Redistribution of Glycolipid Raft Domain Components Induces Insulin-Mimetic Signaling in Rat Adipocytes

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Caveolae and caveolin-containing detergent-insoluble glycolipid-enriched rafts (DIG) have been implicated to function as plasma membrane microcompartments or domains for the preassembly of signaling complexes, keeping them in the basal inactive state. So far, only limited in vivo evidence is available for the regulation of the interaction between caveolae-DIG and signaling components in response to extracellular stimuli. Here, we demonstrate that in isolated rat adipocytes, synthetic intracellular caveolin binding domain (CBD) peptide derived from caveolin-associated pp59^{Lyn} (10 to 100 μ M) or exogenous phosphoinositolglycan derived from glycosyl-phosphatidylinositol (GPI) membrane protein anchor (PIG; 1 to 10 μ M) triggers the concentration-dependent release of caveolar components and the GPI-anchored protein Gce1, as well as the nonreceptor tyrosine kinases pp59^{Lyn} and pp125^{Fak}, from interaction with caveolin (up to 45 to 85%). This dissociation, which parallels redistribution of the components from DIG to non-DIG areas of the adipocyte plasma membrane (up to 30 to 75%), is accompanied by tyrosine phosphorylation and activation of pp59^{Lyn} and pp125^{Fak} (up to 8- and 11-fold) but not of the insulin receptor. This correlates well to increased tyrosine phosphorylation of caveolin and the insulin receptor substrate protein 1 (up to 6- and 15-fold), as well as elevated phosphatidylinositol-3' kinase activity and glucose transport (to up to 7- and 13-fold). Insulin-mimetic signaling by both CBD peptide and PIG as well as redistribution induced by CBD peptide, but not by PIG, was blocked by synthetic intracellular caveolin scaffolding domain (CSD) peptide. These data suggest that in adipocytes a subset of signaling components is concentrated at caveolae-DIG via the interaction between their CBD and the CSD of caveolin. These inhibitory interactions are relieved by PIG. Thus, caveolae-DIG may operate as signalosomes for insulin-independent positive cross talk to metabolic insulin signaling downstream of the insulin receptor based on redistribution and accompanying activation of nonreceptor tyrosine kinases.

Caveolae, caveolin-containing small invaginations of the plasma membrane expressed in many differentiated cells (2, 50, 51, 54), and their biochemical correlate, detergent-insoluble glycolipid-enriched raft domains (DIG) (5, 53), harbor a number of components of various intracellular signal transduction pathways, including G protein-coupled receptors, heterotrimeric and small G proteins, nonreceptor tyrosine kinases (NRTK), components of the Ras/mitogen-activated protein kinase (MAPK) pathway, protein kinase C isoforms, endothelial nitric oxide synthase (eNOS), and glycosyl-phosphatidylinositol-anchored plasma membrane proteins (GPI proteins; 2, 55, 58, 59, 60). As a consequence, these structures may function as sites for direct physical interaction of signaling components where (positive or negative) cross talk between the corresponding signaling pathways takes place (49). A large body of evidence strongly suggests that caveolin family members (caveolins 1, 2, and 3 and flotillins) (3, 21) operate as scaffolding proteins which organize and concentrate cholesterol and glycosphingolipids as well as lipid-modified signaling proteins within caveolae-DIG, thereby suppressing their activity via direct interaction with caveolin (31, 32, 61). Recently, it was demonstrated that caveolin 1 functionally suppresses the GTPase activity of heterotrimeric G proteins and blocks the activity of eNOS as well as (receptor and nonreceptor) tyrosine kinases

by direct binding of a common domain within the caveolins, the caveolin scaffolding domain (CSD) to the corresponding caveolin binding domain (CBD) of the signaling protein (10, 27, 48). Functional CBDs have also been identified in serine/threonine kinases (51), where they are located within the conserved kinase subdomain IX. Taken together, this argues for caveolin operating as a general kinase inhibitor (11). Compatible with this hypothesis is the finding that synthetic CSD peptide (CSDP) inhibits Src family tyrosine kinases (c-Src/Fyn), epidermal growth factor receptor, MAPK, G protein-coupled receptor kinases, and protein kinases C and A, with similar potencies in vitro (7, 11, 14, 31, 48, 51). However, in contrast to the large body of in vitro evidence for the so-called caveola signaling hypothesis (34), there is only limited demonstration for functional relevance of the CBD-CSD interaction in vivo. Inactivation of the CBD of eNOS by site-directed mutagenesis (within the FSAAPFSGW motif; italics indicate invariant amino acids of the CBD consensus sequence) (see Fig. 1) led to the blockade of the ability of caveolin 1 to inhibit eNOS activity, thus indicating functional relevance of caveolin binding to a signaling protein in vivo (20).

If relevant in vivo, the inhibitory interaction between caveolin and signaling molecules should be accessible for modulation in response to extracellular and intracellular signals. Activation of signaling pathways engaging CBD-harboring components requires their relief from binding to or inhibition by caveolin. The molecular mechanism for long-term response may be based on the regulation of caveolin gene expression. Consistent with this hypothesis, caveolin 1 mRNA and protein

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FIG. 1. Structural and functional organization of caveolin 1 α and the amino acid sequences of the wild-type or mutant CSDP derived from caveolin 1 α , 2, and 3 as well as CBDP derived from pp59^{Lyn}. The CBD consensus sequence for functional interaction of signaling proteins with caveolin is also given. Palmitoylation at the carboxy-terminal domain of caveolin 1 α is thought to support caveolar assembly (56, 60).

expression as well as the number of caveolae are dramatically diminished upon cell transformation by activated oncogenes, such as H-Ras (28). In these transformed cells, ectopic caveolin 1 expression and concomitant formation of caveolae prevented the transformed phenotype, accompanied by downregulation of the MAPK pathway (15). Conversely, anti-sense-mediated reduction in caveolin 1, but not caveolin 2, expression in NIH 3T3 cells led to oncogenic transformation and constitutive activation of the MAPK cascade (19). Interestingly, caveolins 1 and 2 are coexpressed and form heterooligomeric DIG via their membrane-spanning domains in most cell types (12, 56). Thus, in addition to the absolute caveolin 1 level, the stoichiometry of caveolins 1 and 2 protein expression could affect the inhibitory potency by the masking of CSD of caveolin 1 by CSD of caveolin 2 within the assembled heterooligomeric caveolae-DIG.

Here, we demonstrate that in isolated rat adipocytes, two NRTK are downregulated by localization in DIG and by binding to caveolin and upregulated by release from DIG and by dissociation from caveolin in response to phosphoinositolygans (PIG), cleavage products of GPI protein anchors (18, 26, 41). Short-term redistribution of these NRTKs from caveolae-DIG induces potent insulin-independent activation of metabolic insulin signaling.

MATERIALS AND METHODS

Materials. Radiochemicals and chemiluminescent reagents (Renaissance Chemiluminescence Detection System) were bought from NEN/DuPont (Bad Homburg, Germany); PIG 41, 37, 7, and 1 (18) were made available by Jochen Bauer and Andrea Bauer (Department of Medicinal Chemistry of Aventis Pharma, Frankfurt am Main, Germany); and antibodies were obtained as follows. For immunoprecipitation, antibodies against pp59^{Lyn} (clone 42), pp125^{Fak} (clone 77), and caveolin 1 (rabbit) were from Transduction Laboratories (Lex-

ington, Ky.); antibodies against total human recombinant (insect cells; purified by gel filtration) insulin receptor substrate protein 1 (IRS-1) and IRS-2 (rabbit) were from Biotrend (Cologne, Germany); and antibodies against human insulin receptor β -subunit (IR β ; monoclonal) were from Upstate Biotechnology (Lake Placid, N.Y.). For immunoblotting, antibodies against phosphotyrosine (clone PY20) were from Transduction Laboratories; those against phosphotyrosine (clone 4G10) were from Upstate Biotechnology; those against synthetic peptide corresponding to the carboxy-terminal sequence comprising amino acids 1223 to 1235 of rat IRS-1 (rabbit) were from Suzanne Dalle (Humboldt University, Berlin, Germany); antibodies against paxillin (clone 165), caveolin (rabbit), pp59^{Lyn} (clone 32), and pp125^{Fak} (clone 197) were from Transduction Laboratories; those against human integrin β_1 and IR β were from Upstate Biotechnology; and those against rat glucose transporter isoform 1 (Glut1) and Glut4 (rabbit) were from Biotrend. Rabbit muscle enolase, 1-methyl-2-phenylethyladenosine, fatty-acid-free bovine serum albumin (BSA; fraction V) and *N*-ethylmaleimide were purchased from Sigma (Deisenhofen, Germany); protein A- and G-Sepharose were delivered by Pharmacia/Upjohn (Freiburg, Germany); proteinase inhibitors and adenosine deaminase were from Roche Molecular Biochemicals (Mannheim, Germany); precast gels were purchased from Novex (San Diego, Calif.); and polyvinylidene difluoride membranes were obtained from Millipore, Eschborn, Germany.

Synthesis of peptides. The wild-type and mutant CBD peptides (CBDP) derived from pp59^{Lyn} as well as CSDP (Fig. 1) were synthesized on an Applied Biosystems Model 431A Peptide Sequencer by using 9-fluorenylmethoxy carbonyl as the α -amino protecting group. Coupling of amino acids to the nascent peptide was carried out in *N*-methylpyrrolidone using HOBT-HBTU [*N*-hydroxybenzotriazole-2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate]. 9-Fluorenylmethoxy carbonyl was removed by treatment of the peptide with 15% piperidine in *N*-methylpyrrolidone. Cleavage of the peptide from the resin and deprotection of amino acid chains was carried out by reaction with 95% trifluoroacetic acid (3 h, room temperature) in water containing phenol, ethanedithiol, and thioanisole as scavengers. Peptide was removed from resin by filtration and precipitated in diethyl ether. Peptides were purified by reversed-phase high-performance liquid chromatography, and their molecular weights were confirmed by mass spectroscopy.

Preparation of rat adipocytes and incubation with PIG. Adipocytes were isolated by collagenase digestion from epididymal fat pads of male Wistar rats (140 to 160 g, fed ad libitum) as described previously (40). At a final concentra-

tion of 100 μ l of packed cell volume per ml (determined by aspiration of small aliquots into capillary hematocrit tubes and centrifugation for 90 s in a micro-hematocrit centrifuge in order to measure the fractional occupation of the suspension by the adipocytes; 10% cytocrit corresponds to about 1.5×10^6 cells/ml), cells were incubated in HEPES-based Krebs-Ringer solution (KRH; 0.12 M NaCl, 4.7 mM KCl, 2.5 mM CaCl_2 , 1.2 mM MgSO_4 , 1.2 mM KH_2PO_4 , 20 mM HEPES-KOH, pH 7.4) containing 2% (wt/vol) BSA, 100 μ g of gentamicin/ml, 100 nM 1-methyl-2-phenylethyladenosine, 0.5 U of adenosine deaminase/ml, 0.5 mM sodium pyruvate, and 5 mM D-glucose in the presence of PIG (dissolved in 20 mM HEPES-KOH, pH 7.4) at 37°C in a shaking water bath with constant bubbling with 5% CO_2 -95% O_2 for the periods indicated.

Electroporation of isolated rat adipocytes. Electroporation was performed as described previously (44) by incubating (at 25°C) the adipocytes (25% cytocrit) in a cuvette in 0.8 ml of 4.74 mM NaCl, 118 mM KCl, 0.38 mM CaCl_2 , 1 mM EGTA, 1.19 mM MgSO_4 , 1.19 mM KH_2PO_4 , 25 mg of BSA/ml, 3 mM sodium pyruvate, and 25 mM HEPES-KOH (pH 7.4) in the presence of CDBP-CSDP (dissolved in dimethyl sulfoxide at 30 mM; final dimethyl sulfoxide concentration of 1%, which was also contained in control incubations and did not affect adipocyte viability) at the concentrations indicated and using a Gene Pulser Transfection Apparatus (Bio-Rad, Munich, Germany; set at a capacitance of 25 μ F and voltage of 2 kV/cm) for six shocks. The cells from five electroporations were pooled and centrifuged ($200 \times g$, 1 min, swing-out rotor). After aspiration of the infranatant, the cells were washed once with 40 ml of the above buffer containing 4% BSA, suspended in 20 ml of Dulbecco's minimal essential medium containing 5 mM glucose, 0.5 mM sodium pyruvate, 4 mM L-glutamine, 200 nM 1-methyl-2-phenylethyladenosine, 100 μ g of gentamicin/ml, 1% BSA, and 25 mM HEPES-KOH (pH 7.4), and then incubated (1 h, 37°C) under 5% CO_2 -95% O_2 prior to stimulation with PIG.

Preparation of total-cell lysate and plasma membranes. After stimulation and/or electroporation, rat adipocytes (5×10^7 cells) were washed once with KRH containing 0.25 M sucrose and 2 mM sodium pyruvate by flotation ($200 \times g$, 2 min) and aspiration of the infranatant and were immediately homogenized in 10 ml of lysis buffer (25 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 0.25 mM EGTA, 0.25 M sucrose, 50 mM NaF, 5 mM sodium pyrophosphate, 25 mM glycerol-3-phosphate, and 1 mM sodium orthovanadate, supplemented with protease inhibitors [10 μ g of leupeptin/ml, 2 μ M pepstatin, 10 μ g of aprotinin/ml, 5 μ M antipain, 5 mM iodoacetate, 100 μ M phenylmethylsulfonyl fluoride, 4 mM benzamide]) by using a motor-driven Teflon-in-glass homogenizer (10 strokes with a loosely fitting pestle) at 22°C. The following procedures were performed at 4°C (43). After centrifugation ($1,500 \times g$, 5 min), the postnuclear infranatant was separated from the fat cake, and the pellet fraction (containing adipocyte ghosts and cell debris) was removed by suction with a needle. For preparation of plasma membranes, the postnuclear infranatant was centrifuged ($12,000 \times g$, 15 min). The pellet was suspended in 10 ml of homogenization buffer and recentrifuged ($1,000 \times g$, 10 min). The supernatant was centrifuged ($12,000 \times g$, 20 min). The washed pellet was suspended in 1 ml of homogenization buffer, layered onto a 5-ml cushion of 38% (wt/vol) sucrose, 25 mM Tris-HCl (pH 7.4), and 1 mM EDTA, and centrifuged ($110,000 \times g$, 1 h). The membranes at the interface between the two layers (0.5 ml) were removed by suction, diluted with four volumes of homogenization buffer, and layered on top of an 8-ml cushion of 28% Percoll, 0.25 M sucrose, 1 mM EDTA, and 25 mM Tris-HCl (pH 7.0). After centrifugation ($45,000 \times g$, 30 min), the plasma membranes were withdrawn from the lower third of the gradient (0.5 ml) with a Pasteur pipette, diluted with 10 volumes of homogenization buffer, and centrifuged ($200,000 \times g$, 90 min). The washed pellet was suspended in the same buffer at 0.5 mg of protein/ml. For solubilization of plasma membranes and preparation of total-cell lysate, plasma membranes and postnuclear infranatant, respectively, were supplemented with deoxycholate and Nonidet P-40 (final concentrations, 0.3 and 0.2%, respectively), and after incubation (1 h, 4°C), the solubilized plasma membranes and total-cell lysates were cleared from insoluble material by centrifugation ($100,000 \times g$, 1 h, 4°C) and used for photoaffinity labeling and (co)immunoprecipitation.

Preparation of DIG. (i) **Detergent method.** Washed adipocytes (3.5×10^6) were suspended in 1.5 ml of lysis buffer (25 mM morpholinethanesulfonic acid [MES], pH 6.0, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.2 mM sodium orthovanadate, and protease inhibitors) and incubated for 20 min on ice. The cells were lysed with 10 strokes in a manual Teflon-in-glass homogenizer over the course of 1 h at 4°C. The lysate was centrifuged ($1,300 \times g$, 5 min) to pellet unbroken cells, cellular debris, nuclei, and large insoluble material. A 1-ml volume of the postnuclear supernatant was subjected to sucrose gradient centrifugation by mixing with an equal volume of 85% sucrose, 25 mM MES (pH 6.0), 150 mM NaCl, and 5 mM EDTA at the bottom of a 12-ml centrifuge tube which was overlaid with 5.5 ml of 35% sucrose and then 3.5 ml of 5% sucrose in the same medium. After centrifugation ($230,000 \times g$, Beckman SW41 rotor, 18 h,

4°C), 0.9-ml fractions were collected from top to bottom and termed fractions 1, 2, 3, etc. The bottom fraction was fraction 12. Fraction 5 appeared as a white, light-scattering band under illumination located at 5 to 7% sucrose at the 35% sucrose interface. DIG contained in fraction 5 were pelleted by dilution of the sucrose with 5 volumes of 50 mM HEPES-KOH containing protease inhibitors and centrifugation ($200,000 \times g$, 2 h).

(ii) **Carbonate method.** Plasma membranes (200 μ g) were pelleted ($200,000 \times g$, 90 min), suspended in 1.5 ml of 0.5 M Na_2CO_3 (pH 11) containing protease inhibitors, and sonicated (three 30-s bursts with 1-min intervals on ice; Branson B-12, power stage 4). The suspension was then adjusted to 45% sucrose in a medium containing 15 mM MES-KOH (pH 6.5), 75 mM NaCl, and 0.25 M Na_2CO_3 overlaid with 2 ml each of 35, 25, 15, and 5% sucrose in the same medium and was centrifuged ($230,000 \times g$, Beckman SW41 rotor, 18 h). The light-scattering band of flocculent material just below the 15 to 25% sucrose interface was collected as DIG using a 19-gauge needle and a syringe (about 1.5 ml). Alternatively, washed adipocytes (0.5×10^7 cells) were suspended in 2 ml of sodium carbonate buffer and homogenized sequentially using a loosely fitting Dounce homogenizer (10 strokes) and a sonicator (three 20-s bursts). The homogenate (2 ml) was then adjusted to 45% sucrose by addition of 2 ml of 90% sucrose, 50 mM MES-KOH (pH 6.5), and 150 mM NaCl (final pH of the mixture, 10.2). A discontinuous sucrose gradient was formed by overlaying this solution with 4 ml of 35% sucrose and 4 ml of 5% sucrose, both in the same buffer containing 0.25 M Na_2CO_3 . After centrifugation (see above), 0.85-ml gradient fractions were collected to yield a total of 14 fractions. The individual gradient fractions were pooled into DIG (fractions 4 to 7) and non-DIG areas (fractions 10 to 14). The membranes from each of the pooled gradient fractions obtained by either method were diluted two- to threefold with 25 mM MES (pH 6.5), 150 mM NaCl, and 1% Triton X-100, collected by centrifugation ($50,000 \times g$, 30 min, 4°C), resuspended in nondissociating buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, 0.5 mM EGTA, 1 mM sodium orthovanadate, 50 mM NaF, and protease inhibitors) or dissociating buffer (composed of nondissociating buffer supplemented with 60 mM β -octylthioglycoside, 0.3% deoxycholate) as indicated, incubated (1 h, on ice), and used for (co)immunoprecipitation, immunoblotting, or photoaffinity labeling.

Immune complex kinase assays. Immune complex kinase assays were performed as described previously (17, 44) with minor modifications. Briefly, pp59^{Lyn} or pp125^{Fak} immune complexes were suspended in 30 μ l of kinase buffer (50 mM HEPES-KOH, pH 7.4, 100 mM NaCl, 1.25 mM MnCl_2 , 12.5 mM MgCl_2 , 1.25 mM EGTA, 0.5 mM dithiothreitol, 1 mM Na_3VO_4) containing [γ -³²P]ATP (final concentrations: pp59^{Lyn}, 40 μ M, 0.2 mCi/ml; pp125^{Fak}, 100 μ M, 0.5 mCi/ml) or 1 mM ATP and were incubated (pp59^{Lyn}, 15 min; pp125^{Fak}, 3 min; 22°C) in the presence of 1 μ g of heat-denatured enolase. Phosphorylation was terminated by addition of 10 μ l of fourfold-concentrated Laemmli buffer and boiling. The phosphoproteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% Bis-Tris resolving gel, morpholinopropanesulfonic acid [MOPS]-SDS running buffer) and analyzed for phosphotyrosine by phosphorimaging ([γ -³²P]ATP) or immunoblotting (ATP). Under these conditions, the kinase reactions were linear with time for the assay period.

Miscellaneous. Phosphatidylinositol 3'-kinase (PI-3'K) assay, photoaffinity labeling of Gce1 with 8-N₃-[³²P]cyclic AMP (cAMP), immunoprecipitation, and immunoblotting were performed as described previously (17, 39, 43, 44). Protein concentration was determined using the bicinchoninic acid protein determination kit from Pierce (Rockford, Ill.) and BSA as a calibration standard. Autoradiographs and direct photoimages were processed and quantified by computer-assisted video densitometry using the Storm 860 PhosphorImager system (Molecular Dynamics, Gelsenkirchen, Germany). Figures of autoradiographs and photoimages were constructed using the Adobe Photoshop software (Adobe Systems Inc., Mountain View, Calif.). The recovery in the amount of immunoprecipitated protein was corrected (by fold increase or percent stimulation) for the amount of protein actually applied onto the gel as revealed by homologous immunoblotting. Each experiment and incubation was performed with different batches of adipocytes (see figure legends), with two to four independent immunoprecipitation, kinase assay, immunoblotting, or photoaffinity labeling analyses.

RESULTS

Characterization of DIG in rat adipocytes. DIG are assumed to represent the biochemical correlate for (certain subsets of) the morphologically defined caveolae and/or precaveolae prior to their invagination or budding from the plasma membrane (49, 60). The protein composition of DIG and non-

TABLE 1. Characterization of DIG and non-DIG areas prepared by the carbonate and detergent methods^a

Plasma membrane protein	Separation method	Fold enrichment of DIG area	Relative amt of protein of DIG area	Fold enrichment of non-DIG area	Relative amt of protein from non-DIG area
Caveolin	Carbonate	10.5 ± 1.50	85 ± 7.5	0.09 ± 0.02	7 ± 2.1
	Detergent	14.5 ± 2.34	90 ± 6.6	0.07 ± 0.02	4 ± 1.3
5'-Nucleotidase	Carbonate	7.4 ± 0.85	60 ± 6.3	0.08 ± 0.01	6 ± 1.2
	Detergent	11.6 ± 1.22	72 ± 7.9	0.17 ± 0.04	10 ± 3.0
Gce1	Carbonate	8.9 ± 1.10	72 ± 9.2	0.07 ± 0.01	5 ± 1.2
	Detergent	13.1 ± 1.94	81 ± 7.5	0.13 ± 0.03	8 ± 2.3
pp59 ^{Lyn}	Carbonate	5.1 ± 0.60	41 ± 5.6	0.60 ± 0.06	43 ± 7.5
	Detergent	5.6 ± 0.81	35 ± 7.8	0.94 ± 0.13	56 ± 9.8
Clathrin α	Carbonate	0.3 ± 0.08	2 ± 0.4	0.73 ± 0.15	52 ± 9.5
	Detergent	0.6 ± 0.11	4 ± 2.1	0.52 ± 0.22	31 ± 4.0
Glut1	Carbonate	0.5 ± 0.03	4 ± 0.6	1.06 ± 0.13	76 ± 5.2
	Detergent	0.9 ± 0.08	12 ± 3.0	1.45 ± 0.19	86 ± 6.1
Glut4	Carbonate	0.8 ± 0.10	6 ± 1.1	0.96 ± 0.12	69 ± 8.9
	Detergent	0.8 ± 0.14	5 ± 1.3	1.29 ± 0.14	77 ± 6.8
IR β	Carbonate	0.4 ± 0.07	3 ± 0.5	0.82 ± 0.08	59 ± 7.4
	Detergent	1.1 ± 0.11	7 ± 2.4	1.22 ± 0.10	73 ± 9.8
Na ⁺ /K ⁺ ATPase	Carbonate	0.2 ± 0.04	2 ± 0.4	1.42 ± 0.25	102 ± 9.2
	Detergent	0.5 ± 0.06	3 ± 0.7	1.58 ± 0.11	94 ± 8.1
COP1B	Carbonate	0.2 ± 0.05	2 ± 0.2	0.72 ± 0.09	50 ± 7.7
	Detergent	0.3 ± 0.08	2 ± 0.4	0.54 ± 0.07	31 ± 5.5
β -Actin	Carbonate	0.5 ± 0.05	4 ± 0.3	0.54 ± 0.07	39 ± 6.9
	Detergent	0.4 ± 0.09	2 ± 0.3	0.63 ± 0.06	37 ± 5.2

^a Total plasma membranes (upper values) and total-cell lysates (lower values) were prepared from isolated rat adipocytes and then separated into DIG and non-DIG areas by the carbonate and detergent methods, respectively. DIG and non-DIG areas were subjected to immunoblotting and chemiluminescent detection. Protein enrichment in DIG and non-DIG areas versus total plasma membranes was calculated as the relative amount per milligram of protein, which was set at 1 for total plasma membranes in each case. The relative amount of protein recovered with DIG and non-DIG areas is given as the percent of total plasma membranes, which was set at 100% in each case (carbonate method: fold enrichment, 8.1 ± 1.8 , and relative amount of protein, $71.5\% \pm 8.2\%$; detergent method: fold enrichment, 6.2 ± 1.5 , and relative amount of protein, $59.5\% \pm 7.7\%$). Quantitative evaluations of four different adipocyte incubations with measurements taken in triplicate are given for each (mean $\pm$ standard deviation).

DIG areas prepared by the carbonate method from purified plasma membranes or by the detergent method from total-cell lysates of untreated isolated rat adipocytes was analyzed by immunoblotting. The caveolar structural and marker protein, caveolin 1, the GPI proteins Gce1 (glycolipid-anchored cAMP-binding ectoprotein) and 5'-nucleotidase (39, 43, 46), and the dual-acylated NRTK of the Src-class, pp59^{Lyn} (52), were significantly enriched with DIG (versus total plasma membranes) prepared by either method (Table 1). The typical plasma membrane proteins, glucose transporter isoforms Glut1 and Glut4, IR β , and Na⁺/K⁺-ATPase were deprived from DIG areas (versus plasma membranes) and recovered mainly from non-DIG areas. Previous studies provided controversial data about the presence of Glut4 and the insulin receptor in caveolae-DIG from 3T3-L1 and rat adipocytes (24, 57). Furthermore, the present study revealed deprivation of Golgi-derived vesicles and clathrin-coated pits from both DIG and non-DIG areas based on their low content of COPII vesicle protein β and clathrin α -chain, respectively, relative to total plasma membranes (Table 1). Thus, cytoskeletal components, which are characterized by detergent insolubility, did not contaminate the DIG prepared by both methods to any significant degree. Based on the comparable enrichment and deprivation factors, DIG areas (and non-DIG areas) of qualitatively similar protein composition can be prepared by the carbonate and detergent methods.

After dissociation of the DIG (detergent method) with octylglucoside plus deoxycholate (see Materials and Methods), 65 to 85% of pp59^{Lyn} and pp125^{Fak} present in DIG from basal adipocytes (as detected by immunoblotting of 1% SDS-solubilized DIG) was immunoprecipitated with caveolin 1. In con-

trast, less than 5% of Gce1 present in SDS-solubilized DIG was recovered with caveolin 1 immunoprecipitates from octylglucoside plus deoxycholate-dissociated DIG. This suggests a direct interaction between pp59^{Lyn}-pp125^{Fak} and caveolin 1 within caveolae-DIG which resists their dissociation by octylglucoside plus deoxycholate to a considerable degree but is disrupted by 60 mM octylglucoside plus 1% Nonidet P-40 (5 to 8% coimmunoprecipitation) or by 1% SDS (0.5 to 1% coimmunoprecipitation). In contrast, the localization of GPI proteins, such as Gce1, in caveolae-DIG apparently does not rely on direct binding to caveolin 1 but requires the intact structural organization of caveolae-DIG (in the presence of 1% Nonidet P-40 only). Thus, caveolin 1 (co)immunoprecipitates from non-dissociated DIG harbor caveolar-DIG components irrespective of the molecular mechanism of their retention. Caveolin 1 immunoprecipitates from dissociated DIG recover only caveolin-interacting caveolar-DIG components. The conditions used for both immunoprecipitations result in efficient removal of noncaveolar plasma membrane polypeptides.

CBDP and PIG abrogate caveolin-pp59^{Lyn} interaction and stimulate pp59^{Lyn} and insulin-mimetic signaling. For demonstration of functional interaction between caveolin and NRTKs in DIG of the adipocyte plasma membrane, possibly mediated by the CSD and CBD, respectively (see Introduction), an excess of synthetic CBDP derived from pp59^{Lyn} (Fig. 1) (41, 49) was introduced into isolated rat adipocytes by electroporation and analyzed for the effect on the association between caveolin and pp59^{Lyn} residing in DIG (Fig. 2). The wild-type but not the mutant CBDP (Fig. 1) reduced in a concentration-dependent and drastic fashion the amount of caveolin which was coimmunoprecipitated with pp59^{Lyn} and, vice versa, the amount of

FIG. 2. Intracellular CDBP causes dissociation of pp59^{Lyn} and caveolin in DIG. Isolated rat adipocytes were electroporated in the absence or presence of different concentrations of wild-type or mutant CDBP derived from pp59^{Lyn}. From portions of total-cell lysates, DIG were prepared (detergent method) and used for immunoprecipitation (IP) (dissociating conditions) of pp59^{Lyn} and caveolin. From other portions of the lysates, pp125^{Fak} and IRS-1 were immunoprecipitated. The immunoprecipitates were immunoblotted (IB) for caveolin, pp59^{Lyn}, and phosphotyrosine by immunoblotting using chemiluminescent detection. Shown are phosphorimages of a typical experiment that was repeated three times, with similar results.

pp59^{Lyn} which was coimmunoprecipitated with caveolin (Fig. 2). The apparent dissociation of pp59^{Lyn} from caveolin residing in DIG was also reflected in the loss of pp59^{Lyn} from total adipocyte caveolin immunoprecipitates as well as total (non-dissociated) DIG in response to increasing concentrations of CDBP (Fig. 3A). It correlated well to pronounced increases in tyrosine phosphorylation and activity (toward exogenous substrate) of total immunoprecipitated pp59^{Lyn} (Fig. 3B) as well as tyrosine phosphorylation of total cellular IRS-1 and pp125^{Fak} with about the same concentration dependence (apparent 50% effective concentration [EC₅₀] = 10 to 30 μM; Fig. 2). Homologous immunoblotting of the caveolin immunoprecipitates demonstrated similar efficiency of the immunoprecipitations. Stimulation of total IRS-1 tyrosine phosphorylation by wild-type but not mutant CDBP was strongly correlated to IRS-1-associated PI-3'K activity and glucose transport (Fig. 4). As expected, a control incubation of intact rat adipocytes with 300 μM CDBP omitting electroporation failed to induce pp59^{Lyn}-caveolin dissociation and insulin-mimetic signaling, arguing for an intracellular site of action of CDBP.

Interestingly, a similar decline in the interaction between pp59^{Lyn} and caveolin in DIG and a concomitant increase in tyrosine phosphorylation and activation of total immunoprecipitated pp59^{Lyn} was observed after incubation of rat adipocytes with the active insulin-mimetic PIG 41 (17, 18, 45) but not the inactive PIG 1 (Fig. 3B). However, about 100-fold-lower concentrations of PIG 41 than of wild-type CDBP were sufficient. This suggests that intracellular CDBP derived from pp59^{Lyn} and exogenous active PIG trigger insulin-mimetic signaling and metabolic action in isolated rat adipocytes by similar mechanisms involving dissociation of pp59^{Lyn} from caveolin in DIG. To test this hypothesis, DIG of isolated rat

adipocytes incubated with structurally different PIG were assayed for caveolin-associated pp59^{Lyn}, pp125^{Fak} (by immunoblotting), and Gce1 (by photoaffinity labeling with 8-N₃-[³²P] cAMP; Fig. 5). The amounts of pp125^{Fak}, pp59^{Lyn}, and Gce1 recovered with caveolin immunoprecipitates from DIG were reduced by up to 60 and 80% in response to PIG 41 and 37, respectively (Fig. 5A and B). PIG 7 and 1 were less efficient (pp59^{Lyn} and Gce1) or even inactive (pp125^{Fak}). Comparable efficiency of caveolin immunoprecipitation was shown by homologous immunoblotting. Thus, the insulin-mimetic potency of active PIG correlates with their ability to disrupt the caveolin-pp59^{Lyn}, -pp125^{Fak}, and -Gce1 interactions.

Dissociation of this interaction by CDBP may be overcome by an excess of intracellular CSDP. In fact, the almost complete loss of Gce1 and pp125^{Fak} along with pp59^{Lyn} from caveolin residing in DIG upon introduction of CDBP into adipocytes was completely blocked by a simultaneous electroporation of a threefold molar excess of CSDP (Fig. 6). This argues for the neutralization of CDBP action by direct binding to CSDP. Apparently, CSDP not bound to CDBP does not compete with endogenous caveolin for interaction with CDBP of pp59^{Lyn}. This may be explained by a higher affinity of CSD in the context of native DIG-embedded caveolin. Excess of CSDP (up to 100-fold) impaired the PIG 41-induced release from DIG-associated caveolin of pp125^{Fak} to only a low degree and that of pp59^{Lyn} and Gce1 not at all (Fig. 6). Thus, CDBP and active PIG apparently differ in the mechanism interfering with the caveolin-signaling component interaction, PIG indirectly (possibly via signaling processes within caveolae-DIG) rather than physically.

Tyrosine phosphorylation of caveolin and its dissociation from pp59^{Lyn}, pp125^{Fak}, and Gce1 are correlated. The concen-

FIG. 3. CBDP and PIG affect pp59^{Lyn} tyrosine phosphorylation and its activity and interaction with caveolin in a similar fashion. Isolated rat adipocytes were electroporated or incubated (20 min, 37°C) in the absence or presence of increasing concentrations of wild-type or mutant CBDP derived from pp59^{Lyn} (A, B) or PIG 41 and PIG 1 (B). From portions of total-cell lysates, DIG were prepared (detergent method) and used for direct immunoblotting (IB) of pp59^{Lyn} (A) or immunoprecipitation (IP) (nondissociating conditions) of caveolin (B). From other portions of the lysates, caveolin (A) and pp59^{Lyn} (B) were immunoprecipitated. The immunoprecipitates were immunoblotted for pp59^{Lyn} and phosphotyrosine (pY) or measured for pp59^{Lyn} activity by the immune complex kinase assay. (A) Shown are phosphorimages of a typical experiment that was repeated two times, with similar results. (B) Quantitative evaluations of three different adipocyte incubations with measurements in triplicate each are given as the percentage of maximum or fold stimulation (mean plus standard deviation), with basal values set at 100% or 1.

tration-dependent dissociation of pp59^{Lyn}, pp125^{Fak}, and Gce1 from caveolin in DIG can also be followed with total adipocyte caveolin (Fig. 7). Upon treatment of adipocytes with different PIG, the decline in interaction correlated well to the increase in tyrosine phosphorylation of caveolin immunoprecipitated

from total-cell lysates both with regard to the similar EC₅₀ values (1 to 3 μM) and with the ranking between PIG 41 and 37. PIG 1 did not elicit caveolin tyrosine phosphorylation. Thus, in adipocytes, tyrosine phosphorylation of caveolin may be causally related to its dissociation from pp59^{Lyn}, pp125^{Fak},

FIG. 4. Intracellular CBDP causes stimulation of IRS-1 tyrosine phosphorylation, PI-3'K, and glucose transport. Isolated rat adipocytes were electroporated in the absence or presence of increasing concentrations of wild-type CBDP derived from pp59^{Lyn}. From portions of the cells, total lysates were prepared and used for immunoprecipitation (IP) of IRS-1. The immunoprecipitates were immunoblotted (IB) for phosphotyrosine (pY) or assessed for associated PI-3'K activity by the immune complex kinase assay. Other portions of the cells were assayed for 2-deoxyglucose transport. Quantitative evaluations of three different adipocyte incubations with measurements in quadruplicate each are given as fold stimulation (mean $\pm$ standard deviation), with basal values set at 1.

and Gce1. Candidate kinases are Src family members since insulin-dependent caveolin tyrosine phosphorylation by pp60^{v-src} and pp59^{Fyn} has been described (22, 33, 36). In this case, a positive feedback loop between kinase activation and dissociation from caveolin and tyrosine phosphorylation of caveolin may be initiated.

CSDP blocks PIG-induced tyrosine phosphorylation and glucose transport activation. In agreement with the caveolin signaling hypothesis (34, 49), the above data hint to a negative regulatory function of caveolin on pp59^{Lyn}. To further substantiate the involvement of caveolin in pp59^{Lyn} inhibition and its antagonism by active PIG, we studied the effect of CSDP on pp59^{Lyn} tyrosine phosphorylation (Fig. 8A) and downstream signaling events (Fig. 8B) after introduction into isolated rat adipocytes. As demonstrated previously (44), PIG 7, 37, and 41 induced tyrosine phosphorylation of total immunoprecipitated pp59^{Lyn} in a concentration-dependent fashion, with PIG 41 being most effective, followed by PIG 37 and, lastly, PIG 7. PIG-induced tyrosine phosphorylation was reduced by wild-type CSDP in a concentration-dependent fashion, with a 50% inhibitory concentration of 30 μ M irrespective of the PIG concentration used (Fig. 8A and B). Inhibition of tyrosine phosphorylation of total immunoprecipitated pp59^{Lyn}, pp125^{Fak}, and IRS-1 as well as glucose transport in response to PIG 41 (2 μ M) by CSDP were well correlated (Fig. 8B). Thus, cytoplasmic wild-type CSDP can act as an efficient inhibitor of PIG stimulation of pp59^{Lyn}, insulin-mimetic signaling (IRS-1 tyrosine phosphorylation), and insulin-mimetic action (glucose transport based on translocation of Glut4 and Glut1 from intracellular vesicles to the plasma membrane as reported previously [18]). Since CSDP does not interfere with PIG-induced release of pp59^{Lyn} and pp125^{Fak} from caveolin (Fig. 6), it

presumably acts via dominant-negative binding to pp59^{Lyn} upon relief of the latter from inhibition by endogenous caveolin.

PIG trigger redistribution of pp59^{Lyn}, pp125^{Fak}, and Gce1 from DIG to non-DIG areas. The observation that active PIG trigger the dissociation of a specific set of proteins from caveolin in adipocytes raised the question about their destination. Therefore, we studied whether these polypeptides leave the DIG and move to other regions of the plasma membrane which do not harbor DIG according to the criterion of detergent or carbonate insolubility (non-DIG areas) or translocate to intracellular membranes or the cytosol. The non-DIG areas are characterized by significant deprivation of caveolin, 5'-nucleotidase, Gce1, and pp59^{Lyn} versus both total plasma membranes and DIG, as well as significant enrichment of Na⁺/K⁺-ATPase and β -actin versus DIG (Table 1). Glut1, Glut4, and IR β were distributed about equally between DIG and non-DIG areas. Thus, in isolated rat adipocytes, non-DIG areas represent the typical overall plasma membranes harboring the majority of plasma membrane proteins but lacking typical caveolar or DIG components. Figure 9A demonstrates that the concentration-dependent decrease in the amount of Gce1, pp59^{Lyn}, and pp125^{Fak} in DIG in response to PIG 41 was in parallel to their increase in non-DIG areas isolated from the same adipocytes. Quantitative evaluation confirmed the inverse relationship between the disappearance and appearance of Gce1, pp59^{Lyn}, and pp125^{Fak} in DIG and non-DIG areas, respectively, in response to PIG 41 with similar EC₅₀ (0.3 to 1 μ M) for all three proteins (Fig. 9B). PIG treatment did not affect the distribution of caveolin, IR β , and Glut4 between DIG (Fig. 9A) and non-DIG areas and was not associated with a considerable shift of Gce1, pp59^{Lyn}, and pp125^{Fak} to intracellular membranes or the cytosol (data not shown). In agree-

FIG. 5. PIG causes dissociation of pp125^{Fak}, pp59^{Lyn}, and Gce1 from caveolin in DIG. Isolated rat adipocytes were incubated (20 min, 37°C) with 10 μ M PIG 41, 37, 7, or 1. DIG were prepared from total-cell lysates (detergent method) and used for immunoprecipitation (IP) (nondissociating conditions) of caveolin. The immunoprecipitates were immunoblotted (IB) for pp125^{Fak}, pp59^{Lyn}, and caveolin or assayed for Gce1 by photoaffinity labeling with 8-N₃-[³²P]cAMP. (A) Shown are phosphorimager images of a typical experiment that was repeated two times, with similar results. (B) Quantitative evaluations of three different adipocyte incubations with measurements in triplicate each are given as the percent of maximal response (mean $\pm$ standard deviation), with basal values (absence of PIG) set at 100%.

ment with the failure of CSDP to impair the PIG-induced dissociation of pp59^{Lyn} from caveolin (Fig. 6), a 30-fold molar excess of CSDP over PIG 41 did not affect redistribution of pp59^{Lyn} from DIG to non-DIG areas (Fig. 10). Taken together, active PIG trigger pronounced redistribution of Gce1, pp59^{Lyn}, and pp125^{Fak} from DIG to non-DIG areas of the adipocyte plasma membrane.

pp125^{Fak} interacts with pp59^{Lyn}, IRS-1, and Gce1 during PIG-induced redistribution. Previous studies on the mechanism of insulin receptor-independent tyrosine phosphorylation of IRS-1 in response to PIG showed that pp125^{Fak} acts as a platform molecule for PIG-dependent recruitment of pp59^{Lyn} and IRS-1 (44). The present observation of PIG-dependent redistribution of pp59^{Lyn}, Gce1, and pp125^{Fak} raised the pos-

FIG. 6. CSDP blocks CBDP-induced but not PIG-induced dissociation of Gce1, pp59^{Lyn}, and pp125^{Fak} from caveolin. Isolated rat adipocytes were electroporated in the absence or presence of various concentrations of CSDP and/or CBDP and then were incubated (20 min, 37°C) with or without 3 μM PIG 41 as indicated. DIG were prepared from total-cell lysates (detergent method) and used for immunoprecipitation (IP) (nondissociating conditions) of caveolin. The immunoprecipitates were assessed for the presence of Gce1 by photoaffinity labeling and assessed for the presence of pp59^{Lyn} and pp125^{Fak} by immunoblotting (IB) using chemiluminescent detection. Shown are phosphorimages of a typical experiment that was repeated two times, with similar results.

sibility of their interaction after arrival at non-DIG areas. In fact, treatment of isolated rat adipocytes with PIG 41 increased, in a concentration-dependent manner, the amounts of pp59^{Lyn}, Gce1, and IRS-1 which were coimmunoprecipitated

with pp125^{Fak} from non-DIG areas (Fig. 11). The efficiency of pp125^{Fak} immunoprecipitation did not vary significantly. The (small) amounts of β₁-integrin, paxillin (a major cytoskeletal substrate of pp125^{Fak}), and IRβ recovered with pp125^{Fak} in

FIG. 7. PIG-induced dissociation of Gce1, pp125^{Fak}, and pp59^{Lyn} from caveolin correlates to caveolin tyrosine phosphorylation. Isolated rat adipocytes were incubated (20 min, 37°C) with increasing concentrations of PIG 41, 37, or 1. From total-cell lysates, caveolin was immunoprecipitated (IP). The immunoprecipitates were assayed for the presence of Gce1 by photoaffinity labeling or for the presence of pp125^{Fak}, pp59^{Lyn}, phosphotyrosine (pY), and caveolin by immunoblotting (IB). Shown are quantitative evaluations of three different adipocyte incubations with measurements taken in quadruplicate, and results are given as a ratio of the relative amount of caveolin (mean ± standard deviation) or as the fold stimulation (mean ± standard deviation), with basal values set at 1.

FIG. 8. CSDP impairs PIG-induced tyrosine phosphorylation of pp59^{Lyn} (A) and tyrosine phosphorylation of pp125^{Fak} and IRS-1 and glucose transport (B). Isolated rat adipocytes were electroporated in the absence of CSDP or presence of increasing concentrations of CSDP and then incubated (20 min, 37°C) with increasing concentrations of PIG 41, 37, and 7 (A) or 2 μM PIG 41 (B). From portions of the cells, pp59^{Lyn} (A, B) and pp125^{Fak} and IRS-1 (B) were immunoprecipitated (IP) from total-cell lysates and were immunoblotted (IB) for phosphotyrosine (pY). (A) Shown are phosphorimages of a typical experiment that was repeated three times, with similar results. (B) Other portions of the cells were assayed for 2-deoxyglucose transport. Shown are quantitative evaluations of four different adipocyte incubations with measurements in duplicate, and results are given as the percent of PIG response (mean ± standard deviation), set at 100% in the absence of CSDP.

non-DIG areas did not elevate during the course of PIG treatment. Thus, PIG-stimulated redistribution of pp59^{Lyn} and Gce1 from DIG to non-DIG areas is accompanied by their association with pp125^{Fak} and IRS-1. This provides further evidence for pp125^{Fak} functioning as a recruitment platform during PIG signaling.

CSDP does not block insulin signaling and action. Insulin has been reported to cause partial activation of NRTK of the Src family (pp59^{Fyn}) in 3T3-L1 adipocytes (29, 36). However, its relevance for metabolic insulin action remained unclear. It

was conceivable that insulin and PIG activation of PI-3'K operate in part via the same molecular mechanism. Therefore, we studied whether CSDP manages to inhibit insulin stimulation of glucose transport and IRS-1 tyrosine phosphorylation (Table 2). As expected, electroporation of isolated rat adipocytes with CSDP inhibited, in a concentration-dependent manner, IRS-1 tyrosine phosphorylation and glucose transport activation in response to both CSDP and PIG 41. In contrast, CSDP failed to suppress insulin signaling and metabolic action to any significant degree, even at 300 μM and at submaximal insulin

A Design: Stimulation of Isolated Rat Adipocytes with PIG 41 → Preparation and Purification of DIGs and non-DIG areas → IB : pp59^{Lyn}/pp125^{Fak}/Caveolin/IRβ/Glut 4; Photoaffinity Labeling : Gce1

FIG. 9. PIG induces redistribution of Gce1, pp59^{Lyn}, and pp125^{Fak} from DIG to non-DIG areas of the plasma membrane. Isolated rat adipocytes were incubated (20 min, 37°C) with increasing concentrations of PIG 41. From total plasma membranes, DIG and non-DIG areas were prepared (carbonate method), purified (sucrose gradient centrifugation), and then immunoblotted (IB) for pp59^{Lyn}, pp125^{Fak}, caveolin, IRβ, and Glut4 or assayed for Gce1 by photoaffinity labeling. (A) Shown are phosphorimages of a typical experiment that was repeated two times, with similar results. (B) Shown are quantitative evaluations of three different adipocyte incubations with measurements in duplicate, and results are given as the percentage of total material contained in DIG (○) plus non-DIG (◼) areas (mean ± standard deviation), with basal values (absence of PIG 41) set at 100%.

concentrations. This argues for the specificity of the CSDP inhibitory action and strongly suggests that insulin does not use the same signaling pathway upstream of IRS-1 as active PIG involving the modulation of the caveolin-pp59^{Lyn} interaction.

DISCUSSION

It has been proposed that caveolins and, by implication, caveolae and DIG may act to coordinate the interaction of receptors and a variety of downstream signal-transducing mol-

FIG. 10. CSDP does not interfere with PIG-induced redistribution of pp59^{Lyn} from DIG to non-DIG areas. Isolated rat adipocytes were electroporated in the absence or presence of 300 μM CSDP and then incubated (20 min, 37°C) with increasing concentrations of PIG 41. From total plasma membranes, DIG and non-DIG areas were prepared (carbonate method), purified (sucrose gradient centrifugation), and then immunoblotted (IB) for pp59^{Lyn}. Shown are phosphorimages of a typical experiment that was repeated once, with similar results.

ecules that localize to the plasma membrane following cell stimulation (41, 49, 60). Agonist stimulation has been demonstrated to result in redistribution of receptors for contractile agonists (e.g., bradykinin [13] and acetylcholine [16]), hormones (e.g., insulin [24] and angiotensin II [25]), and growth factors (e.g., epidermal growth factor [11]), as well as of downstream elements (e.g., protein kinase Cα [37] and *rhoA* [63]) to caveolin-containing subcellular fractions, which for *rhoA* was shown to be inhibited by the CSDP (63).

Our studies with isolated rat adipocytes have revealed the induction and regulation of the opposite movement of signaling components. The NRTK, pp59^{Lyn}, pp125^{Fak}, and the GPI protein, Gce1, translocate from DIG to non-DIG areas of the plasma membrane in response to (i) an extracellular stimulus, insulin-mimetic PIG molecules, and (ii) intracellular accumulation of CDBP derived from pp59^{Lyn}. This movement is based on interference (indirectly or directly, respectively) with the caveolin(CSD)-NRTK(CBD) interaction. It correlates well to increased tyrosine phosphorylation and activity of pp59^{Lyn} and pp125^{Fak} and is accompanied by elevated tyrosine phosphorylation of IRS-1, PI-3'K activity, and glucose transport. In contrast, CSDP blocked PIG-induced tyrosine phosphorylation of pp59^{Lyn}, pp125^{Fak}, and IRS-1 as well as glucose transport and can therefore be used as a specific inhibitor to test for involvement of the PIG signaling pathway. These findings suggest a causal relationship between the interaction with caveolin, localization in DIG-caveolae, and low activity of Gce1, pp59^{Lyn}, and pp125^{Fak} and vice versa between their dissociation from caveolin, redistribution from DIG-caveolae to plasma membrane non-DIG areas, and insulin-mimetic signaling.

In vivo, PIG molecules as a putative physiological signal may be generated by lipolytic cleavage of GPI proteins at the outer face of the plasma membrane, which, in fact, has been reported for primary and cultured adipose, muscle, and endothelial cells in response to a number of hormones and growth factors, such as insulin and nerve growth factor (38, 39), glucose (1, 39), and the drug glimepiride (38, 39). Alternatively, degradation products of free GPI lipids concentrated in DIG-caveolae may act in a similar fashion as the synthetic PIG used in the present study. In different cellular systems, lipolytic GPI turnover is modulated by a variety of hormones, cytokines, and growth factors, such as insulin, interleukin-2, epidermal growth factor, and erythropoietin (4, 9, 26). Treatment of intact rat adipocytes with bacterial (G)PI-specific phospholipase C induces some insulin effects (35) and tyrosine phosphorylation of caveolin but not of the insulin receptor (42). However, redistribution of caveolar components in response to GPI cleavage remains to be studied.

pp125^{Fak} released from DIG-caveolae is phosphorylated at tyrosines, presumably in an autophosphorylation reaction (Tyr397) and by pp59^{Lyn} (Tyr576, Tyr577). This ensures proper interaction of the two kinases (6) and maximal activation of pp125^{Fak} (44), respectively. pp125^{Fak} is known to function as a platform molecule for signal-dependent recruitment of a number of signaling molecules, including IRS-1 and IRS-2 (62). This finding was confirmed and extended here by the demonstration of complex formation between pp125^{Fak}, pp59^{Lyn}, IRS-1, and Gce1, but not the cytoskeletal proteins, paxillin, and β₁-integrin, in response to PIG. Thus, Gce1, pp59^{Lyn}, and pp125^{Fak} together with IRS-1 and IRS-2 may be redistributed

FIG. 11. PIG induces complex formation between pp125^{Fak}, pp59^{Lyn}, IRS-1, paxillin, and Gce1. Isolated rat adipocytes were incubated (20 min, 37°C) in the absence or presence of increasing concentrations of PIG 41. From total-cell lysates, non-DIG areas were prepared (detergent method) and then used for immunoprecipitation (IP) (nondissociating conditions) of pp125^{Fak}. The immunoprecipitates were subsequently immunoblotted (IB) for pp125^{Fak}, β₁-integrin, pp59^{Lyn}, IRS-1, paxillin, and IRβ or assayed for Gce1 by photoaffinity labeling. Shown are phosphorimages of a typical experiment that was repeated two times, with similar results.

from caveolae to a multicomponent signaling module where tyrosine-phosphorylated and activated pp125^{Fak} may present IRS-1 and IRS-2 for phosphorylation at specific tyrosine residues by activated pp59^{Lyn}. This in turn initiates signaling to the Glut4 and Glut1 translocation apparatus and other metabolic effector systems which also receive their insulin signals from the IRS proteins (23, 47). Apparently, IRS-1 acts as site of convergence of the signaling pathways initiated by insulin-mimetic PIG and insulin. These are clearly separate upstream of IRS-1 based on the ability of CSDP to block PIG but not insulin signaling and action. It is clear from studies with CSDP that relief of pp59^{Lyn} from interaction with or inhibition by caveolin is required for insulin-mimetic PIG signaling and action. However, the signal emerging from this pathway and

ultimately resulting in potent PI-3'K activation may not be sufficient for glucose transport stimulation by PIG. Furthermore, the PIG pathway may operate not only in adipose but also muscle cells since they both express a high number of caveolae (2), which is a prerequisite and may guarantee specificity for metabolic coupling. Future experiments have to address these possibilities.

The PIG signaling module upstream of IRS-1 seems to be used by other ligands or pathways since clustering of the adipocyte-specific integrins α₅ and β₁ by fibronectin plus activating anti-β₁-antibody blocked PIG-induced tyrosine phosphorylation of pp59^{Lyn} and IRS-1 via interference at pp125^{Fak} (44). Thus, the Gce1–pp125^{Fak}–pp59^{Lyn}–IRS-1–IRS-2 complex may represent the site for the integration of at least two pathways

TABLE 2. Effect of CSDP on glucose transport stimulation by PIG, CBDP, and insulin^a

Peptide effect	Fold stimulation (mean ± SD) by CSDP							
	No CSDP		30 μM CSDP		100 μM CSDP		300 μM CSDP	
	Glucose transport	IRS-1 phosphorylation	Glucose transport	IRS-1 phosphorylation	Glucose transport	IRS-1 phosphorylation	Glucose transport	IRS-1 phosphorylation
Control	100 ± 13	1 ± 0.2	112 ± 18	0.8 ± 0.1	137 ± 22	1.2 ± 0.1	87 ± 19	1.1 ± 0.2
0.05 nM insulin	231 ± 48	4.1 ± 0.6	205 ± 35	4.3 ± 0.3	263 ± 50	4.7 ± 0.5	245 ± 29	4.2 ± 0.5
0.2 nM insulin	608 ± 77	9.9 ± 1.3	692 ± 103	10.7 ± 1.9	641 ± 89	10.9 ± 1.6	695 ± 63	10.2 ± 0.9
5 nM insulin	1,089 ± 204	18.6 ± 1.7	960 ± 163	20.3 ± 2.1	1,189 ± 214	19.4 ± 2.3	947 ± 151	21.4 ± 2.0
0.3 μM PIG 41	396 ± 41	2.2 ± 0.3	342 ± 38	2.4 ± 0.1	269 ± 17	1.9 ± 0.2	147 ± 20	1.2 ± 0.2
1 μM PIG 41	578 ± 53	3.4 ± 0.2	518 ± 40	3.3 ± 0.2	380 ± 39	2.6 ± 0.3	188 ± 42	1.8 ± 0.2
10 μM CBDP	402 ± 34	1.8 ± 0.2	352 ± 24	1.8 ± 0.1	244 ± 28	1.2 ± 0.1	89 ± 11	0.8 ± 0.1
30 μM CBDP	529 ± 62	2.7 ± 0.3	489 ± 41	2.3 ± 0.2	297 ± 21	1.7 ± 0.2	95 ± 12	0.9 ± 0.1

^a Isolated rat adipocytes were electroporated in the absence of CSDP or in the presence of increasing concentrations of wild-type CSDP derived from pp59^{Lyn}, then incubated (10 min, 37°C) with different concentrations of PIG, CBDP, or insulin, and subsequently assayed for IRS-1 tyrosine phosphorylation (by immunoblotting and chemiluminescent detection) and 2-deoxyglucose transport. Quantitative evaluations of four different adipocyte incubations with measurements in triplicate each are given as fold stimulation (mean ± standard deviation), with basal values set at 100 (glucose transport) and 1 (IRS-1 phosphorylation), respectively, in the absence of CSDP and insulin.

for extracellular signals leading to insulin-mimetic metabolic signaling. One of the pathways acts from GPI proteins via caveolae-DIG in a positive fashion, and the other pathway acts from the extracellular matrix via integrins in a negative fashion. Localization of the activated signaling module at sites different from both caveolae-DIG and integrins at non-DIG areas may favor this cross talk.

Our finding may be of relevance for the elucidation of novel approaches for the therapy of insulin-resistant states which are manifested during metabolic syndrome and type II diabetes mellitus (8). Reduced responsiveness and sensitivity of components of the insulin signaling pathway toward insulin can be observed already immediately downstream of the insulin receptor at the level of tyrosine phosphorylation of IRS-1 and IRS-2 (30). Consequently, insulin receptor-independent tyrosine phosphorylation of IRS-1 and IRS-2 mediated by cross talk from the GPI protein-DIG-caveola-pp59^{Ln} pathway should induce insulin-mimetic effects, such as stimulation of glucose uptake, in insulin-resistant adipose and muscle cells. This has been demonstrated so far for PIG in adipocytes isolated from obese rats (18). Thus, small molecules which cause redistribution of caveolar or DIG components may be useful for the therapy of metabolic syndrome and type II diabetes mellitus.

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