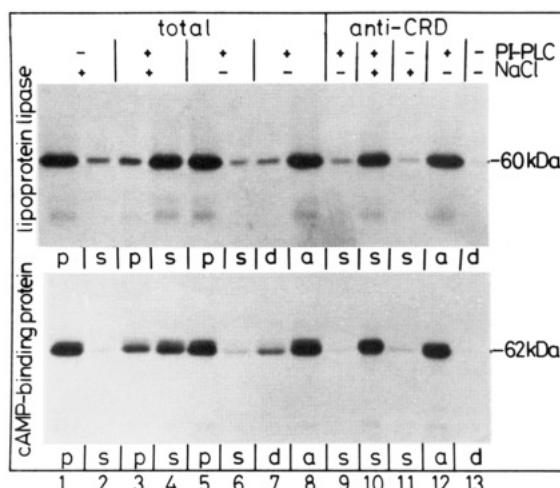


FIGURE 3: Digestion of LPL and Gce1 with PI-PLC. Adipocytes were metabolically labeled with myo-[¹⁴C]inositol and subsequently incubated in the absence or presence of PI-PLC (*B. thuringiensis*). Plasma membranes were prepared and aliquots (75 µg of protein in 300-µL total volume) incubated (30 min, 30 °C) in the absence or presence of 2 M NaCl and centrifuged through a cushion of sucrose (lanes 1–6, 9–11) or subjected to TX-114 partitioning (lanes 7, 8, 12, 13). The pellet (p) and supernatant (s) fractions or detergent (d) and aqueous (a) phases, respectively, were subjected to immunoprecipitation with anti-LPL antibodies or to affinity purification on cAMP-Sepharose. One half of the samples was analyzed directly by SDS-PAGE and fluorography (lanes 1–8) and the other half after immunoprecipitation with anti-CRD antibodies (lanes 9–13). Molecular masses of marker proteins are indicated in the right margin.

described for LPL in 3T3 adipocytes (Chan et al., 1988; Müller et al., 1993). Only marginal amounts were recovered from the supernatant fraction (lane 2) and aqueous phase (not shown), suggesting a tight association of amphiphilic LPL with the adipocyte plasma membrane.

The GPI Anchor of Gce1 and LPL Is Cleaved by Bacterial PI-PLC. To demonstrate that Gce1 and LPL are anchored to the adipocyte plasma membrane via covalently attached GPI moieties, we tested their accessibility to cleavage by bacterial PI-PLC. Adipocytes were metabolically labeled with myo-[¹⁴C]inositol and then incubated with PI-PLC (*B. thuringiensis*). Isolated plasma membranes were treated with high salt, subjected to centrifugation or TX-114 partitioning, and then assayed for the presence of LPL and Gce1. Figure 3 shows that incubation of intact adipocytes with PI-PLC led to conversion of the major portion of ¹⁴C-labeled amphiphilic LPL (upper panel) and Gce1 (lower panel) into their hydrophilic forms which now partitioned into the aqueous phases (compare lanes 7 and 8). However, release of the hydrophilic versions of LPL and Gce1 from plasma membranes as assayed by centrifugation through a cushion of sucrose required the presence of high salt subsequent to the PI-PLC treatment (compare lanes 4 and 6). In the absence of salt the major fraction of LPL and Gce1 remained associated with plasma membranes despite cleavage by PI-PLC (lanes 5). This pointed to the existence of bipolar interactions between the hydrophilic protein moieties of these GPI-proteins and the membrane surface.

Lipolytic cleavage of the GPI anchors of LPL and Gce1 was confirmed by immunoprecipitation of [¹⁴C]inositol-labeled LPL and Gce1 with anti-CRD antibodies from the supernatant (after centrifugation) or aqueous phase (after TX-114 partitioning). These antibodies exclusively recognize the PLC-cleaved form of GPI-proteins, the major epitopes (CRD) residing in the inositol 1,2-cyclic monophosphate moiety formed during cleavage (Zamze et al., 1988). In addition,

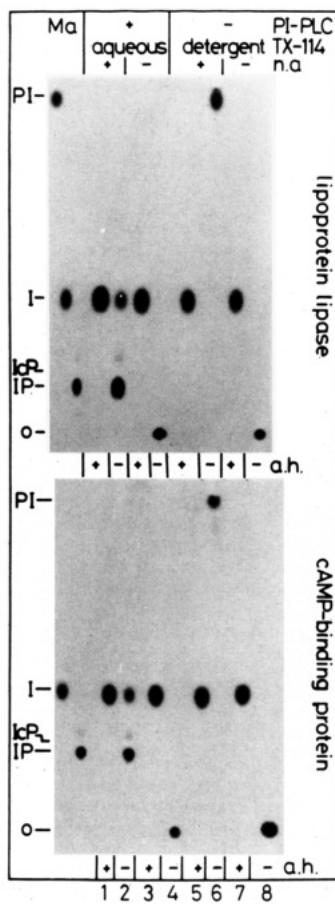


FIGURE 4: TLC Analysis of the GPI anchor of LPL and Gce1. Adipocytes were metabolically labeled with *myo*-[¹⁴C]inositol and subsequently incubated in the absence or presence of PI-PLC (*B. thuringiensis*). LPL and Gce1 were purified from solubilized plasma membranes by immunoprecipitation with anti-LPL antibodies or affinity purification on cAMP-Sepharose, respectively, and then subjected to TX-114 partitioning. Half of the aqueous and detergent phases was treated with nitrous acid (n.a.), and the other half was left untreated. Each sample was divided again and subjected to acid hydrolysis (a.h.) or left untreated and subsequently analyzed by TLC and fluorography. The positions of radiolabeled inositol 1-monophosphate (IP), *myo*-inositol (I), and phosphatidylinositol (PI) run in parallel as markers are indicated in the left margin (Ma). The position of inositol 1,2-cyclic monophosphate (IcP) was derived from the *R*_f value as determined from a parallel TLC run with unlabeled compound which was identified by staining with 1 N H₂SO₄. o, origin.

inositol monophosphate is recognized, albeit with lower efficiency. As can be seen from Figure 3, 80–90% of total radiolabeled hydrophilic (lanes 12) or soluble (lanes 10) LPL and Gce1 was immunoprecipitated by anti-CRD antibodies, whereas the amphiphilic (lanes 13) and membrane-associated versions (lanes 11) were not recognized. In agreement with the failure to release the two GPI-proteins from the adipocyte plasma membrane solely by cleavage of their GPI anchor (see above), inclusion of high salt prior to centrifugation was required for subsequent immunoprecipitation of LPL and Gce1 with anti-CRD antibodies from the supernatant (compare lanes 9 and 10).

The ¹⁴C-radiolabel of the PI-PLC-cleaved LPL and Gce1 from adipocytes metabolically labeled with *myo*-[¹⁴C]inositol is in agreement with lipolytic processing of their amphiphilic versions by PI-PLC. To confirm the identity of [¹⁴C]inositol and to prove the cleavage specificity of the phospholipase used, we analyzed the acid hydrolysis and nitrous acid deamination products of the amphiphilic and hydrophilic forms of [¹⁴C]-inositol-labeled LPL and Gce1. Figure 4 shows that the major fraction of the radiolabel incorporated into LPL and Gce1

from untreated adipocytes comigrated with inositol in TLC after acid hydrolysis of amphiphilic (lanes 7) or of amphiphilic deaminated material (lanes 5; compare with amphiphilic nonhydrolyzed material in lanes 8), but with phosphatidylinositol after nitrous acid deamination alone (lanes 6). Thus there was no significant conversion of *myo*-[¹⁴C]inositol into other components in rat adipocytes during the period of metabolic labeling. Nitrous acid deamination of [¹⁴C]inositol-labeled hydrophilic LPL and Gce1 isolated from PI-PLC-treated adipocytes released 65–70% of the total radiolabel as inositol monophosphate and 20–30% as inositol (lanes 2; compare with nonhydrolyzed hydrophilic material in lanes 4). Acid hydrolysis of the deamination products identified [¹⁴C]inositol as the only radiolabel (lanes 1). The presence of inositol monophosphate after nitrous acid deamination of the PLC-cleaved proteins confirms the specificity of the PI-PLC (*B. thuringiensis*) used. The failure to detect inositol 1,2-cyclic phosphate, which can be discriminated from inositol monophosphate by the TLC system used (as indicated by the position of unlabeled stained inositol 1,2-cyclic monophosphate), may be due to the limited lifetime of this reaction intermediate of bacterial PI-PLC action (Lewis et al., 1993) or to cyclic phosphodiesterase activity. Most likely, the immunodetection of hydrophilic LPL and Gce1 by anti-CRD antibodies (see Figure 3) resulted from crossreactivity with an inositol monophosphate-containing epitope. The release of free inositol by nitrous acid deamination alone points to additional phosphatase action during the incubation of adipocytes or subsequent purification of the GPI-proteins.

Taken together, metabolic labeling with *myo*-[¹⁴C]inositol, accessibility to PI-PLC cleavage, and crossreactivity with anti-CRD antibodies strongly argue for anchorage by GPI of LPL and Gce1. We therefore name the rat adipocyte cAMP-binding ectoprotein “Gce1” (glycolipid-anchored cAMP-binding ectoprotein). These findings do not exclude that a fraction of LPL is associated with the adipocyte plasma membrane via interaction with heparan sulfate proteoglycan which is also anchored in the membrane by GPI (Ishihara et al., 1987). This form of LPL can be released by heparin as has been shown by Chajek-Shaul et al. (1989) for rat heart cell cultures. However, we found that only 30–35% of the total LPL activity of rat adipocytes can be released by heparin (G. Müller, unpublished results). A total of 70–85% of the non-heparin-releasable LPL can be removed by subsequent incubation with bacterial PI-PLC, suggesting that both types of membrane anchorage of LPL coexist in rat adipocytes.

The Anti-CRD Epitope Is Masked in PI-PLC-Cleaved Solubilized Gce1. The observed membrane release of LPL and Gce1 via the concerted action of PI-PLC and high salt, but not by either procedure alone, is compatible with membrane association of the two proteins via their GPI moieties and additional bipolar interactions which may involve the specific recognition of the anti-CRD epitope generated by PI-PLC action by (a) receptor protein(s) of the cell surface. This type of interaction may result in masking of the anti-CRD epitope. We tested this hypothesis for Gce1 by performing immunoprecipitation studies with anti-CRD antibodies using photoaffinity-labeled or metabolically labeled and affinity-purified Gce1 from PI-PLC-treated and subsequently solubilized (0.1% BATC) plasma membranes which have been exposed to different conditions affecting receptor–ligand interactions. Figure 5 (upper panel) shows that the amounts of solubilized total photoaffinity-labeled (lanes 1–4) or cAMP-affinity-purified metabolically labeled (lanes 5–8) Gce1 treated in a different manner prior to precipitation with PEG 6000 were

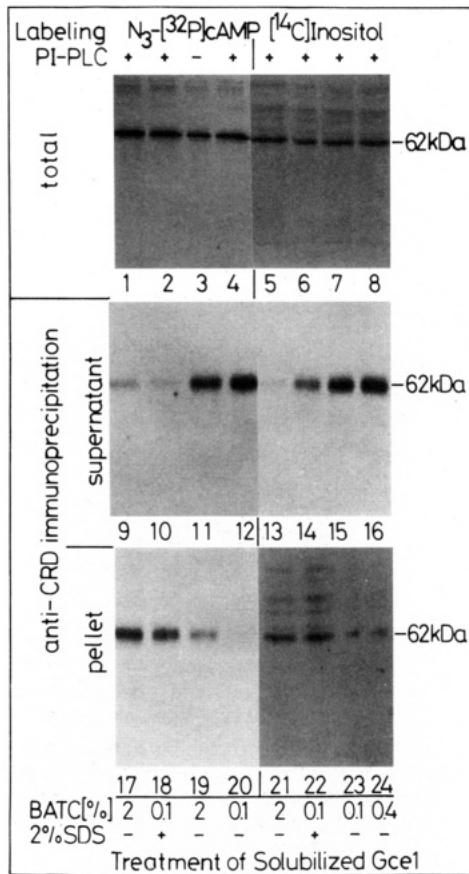


FIGURE 5: Immunoprecipitation of PI-PLC-cleaved Gce1 with anti-CRD antibodies from solubilized membranes. Adipocytes were metabolically labeled with *myo*-[¹⁴C]inositol (lanes 5–8, 13–16, 21–24) or photoaffinity-labeled with 8-N₃-[³²P]cAMP (lanes 1–4, 9–12, 17–20) and then incubated in the absence (lanes 3, 11, 19) or presence of PI-PLC (*B. thuringiensis*). Plasma membranes were prepared, solubilized with 0.1% BATC, and centrifuged through a cushion of sucrose. The supernatant from the metabolically labeled cells was affinity-purified on cAMP-Sepharose, and the supernatant from the photoaffinity-labeled cells was left untreated. Aliquots of the two supernatants were adjusted to a final concentration of 0.4% or 2% BATC or 2% SDS or left untreated as indicated. One half of each sample was precipitated with 8% PEG 6000 (total; upper panel). The other half of each sample was diluted to a final detergent concentration of 0.1% and then subjected to immunoprecipitation with anti-CRD antiserum preadsorbed to protein A-Sepharose. The immunoprecipitates were collected by centrifugation and washed twice with immunoprecipitation buffer containing 2% NP-40, twice with buffer containing 0.1% NP-40, and once with buffer lacking detergent (pellet; lower panel). The supernatants of the protein A-Sepharose beads were precipitated with 8% PEG 6000 (middle panel). The PEG-precipitated or immunoprecipitated materials were suspended in sample buffer, heated, and centrifuged. The supernatants were analyzed by SDS-PAGE and fluorography.

comparable. In contrast, anti-CRD antibodies immunoprecipitated (lower panel) significant amounts of the photolabeled or metabolically labeled Gce1 only if it had been treated with 2% BATC (50–70%; lanes 17 and 21) or SDS (70–90%; lanes 18 and 22) prior to incubation with the antibodies. In agreement, under these conditions only minor amounts of total labeled Gce1 (2–20%; lanes 9, 10, 13, and 14) were left in the supernatants of the corresponding anti-CRD/protein A-Sepharose complexes which are shown as a control for the efficiency of the immunoprecipitation (middle panel). Solubilized Gce1 maintained at 0.1% BATC throughout the immunoprecipitation protocol did hardly react with anti-CRD antibodies (1–5%; lanes 20 and 23). A small increase of immunoreactive Gce1 (10%; lane 24) was detected by prior incubation with 0.4% BATC. A limited portion of photoaf-

Table 1: Release of PI-PLC-Cleaved LPL and Gce1 from Rat Adipocyte Plasma Membranes by Salt and Sugar Compounds^a

compd added	% released PI-PLC-cleaved GPI-protein		compd added	% released PI-PLC-cleaved GPI-protein	
	LPL	Gce1		LPL	Gce1
-	22.1 ± 4.8	36.3 ± 6.9	GlcN	43.6 ± 10.1	54.8 ± 23.1
NaCl	94.2 ± 27.8	91.9 ± 32.5	Man	28.5 ± 9.5	40.2 ± 11.4
I	41.7 ± 12.3	58.4 ± 15.7	ManP	48.7 ± 15.2	58.5 ± 15.9
IP	86.2 ± 34.5	84.1 ± 28.6	Gal	16.8 ± 8.5	33.1 ± 7.7
IcP	92.3 ± 17.8	90.4 ± 15.8	Glc	27.6 ± 7.4	30.3 ± 10.9
IP ₃	61.7 ± 35.5	65.1 ± 25.2			

^a Rat adipocytes were metabolically labeled with *myo*-[¹⁴C]inositol. Plasma membranes were isolated and treated with PI-PLC. 400-μL aliquots (200 μg of protein) were supplemented with 400 μL of 2-fold membrane buffer (40 mM Tris-HCl, pH 7.4, 2 mM EDTA, 0.5 mM DTT, 0.4 M sucrose, 200 μM PMSF) and then incubated (5 min, 4 °C) with 2 M NaCl or 1 mM compound in a total volume of 1 mL. Subsequently, the plasma membranes were centrifuged through a cushion of sucrose. One half of the supernatant was used for immunoprecipitation of LPL and the other half for affinity purification of Gce1. The proteins were analyzed by SDS-PAGE. The sliced gel bands corresponding to LDL (60 kDa) and Gce1 (62 kDa) were counted for radioactivity. The total amount of PI-PLC-cleaved LPL and Gce1 recovered from the aqueous phase after TX-114 partitioning of plasma membranes (omitting centrifugation) was set at 100% in each case. The values are the means of three independent experiments (±SD). I, *myo*-inositol; IP, *myo*-inositol 1-monophosphate; IcP, *myo*-inositol 1,2-cyclic monophosphate; IP₃, inositol 1,4,5-trisphosphate; GlcN, nonacetylated glucosamine; Man, mannose; ManP, mannose 6-phosphate; Gal, galactose; Glc, glucose.

finity-labeled uncleaved Gce1 was immunoprecipitated by anti-CRD antiserum after incubation with 2% BATC (10–15%; lane 19). This is largely explained by the portion of hydrophilic Gce1 generated by an endogenous adipocyte PLC activity (see above). In each case Gce1 not immunoprecipitated by anti-CRD antibodies was recovered in the supernatant of the anti-CRD/protein A-Sepharose beads (compare middle and lower panels). These data suggest that the anti-CRD epitope of PI-PLC-cleaved Gce1 which has been solubilized under mild conditions (0.1% BATC) is masked by a receptor protein of the adipocyte plasma membrane. This interaction seems to be destroyed by high concentrations of detergent or denaturing conditions (2% SDS) and may be responsible for the retention of lipolytically cleaved GPI-proteins at the adipocyte cell surface.

Inositol Monophosphates Interfere with Membrane Association of PI-PLC-Cleaved Gce1 and LPL. To characterize the nature of the bipolar interactions in greater detail, [¹⁴C]-inositol-labeled plasma membranes were treated with PI-PLC and then incubated with 1 mM of various sugar compounds (Table 1). The amount of both Gce1 and LPL released into the supernatant after centrifugation was highest with inositol 1,2-cyclic monophosphate and inositol 1-monophosphate approaching the value for high-salt treatment. Inositol 1,4,5-trisphosphate, mannose 6-phosphate, glucosamine, and inositol were significantly less efficient whereas mannose, glucose, and galactose were almost ineffective. About 20–40% of the total PI-PLC-cleaved hydrophilic form of LPL and Gce1 was released from the plasma membranes in the absence of salt or sugar compound. This fraction may comprise molecules which have lost the structural determinants necessary for the bipolar interaction with the membrane.

Released PLC-Cleaved Gce1 and LPL Reassociate with Isolated Adipocyte Plasma Membranes. The dissociation of PI-PLC-cleaved Gce1 and LPL from the membrane by inositol monophosphate suggests interaction of the terminal inositol (cyclic) monophosphate moiety of the residual GPI anchor with a component of the adipocyte plasma membrane. To

Table 2: Reassociation of PI-PLC-Cleaved LPL and Gce1 with Plasma Membranes^a

compd added	% reassociated PI-PLC-cleaved GPI-protein		compd added	% reassociated PI-PLC-cleaved GPI-protein	
	LPL	Gce1		LPL	Gce1
-	65.5 ± 14.3	57.8 ± 21.5	Man	61.4 ± 14.8	53.1 ± 17.6
NaCl	8.8 ± 3.9	6.3 ± 2.4	ManP	45.6 ± 12.4	40.8 ± 20.4
I	31.5 ± 8.5	35.7 ± 12.4	Gal	73.5 ± 19.7	68.4 ± 16.3
IP	12.4 ± 5.7	16.7 ± 6.8	Glc	71.2 ± 28.5	62.5 ± 17.6
IcP	6.1 ± 3.5	4.3 ± 2.5	anti-CRD	13.5 ± 8.9	10.5 ± 4.6
IP ₃	56.9 ± 19.3	49.6 ± 13.5	preimmune serum	69.5 ± 17.7	55.6 ± 21.3
GlcN	71.0 ± 23.5	61.5 ± 18.5	serum		

^a Rat adipocytes were metabolically labeled with *myo*-[¹⁴C]inositol. Plasma membranes were isolated, treated with PI-PLC, and subjected to TX-114 partitioning. Proteins from the aqueous phase were precipitated with PEG. The pellet was suspended in membrane buffer (2000–3500 dpm/100 μL). 100-μL samples, dialyzed against membrane buffer, were incubated (5 min, 4 °C) with 150 μL of untreated plasma membranes (1 mg of protein/mL of membrane buffer) in the absence or presence of 2 M NaCl or 1 mM sugar compound or 25 μL of anti-CRD antiserum or preimmune serum in a total volume of 500 μL. Subsequently, the plasma membranes were centrifuged through a cushion of sucrose. Purification of LPL and Gce1 from the membranes and quantification of the radiolabel were performed as described above. The amount of radiolabeled PI-PLC-cleaved GPI-protein added to the plasma membranes was set at 100%. Each value represents the mean of three independent experiments (±SD). For abbreviations see legend to Table 1.

substantiate this interpretation, the interaction was reconstituted by incubation of isolated plasma membranes with [¹⁴C]inositol-labeled PI-PLC-cleaved GPI-proteins in the presence of NaCl or certain sugar compounds. In the absence of competitors 150 μg of membrane protein bound up to 70% of the total hydrophilic radiolabeled LPL and Gce1 added as assayed in the supernatant after centrifugation (Table 2). The amount of reassociated GPI-protein decreased to 6–12% in the presence of 2 M NaCl or 1 mM inositol 1,2-cyclic monophosphate and to a lower extent in the presence of 1 mM inositol 1-monophosphate. 30–50% reductions were observed with 1 mM inositol, inositol trisphosphate, and mannose 6-phosphate, whereas mannose, glucosamine, glucose, and galactose were completely ineffective. There was no significant difference in the ranking orders of the sugar compounds between their efficiency to displace either LPL or Gce1 from native adipocyte plasma membranes (Table 1) and their ability to inhibit the reassociation with the membranes (Table 2), suggesting that the same type of interaction between GPI-proteins and the membrane is effective in the two systems.

For quantitative evaluation of this difference the reassociation experiment was repeated using decreasing concentrations of displacing sugar compound (Figure 6). The IC₅₀ values of the various effective sugar compounds calculated from the displacement curves for both LPL and Gce1 confirmed their ranking order as defined in the previous experiment. However, the IC₅₀ values for the inhibition of reassociation of Gce1 (inositol 1,2-cyclic monophosphate: 8 μM; inositol 1-monophosphate: 21 μM) were lower compared to those for LPL (inositol 1,2-cyclic monophosphate: 42 μM; inositol 1-monophosphate: 85 μM). Thus, the binding affinity of PI-PLC-cleaved LPL seems to be higher than that of Gce1.

The efficiency of the inositol monophosphate compounds in inhibiting the reassociation suggested that these sugars compete with the terminal inositol monophosphate moiety of PI-PLC-cleaved LPL and Gce1 for binding to a plasma membrane receptor protein. We tested this possibility by assaying the reassociation of the hydrophilic versions of the two GPI-proteins for which the terminal inositol monophos-

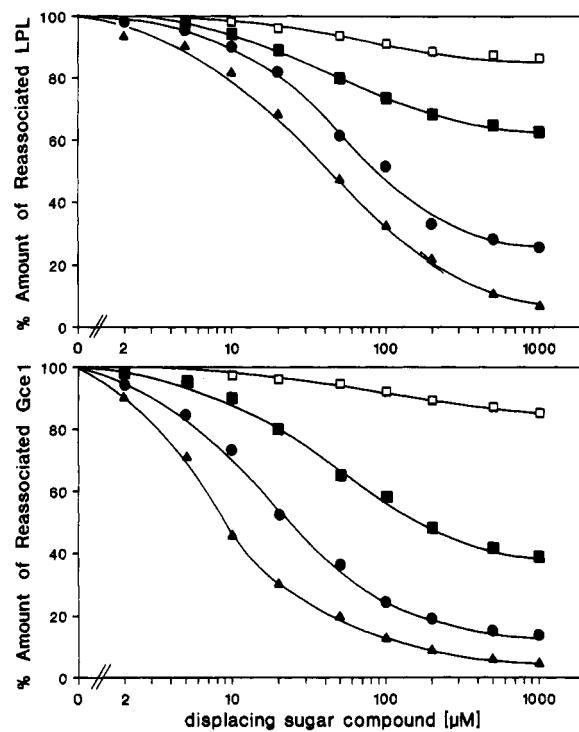


FIGURE 6: Concentration-dependent inhibition by sugar compounds of the reassociation of PI-PLC-cleaved GPI-proteins. The experiment was performed as described in the legend to Figure 7 except that for the reassociation 150 μg of adipocyte plasma membranes were incubated in the presence of increasing concentrations of sugar compound. The radioactivity measured in the presence of 2 M NaCl was subtracted from each value. The specific association of radiolabeled Gce1 and LPL with the membranes in the absence of sugar was set at 100%. Each point represents the means of three independent experiments. ▲, *myo*-inositol 1,2-cyclic monophosphate; ■, *myo*-inositol 1-monophosphate; □, mannose.

Table 3: Reassociation of PLD-Cleaved or Deaminated LPL and Gce1 with Plasma Membranes

compd added	% reassociated PLD-cleaved GPI-protein	
	LPL	Gce1
-	38.4 ± 14.7	29.9 ± 11.9
NaCl	4.8 ± 2.1	6.7 ± 3.0
IcP	8.6 ± 1.9	11.3 ± 4.5
GlcN	40.7 ± 14.2	34.7 ± 18.5

	% reassociated deaminated GPI-protein	
	LPL	Gce1
-	15.2 ± 7.3	11.2 ± 5.8
NaCl	6.4 ± 2.8	7.5 ± 2.4
IcP	17.9 ± 8.7	13.4 ± 5.8

^a The experiment was carried out as described in the legend to Table 2 with the exception that plasma membranes from which LPL and Gce1 were isolated subsequently had been treated with GPI-PLD or nitrous acid. Each value represents the mean of four independent experiments (±SD).

phate moiety had been removed by GPI-PLD digestion or nitrous acid deamination (Table 3) or had been masked by preincubation with excess of anti-CRD antibodies (Table 2). Only a limited fraction (30–40%) of the total amount of PI-PLC-cleaved hydrophilic LPL and Gce1 was able to reassociate with the membranes. This amount was drastically diminished in the presence of 2 M NaCl or 1 mM inositol 1,2-cyclic monophosphate but not of glucosamine. There was only a marginal reassociation of the deaminated GPI-proteins which was not inhibited by inositol 1,2-cyclic monophosphate.

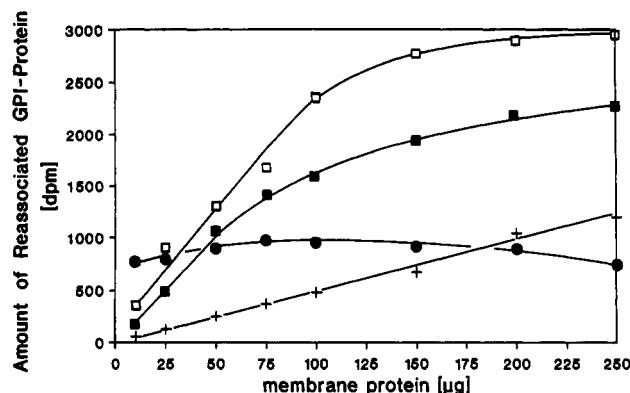


FIGURE 7: Dependence of the reassociation of PI-PLC-cleaved GPI-proteins on the amount of membrane protein. Metabolically labeled proteins from PI-PLC-treated adipocyte plasma membranes (prepared as described in legend to Table 2) were incubated with various amounts of untreated or trypsin-treated (1 mg/mL trypsin for 60 min at 15 °C followed by addition of 2 mg/mL soybean trypsin inhibitor and 200 μM PMSF) adipocyte plasma membranes or of untreated 3T3 fibroblast membranes in the absence or presence of 2 M NaCl. The membranes were spun through a cushion of sucrose. The amount of radiolabeled LPL and Gce1 purified from the suspended pellet fractions was determined after SDS-PAGE and densitometry of the fluorograms. For calculation of the specific association, the radioactivity measured in the presence of 2 M NaCl (unspecific association) was subtracted from the value measured in the absence of NaCl (total association). Each point represents the means of three to four independent experiments. □, LPL associated with adipocyte plasma membranes; ■, Gce1 associated with adipocyte plasma membranes; +, Gce1 associated with fibroblast membranes; ●, Gce1 associated with trypsin-treated adipocyte plasma membranes.

Furthermore, anti-CRD antibodies blocked the reassociation almost to the same degree as 2 M NaCl or 1 mM inositol monophosphate whereas preimmune serum did not interfere. These findings strongly argue that the binding of hydrophilic GPI-proteins to rat adipocyte plasma membranes involves the terminal inositol monophosphate.

Soluble PLC-Cleaved Gce1 and LPL Compete for Binding to a Plasma Membrane Receptor Protein. The low concentrations of inositol monophosphate compounds necessary for displacement of PI-PLC-cleaved GPI-proteins from the adipocyte plasma membrane hint to the existence of a limited number of binding sites in the membrane. Consequently, we tested the dependence of the reassociation of PI-PLC-cleaved LPL and Gce1 on the amount of membrane protein. Figure 7 shows that with a constant amount of LPL and Gce1 the fraction of reassociated material increased with increasing amounts of membrane protein up to 75–100 μg in a linear manner, leading to 55–65% association. The maximum association did not exceed 70–80%, which may rely on a certain fraction of PLC-cleaved GPI-proteins having lost their ability to recognize the binding site. Membranes prepared from 3T3 fibroblasts also exhibited binding of PI-PLC-cleaved Gce1, albeit to a significantly lower degree than rat adipocyte plasma membranes. Linearity was observed with more than 250 μg of fibroblast membrane protein, indicating complete saturation of all binding sites by an amount of cleaved GPI-protein which was sufficient for the saturation of 75 μg of adipocyte plasma membrane protein, only. This observation demonstrates a drastically lower number of binding sites in fibroblasts compared to adipocytes.

To test whether the binding sites were constituted by membrane proteins, adipocyte plasma membranes were pretreated with trypsin followed by the addition of trypsin inhibitor, and variable amounts were assayed for reassociation of PI-PLC-cleaved Gce1 and LPL (Figure 7). A constant amount of Gce1 and LPL (not shown) was recovered with

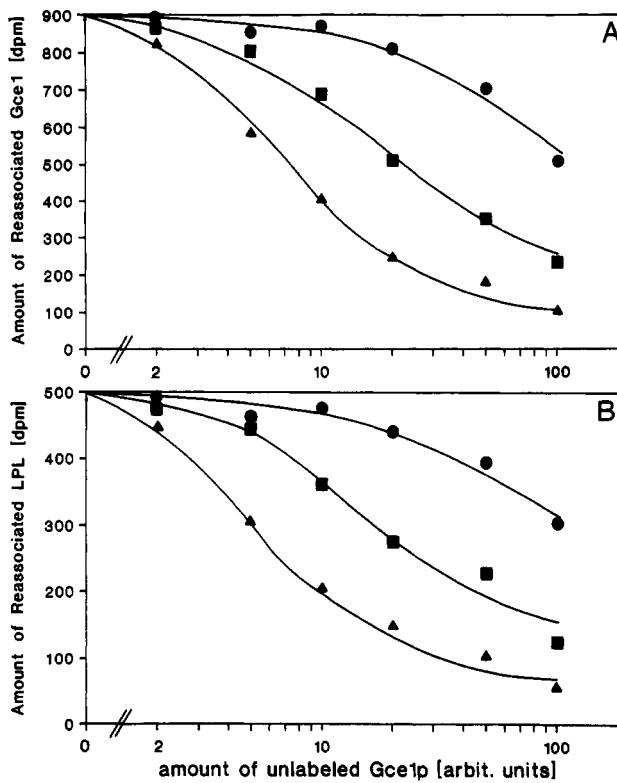


FIGURE 8: Competition of Gce1 and LPL for reassociation. Rat adipocytes were metabolically labeled with myo-[¹⁴C]inositol. Plasma membranes were isolated, treated with PI-PLC, and subjected to TX-114 partitioning. Proteins from the aqueous phase (3000–4000 dpm per 100 μL) were incubated (5 min, 4 °C) in a total volume of 500 μL with 25 μg of untreated plasma membranes in the absence or presence of various amounts of PI-PLC-cleaved and affinity-purified unlabeled Gce1p from *S. cerevisiae* (the amount of Gce1p isolated from plasma membranes of 10⁸ yeast spheroplasts was arbitrarily set at 1 unit). Yeast Gce1p had been pretreated with PI-PLC (*B. thuringiensis*) (▲) or GPI-PLD (rabbit serum) (■) or nitrous acid (●). After incubation with plasma membranes, the mixture was centrifuged through a cushion of sucrose. Gce1 (panel A) and LPL (panel B) were purified from the resuspended membranes and assayed for radioactivity as described in Figure 7. Each point represents the means of two independent experiments.

varying amounts of trypsin-treated membranes. This association was not reduced in the presence of 2 M NaCl or 1 mM inositol 1,2-cyclic monophosphate (data not shown). As shown above, these conditions diminished the reassociation of Gce1 and LPL with even the highest amount of untreated adipocyte and fibroblast membranes to 5–10%. Thus, trypsin treatment seems to destroy specific binding sites for PI-PLC-cleaved GPI-proteins.

The inhibition of binding of PI-PLC-cleaved Gce1 and LPL by various sugar compounds with the same ranking order and comparable efficiencies suggests interaction of Gce1 and LPL with the same receptor protein. Further evidence for this possibility was derived from competition of the reassociation of PI-PLC-cleaved rat adipocyte Gce1 and LPL by excess of purified Gce1p from *S. cerevisiae*. For this, PI-PLC-cleaved [¹⁴C]inositol-labeled Gce1 and LPL were incubated with a limited and constant amount of untreated plasma membranes in the presence of increasing amounts of PI-PLC-cleaved affinity-purified unlabeled Gce1p isolated from plasma membranes of *S. cerevisiae*. As controls, Gce1p was prepared after cleavage with GPI-PLD or deamination with nitrous acid. Quantitative evaluation of the radiolabeled membrane-associated GPI-proteins after centrifugation (Figure 8, panel A) shows that PI-PLC-cleaved Gce1p from yeast efficiently

competed for the reassociation of the radiolabeled PI-PLC-cleaved Gce1 from adipocytes. The competition was significantly less pronounced when GPI-PLD-cleaved yeast Gce1p was used and almost abolished with the deaminated protein.

Analysis of the supernatant for radiolabeled LPL revealed its displacement by the various versions of hydrophilic yeast Gce1p in a concentration-dependent manner. The ranking order and efficiencies of three versions of yeast Gce1p to displace LPL from rat adipocyte plasma membranes were the same as observed for adipocyte Gce1 (panel B). The IC₅₀ values for PI-PLC-cleaved Gce1p for displacement of adipocyte LPL and Gce1 were comparable. These data provide strong evidence for interaction of the two GPI-proteins with the same adipocyte receptor protein presumably *via* their common terminal inositol (cyclic) monophosphate residues.

DISCUSSION

In this study we report that LPL and a cAMP-binding ectoprotein, now termed Gce1, are associated with plasma membranes of rat adipocytes *via* GPI structures. GPI-anchored LPL and Gce1 have first been described in 3T3 adipocytes (Chan et al., 1988; Müller et al., 1993) and in the yeast *S. cerevisiae* (Müller & Bandlow, 1991). The mammalian and yeast Gce1 proteins (Müller et al., 1992) share the glycoprotein nature, the GPI anchorage, and the topology, with the protein domain being located at the outer face of the plasma membrane. It is tempting to speculate about a common function of this protein in lower eucaryotes and mammalian cells as distantly related as yeast and rat adipocytes. A role in nutritional regulation has been suggested based on the observation that the GPI membrane anchors in both cell types are cleaved by an endogenous phospholipase in response to nutritional signals like glucose and insulin (Müller & Bandlow, 1993; Müller et al., in press).

In rat adipocytes, cleavage of the GPI anchor by bacterial PI-PLC does not result *per se* in the release of LPL and Gce1 from the cell surface. Our data strongly argue for specific recognition of the inositol (cyclic) monophosphate moiety of the PI-PLC-cleaved GPI anchor of both LPL and Gce1 by a receptor protein at the outer face of the plasma membrane. Consequently, GPI-proteins with intact GPI anchor do not interact with this receptor and, *vice versa*, binding of GPI-proteins to the receptor requires lipolytic cleavage of their GPI anchors.

Evidence for the cleavage of GPI-proteins by specific phospholipases induced in response to hormonal and nutritional signals has been presented for a number of different cell types including cultured hepatocytes (Ishihara et al., 1987), 3T3-L1 adipocytes (Chan et al., 1988; Lisanti et al., 1989; Müller et al., 1993), BC₃H1 myocytes (Romero et al., 1988), and yeast (Müller & Bandlow, 1993). Recent experiments revealed that in isolated rat adipocytes LPL and Gce1 are cleaved by an endogenous PLC in response to insulin and the sulfonylurea drug, glimepiride (Müller et al., in press). Preliminary evidence suggests that the generated hydrophilic GPI-proteins are not released from the surface of rat adipocytes or yeast spheroplasts (Müller & Bandlow, 1993). Thus the retention mechanism for lipolytically cleaved GPI-proteins studied here with bacterial PI-PLC-cleaved LPL and Gce1 may also function *in vivo*. Interestingly, in 3T3 adipocytes and BC₃H1 myocytes PLC-cleaved GPI-proteins were recovered as soluble molecules in the culture medium (Chan et al., 1988; Romero et al., 1988; Müller et al., 1993). This lack of membrane association may be due to a limited number of binding sites and their saturation by cleaved GPI-proteins

soon after stimulation of the endogenous PLC. In favor of this assumption, we observed that membranes of cultured 3T3 fibroblasts contain a significantly lower number of binding sites for PI-PLC-cleaved GPI-proteins than isolated adipocytes.

The peripheral association *via* bipolar interactions with a plasma membrane receptor protein may have an important regulatory role *in vivo* for those GPI-proteins which are destined to change their topology or location (within multicellular organisms) in response to extracellular signals. Such signals first will lead to cleavage of their GPI anchors as is the case for LPL: LPL is synthesized by adipocytes but finally will be transported to the luminal endothelial cell surface where it interacts with circulating lipoproteins and chylomicrons. During starvation a dramatic shift of LPL activity from adipose to muscle tissue ensues [for a review see Smith and Pownall (1984)]. The first step in the extracellular LPL transport pathway is the release of LPL from the surface of adipocytes (Spooner et al., 1979) which may be initiated by circulating lipolysis products from triglyceride-rich lipoproteins (Sasaki & Goldberg, 1992). Lipolytic cleavage of the GPI anchor of LPL in response to insulin, secreted during a previous period of food intake, will provide the pool of releasable cell surface-exposed LPL. In agreement with this hypothesis, LPL activity is low in plasma and muscle tissue of patients with severe uncontrolled diabetes which is always characterized by hypertriglyceridemia but returns to normal levels after administration of insulin or sulfonylurea (Bagdade et al., 1967). This indicates that lipolysis products *per se* are not sufficient to elicit dissociation of LPL from the membrane but require prior lipolytic cleavage of its GPI anchor and, *vice versa*, that PLC activation *per se* does not cause LPL release from the adipocyte surface. The lipolysis products may induce LPL release and thus ultimately regulate the flux of LPL from fat to muscle tissues by interference with the recognition of the inositol (cyclic) monophosphate residue of LPL by the adipocyte plasma membrane receptor.

A second functional role for the binding of PLC-cleaved GPI-proteins to the plasma membrane receptor may rely on mediation of the cellular uptake of GPI-proteins. In agreement with this possibility, it has been demonstrated that, after cleavage of its GPI membrane anchor by an insulin-activated PLC, heparan sulfate proteoglycan is rapidly internalized *via* a plasma membrane receptor which specifically recognizes the inositol monophosphate moiety of the residual GPI structure (Ishihara et al., 1987). Apart from pinocytosis which involves the uptake of GPI-proteins *via* caveolae [for recent reviews see Anderson (1992) and Anderson et al. (1993)] and endocytosis *via* clathrin-coated vesicles which depends on the binding of GPI-proteins to certain transmembrane proteins as has been shown for alkaline phosphatase (Makiya et al., 1992) and urokinase-type plasminogen activator receptor (Nykjaer et al., 1992), this could represent a third mode for internalization of GPI-proteins. However, it would apply to PLC-cleaved molecules, exclusively.

Internalization of PLC-cleaved GPI-proteins may be a prerequisite for a subsequent second processing step to occur within the residual GPI anchor structure which has been demonstrated recently for Gce1p of the yeast *S. cerevisiae* (Müller et al., in preparation). Interestingly, anti-CRD antibodies drastically impair the second cleavage reaction and, as shown in this report, also interfere with the association of PLC-cleaved Gce1 with the adipocyte plasma membrane. The possible relationship between binding to the receptor and the uptake/processing of GPI-proteins is under current investigation.

tion. Taken together, binding of PLC-cleaved GPI-proteins to a cell surface receptor potentially serves two purposes: (i) storage until release into the extracellular space and (ii) storage until and mediation of internalization (and secondary processing). The structural features of the anchors or proteins for being channeled into one pathway or the other and the signals causing either release or uptake are unknown at present.

In addition, it must be taken into consideration that the retention mechanism may recognize (and mediate internalization of) not only PLC-cleaved GPI-proteins but also phosphoinositol-glycan structures which are generated in mammalian cells from free glycosylphosphatidylinositol glycolipids in response to certain growth hormones, like insulin, by activation of an endogenous PLC [for a review see Saltiel (1990)]. These molecules isolated from insulin-stimulated cells or PI-PLC-treated plasma membranes possess a terminal inositol (cyclic) monophosphate residue and can mimic certain insulin actions if incubated with intact insulin-sensitive cells (Kellerer et al., 1993; Gottschalk & Jarett, 1988). It has been documented that the stimulation of lipogenesis by phosphoinositol-glycans in rat adipocytes is antagonized by inositol monophosphate in a concentration-dependent manner (Machicao et al., 1990). Since phosphoinositol-glycans but not inositol monophosphate are transported into cells (Alvarez et al., 1991; Stralfors & Alemany, 1990), the inhibitory effect of exogenous inositol monophosphate appears to be exerted at the step of cellular uptake which may involve specific plasma membrane binding sites for inositol monophosphate-containing molecules as described in this study.

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