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Review

Signalling via caveolin: involvement in the cross-talk between phosphoinositolglycans and insulin

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Abstract. In recent years, a number of cross-talk systems have been identified which feed into the insulin signalling cascade at the level of insulin receptor substrate (IRS) tyrosine phosphorylation, e.g., receptor and non-receptor tyrosine kinases and G-protein-coupled receptors. At the molecular level, a number of negative modulator and feedback systems somehow interacting with the β-subunit (catecholamine-, phorbolester-, or tumor necrosis factor-α-induced serine/threonine phosphorylation, carboxy-terminal trimming by a thiol-dependent protease, association of inhibitory/regulatory proteins such as RAD, PC1, PED, α_2-HS-glycoprotein) have been identified as candidate mechanisms for the impairment of insulin receptor function by elevations in the activity and/or amount of the corresponding modification enzymes/inhibitors. Both decreased responsiveness and sensitivity of the insulin receptor β-subunit for insulin-induced tyrosine autophosphorylation have been demonstrated in several cellular and animal models of metabolic insulin resistance as well as in the adipose tissue and skeletal muscle of diabetic patients and obese Pima Indians compared to non-obese subjects. Therefore, induction of the insulin signalling cascade by bypassing the defective insulin receptor kinase may be useful for the therapy of non-insulin dependent diabetes mellitus. During the past two decades, phosphoinositolglycans (PIGs) of various origin have been demonstrated to exert potent insulin-mimetic metabolic effects upon incubation with cultured or isolated muscle and adipose cells. However, it remained to be elucidated whether these compounds actually manage to trigger insulin signalling and if so at which level of hierarchy within the signalling cascade the site of interference is located. Recent studies using isolated rat adipocytes and chemically synthesized PIG compounds point to IRS1/3 tyrosine phosphorylation by p59Lyn kinase as the site of cross-talk, the negative regulation of which by interaction with caveolin is apparently abrogated by PIG. This putative mechanism is thus compatible with the recently formulated caveolin signalling hypothesis, the supporting data for which are reviewed here. Though we have not obtained experimental evidence for the involvement of PIG in physiological insulin action, the potential cross-talk between insulin and PIG signalling, including the caveolae/detergent-insoluble glycolipid-enriched rafts as the compartments where the corresponding signalling components are concentrated, thus represent novel targets for signal transduction therapy.

Key words. Insulin receptor and signalling; insulin receptor substrate proteins; insulin resistance; glycosyl-phosphatidylinositol; caveolin; detergent-insoluble glycolipid-enriched rafts.

The insulin signalling cascade

Metabolic and mitogenic signalling by insulin seems to operate in a bipartite fashion, since two pathways within the insulin signalling pathway are thought to mediate the different biological functions of the hormone (fig. 1): (i) activation of the phosphatidylinositol-

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3′-kinase (PI 3-K) plays a pivotal role for regulation of glucose transport and cellular trafficking [for reviews see refs 1–3] as well as glycogen synthesis and lipolysis by insulin [for reviews see refs 4–6]; (ii) formation of the Shc-Grb2 complex leads to activation of the Ras pathway which has been linked to insulin regulation of both cell growth and gene expression, although this has been questioned recently. Many of the proteins involved in these two pathways have been identified at the molecular level [for reviews see refs 7–11]. The insulin receptor is a heterotetramer consisting of two α- and two β-chains (125 and 95 kDa, respectively). The transmembrane β-chain functions as a tyrosine kinase which is regulated by the disulfide-linked insulin-binding α-chain. When derepressed, i.e., activated by insulin binding, the β-subunits undergo rapid trans-autophosphorylation and phosphorylate a number of intracellular substrates, among them one or more 50- to 60-kDa proteins including Shc, a 15-kDa fatty-acid-binding protein, the Gab-1 adaptor protein and the insulin receptor substrate (IRS)-1/2/3/4 polypeptides. Following tyrosine phosphorylation, Shc, Gab-1 and IRS-1 to 4 act as docking proteins for several Src homology 2 (SH2) domain-containing adaptor molecules and enzymes, including PI 3-K, Grb2, SHP2, Nck, Fyn, and non-receptor tyrosine kinases. The interaction between the IRS proteins and PI 3-K occurs through the p85 regulatory subunit of the enzyme and results in an increase in catalytic activity of the p110 subunit. In all cases in which there is stimulation of tyrosine phosphorylation of IRS proteins, there is concomitant docking of these proteins to the p85 subunit of PI 3-K and, with the exception of the cross-talk between the insulin and angiotension signalling systems [12], this docking is associated with stimulation of PI 3-K activity.

It is generally accepted that one branch of acute metabolic insulin signalling is initiated by activation of PI 3-K (fig. 1). This may be necessary for insulin stimulation of glycogen synthase and anti-lipolysis [13, 14] as well as glucose transport [15–17] and may even be sufficient for the latter [18, 19; for reviews see refs 20, 21]. Further, strong experimental evidence argues for protein kinase B (PKB) as an element of the signalling cascade leading from PI 3-K to activation of glycogen synthase and glucose transport in isolated rat adipocytes, various cell culture models, and human skeletal muscle [22–24]. Currently, a model is favored with the following sequence of events [for reviews see refs 25–27]: PI 3-K activation results in the production of phosphatidylinositol (3,4,5)-trisphosphate and phosphatidylinositol (3,4)-bisphosphate. The binding of the pleckstrin-homology domain of PKB to these phosphoinositides both recruits PKB to the plasma membrane and directly and/or indirectly (via phosphorylation by the membrane-associated phosphoinositide-dependent serine/threonine protein kinases, PDK1/2) stimulates its kinase activity [for a review see ref. 28]. Activated PKB

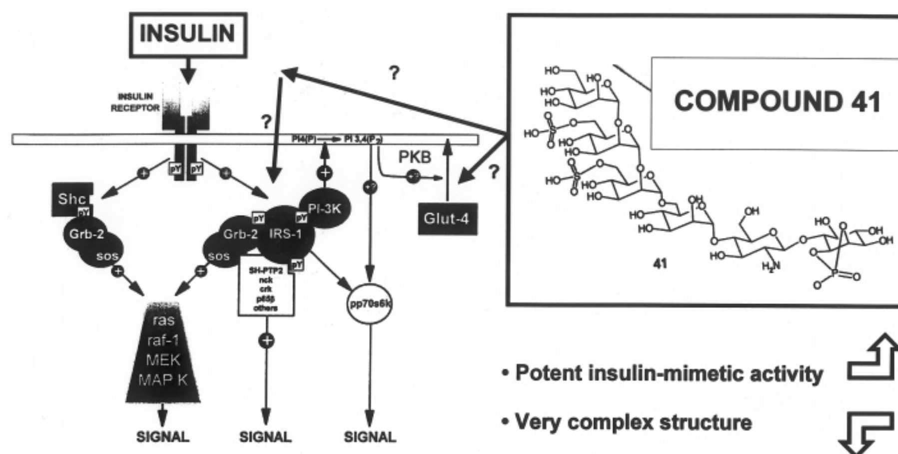

Figure 1. Insulin signalling and its modulation by phosphoinositolglycan (PIG) molecules. The molecular components of the bipartite insulin signalling cascade from the insulin receptor via tyrosine-phosphorylated Shc and the Ras/MAPK pathway to MAPK (gene expression) and via tyrosine-phosphorylated insulin receptor substrate (IRS) proteins and the PI 3-K/PKB pathway to the GLUT4 translocation apparatus (glucose transport) are depicted. Additional signalling routes emerging from the IRS proteins, e.g., via the SH2-containing protein tyrosine phosphatase, SH-PTP2 or Syp (DNA synthesis), and via the serine/threonine kinase, pp70S6 (protein synthesis), are also indicated although their functional relevance is less clear. The primary target(s) of PIG compounds may be located at the level of the cell surface, the intracellular signalling components, or the GLUT4 translocation apparatus. Of course, these possibilities are not mutually exclusive.

phosphorylates substrate proteins at serine/threonine residues, e.g., glycogen synthase kinase 3 (GSK-3, which is thereby inhibited, leading to dephosphorylation and activation of glycogen synthase [for a review see ref. 29]), resulting in a variety of biological effects possibly including stimulation of glycogen synthesis and glucose transporter isoform 4 (GLUT4) translocation. However, the importance of GSK-3 in insulin activation of glycogen synthase remains unclear since (i) inactivation of GSK-3 by isoproterenol in rat adipocytes equalled that of insulin, yet had no effect on glycogen synthase activity [30], (ii) overexpression of active PI 3-K had no effect on basal or insulin-stimulated glycogen synthase activity [13], and (iii) the phosphorylation state of glycogen synthase is (in addition) regulated by the insulin-stimulated protein phosphatase 1 [for a review see ref. 31]. In any case, the insulin-induced inactivation of GSK-3 has been described in adipocytes and CHO cells [32]. Taken together, GSK-3 is clearly a target for insulin signalling, possibly primarily involved in the insulin regulation of protein synthesis and cytoskeletal reorganization rather than of glycogen synthesis. Distal signalling pathways are difficult to dissect because branching paths emerge from downstream effectors and several upstream inputs converge upon branch points. Thus, insulin action is determined by complicated integrated signalling networks rather than simple linear pathways.

Although multiple downstream effectors of insulin action are shared by many receptor tyrosine kinases, there are potential mechanisms for incorporating specificity at each step in the insulin signal transduction pathway. Mechanisms of specificity of insulin signalling may act at the level of (i) the receptor substrate, (ii) compartmentalization of signalling complexes, (iii) tissue-specific expression of key effectors, (iv) modulation of signal frequency and amplitude, and (v) feedback regulation. In this regard, the function of end-products to dampen or amplify signals from upstream pathways has been recognized as a common paradigm. It seems possible that specificity in insulin receptor tyrosine kinase signal transduction is also determined, in part, by positive or negative feedback. In the case of insulin signalling, it was recently shown that GSK-3 can phosphorylate IRS-1 at serine/threonine residues, impairing its function as receptor substrate and adaptor protein but inducing a role as inhibitor for the receptor kinase activity [33]. Similary, PI 3-K has serine/threonine kinase activity in addition to its lipid kinase activity that can phosphorylate IRS-1 and may modulate IRS-1 function [34]. In addition, there is evidence that PI 3-K has functional interactions both upstream and downstream from Ras suggesting another feedback loop that may be involved with insulin signalling [35, 36].

In addition to the identification of the signal transduction pathways directly leading from the insulin receptor to downstream targets, several cross-talks have been delineated between signal transmission by insulin and other hormones/growth factors [e.g., catecholamines, tumor necrosis factor (TNF)-α, angiotensin II, leptin, interleukins, interferon, growth hormone] which either mimic (to a certain degree) or modulate in a positive or negative fashion metabolic and/or mitogenic insulin action in various cellular systems [37; for reviews see refs 7, 8, 11, 38–40]. For example, there is evidence for cross-talk between insulin and platelet-derived growth factor or G-protein-coupled receptor (such as the angiotensin II receptor) signalling with respect to interactions of IRS-1 and PI 3-K [41]. Stimulation of $\alpha_5\beta_1$-integrins, which bind to particular extracellular matrix proteins, by fibronectin enhances insulin receptor and IRS-1 phosphorylation [42]. Moreover, insulin promotes association of the $\alpha_v\beta_3$-integrin (a vitronectin receptor) with IRS-1 [43]. As a result, the interaction between integrin and IRS-1 may be a mechanism for the synergistic action of growth-promoting and extracellular matrix receptors. Thus, a plethora of extracellular signals as well as the extracellular matrix surrounding the cell may influence the specificity of signalling by the insulin receptor. Since none of the stimulatory factors so far characterized activates the insulin receptor kinase directly, their signalling pathways seem to converge with that of insulin at a more distal signalling step. Soluble phosphoinositolglycan (PIG) or phosphoinositolglycan-peptide (PIG-P) molecules which have been shown to exert partial insulin-mimetic effects in diverse cellular and subcellular systems can also be classified into this expanding list of molecules which manage to cross-talk to the insulin signalling cascade via a complex mechanism.

Structure of PIG molecules

Initially, PIG substances isolated from natural sources were thought to be of peptidic nature [for a review see ref. 44]. However, subsequent analysis revealed that they can be generated from lipidic precursor structures [45] as the polar headgroups consisting of PIG moieties [46, 47] which may harbor *chiro*- or *myo*-inositol [48]. Whereas the existence and detailed structure of the insulin-sensitive PIG molecules with physiological function in insulin signalling ('mediator or second messenger') is still a matter of dispute, a minimal consensus on the composition of PIG compounds with partial insulin-mimetic activity (in particular based on analytical methods like nuclear magnetic resonance, mass spectroscopy, and metabolic labelling studies with radiolabelled carbohydrates, as well as on chemical and enzymatic mod-

Analytic and metabolic labeling studies

myo/chiro *inositol, amino sugar, mannose, galactose*
phosphate (hydrogen fluoride cleavage = HF)

Chemical and enzymatic modifications

phosphoinositol glycosidically linked to glucosamine (nitrous acid deamination = NA)

glucosamine coupled to additional monosaccharides (trifluoroacetic acid cleavage = TFA)

⇨ Phosphoinositolglycans ("PIG" molecules)

Similarity of PIG molecules to the glycan portion of glycosyl-phosphatidylinositol (GPI) lipids or GPI membrane protein anchors?

Figure 2. Summary of the structural features of PIG molecules prepared from natural sources as soluble entities (from plasma, urine, or hemodialysate), or as lipolytic cleavage products (from plasma membranes or glycolipid fractions derived from insulin-sensitive tissues or cells), and some of the methods used for their elucidation.

ifications and cleavages) has developed (fig. 2). PIG compounds appear to consist of a core structure of phosphorylated *myo*/*chiro*-inositol glycosidically linked to non-acetylated hexosamine (glucosamine, galactosamine) which is coupled to an oligosaccharide of varying composition, often containing galactose and mannose residues [for reviews see refs 49–52]. Thus, PIG molecules resemble the polar core glycan head groups of both free glycosyl-phosphatidylinositol (GPI) lipids and GPI membrane anchors of GPI-modified cell surface proteins (GPI proteins) (fig. 3a,b), which are embedded in the outer leaflet of the plasma membrane by a covalently bound glycolipid of the GPI type [for reviews see refs 53–55]. The PIG portion of GPI anchors consists of phospho-*myo*-inositol, non-acetylated glucosamine, and an oligosaccharide which in all GPI proteins investigated so far from yeast (fig. 3c) and parasites to humans (fig. 3b) consists of three mannose residues linked in a characteristic glycosidic manner. The terminal mannose residue is coupled via a phosphodiester bridge to ethanolamine which forms an amide bond to the carboxy-terminal amino acid of the polypeptide portion of the GPI protein. Unfortunately, the detailed structure of a PIG molecule from insulin-sensitive tissues exhibiting insulin-mimetic activity has not yet been elucidated, and thus the extent of structural similarity between GPI anchors/lipids and insulin-sensitive PIG precursor lipids remains speculative.

Activity of PIG molecules

During the past two decades, a large body of evidence has accumulated that PIG molecules can exert some insulin-mimetic activity on glucose and lipid metabolism in insulin target cells in vitro. They have been demonstrated to stimulate glucose transport, glycolysis, glycogen synthesis, and lipid synthesis, and to inhibit glucagon-induced gluconeogenesis and isoproterenol-induced lipolysis in isolated or cultured muscle, fat, and liver cells to a partial degree [for reviews see refs 44, 49–51]. The molecular basis of these PIG actions seems to rely on the appropriate modulation of the activity of the corresponding key metabolic and regulatory enzymes/proteins, such as pyruvate dehydrogenase, glycogen synthase, glycerol-3-phosphate acyltransferase, glycogen phosphorylase, protein kinase A, cGMP-inhibitable cAMP-specific phosphodiesterase [for reviews see refs 50, 51], and protein phosphatase 1 and 2C [56]. This partial insulin-mimetic metabolic activity together with the mechanistic findings that (i) some metabolic insulin actions can be inhibited by anti-PIG antibodies in intact BC_3H-1 myocytes [57], (ii) insulin stimulation of glycogen synthesis is blocked in mutant K562 erythroleukemia cells which are totally deficient in GPI synthesis [58], (iii) isolated PIG can exert acute hypoglycemic activity in normal and streptozotocin diabetic rats [59, 60], (iv) PIG can be isolated from normal subjects and in reduced amounts from non-insulin-dependent diabetes mellitus (NIDDM) patients [61], and (v) stimulation of the GPI-specific phospholipase is impaired in insulin-resistant rat adipocytes [62] suggest but do not prove the involvement of GPI structures, GPI cleavage, or PIG molecules in metabolic insulin signalling. The partial insulin-mimetic activity of PIG structures in cellular and cell-free assay systems [for reviews see refs 49–51, 63, 64] in combination with the possibility of the generation of PIG molecules from GPI structures by regulated lipolytic or combined lipolytic/proteolytic cleavages [62, 65–67] prompted P. Cuatrecasas, J. Larner, M. G. Low, J. Mato, and A. Saltiel in the late 1980s to assign PIG molecules a function as soluble mediators of metabolic insulin action [45, 52, 65, 68–70]. According to this hypothesis, PIG molecules are generated in response to insulin through lipolytic cleavage of free GPI lipids and/or GPI proteins by a phospholipase/protease at the extracellular face of the plasma membrane of insulin-sensitive cells and are then transported into the cytosol, where they directly affect key metabolic enzymes and/or their regulatory proteins in an allosteric fashion (fig. 4).

Despite this attractive and challenging explanation for the pleiotropic effects of insulin on glucose and lipid metabolism, essentially no data concerning the targets of and signalling events triggered by PIG molecules at the molecular level were reported in the following years. Such an analysis has been hampered by limited amounts (natural sources, no chemical synthesis) and quality (heterogeneous nature, chemically ill-defined structure, concentration estimated or unknown) of the PIG preparations available at that time. As a source for

PIG molecules of defined structure, we and others used the polar core glycan head groups generated by proteolytic/lipolytic digestion in vitro of a single purified GPI protein [71–74]. In particular, it turned out that the preparation of such structures from the GPI protein, Gce1p, of the yeast *Saccharomyces cerevisiae* [74] has some advantages in that (i) Gce1p can be isolated and purified in sufficient quantity [73], (ii) the structure of its GPI anchor is known, the glycan core being identical with that of mammalian GPI anchors [74], (iii) 3T3 adipocytes and rat adipocytes contain a GPI protein (named Gce1) similar to Gce1p from yeast [62, 66, 75], (iv) the GPI anchors of Gce1p and Gce1 are susceptible to regulated lipolytic/proteolytic processing in yeast [76] and to lipolytic cleavage in 3T3 and rat adipocytes [62, 66] in vivo. The resulting PIG-P could be isolated in sufficient amounts with limited expenditure according to the scheme given in figure 5 and thereafter exhibited high purity and homogeneity. The data on the molecular mode of action of these PIG-Ps obtained in detailed studies with isolated rat adipocytes, cardiomyocytes, and diaphragms [73, 77, 78] are summarized in figure 6a and led to a first simple working model presented in figure 6b.

Synthetic PIG compounds

As an alternative source for PIG molecules with defined structure, we at Hoechst Marion Roussel, Germany, decided to synthesize complete PIG molecules by chemical means, which required a very complex synthesis strategy [79]. PIG 41 (fig. 7), the synthesis of which required 52 steps in total, exhibits the complete authen-

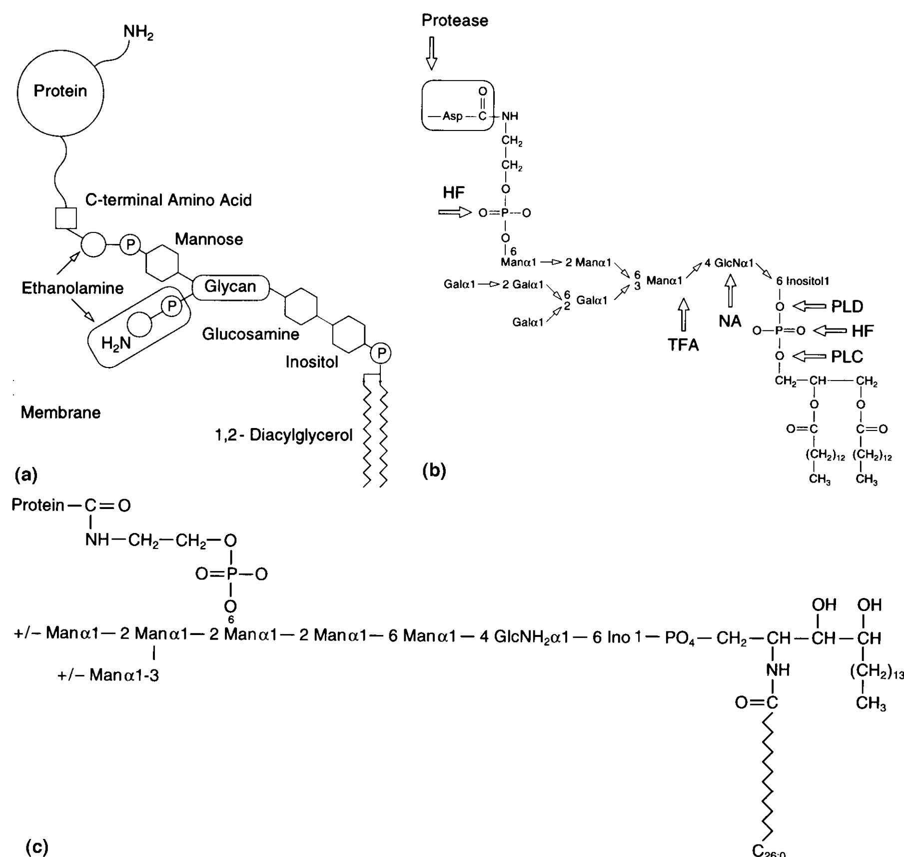

Figure 3. Structures of the GPI anchors of typical GPI proteins. A simplified general scheme (*a*) shows the constituent components (including an additional phosphoethanolamine moiety which has been found attached to the glycan core in some mammalian GPI anchors) without the type of glycosidic linkages between them. The lipid portion is most often diacylglycerol or alkylacylglycerol. A detailed scheme of the human erythrocyte acetylcholinesterase GPI anchor (*b*) indicates the cleavage sites for proteases (at the carboxy-terminus of the protein portion), aqueous hydrogen fluoride (HF), trifluoroacetic acid (TFA), nitrous acid (NA), and GPI-specific phospholipases C (PLC) and D (PLD). A detailed scheme (*c*) presents the GPI structure obtained for the yeast *Saccharomyces cerevisiae*. The lipid portion consists of a ceramide moiety. The glycan core (types of sugars and linkages) as well as its coupling to the carboxy-terminal amino acid of the protein portion via ethanolamine and a phosphodiester bridge are identical between mammals and yeast.

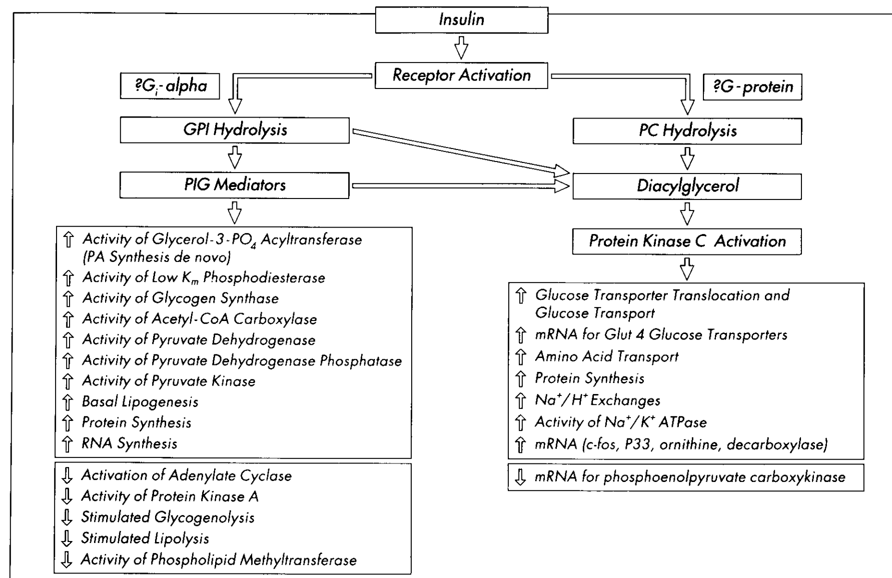

Figure 4. Compilation of the metabolic effects on key metabolic enzymes and pathways (activation ⇑, inhibition ⇓) by PIG molecules (left part) and diacylglycerol (which is generated in the course of lipolytic hydrolysis of GPI molecules by GPI-specific PLC or of phosphatidylcholine (PC) by PC-specific PLC, right part) as has been found in numerous studies in cell-free and cellular assay systems. The hydrolytic cleavages by the PLCs are assumed to be regulated by G-proteins (e.g. the alpha-subunit of an inhibitory G-protein G_i) although experimental evidence for this is limited.

tic structure of the polar core glycan head group of a typical yeast GPI anchor (compare with fig. 3c) and represents the first chemically synthesized PIG molecule with proven potent biological activity (see below). Furthermore, a number of structural derivatives were synthesized (see fig. 7) with exchanges/elongations/truncations with regard to the sugars, and variations in their linkages within both the core glycan chain [e.g., presence or absence of terminal (cyclic) phosphate and ethanolamine] and the carbohydrate side chain(s) (e.g., mannose or galactose branches and branch side number). They were all assayed for insulin-mimetic metabolic activity with respect to stimulation of lipogenesis in isolated rat adipocytes and glycogen synthesis in isolated rat diaphragms [79]. PIG 41 and 37 stimulated lipogenesis and glycogen synthesis up to 90% (at 20 μM) of the maximal insulin response (MIR) but with twofold lower EC_{50} values for PIG 41 versus 37. PIG 7 was clearly less potent (20% of MIR at 100 μM), whereas PIG 1 was almost inactive. This relative ranking in the insulin-mimetic potency between members of the PIG classes (i.e. $41 > 37 \gg 7 > 1$) was also observed for (i) activation of glucose transport in isolated normal and insulin-resistant adipocytes and (ii) inhibition of lipolysis in adipocytes. The analysis of the relationship between structure and activity suggested that the complete glycan core structure (Man_3-GluN) of typical GPI anchors including a mannose side chain and the inositol-phosphate moiety is required for maximal insulin-mimetic activity of PIG molecules with some minor variations possible with respect to the type of residues coupled to the terminal mannose/inositol as well as the type of linkages involved. These data argue for the potency and specifity of the interaction of active PIG compounds with a putative signalling component(s) in adipose and muscle cells which finally leads to insulin-mimetic metabolic activity, even in insulin-resistant states. In agreement with the necessity for certain structural complexity for pronounced insulin-mimetic activity is our finding that the activities of simple PIG derivatives consisting of (phospho)disaccharides, only were very low compared to PIG compounds harboring the complete core glycan. Despite these obvious limitations to simplifying the PIG structure while retaining almost the full insulin-mimetic activity, we succeeded in considerably reducing the structural complexity and thereby the synthesis expenditure for compounds with quite pronounced activity (see below). Insulin stimulates glucose transport by increasing the cell surface expression of GLUT4 in muscle and fat cells [for reviews see refs 1, 2]. However, in insulin- resistant adipocytes derived from the Zucker fatty rat, a typical animal model for insulin resistance associated with NIDDM, insulin only provoked a moderate increase in cell surface expression of GLUT4, in comparison to PIG 41 which was far more potent, followed by PIG 37 and 7, whereas PIG 1 was inactive and served as a negative control compound in the following studies [80].

Obviously, the synthetic PIG compounds stimulate GLUT4 cell surface expression and thereby glucose transport even in insulin-resistant cells.

Convergence of PIG and insulin signalling

Insulin initiates signalling to the glucose transport system by binding to the insulin receptor and activating its intrinsic tyrosine kinase (see above). Under conditions which led to a circa 11-fold increase in β-subunit tyrosine phosphorylation by insulin in isolated rat adipocytes, PIG 41 was virtually inactive [79, 80]. Insulin and PIG 41 induced tyrosine phosphorylation of IRS-1, and to a lower degree of IRS-2, and association of p85 with IRS-1 and, to a limited extent, of IRS-2 in rat adipocytes. The fat-cell-specific IRS-3, which could

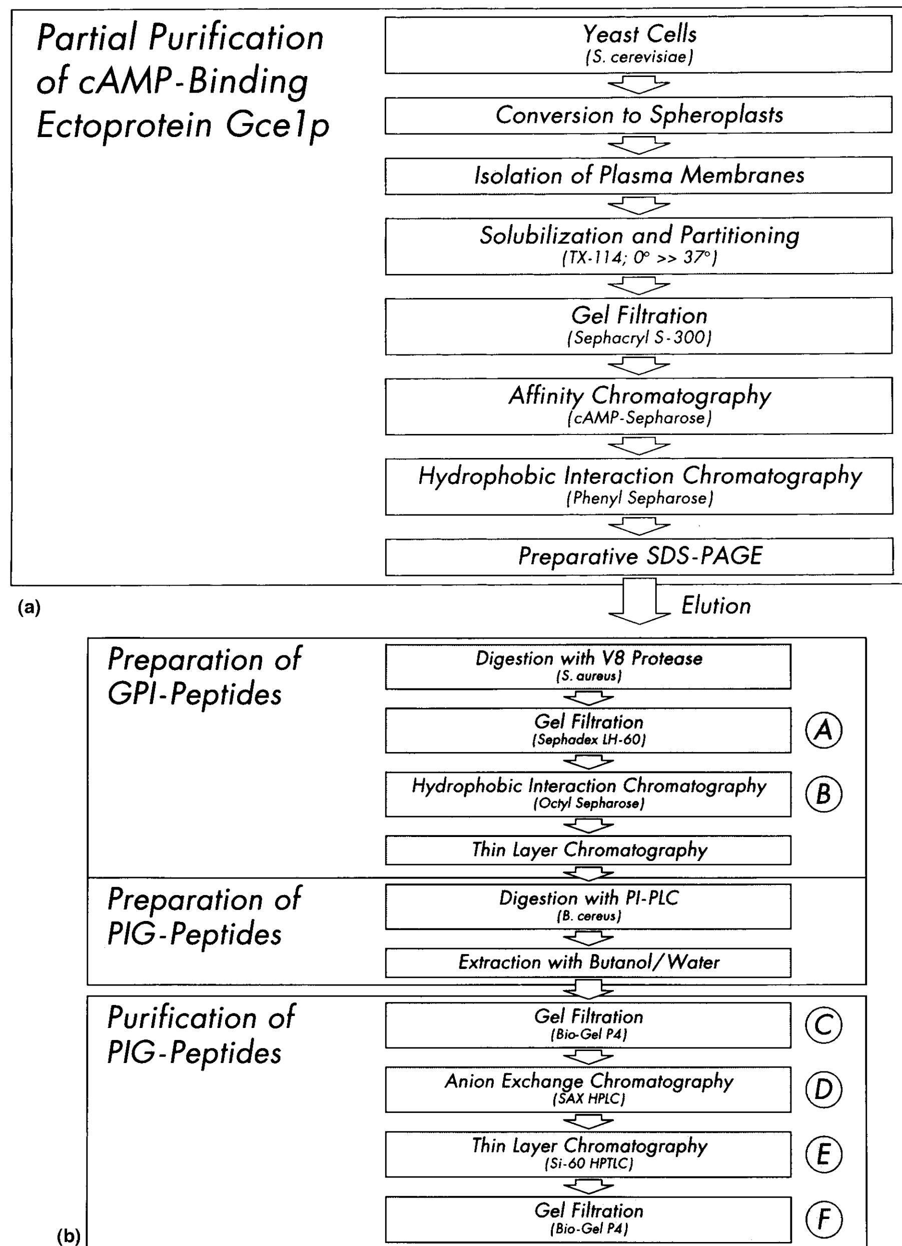

Figure 5. Scheme for the preparation of PIG-P molecules from the GPI anchor and carboxy-terminus of a yeast GPI protein by sequential partial purification of Gce1p (*a*), preparation of GPI peptides by limited proteolytic digestion (for removal of the protein portion except the carboxy-terminal anchor peptide), preparation of PIG peptides by lipolytic digestion with bacterial PI-specific PLC (for removal of diacylglycerol), and final purification of the PIG peptides to homogeneity (*b*).

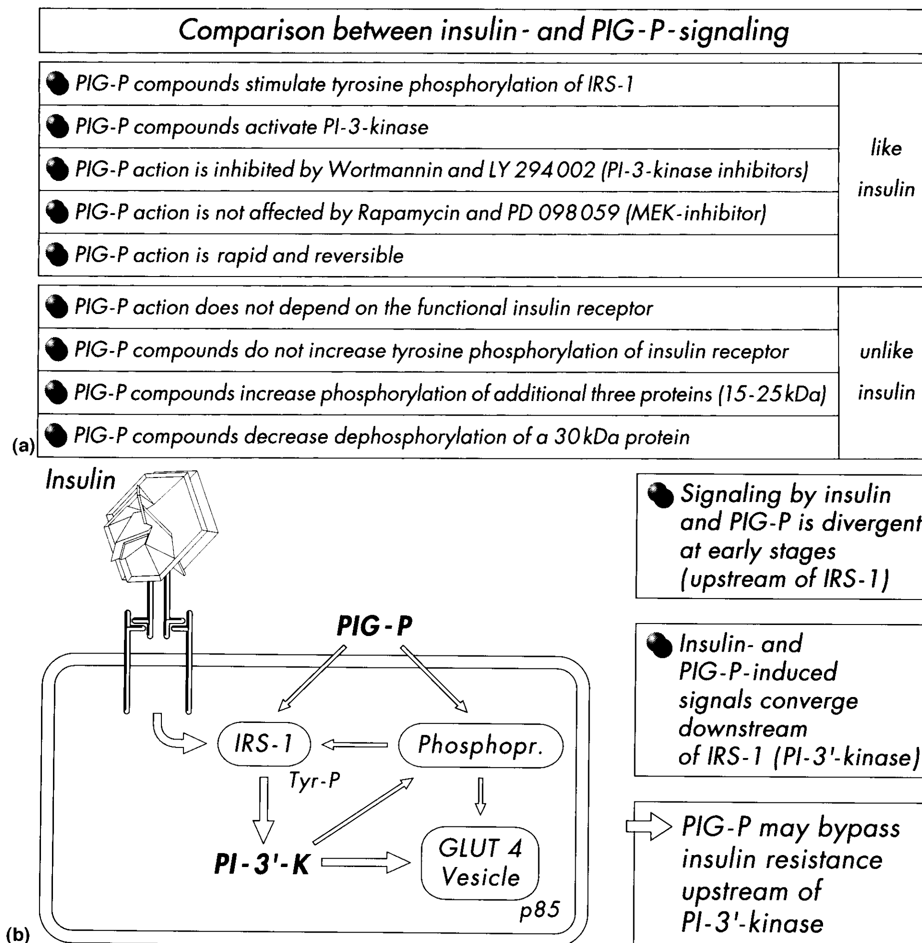

Figure 6. Summary of the experimental data obtained with PIG-P prepared from yeast on signalling in isolated rat adipocytes, cardiomyocytes, and diaphragms (*a*) (see text for details) and the resulting working model for a typical insulin target cell (*b*). PIG-P provokes tyrosine phosphorylation of IRS-1 and activation of PI 3-K (as does insulin) and, in addition, induces alterations in the phosphorylation state of polypeptides (phosphopr.) which are not affected by insulin. The two pathways together may be required or even be sufficient for initiation of the movement of GLUT4-containing vesicles from the cytoplasm to the plasma membrane. For this so-called GLUT4 translocation, the described association of PI 3-K (p85) with GLUT4-containing vesicles in adipocytes may be a prerequisite.

be identified as a 60-kDa polypeptide in anti-phosphotyrosine immunoprecipitates, was phosphorylated at tyrosine residues to almost MIR [80]. Corresponding to the PIG-induced association of IRS proteins and p85, PIG 41, 37, and 7 increased PI 3-K activity in a concentration-dependent manner, with PIG 41 approaching 90% of MIR at 10 μM [80]. The inactive PIG 1 had no effect. Thus PIG compounds manage to increase cell surface expression of GLUT4 in isolated rat adipocytes, presumably by inducing tyrosine phosphorylation of IRS proteins and stimulating PI 3-K without preceding/concomitant activation of the insulin receptor (fig. 1).

Not only the insulin and IGF-1 receptors, but also several cytokine, interleukin, and interferon receptors can induce tyrosine phosphorylation of IRS proteins [81, 82; for reviews see refs 7, 8]. In response to ligand binding, these receptors recruit and activate cytosolic tyrosine kinases of the Janus kinase family, which in turn associate and phosphorylate IRS-1 and IRS-2. IRS-1 has also been shown to be phosphorylated on tyrosine residues in response to angiotension II [83, 84] and gastrin [85], ligands that bind to specific seven-transmembrane domain G-protein-coupled receptors. Ligand-dependent tyrosyl phosphorylation of proteins in the size range (160–170 kDa) appropriate for IRS-1/2 has also been observed for growth hormone and a number of other ligands that bind to members of the hematopoietin [86] and erythropoietin receptors [87]. The combined data suggest that these ligands might utilize IRS-1/2 as common signalling molecules. The present data are compatible with the view that different signalling pathways can use IRS proteins, in general, possibly as the site for cross-talk. The apparent correla-

tion between the relative increment in IRS-1/3 tyrosine phosphorylation and PI 3-K activation for the synthetic PIG compounds studied as well as the identical ranking between different molecules for both processes strongly argues that PIG molecules exert (part of) their insulin-mimetic metabolic signalling via both IRS-1 and IRS-3.

To support the assumption that IRS proteins and PI 3-K are the point of convergence between the insulin and PIG signalling cascades, downstream-located elements should be affected in a similar manner by both stimuli. This was confirmed by the following findings [80]. In rat adipocytes, PIG 41, 37, and 7 increased the activity and phosphorylation state of immunoprecipitated PKB, and reduced GSK-3 activity in a concentration-dependent manner as did insulin. PIG 41 was most efficient followed by PIG 37. PIG 1 was ineffective. Thus the ranking order between the PIG compounds for PI 3-K activation, PKB stimulation, GSK-3 inactivation, and the insulin-mimetic metabolic activity with respect to MIR, as well as their relative ranking in potency, is identical, compatible with a role for the PI 3-K pathway in the metabolic activity of PIG compounds.

At present there is broad consense that the PI 3-K pathway is more strictly coupled to metabolic insulin signalling than the mitogen-activated protein kinase (MAPK) pathway. This is in agreement with data showing that PIG 41 and 37 increased MAPK activity in rat adipocytes to 30–50% of MIR only, relative to the PI 3-K pathway (90–100% of MIR). This suggests a certain selectivity of PIG compounds for inducing metabolic signalling, as also reflected in the preferential activation of glucose and lipid metabolism (80–90% of MIR) rather than protein synthesis (30% of MIR) in rat adipocytes.

The involvement of certain tyrosine kinases and the PI 3-K pathway but not of the MAPK pathway in the insulin-mimetic action of PIG molecules was strengthened by the finding that inhibition of PI 3-K by LY249002 or wortmannin and the inhibition of tyrosine phosphorylation by genistein interfered with stimulation of lipogenesis and glycogen synthase by both insulin and PIG compounds, in contrast to the blockade of the MAPK pathway by PD98059 and of both PKC and calmodulin-dependent kinase II by the (general PKC) inhibitor, rottlerin, which were both ineffective [80]. The increased sensitivity of the insulin effects towards genistein compared to PIG action was the first evidence for distinct tyrosine kinases involved in insulin and PIG signalling (e.g., for phosphorylation of IRS proteins). It may be speculated that PIG compounds bind to and/or activate a receptor/non-receptor tyrosine kinase distinct from the insulin receptor. Taken together, the insulin-receptor-independent stimulation of PI 3-K by PIG leads to insulin-like modulation of the activity of all the typical components along the metabolic insulin signalling cascade to the GLUT4 translocation apparatus (including the small G-protein, Rab4) so far characterized.

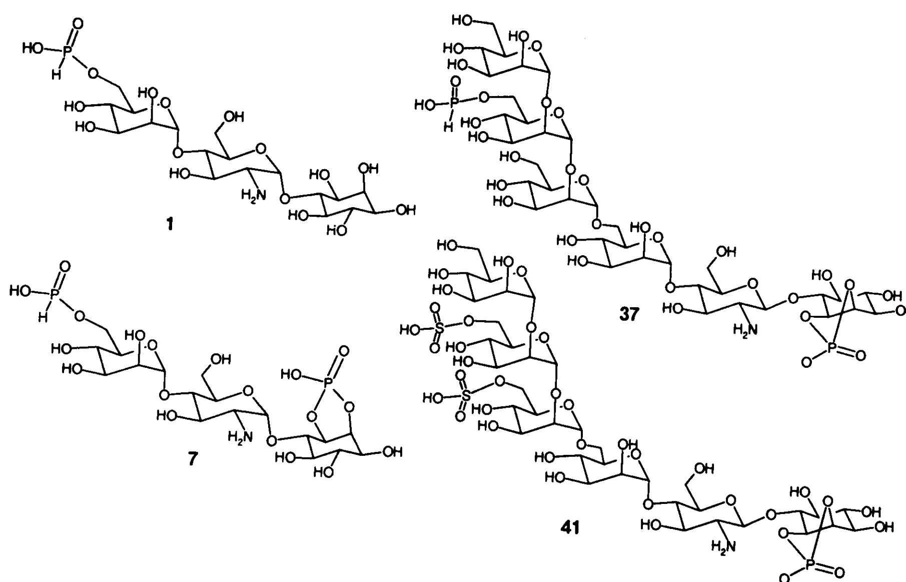

Figure 7. Structure of four chemically synthesized PIG molecules, PIG 1, 7, 37 and 41.

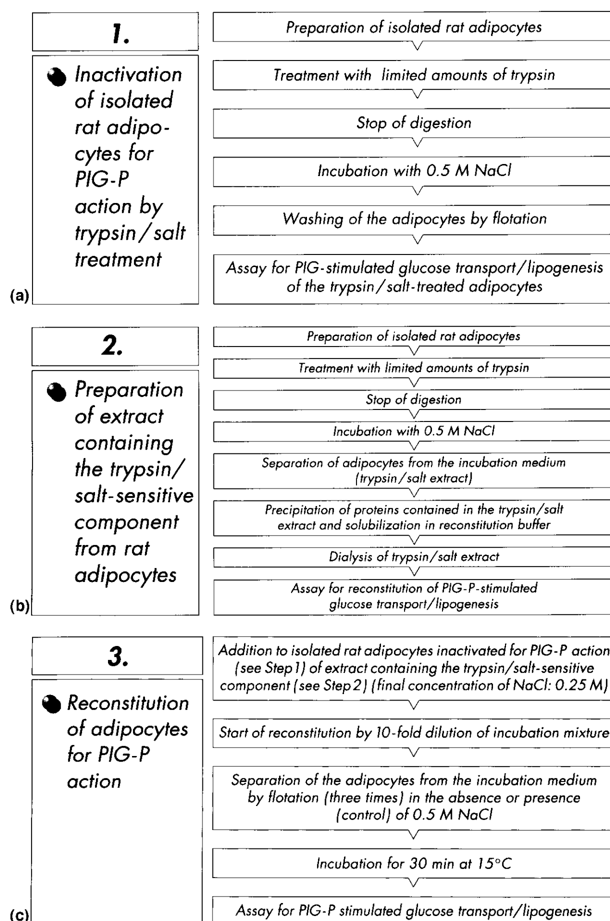

Figure 8. Scheme of the experimental protocols for (1) trypsin/salt treatment of isolated rat adipocytes for desensitization toward PIG(-P) compounds (*a*), (2) preparation of the trypsin/salt extract containing the trypsin/salt/NEM-sensitive component (*b*), and (3) reconstitution of the trypsin/salt-treated adipocytes with trypsin/salt extract for restoration of responsiveness/sensitivity toward PIG(-P) compounds (*c*). See text for details.

Divergence of PIG and insulin signalling

For a first characterization of the molecular mechanism of the apparent bypass of the insulin receptor by PIG, we studied whether the primary target of PIG is located intra- or extracellularly (see fig. 1) by establishing conditions for desensitization of isolated rat adipocytes toward PIG action using limited trypsin digestion (fig. 8a) [78]. Trypsin (up to 100 μg/ml) alone had no effect. However, NaCl washing of the adipocytes subsequent to the trypsin digestion almost completely abolished the PIG response. Obviously, the combined trypsin/salt treatment removed a proteinaceous component from the extracellular face of the plasma membrane of intact adipocytes which is required for PIG action. One possibility is that this component is anchored in the plasma membrane via a transmembrane domain and additional bipolar interactions between its extracellular domain and (phospholipids of) the plasma membrane.

To substantiate this hypothesis, we tried to restore the sensitivity of trypsin/salt-treated adipocytes toward PIG by addition of the trypsin/salt extract which may contain the extracellular domain of the component clipped off by the trypsin/salt treatment (fig. 8b,c). In fact, the addition of increasing volumes of trypsin/salt extract to the inactivated adipocytes caused a partial restoration of PIG-stimulated lipogenesis. Extract prepared by trypsin or salt treatment alone or boiled trypsin/salt extract completely failed to reconstitute PIG action in trypsin/salt-treated adipocytes. The concentration-response curve for stimulation of lipogenesis with reconstituted adipocytes closely resembled that with untreated adipocytes (50–60% of the maximal response, EC_{50} value about 2 μM). Interestingly, the reconstituted adipocytes could be inactivated again by salt washes only without the need for additional trypsin treatment. This can be explained by the binding of a component contained in the trypsin/salt extract to the cell surface of intact adipocytes by bipolar interactions, which is apparently sufficient for its functionality in the reconstitution reaction. The reconstitution activity of the component contained in the trypsin/salt extract could be completely abolished by replacement of trypsin by V8 protease during preparation of the extract as well as by treatment of the trypsin/salt extract prior to the reconstitution assay with heat, proteinase K or the SH-alkylating agent, *N*-ethylmaleimide (NEM). Furthermore, the component was not dialyzable. These data suggest that the component released from the adipocytes by trypsin/salt treatment and required for PIG action is a protein or a protein fragment. By analysis of the protein pattern of the trypsin/salt extract prepared from metabolically labelled rat adipocytes, we identified two polypeptides of 115 and 49 kDa which were released from the cell surface only by a combined trypsin/salt treatment but not by either treatment alone [78]. Only the 115-kDa polypetide could be labelled in vitro with ^{14}C-labelled NEM. Thus, the 115-kDa polypeptide is a candidate for the trypsin/salt/NEM-sensitive component of the adipocyte cell surface required for PIG action. Recent localization experiments strongly suggest that this protein is a constituent of detergent-insoluble glycolipid-enriched rafts (DIGs), which resist solubilization by 1% TX-100 (but not by 60 mM octyl glucoside) in the cold and can be purified by sucrose gradient centrifugation due to their low buoyant density [88, for a review see ref. 89]. Furthermore, the soluble (extracellular) tryptic fragment of the 115-kDa component per se binds to isolated DIGs in a salt-sensitive manner (S. Welte and G. Müller, unpublished data).

DIGs and caveolae as signalling compartments for PIG molecules

These DIGs may be structurally related to or even represent a subpopulation of caveolae [for reviews see refs 90–92]. Caveolae or plasmalemmal vesicles are small bulb/flask-shaped non-clathrin-coated invaginations or subcompartments/microdomains of the plasma membrane of about 50 nm diameter, with a very characteristic striated appearance. They are found in almost all types of cells, but are highly abundant in fibroblasts, adipocytes (20% of the total plasma membrane surface area), endothelial cells, epithelial cells, and smooth and skeletal muscle cells. They are coated at the cytosolic face with the 21-kDa membrane protein, caveolin or VIP21 [93, 94] which fulfils all the criteria required for classification as an integral membrane protein: it cannot be extracted from membranes by either high-salt or carbonate treatment and partitions into the detergent phase of the TX-114 extract.
The mammalian caveolin gene family consists of caveolin-1 and -2 (coexpressed in most cell types) and caveolin-3 (muscle specific). The amino acid sequence of caveolin-1 (178 residues) predicts no cleavable signal peptide but a single long stretch of 33 hydrophobic residues (102–134) which forms a loop within the membrane with both termini facing the cytosol. The morphological appearance of caveolae directly correlates with caveolin expression levels (e.g., during differentiation of 3T3-L1 fibroblasts into adipocytes [95]). The formation of caveolae may be promoted by the capacity of the caveolins to form homo- (caveolin-3) and hetero-oligomers (caveolin-1 and -2) by self-association via their amino-terminal and carboxy-terminal cytoplasmic domains [96, 97]. It is thought that the membrane-spanning domain of the caveolins forms a hairpin-like structure that does not traverse the lipid bilayer entirely. Such an arrangement would bring the amino- and carboxy-terminal regions near the membrane into close juxtaposition, forming a common caveolin-1-binding domain. In addition, the protein-protein interactions between the membrane-spanning domains of both caveolin-1 and -2 are involved in mediating their ability to interact with each other and in driving caveolae formation in vivo [96]. Each caveolin homo- or hetero-oligomer is linked side by side within caveolae membranes to its neighbors through multiple carboxy-terminal-carboxy-terminal interaction between the constituent caveolins [97; for a review see ref. 92].
Caveolae are thought to be involved in various cellular transport processes [transcytosis, potocytosis (receptor-mediated uptake of small molecules), endocytosis (alternative pathway to clathrin-coated vesicles), cholesterol flux, (constant flow of cholesterol/sphingolipid-enriched rafts from the endoplasmic reticulum via the Golgi apparatus to the plasma membrane for replenishment of HDL cholesterol), apical/basolateral membrane protein sorting] and in transmembrane signal transduction events (regulation of intracellular calcium concentration, generation, concentration and regulated release of messenger molecules such as inositol-trisphosphate (IP_3), ‘caveolae signalling hypothesis’; [for reviews see refs 98–101]). An impressive and growing body of biochemical and morphological evidence indicates that a variety of molecules that function directly or indirectly in signal transduction are enriched in caveolae. These data have given rise to the signalling hypothesis, in which compartmentation of certain signalling molecules to caveolae could allow efficient and rapid coupling of activated receptors to one or more effector systems. The local concentration, specificity, and efficiency of interaction of signal transduction components would be increased by their residence within the specialized lipid environment of caveolae. Methods that allow the isolation of low-buoyant-density, detergent-insoluble caveolae-like membranes from cultured cells revealed the cofractionation of (multiple) fatty acylated proteins. In addition to the ‘marker, coat or scaffolding’ protein, caveolin (three different tissue-specific isoforms 1–3 have been identified so far, with caveolin-1α and -1β exhibiting two different translation initiation sites and triple palmitoylation), the recently discovered flotillin, a predominant 45-kDa integral membrane protein [102], may play a role in caveolae biogenesis and structure. Coexpression of caveolins and flotillins in A498 kidney cells leads to the formation of a stable hetero-oligomeric caveolar complex between them, and heterologous expression of murine flotillin-1 in Sf21 insect cells is sufficient to drive the formation of caveolae-like vesicles [103].
Caveolae harbor a number of important signalling components, such as some protein kinase C isoforms, certain receptors (e.g., for IP_3), channels (e.g., for Ca^{2+}) and transporters (e.g., for folate, Ca^{2+}), and lipid-modified signalling molecules, such as small and heterotrimeric G-proteins as well as non-receptor tyrosine kinases of the Src family [98, 99]. Both myristoylation and palmitoylation are important for their correct caveolar localization. A sequence motif that serves as a predictor for a subset of (dually) acylated proteins is Met-Gly-Cys-X-X-Ser/Cys at the amino-terminus of a protein [for a review see ref. 104]. This motif is found in the G_i subfamily of G-protein α-subunits and in (the SH4 domain of) some non-receptor tyrosine kinases of the Src-related family kinases such as Hck, Lck, p59Lyn and p60Fyn, as well as in endothelial nitric oxide synthase (eNOS). In these proteins, the 14-carbon saturated fatty acid, myristate, is joined by a stable amide linkage to the amino-terminal glycine residue (following cleavage of the initiator methionine) and the adjacent cysteine is palmitoylated via a thioester bond. The two fatty acyl chains partition into the inner leaflet of the plasma membrane corresponding to the area of

caveolae and thereby participate in the formation of DIGs.

Interestingly, the carboxy-terminal domain of caveolin-1 also undergoes palmitoylation on three residues (see below). The close association with GPI proteins, caveolin, glycosphingolipids, glycolipids, and cholesterol enables the recovery of Src family kinases together with these components in DIGs [for a review see ref. 105]. The basic forces driving this raft formation are transient and weak lipid interactions leading to the Triton-insoluble microenvironment, termed a liquid-ordered phase [106–111]. DIGs possibly represent the biochemical equivalent of caveolar structures, although this remains a matter of controversy. Lisanti and coworkers recently suggested a true functional relationship between DIGs and mature caveolae, in that during the biogenesis of the latter, DIGs would need to exist as precursors for proper insertion of caveolins into membranes [100]. In fact, DIGs could be reconstituted in vitro by mixing cholesterol, glycosphingolipids, and phospholipids in the appropriate ratio [89], insertion of caveolin-1 into model lipid membranes required a high local concentration of cholesterol [112], and expression of caveolin-1 in cells lacking morphologically detectable caveolae and caveolin led to the formation of mature invaginated caveolae [113]. These findings indicate that cells synthesize all the components required for the formation of 'precaveolae' or DIGs, insertion of caveolin proteins into these biogenetic precursors of caveolae being only a late process which occurs in the trans-Golgi network.

In any case, the following lines of evidence hint at a role for caveolin, caveolae, and DIGs in mediating or modulating signalling events by insulin, PIG, and GPI molecules: (i) caveolae are present at high numbers in typical peripheral insulin target cells (e.g., skeletal muscle cells [114]), (ii) the expression level of caveolin directly correlates with the morphological appearance of caveolae during differentiation of 3T3-L1 fibroblasts into adipocytes which is accompanied by gain of insulin sensitivity [95], (iii) GLUT4 is associated with caveolin-enriched vesicles in 3T3-L1 adipocytes [95], (iv) GPI lipids are localized in specific plasma membrane structures, referred to as caveolae in 3T3-L1 adipocytes [115], (v) GLUT4 seems to be translocated to caveolae of the plasma membrane in response to insulin in 3T3-L1 adipocytes [95, 116] (which is, however, still a matter of dispute [117, 118]), (vi) the activated/autophosphorylated insulin receptor and other components of the insulin signalling cascade (IRS-1, Shc, Grb-2, Sos, SHP2, PI 3-K, MAPK) appear to be enriched in caveolin-enriched fractions of H35 hepatoma cells [119], (vii) in these cells, insulin-induced tyrosine phosphorylation of the insulin receptor and IRS-1 was more pronounced in caveolin-enriched than in caveolin-poor plasma membrane fractions [119], (viii) insulin, but not epidermal growth factor or platelet-derived growth factor, stimulates tyrosine phosphorylation of caveolin in 3T3-L1 adipocytes [120] and isolated rat adipocytes [121].

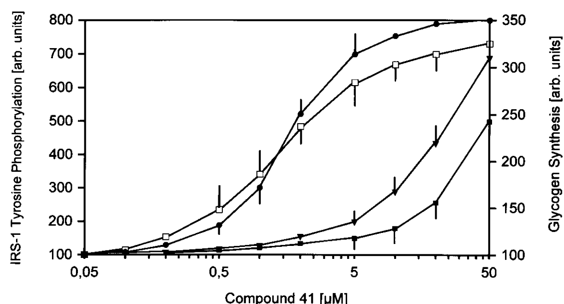

Figure 9. Involvement of p59Lyn kinase in insulin-mimetic signalling and action by PIG. Isolated rat adipocytes (prepared by collagenase digestion of epididymal fat pads from 140- to 160-g male rats) were electroporated in the presence of neutralizing anti-p59Lyn antibodies (triangles, filled squares) or non-immune control IgG (circles, open squares) and then incubated (30 min, 37 °C) with increasing concentrations of PIG 41. From portions of the total cellular defatted extract (prepared by homogenization in the presence of 1 mM sodium vanadate, 100 mM NaF, 25 mM pyrophosphate and protease inhibitors, subsequent solubilization in the presence of 60 mM octyl glucoside and centrifugation at 25,000 g for 20 min), IRS-1 was immunoprecipitated with anti-IRS-1 antibodies and then immunoblotted with anti-phosphotyrosine antibodies. Tyrosine phosphorylation of IRS-1 was detected by chemiluminescence, quantitated by phosphorimaging and corrected for the amount of IRS-1 by homologous immunoblotting (circles, triangles). Other portions of the total cellular extract were incubated (15 min, 30 °C) with UDP-[^{14}C]glucose in the presence of 0.1 mM glucose-6-phosphate, 0.5 mg/ml glycogen and 100 mM NaF. The amount of glycogen synthesized was determined by ethanol precipitation and liquid scintillation counting (open and filled squares) (mean ± SD, n = 5–7).

Involvement of non-receptor tyrosine kinases in PIG signalling

The marked and rapid induction of tyrosine phosphorylation of IRS-1 and of phosphorylation of additional proteins in response to PIG [77, 80] raises the question of the kinase(s) involved. A member of the Src family, p59Lyn kinase [for reviews see refs 122, 123], which is expressed in rat adipocytes, seems to play a prominent role in PIG signalling in rat adipocytes according to our recent findings [124]. Introduction of p59Lyn-neutralizing antibodies into isolated rat adipocytes by electroporation led to blockade of tyrosine phosphorylation of IRS-1 (assayed by immunoblotting with anti-phosphotyrosine antibodies of the anti-IRS-1 immunoprecipitates from total cell lysates of stimulated adipocytes) in response to PIG 41. This was revealed by the significant rightward shift and reduction in the maximal response of the concentration-response curve in the presence of the inhibitory antibodies versus non-immune IgG (fig.

9). The blockade of IRS-1 tyrosine phosphorylation was correlated with inhibition of PIG-41-driven glycogen synthesis activation, a typical metabolic insulin effect (as assayed by the incorporation of radiolabelled glucose into ethanol-precipitable glycogen), by the anti-p59Lyn antibodies. The involvement of Lyn kinase in PIG signalling was further strengthened by the finding that incubation of rat adipocytes with PIG compounds increased Lyn kinase activity [124]. Caveolin was also tyrosine phosphorylated upon exposure of intact adipocytes to insulin or PIG compounds. In this experiment, the adipocytes were incubated with insulin or PIG-P in the presence of radiolabelled phosphate. Caveolin immunoprecipitated from DIGs and then immunoblotted with anti-phosphotyrosine antibodies showed concentration-dependent tyrosine phosphorylation in response to insulin and PIG [121]. This result supports the involvement of caveolin (phosphorylation) in signal transduction via PIGs. Tyrosine phosphorylation of caveolin had also been observed previously after incubation in vitro of DIGs isolated from insulin-stimulated adipocytes with Mg^{2+}ATP [120]. Since insulin receptors could not be detected in these complexes, caveolin must have been phosphorylated by a non-receptor tyrosine kinase associated with the complexes. One kinase identified was p60Fyn, the homolog of rat adipocyte p59Lyn in 3T3-L1 adipocytes [123]. Remarkably, in an effort to identify novel IRS-1-binding proteins by screening a mouse embryo expression library with recombinant [^{32}P]IRS-1, a specific association between p60Fyn and IRS-1 was detected [125]. By comparison with the insulin receptor, p60Fyn phosphorylated a unique cohort of tyrosine residues in IRS-1. Recently, a GPI anchor alone was shown in macrophages to induce a rapid onset of tyrosine phosphorylation of proteins, which may be involved in regulating the expression of interleukin-1α and TNF-α, among them polypeptides of the size of caveolin [126]. Furthermore, it may be speculated that certain receptor tyrosine kinases, cytokine receptors, and G-protein-coupled receptors mediate tyrosine phosphorylation of IRS-1 in some cell types (see above) by coupling directly or indirectly to the trypsin/salt/NEM-sensitive component, thereby activating the same acylated non-receptor tyrosine kinase(s) as used by the PIG, i.e., presumably p59Lyn.

G-protein-coupled receptors would be particularly appropriate for specific binding of PIG based on the remarkably diverse array of stimuli identified so far. It has been demonstrated that stimulation of many heptahelical receptors can lead to the activation of MAP kinases which in many cases depends on activation of both G-proteins and tyrosine kinases of the Src family [127]. The most recent findings reveal that Src associates in cells with agonist-activated β_2-adrenergic receptors, as assessed by immunofluorescence and coimmunoprecipitation [128]. The recruitment of cellular Src to β_2-adrenergic receptors is potentiated by overexpression of β-arrestin, and in vitro fusion protein pull-down studies revealed a direct high-affinity association of Src and β-arrestin. β-Arrestin-mediated association of Src with β_2-adrenergic receptors is a key step in mitogenic signalling by these receptors, since inhibition of the binding of β-arrestin to either the β_2-adrenergic receptor or Src attenuates β_2-adrenergic receptor activation of MAPK. These results indicate that the association of arrestins with heptahelical receptors induces a switch in receptor signalling from classical second-messenger-generating G-protein-mediated pathways to other pathways such as those involving kinases of the Src family. In this context, β-arrestin can act as an adaptor protein to recruit Src into a signalling complex organized around the β_2-adrenergic receptor. Although completely speculative at present, G-protein-coupled receptors and arrestins thus represent candidate components of the PIG signalling cascade.

The focal adhesion kinase, p125^{FAK} is a cytosolic tyrosine kinase, which upon engagement of integrins, transmembrane proteins that play an important role in adhesion between cells and extracellular matrix, becomes tyrosine-phosphorylated and binds to Src. Coexpression of IRS-1 in 293 cells together with p125^{FAK} led to their association, IRS-1 tyrosine phosphorylation, interaction of IRS-1 with SH2-domain-containing signalling proteins such as Src and PI 3-K, and PI 3-K activation [129]. Thus p125^{FAK} seems to act as a signalling platform for IRS-1 and non-receptor tyrosine kinases of the Src class. Consequently, we studied p125^{FAK} involvement in the PIG signalling pathway leading to Lyn-mediated IRS-1 tyrosine phosphorylation [130]. Clustering of β1-integrin by fibronectin plus non-blocking monoclonal anti-β1 antibodies led to activation of p125^{FAK} in isolated rat and non-adherent 3T3-L1 adipocytes but inhibited IRS-1 tyrosine phosphorylation and glucose transport activation by 20 μM PIG by about 70 ± 14 and $55 \pm 10\%$, respectively, compared to incubation of the cells with poly-L-lysine plus anti-β3 antibodies (19 ± 7 and $11 \pm 5\%$, respectively). Treatment of isolated rat adipocytes with PIG (0.1–20 μM) concentration dependently induced tyrosine phosphorylation of p125^{FAK} to up to 4.5 ± 0.8-fold and of paxillin to up to 6.8 ± 1.3-fold, as well as association of IRS-1 with p125^{FAK} to up to 3.2 ± 0.5-fold over basal. In adherent 3T3-L1 adipocytes, these PIG effects were considerably diminished. Introduction of a synthetic peptide containing two twin tyrosine residues, Tyr-576 and Tyr-577, which are localized in the regulatory loop of the FAK kinase domain and can be phosphorylated by the insulin receptor and Src kinases in vivo and in vitro, into isolated rat adipocytes by electroporation

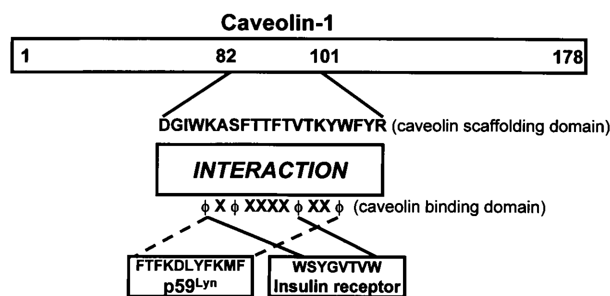

Figure 10. Structures of the caveolin-scaffolding domain as well as its position within caveolin-1, and of the caveolin-binding domains of two signalling components, p59Lyn and the insulin receptor. ϕ represents an aromatic amino acid (Trp, Tyr, or Phe). See text for details.

inhibited tyrosine phosphorylation of both p125^{FAK} and IRS-1 in response to PIG by up to 60 ± 19 and 72 ± 13%, respectively. This was correlated with blockade of glucose transport and lipogenesis by up to 45 ± 14 and 58 ± 8%, respectively, compared to a non-related control peptide, but did not affect the PIG-stimulated association of p125^{FAK} and IRS-1. In contrast, a peptide corresponding to the FAK major autophosphorylation and SH2 docking site, Tyr-397, did not impair PIG-induced pp125^{FAK} and paxillin tyrosine phosphorylation but reduced IRS-1 tyrosine phosphorylation and lipogenesis (to up to 1.9 ± 0.5-fold over basal and 24 ± 8% of the maximal insulin effect) upon introduction into adipocytes [130].

Previous studies have suggested that the recruitment of the adaptor protein Shc by activated β1 and αv is indirect and possibly mediated by caveolin. Interestingly, in WI-38 fibroblasts, a TX-100-soluble fraction of caveolin-1 physically and functionally links the integrin α-subunit to p60Fyn in vitro and in vivo [131]. Upon integrin ligation, p60Fyn is activated and binds via its SH3 domain to Shc. This sequence of events is necessary to couple integrins to the Ras-ERK pathway and promote cell cycle progression. It is now clear that integrins activate common as well as subgroup-specific signalling pathway (e.g., anchorage-dependent cell growth) [for a review see ref. 132]. In 293 cells, integrin engagement induces IRS-1 tyrosine phosphorylation [129]. This opens the possibility that both β1-integrin clustering and PIG action induce tyrosine phosphorylation of IRS-1 and initiate downstream metabolic insulin-mimetic signalling by recruiting IRS-1 and a non-receptor tyrosine kinase, possibly p60Fyn, via the alternative but not mutually exclusive signalling platform molecules, pp125^{FAK} and caveolin. This implies similar mechanisms of cross-talk of extracellular matrix proteins and of extracellular PIG (and potentially GPI) molecules with (metabolic) insulin signalling based on the integrin system and dependent on cell architecture.

Caveolin and the modulation of signalling pathways

Lisanti and coworkers put forward the hypothesis that caveolae may function as a type of signalling organelle or compartment based on the concentration of certain (inactive, but not mutationally activated versions of) signalling proteins in this specialized area of the plasma membrane, including G-protein α-subunits, Ha-Ras, Src family tyrosine kinases, eNOS, certain receptor tyrosine kinases, and protein kinase C isoforms, where they may be kept in an off state [99, 100]. Using truncated versions of caveolin, they identified a discrete region, the so-called caveolin scaffolding domain (residues 82–101) which interacts with high specificity with G-protein α-subunits, H-Ras, and the kinase domains of many distinct families of tyrosine and serine/threonine protein kinases (Src family kinases, PKCα, MAPK, epidermal growth factor receptor, insulin receptor, platelet-derived growth factor receptor) [133–135]. The corresponding caveolin-binding domain responsible for binding of caveolin to the various signalling proteins could be identified by means of a phage display library for random peptides and the caveolin-scaffolding domain peptide as the receptor [136] (fig. 10). It is this interaction between the caveolin-scaffolding domain and the caveolin-binding domain which may keep some signalling proteins in the inactive basal state, competent however, for future activation. This may represent a general mechanism for caveolin-mediated sequestration and inactivation of a diverse group of signalling molecules within caveolae membranes for regulated activation by receptor ligands [for a review see ref. 100]. For example, the caveolin-scaffolding domain peptide derived from caveolin-1 suppressed the GTPase activity of the G-protein α-subunit and so held the trimeric G-protein in an inactive state (analogous to the effects of GDP-dissociation inhibitor proteins of small G proteins; GDIs). This contrasted with the effects of the corresponding scaffolding domain peptide of caveolin-2 which stimulated the GTPase activity of the α-subunit G_o and could therefore act as a GTPaseactivating protein (GAP). The analogous scaffolding-domain peptide of caveolin-3 suppressed or stimulated GTPase activity depending on the peptide concentration, and this was interpreted as evidence for both GAP and GDI activity [133, 134]. Thus caveolin-3 may interact with both GTP-bound and GDP-bound forms of α-subunits. However, both molecular mechanisms of stimulating the GDI or GAP activities actively hold or lead to the G α-subunit in the GDP-loaded inactive state. By analogy, interaction of the caveolin-scaffolding domain with the caveolin-binding domains

located within the kinase domains of PKC and the epidermal growth factor receptor inhibits their kinase activity. In all cases examined, the caveolin-binding domain is located within the enzymatically active catalytic domain of a given signalling molecule. For example, in the case of tyrosine and serine/threonine kinases, a kinase domain consists of 11 conserved subdomains (I–XI) [137, 138]. The caveolin-binding domain is located within conserved kinase subdomain number IX, suggesting that caveolin could function as a 'general kinase inhibitor' [100]. This hypothesis has been substantiated by the observation that the caveolin-scaffolding domain inhibits Src family tyrosine kinases (c-Src/Fyn), epidermal growth factor receptor, Neu and PKC with similar potencies [139].

In contrast, other signalling proteins, like the insulin receptor, become activated by interaction of their caveolin-binding domain with the caveolin-scaffolding domain of caveolin [140]. Overexpression of caveolin-3 enhanced insulin-induced tyrosine phosphorylation of IRS-1 in 293T cells without an accompanying effect on the phosphorylation state of the insulin receptor. Synthetic caveolin-1/3-scaffolding domain peptide increased phosphorylation of recombinant IRS-1 or a Src-derived peptide by the purified insulin receptor up to 17-fold, whereas the caveolin-2-derived peptide was ineffective. The (functional) interaction between the insulin receptor and caveolin was also reflected in binding of the receptor to immobilized caveolin peptides which could be competed for by excess caveolin-3-scaffolding domain peptide [140]. As a molecular mechanism, it is assumed that the putative caveolin-binding domain of the insulin receptor (1175–1192) upon occupancy by the caveolin scaffolding domain stabilizes the activated conformation by binding to the autoinhibitory kinase regulatory loop located nearby, preventing it from sitting over and blocking the kinase domain and thereby enabling substrate access to the catalytic site and kinase activity. Taking all the experimental evidence together, caveolin may selectively (in a subtype-specific fashion) regulate the function of signalling molecules accumulated in caveolae by either increasing or suppressing their enzymatic activity or capability for protein interacting with additional proteins.

The caveolin-scaffolding domain (82–101) overlaps with a region of caveolin (61–101) required for its homo-oligomerization into 300- to 400-kDa complexes; the functional significance of this close spatial relationship remains unclear [96, 97, 134–136]. Interestingly, recent studies with expression of fusion proteins consisting of the wild-type or mutated amino-terminus of $G_{i1\alpha}$ and the green fluorescence reporter protein as well as expression of caveolin-1 lacking the carboxy-terminal palmitoylation sites in COS-7 cells revealed that dual acylation of the amino-terminus of $G_{i1\alpha}$ is necessary and sufficient and palmitoylation of caveolin-1 required for efficient targeting of the fusion protein to caveolin-enriched plasma membrane domains [141]. Remarkably, the caveolin-binding domain of $G_{i1\alpha}$ fused to the amino-terminus of the reporter protein did not function as a targeting signal for caveolae in COS-7 cells. The authors favor a model for caveolar targeting of $G_{i1\alpha}$ in which during a first step, the amino-terminal myristic and palmitic acids of signalling proteins initiate binding to the plasma membrane bilayer and to caveolin-1 oligomers, the carboxy-terminal palmitic acid residues of which directly interact with the fatty acyl chains of the signalling protein. In a second step, the caveolin-binding domain of the signalling protein interacts with the scaffolding domain of caveolin-1, stabilizing the mutual interaction, increasing its specificity, and modulating the activity state of the signalling protein [141]. Recent findings provide compelling evidence that the caveolin-binding domain is relevant and functional in vivo. Sessa and colleagues have performed site-directed mutagenesis to modify the predicted caveolin-binding domain (from $\underline{F}$SAAP$\underline{F}$SG$\underline{W}$ to $\underline{A}$SAAP$\underline{A}$SG$\underline{A}$) within eNOS [133]. It is known from in vitro studies that aromatic residues (W, F, or Y) are required for proper recognition of the caveolin-scaffolding domain. In their work, they show that mutation of the caveolin-binding domain within eNOS blocks the ability of caveolin-1 to inhibit eNOS activity in vivo [142]. The functional role of caveolins in regulating signalling along the MAPK cascade was examined by the Lisanti group [143]. Coexpression with caveolin-1 dramatically inhibited signalling from the epidermal growth factor receptor to the nucleus in vivo. Using a variety of caveolin-1 deletion mutants, they mapped this in vivo inhibitory activity to caveolin-1 residues 32–95, thus overlapping with the caveolin-scaffolding domain as defined by inhibiting the kinase activity of purified extracellular signal regulated kinase-2 (ERK-2) as well as MAPK- and ERK-kinase-1 (MEK-1) in vitro [143]. Thus caveolin-1 can inhibit signal transduction by the p42/p44 MAPK cascade both in vivo and in vitro by acting as a natural inhibitor of both MEK and ERK.

We have now obtained evidence that the introduction of an excess of synthetic caveolin-scaffolding domain peptide (see fig. 10) into isolated rat adipocytes by electroporation causes the dissociation of Lyn kinase from caveolin as determined by the concentration-dependent reduction in the amount of Lyn kinase coimmunoprecipitated with anti-caveolin antibodies [144, 145]. Lyn kinase was detected by homologous immunoblotting and evaluated by phosphorimaging (fig. 11, left panel). This interference with the Lyn kinase-caveolin interaction correlated perfectly with inhibition of glucose transport activation by PIG 41 (2 μM) in the presence of increasing amounts of intracellular caveolin-scaffolding domain peptide. Furthermore, PIG 41

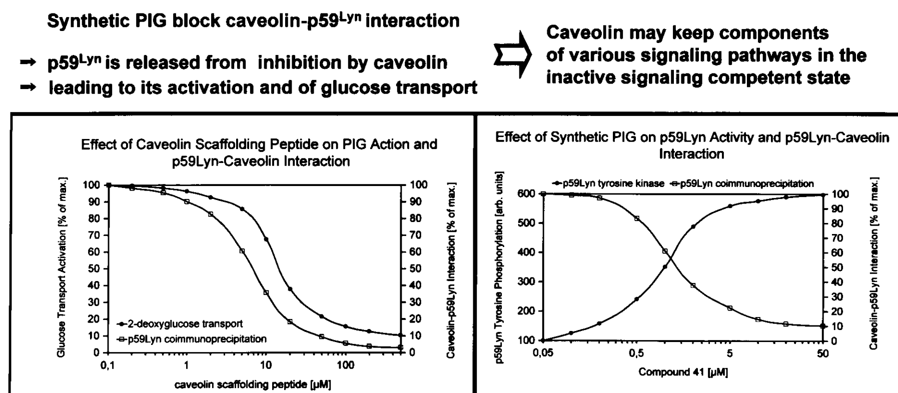

Figure 11. Interference of the Lyn-caveolin interaction by caveolin-scaffolding domain peptide and PIG. Left panel, isolated rat adipocytes were electroporated in the presence of increasing amounts of caveolin-scaffolding domain peptide (see fig. 10; dissolved in DMSO, final concentration 2% which did not affect cell viability). Portions of the cells were used for measurement of cytochalasin-B-inhibitable 2-deoxyglucose transport using the oil centrifugation method (circles). From other portions, total cellular defatted extracts were prepared, solubilized with 60 mM octyl glucoside and used for immunoprecipitation with anti-caveolin antibodies. p59Lyn was demonstrated in the anti-caveolin immunoprecipitates by immunoblotting with anti-p59Lyn antibodies and chemiluminescence detection followed by phosphorimaging (squares). The values were corrected for the amount of immunoprecipitated caveolin by homologous immunoblotting ($n = 3-5$). Right panel, isolated rat adipocytes were incubated (15 min, 37 °C) with increasing concentrations of PIG 41. From portions of the total cellular defatted extract, p59Lyn was immunoprecipitated with anti-p59Lyn antibodies. Autophosphorylated Lyn was demonstrated by immmunoblotting of the immunoprecipitates with anti-phosphotyrosine antibodies, chemiluminescence detection, and phosphorimaging (circles). The values were corrected for the amount of p59Lyn by homologous immunoblotting. Other portions of the extract were used for coimmunoprecipitation of p59Lyn with anti-caveolin antibodies and subsequent immunoblotting for p59Lyn (see left panel, squares) ($n = 4-5$).

itself triggered the dissociation of Lyn kinase from caveolin in a concentration-dependent manner, again assayed as failure to coimmunoprecipitate Lyn kinase with anti-caveolin antibodies (fig. 11, right panel). This was inversely correlated to Lyn kinase activation/autophosphorylation by PIG 41. PIG 37 exhibited lower activity, while PIG 1 was inactive, thus reflecting the same ranking order between structurally different PIG molecules in efficiency of glucose transport activation, dissociation of Lyn from caveolin, and Lyn kinase activation. The causal relationship between the Lyn kinase-caveolin interaction, Lyn kinase activation, and metabolic signalling was highlighted by the introduction of an excess of the counterpart of the caveolin-scaffolding domain peptide, the caveolin-binding domain peptide of Lyn kinase (see fig. 10) into rat adipocytes by electroporation (fig. 12). As observed for the scaffolding domain peptide (see above), the caveolin-binding domain peptide triggered the dissociation of Lyn from caveolin as assayed as loss of Lyn protein (detected by homologous immunoblotting) from anti-caveolin immunoprecipitates which had been prepared from the isolated DIGs (fig. 12). However, in contrast to the inhibitory action of the scaffolding domain peptide, the caveolin-binding domain peptide induced a concentration-dependent activation of both Lyn kinase measured in vivo as autophosphorylation (fig. 12) as well as tyrosine phosphorylation of IRS-1 and glucose transport [144, 145]. We assume that PIG compounds induce liberation of Lyn protein from binding to caveolin, thereby relieving Lyn kinase from inhibition by caveolin. Activated Lyn kinase phosphorylates IRS proteins and other signalling proteins, like caveolin, at tyrosine residues, thereby initiating metabolic signalling.

Recently, another example for the reversible interaction/inhibition of a signalling protein with/by caveolin in response to an external stimulus has been described in 3Y1 rat fibroblasts [146]. Phospholipase D1 (PLD1) was demonstrated to interact with caveolin via the caveolin-scaffolding domain. Excess of the corresponding peptide blocked basal and PKCα-stimulated PLD1 activity. Activation of PKCα by phorbolester caused its translocation to caveolae, displacement of caveolin from PLD1, interaction of PKCα with PLD1, and its stimulation through phosphorylation. Thus phorbolester apparently induces release of caveolin from PLD1 enabling its interaction with and activation by PKCα, just as PIGs promote liberation of caveolin from Lyn leading to its direct activation. As far as we know, phorbolester and PIG compounds represent the first small signalling factors regulating (in a negative fashion) the interaction between caveolin and signalling proteins of the corresponding signalling pathways.

Phosphorylation of caveolin (at tyrosine as observed after treatment of insulin-sensitive cells with insulin, sulfonylureas, PI-specific phospholipases, as well as PIG compounds [121], and during oncogenic transformation of NIH3T3 cells with Rous sarcoma virus [147] or

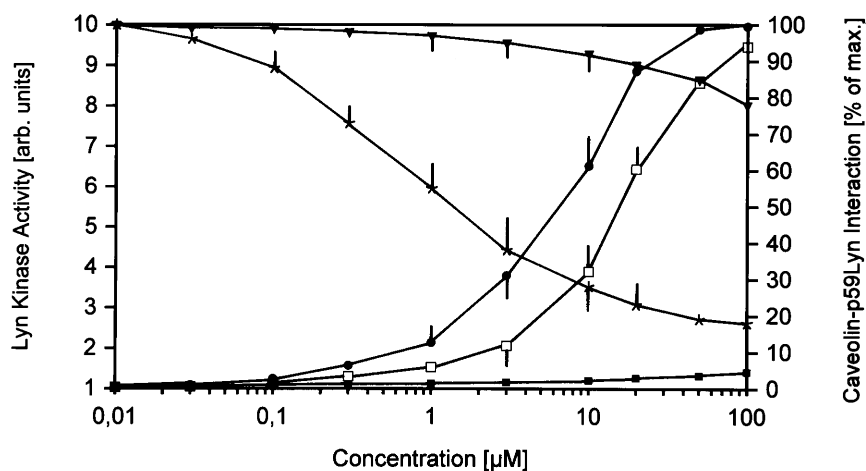

Figure 12. Mimicking of PIG effects on Lyn-caveolin interaction and Lyn kinase activity by caveolin-binding domain peptide in rat adipocytes. Isolated rat adipocytes were electroporated in the presence of increasing concentrations of the authentic caveolin-binding domain peptide (see fig. 10; dissolved in DMSO, final concentration 1%) derived from p59Lyn (circles, stars), a truncated version lacking the amino-terminal three amino acids (open squares), and a mutant version with serine residues replacing the three aromatic amino acids (closed squares, triangles). Portions of the total cellular defatted extracts were used to determine Lyn-caveolin interaction (stars, triangles; see fig. 11, left panel) and Lyn kinase activity measured as Lyn autophosphorylation (circles, open and closed squares; see fig. 11, right panel). The total amount of p59Lyn coimmunoprecipitated with anti-caveolin antibodies was set at 100%. Each value was corrected for the amount of caveolin by homologous immunoblotting (mean $\pm$ SD, n = 4–9).

additionally/alternatively at serine as may occur during phorbolester treatment in rat fibroblasts) could trigger the release of interacting signalling proteins from caveolin and, thereby, their activation. This will initiate the corresponding signalling pathways (i.e., IRS-1 tyrosine phosphorylation, MAPK and PLD1 activation, respectively). However, experimental elucidation of the role of caveolin (tyrosine) phosphorylation has yet to be tackled. Furthermore, it is now important that this novel concept for the regulation of caveolin signalling activity demonstrate its operation for physiological stimuli, such as growth factors and hormones.

Redistribution of caveolar/DIG components during PIG signalling

The PIG-induced release of Lyn kinase from interaction with caveolin, a resident protein of DIGs/caveolae, seems to result in redistribution of Lyn from DIGs into other areas of the adipocyte plasma membrane [144, 145]. This was demonstrated by preparing DIGs from isolated rat adipocytes which had been exposed to PIG compounds. Appropriate low-buoyant-density fractions obtained by sucrose gradient centrifugation of DIGs were assayed for the amounts of Lyn kinase and caveolin by sequential homologous immunoprecipitation and immunoblotting and quantitative evaluation by phosphorimaging (fig. 13). PIG 41 and 37 but not 1 caused considerable reductions in the amount of Lyn protein relative to caveolin recovered with the DIGs (fig. 13). Thus Lyn was depleted from DIGs relative to caveolin, but was enriched in heavy-buoyant-density fractions of the plasma membrane.

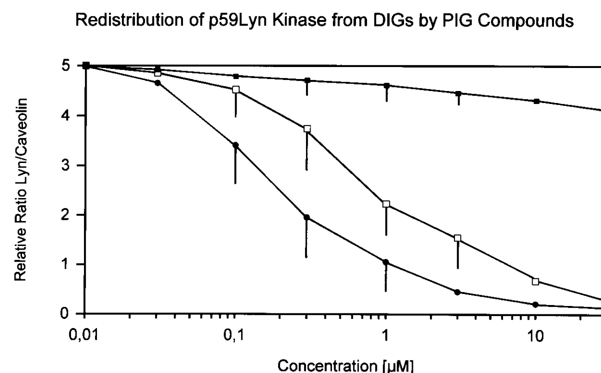

Figure 13. PIG-induced redistribution of p59Lyn from DIGs in rat adipocytes. Isolated rat adipocytes were incubated (15 min, 37 °C) with increasing concentrations of PIG 41 (circles), 37 (open squares), or 1 (closed squares). DIGs were prepared by treatment with 1% TX-100 at 4 °C and purified by sucrose gradient centrifugation according to published procedures. The proteins contained in appropriate low-buoyant-density fractions were sequentially immunoprecipitated and immunoblotted with corresponding homologous antibodies for caveolin and p59Lyn. The relative ratio between caveolin and p59Lyn in the absence of compound was set at 5 (mean $\pm$ SD, n = 5–8).

The PIG-induced redistribution of DIG constituents into non-caveolar areas of the adipocyte plasma membrane was also observed using the same methods for a typical GPI protein and a signalling protein. It is interesting to note that in many cell types, including insulin-sensitive ones, a variety of GPI proteins, such as Thy-1 in lymphocytes and T cells [106–111, 148], and the bulk of GPI lipids are concentrated within DIGs/caveolae [for a review see ref. 105]. For example, in immune complex kinase assays, antibodies directed against Thy-1 recovered tyrosine protein kinase activity from mouse thymocytes, and this was shown to rely on p60Fyn associated with the immune complexes or low-buoyant-density DIGs [148]. The GPI protein, Gce1, a fraction of which colocalizes with DIGs from rat adipocytes, was reduced in amount in DIGs relative to caveolin upon stimulation with PIG 41 and 37 but not 1. The same was true for the adipocyte-specific IRS-3, a small fraction of which can be recovered with DIGs from unstimulated isolated rat adipocytes [141]. PIG 41 and 37 induced a significant decline in the amount of immunoblotted IRS-3 in anti-IRS-3 immunoprecipitates from total cell lysates prepared from stimulated cells [145]. Compatible with our findings of localization of signalling proteins such as Lyn kinase and IRS-3 in DIGs/caveolae in the basal state only, but of their redistribution in PIG-stimulated cells is the previous demonstration of recruitment to caveolar membranes of the inactive or basally active versions of G-proteins and non-receptor tyrosine kinases exclusively. Inactive H-Ras preferentially interacted with caveolin both in vitro and in vivo, whereas the mutationally activated H-Ras^{G12V} failed to form a stable complex with caveolin via binding to the caveolin-scaffolding domain [135]. Similarly, the caveolin-scaffolding domain has been observed to stably interact with c-Src but not with the constitutively active v-Src [134]. Furthermore, it was found that 15–20% of wild-type (inactive) $G_{s\alpha}$ copurifies (by coimmunoprecipitation) with caveolin from Madin Darby canine kidney cells resulting in a specific 20- to 40-fold enrichment with caveolar membranes relative to plasma membranes, whereas the constitutively active mutant $G_{s\alpha}^{Q227L}$ is quantitatively excluded from caveolae-rich fractions [133, 134]. Components of the MAPK pathway provide further evidence for the concentration in and physiological release from caveolae of signalling proteins. The structural and functional integrity of caveolae depends critically on the cholesterol-to-protein ratio of the caveolae fraction which is four to five times higher than the surrounding plasma membrane [149]. The maintenance of this level of cholesterol in the caveolae fraction of fibroblasts and transformed lymphocytes appears to rely on the functional caveolin-1 shuttle [149]. Experimental lowering of the cholesterol level in the caveolar fraction disrupts the molecular organization of the domain and inhibits internalization of both molecular [150] and particulate material by potocytosis [151]. Treatment of cells with cholesterol-binding drugs such as filipin appears to have a similar effect [152]. Acute deprivation of cholesterol from caveolae by incubating Rat-1 cells in the presence of either cyclodextrin or progesterone caused a rapid decrease in the number of caveolae and in parallel in the amount of several key elements of the MAPK pathway, including Ras, Grb-2 and MAPK (p42) associated with the plasma membrane [153]. This was accompanied by an increase in the amount of activated p42 in the cytoplasm and a twofold stimulation of DNA synthesis in response to epidermal growth factor. Cholesterol depletion alone triggered MAPK activation and mitogenesis [153]. This strongly suggests that one of the main functions of caveolin is the sequestration of inactive signalling molecules, like G-proteins, Src family tyrosine kinases, and MAPK, until they are forced to relay a message in response to an exogenous stimulus, like PIG, transmitted to caveolin by the corresponding (PIG) signalling cascade. Thus, release of these signalling components from caveolae by (PIG-)signal-induced dissociation of the interaction between their caveolin-binding domains and the caveolin-scaffolding domain or by transient destabilization/disassembly of caveolae relieves the signalling components from their basal off-state. Certainly, these two mechanisms of redistribution of caveolar components are not mutually exclusive but may be causally related, i.e., the (PIG) signal-induced abrogation of the interaction between caveolin and Lyn/Gce1/IRS-3 and possibly other caveolar constituent components may lead to reversible reorganization or even transient disappearance of caveolae and, vice versa, in cholesterol-depleted (remnant) caveolar structures caveolin may fail to interact with the signalling proteins.

Recent experiments have provided some evidence for regulated release of signalling components from caveolae and the underlying molecular mechanism. (i) Double immunofluorescence studies of cells transfected to express wild-type eNOS or the non-palmitoylated mutant reveal colocalization of the wild-type eNOS, but not the mutant, with endogenous caveolin [142]. These data imply that when an agonist triggers activation of eNOS (or a G-protein), the ensuing depalmitoylation may allow the deacylated protein to either partition out of caveolae to the noncaveolar plasma membrane or be released into the cytoplasm. (ii) Consistent with this model, Lisanti and coworkers found that mutational or pharmacological activation of $G_{S\alpha}$ prevented its cofractionation with caveolin [133]. (iii) Under certain conditions, pharmacological or mutational activation of G-proteins can result in the release of G_α-subunits from membranes [154]. Observed release of α-subunits is slow and has been correlated with the absence of palmitate

[155]. These findings support a role for palmitoylation/depalmitoylation as mechanisms for directing/releasing signalling components to/from caveolar-like membranes. Although depalmitoylation and release from the membrane is an attractive hypothesis, a model is preferred in which a cycle of acylation and deacylation may regulate the lateral translocation of signalling proteins between (caveolar and non-caveolar) subdomains of the plasma membrane. (iv) Quantitative double-immunogold labelling of MA104 cells and fibroblasts with caveolin-specific antibody and α_i- or β-subunit-reactive antibodies led to colabelling of caveolae [156]. Since the distribution of α_i was clearly not restricted to caveolae, it seems likely that α-subunits may partition into and out of these membrane specializations. Dynamic palmitoylation is an attractive means by which such redistribution among membrane compartments could be regulated. (v) Shenoy-Scaria et al. [108, 157] have recently shown that the partitioning of $p56^{lck}$ into DIGs depends on Cys-3 (and presumably palmitoylation). The importance of Cys-3 in the distribution of the kinases is underscored by a mutation in $p60^{src}$ that causes a gain of function. Replacement of Ser-3 in wild-type $p60^{src}$ with Cys allowed the protein to incorporate radiolabelled palmitate, to interact with a GPI protein, and to partition into DIGs (unlike the unpalmitoylated wild-type protein) [158]. It was concluded that the partitioning of palmitoylated Src-related kinases into DIGs represents a distribution of these proteins to caveolae. The dual acylation and the DIG fractionation similarities between α_i and the kinases led the authors to propose that dynamic palmitoylation of α-subunits may regulate α distribution into and out of caveolae. (vi) Recently, Anderson and colleagues have shown that the pool of ERK-1 that localizes to caveolae is initially inactive and can be activated by regulated stimulation with growth factor ligands, such as platelet-derived growth factor [159]. After such stimulation, ERK-1 is activated and translocates from caveolae membranes to the cytosol, suggesting that ERK-1 is activated as it leaves the caveolae membranes [159]. These results are consistent with the findings that down-regulation of caveolin-1 expression constitutively activates signalling from MEK and ERK in vivo, perhaps by prematurely releasing activated ERK-1/2 and other components of the p42/44 MAP kinase cascade into the cytosol so that they may accumulate within the nucleus.

In light of these findings, the PIG-induced redistribution of Gce1, Lyn, and IRS-3 may be based on (or on several of) the following molecular mechanisms: (i) lipolytic removal of the GPI anchor of Gce1 which, however, was not observed in recent experiments (S. Welte and G. Müller, unpublished data) compatible with cofractionation of amphiphilic Gce1 with non-caveolar plasma membranes but not with the cytoplasm (see above); (ii) depalmitoylation of Lyn, in addition to the demonstrated abrogation of the interaction between Lyn and caveolin, which remains to be studied; (iii) release of IRS-3 from interaction (via its amino-terminal phosphotyrosine-binding and/or pleckstrin homology domains) with constituent components of caveolae, i.e., the insulin receptor (the accumulation of which has been shown in caveolae [119]) and/or phosphatidylinositol, respectively. Alternatively, a straightforward mechanism for the coordinated redistribution of the three proteins from caveolae, which are directed to caveolar membranes by different modes (glypiation, fatty acylation, protein-protein/lipid interaction), may rely on profound structural rearrangement of caveolae/DIGs in response to PIG. We are currently tackling this possibility using both morphological and biochemical criteria.

A working model for PIG signalling via caveolin

We propose the following working hypothesis for metabolic signalling by PIG molecules and their cross-talk to the insulin signalling pathway (fig. 14). PIG binds to the trypsin/salt/NEM-sensitive component of the adipocyte cell surface which is a constituent of DIGs/caveolae and is possibly identical to the 115-kDa polypeptide (see above). This component has a transmembrane domain which can be clipped off by trypsin and an extracellular domain which interacts via salt bridges with another transmembrane protein. This interaction, which might guarantee peripheral association of the extracellular domain of the component with the cell surface upon trypsin cleavage of its membrane anchor, seems to be sufficient for mediating PIG action and may form the molecular basis for reconstitution of PIG activity in trypsin/salt-treated adipocytes upon addition of the trypsin/salt extract. Whether the extracellular domain of this component itself functions as the receptor for the PIG or the glycan core of GPI lipids (as assumed in the figure) or whether it receives the 'PIG signal' from a distinct binding protein remains to be elucidated. In any case, the PIG signal is transmitted from this component via interaction with a transmembrane protein, called the 'bridge' protein. Integrins represent candidate proteins for this function although only about 2% of total cellular integrins are usually found associated with DIGs/caveolae in rat adipocytes (G. Müller and S. Welte, unpublished results). The bridge protein transduces (presumably via a conformational change) the signal across the caveolar plasma membrane to the inner face of the membrane where the acylated caveolar signalling proteins are concentrated. This causes dissociation of Lyn kinase from caveolin which is thereby activated. The activated and autophosphorylated Lyn tyrosine kinase will phosphorylate the

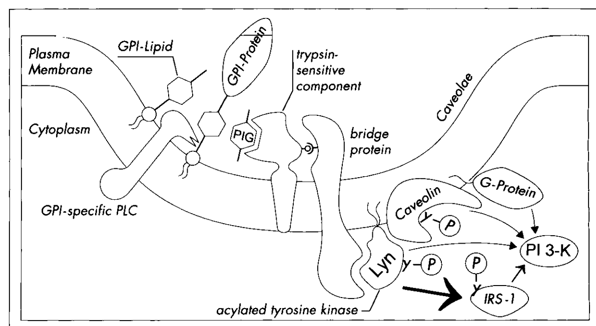

Figure 14. Hypothetical model for metabolic signalling by PIG via caveolae/DIGs. In a typical peripheral insulin target cell, i.e., muscle and adipose cell, GPI lipids, GPI proteins, GPI-specific PLC, the trypsin/salt/NEM-sensitive (115-kDa?) component, the unidentified bridge protein, dual acylated non-receptor tyrosine kinases, caveolin, and G-proteins are concentrated in special areas of the plasma membrane, the caveolae/DIGs (of which other constituent components, like cholesterol, are not indicated). PIG compounds will compete the interaction between the GPI lipids/proteins, which may function as the physiological ligands of the 115-kDa component. This triggers the generation of a signal which is transmitted via the non-covalently (electrostatically) associated bridge protein across the caveolar plasma membrane to its cytoplasmic face where caveolin and p59Lyn interact with one another. The signal-induced weakening of this interaction relieves the inhibition of Lyn kinase. Activated and autophosphorylated Lyn kinase phosphorylates caveolin and IRS-1 at tyrosine residues which will lead to PI 3-K stimulation and initiation of metabolic signalling. Other modes of PI 3-K activation independent of the insulin receptor may involve lipid-modified heterotrimetric G-proteins, known to be negatively regulated by caveolin, which may be released from inhibitory caveolin in response to PIG for subsequent stimulatory interaction with PI 3-K. Consequent to the dissociation of Lyn and caveolin, Lyn kinase and GPI proteins are redistributed from DIGs/caveolae into other areas of the plasma membrane. This is also true for that small fraction of IRS protein which is localized in DIGs/caveolae (for the sake of clarity, not indicated in this scheme).

IRS proteins (as shown), caveolin and possibly other signalling proteins, like cytoplasmic tyrosine kinases and G-proteins. Tyrosine phosphorylation of IRS-1/3 may initiate downstream signalling to the glucose transport system and both glucose and lipid metabolism via PI 3-K and complex yet ill-understood lipid and serine/threonine kinase cascades. Tyrosine phosphorylation of caveolin may even function as the trigger for redistribution of constituent components of DIGs/caveolae, GPI proteins, and of certain signalling proteins like IRS-3 into non-caveolar areas of the plasma membrane or the cytoplasm, respectively, where they can fulfil (additional) specific signalling functions. According to this speculation, tyrosine-phosphorylated caveolin causes disassembly of DIGs/caveolae which, in fact, have been demonstrated to be dynamic, mobile rather than static structures. Importantly, oncogenic transformation of fibroblasts with Rous sarcoma viral oncogene correlated with tyrosine phosphorylation of caveolin [147, 160], decreased caveolin-1 expression and a reduced number of caveolae [161]. In general, caveolin-1 mRNA and protein expression are lost or reduced during cell transformation by activated oncogenes. Downregulation of caveolin-1 (e.g., by an antisense approach) is sufficient to mediate cell transformation, hyperactivation of the p42/44 MAPK pathway, anchorage-independent growth (e.g., of NIH3T3 cells), or tumorgenicity (e.g., in immunodeficient mice), while, conversely, upregulation of caveolin-1 (e.g., by loss of the caveolin-1 antisense vector) seems to be important in mediating contact inhibition of growth and negatively regulates the activation state of the MAPK cascade [162]. This may explain the localization of the caveolin-1 gene to a suspected tumor suppressor locus (7q31.1) [163].

In adipocytes, the trypsin/salt/NEM-sensitive component may normally function as a binding/receptor protein for certain GPI lipids and GPI membrane protein anchors, thereby contributing to the localization/concentration (via cross-linking) of (some) GPI lipids and GPI proteins (but not of others) in DIGs/caveolae (see fig. 14). The addition of excess soluble PIG molecules to the surface of intact adipocytes will compete for this interaction thereby causing the release of GPI lipids and GPI proteins from DIGs/caveolae into other areas of the plasma membrane. These topological rearrangements may also occur after cross-linking of GPI proteins or GPI lipids with antibodies (known to elicit biological effects in T cells and neutrophils [106, 107]) or with multivalent natural yet unidentified ligands or, alternatively, dissociation of GPI structures from the trypsin/salt/NEM-sensitive component by their lipolytic cleavage (treatment of intact cells with PI-specific PLC is known to induce biological effects [164]).

According to the latter possibility, the release of GPI lipids and GPI membrane protein anchors from the surface of intact insulin-sensitive cells by lipolytic cleavage may lead to displacement of the postulated endogenous ligand (i.e., the PIG portions of the GPI lipids and membrane protein anchors) from their cell surface receptor (i.e., the trypsin/salt/NEM-sensitive component) initiating the same cascade of events as alternatively provoked by the addition of excess soluble PIG. This molecular view of PIG action in insulin-sensitive cells differs fundamentally from the classical 'intracellular mediator or second messenger concept' (see fig. 4). Interestingly, we previously observed that lipolytic cleavage of the GPI anchors of Gce1 and lipoprotein lipase by bacterial PI-specific PLC did not result in release of the hydrolphilic anchorless versions of these proteins from the surface of intact rat adipocytes into the incubation medium; they remained attached to the cell [75]. Excess inositol-cyclic phosphate added to the intact cells displaced the polypeptides into the medium.

This argues for the existence of a saturable receptor for lipolytically cleaved GPI anchors which recognizes the inositol-cyclic phosphate and presumably additional epitopes. Whether this protein is identical with the trypsin/salt/NEM-sensitive component remains to be clarified.

The redistribution of structural constituents of DIGs/caveolae may be accompanied by or may even lead to the redistribution of signalling components concentrated in DIGs/caveolae in the basal state into other areas of the plasma membrane (e.g., Lyn) or cytoplasm (e.g., IRS-3) and ultimately cause alterations in their functional states. With respect to the putative mechanism of competition, the PIG compounds derived from the GPI protein, Gce1p, of *S. cerevisiae* may be particularly efficient in interacting with the trypsin/salt/NEM-sensitive component and thus in causing redistribution of constituent components of DIGs/caveolae, compared to other PIG structures or complete GPI anchors. The finding that PIG-P molecules, which have been either prepared from the GPI protein, Gce1p, from *S. cerevisiae*, or chemically synthesized according to the structure of the GPI anchor of Gce1p including its carboxy-terminus, are most efficient in cross-talking to the insulin signalling cascade in insulin-sensitive mammalian cells have to be considered in light of the recent demonstration of the existence of signal transduction mechanisms even in unicellular eukaryotes, like yeast, which respond to human insulin [165–167].

Potential use of PIG-like molecules in signal transduction therapy

In conclusion, the data presented in this review strongly indicate that caveolin regulates the activity of diverse signalling pathways, (the characterization of which has started only recently), by interaction with key components. Experimental evidence is accumulating that this protein-protein interaction can be modulated efficiently by small signalling factors. This opens fascinating perspectives for future signal transduction therapy with regard to signalling pathways emenating from caveolae/DIGs. For example, natural or synthetic PIG, PIG-P or PIG-like molecules seem to harbor all the structural requirements needed for metabolic insulin signalling independent of the insulin receptor by triggering tyrosine phosphorylation of IRS proteins and activation of the PI 3-K pathway. These molecules may thereby manage to regulate cellular glucose and lipid metabolism in insulin target tissues in insulin-like fashion. This characteristic makes them potentially interesting for the design of drugs for the treatment of diseases like metabolic syndrome, obesity, and other insulin-resistant states [for reviews see refs 168, 169], of which all are characterized by impaired insulin receptor tyrosine kinase activation [170–177]. Moreover, in the face of decreased peripheral glucose utilization, the insufficient insulin secretion from β-cells results in glucose intolerance and frank diabetes. The recent findings in mice with β-cell-specific disruption of the insulin receptor suggest that impaired insulin secretion might be a result of insulin resistance in the β-cells themselves [178]. The loss of first-phase insulin secretion after glucose challenge seen in these mice resembles that seen in human type II diabetes [178]. These data suggest that signals mediated by the insulin receptor are essential for the release of insulin secretory vesicles. Indeed, a combination of impaired insulin receptor kinase action in muscle, adipose, and β-cells may create the phenotype of type II diabetes. Consequently, PIG-like molecules may have the potential to bypass the insulin receptor blockade in the β-cell, thereby triggering insulin release.

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