Insulin-like Signaling in Yeast: Modulation of Protein Phosphatase 2A, Protein Kinase A, cAMP-Specific Phosphodiesterase, and Glycosyl-phosphatidylinositol-specific Phospholipase C Activities

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ABSTRACT: Previously, we have described significant effects of human insulin on glucose metabolism in the yeast *Saccharomyces cerevisiae* under conditions of growth limitation. These regulations apparently rely on a transmembrane receptor capable of binding human insulin and responding by tyrosine/serine phosphorylation of a specific set of polypeptides [Müller, G., Rouveyre, N., Crecelius, A., and Bandlow, W. (1998) *Biochemistry* 37, 8683−8695; Müller, G., Rouveyre, N., Upshon, C., Gross, E., and Bandlow, W. (1998) *Biochemistry 37*, 8696−8704; Müller, G., Rouveyre, N., Upshon, C., and Bandlow, W. (1998) *Biochemistry 37*, 8705−8713]. To characterize the molecular link between the initial steps in insulin-like signaling in yeast and the changes in the activities of glycogen synthase and glycogen phosphorylase, we examined here the effects of human insulin on a set of key regulatory enzymes of glycogen metabolism, protein phosphatase 2A (PP2A), cAMP-specific phosphodiesterase (cAMP-PDE), and protein kinase A (PKA). PP2A was activated about 2-fold by insulin in spheroplasts and in intact cells, whereas the fraction of active PKA was significantly reduced in a cAMP-independent manner as well as through a subsequent up to 3-fold increase in particulate cAMP-PDE activity accompanied by a 50% decrease in cytosolic cAMP levels. In addition, glycosyl-phosphatidylinositol-specific phospholipase C (GPI-PLC), which in isolated rat adipocytes is activated by insulin, was stimulated to up to 5-fold by glucose and 10-fold by glucose *plus* insulin in both yeast spheroplasts and intact cells leading to a concentration-dependent leftward shift of the glucose-response curve for activation of the GPI-PLC. GPI-PLC was most pronouncedly stimulated by authentic human insulin compared to various insulin analogues and insulin-like growth factor I. In addition to lipolytic cleavage by GPI-PLC, the GPI anchor of the cAMP-binding ectoprotein, Gce1p, was secondarily processed by a rapid proteolytic event. As the GPI-PLC reaction is rate limiting, the efficiency of the two-step anchor cleavage was significantly increased when insulin was present together with glucose as compared to glucose alone. The insulin concentrations effective in modulating PP2A, PKA, cAMP-PDE, and GPI-PLC activities correlate well with those required for half-saturation of the specific binding sites as well as for stimulation of protein phosphorylation and glycogen accumulation. The data suggest that mammalian insulin-sensitive cells and yeast share (part of) the key regulatory mechanism (consisting of PP2A, PKA, cAMP-PDE, and GPI-PLC) involved in the transduction of the insulin signal from the respective receptor systems to glycogen synthase and phosphorylase.

The molecular mechanisms by which nutrients control yeast physiology, in general, and glycogen metabolism, in particular, are far from clear. Genetic and biochemical evidence suggests that, in yeast as in vertebrates, glycogen phosphorylase and glycogen synthase activities can be modulated at the transcriptional level (*1*, *2*) and, in addition, through both allosteric and phosphorylation/dephosphorylation-dependent mechanisms (*3*−*6*). Phosphorylation inactivates glycogen synthase and activates glycogen phosphorylase, whereas dephosphorylation has the opposite effects. The activity of GSY-2, the rate-limiting enzyme of the two isoforms of glycogen synthase, increases during the growth cycle in batch culture on glucose medium in parallel with the accumulation of glycogen (*2*). GSY-2 is thought to be regulated posttranslationally by phosphorylation at three sites close to the carboxy-terminus of the protein (*7*). Mutation of these serine residues to alanine or removal of all three sites by truncation leads to a constitutively hyperactive GSY-2 protein that will overaccumulate glycogen even in the presence of glucose by bypassing phosphorylation controls. These results indicate the importance of phosphorylation/dephosphorylation in the regulation of GSY-2. However, little is known about how the phosphorylation state is linked to the sensing of the nutrient supply and about the signal transduction chains involved.

In addition to protein phosphatase type 1, type 2A phosphatases (PP2A)[1] have been implicated in the regulation of glycogen metabolism in higher eukaryotic cells (*8*), and a type 2A activity was identified in vitro as a glycogen

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synthase phosphatase in yeast (*9*). In *Saccharomyces cerevisiae*, two genes (PPH21 and PPH22) encode two closely related proteins that account for most of the PP2A activity in yeast cells and strongly resemble the catalytic subunits of mammalian type 2A phosphatases (*10*, *11*). As in mammals, the yeast catalytic subunits probably interact with at least two regulatory subunits, encoded by the genes TPD3 (similar to the mammalian A subunit) and CDC55 (similar to the mammalian B subunit) (*12*, *13*). The decrease in total PP2A activity by controlled depletion of the PPH22 gene product after switch off of the PPH21 gene's trancriptional activity in a mutant yeast strain correlated with reduced accumulation of glycogen and a more pronounced inactivation of glycogen synthase. On the other hand, under the same circumstances, glycogen phosphorylase became more resistant to inactivation (*14*). In addition, deletion of the SIT4 gene in yeast, which codes for a protein distantly related to the catalytic subunit of mammalian PP2A, likewise resulted in activation of glycogen phosphorylase and inactivation of synthase (*15*). These observations strongly suggest a role of PP2A in the control of the activation states of both enzymes in yeast.

The cAMP pathway is known to counteract PPA2 in the control of the coupling of glycogen storage to the supply with nutrients in yeast presumably also at the level of regulation of the phosphorylation states of GSY-2 and glycogen phosphorylase. Defects in the BCY1 gene, which encodes the regulatory subunit of PKA, led to a constitutively active kinase and resulted in hypersensitivity to starvation and inability to sporulate (*16*). In contrast, mutants with an attenuated cAMP pathway (e.g., in a ras2 genetic background) sporulated even in rich media (*17*). These strains also displayed aberrant glycogen accumulation, with ras2 mutants showing hyperaccumulation and bcy1 mutants being unable to synthesize glycogen. Consequently, it has been suggested that the glycogen accumulation phenotypes of cAMP pathway mutants reflect posttranslational controls of yeast glycogen synthase by PKA (*15*, *16*, *18*).

In mammalian adipose and muscle cells, insulin is a key player in the regulation of glycogen metabolism by simultaneously triggering activation of glycogen synthase and inactivation of glycogen phosphorylase through several mechanisms finally leading to their dephosphorylation (*19*, *20*). There is some experimental evidence that turnover of GPI plasma membrane lipids elicited by the collaborative result of biosynthesis and lipolytic cleavage through a GPI-PLC is required for transmission of the insulin signal from the insulin receptor to glycogen synthase (*21*). In fact, it has been demonstrated that insulin stimulates a GPI-PLC in cultured and isolated adipose as well as muscle cells in a concentration-dependent and rapid manner (*22*−*24*). Recently, a potent cross-talk of synthetic PIG compounds, which represent the polar core glycan headgroups of GPI lipids generated by GPI-PLC cleavage, to the insulin signaling cascade at the level of tyrosine phosphorylation of the IRS proteins has been demonstrated in isolated rat adipocytes and cardiomyocytes (*25*−*27*). PIG molecules designed according to the consensus structure of yeast GPI anchors were particularly efficient in this insulin-like signaling and provoking of metabolic activity changes, such as stimulation of glycogen synthesis in isolated rat adipocytes (*28*). Interestingly, *S. cerevisiae* harbors a GPI-PLC activity which markedly increases upon glucose induction of spheroplasts or glucose exhaustion of intact cells (*29*, *30*). These findings raised the possibility that in yeast glycogen accumulation may be regulated (in part) via a mechanism encompassing the generation of PIG molecules through the action of an inducible GPI-PLC. In yeast, after the priming lipolytic cleavage, a proteolytic processing enzyme acts on the lipolytically cleaved GPI anchor completely cleaving off the polar headgroup together with a short carboxy-terminal peptide (*31*). The glucose-induced 2-fold processing of GPI anchors in *S. cerevisiae* seems to comprise a step during the biogenesis of some cell wall mannoproteins (*31*, *32*). However, the role of its stimulation by glucose in nutritional signaling remains an enigma so far.

Here we characterize the molecular basis for the recent provocative finding that human insulin stimulates glycogen synthesis in *S. cerevisiae*, which is based on both activation of glycogen synthase and inhibition of glycogen phosphorylase (*33*). For this, the effect of human insulin on the activity of PP2A, PKA, cAMP-PDE, and the GPI-PLC was studied under two distinct conditions of growth limitation, which support glycogen storage in yeast. The data suggest that these enzymes may function as key regulators in mediating the insulin-like effects on glycogen metabolism in yeast and that yeast is likely to use a signal transfer chain to control these activities which strikingly resembles that of mammalian insulin-responsive cells.

EXPERIMENTAL PROCEDURES

Materials. [2,8-^{3}H]cAMP (15 Ci/mmol) and [γ-^{32}P]ATP (5000 Ci/mmol) were bought from NEN/DuPont (Bad Homburg, Germany). 8-N_3-[^{32}P]cAMP (750 Ci/mmol) was purchased from ICN (Eschwege, Germany). [U-^{14}C]Leucine (300 mCi/mmol) and *myo*-[U-^{14}C]inositol (250 mCi/mmol) were delivered by Biotrend (Köln, Germany). 2-Deoxyglucose, 3-*O*-methylglucose, IBMX, poly(ethylene glycol) 6000, 5′-nucleotidase from *Crotalus atrox* (grade IV), myelin basic protein, bovine pancreatic trypsin and soybean trypsin inhibitor and Dowex 1X8-400 were obtained from Sigma (Deisenhofen, Germany). cAMP Sepharose and protein A-Sepharose were provided by Pharmacia/LKB (Freiburg, Germany). Anti-CRD serum from rabbits immunized with ILTat1.21 s-VSG was from Oxford GlycoSystems (Abingdon, U.K.). Recombinant human glucagon, Kemptide, cAMP (sodium salt), okadaic acid (sodium salt), octyl glucoside, and TX-114 were bought from Calbiochem (Bad Soden, Germany). Protease inhibitors and recombinant human IGF-I and EGF were purchased from Boehringer Mannheim (Germany). Recombinant human insulin and insulin analogues were made available by the Pharmaceutical Synthesis Department of Hoechst Marion Roussel Deutschland GmbH (Frankfurt, Germany). All other materials were purchased as described in previous papers (*33*−*36*).

[1] Abbreviations: cAMP-PDE, cAMP-specific phosphodiesterase; CRD, cross-reactive determinant; EC_{50}, effective concentration for half-maximal activation; EGF, epidermal growth factor; Gce1p, glycolipid-anchored cAMP-binding ectoprotein; GPI, glycosyl-phosphatidylinositol; GPI-PLC, GPI-specific phospholipase C; GPI-protein, GPI-anchored membrane protein; IBMX, 3-isobutyl-1-methylxanthine; IGF-I, insulin-like growth factor I; IRS, insulin receptor substrate; PIG, phosphoinositolglycan; PKA, cAMP-dependent protein kinase A; PP2A, protein phosphatase 2A; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TX-114, Triton X-114.

Growth Conditions and Incubation with Insulin. S. cerevisiae strain W303-1A was used for analysis of PP2A and GPI-PLC activity, strain X-2180 for studies on insulin binding and cross-linking. Yeast cells were grown in 1% yeast extract, 2% bacto-peptone, and 2% glucose. For analysis of the effect of insulin in intact cells after exhaustion of a carbon source (glucose or lactate medium) during saturation of the culture, cells were harvested by rapid vacuum filtration at different time points during transition from exponential growth to stationary phase. Human insulin (at the final concentration indicated) was added to the medium at the start of the culture and at 3 h intervals throughout the total culture period. For analysis of insulin effects in glucose-induced spheroplasts, logarithmically grown cells were converted to spheroplasts, which were then incubated in osmotically stabilized succinate containing medium for 1 h followed by addition of glucose (100 mM final concentration) and further incubation for variable periods prior to rapid centrifugation through a cushion of Ficoll/sucrose (for details, see ref *33*). Human insulin (1 μM final concentration) was added to the spheroplasts in one portion together with the glucose.

PP2A Activity. The cell cake (approximately 300−500 mg wet weight) or spheroplast pellet (70−100 mg wet weight) was washed once with 25 mL of ice-cold 100 mM Tris/HCl (pH 8.5), 10 mM EDTA, and 25 mM DTT (osmotically stabilized by 1.2 M sorbitol in the case of spheroplasts) and immediately resuspended in 0.5 mL of 100 mM Tris/HCl (pH 7.0), 2 mM EDTA, 10 mM DTT, 0.5 mM benzamidine, 0.2 mM PMSF, 2 μg/mL leupeptin, and 5 μg/mL pepstatin. After addition of the same volume of ice-cold glass beads, cell extracts were prepared by vigorous vortexing (five times 5 s each with cooling intervals on ice) and centrifuged (13000*g*, 5 min, 4 °C). The supernatant (S13) was diluted with 3 vol of the same buffer containing 1% bovine serum albumin. Protein phosphatase activity was assayed as the ability to dephosphorylate ^{32}P-labeled myelin basic protein. A total of 10 μL of S13 was added to an assay mixture (total volume 50 μl) containing 50 mM Tris/HCl (pH 7.5), 1 mM EDTA, 0.5 mM EGTA, and 0.2 nmol of ^{32}P-labeled myelin basic protein (900 dpm/pmol) for 20 min at 30 °C in the absence or presence of 2 nM okadaic acid. The reaction was terminated by the addition of 50 μL of ice-cold 10% TCA. After incubation for 15 min on ice and centrifugation (12000*g*, 5 min, 4 °C), the supernatant was neutralized with 1 N NaOH and measured for radioactivity by liquid scintillation counting (10 mL of Aquasol, Beckman, Munich, Germany). PP2A activity was determined as the difference between ^{32}P-radiolabel measured in the presence of okadaic acid corresponding to the PP2A independent phosphatase portion and the label determined after uninhibited incubation corresponding to the total phosphatase activity. Under these conditions, release of $^{32}P_i$ was linear for 10 min. One unit of activity is defined as the amount of enzyme that catalyzes the release of 1 μmol of $^{32}P_i$-radiolabel from labeled myelin basic protein in 1 min under conditions of the standard assay.

PKA Activity. S13 was prepared from the spheroplast pellet as described for the PP2A assay. PKA activity was measured as the ability to phosphorylate the synthetic substrate peptide, Kemptide: 100 μL of S13 was added to 100 μL of prewarmed assay buffer containing 1 μM Kemptide, 50 mM Tris/HCl (pH 7.2), 5 mM DTT, 25 mM $MgCl_2$, 100 μM PMSF, 1 mM IBMX, 100 μM [γ-^{32}P]ATP (10 μCi) and incubated in the absence or presence of 1 μM cAMP (10 min, 30 °C). Subsequently, the reaction mixture was chilled on ice, supplemented with 0.8 mL of 25 mM ATP, 100 mM NaF, 200 mM $NaPP_i$, 10 mM glycerol-3-phosphate, 100 nM okadaic acid and spotted on phosphocellulose filters (Whatman P18). The filters were washed extensively (500 mL for 50 filters for each cycle) with 200 mM $NaPP_i$ (two times, 30 min each) and 0.3 M phosphoric acid (two times, acetone once), dried, and measured for radioactivity by Cerenkov counting. All values were corrected for a blank value obtained in the absence of S13. The PKA activity was expressed as the ratio of ^{32}P-incorporation into Kemptide in the absence and presence of cAMP (set at 100%). This activity ratio reflects the portion of PKA active at the time point of homogenization to total cellular PKA content.

cAMP-PDE Activity. The cell cake (approximately 300−500 mg wet weight) obtained by rapid filtration was washed once with 25 mL of ice-cold 100 mM Tris/HCl (pH 8.5), 10 mM EDTA, 25 mM DTT, and immediately resuspended in 0.5 mL of 100 mM Tris/HCl (pH 7.0), 2 mM EDTA, 10 mM DTT, 0.5 mM benzamidine, 0.2 mM PMSF, 2 μg/mL leupeptin, 5 μg/mL pepstatin, and 20 μg/mL antipain. After addition of the same volume of ice-cold glass beads, cell extracts were prepared by vigorous vortexing (five times 5 s each with cooling intervals on ice) and centrifuged (5000*g*, 5 min, 4 °C). The supernatant was diluted with 5 vol of the same buffer and centrifuged (100000*g*, 15 min, 4 °C). The pellet was resuspended in the same volume of buffer containing 140 mM NaCl by homogenization with a Teflon-in-glass homogenizer. After recentrifugation (50000*g*, 15 min, 4 °C), the pellet (P50) fraction was suspended in assay buffer (40 mM Tris/HCl, pH 8.0, 10 mM DTT, 20 mM $MgCl_2$, and 0.2 mM EDTA) at 3−5 mg of protein/mL and immediately assayed for cAMP-PDE activity. A total of 200 μL of sample was incubated (10 min, 37 °C) with 200 μL of 2 μM [^{3}H]cAMP (0.1 μCi in assay buffer). After termination of the reaction by heating at 95 °C for 45 s, 100 μL of 5′-nucleotidase (0.02 units in assay buffer) was added. After incubation (10 min, 37 °C) and addition of 1 mL of Dowex 1X8-400 (10 mg in methanol after equilibration), the incubation was continued for 15 min on ice with several cycles of vortexing. The mixture was centrifuged (20000*g*, 5 min, 4 °C). Aliquots of 0.5 mL of the supernatant were supplemented with 5 mL of scintillation cocktail (Readysafe, Beckman, Munich, Germany) and counted for radioactivity. A blank value obtained in the absence of enzyme was subtracted in each case. cAMP-Specific cAMP-PDE was calculated as the difference between the radioactivity measured in the absence and presence of 1 mM IBMX during the cAMP-PDE assay. One unit is defined as 1 nmol of cAMP cleaved during 10 min of incubation under the standard conditions.

GPI-PLC Activity. GPI-PLC was determined as lipolytic cleavage of the GPI anchor of the GPI-protein, Gce1p, and thereby conversion of the amphiphilic form harboring the intact anchor, into its hydrophilic form lacking the anchor (according to refs *37* and *38* with the following modifications). (i) For analysis under conditions of glucose repression, aliquots of yeast spheroplasts (3×10^8 cells) grown in succinate medium were collected by centrifugation through a cushion of Ficoll/sucrose, suspended, and metabolically

labeled with *myo*-[^{14}C]inositol or [^{14}C]leucine for labeling of the anchor portion and protein moiety, respectively, of Gce1p prior to addition of glucose. After various periods of time, the spheroplasts were again harvested by centrifugation through a cushion of Ficoll/sucrose and homogenized, and plasma membranes were prepared. (ii) For analysis under conditions of release from glucose repression, aliquots of intact yeast cells (1.5×10^8 cells) grown in glucose medium for various periods of time were collected by rapid filtration. The cells were converted to spheroplasts and photoaffinity labeled with 8-N_3-[^{32}P]cAMP. After homogenization of the spheroplasts (10 strokes in a tight-fitting Teflon-in-glass homogenizer), total proteins were successively precipitated with poly(ethylene glycol) 6000 (12%, by vol), immunoprecipitated with anti-CRD antibodies, and delipidated (chloroform:methanol:water, 10:10:3, by vol). After centrifugation (10000*g*, 5 min), the precipitated proteins were recovered from the interface and extracted three times with the same solution and once with chloroform:methanol (1:1, by vol). Pelleted plasma membranes (i) or the dried protein pellet (ii) were suspended in 1.5 mL of 2% TX-114, 140 mM NaCl, and 10 mM Tris/HCl (pH 7.4), incubated (10 min, 4 °C), and then centrifuged (4000*g*, 2 min, 4 °C). The supernatant was subjected to phase separation by warming up to 37 °C for 5 min (*39*). Hydrophilic Gce1p, remaining associated with the plasma membrane after lipolytic cleavage of its GPI anchor via bipolar interactions, is recovered in the aqueous phase and thereby separated from the uncleaved amphiphilic version which remains in the TX-114 phase. For detection of metabolically labeled Gce1p, the aqueous phase containing lipolytically cleaved Gce1p was affinity-purified using cAMP-Sepharose chromatography. Metabolically labeled or photoaffinity-labeled Gce1p partitioned into the aqueous phase was precipitated, separated by SDS−PAGE and analyzed by fluorography or phosphorimaging (Molecular Dynamics Storm 840).

Analysis of Double Processing of the GPI Anchor of Gce1p. Conversion of the uncleaved u-form of Gce1p with the complete GPI anchor still attached into the lipolytically cleaved l-form and proteolytically cleaved p-form was followed in spheroplasts as described previously (*31*, *32*). Briefly, aliquots of yeast spheroplasts (9×10^8 cells) incubated in succinate medium containing 0.05% yeast extract for 60 min were collected by centrifugation through a cushion of Ficoll/sucrose, suspended in 10 mL of succinate medium containing 0.01% yeast extract, and incubated with 50 μCi of [^{14}C]leucine (300 mCi/mmol) for 2 h at 30 °C. After termination of the labeling by addition of 1 mL of 200 mM leucine, glucose was added (final concentration, 100 mM), and the incubation continued. After various periods, spheroplasts were harvested by centrifugation through a cushion of Ficoll/sucrose. Plasma membranes were prepared and subjected to TX-114 partitioning. Proteins from the aqueous and detergent phases were subjected to affinity purification on cAMP Sepharose, precipitated with poly(ethylene glycol) 6000, treated with phosphodiesterase and alkaline phosphatase, again precipitated, and finally analyzed by isoelectric focusing and fluorography.

Statistical Analysis. Data are expressed as means ± SD (halves of the error bars are shown, only, for the sake of clearness). The untreated control cells or spheroplasts were compared with the insulin-treated ones by two-way Anova followed by the unpaired Student's *t*-test. When the difference between these two groups was significant, a multiple comparison was performed by Dunnett's test to compare each insulin-treated group with the untreated control group. Calculations of the 95, 98, and 99% confidence intervals were performed by nonlinear least-squares analysis using a four-parameter logistic model. A *p*-level of <0.05 was accepted as statistically significant.

Miscellaneous. Protein determination was performed using the Bradford procedure (*37*) with bovine serum albumin as standard. Published procedures were used for determination of cAMP levels (*33*), metabolic labeling of yeast cells with [^{14}C]leucine (*31*) or of spheroplasts with *myo*-[^{14}C]inositol (*30*) and [^{14}C]leucine (*31*), photoaffinity labeling of spheroplasts with 8-N_3-[^{32}P]cAMP, centrifugation of spheroplasts through Ficoll/sucrose and subsequent incubation with nonfermentable carbon sources, preparation of plasma membranes and TX-114 partitioning of plasma membrane proteins (*29*), affinity purification of hydrophilic Gce1p (*38*), immunoprecipitation of Gce1p with anti-CRD antibodies (*39*), precipitation of proteins (*40*), isoelectric focusing including sample preparation (*31*) and SDS−PAGE including fluorography (*41*).

RESULTS

Insulin Stimulates PP2A Activity in Glucose-induced Spheroplasts and in Glucose-Exhausted Cells. Some evidence has been obtained that, in *S. cerevisiae* SNF1 kinase, the key element in glucose repression/derepression, controls the phosphorylation state and thereby the activity of glycogen synthase and glycogen phosphorylase via activating the corresponding protein phosphatases, such as PP2A (see the introductory portion of this paper). We have shown that insulin increases SNF1 activity in glucose-induced spheroplasts and glucose-exhausted intact cells (*33*). This implies the involvement of PP2A in the activation of nonoxidative glucose metabolism by insulin. To examine this possibility, PP2A activity was measured as dephosphorylation of ^{32}P-labeled myelin basic protein using the S13 fraction prepared from total homogenates of either spheroplasts (collected by rapid centrifugation through a cushion of Ficoll/sucrose) or intact cells (collected by rapid filtration). After induction of yeast spheroplasts with 100 mM glucose or the nonmetabolizable glucose analogues, 2-deoxyglucose or 3-*O*-methylglucose, PP2A activity did not increase within the next 60 min of incubation irrespective of which of the three carbohydrates had been added (Figure 1A). However, human insulin (1 μM) present during glucose induction yielded a rapid and marked elevation of PP2A activity with maximal stimulation reached at 15 min and thereafter remaining constant. Insulin was more efficient in activating PP2A if combined with glucose (max. 1.9-fold vs basal) compared to 2-deoxyglucose (max. 1.6-fold vs basal) and 3-*O*-methylglucose (1.3-fold vs basal). This relative ranking parallels that observed for insulin stimulation of glycogen synthesis in yeast spheroplasts induced with glucose, 2-deoxyglucose, or 3-*O*-methylglucose (*33*). Nonfermentable carbon sources, like lactate or succinate, did not support activation of PP2A and glycogen synthase by insulin. This correlation of the efficiency of various carbon sources in stimulating PP2A activity and glycogen synthesis in the presence of insulin is compatible with participation of PP2A in the

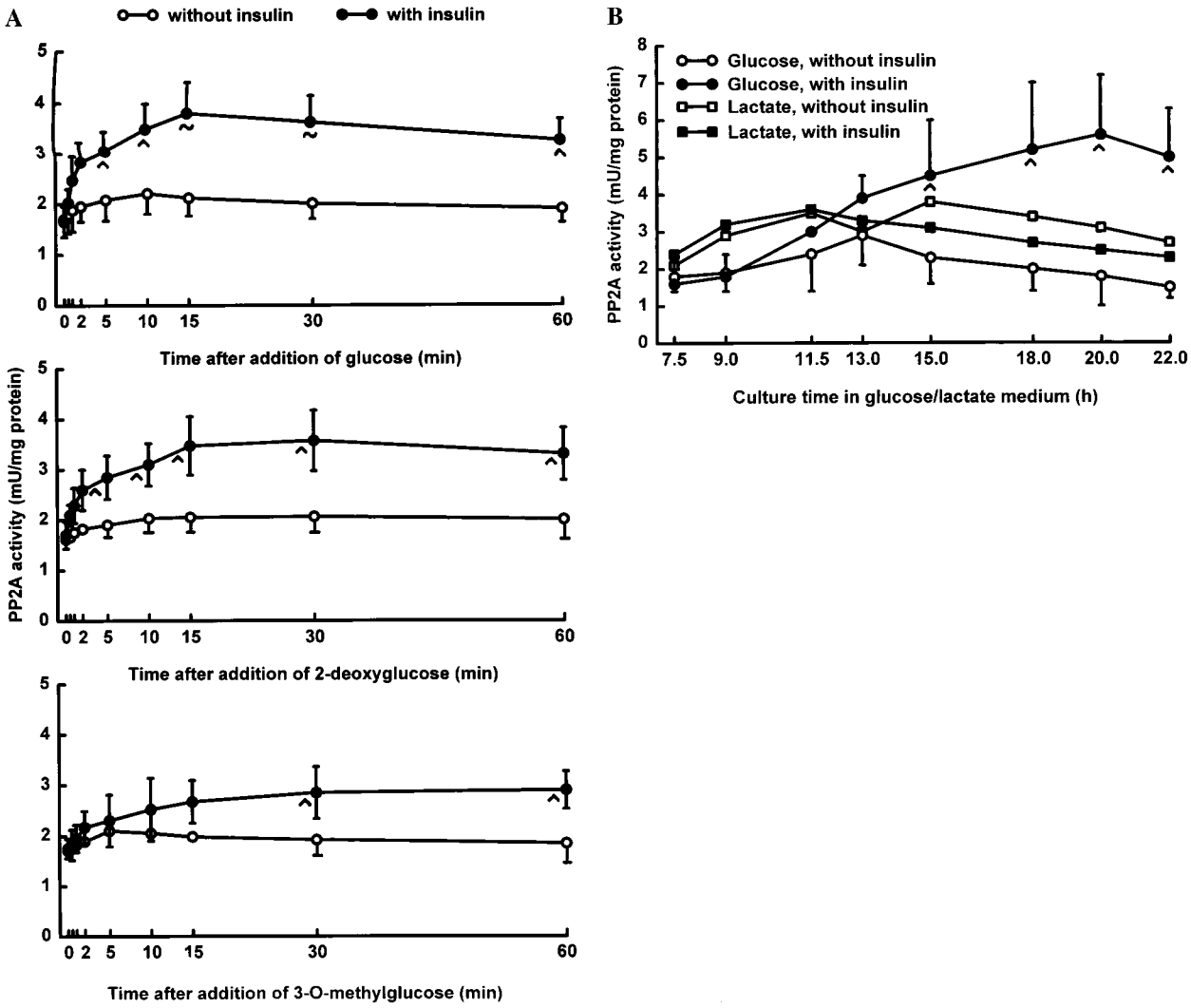

FIGURE 1: Effect of insulin on PP2A activity in yeast spheroplasts and intact cells. (A) Spheroplasts derived from logarithmically grown yeast cells were incubated at a density of 10−15 mg wet mass/mL for 60 min in succinate medium as described in the Experimental Procedures prior to addition of glucose (top), 2-deoxyglucose (center), or 3-*O*-methylglucose (bottom) at a final concentration of 100 mM (open circles), or glucose/derivative *plus* insulin (1 μM final concentration; filled circles). After the incubation periods indicated, aliquots of the spheroplasts were collected by centrifugation through Ficoll/sucrose, and S13 extracts were prepared and assayed for PP2A activity. The points represent means ± SD of at least 5 independent incubations with triplicate determinations, each. ($\wedge$) $p < 0.05$; ($\sim$) $p < 0.02$; ($\vee$) $p < 0.01$, presence vs absence of insulin. (B) Yeast cells were grown to saturation in YPD medium. Aliquots of 0.2−1 mL were reinoculated into 750 mL YPD medium (circles) or lactate medium (squares) lacking (open symbols) or containing 10 μM insulin (filled symbols). Growth was continued for different periods of time after which the cells were collected by rapid filtration. S13 supernatant fractions were prepared and assayed for PP2A activity. The points represent means ± SD of at least six independent incubations with determinations in triplicate, each. ($\wedge$) $p < 0.05$; ($\sim$) $p < 0.02$; ($\vee$) $p < 0.01$ glucose, presence vs absence of insulin.

transmission of the insulin effect on glycogen synthase and phosphorylase.

During transition of intact yeast cells from exponential growth on glucose to stationary phase (in the interval between 7.5 and 22 h of culture), PP2A activity did not change significantly and was comparable between growth on lactate and glucose medium (Figure 1B). This is in agreement with previous findings by others that PP2A expression is not modulated by the carbon source (*14*). When human insulin (10 μM) was present in the culture medium, PP2A activity increased continuously from 7.5 to 20 h with a significant difference compared to the absence of insulin becoming established at 15 h (1.9-fold vs basal). Maximal stimulation was reached between 20 and 22 h (2.7−3-fold vs basal; Figure 1B). Insulin had no effect on PP2A in cells grown on lactate medium. Subsequent experiments showed that the efficiency of insulin in activating PP2A and glycogen synthase as well as in inducing glycogen accumulation in intact cells was comparable if insulin was added to the glucose medium between time point 0 or 7 h after start of the culture, but was markedly reduced when insulin was supplemented at later time points (N. Hanekop and G. Müller, unpublished data). This suggests that yeast develops insulin sensitivity (at least for stimulation of glycogen synthesis and PP2A activity) during a distinct phase of the growth cycle, i.e., in late exponential phase and at the beginning of saturation of the culture, but prior to complete exhaustion of glucose in the medium.

Insulin Inhibits PKA Activity in Glucose-Exhausted Cells. PKA is one of the kinases which is assumed to counteract PP2A with respect to regulation of glycogen synthase and phosphorylase activities in yeast. Therefore, we studied whether PKA is inhibited in response to insulin which would enhance the positive impact of insulin on PP2A activity. In

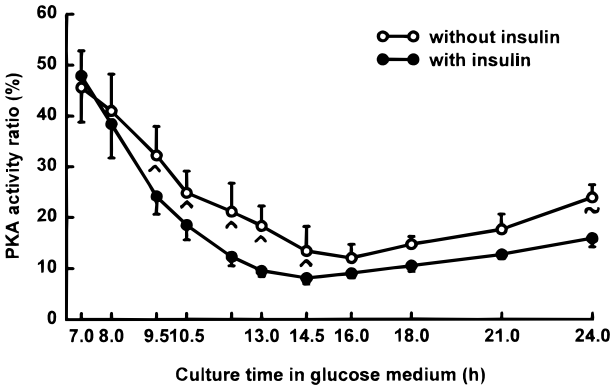

FIGURE 2: Effect of insulin on PKA activity in intact yeast cells during glucose exhaustion. Yeast cells were grown in YPD medium to saturation. Aliquots of 0.2−1 mL were reinoculated into 750 mL of YPD medium lacking (open circles) or containing 10 μM insulin (filled circles) and growth resumed. At different time points, cells were collected by rapid filtration, and S13 supernatant fractions were prepared and assayed for PKA activity ratio (active PKA per total PKA). The points represent means ± SD of at least four independent incubations with determinations in quadruplicate, each. (∧) $p < 0.05$; (∼) $p < 0.02$; (∨) $p < 0.01$, presence vs absence of insulin

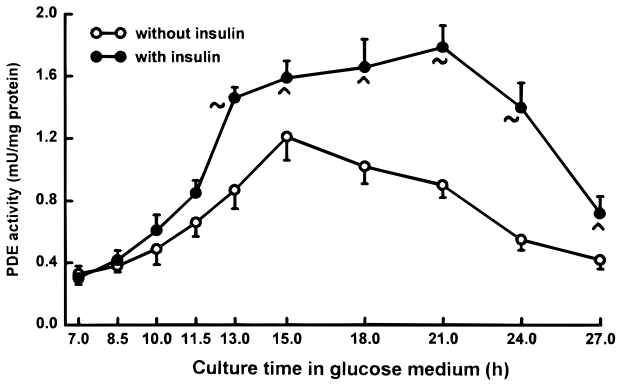

FIGURE 3: Effect of insulin on cAMP-PDE activity in intact yeast cells during glucose exhaustion. Yeast cells were grown to saturation in YPD medium. Aliquots of 0.2−1 mL were reinoculated into 750 mL YPD medium lacking (open circles) or containing 10 μM insulin (filled circles) and growth resumed. At different time points, the cells were collected by rapid filtration, and P50 pellet fractions were prepared and assayed for cAMP-PDE activity in the absence or presence of IBMX as described in the Experimental Procedures. The points represent means ± SD of at least five independent cultures with determinations in triplicate, each. (∧) $p < 0.05$; (∼) $p < 0.02$; (∨) $p < 0.01$, presence vs absence of insulin.

yeast cells grown in glucose medium, the PKA activity ratio (absence of extrinsic cAMP over presence of excess cAMP, i.e., active PKA over total PKA; see the Experimental Procedures) decreased markedly during middle to late exponential growth from about 50 to 10% at 14.5 h after start of the culture and then increased slightly to 20% when stationary phase was reached (Figure 2). The PKA activity ratio of cells grown in the presence of 10 μM human insulin was statistically significantly lower during 9.5 and 14.5 h of growth in glucose. Total PKA activity (determined in the presence of 1 μM cAMP) and thus cellular PKA content did not differ between insulin-treated and basal cells during the entire culture period. Thus, the modest insulin-induced inhibition of PKA during saturation of the culture is apparently due to reduction of its intrinsic activation state and/or of the cytosolic concentration of cAMP. No impact of insulin on the PKA activity ratio in glucose-induced yeast spheroplasts (up to 60 min after glucose addition) was observed (N. Hanekop and G. Müller, unpublished data). This suggests that modulation of PKA intrinsic activity, as a part of insulin's molecular mechanism by which it stimulates glycogen metabolism, operates during long-term culture under glucose limitation, only.

Insulin Stimulates cAMP-PDE Activity in Glucose-Exhausted Cells. Next we studied whether a reduction in the cytosolic cAMP concentration due to increased cAMP-PDE activity contributes to the diminished PKA activity ratio in glucose-exhausted intact cells in response to insulin. The cAMP-PDE activity increased from middle to late exponential growth to up to 3−4-fold at 15 h after start of the culture both in the absence (3-fold) and presence of insulin (4-fold, Figure 3). However, in the absence of insulin, the cAMP-PDE activity declined to the initial value during the next 12 h of glucose exhaustion, whereas in the presence of insulin, it increased further with a maximum after 21 h and subsequent activity loss. During the period between 13 and 27 h of glucose exhaustion, cAMP-PDE activity in insulin-induced cells was significantly elevated by 133−200% compared to that in control cells (Figure 3). Thus, the time course for the stimulating effect of insulin on cAMP-PDE is significantly delayed in comparison to its inhibitory effect on PKA (see Figure 2). In glucose-induced spheroplasts insulin did not significantly modulate cAMP-PDE activity (N. Hanekop and G. Müller, unpublished data), which is in agreement with the previously observed failure of insulin to block the transient increase in the intracellular cAMP levels peaking shortly after addition of glucose to spheroplasts (*33*). Taken together, the insulin-induced PKA inhibition during glucose exhaustion of intact yeast cells seems to be based on both a cAMP-independent reduction of the enzyme's intrinsic activity (during the initial phase) and a lowering of the cytosolic cAMP pool due to cAMP-PDE activation (during the subsequent phase).

Table 1: Effect of Insulin on Intracellular cAMP Levels in Intact Cells[a]

culture time in glucose medium (h)	cAMP (nmol/g wet mass of cells)		
	without insulin	1 μM insulin	10 μM insulin
10	4.4 ± 1.1	4.0 ± 0.6	4.8 ± 0.7
11.5	3.8 ± 0.6	3.3 ± 0.5	2.5 ± 0.5 (∧)
13	2.5 ± 0.6	2.3 ± 0.5	1.6 ± 0.5 (∧)
15	2.0 ± 0.5	1.6 ± 0.4	0.9 ± 0.3 (∼)
18	3.3 ± 0.6	2.1 ± 0.4 (∧)	1.4 ± 0.4 (∼)
21	6.9 ± 0.7	4.2 ± 0.8 (∼)	3.3 ± 0.7 (∼)
24	8.9 ± 0.9	7.0 ± 0.5 (∧)	6.0 ± 0.6 (∧)
27	9.4 ± 1.0	8.8 ± 0.6	8.5 ± 0.7

[a] Yeast cells were grown to saturation in YPD medium. Aliquots of 0.2−1 mL were reinoculated into 750 mL YPD medium (circles) or lactate medium lacking or containing 1 or 10 μM human insulin. Growth was continued for different periods of time after which the cells were collected by rapid filtration. S13 supernatant fractions were prepared, and assayed for cAMP. The points represent means ± SD of at least four independent incubations with determinations in triplicate, each. (∧) $p < 0.05$; (∼) $p < 0.02$, presence vs absence of insulin.

Supporting evidence for the latter mode of action came from analysis of the intracellular cAMP concentrations in intact yeast cells upon response to insulin (Table 1). From 10 to 15 h after addition of glucose, cAMP concentrations declined steadily to up to 50% of the initial value, which

was further significantly enhanced in the presence of 10 μM insulin to up to 20%. These time courses and the insulin dependence correlated well with the observed activity states of cAMP-PDE during this growth period (see Figure 3). From 15 to 27 h of culture in glucose medium, the cAMP levels increased to up to 200% of the initial value in both the absence and presence of insulin (Table 1). However, the time course for the accumulation of cAMP was significantly delayed in cells treated with insulin in a concentration-dependent fashion. Apparently, the lowered cAMP levels from 15/18 to 24 h in insulin-treated cells are based (at least in part) on the maintenance of high cAMP-PDE activity during this period in the presence of insulin, whereas in its absence, cAMP-PDE was downregulated continuously during this period (see Figure 3). Interestingly, insulin failed to modulate cytosolic cAMP levels in intact yeast cells grown in lactate or succinate medium (N. Hanekop and G. Müller, unpublished data). Thus, the effect of human insulin on cAMP levels in yeast can be explained by long-term upregulation of cAMP-PDE in the glucose-repressed state. A putative inhibition of adenylate cyclase by insulin (well established in mammalian cells) as an additional mechanism to lower cAMP in yeast remains to be studied.

Insulin Increases GPI-PLC Activity in Glucose-Induced Spheroplasts and in Glucose-Exhausted Intact Cells. The putative involvement of GPI metabolism in the regulation of glycogen synthesis in mammals and yeast (see the introductory portion of this paper) prompted us to study the effect of insulin on GPI-PLC activity in glucose-induced yeast spheroplasts and glucose-exhausted intact cells. Spheroplasts were incubated with *myo*-[^{14}C]inositol and [^{14}C]leucine for labeling of the GPI anchor and protein moiety of the GPI-protein, Gce1p, respectively, in succinate medium and then induced with glucose in the absence or presence of 1 μM human insulin for various periods. Hydrophilic Gce1, generated by lipolytic cleavage by GPI-PLC, has been shown previously to remain associated with the yeast plasma membrane via bipolar interactions (*29*). It was partially purified by cAMP affinity chromatography from the aqueous phase after TX-114-partitioning of the isolated plasma membranes and analyzed by SDS−PAGE and fluorography (Figure 4A). With increasing incubation time, the amount of hydrophilic Gce1p with the GPI anchor lipolytically cleaved (as demonstrated by retention of the inositol label; section a, lanes 1−6) and harboring the inositol-1,2-cyclic phosphate moiety (as demonstrated by crossreactivity with anti-CRD antiserum, which recognizes predominantly this epitope; section b, lanes 1−6) as well as the amount of total hydrophilic Gce1p (as demonstrated by the leucine label; section c, lanes 1−6) increased steadily up to 45 min after glucose addition reflecting glucose-dependent activation of the GPI-PLC. Insulin added together with glucose to the spheroplasts caused a further increase in the amounts of inositol-labeled (section a, lanes 7−12), anti-CRD immunoprecipited (section b, lanes 7−11) and leucine-labeled (section c, lanes 7−12) hydrophilic Gce1p, indicating stimulation of the GPI-PLC by insulin. Quantitative evaluation of the amount of hydrophilic, inositol-labeled Gce1p generated during 30 min incubation in glucose medium (calculated as difference between time points X and 0), revealed a significant (from 5 to 30 min) increase in the presence of 1 μM insulin compared to its absence, which reached 40% at 10 min (Figure 4B). The rates for the amphiphilic/hydrophilic conversion of Gce1p declined as early as 2 min after addition of glucose both in the presence and absence of insulin leading to a plateau level between 20 and 30 min. The rapid and transient nature of the GPI-PLC action was confirmed by measurement of the velocity of the GPI-PLC reaction for a 1 min interval at each time point after glucose induction of the spheroplasts (Figure 4C). Maximal rates of GPI-PLC activity were observed at 10 min in the absence and between 2 and 5 min in the presence of 1 μM insulin with a rapid half-maximal decline within the next 5−10 min. At 2 and 5 min, the insulin effect was significantly more pronounced with a 3-fold stimulation vs basal irrespective of whether glucose or an analogue was contained in the medium. Supplementation of glucose (final concentration, 100 mM) to spheroplasts after 60 min of the initial glucose addition, i.e., at a time point when the GPI-PLC activity had already returned to near basal values (see Figure 4C) and the glucose levels in the medium had fallen below 20 mM, triggered another rapid and transient activation of the GPI-PLC, the basal and insulin-induced values of which were comparable with those after the initial glucose induction. This suggests that, in yeast spheroplasts, the rapid and transient action of the GPI-PLC in response to glucose and insulin is based on a direct regulation of the enzyme's activity rather than on limited availability of the substrate, i.e., the cleavable GPI anchors at the plasma membrane. The rapid kinetics of the glucose and insulin effects and the ranking in efficiency (although the differences were not statistically significant) of nonmetabolizable glucose analogues (glucose > 2-deoxyglucose > 3-*O*-methylglucose) in spheroplasts were the same for stimulation of GPI-PLC (Figure 4C) and PP2A (Figure 1A) arguing in favor of a mechanistic coupling of these two processes.

The constitutive activity of the GPI-PLC measured when spheroplasts were incubated in nonfermentable carbon sources, like succinate, was less than 20% of the one observed after induction by glucose. It did also not rise upon addition of insulin. The generation of hydrophilic inositol-labeled Gce1p was strongly dependent on the concentration of glucose added to the succinate medium with maximal activation of the GPI-PLC at 5% and half-maximal activation at about 1.5% initial glucose concentration (Figure 4D). Insulin caused a significant leftward shift of the glucose concentration−response curve for GPI-PLC activation reducing the initial glucose concentration required for its half-maximal activation to about 0.9% at 0.1 μM and 0.4% at 1 μM insulin. Thus, insulin efficiently increased the sensitivity of the yeast GPI-PLC for induction by glucose in a concentration-dependent fashion. It had no effect per se (i.e., in cells grown on nonfermentable carbon sources).

The second mode of growth restriction, glucose exhaustion of intact yeast cells during saturation of the culture, which provoked insulin sensitivity of glucose metabolism (*33*) and PP2A (see above), was also tested for supporting insulin sensitivity of the glucose-induced GPI-PLC. For this, spheroplasts were prepared from cells collected after various periods of growth in glucose medium and photoaffinity labeled with 8-N_3-[^{32}P]cAMP for labeling of the protein moiety of Gce1p. Hydrophilic Gce1p was recovered from the aqueous phase after TX-114 partitioning of isolated plasma membranes and analyzed by SDS−PAGE and phosphorimaging. The quan-

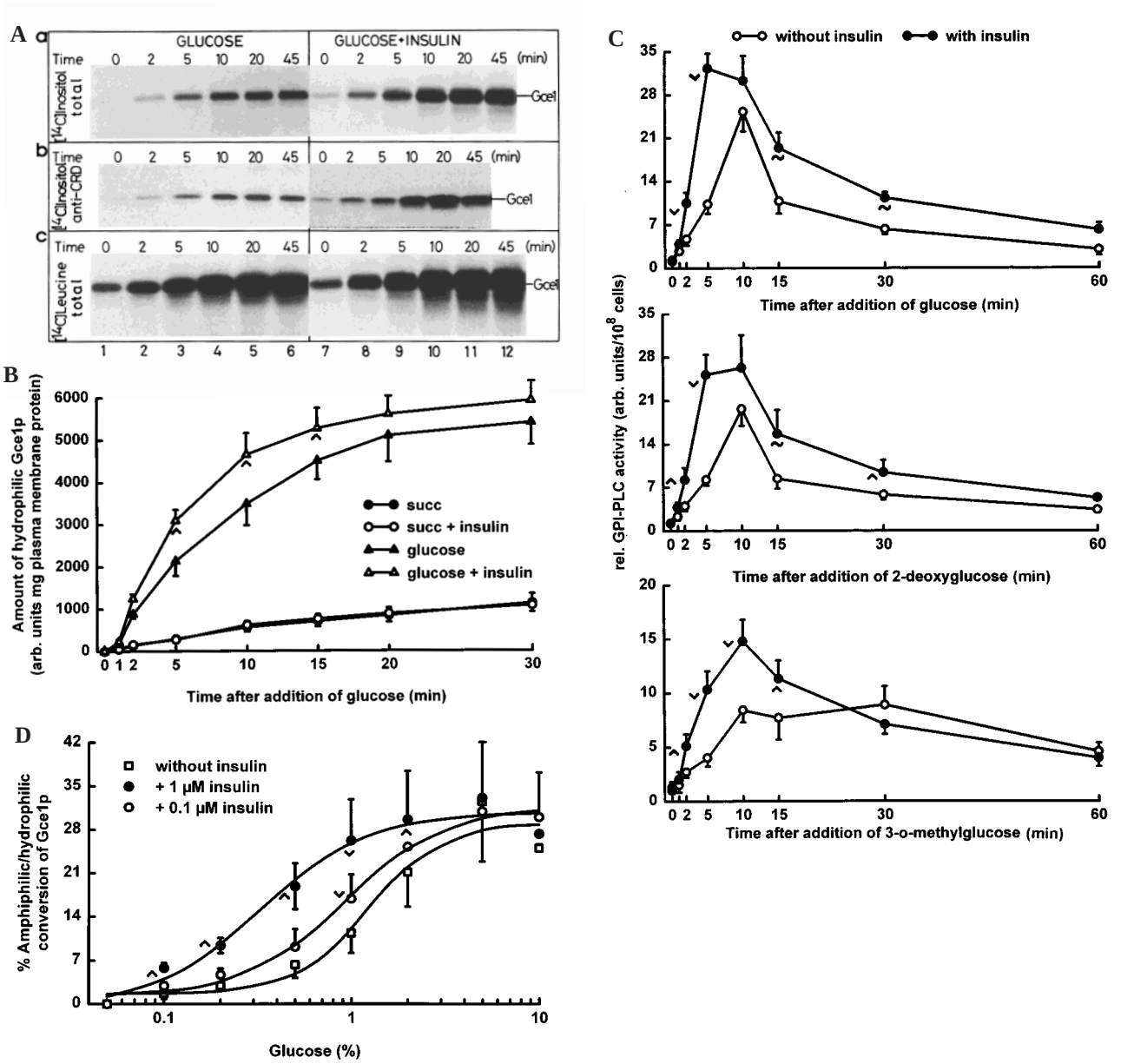

FIGURE 4: Effect of insulin on GPI-PLC activity in glucose-induced yeast spheroplasts. (A) Spheroplasts from logarithmically grown yeast cells were incubated at a density of 5−8 mg wet mass/mL for 60 min in succinate medium as described in the Experimental Procedures prior to addition of *myo*-[^{14}C]inositol (sections a and b) or [^{14}C]leucine (section c). After further incubation for 15 min, glucose (at a final concentration of 100 mM; lanes 1−6), or glucose *plus* insulin (at a final concentration of 1 μM) was added (lanes 7−12). Aliquots of the spheroplast suspension were taken at the time points indicated. Plasma membranes were prepared from the spheroplasts, which had been collected by centrifugation through a cushion of Ficoll/sucrose and partitioned between a TX-114 phase and an aqueous phase. Hydrophilic Gce1p was partially purified from the aqueous phase by affinity chromatography on cAMP-Sepharose. Total [^{14}C]leucine-labeled protein (section c) and halves of the [^{14}C]inositol-labeled protein were quantitatively precipitated either directly (section a) or after immunoprecipitation with anti-CRD antibodies (section b) and then analyzed by SDS−PAGE and fluorography. The position of solubilized Gce1p photoaffinity labeled with 8-N_3-[^{32}P]cAMP and run in parallel on the same gel is indicated on the right margin. The experiment was repeated two times with similar results. (B−D) The experiments were performed as described for panel A with the following incubations of the metabolically labeled (with *myo*-[^{14}C]inositol) spheroplasts: (B) absence (filled symbols) or presence (open symbols) of 1 μM (final concentration) human insulin after glucose supplementation (triangles) or its omission (circles) for the periods indicated, ($\wedge$) $p < 0.05$; ($\sim$) $p < 0.02$; ($\vee$) $p < 0.01$ glucose, presence vs. absence of insulin; (C) absence (open circles) or presence (filled circles) of 1 μM (final concentration) insulin in the presence of 50 mM (final concentration, each) glucose (upper section), 2-deoxyglucose (middle section), or 3-*O*-methylglucose (lower section) for various periods of time, ($\wedge$) $p < 0.05$; ($\sim$) $p < 0.02$; ($\vee$) $p < 0.01$, presence vs absence of insulin; (D) absence (squares) or presence of insulin (0.1 μM, open circles; 1 μM, filled circles) after supplementation of glucose (final concentration 0.05−10%) for 10 min. After TX-114 partitioning of the isolated plasma membranes, the fraction of hydrophilic [^{14}C]inositol-labeled Gce1p was affinity-purified from the aqueous phase and separated by SDS−PAGE. The amount of hydrophilic Gce1p recovered at each time point was quantitatively evaluated by phosphorimaging of the dried gel. The relative activity of the GPI-PLC at the time points indicated was calculated as the difference between the amounts (arbitrary units per 10^8 cells as determined by phosphorimaging) of hydrophilic Gce1p measured at that time point and at 1 min before (C). The points represent means ± SD (arbitrary units per 10^8 spheroplasts) of three to five independent metabolic labelings and incubations with determinations of hydrophilic Gce1p in triplicate, at least, each. ($\wedge$) $p < 0.05$; ($\sim$) $p < 0.02$; ($\vee$) $p < 0.01$, presence vs absence of 1 μM insulin.

titative evaluation (Figure 5A) demonstrates that the percentage of hydrophilic relative to total photoaffinity-labeled Gce1p (indicative of lipolytic cleavage by the GPI-PLC) rose from the mid-exponential growth (10 h after start of culture)

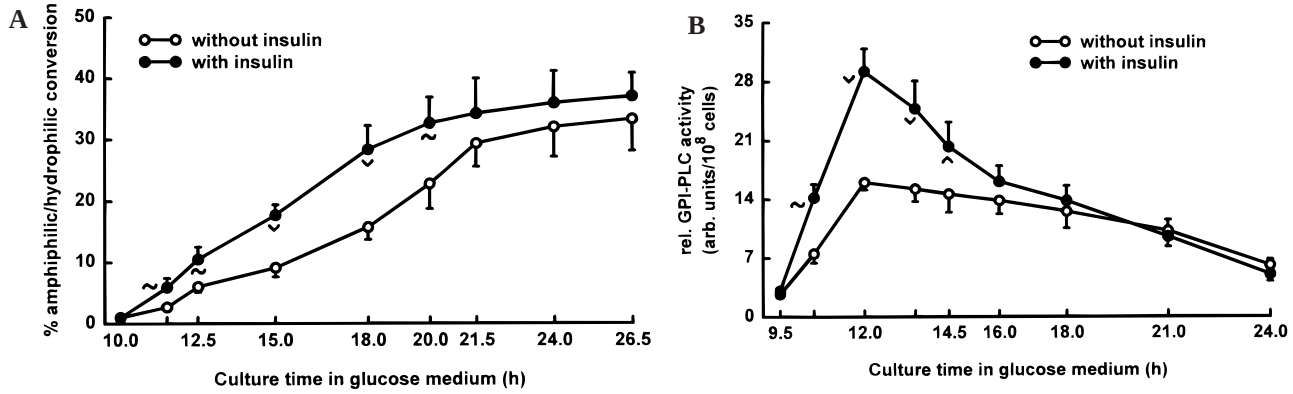

FIGURE 5: Effect of insulin on GPI-PLC activity in intact cells during glucose exhaustion. Cells were grown to saturation in YPD medium. Aliquots of 0.2−1 mL aliquots were reinoculated into 750 mL of YPD medium containing 10 μM insulin (filled circles) or lacking further additions (open circles) and growth resumed. Samples were taken at the time points indicated and 30 min before. Cells were collected by rapid filtration and converted to spheroplasts. After photoaffinity labeling of the spheroplasts with 8-N_3-[^{32}P]cAMP, lipolytic cleavage of Gce1p was followed as described in the Experimental Procedures. (A) The percent amphiphilic to hydrophilic conversion at each time point was calculated as the ratio between the amounts of hydrophilic Gce1p (as determined by phosphorimaging) and of total Gce1p (as determined prior to phase separation and set at 100%). The points represent means ± SD of four independent incubations with determinations of hydrophilic Gce1p in duplicate each. (B) The relative activity of GPI-PLC at the indicated time points was calculated as the difference between the amounts (arbitrary units per 10^8 cells as determined by phosphorimaging) of hydrophilic Gce1p measured at that time point and 30 min before. The points represent the means ± SD of six independent incubations and determinations in duplicate, each. (∧) $p < 0.05$; (∼) $p < 0.02$; (∨) $p < 0.01$ presence vs absence of insulin.

to the beginning stationary phase (20 h) and then remained constant during saturation. When human insulin (10 μM) had been added to the glucose medium, the GPI-PLC activity was significantly elevated compared to basal cells at each time point from 11.5 to 20 h after culture start with a maximal increment of 80−90% at 18 h. For a more precise (kinetic) analysis, the specific activity of the GPI-PLC (arbitrary units/10^8 cells and 30 min) was assayed at various time points after growth in the presence or absence of insulin. The amount of hydrophilic Gce1p was determined (as above) at 30 min intervals throughout 24 h after start of the culture. The relative GPI-PLC activity was calculated for the time points indicated as the increase of hydrophilic Gce1p and thus reflects the activity state of the GPI-PLC at these times more accurately than the amount or percentage of hydrophilic Gce1p accumulated up to these times (see "end-point measurements" of Figure 5A). Maximal relative GPI-PLC activity was observed 12 h after start of the culture in glucose medium (14.1 vs 1.5 arbitrary units in succinate medium) with an additional significant 2-fold stimulation elicited by 10 μM insulin at 10.5 and 12 h (Figure 5B). Thereafter, the relative GPI-PLC activity dropped significantly more rapidly in insulin-induced compared to basal cells, resulting in comparable activities in both cultures after 16 h. In conclusion, exhaustion of glucose (but not of nonfermentable carbon sources such as lactate, see also Figure 6) by intact yeast cells caused a drastic and transient activation of the GPI-PLC which was further enhanced by insulin. This significant potentiating effect was specific for insulin (up to 3-fold vs glucose alone) since the presence of a monoclonal neutralizing anti-insulin α-chain-specific antibody during insulin incubation of intact cells or preincubation of insulin with trypsin prior to addition to the cells completely blocked further activation of the GPI-PLC during glucose exhaustion (Figure 6). Furthermore, EGF and glucagon failed to significantly potentiate GPI-PLC activation during late logarithmic to stationary phase transition in glucose culture. IGF-I, the structure of which resembles proinsulin, provoked

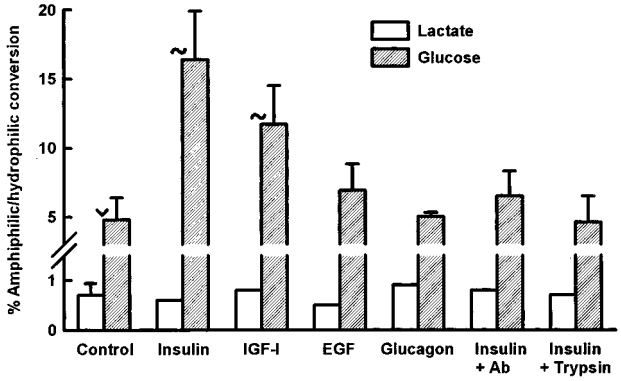

FIGURE 6: Effect of peptide hormones on GPI-PLC activity in intact yeast cells during glucose exhaustion. Yeast cells were grown to saturation in YPD medium. Aliquots of 0.2−1 mL were reinoculated into 750 mL YPD medium (filled columns) or lactate medium (open columns) lacking or containing 10 μM human insulin (in the absence or presence of monoclonal anti-human insulin antibody (1:200; Sigma, Deisenhofen, clone no. HG-36) or pretreated with trypsin (250 μg/mL for 1 h at 4 °C, followed by addition of 250 μg/mL trypsin inhibitor and 0.1 mM PMSF), 10 μM IGF-I, 10 μM EGF, and 10 μM glucagon as indicated. Growth was continued for 20 h. Then cells were collected by rapid filtration and converted to spheroplasts. After photoaffinity labeling of the spheroplasts with 8-N_3-[^{32}P]cAMP, lipolytic cleavage of Gce1p was followed as described in the Experimental Procedures. The percent amphiphilic to hydrophilic conversion at each time point was calculated as described in Figure 5A. The points represent means ± SD of three independent incubations, at least, with determinations in triplicate, each. (∧) $p < 0.05$; (∼) $p < 0.02$; (∨) $p < 0.01$, glucose, treatment vs control incubation.

about 50% of the maximal insulin effect (2-fold vs glucose alone, Figure 6).

Insulin Increases the Rate of Double-Processing of Gce1p in Glucose-Induced Spheroplasts. It has been shown previously that stimulation of the GPI-PLC by glucose in yeast spheroplasts leads to conversion of the amphiphilic uncleaved u-form of Gce1p to the hydrophilic lipolytically cleaved l-form (*29*, *30*). This primary cleavage is followed by a secondary cleavage event presumably by a protease, which removes the complete glycan portion of the l-form of the

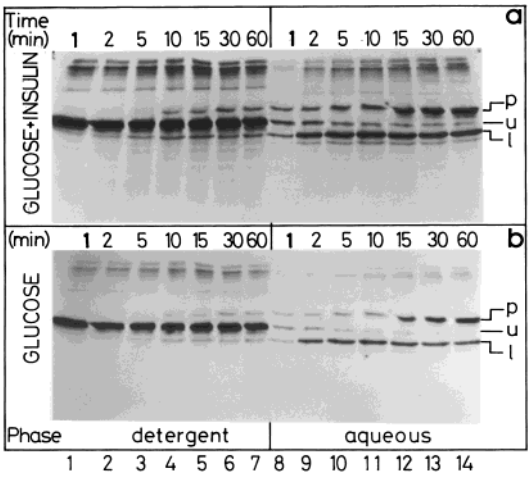

FIGURE 7: Effect of insulin on glucose-induced double-processing of Gce1p. Spheroplasts from logarithmically grown yeast cells were incubated at a density of 5−8 mg wet mass/mL for 60 min in succinate medium as described in the Experimental Procedures prior to addition of [^{14}C]leucine. After further incubation for 15 min, glucose (final concentrated 50 mM; section b), or glucose *plus* insulin (final concentrated 1 μM) were added (section a). Aliquots were taken at the time points indicated (a) or at 30 min (b). Plasma membranes were prepared from the spheroplasts which had been collected by centrifugation through a cushion of Ficoll/sucrose and partitioned between a detergent (TX-114) phase and an aqueous phase. Hydrophilic and amphiphilic Gce1p was partially purified from the detergent (lanes 1−7) and aqueous phases (lanes 8−14), respectively, by affinity chromatography on cAMP−Sepharose. All samples were precipitated and then analyzed by isoelectric focusing and fluorography. The positions of uncleaved (u), lipolytically cleaved (l), and proteolytically cleaved (p) versions of Gce1p are indicated on the right margin. The experiment was repeated three times with similar results.

GPI anchor together with the ethanolamine residue and a few carboxy-terminal amino acids of the protein moiety of Gce1p. Thereby, the hydrophilic proteolytically cleaved p-form is generated (*31*, *32*). We studied whether insulin has some impact on the primary, only, or on both cleavage reactions in the double-processing of Gce1p. For this, spheroplasts were incubated with [^{14}C]leucine in succinate medium to label the protein portion of all three forms of Gce1p prior to addition of glucose (100 mM) and human insulin (1 μM) as indicated (Figure 7). Hydrophilic and amphiphilic versions of Gce1p were partially purified by affinity purification from the aqueous and detergent phases after TX-114 partitioning of isolated plasma membranes, subjected to alkaline phosphatase treatment (for removal of the terminal cyclic phosphate moiety) and then analyzed by isoelectric focusing which separates the u-, l-, and p-forms from one another due to charge differences (for a detailed description of the procedure see ref *31*). After addition of glucose (absence of insulin; Figure 7, section b), the amount of u-form recovered from the detergent phase declined with time (lanes 1−7) accompanied by the emergence of the l- and p-forms in the aqueous phase (lanes 8−14). This substrate/product relationship reflects the action of GPI-PLC on Gce1p in response to glucose. The amount of the intermediate l-form rapidly accumulated during up to 10 min after start of the induction (lanes 8−11) and then decreased due to the slower generation of the l-form (lanes 12−14) compatible with the transient activation of GPI-PLC (see time course in Figure 4C). This precursor−product relationship between the l- and p-forms is compatible with conversion of lipolytically cleaved Gce1p into Gce1p having lost the complete GPI anchor by the secondary proteolytic processing event. When insulin had been added to the glucose medium, the amounts of both l- and p-forms of Gce1p, which had been accumulated at each time point during the 60 min period of induction, were markedly elevated (section a, lanes 8−14). The quantitative evaluation of a number of different experiments (Figure 8A) revealed that, in the time interval from 2 to 10 min, the amounts of l- and p-forms significantly increased to up to 6.6- and 5.1-fold, respectively, in insulin-induced spheroplasts compared to 3.4- and 2.5-fold, respectively, with glucose alone. Insulin stimulation of proteolytic processing of Gce1p was most pronounced (3.1-fold) at 10 min and declined thereafter, whereas maximal insulin-induced lipolytic processing (3.6-fold) was observed between 2 and 5 min. The decline of the l-form from 10 to 60 min followed a similar kinetics in the presence and absence of insulin and was inversely correlated to a continuous increment in the amount of p-form in both insulin-induced and basal spheroplasts (Figure 8A). Nevertheless, from 2 and 10 min, respectively, to 45 min, the amounts of l-form and p-form, respectively, were significantly higher in insulin-induced vs control spheroplasts.

Analysis of the dependence of the proteolytic processing of Gce1p measured both as decrease in the amount of l-form and as increase in the amount of p-form on the concentration of human insulin in spheroplasts (Figure 8B) demonstrated close similarity with the corresponding concentration−response curves for lipolytic processing of Gce1p (G. Müller and S. Grey, unpublished data). The EC_{50} for insulin stimulation was 0.2−0.3 μM for the proteolytic processing and 0.3−0.5 μM for lipolytic processing with similar sigmoidal curve shapes resulting from semilogarithmic plotting (the difference in the EC_{50} values did not reach statistic significance). Significant insulin effects were observed at concentrations as low as 0.1 μM. The apparent discrepancy between the EC_{50}-values for insulin signaling/action between yeast and mammals (about 3 orders of magnitude) may be explained (in part) by the evolutionary distance and furthermore be taken as a first hint to the existence of a structurally less conserved insulin-like molecule in *S. cerevisiae* (see Discussion). Incubation of yeast spheroplasts in succinate medium did not support proteolytic cleavage (as well as lipolytic cleavage; see above) to any significant degree irrespective of the amount of insulin present (Figure 8B). Altogether, these data suggest that GPI-PLC, which catalyzes the first processing event of Gce1p, is markedly stimulated by insulin on top of the activation by glucose alone. In consequence, the efficiency of the second proteolytic event is markedly increased in response to insulin, although insulin has no direct impact on the putative protease involved in this secondary processing reaction. Since previous studies suggested that GPI-PLC catalyzes the rate-limiting reaction in the overall processing of Gce1p (*31*), it is evident that insulin further relieved the rate-limiting step in this processing pathway without affecting the proteolytic cleavage step significantly.

Similar Insulin Concentrations Are Required for the Modulation of PP2A, PKA, cAMP-PDE, GPI-PLC, and Glycogen Synthase Activities and Initial Signaling Events. Finally, we attempted to provide support for the view that the 58 kDa insulin-binding protein, identified by equilibrium

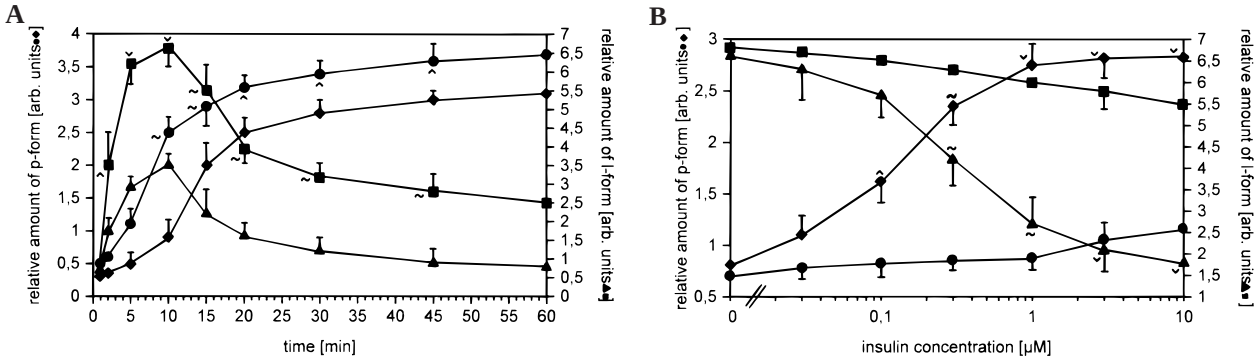

FIGURE 8: Effect of insulin on proteolytic processing of Gce1p. Spheroplasts from logarithmically grown yeast cells were incubated at a density of 5−8 mg wet mass/mL for 60 min in succinate medium as described in the Experimental Procedures prior to addition of [^{14}C]-leucine. Thereafter portions of the cultures were supplemented with glucose (final concentration 50 mM; panel A and panel B, triangles, diamonds) or succinate (panel B, circles, squares). After 15 min of incubation, the cultures received human insulin at increasing concentrations as indicated (B) or at 1 μM (final concentration, Panel A, circles, squares) or the cultures were left untreated (panel A, diamonds, triangles). Aliquots of the spheroplast suspension were taken at 10 min (B) or at the time points indicated (A). Hydrophilic Gce1p was partially purified from the aqueous phases of partitioned plasma membranes, precipitated, and then analyzed by isoelectric focusing and fluorography as decribed under Figure 7. The relative amount of the l-form (triangles, squares) and p-form (circles, diamonds) of Gce1p was evaluated by phosphorimaging of the corresponding gel bands resolved by isoelectric focusing. The points represent means ± SD of four independent incubations, at least, with determinations in quadruplicate, each. The amount of l-form recovered at one min in the presence of 1 μM insulin (see panel B) was set at 1 arbitrary unit. ($\wedge$) $p < 0.05$; ($\sim$) $p < 0.02$; ($\vee$) $p < 0.01$ presence vs absence of 1 μM (A) or increasing concentrations (B) of human insulin.

Table 2: Correlation of Effects on Various Key Regulatory Enzymes and the 58 kDa Insulin-Binding Protein in Glucose-Induced Yeast Spheroplasts between Insulin, Some Insulin Analogues and Other Hormones[a]

	glycogen synthase	PKA	PP2A	GPI-PLC	cAMP−PDE	insulin binding	serine/threonine phosphorylation
human insulin	100	100	100	100	100	100	100
analogue I	45 ± 8 ($\sim$)	69 ± 9 ($\wedge$)	62 ± 10 ($\sim$)	77 ± 8	93 ± 11	81 ± 7	71 ± 7
analogue II	25 ± 6 ($\vee$)	38 ± 11 ($\sim$)	29 ± 8 ($\vee$)	53 ± 10 ($\sim$)	64 ± 13 ($\wedge$)	49 ± 5 ($\sim$)	44 ± 5 ($\sim$)
analogue III	10 ± 3 ($\vee$)	16 ± 8 ($\vee$)	8 ± 5 ($\vee$)	15 ± 7 ($\vee$)	19 ± 9 ($\sim$)	6 ± 2 ($\vee$)	14 ± 3 ($\vee$)
analogue IV[b]	0	3 ± 1	0	0	4 ± 2	0	4 ± 3
IGFI	30 ± 9 ($\vee$)	44 ± 15 ($\sim$)	22 ± 7 ($\vee$)	55 ± 13 ($\sim$)	71 ± 9 ($\wedge$)	33 ± 8 ($\vee$)	59 ± 6 ($\sim$)
human proinsulin	35 ± 7 ($\vee$)	55 ± 10 ($\wedge$)	49 ± 11 ($\sim$)	63 ± 11 ($\wedge$)	42 ± 8 ($\sim$)	26 ± 5 ($\vee$)	37 ± 8 ($\sim$)
EGF[b]	5 ± 2	8 ± 3	0	10 ± 3	3 ± 2	2 ± 2	2 ± 1

[a] Spheroplasts from logarithmically grown yeast cells were incubated at a density of 5−8 mg wet mass/mL for 60 min in succinate medium as described in the Experimental Procedures. After further incubation for 15 min, glucose (final concentration 50 mM) was added and the incubation was continued for 15 min in the absence or presence of the corresponding hormone (1 μM final concentration). Aliquots of the spheroplast suspension were taken at the time points indicated. Glycogen synthase activity and competition of binding of human iodinated insulin to the partially purified 58 kDa insulin-binding protein as well as its insulin-dependent phosphorylation were assayed as described previously (*34*, *35*). Specific binding is given as displacement of trace amounts of [^{125}I]monoiodo[B_{26}]insulin by 10 μM unlabeled competitor. The amounts of radiolabeled insulin displaced by human insulin (difference between total associated insulin and insulin associated in the presence of competitor) and of the ^{32}P-labeled 58-kDa insulin-binding protein autophosphorylated in the presence of 1 μM human insulin are set at 100%. PP2A, PKA, cAMP-PDE, and GPI-PLC activities were measured as described in the Experimental Procedures. GPI-PLC activity reflects the amount of total hydrophilic Gce1p produced. In each case the difference between the activity measured for hormone-treated and basal spheroplasts is given as percentage of the maximal insulin effect (set at 100%). Each value represents the mean ± SD of three to four independent incubations with determinations in quadruplicate. ($\wedge$) $p < 0.05$; ($\sim$) $p < 0.02$; ($\vee$) $p < 0.01$ vs human insulin. [b] *p*-Levels for both analogue IV and EGF vs basal were >0.05 in each case. The insulin analogues have the following deviations from the amino acid sequence of human insulin with the EC_{50}-values (nM ± SEM, $n = 5$) determined from the corresponding concentration (0.01−100 nM)-response curves for stimulation of lipogenesis in isolated rat adipocytes as indicated: human insulin, 0.12 ± 0.05; analogue I, Lys(B3)-Glu(B29), 0.10 ± 0.04; analogue II, Gly(A21)-diLys(B31), 0.65 ± 0.19; analogue III, Gly(A21)-His(B1)-His(B3)-diArg(B31), 3.77 ± 0.38; analogue IV, Met(A3)-Gly(A21)-His(B31)-Ala(B32)-Ala(B33)-Arg(B34), >10 ± 4.

binding, affinity cross-linking, and insulin-induced serine/threonine-specific phosphorylation (*34*, *35*), is involved in mediating insulin stimulation of glycogen synthesis via a signaling cascade consisting of the elements PP2A, PKA, cAMP-PDE, and GPI-PLC. For this, we compared the efficiencies of four different analogues of insulin (which exhibit different EC_{50}-values for activation of lipogenesis in isolated rat adipocytes, see ref *33*), proinsulin, IGF-1, and EGF in (i) binding to the partially purified insulin-binding protein, (ii) increasing the serine/threonine phosphorylation of the binding protein, and (iii) stimulating glycogen synthase with those required for modulating PP2A, PKA, cAMP-PDE, and GPI-PLC (Table 2). The functional assays were performed with glucose-induced spheroplasts incubated in the presence or absence of 1 μM hormone. The ranking in the relative efficiencies of the various insulin analogues in competing insulin binding and activating glycogen synthase (human insulin > analogue I > analogue II > analogue III > analogue IV) was consistently reflected in both modulating the key regulatory enzymes PP2A, PKA, cAMP-PDE, and GPI-PLC and inducing serine/threonine phosphorylation of the 58 kDa insulin-binding protein. IGF-1 and the structurally related proinsulin exerted moderate effects comparable to those of analogue II. Analogue IV and EGF were almost inactive in each assay studied in line with their very low insulin-mimetic activity in isolated rat adipocytes (*33*). This

perfect correlation between the ranking of their affinity to the binding protein as well as their ability to trigger its phosphorylation and the ranking of their relative potencies in modulating PP2A, PKA, cAMP-PDE, and GPI-PLC activities strongly argues for involvement of these components in mediating the effect of insulin on glycogen metabolism in *S. cerevisiae*.

DISCUSSION

Insulin is produced in large amounts in β-cells of the pancreas in mammals, but also occurs in the most primitive vertebrates and complex invertebrates, which in terms of evolution are about 500 million years old (*42*). The existence of material extremely similar to mammalian insulin with regard to specific cross-reactivity in the insulin radioimmunoassay and in the insulin bioassay has also been reported for three unicellular eukaryotes, such as *Tetrahymena pyriformis*, *Neurospora crassa*, *Aspergillus fumigatus* (for a review, see ref *43*). Thus, accumulating evidence hints to the origin of the insulin molecule about 1 billion years ago (*44*). However, data on the presence of insulin-like material in yeast is lacking so far. Although the genome of baker's yeast has been completely sequenced, searches for insulin-like sequences in the databases have been unsuccessful probably due to one or both of two reasons: (i) insulin-like material identified in sponges and other lower eukaryotes reveals little sequence similarity with mammalian insulin so that comparative sequence analysis of the yeast genome has left the presumptive insulin gene undetected; (ii) since the presumptive insulin gene encodes a polypeptide of, likely, less than a hundred amino acids in length, it may not have been assigned an open reading frame in the systematic sequence analysis.

Recently, we reported the surprising finding that human insulin exerts marked and specific effects on oxidative and nonoxidative glucose metabolism in *S. cerevisiae* (*33*). Moreover, yeast cells have been reported to harbor a saturable number of high-affinity binding sites for human insulin at their surface, and human insulin could be chemically cross-linked to a unique plasma membrane protein of yeast (*34*). The much lower molecular mass of the cross-linked polypeptide as determined by SDS−PAGE (54 kDa) compared to the binding subunit of mammalian insulin receptors (125 kDa) argues that the mechanisms of signal perception and transmission might be different and analogous at best. Nevertheless, it has been reported that human insulin exerts a large number of effects on carbohydrate metabolism in a fashion similar to mammals arguing that yeast cells produce, and respond to, insulin-like material and that this signaling pathway plays a role in the modulation of glucose repression/derepression. In detail, glycogen storage under conditions of growth limitation in glucose medium (i.e., transition of yeast cells from late logarithmic to stationary phase or incubation of spheroplasts in synthetic glucose medium) was markedly elevated in the presence of human insulin. Insulin seemed to effect an increase in sensitivity of both glycogen synthase for activation by glucose and glycogen phosphorylase for inactivation by glucose. The activity of both enzymes is regulated by phosphorylation/dephosphorylation. It is generally assumed that their phosphorylation state is controlled by a complex interplay between PKA and serine/threonine-specific phosphatases, like PP2A (*14*), which are activated directly or indirectly by SNF1 kinase (*45*). SNF1 kinase has been demonstrated to be induced under conditions of glycogen accumulation in yeast (*46*, *47*) and to be further enhanced in the presence of insulin (*33*). These data argue that an insulin-like signaling system acts and primarily modulates carbohydrate metabolism in yeast and raises the possibility that human insulin is recognized by, and acts on top of, an intrinsic insulin-like signaling system. In agreement with the proposed linkage between glycogen storage and SNF1, PP2A, and PKA activities, the present study revealed in both glucose-exhausted cells and glucose-induced spheroplasts that the presence of human insulin significantly (i) increases PP2A activity, (ii) reduces PKA activity in a rapid fashion, and (iii) elevates cAMP-PDE activity after longer periods of treatment (which can be performed with intact cells, only). In addition to the long-term degradation of cytosolic cAMP by cAMP-PDE, a direct inhibition of PKA by an insulin-dependent but cAMP-independent covalent and/or allosteric mechanism is likely to operate as judged from the evident kinetic differences in inhibition of PKA and activation of cAMP-PDE by human insulin. The existence of allosteric effectors of protein phosphatases in mammalian cells has been postulated, albeit evidence is indirect so far, only (see below).

Phosphoinositolglycan peptides prepared in vitro from the GPI-proteins, Gce1p, of *S. cerevisiae* and, VSG, of *Trypanosoma brucei* as well as peptide-free PIG molecules have been demonstrated to mimick the phosphorylation control elicited by insulin on a number of phosphoproteins upon incubation with isolated rat adipocytes (*48*−*51*) and to exhibit potent insulin-mimetic effects in isolated rat adipocytes, cardiomyocytes, and diaphragms (*25*, *52*). Furthermore, from structurally related molecules prepared from isolated and cultured fat and muscle cells, activation of nonoxidative and oxidative glucose metabolism has been reported, relying presumably on the induction of phosphorylation/dephosphorylation of glycogen synthase, glycogen phosphorylase, and pyruvate dehydrogenase as well as inhibition of PKA (*53*−*56*). In contrast to former speculations that these compounds directly alter the activity of protein kinases and phosphatases involved in the regulation of glucose and lipid metabolism in an allosteric manner (*53*, *57*), we recently obtained strong experimental evidence for isolated rat adipocytes in favor of an efficient cross-talk of PIG molecules with the insulin signaling cascade downstream of the insulin receptor at the level of tyrosine phosphorylation of the IRS proteins (*26*, *27*). These data show that in insulin-responsive mammalian cells GPI anchor structures are likely to fulfill a role in the modulation of metabolic insulin effects.

In yeast, the generation by endogenous mechanisms and physiological role in response to an exogenous stimulus (e.g., glucose repression) of PIG structures have not yet been documented. However, yeast contains the processing machinery for lipolytic cleavage of GPI lipids and GPI proteins as well as double lipolytic/proteolytic cleavage of GPI proteins, which is a prerequisite for the release of PIG molecules (*29*−*31*). The present observation that, in *S. cerevisiae* cells and spheroplasts, human insulin accelerates and stimulates the GPI-PLC and increases the efficiency of double processing of GPI-proteins at concentrations and under growth conditions, which favor glycogen synthesis,

is compatible with the assumption that the GPI-PLC is one of the key players regulating glycogen synthase activity in yeast in concert with PP2A, PKA, and cAMP-PDE. The identical ranking in the efficiency of various insulin analogues in activating glycogen synthase, PP2A, PKA, cAMP-PDE, and GPI-PLC supports this view. It is tempting to speculate that the GPI-PLC may be epistatic to PP2A, PKA, and cAMP-PDE in controlling their activity via a molecular mechanism involving soluble PIG molecules which induce phosphorylation of common key signaling components as has been demonstrated recently with PIG-induced tyrosine phosphorylation of IRS proteins via the nonreceptor tyrosine kinases, $pp59^{Lyn}$ and $pp125^{FAK}$ in isolated rat adipocytes (*58*, *59*).

The present findings suggest that a signal transduction cascade linking the initial events of specific insulin binding and phosphorylation of the corresponding insulin-binding protein to the metabolic end effector systems, such as glycogen synthase, via modulation of the corresponding key regulatory enzymes, PP2A, PKA, cAMP-PDE, and GPI-PLC has not evolved primarily for the typical (neuro)endocrinous system of multicellular invertebrates and vertebrates. Rather, the insulin-signaling cascade seems to be evolutionary much more ancient than has been thought and may orginally constitute the regulatory component of a metabolic, biosynthetic or proliferative pathway characteristic for the physiology of yeast. In this sense, it may guarantee a more efficient storage of glycogen under conditions of limited glucose availability. Alternatively, it may have evolved in this low unicellular eukaryotic organism for glucose regulation of cell wall biosynthesis. Compatible with this admittedly very speculative view is the finding that, under conditions of growth restriction in glucose medium, insulin promotes double processing of GPI protein anchors, which presumably is prerequisite for anchorage of certain mannoproteins to the cell surface of yeast (*31*). Furthermore, in recent years, *S. cerevisiae* and *Tetrahymena thermophila* have been studied for a potential role of insulin in regulating cell proliferation in unicellular eukaryotes (*60*). In an in vivo assay system containing diluted Wickerham's medium with 2% glycerol as the only carbon source, addition of low concentrations of insulin significantly reduced the lag phase of growth of *S. cerevisiae* (*61*). Interestingly, activators of protein kinase C, phorbol ester (PMA), or oleyl acetyl glycerol (DAG), in combination with bradykinin, a mammalian intercellular signaling molecule, which leads to a rise in intracellular Ca^{2+} (*62*), caused a considerable shortening of the lag phase in a similar fashion as insulin, whereas PMA and DAG alone were ineffective (*61*, *63*). Furthermore, PMA and insulin induced a synergistic effect, enabling cells to start proliferation after very short lag (*61*). These results hint to insulin stimulation of the transition of cells from the lag phase to proliferation via participation of a Ca^{2+}-dependent protein kinase C isoform. Altogether these findings suggest that an insulin-like signal transduction cascade is somehow involved in the regulation of metabolic, cell wall biosynthetic and/or proliferative pathways in unicellular eukaryotes, such as yeast, which uses components analoguous to those operating in the different branches of metabolic and mitogenic insulin signaling in mammalian tissues, such as PP2A, PKA, cAMP-PDE, GPI-PLC, and protein kinase C. The specificity and relative potency of human insulin on a set of typical signaling processes in *S. cerevisiae* all related to sensing of nutrient availability argues for the presence of insulin-like signaling molecules and the corresponding insulin-like signal transduction machinery in yeast. During evolution, this putatively very ancient pathway for nutrient signaling may have been adapted by multicellular invertebrates and vertebrates predominantly to control blood glucose homeostasis. Certainly, the physiological relevance of the apparent insulin-like signal transduction cascade in yeast will only be elucidated by the identification of the genes involved and of naturally occurring endogenous/exogenous factor(s)/stimuli modulating the operation of this pathway. This emphasizes the next level of experimental studies we are currently addressing.

REFERENCES

1. Hwang, P. K., Tugendreich, S., and Fletterick, R. J. (1989) *Mol. Cell. Biol. 9*, 1659−1666.
2. Farkas, I., Hardy, T. A., Goebl, M. G., and Roach, P. J. (1991) *J. Biol. Chem. 266*, 15602−15607.
3. Rothman-Denes, L. B., and Cabib, E. (1970) *Proc. Natl. Acad. Sci. U.S.A. 66*, 967−974.
4. Rothman-Denes, L. B., and Cabib, E. (1971) *Biochemistry 10*, 1236−1242.
5. Huang, K.-P., and Cabib, E. (1972) *Biochem. Biophys. Res. Commun. 49*, 1610−1616.
6. Becker, J. U., Wingender-Drissen, R., and Schiltz, E. (1983) *Arch. Biochem. Biophys. 225*, 667−678.
7. Hardy, T. A., and Roach, P. J. (1993) *J. Biol. Chem. 268*, 23799−23805.
8. Ingebritsen, T. S., Foulkes, G., and Cohen, P. (1983) *Eur. J. Biochem. 132*, 263−274.
9. Peng, Z.-Y., Wang, W., Wilson, S. E., Schlender, K. K., Trumbly, R. J., and Reimann, E. M. (1991) *J. Biol. Chem. 266*, 10925−10932.
10. Sneddon, A. A., Cohen, P. T. W., and Stark, M. J. R. (1990) *EMBO J. 9*, 4338−4346.
11. Ronne, H., Carlberg, M., Hu, G.-Z., and Nehlin, J. O. (1991) *Mol. Cell. Biol. 11*, 4876−4884.
12. van Zyl, W., Huang, W., Sneddon, A. A., Stark, M., Carnier, S., Werner, M., Marck, C., Sentenac, A., and Broach, J. R. (1992) *Mol. Cell. Biol. 12*, 4946−4959.
13. Healy, A. M., Zolnierowicz, S., Stapleton, A. E., Goebl, M., DePaoli-Roach, A. A., and Pringle, J. R. (1991) *Mol. Cell. Biol. 11*, 5767−5780.
14. Clotet, J., Posas, F., Hu, G.-Z., Ronne, H., and Arino, J. (1995) *Eur. Biochem. J. 229*, 207−214.
15. Posas, F., Clotet, J., and Arino, J. (1991) *FEBS Lett. 279*, 341−345.
16. Cannon, J. F., and Tatchell, K. (1987) *Mol. Cell. Biol. 7*, 2653−2663.
17. Toda, T., Uno, I., Ishikawa, T., Powers, S., Kataoka, T., Broek, D., Cameron, S., Broach, J., Matsumoto, K., and Wigler, M. (1985) *Cell 40*, 27−36.
18. Farkas, I., Hardy, T. A., DePaoli-Roach, A. A., and Roach, P. J. (1990) *J. Biol. Chem. 265*, 20879−20886.
19. Larner, J. (1994) *Endocr. J. 2*, 167−171.
20. Saltiel, A. R. (1994) *FASEB J. 8*, 1034−1040.
21. Lazar, D. F., Knez, J. J., Medof, M. E., Cuatrecasas, P., and Saltiel, A. R. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 9665−9669.
22. Romero, G., Luttrell, L., Rogol, A., Zeller, K., Hewlett, E., and Larner, J. (1988) *Science 240*, 509−511.
23. Chan, B. L., Lisanti, M. P., Rodriguez-Boulan, E., and Saltiel, A. R. (1988) *Science 241*, 1670−1672.
24. Müller, G., Dearey, E.-A., Korndörfer, A., and Bandlow, W. (1994) *J. Cell Biol. 126*, 1267−1276.
25. Müller, G., Wied, S., Crecelius, A., Kessler, A., and Eckel, J. (1997) *Endocrinology 138*, 3459−3475.
26. Müller, G., Wied, S., Piossek, C., Bauer, A., Bauer, J., and Frick, W. (1998) *Mol. Med. 4*, 299−323.

27. Frick, W., Bauer, A., Bauer, J., Wied, S., and Müller, G. (1998) *Biochem. J.* 336, 163−181.
28. Frick, W., Bauer, A., Bauer, J., Wied, S., and Müller, G. (1998) *Biochemistry 38*, 13421−13436.
29. Müller, G., and Bandlow, W. (1993) *J. Cell Biol. 122*, 325−336.
30. Bandlow, W., Wied, S., and Müller, G. (1996) *Arch. Biochem. Biophys. 324*, 300−316.
31. Müller, G., Gross, E., Wied, S., and Bandlow, W. (1996) *Mol. Cell. Biol. 16*, 442−456.
32. Niemer, I., Müller, G., Strobel, G., and Bandlow, W. (1997) *Curr. Genet. 32*, 41−51.
33. Müller, G., Rouveyre, N., Crecelius, A., and Bandlow, W. (1998) *Biochemistry 37*, 8683−8695.
34. Müller, G., Rouveyre, N., Upshon, C., Gross, E., and Bandlow, W. (1998) *Biochemistry 37*, 8696−8704.
35. Müller, G., Rouveyre, N., Upshon, C., and Bandlow, W. (1998) *Biochemistry 37*, 8705−8713.
36. Müller, G., and Bandlow, W. (1989) *Biochemistry 28*, 9957−9967.
37. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
38. Müller, G., Schubert, K., Fiedler, F., and Bandlow, W. (1992) *J. Biol. Chem. 267*, 25337−25346.
39. Müller, G., and Bandlow, W. (1991) *Eur. J. Biochem. 202*, 299−308.
40. Wessel, D., and Flügge, U. I. (1984) *Anal. Biochem. 138*, 141−143.
41. Müller, G., and Zimmermann, R. (1987) *EMBO J. 6*, 2099−2107.
42. Conlon, J. M., Reinecke, M., Thorndyke, M. C., and Falkmer, S. (1988) *Horm. Metabol. Res. 20*, 406−410.
43. Lenard, J. (1992) *Trends Biol. Sci. 17*, 147−150.
44. Le Roith, D., Shiloach, J., Roth, J., and Lesniak, M. A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 6184−6188.
45. Hardy, T. A., Huang, D., and Roach, P. J. (1984) *J. Biol. Chem. 269*, 27907−27913.
46. Celenza, J. L., and Carlson, M. (1986) *Science 233*, 1175−1180.
47. Celenza, J. L., and Carlson, M. (1989) *Mol. Cell. Biol. 9*, 5034−5044.
48. Kessler, A., Müller, G., Wied, S., Crecelius, A., and Eckel, J. (1998) *Biochem. J. 330*, 277−286.
49. Misek, D. E., and Saltiel, A. R. (1994) *Endocrinology 135*, 1869−1876.
50. Alemany, S., Mato, J. M., and Stralfors, P. (1987) *Nature 330*, 77−79.
51. Stralfors, P., and Alemany, S. (1990) *FEBS Lett. 268*, 169−172.
52. Misek, D. E., and Saltiel, A. R. (1992) *J. Biol. Chem. 267*, 16266−16273.
53. Villar-Palasi, C., and Zhang, C. (1990) *Abstracts Annual Meeting of American Society of Biochemistry and Molecular Biology*, New Orleans, pA 2082.
54. Deeg, M. A., Murray, N. R., and Rosenberry, T. L. (1992) *Diabetes 42*, 1318−1323.
55. Jones, D. R., and Varela-Nieto, I. (1998) *Int. J. Biochem. Cell Biol. 30*, 313−326.
56. Varela-Nieto, I., Leon, Y., and Caro, H. N. (1996) *Comp. Biochem. Physiol. 115B*, 223−241.
57. Larner, J. (1987) *Diabetes 37*, 262−275.
58. Müller, G., Welte, S., Bauer, A., and Frick, W. (1999) *Diabetes 48* (Suppl. 1, abstract), A219−A220.
59. Müller, G., Welte, S., Wied, S., Jung, C., and Frick, W. (1999) *Diabetologia 42* (Suppl. 1, abstract), A66.
60. Christensen, S. T., Leick, V., Rasmussen, L., and Wheatley, D. N. (1998) *Int. Rev. Cytol. 177*, 181−253.
61. Friis, J., Szablewski, L., Christensen, S. T., Schousboe, A. L., and Rasmussen, L. (1994) *FEMS Microbiol. Lett. 123*, 33−36.
62. Roberts, R. A., and Gullick, W. J. (1989) *J. Cell Sci. 94*, 527−535.
63. Overgaard, A. K., Friis, J., Christensen, L., Christiansen, H., and Rasmussen, L. (1995) *FEMS Microbiol. Lett. 132*, 159−163.

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