Stimulation of a Glycosyl–Phosphatidylinositol-specific Phospholipase by Insulin and the Sulfonylurea, Glimepiride, in Rat Adipocytes Depends on Increased Glucose Transport

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Abstract. Lipoprotein lipase (LPL) and glycolipid-anchored cAMP-binding ectoprotein (Gcel) are modified by glycosyl–phosphatidylinositol (GPI) in rat adipocytes, however, the linkage is potentially unstable. Incubation of the cells with either insulin (0.1–30 nM) or the sulfonylurea, glimepiride (0.5–20 μM), in the presence of glucose led to conversion of up to 35 and 20%, respectively, of the total amphiphilic LPL and Gcel to their hydrophilic versions. Inositol–phosphate was retained in the residual protein-linked anchor structure. This suggests cleavage of the GPI anchors by an endogenous GPI-specific insulin- and glimepiride-inducible phospholipase (GPI–PL). Despite cleavage, hydrophilic LPL and Gcel remained membrane associated and were released only if a competitor, e.g., inositol–(cyclic)monophosphate, had been added. Other constituents of the GPI anchor (glucosamine and mannose) were less efficient. This suggests peripheral interaction of lipolytically cleaved LPL and Gcel with the adipocyte cell surface involving the terminal inositol–(cyclic)monophosphate epitope and presumably a receptor of the adipocyte plasma membrane. In rat adipocytes which were resistant toward glucose transport stimulation by insulin, the sensitivity and responsiveness of GPI–PL to stimulation by insulin was drastically reduced. In contrast, activation of both GPI–PL and glucose transport by the sulfonylurea, glimepiride, was not affected significantly. Inhibition of glucose transport or incubation of rat adipocytes in glucose-free medium completely abolished stimulation of GPI–PL by either insulin or glimepiride. The activation was partially restored by the addition of glucose or nonmetabolizable 2-deoxyglucose. These data suggest that increased glucose transport stimulates a GPI–PL in rat adipocytes.

A great body of information exists regarding the structural diversity as well as the biosynthesis and post-translational attachment of glycosyl-phosphatidylinositol (GPI)[1] structures (for recent reviews see Low, 1989; McConville et al., 1993). However, the functional significance of membrane anchorage via GPI structures versus *trans*membrane polypeptide domains is still a matter of debate. The accessibility of GPI molecules to cleavage by phospholipase [(G)PI-PL] opens the possibility of a regulated release of the polypeptide portion of proteins which have been synthesized with a GPI membrane anchor (GPI-proteins) from the cell surface (for a review see Low and Saltiel, 1988; Cross, 1990). Experimental evidence has been presented that the released protein may exhibit altered functional characteristics (e.g., Müller and Bandlow, 1994) and/ or, in the case of multicellular organisms, may fulfill a special physiological role at its new location (e.g., Smith and Pownall, 1984).

Whereas the number of studies on constitutive membrane release of GPI-proteins is steadily increasing (e.g., Almqvist and Carlsson, 1988; Furley et al., 1990; Hortsch and Goodman, 1990), only limited experimental evidence is available for regulated release so far. In a rat hepatocyte cell line it has been demonstrated that the linkage between the protein moiety of heparin sulfate proteoglycan and its GPI anchor is cleaved by an insulin-activated PLC (Ishihara et al., 1987). Short-term incubation of 3T3-L1 adipocytes and BC₃H1 myocytes with insulin or fetal serum causes release of some GPI-anchored ectoproteins. Among the proteins liberated from the cell surface into the culture medium are lipoprotein

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1. *Abbreviations used in this paper*: CRD, cross-reacting determinant; Gcel, glycolipid-anchored cAMP-binding ectoprotein; GLUT4, glucose transporter isoform 4; (G)PI-PL(C/D), (glycosyl)–phosphatidylinositol-specific phospholipase (C/D); GPI, glycosyl-phosphatidylinositol; GPI-protein, proteins which have been synthesized with a GPI membrane anchor irrespective of whether this moiety is preserved or cleaved; LDM, low density microsomes; LPL, lipoprotein lipase; TX-114, Triton X-114; VSG, variant surface glycoprotein.

lipase (LPL) (Spooner et al., 1979; Chan et al., 1988), alkaline phosphatase (Romero et al., 1988), and a set of additional unidentified GPI-proteins (Lisanti et al., 1989). In the case of LPL, retention of radiolabeled *myo*-inositol in the released molecules suggested lipolytic cleavage of the GPI moiety in response to insulin (Chan et al., 1988). Furthermore, treatment of isolated rat diaphragm with insulin leads to a decreased plasma membrane-associated 5'-nucleotidase activity which has been interpreted as an insulin-induced release of the GPI-modified isoform of this enzyme from the plasma membrane (Klip et al., 1988). These observations have been interpreted in terms of a mechanistic coupling and direct signaling between the insulin (growth factor) receptors and GPI-PL's in mammalian cells. Finally, it has been demonstrated recently that glimepiride, a novel sulfonylurea drug (Geisen, 1988) which stimulates glucose transport in insulin-sensitive isolated and cultured cells (Müller and Wied, 1993), induces lipolytic cleavage of the GPI anchors of LPL, 5'-nucleotidase and a cAMP-binding ectoprotein (Gce1), in cultured 3T3 adipocytes in a concentration-dependent manner (Müller et al., 1993). In parallel to these observations with vertebrate cells, it has been described with lower eukaryotes that a nutritional upshift of spheroplasts from the yeast *Saccharomyces cerevisiae* from lactate to glucose medium activates a GPI-PLC which cleaves the GPI anchor of a cAMP-binding ectoprotein dependent on the concentration of glucose (Müller and Bandlow, 1993). These data suggest the existence of similar signaling cascades for the activation of GPI-PLs in lower eukaryotes and mammalian cells with multiple entry sites for hormonal, drug-induced, or nutritional signals.

In an attempt to elucidate the molecular mechanism which links these various signals to the lipolytic cleavage of certain GPI-proteins, we first studied whether isolated rat adipocytes, which are characterized by exquisite sensitivity and responsiveness toward hormones (like insulin), certain drugs (like sulfonylureas), and the nutritional situation, can be used as a model system for regulated GPI-protein release. We found that the GPI anchors of two GPI-proteins, LPL and the cAMP-binding ectoprotein, Gce1, are cleaved by a GPI-PL after stimulation of glucose transport by insulin and glimepiride which seems to funnel a signaling cascade leading to activation of the GPI-PL.

Materials and Methods

Materials

Affinity-purified ^{125}I-labeled anti-rabbit IgG from sheep and AMPLIFY were obtained from Amersham-Buchler (Braunschweig, Germany); reagents for cell culture were obtained from GIBCO BRL (Eggenstein-Leopoldshafen, Germany); protein A-Sepharose CL-4B was purchased from LKB/Pharmacia (Freiburg, Germany); 2-deoxyglucose, cytochalasin B, phloretin, and polymyxin B were bought from Sigma (Deisenhofen, Germany); protease inhibitors were bought from Boehringer Mannheim GmbH (Mannheim, Germany); partially purified PI-PLC (*Bacillus thuringiensis*) was delivered by Biotrend (Köln, Germany); recombinant human insulin and glimepiride (HOE 490) were prepared by the synthesis department of Hoechst AG (Frankfurt a.M., Germany); male Wistar rats were bred and delivered by Hoechst AG (Tierzucht Kastengrund, Germany); affinity-purified polyclonal anti-glucose transporter isoform 4 (GLUT4) antibodies were obtained from rabbits injected with a peptide corresponding to the COOH-terminal 16 amino acids of rat GLUT4 and coupled to keyhole limpet hemocyanin; all other radiochemicals, chemicals, and antibodies were obtained as described previously (Müller et al., 1993; Müller and Wied, 1993).

Primary Culture of Adipocytes

Freshly prepared adipocytes were washed twice by flotation with 10 vol of HBSS (Marshall et al., 1991) containing 25 U/ml penicillin and 50 μg/ml streptomycin. After the final wash, 1 ml of suspension (2 × 10^5 cells/ml) was added to 3 ml of HBSS containing 5 mM glucose (control) or 25 mM glucose, 10 nM insulin, 16 mM glutamine (for desensitization of the glucose transport system) and incubated (20 h, 37°C) in sterile 50-ml polystyrene tubes as described (Traxinger and Marshall, 1989). After incubation, control and desensitized adipocytes were washed by flotation, twice with 40 ml of HBSS containing 0.5% BSA and two times with 4 ml of DMEM containing 0.5 mM glucose, 1% BSA, 2% fetal calf serum, and 25 mM Hepes (pH 7.4). Then the cells were suspended at 0.5 × 10^5 cells/ml in the same medium and incubated (30 min, 37°C, gentle stirring) to allow deactivation of the glucose transport system of insulin-stimulated cells to near basal values before subsequent incubation with glimepiride or insulin. Control and desensitized adipocytes were derived from the same pool of commonly isolated and washed cells, subjected to an equal number of washes so that any losses of cells were identical with the two cell populations. Subsequent procedures (photoaffinity and metabolic labeling, incubation with glimepiride and insulin) were performed in parallel for both groups of cells.

Incubation of Adipocytes with Insulin and Glimepiride

Freshly isolated or primary-cultured adipocytes were incubated (37°C) for various periods with the indicated concentrations of insulin and glimepiride in 1 ml of DME medium at 2 × 10^5 cells/ml in 10-ml scintillation vials under very gentle agitation with the cells floating on top of the medium in a thin cell layer. Insulin was added from a stock solution made daily by dissolving 0.5 mg insulin in 150 μl aqua bidest./150 μl of 0.1 N HCl and then supplementing 200 μl of twofold KRH buffer lacking glucose; glimepiride was added from a 10 mM stock solution prepared daily by suspending 9.95 mg glimepiride in 1.94 ml aqua bidest., subsequently 60 μl of 1 N NaOH was added and it was warmed up to 50–70°C. Following incubation, the cells were washed by flotation two times with 5 ml each of HBSS containing 1 mM sodium pyruvate, 0.2% BSA, 1 mM ETDA, 200 μM PMSF, 100 μM benzamidine, 20 μg/ml of leupeptin, pepstatin, aprotinin, and antipain and finally concentrated fivefold (1 × 10^6 cells/ml) in the same buffer lacking BSA for subsequent analysis of GPI-proteins and preparation of plasma membranes.

Miscellaneous Procedures

Published procedures were used for immunoblotting of GLUT4 using antibodies raised in rabbits against rat GLUT4 and ^{125}I-labeled anti-rabbit IgG from sheep (Towbin et al., 1979; Müller and Wied, 1993); determination of 2-deoxyglucose transport in rat adipocytes (Foley and Gliemann, 1981; Müller and Wied, 1993); isolation (Rodbell, 1964), metabolic labeling with *myo*-[U-14C]inositol (Müller et al., 1994b), photoaffinity labeling with 8-N_3-[32P]cAMP (Pomerantz et al., 1975; Müller and Bandlow, 1991), and subcellular fractionation of rat adipocytes (Müller et al., 1994a); centrifugation of plasma membranes through a cushion of sucrose (Müller, G., E.-M. Wetekam, C. Jung, and W. Bandlow, manuscript submitted for publication); purification of Gce1 by affinity chromatography on N^6-(2-aminoethyl)cAMP-Sepharose (Müller et al., 1992, 1993); immunoprecipitation of LPL using a polyclonal rabbit antiserum raised against human milk LPL and protein A-Sepharose (Müller et al., 1993); Triton X-114 (TX-114) partitioning (Bordier, 1981); TLC analysis (Müller et al., 1994b); protein determination (Popov et al., 1975); SDS-PAGE and fluorography using AMPLIFY (Müller and Zimmermann, 1987).

Results

Hydrophilic Membrane-associated Versions of Gce1 and LPL Are Generated in Response to Insulin and Glimepiride

We first examined whether incubation of isolated rat adipocytes with glucose, insulin or glimepiride, a representative of the sulfonylurea drugs, for which glucose transport stimulation in insulin-sensitive cells in vitro has been amply documented (Jacobs et al., 1987; Rogers et al., 1987; Farese et al., 1991), causes conversion of amphiphilic LPL and
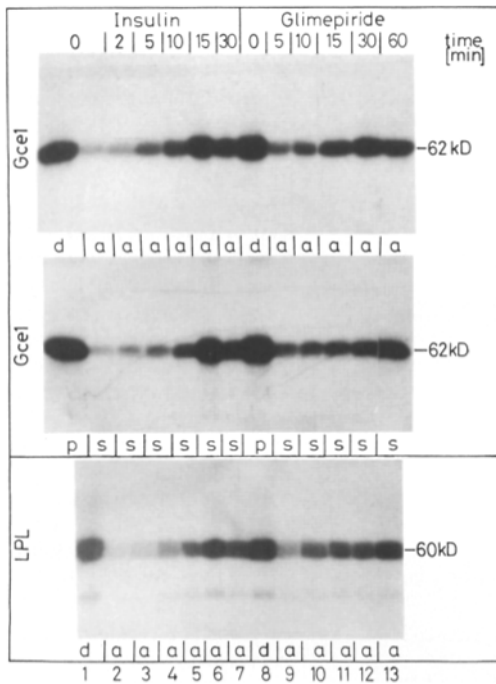
Figure 1. Lipolytic cleavage of the GPI anchors of LPL and Gcel in response to insulin and glimepiride. Adipocytes were metabolically labeled with *myo*-[^{14}C]inositol and subsequently incubated with 10 μM glimepiride or 10 nM insulin for the periods indicated. Plasma membranes were prepared and subjected to TX-114 partitioning (*top* and *bottom*) or centrifugation through a sucrose cushion in the presence of 0.5 M NaCl (*middle*). Gcel and LPL were purified by affinity-chromatography or immunoprecipitation, respectively, from the detergent (*d*) and aqueous (*a*) phases or from the pellet (*p*) and supernatant fractions (*s*) and then analyzed by SDS-PAGE and fluorography. Molecular masses indicated on the right margin were derived from marker proteins run in parallel.

Gcel into their hydrophilic forms. Adipocytes were metabolically labeled with *myo*-[^{14}C]inositol, then incubated with insulin or glimepiride in the presence of glucose, and subsequently subjected to amphiphilic/hydrophilic partitioning using TX-114 or to centrifugation through a sucrose cushion. LPL was purified by immunoprecipitation with monospecific antiserum and Gcel by cAMP-affinity chromatography. The fluorogram of the SDS-PAGE (Fig. 1, *top* and *bottom*) shows that both, insulin and glimepiride, caused a time-dependent conversion of amphiphilic LPL and Gcel (partitioning into the detergent phase; Fig. 1, lanes *1* and *8*) into the hydrophilic forms (partitioning into the aqueous phase; lanes *2–7* and *9–13*). Significant amounts of hydrophilic Gcel and LPL were detectable already 2 and 5 min after the addition of insulin or glimepiride, respectively (Fig. 1, lanes *3* and *9*). After 15 or 30 min, respectively, the amount of hydrophilic GPI-proteins reached a maximum (Fig. 1, lanes *6* and *12*) accounting for up to 35% (*Gcel*) and 25% (*LPL*) of the total amount of amphiphilic GPI-proteins in nonstimulated cells (compare with lanes *1* and *8*). Thereafter, the amount of hydrophilic GPI-proteins declined again which was more obvious for insulin (lane *7*) than glimepiride (lane *13*).

Since the hydrophilic forms of LPL and Gcel were recovered from isolated plasma membranes of insulin- and glimepiride-stimulated cells we next studied the nature of their membrane association. Fig. 1 (*middle*) reveals that incubation with 0.5 M NaCl and subsequent centrifugation through sucrose of plasma membranes isolated from insulin- and glimepiride-treated cells led to almost quantitative recovery of LPL and Gcel in the supernatant fraction (shown only for Gcel). Omission of the salt prevented the dissociation from the membrane indicating bipolar interactions as found recently after incubation of rat adipocytes with bacterial PI–PLC (Müller, G., E.-M. Wetekam, C. Jung, and W. Bandlow, manuscript submitted for publication). The kinetics and efficiency of the insulin- and glimepiride-induced membrane release and amphiphilic to hydrophilic conversion were very similar (Fig. 1, lanes *2–7* and *9–13*, compare *top* and *middle*). These data indicate that short term treatment of rat adipocytes with insulin and glimepiride stimulates cleavage of the GPI membrane anchors of LPL and Gcel as has been described for 3T3 adipocytes, but in contrast, does not cause dissociation from the membrane of the cleaved versions due to bipolar interactions with the cell surface.

Recent experimental evidences suggest that some lipolytically cleaved GPI-proteins are recognized by a receptor of hepatocyte and adipocyte plasma membranes (Ishihara et al., 1987; G. Müller, unpublished observations). The PLC-cleaved proteins can be released from isolated membranes by incubation with certain sugar constituents of the GPI structure like inositol–(cyclic)monophosphate. Consequently, we studied whether this type of interaction also applied to the hydrophilic forms of LPL and Gcel generated in response to insulin or glimepiride. Adipocytes, metabolically labeled with *myo*-[^{14}C]inositol, were incubated with 10 nM insulin or 10 μM glimepiride. Isolated plasma membranes were incubated with increasing concentrations of sugar compound and then spun through a cushion of sucrose. The pellet fractions were subjected to TX-114 partitioning. The aqueous phases and supernatant fractions were used for purification and SDS-PAGE of Gcel and LPL. The amounts of hydrophilic membrane-associated or soluble Gcel and LPL were determined by liquid scintillation counting of the corresponding excised gel regions. Fig. 2 shows that the amount of membrane-associated hydrophilic Gcel (*a*) and LPL (*b*) decreased with increasing concentrations of inositol-1,2-cyclic phosphate, inositol-1-monophosphate and glucosamine but was not significantly affected by mannose. Simultaneously, the amount of the hydrophilic forms increased in the supernatant fractions (data not shown). 85–90% of the total hydrophilic Gcel and LPL generated in response to either insulin or glimepiride could be displaced as a maximum. As expected, the IC$_{50}$ values of the sugar compounds were very similar for displacement of the GPI-proteins from insulin- and glimepiride-treated cells, inositol-1,2-cyclic monophosphate being most effective. But Gcel was generally released with lower concentrations of the sugar compounds (IC$_{50}$ values; inositol-1,2-cyclic phosphate: 90–110 μM; inositol-monophosphate: 200–220 μM; glucosamine: 1.0–1.5 mM) than LPL (IC$_{50}$ values; inositol-1,2-cyclic phosphate 400–500 μM; inositol-monophosphate: 750–900 μM; glucosamine: 3–5 mM). These findings suggest that the major fraction of both Gcel and LPL converted from the amphiphilic into their hydrophilic forms in response to insulin and glimepiride was retained at the adipocyte cell surface via

a

b
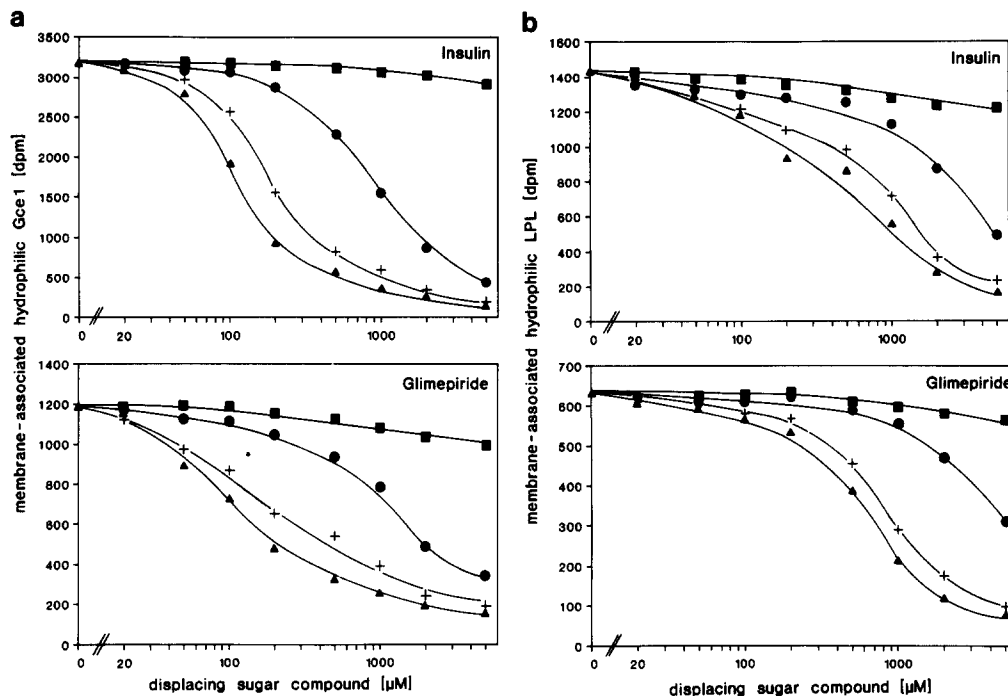
Figure 2. Dissociation from the membrane of lipolytically cleaved LPL and Gce1 with sugar compounds. Adipocytes were metabolically labeled with *myo*-[^{14}C]inositol and subsequently incubated (30 min, 37°C) with insulin (10 nM) or glimepiride (10 µM). Plasma membranes were prepared, incubated (15 min, 4°C) in the absence or presence of the indicated concentrations of inositol-1,2-cyclic monophosphate (▲), inositol-1-monophosphate (+), glucosamine (●), and mannose (■) and then centrifuged through a cushion of sucrose. The plasma membranes recovered in the pellet were subjected to TX-114 partitioning. Hydrophilic Gce1 (*a*) and LPL (*b*) were purified from the aqueous phase by affinity purification or immunoprecipitation, respectively. All samples were separated by SDS-PAGE and fluorography. The excised gel regions containing membrane-associated hydrophilic LPL and Gce1 were measured for radioactivity. The amounts of radiolabeled total hydrophilic LPL or Gce1 determined prior to centrifugation were as follows: Gce1 (+ insulin), 4951 dpm; Gce1 (+ glimepiride), 2031 dpm; LPL (+ insulin) 2265 dpm; LPL (+ glimepiride), 941 dpm.

binding to similar or identical plasma membrane receptors which mainly recognize the terminal inositol–(cyclic)monophosphate epitope and, possibly, the glucosamine moiety of the residual GPI anchor structures.

The GPI-anchors of Gce1 and LPL Are Cleaved by a Phospholipase in Response to Insulin and Glimepiride

The retention of *myo*-[^{14}C]inositol radiolabel in the hydrophilic and soluble versions of LPL and Gce1 is compatible with the action of a phospholipase which is controlled by insulin and glimepiride. To confirm this and to gain a first insight into its cleavage specificity, we analyzed the cleavage site. Adipocytes were labeled with [^{14}C]inositol and then stimulated with insulin (10 nM) or glimepiride (10 µM) for 30 min. Hydrophilic LPL and Gce1 were purified from isolated plasma membranes and then subjected to acid hydrolysis or nitrous deamination. Fig. 3 shows the TLC analysis and fluorography of the degradation products. After acid hydrolysis most of the radiolabel of the GPI-proteins from insulin- and glimepiride-treated adipocytes was recovered as inositol (Fig. 3, lanes *1* and *4*) excluding insulin- and glimepiride-induced conversion of *myo*-inositol into other sugar components. Nitrous acid deamination generated about equal amounts of radiolabeled inositol-phosphate and inositol from both GPI-proteins after insulin as well as glimepiride stimulation (Fig. 3, lanes *2* and *5*). The inositol–phosphate was hydrolyzed to inositol by subsequent treatment with HCl (Fig. 3, lanes *3* and *6*) proving its identity. The identification of inositol-monophosphate in the TLC patterns provides the first evidence for cleavage of the GPI anchors by an insulin- and glimepiride-activated GPI-PLC, but definite proof for this specificity will require

further experimental support. The failure to detect inositol-1,2-cyclic phosphate and the appearance of inositol after deamination may be due to either intrinsic phosphodiesterase and phosphatase activities in the adipocyte plasma mem-
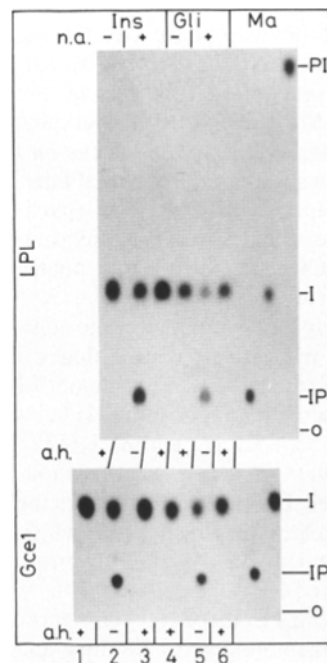
Figure 3. TLC analysis of the GPI anchor of hydrophilic LPL and Gce1. Adipocytes were metabolically labeled with *myo*-[^{14}C]inositol and subsequently incubated (30 min, 37°C) with 10 nM insulin (*Ins*) or 10 µM glimepiride (*Gli*). Plasma membranes were prepared and subjected to TX-114 partitioning. Hydrophilic Gce1 and LPL were purified from the aqueous phase by affinity chromatography or immunoprecipitation, respectively, and then subjected to nitrous acid deamination (*n.a.*) or left untreated. The untreated and one half of the deaminated samples were hydrolyzed with HCl (*a.h.*), the other half was left untreated. All samples were analyzed by TLC and fluorography. The positions of radiolabeled markers, inositol-1-monophosphate (*IP*), *myo*-inositol (*I*) and phosphatidylinositol (*PI*), run in parallel, are indicated on the right margin (*Ma*).
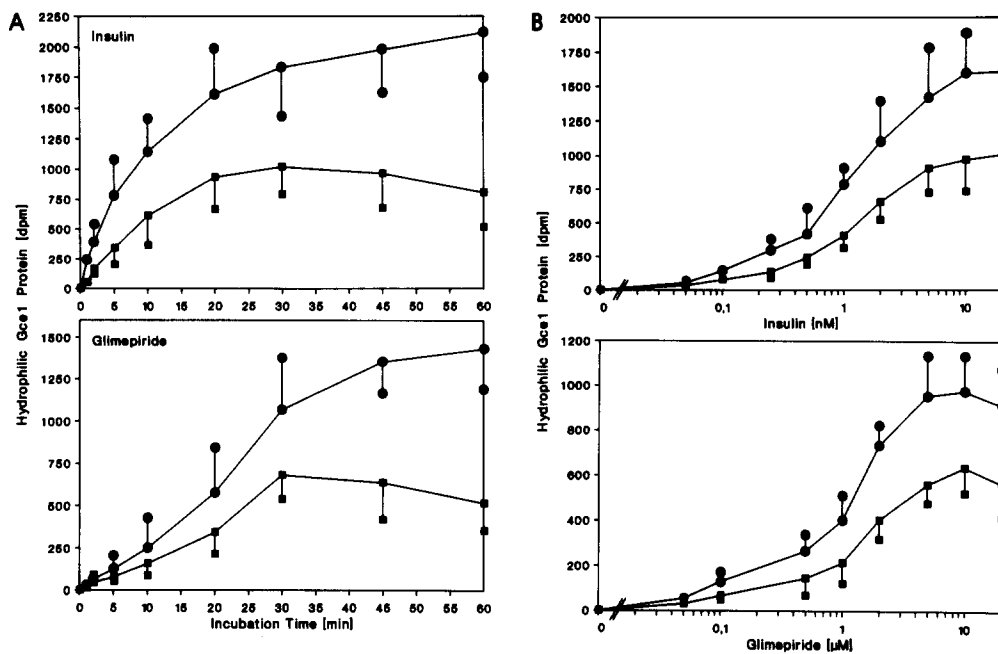
Figure 4. Immunoprecipitation of hydrophilic Gce1 with anti-CRD antibodies. Adipocytes were photoaffinity-labeled with 8-N₃-[³²P]cAMP and then incubated in the absence or presence of 10 nM insulin or 10 μM glimepiride for the periods indicated (A) or in the absence or presence of various concentrations of insulin and glimepiride for 20 min (B). The incubation was terminated by the addition of TX-114 (2% final concentration) and phase separation. Hydrophilic Gce1 was purified from the aqueous phase by affinity chromatography. Subsequently, one half of the samples was subjected to quantitative immunoprecipitation using anti-CRD antibodies (■), the other half was left (●). Total and immunoprecipitated samples were analyzed by SDS-PAGE and autoradiography or fluorography, respectively. The radioactivity (dpm values) contained in the gel region corresponding to 62 kD (Gce1) was determined by a Berthold radioactivity scanner and calculated for each time point or concentration as the difference between radioactivity measured for incubation with insulin or glimepiride or without additions (basal). The values represent the means of six determinations using two different cell preparations ($n = 12$, ±SD).

brane or to an enzymic mechanism of the putative adipocyte GPI-PL which differs from that of bacterial PI-PLCs (Lewis et al., 1993). Furthermore, GPI-PLD activity associated with adipocyte plasma membranes which may be due to an endogenous enzyme or originate from the serum cannot be excluded. The existence of a GPI-PLD in mammalian serum and tissues has well been documented (Cardoso de Almeida et al., 1988; Low and Prasad, 1988; Davitz et al., 1989; Hoener et al., 1990; Huang et al., 1990).

To gain information about the physiological significance of the adipocyte GPI-PL, we studied the time course and concentration dependence of the stimulation of this enzyme by insulin and glimepiride. For this, the hydrophilic Gce1 was purified from the aqueous phase after TX-114 partitioning of total lysates from adipocytes which had been photoaffinity labeled with 8-N₃-[³²P]cAMP and then incubated with insulin or glimepiride. To assay for the cleavage of the GPI anchor, half of the hydrophilic Gce1 was tested for anti-cross-reacting determinant (CRD) immunoreactivity by subsequent immunoprecipitation with anti-CRD antibodies. These antibodies exclusively recognize determinants in the (glycosyl)-phosphatidylinositol-specific phospholipase [(G) PI-PLC]-cleaved anchors and do not cross-react with protein epitopes (Cardoso de Almeida et al., 1988; Zamze et al., 1988). Fig. 4 shows that there is a difference between the amounts of total and immunoprecipitated hydrophilic [³²P]cAMP-labeled Gce1 purified from insulin- or glimepiride-stimulated and nonstimulated adipocytes. From the time course (Fig. 4 *a*) it can be seen that the amount of total hydrophilic form increased up to 45 min for insulin (*upper half*) and glimepiride stimulation (*lower half*), followed by a plateau for the next 15–60 min (not shown). However, at each time point anti-CRD antibodies recognized only a fraction of the total hydro-

Table I. Generation of Hydrophilic Gce1 Lacking the Anti-CRD Epitope in Response to Insulin or Glimepiride

	Incubation Time min	Gce1 lacking anti-CRD epitope	
		Total Amount arb. units	Fraction % total
Insulin	1	1.86	47.8
	2	2.22	57.1
	5	4.36	56.1
	10	5.33	46.6
	20	6.79	42.1
	30	8.09	44.2
	45	10.37	51.8
	60	13.11	62.0
Glimepiride	1	0.67	46.2
	2	1.64	55.0
	5	2.42	50.1
	10	2.78	40.7
	20	3.95	40.8
	30	5.15	43.2
	45	7.41	54.7
	60	8.38	61.8

The dpm values measured for total and anti-CRD immunoreactive hydrophilic [³²P]cAMP photoaffinity-labeled Gce1 were derived from Fig. 7 *a*. The amount of hydrophilic Gce1 lacking anti-CRD epitope was calculated as the difference between hydrophilic total and anti-CRD immunoreactive protein (*arb. units*) and expressed as fraction of the hydrophilic total protein at each time point (set at 100%; Fig. 7 *b*).

philic Gce1 which declined after 30 min. Thus, a considerable fraction of the hydrophilic form of the GPI-protein did not harbor the major epitope, the terminal inositol-(cyclic)-monophosphate residue. Gce1 protein lacking this epitope accumulated during the incubation resulting in a drastic in-
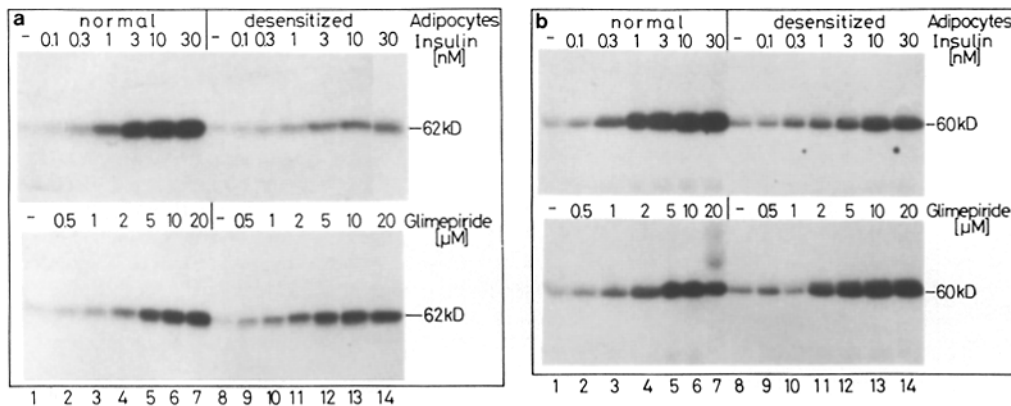
Figure 5. Lipolytic cleavage of LPL and Gcel in response to insulin and glimepiride in normal and desensitized adipocytes. Adipocytes were incubated (20 h, 37°C) in primary culture in the presence of 0.5 mM glucose (*normal*) or 20 mM glucose plus 16 mM glutamine plus 10 nM insulin (*desensitized*). Subsequently, the washed cells were metabolically labeled with *myo*-[^{14}C]inositol and then incubated (30 min, 37°C) with various concentrations of insulin or glimepiride. Plasma membranes were prepared and subjected to TX-114 partitioning. Hydrophilic Gcel (62 kD) and LPL (60 kD) were purified from the aqueous phase by affinity chromatography (*a*) or immunoprecipitation (*b*), respectively. All samples were analyzed by SDS-PAGE and fluorography. The molecular masses were derived from marker proteins run in parallel.

crease of its total amount with time (Table I). The ratio with regard to total hydrophilic protein increased only at later incubation times. This time course is compatible with the observed reduction of the amount of hydrophilic *myo*-[^{14}C]inositol-labeled LPL and Gcel after 15 min of stimulation by insulin and 30 min by glimepiride (see Fig. 1). But, these data suggest that, in addition to the lipolytic cleavage, a second hydrolytic processing event occurs within the GPI structure of certain GPI-proteins in consequence of an incubation of rat adipocytes with insulin or glimepiride and removes the epitope(s) recognized by the anti-CRD antiserum. From the concentration dependence of short term stimulation (20 min) (Fig. 4 *b*) of the PL activity (reflected by the amount of hydrophilic anti-CRD immunoreactive Gcel) and of the additional hydrolytic activity (reflected by the difference between total and anti-CRD immunoreactive hydrophilic protein), we calculated for insulin a maximal effect at 10 nM for both activities and ED$_{50}$ values of 1.2 and 2.1 nM, respectively, and for glimepiride a maximal effect at 10 μM and ED$_{50}$ values of 1.7 and 2.5 μM, respectively. Thus, the concentrations of insulin and glimepiride required for stimulation of the PL and the second hydrolytic activity are very similar. These observations are compatible with the possibility that the lipolytic cleavage reaction is rate limiting for the subsequent hydrolytic processing but do not exclude two independent processing mechanisms with similar dose response characteristics. The effective concentrations of insulin and glimepiride lie in the range necessary for glucose transport stimulation by insulin and glimepiride in rat adipocytes (Muller and Wied, 1993). This correlation provides a first hint that glucose transport is required for activation of the GPI-PL.

## Impairment of Glucose Transport Interferes with Activation of the GPI-PL

To gain further evidence for this conclusion we used rat adipocytes which exhibited an impaired responsiveness and sensitivity of glucose transport stimulation by insulin but not by glimepiride. Adipocytes were incubated with high concentrations of insulin, glucose, and glutamine in primary culture which causes desensitization of the glucose transport system towards insulin but not glimepiride. This is reflected

by a 50% reduction of the maximal insulin-stimulated glucose transport velocity and a rightward shift of the dose-response curve of insulin from an ED$_{50}$ of 0.45 nM to 1.85 nM, whereas the maximal glimepiride response and glimepiride dose-response curve hardly were affected (Müller and Wied, 1993). The response of the PL to insulin and glimepiride was studied in desensitized cells after metabolic labeling with *myo*-[^{14}C]inositol. The fluorograms after the SDS-PAGE of LPL (Fig. 5 *a*) and Gcel (*b*), lipolytically cleaved under these conditions, show that the amounts of the hydrophilic forms recovered at each insulin concentration (*upper halves*) was considerably lower in desensitized (lanes *9–14*) versus normal adipocytes (lanes *2–7*). This resulted in a reduced maximal responsiveness (15–25% for LPL, 30–35% for Gcel) and sensitivity (rightward shift of the corresponding dose response curves; desensitized: ED$_{50}$ = 7.5 nM for LPL, 4.7 nM for Gcel; normal: ED$_{50}$ = 2.2 nM for LPL, 1.7 nM for Gcel) of the PL toward stimulation by insulin. In contrast, its maximal responsiveness and sensitivity toward stimulation by glimepiride (*lower halves*) remained largely unaffected (Fig. 5, lanes *9–14*). The same behavior holds for glucose transport: the activation by insulin is reduced (data

Table II. Inhibition of Glucose Transport in Rat Adipocytes

Stimulation	Inhibitor			
	Control	Cytochalasin B	Phloretin	Polymyxin B
Basal	254 ± 66	223 ± 81	308 ± 56	198 ± 38
Insulin	3891 ± 431	487 ± 78	571 ± 102	389 ± 61
Glimepiride	1623 ± 210	305 ± 55	413 ± 71	255 ± 41

Adipocytes were incubated (15 min, 37°C) in the absence (*Control*) or presence of 20 μM cytochalasin B, 0.3 mM phloretin, or 0.5 mg/ml polymyxin B and then supplemented with insulin (10 nM final concentration) or glimepiride (10 μM final concentration), or left untreated. After further incubation (30 min), 1 μCi of D-2-[^{3}H]deoxyglucose (0.1 mM final concentration) was added. The uptake reaction (10 min, 25°C) was terminated by addition of cytochalasin B (50 μM final concentration) to all samples and rapid centrifugation of the incubation mixture through dinonylphtalate. The cells were recovered from the oil layer and counted for radioactivity. Each dpm value is corrected for diffusion into cells and entrapping in extracellular spaces of [^{3}H]deoxyglucose by subtracting the value measured for corresponding incubation mixtures in the presence of 0.1 mM L-[^{3}H]deoxyglucose (identical specific radioactivity). The values represent the means of eight determinations from two different cell preparations each (n = 16, ±SD).
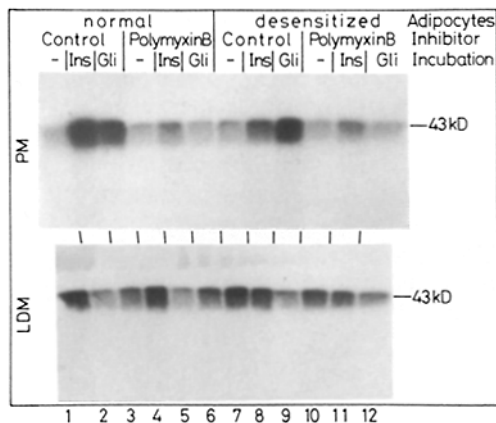
Figure 6. Inhibition of insulin- and glimepiride-induced GLUT4 translocation by polymyxin B. Adipocytes were incubated (20 h, 37°C) in primary culture in the presence of 0.5 mM glucose (*normal*) or 20 mM glucose plus 16 mM glutamine plus 10 nM insulin (*desensitized*). Subsequently, the adipocytes were preincubated (20 min) without (*Control*) or with 0.5 mg/ml polymyxin B and then incubated in the absence or presence of 10 nM insulin (*Ins*) or 10 μM glimepiride (*Gli*). Plasma membranes (*PM*) and low density microsomes (*LDM*) were prepared, separated by SDS-PAGE and analyzed for the presence of GLUT4 by immunoblotting. The autoradiogram of a typical blot is shown. The molecular masses are derived from marker proteins run in parallel.

not shown here; see Müller and Wied, 1993), whereas stimulation by glimepiride is unchanged. Thus desensitization of the glucose transport system in rat adipocytes is accompanied by decreased activation of a GPI-PL or, in other words, the activity states of the glucose transport system and the PL seem to correlate. Moreover, this correlation can be extended to the translocation of GLUT4 from tubulovesicular structures of the *trans*-Golgi network (which fractionate as low density microsomes [LDM]) to plasma membranes. GLUT4 translocation represents the molecular basis for the increase of glucose transport by insulin or glimepiride (Cushman and Wardzala, 1980; Suzuki and Kono, 1980; James et al., 1989; Slot et al., 1991; Müller and Wied, 1993) and is impaired in desensitized adipocytes toward stimulation by insulin but not glimepiride (see below, Fig. 6).

Next, we tested the effect of inhibitors of glucose transport

on the activation of the GPI-PL. Table II shows that 20 μM cytochalasin B, 0.3 mM phloretin or 0.5 mg/ml polymyxin B completely inhibited the insulin- and glimepiride-stimulated glucose transport in rat adipocytes. Cytochalasin B and phloretin bind directly to GLUT1 and GLUT4 (for a review see Gould and Holman, 1993), thereby interfering with the facilitated diffusion of glucose. Polymyxin B inhibits insulin stimulation of glucose transport by blocking the insulin-induced translocation of GLUT4 (Cormont et al., 1992). This was demonstrated by successive incubation of normal and desensitized adipocytes with polymyxin B and insulin and subsequent fractionation into LDM and plasma membranes which were assayed for GLUT4 by immunoblotting. The autoradiogram of the blot (Fig. 6) shows that in normal adipocytes insulin (lanes 2) and, to a lesser degree, glimepiride (lanes 3) caused a decrease of immunoreactive GLUT4 (43 kD) in LDM and a corresponding increase in the plasma membrane reflecting translocation of GLUT4 from LDM to the plasma membrane. Preincubation of normal adipocytes with polymyxin B or desensitization of adipocytes diminished insulin-induced GLUT4 translocation to 15–10% (Fig. 6, lane 5, *top*) or 50–40% (lane 8, *top*), respectively. Despite this block in translocation, incubation with polymyxin B led to a significant decline of GLUT4 in LDM (Fig. 6, lane 5, *bottom*). This is in contrast to desensitized cells (lane 8, *bottom*) and may be caused by targeting of LDM to an unidentified intracellular storage compartment or by inhibition of fusion of LDM with the plasma membrane. Similar observations have been reported previously by others (Cormont et al., 1992). In desensitized adipocytes glimepiride caused 65–70% translocation of GLUT4 to the plasma membrane (Fig. 6, lanes 9) compared to normal cells (lanes 6). This translocation was reduced by polymyxin B to 25–15% despite significant loss of GLUT4 from LDM (Fig. 6, lanes 6 and 12).

Amphiphilic and hydrophilic LPL and Gce1 were purified from plasma membranes of adipocytes, which had been metabolically labeled with myo-[^{14}C]inositol and then incubated with the respective inhibitor followed by exposure to insulin (Fig. 7 a) or glimepiride (b). The analysis by SDS-PAGE and fluorography shows that cytochalasin B, phloretin and polymyxin B (data not shown) did not affect the distribution between the amphiphilic and hydrophilic versions of the two GPI-proteins in nonstimulated control cells (Fig. 7 a,
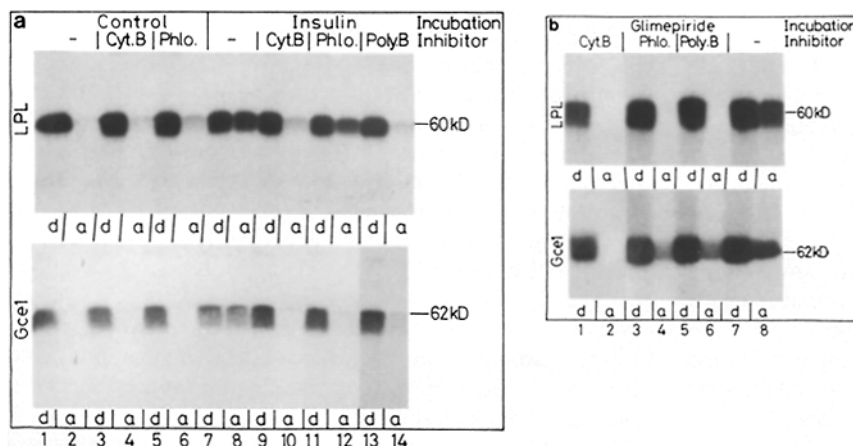
Figure 7. Effect of glucose transport inhibitors on the lipolytic cleavage of LPL and Gce1. Adipocytes were metabolically labeled with myo-[^{14}C]inositol and subsequently incubated (20 min, 37°C) in the absence or presence of 20 μM cytochalasin B (*Cyt.B*), 0.3 mM phloretin or 0.5 mg/ml polymyxin B (*Poly.B*). The incubation was continued (30 min) without (*Control*) or with 10 nM insulin (a) or 10 μM glimepiride (b). Plasma membranes were prepared and subjected to TX-114 partitioning. Hydrophilic Gce1 and LPL were purified from the aqueous (a) and detergent (d) phases by affinity purification or immunoprecipitation, respectively. All samples were analyzed by SDS-PAGE and fluorography. Molecular masses were derived from marker proteins run in parallel.
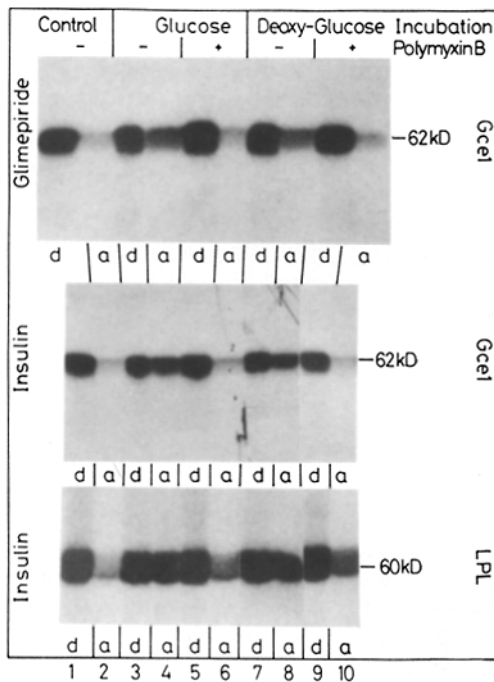
Figure 8. Effect of 2-deoxyglucose on the lipolytic cleavage of LPL and Gcel. Adipocytes were metabolically labeled with *myo*-[^{14}C]inositol, washed and then incubated (60 min, 37°C) with 5 mM sodium pyruvate in DME medium lacking glucose. One half of the cells was incubated (20 min) with 0.5 mg/ml polymyxin B, the other half was left untreated. Subsequently, aliquots of the cells were incubated (5 min, 37°C) in the absence (*Control*, lanes *1* and *2*) or presence of 2 mM glucose (lanes *3–6*) or 2-deoxyglucose (lanes *7–10*) and then supplemented with glimepiride (10 μM final concentration, shown only for *Gcel*) or insulin (10 nM final concentration). After further incubation (30 min), plasma membranes were prepared and subjected to TX-114 partitioning. Gcel and LPL were purified and analyzed as in Fig. 7.

lanes *1–6*). In contrast, each of the three inhibitors almost completely blocked the activation of the PL by insulin (Fig. 7 *a*, lanes *9–14*, compare with lanes *7* and *8*) and glimepiride (*b*, lanes *1–6*, compare with lanes *7* and *8*). These data demonstrate that stimulation of glucose transport is required for activation of the GPI-PL in rat adipocytes.

Transport of Deoxyglucose into Rat Adipocytes Supports the Activation of the GPI-PL

Finally we asked the question whether metabolism of glucose is required for activation of the GPI-PL. For this, the amphiphilic to hydrophilic conversion of LPL and Gcel was monitored in adipocytes which had been metabolically labeled with *myo*-[^{14}C]inositol in glucose-free pyruvate-containing medium and then incubated with glimepiride (Gcel) or insulin in the presence of glucose or the non-metabolizable glucose analogue, 2-deoxyglucose. The fluorography of the SDS-PAGE (Fig. 8) of the amphiphilic and hydrophilic GPI-proteins shows that in the absence of glucose (and presence of sodium pyruvate) both, insulin and glimepiride, failed to support lipolytic cleavage of LPL and Gcel (lane 2). Supplementation of glucose restored the activation of the GPI-PL by both agents (Fig. 8, lane *4*) which was blocked (to 10–15% for Gcel and 25–35% for LPL) by

polymyxin B (lane *6*). Interestingly, addition of 2-deoxyglucose instead of glucose to the washed adipocytes resulted in stimulation of the lipolytic cleavage by both insulin and glimepiride to 60–75% observed for the corresponding values with glucose (Fig. 8, compare lanes *8* and *4*). Again, the GPI-PL was hardly activated when polymyxin B was also present (Fig. 8, lane *10*). These results confirm that increased glucose flux into adipocytes is prerequisite for stimulation of the GPI-PL and furthermore suggest that glucose metabolism beyond the phosphorylation step is not necessary.

Discussion

A functional role of GPI anchorage of LPL and Gcel in insulin-sensitive cells, such as isolated or cultured adipocytes, may rely on their accessibility to specific cleavage by an insulin-inducible GPI-PL: short term incubation of rat adipocytes with physiological concentrations of insulin or with the sulfonylurea, glimepiride, causes the lipolytic cleavage of their GPI anchors as revealed by the retention of the *myo*-inositol radiolabel and anti-CRD cross-reactivity. This suggests stimulation of a GPI-PL by insulin or glimepiride. Since action of a GPI-PLD would destroy the epitope recognized by anti-CRD antibodies and since phosphate is apparently retained with the inositol after cleavage, the insulin-activated PL is presumably of type C.

Lipolytic cleavage of the GPI anchor and membrane release of LPL may be physiologically related to the dramatic shift of LPL activity from adipose to muscle tissue observed during certain nutritional situations in man (for a review see Smith and Pownall, 1984). In contrast, the physiological role for the analogous lipolytic processing of Gcel remains unclear so far. We were unable to detect cAMP-dependent protein kinase A activity in solubilized plasma membranes from untreated and insulin- or glimepiride-treated adipocytes using the typical protein kinase A substrate Kemptide (Müller, G., unpublished data). This argues against participation of anchor cleavage in promoting cAMP-dependent kinase activation of the membrane-associated or released Gcel protein. Such an activation of a cryptic protein kinase A activity upon lipolytic cleavage of a lipidic membrane anchor has been suggested for a mitochondrial cAMP-binding protein in yeast (Müller and Bandlow, 1989).

It may be of functional significance, that after incubation of rat adipocytes with insulin or glimepiride we observed a time-dependent loss of inositol-phosphate from the lipolytically cleaved GPI anchor of hydrophilic Gcel. This may be taken as evidence for a secondary hydrolytic processing step within the residual GPI structure or near the carboxyl terminus of the GPI-protein. Apparently, this reaction exceeds the cleavage rate of the insulin- or glimepiride-stimulated phospholipase after about 20–30 min of incubation resulting in a decline of the steady state concentration of [^{14}C]inositol-labeled and anti-CRD immunoreactive Gcel protein despite continuous constant lipolytic cleavage. Similar observations have been made with GPI-proteins released from 3T3 adipocytes in response to insulin and glimepiride (Müller et al., 1993) as well as with the corresponding Gcel protein from *Saccharomyces cerevisiae* after glucose-induced lipolytic cleavage of its GPI anchor.

Loss of the inositol residue caused by a secondary process-

ing event at a unique site (presumably within the carboxyl terminus of the GPI-protein) should result in the *en bloc* removal of phosphoinositolglycan fragments from GPI membrane anchors which may function in insulin, sulfonylurea and nutritional signaling. Cleavage products generated by the insulin-stimulated metabolism of GPI anchors have been speculated to act as soluble mediators of certain insulin effects (Larner, 1988). Experimental support for this possibility originated from experiments, where insulin-sensitive rat hepatocytes and adipocytes had been incubated with the PLC-cleaved GPI anchors isolated from the proteolytically digested GPI-proteins, human erythrocyte acetylcholinesterase (Deeg et al., 1993) and trypanosomal soluble variant surface glycoprotein (Misek and Saltiel, 1992). These phosphoinositolglycans, linked via a phosphoethanolamine bridge to the carboxy-terminal amino acids of acetylcholinesterase and variant surface glycoprotein, acted specifically and mimicked some of the insulin actions in a concentration-dependent manner. For the second processing reaction to occur and/or the subsequent uptake of the resulting putative signaling molecule(s) into the cell, peripheral membrane association of lipolytically cleaved GPI-proteins at the cell surface as demonstrated for rat adipocytes in the present study and for yeast spheroplasts previously (Müller and Bandlow, 1993) may be prerequisite.

A participation of a GPI-PL in mediating some of the short term insulin effects is further suggested by the present observations that (a) GPI-PL activation depends on physiological plasma insulin levels and follows a rapid onset (within 2–5 min) characteristic for the regulation of key enzymes of glucose and lipid metabolism by insulin (b) GPI-PL activation by insulin is impaired in rat adipocytes which had been desensitized for glucose transport stimulation by insulin. This desensitization *in vitro* was accomplished by incubation with high concentrations of glucose and insulin (Marshall et al., 1991) thus mimicking the hyperinsulinemic and hyperglycemic situation typical of the insulin resistant state in animal models of type II diabetes and subjects with impaired glucose tolerance. An impairment of the insulin stimulation of a GPI-PL is a candidate mechanism for the postreceptor defect generally found in insulin-resistant states (Macaulay and Larkins, 1988). A block in the activation of this enzyme has also been implied for: (a) insulin-resistant hepatocytes isolated from Streptozotocin-induced diabetic rats since the hydrolysis of free GPI lipids in response to insulin and the activation of glycogen synthesis by equivalent volumes of hydrolysis products were significantly reduced compared to untreated rats (Sanchez-Arias et al., 1992); and (b) for type II diabetic patients since the insulin-mimetic bioactivity and *chiro*-inositol content of a phosphoinositolglycan fraction isolated from equivalent volumes/amounts of hemodialysate, urine and muscle homogenate were markedly reduced compared to those from nondiabetic controls (Asplin et al., 1993).

The following lines of evidence strongly suggest that increased glucose transport mediates the stimulatory effects of insulin and glimepiride on the GPI-PL in rat adipocytes: (a) cells which have been desensitized for insulin stimulation of glucose transport, showed impaired insulin stimulation of the GPI-PL with comparable elevations of the ED$_{50}$ values for both processes. (b) The sulfonylurea glimepiride stimulated glucose transport and GPI-PL activity in desensitized

and normal adipocytes to about the same extent and within the same range of drug concentration (to 25–30% of the maximal insulin response in normal cells). (c) Inhibition of the stimulation of glucose transport was accompanied by an almost complete block of activation of the GPI-PL by insulin and glimepiride. Increased glucose transport is based on GLUT4 translocation and leads to elevated glucose flux and metabolism in the cell. The present data allow to discriminate between these possibilities for the functional role of glucose transport for activation of the GPI-PL. Glucose-free incubation medium does not interfere with GLUT4 translocation but did not support insulin and glimepiride stimulation arguing against a direct coupling of GLUT4 translocation to GPI-PL activation. 3-*O*-methylglucose could not replace D-glucose (G.M., unpublished observations) thus excluding the possibility that glucose flux across GLUT4 *per se* triggers the activation. In contrast, 2-deoxyglucose did substitute for D-glucose. Thus phosphorylation of glucose is necessary and sufficient for activation of the GPI-PL. The latter data are compatible with previous findings showing that release of LPL from cultured adipocytes did not depend on energy supply (Spooner et al., 1979).

In conclusion, stimulation of glucose transport, which is the rate-limiting step for the generation of glucose-6-phosphate seems to represent the common molecular mechanism which links the activation of a GPI-PL to the insulin, sulfonylurea and glucose signaling cascades in organisms as divergent as adipocytes and yeast. Thus, in rat adipocytes the receptors for insulin (and growth factors) and sulfonylureas (Martz et al., 1989) may be coupled to the GPI-PL only indirectly via the formation of glucose-6-phosphate. This situation is reminiscent of the regulation of gene expression of many glycolytic and lipogenic enzymes by insulin and glucose in hepatocytes and adipocytes. Insulin dramatically stimulated the transcription of the genes for fatty acid synthase and acetyl-CoA carboxylase in cultured rat adipose tissue and adult hepatocytes only when glucose or 2-deoxyglucose were present in the culture medium. 3-*O*-methylglucose was ineffective (Foufelle et al., 1992; Fukuda et al., 1992). On the basis of these experiments, the existence of a nuclear factor has been proposed whose activity is controlled by the glucose-6-phosphate level in the cell and which binds to carbohydrate-responsive elements of these genes and regulates their rate of transcription (for recent reviews see Girard et al., 1994; Vaulont and Kahn, 1993). In analogy, it is tempting to speculate about a cytosolic factor which binds to glucose-6-phosphate and to the GPI-PL thereby regulating its activity.

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