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Insulin Signaling in the Yeast *Saccharomyces cerevisiae*. 1. Stimulation of Glucose Metabolism and Snf1 Kinase by Human Insulin

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ABSTRACT: Effects of human insulin on glucose metabolism in the yeast *Saccharomyces cerevisiae* were studied in this report. Under two conditions of growth limitation (glucose-grown cells during transition to stationary phase or spheroplasts during incubation in synthetic glucose medium), human insulin (10 and 1 μM, respectively) enhanced glycogen accumulation and glycogen synthase activity by 40−60% compared to control cells. Glycogen phosphorylase activity was also increased under the same conditions, but this stimulation was diminished by 35−45% in insulin-treated compared to control cells. Thus, under growth limitation, insulin causes glycogen phosphorylase and glycogen synthase to become more sensitive to inactivation and activation, respectively. In glucose-induced spheroplasts, insulin (1 μM), in addition to glycogen accumulation, led to about 2-fold increases of the rates of ethanol production and glucose oxidation compared to control cells, and the maximal concentration of hexose 6-phosphate was increased by 30−40%. In contrast, glucose transport as well as the levels of the allosteric regulators, fructose 2,6-bisphosphate and cAMP, were not altered at all. Snf1 kinase is assumed to be involved in the regulation of glycogen metabolism in yeast, although it does not seem to be modulated directly by the glucose concentration. Snf1 kinase activity was elevated 5−10-fold in response to insulin both during glucose induction of yeast spheroplasts and during transition to stationary phase of glucose-grown cells. We conclude that *Saccharomyces cerevisiae* and insulin-sensitive mammalian cells share some parts of the signaling cascades regulating oxidative and nonoxidative glucose metabolism in response to glucose and insulin.

Maintenance of glucose homeostasis has been established as the major physiological role for insulin in mammals for many years. This function seemed to be tightly coupled to the endocrine pancreas, an organ found only in vertebrates. Several findings have challanged this common view: (i) insulin is synthesized and probably secreted in a wide variety of vertebrate cells not primarily involved in glucose homeostasis (*1*, *2*); (ii) insulin is required for growth by a great number of cell types in defined culture media (*3*); and (iii) insulin-like material has been detected in cells of organisms that lack pancreatic or islet cells, such as insects, molluscs, worms, plants (*4*−*7*), certain lower unicellular eucaryotes (*8*, see also Discussion), and even prokaryotes (*9*, *10*). These findings point to a more fundamental and ancient biological role for insulin (*11*) or to an evolutionary predecessor of insulin.

The occurrence of insulin-like material in invertebrate cells and protozoa has been amply documented (*4*−*8*; for reviews, see refs *12*, *13*), but knowledge on its function in these cells is extremely limited. Partially purified insulin-like material from insects exerts hypoglycemic activity in these animals (*14*, *15*), and mammalian insulin promotes growth and differentiation of cultured *Drosophila melanogaster* cells (*16*). Added to the bathing medium, insulin was shown to stimulate oxygen uptake in *Acetabularia mediterranea* (*17*), and glucose transport in *Tetrahymena pyriformis* (*18*). Even in *Neurospora crassa*, insulin effects, i.e., decrease of adenylate cyclase activity in crude membrane preparations (*19*) as well as increase of metabolism and growth rate, have been reported (see also Discussion).

For yeast, and other microorganisms, the ability to sense the availability of nutrients is vital. It involves signal transduction mechanisms similar, at least in principle, to those operating in mammalian cells in response to hormones, growth factors, and other extracellular regulators. Glycogen metabolism in mammalian cells is regulated primarily by hormones in response to changes in blood glucose levels. In yeast, the exhaustion of nutrients (carbon, nitrogen, sulfur) is signaled to the metabolic program necessary for entry into stationary phase. For example, in batch culture the accumulation of the major carbohydrates, glycogen and trehalose, starts (1) during the late logarithmic growth and at the beginning stationary phase, but prior to the exhaustion of carbon sources (*20*−*22*), or (2) during the logarithmic growth on a nonfermentable carbon source in the absence of nitrogen or sulfur (*22*−*25*). Glycogen synthase and glycogen phosphorylase activities increase in parallel under these conditions.

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Glycogen breakdown is catalyzed by the enzyme glycogen phosphorylase (GPH),[1] encoded by the gene *GPH1* (*26*), whereas two genes, *GSY1* and *GSY2*, code for *Saccharomyces cerevisiae* glycogen synthases (GSY),[1] the key isozymes of glycogen synthesis (*27*, *28*). *GPH1* transcription is induced in late exponential phase (*26*) concomitant with the emergence of glycogen. Approximately at the same time, Gsy2p, the major isoform of GSY, is also induced (*28*). Genetic and biochemical evidence suggest that, in yeast, GPH and GSY activities can be modulated, as in other eucaryotes, through phosphorylation−dephosphorylation mechanisms (*20*, *29*−*32*). Phosphorylation is assumed to inactivate Gsy2p and to activate Gph1p, whereas dephosphorylation effects the opposite. Several reports indicate that PKAs are involved in the control of these enzymatic activities in yeast (*33*). It is generally accepted that phosphorylation of Gsy2p and Gph1p is controlled by the levels of cAMP through the activation of the PKA (for a review, see ref *34*), although cAMP-independent mechanisms could also be important for the regulation of glycogen metabolism in yeast.

The apparent analogy in the molecular regulation of glycogen metabolism between mammals and yeast prompted us to study whether mammalian insulin exerts a stimulatory effect on glycogen accumulation in *Saccharomyces cerevisiae* as it does in insulin-sensitive mammalian cells (adipocytes, muscle cells, hepatocytes). We found that under conditions of limited growth yeast spheroplasts as well as intact cells respond to human insulin with respect to both oxidative and nonoxidative glucose metabolism in a specific manner.

EXPERIMENTAL PROCEDURES

Materials. The wild-type *Saccharomcyes cerevisiae* strain X2180 was used throughout the study. D-[U-^{14}C]Glucose 1-phosphate (180 mCi/mmol), UDP-D-[U-^{14}C]glucose (250 mCi/mmol), 2-deoxy-D-[2,6-^{3}H]glucose (60 Ci/mmol), and the [^{3}H]cAMP assay kit and the ECL detection system were provided by Amersham-Buchler (Braunschweig, Germany). [γ-^{32}P]ATP (3000 Ci/mmol) was from DuPont/NEN (Bad Homburg, Germany). D-[U-^{14}C]Glucose (320 mCi/mmol) and L-[1-^{14}C]glucose (55 mCi/mmol) were bought from Biotrend (Köln, Germany). Glucose, glucose 6-phosphate, 2-deoxyglucose, 3-*O*-methylglucose, BSA (defatted, fraction V), and thiamin hydrochloride were purchased from Sigma (Deisenhofen, Germany). Yeast extract and bacto-peptone were obtained from BRL/Gibco (Eggenstein/Leopoldshafen, Germany). Glycogen from rabbit skeletal muscle was provided by Calbiochem (Bad Soden, Germany). Enzymes were purchased from Boehringer Mannheim (Mannheim, Germany). Semisynthetic human insulin and the recombinant insulin analogues were made available by the Pharma Synthesis Department of Hoechst Marion Roussel Deutschland GmbH (Frankfurt, Germany). Recombinant human IGFI/II and EGF were bought from Boehringer Mannheim. Recombinant human glucagon was delivered by Calbiochem. Other chemicals were from Merck (Darmstadt, Germany).

Growth and Incubation Conditions. Yeast cells were grown at 30 °C on an orbital shaker at 250 rpm in YPD medium containing 1% yeast extract, 2% bacto-peptone, and 2% glucose. Growth was monitored by measuring the absorbance at 660 nm (A_{660}). (1) For studying the insulin effect on glucose induction of spheroplasts, cells were harvested by centrifugation at the logarithmic phase of growth (3×10^7 cells/mL), washed twice with water, and converted to spheroplasts by Zymolyase digestion of the cell walls as described previously (*35*). Spheroplasts were suspended at 2×10^6 cells/mL in medium containing 100 mM succinate, 10 mM KCl, 5 mM $MgCl_2$, 2 mM KH_2PO_4, 5 μM thiamin, 1.1 M sorbitol, and 0.05% yeast extract (pH 5.5) and incubated for 60 min at 30 °C. After addition of glucose at a final concentration of 100 mM together with hormone at the final concentration as indicated, the incubation was continued for variable periods and terminated by centrifugation of the spheroplasts through a cushion of Ficoll/sucrose (*36*). (2) For analysis of the insulin effect on intact cells during the transition from exponential to stationary phase, yeast was grown in YPD medium containing or lacking human insulin at the final concentration indicated in the figure legends (the insulin was supplemented at the beginning and then, in identical amounts, at 3-h intervals throughout the total incubation period). Cells were harvested, when A_{660} was 5−11, i.e., 8−25 h after the glucose was exhausted from the medium. Aliquots of the cells were collected by vacuum filtration (see below).

GSY and GPH Activities. Spheroplasts were collected by centrifugation of the total incubation mixture through a cushion of Ficoll/sucrose (*36*). Intact cells (15-mL samples at 5×10^8 cells/mL) were harvested by filtration under vaccum (Whatman, HWPA filters, 0.45 μM). The spheroplast pellets or cell cakes were scraped off the tube and filter, respectively, with a spatula and immediately frozen in liquid nitrogen. This method of sampling was essential in order to preserve the activity state of GSY and GPH at the time of termination of the culture and to allow detection of rapid changes of activity. A cell-free extract was prepared by vortexing 50- to 100-mg portions of frozen cells in 1 mL of 25 mM HEPES/KOH (pH 7.2), 100 mM KCl, 2% glycogen, 0.25 mM sucrose, 100 mM KF, 0.2 mM PMSF, 0.5 mM benzamidine, 1.5 mM DTT, 2 mM EDTA, 1 mM EGTA with 1 g of ice-cold acid-washed glass beads for six 30-s periods at 4 °C and subsequent centrifugation (10000*g*, 2 min, 4 °C). (i) For measurement of GSY, 15−25 μL of the extract was added to an assay mixture (total volume of 100 μL) containing 50 mM glycylglycine (pH 7.4), 0.50 mM UDP-[U-^{14}C]glucose (2100 dpm/nmol), 15 mM Na_2SO_4, 2 mM EDTA, 2% glycogen. After 20 min of incubation at 30 °C in the absence (for determination of the I-form of GSY) or, alternatively, in the absence and presence of 10 mM glucose 6-phosphate (for determination of the activity ratio, as indicated), an 80-μL portion of the mixture was spotted on ET31 paper (Whatman, Springfields Mills, U.K.). Further processing of the filters, determination of the radioactivity, and calculation of activity ratio were performed as described (*37*). Under these conditions, incorporation of radiolabeled glucose from UDP-glucose into glycogen was linear for 30 min. A unit of activity is defined as the amount of enzyme that catalyzes the transfer of 1 μmol of glucose from UDP-glucose to glycogen in 1 min under conditions of the standard assay. The state of activation of GSY is expressed as the ratio between the activity measured in the

[1] Abbreviations: EGF, epidermal growth factor; IGFI/II, insulin-like growth factor I/II; GSY, glycogen synthase; GPH, glycogen phosphorylase; PKA, cAMP-dependent protein kinase A; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

absence of exogenous glucose 6-phosphate and the activity measured in the presence of 10 mM glucose 6-phosphate. The −/+ glucose-6-P activity ratio is frequently used as a kinetic index of the phosphorylation state. Decreasing values are correlated with increased phosphorylation. Activity measured in the presence of glucose-6-P, designated total activity, reflects the GSY concentration, independent of the phosphorylation state under the conditions of the assay. (ii) For measurement of GPH (in the direction of glycogen synthesis as incorporation of radiolabeled glucose from glucose 1-phosphate into glycogen according to refs *38*, *39*), 10−20 μL of extract was added to an assay mixture (total volume 100 μL) containing 50 mM sodium succinate (pH 5.8), 10 mM [U-^{14}C]glucose 1-phosphate (1100 dpm/nmol), 3% glycogen, 2 mM EDTA. After 30 min of incubation at 30 °C, the reaction was terminated as described for GSY. Under these conditions, the assay was linear for 45 min, and less than 15% of glucose 1-phosphate was consumed. To measure both the active form and the total GPH activity, samples of the cell culture were divided into two parts, and growth was continued for 10 min. One aliquot was adjusted to 2 mM 2,4-dinitrophenol (in dimethyl sulfoxide); the other aliquot received the same volume of dimethyl sulfoxide. After 2 min of incubation under shaking at 30 °C, cells were collected and extracts prepared as described above. GPH activity in 2,4-dinitrophenol-treated cells corresponds to total enzyme activity, and activity measured in untreated cells is regarded as the active form of the enzyme (*30*). One unit is the amount of GSY/GPH that catalyzes the incorporation of 1 μmol of radiolabeled glucose into glycogen in 1 min under the conditions of the assay.

Glucose Oxidation. Spheroplasts in 5 mL of medium containing 100 mM succinate, 10 mM KCl, 5 mM $MgCl_2$, 2 mM KH_2PO_4, 5 μM thiamin (pH 5.5) at 2×10^6 cells/mL were incubated for 60 min at 30 °C. After addition of [U-^{14}C]glucose to a final concentration of 20 mM (1.5 μCi/mL), the flasks were sealed with rubber serum stoppers fitted with hanging glass wells which contained cylinders of Whatman No. 1 paper. At the end of the incubation periods (5, 10, 20, or 30 min), 0.5 mL of hyamine hydroxide was injected onto the filters, and the spheroplast suspension was supplemented with sulfuric acid to a final concentration of 0.5 M. After the flasks had been shaken for 15 min at 25 °C, the filters were transferred into scintillation vials containing 10 mL of toluene-based scintillation cocktail and counted. Under these conditions, [^{14}C]CO_2 release was linear over a 60-min period.

Glucose Transport Assay. (1) Cells, collected by vaccum filtration (see above), were washed twice with 20-mL portions of 0.1 M Tris/citrate (pH 6.0), and suspended in the same buffer at a final concentration of 2−6 mg wet mass/mL. Portions (0.1 mL) were incubated under shaking in 18 × 150 mm tubes at 30 °C. Uptake was initiated by addition of 0.1 mL of 10 mM 2-[^{3}H]deoxyglucose (5 μCi) in Tris/citrate buffer. After incubation for various periods of time at 30 °C, transport was terminated by addition of 10 mL of ice-cold distilled water, and the cells were immediately filtered through GF/C glass-fiber filters (Whatman) at reduced pressure. The filters were washed twice with 10 mL of ice-cold water each and dried. Radioactivity was measured in a liquid scintillation counter using 10 mL of scintillation cocktail (Aquasol, Beckman Instruments, Munich). Specific glucose uptake was calculated as the difference between the total radiolabeled deoxyglucose associated with the cell cake and the radiolabel nonspecifically adsorbed to the filters and cells as determined by dilution of the incubation mixture with 10 mL of water prior to initiation of the uptake. (2) Spheroplasts were collected by centrifugation through Ficoll/sucrose (see above) and resuspended in 0.1 M succinic acid (pH 5.5, adjusted by Trizma base), 0.4 M $MgSO_4$, 20 mM KCl, 1 mM β-mercaptoethanol at 0.1 mg of protein/mL in a final volume of 0.4 mL. Uptake was initiated by addition of 0.1 mL of 20 mM 2-deoxy-[^{3}H]-glucose (2 μCi) and L-[^{14}C]glucose (0.1 μCi). After incubation at 30 °C for the appropriate time, 400 μL of the incubation mixture was layered over 5 mL of ice-cold 10% Ficoll in the medium above and centrifuged (8000*g*, 10 min, 4 °C, swing-out rotor). The upper layer was removed by aspiration, and the upper area of the Ficoll cushion was then washed 2 times by layering water over the Ficoll and removing it by aspiration. Finally, the Ficoll cushion was removed by aspiration. The pelleted spheroplasts were suspended in 1 mL of water, supplemented with 10 mL of Aquasol, and radioactivity was measured by liquid scintillation counting. After correction for counting efficiencies of ^{3}H- and ^{14}C-radioactivities, specific glucose uptake was calculated as the difference between radiolabeled 2-deoxyglucose and L-glucose (corrected for the different specific radioactivities) associated with the spheroplast pellet.

Snf1p Immunoprecipitation and Kinase Assay. Collected cells or spheroplasts were frozen in an ethanol−dry ice bath, and thawed on ice in 25 mM Tris/HCl (pH 7.2), 0.5 mM PMSF (2×10^8 cells/mL). The resuspended cells/spheroplasts were broken by vortexing with 1 g of ice-cold glass beads. The lysates were cleared by centrifugation (2000*g*, 10 min). Two hundred fifty microliters of cell extract (1 mg of protein) was diluted with 250 μL of immunoprecipitation buffer (50 mM Tris/HCl, pH 7.5, 1% TX-100, 0.5 mg of BSA/mL). After incubation on ice for 30 min, the extract was centrifuged (13000*g*, 15 min, 4 °C); 400 μL of the supernatant was diluted with 1 mL of ice-cold 50 mM Tris/HCl (pH 7.5), 0.2% TX-100. Six microliters of affinity-purified anti-Snf1p antibody was added. After 4 h at 4 °C, 50 μL of protein A−Sepharose (50 mg/mL) in immunoprecipitation buffer was added and the incubation continued for 1 h. The adsorbed immune complexes were collected by centrifugation (12000*g*, 2 min), washed twice with immunoprecipitation buffer containing 144 mM NaCl, once with immunoprecipitation buffer lacking BSA, and once with kinase buffer (50 mM Tris/HCl, pH 7.5, 1% TX-100, 10 mM $MgCl_2$), and finally suspended in 50 μL of kinase buffer. The kinase reaction was initiated by the addition of [γ-^{32}P]-ATP (20 μCi, final concentration 200 μM) and terminated after 15 min at 4 °C by the addition of 50 μL of 2-fold Laemmli sample buffer. After heating (95 °C, 2 min), the protein A−Sepharose was removed by centrifugation (12000*g*, 2 min) and the supernatant subjected to SDS−PAGE (7.5%). After electrophoresis, the gel was washed extensively in destaining solution containing 10 mM sodium pyrophosphate, dried, and exposed to Kodak X-Omat AR films with an intensifying screen at −70 °C.

Immunoblotting of Snf1p. Snf1p immunoprecipitates were separated by electrophoresis (7.5% acrylamide) and electroblotted to nitrocellulose (*40*). Snf1p was detected by using

polyclonal rabbit anti-Snf1p serum as described previously (*41*, *42*) except that 5% nonfat dry milk was added to buffers. The primary antibody was detected by using goat anti-rabbit immunoglobulin G (Fc)−horseradish peroxidase conjugate and the ECL chemiluminescence detection system.

Determination of Glycogen, Metabolites and cAMP. Spheroplast pellets or cell cakes, respectively, were prepared as described above. Acid extracts were obtained by mixing frozen spheroplasts or cells (about 200 mg wet mass) with 1 mL of 10% perchloric acid and the same volume of glass beads. The mixture was immediately frozen in liquid nitrogen. Samples were thawed on ice, vigorously vortexed by five pulses for 1 min (with intervals of 1 min on ice), and centrifuged at low speed. Glycogen was determined enzymatically by incubation of 100 μL of the acid extract neutralized with 5 M K_2CO_3 with 100 μL of 73 units/mL amyloglucosidase in 0.4 M acetate (pH 4.8) at 50 °C for 3 h. After the addition of 150 μL of 10% perchloric acid and centrifugation (22000*g*, 15 min, 4 °C), glucose was measured in the supernatant using hexokinase and glucose-6-phosphate dehydrogenase (*43*). Glucose 6-phosphate was determined after centrifugation of the acid extract (22000*g*, 15 min, 4 °C), neutralization of the supernatant (5 M K_2CO_3), incubation (30 min on ice), and additional centrifugation (22000*g*, 15 min) in the resulting supernatant using glucose-6-phosphate dehydrogenase (*43*). Alkaline extracts were prepared by suspending frozen cells (25 mg dry weight) in 1.5 mL of 54 mM NaOH prewarmed to 80 °C, incubation for 15 min at 80 °C, and centrifugation (12000*g*, 5 min). Fructose 2,6-bisphosphate was determined in the alkaline extract (*44*). cAMP was measured using the Amersham assay kit as described (*45*). Concentrations are expressed per gram wet mass of spheroplasts or cells, respectively. Ethanol was determined in the culture medium (*46*).

Miscellaneous. Lipogenesis was measured in isolated rat adipocytes as described previously (*47*). Protein concentration was determined by the method of Bradford (*48*) using bovine serum albumin as a standard.

RESULTS

Insulin Stimulates Oxidative and Nonoxidative Glucose Metabolism in Yeast under Conditions of Growth Limitation. In mammalian muscle and fat cells, insulin stimulates nonoxidative glucose metabolism (glycogen and lipid synthesis) as well as glucose oxidation. To study a putative effect of insulin on carbohydrate metabolism in yeast, we have chosen two different conditions of growth limitation which may increase the sensitivity of the key glucose metabolic pathways to up-regulation. Glycogen tends to accumulate in yeast cells under conditions in which growth is restricted, e.g., in a synthetic medium containing glucose but lacking other essential supplements such as nitrogen, phosphate, or sulfate (*22*−*25*), or in batch culture in the presence of glucose during the transition from exponential to stationary phase of growth (*20*−*22*, *49*). Glycogen accumulates before glucose is completely exhausted. Consequently, we studied whether insulin affects glycogen metabolism (1) during the shift of yeast spheroplasts from a synthetic medium containing a nonfermentable carbon source to a synthetic medium containing glucose but lacking nitrogen and sulfate (glucose induction) and (2) during the transition of yeast cells from exponential growth in rich glucose medium to stationary phase when glucose was almost depleted.

Table 1: Effects of Insulin and Insulin Analogues in Yeast Spheroplasts and Isolated Rat Adipocytes[a]

	yeast spheroplasts glucose oxidation		rat adipocytes lipogenesis
	$[^{14}C]CO_2$ [nmol min^{-1} (mg of protein)$^{-1}$]	% of control	EC_{50} (nM)
control cells	1.12 ± 0.09	100	
0.01 μM insulin (human)	1.05 ± 0.09	94	0.12 ± 0.05
0.1 μM insulin	1.25 ± 0.11	111	
0.5 μM insulin	1.51 ± 0.12	134	
1 μM insulin	1.75 ± 0.21	156	
10 μM insulin	1.93 ± 0.15	172	
100 μM insulin	1.99 ± 0.13	178	
10 μM insulin analogue I	1.85 ± 0.19	165	0.10 ± 0.04
10 μM insulin analogue II	1.61 ± 0.12	144	0.65 ± 0.19
10 μM insulin analogue III	1.39 ± 0.14	124	3.77 ± 0.38
10 μM insulin analogue IV	1.03 ± 0.18	92	>10 ± 4
10 μM insulin analogue V	1.19 ± 0.11	106	inactive
10 μM insulin α-chain	1.01 ± 0.09	90	inactive
10 μM insulin β-chain	1.10 ± 0.08	98	inactive
10 μM hIGFI	1.30 ± 0.11	116	
10 μM hIGFII	1.05 ± 0.13	94	
20 μM glucagon (human)	1.17 ± 0.08	104	
10 μM hEGF	0.95 ± 0.14	85	

[a] Spheroplasts were prepared and incubated in medium containing 20 mM [U-^{14}C]glucose lacking or containing the hormone as indicated for 30 min at 30 °C. $[^{14}C]CO_2$ release was determined (Experimental Procedures). Each value ± SEM was calculated from five independent incubations with determinations in triplicate. Human insulin and insulin analogues (0.01−100 nM) were assayed for stimulation of lipogenesis in isolated rat adipocytes. The EC_{50} values for insulin analogues I−IV exerting the maximal response of human insulin were calculated from the corresponding concentration/response curves. The insulin analogues have the following structures: analogue I, Lys(B3)-Glu(B29); analogue II, Gly(A21)-diLys(B31); analogue III, Gly(A21)-His(B1)-His(B3)-diArg(B31); analogue IV, Met(A3)-Gly(A21)-His(B31)-Ala(B32)-Ala(B33)-Arg(B34); analogue V, proinsulin.

Yeast spheroplasts were prepared from logarithmically grown cells and incubated in a defined medium containing a nonfermentable carbon source (succinate). Supplementation with glucose (100 mM) resulted in the activation of oxidative as well as nonoxidative glucose metabolism. This is reflected by a considerable increase (10−90 min after the transfer) of the amounts of glycogen (Figure 1A) as well as by the accumulation of ethanol in the medium (Figure 1B) and by the enhanced release of CO_2 due to glucose oxidation (Table 1), respectively. This was accompanied by drastic increments of the corresponding allosteric activators, hexose 6-phosphate and fructose 2,6-bisphosphate, in total cell extracts (Figure 1C,D). Insulin present in the glucose medium at 1 μM final concentration significantly stimulated formation of glycogen, ethanol, and CO_2, reaching 162 ± 9%, 181 ± 30%, and 172 ± 23% of the basal levels (glucose, absence of insulin), respectively, 60 min after the addition of glucose (Figure 1A,B, Table 1). Hexose 6-phosphate increased to 134 ± 11% above basal after 2−5 min (Figure 1C). An insulin concentration−response relationship was observed in the range of 0.1−10 μM insulin and various insulin analogues (see below), whereas glucagon and EGF had no effect on both nonoxidative and oxidative glucose metabolism (Table 1, shown for glucose oxidation, only). The concentration of fructose 2,6-bisphosphate (Figure 1D) as well as the transient rise of the intracellular cAMP level

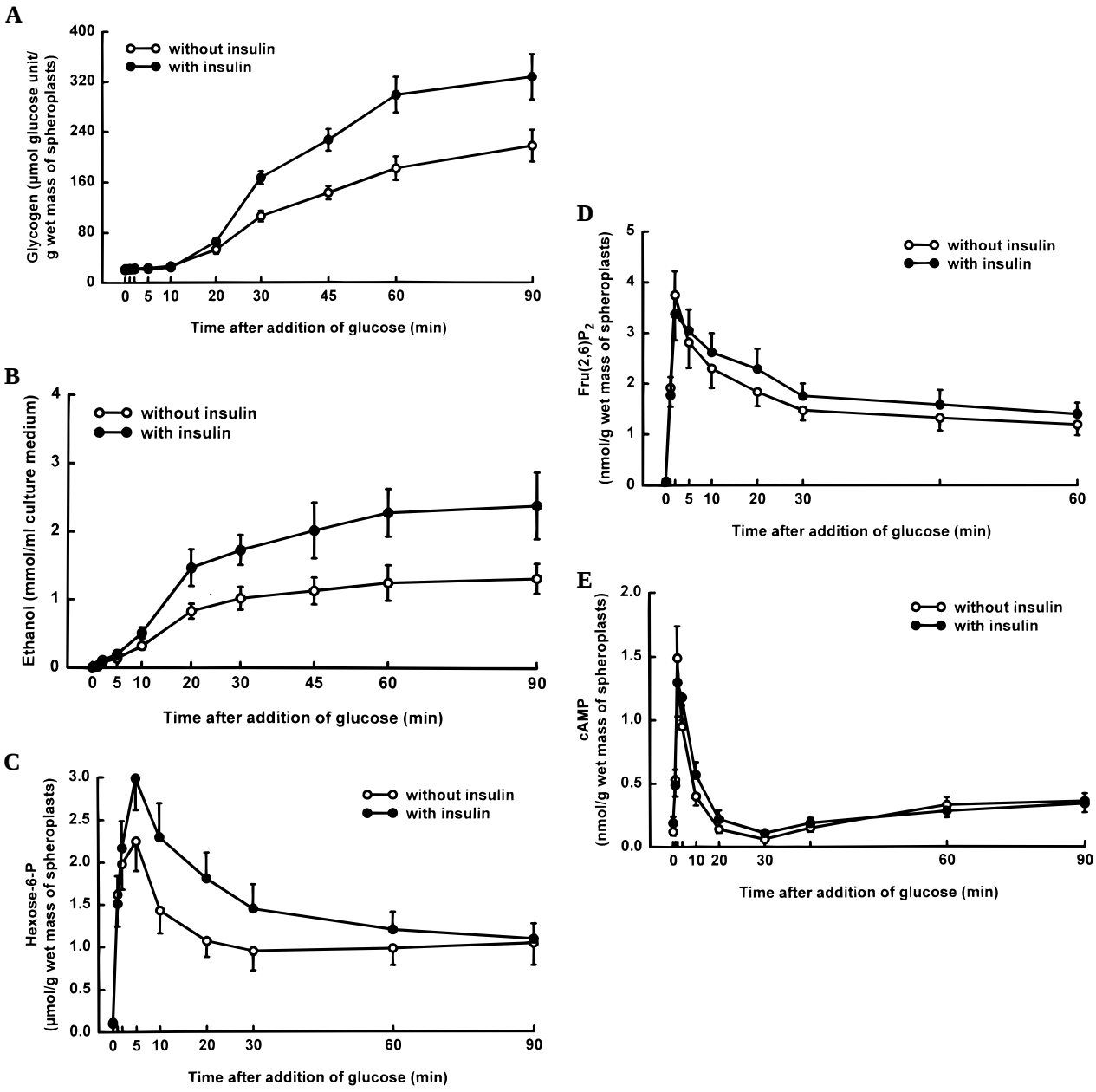

FIGURE 1: Effect of insulin on synthesis of glycogen, ethanol, hexose 6-phosphate, fructose 2,6-bisphosphate, and cAMP in glucose-induced spheroplasts. Spheroplasts derived from logarithmically grown cells were incubated at a density of 10−15 mg wet mass/mL for 60 min in succinate medium (Experimental Procedures) prior to addition of glucose at a final concentration of 100 mM (open circles), or glucose plus insulin (1 μM final concentration; filled circles). After the incubation periods, indicated aliquots of the spheroplast suspension were removed and the spheroplasts collected by rapid centrifugation through Ficoll/sucrose for preparation of cell extracts and determination of glycogen content (panel A), ethanol in the incubation medium containing 10 mg wet mass of cells/mL (panel B), hexose 6-phosphate (panel C), fructose 2,6-bisphosphate (panel D), and cAMP (panel E). The points represent means ± SEM of at least four independent incubations with triplicate determinations each.

(Figure 1E) peaking 5 min after addition of glucose (coinciding with the latency in glycogen synthesis) was not affected by insulin.

Deprivation of the growth medium of glucose during the transition of intact cells from late logarithmic growth to stationary phase induces glycogen synthesis which was increased by 40−60% in the presence of 10 μM insulin. EGF (10 μM) was inactive (Figure 2). Insulin did not increase nonoxidative or oxidative glucose metabolism (1) in yeast spheroplasts or intact cells incubated in nonfermentable carbon sources at any phase of growth or (2) in intact cells grown in continuous culture at a constant glucose concentration (100 mM) or (3) in spheroplasts incubated in rich glucose medium (data not shown). Thus, insulin apparently increases the sensitivity of both nonoxidative and oxidative glucose metabolism in yeast spheroplasts and intact cells toward stimulation by glucose under conditions of restricted growth (nutrient limitation or glucose exhaustion, respectively), but not under conditions permitting continuous growth.

Insulin Activates GSY and Inactivates GPH in Glucose-Induced Spheroplasts and Glucose-Deprived Cells. Next, we studied whether the stimulatory effect of insulin on glucose-regulated glycogen synthesis following glucose induction of yeast spheroplasts or during deprivation of glucose of intact cells is based on the activation of GSY or

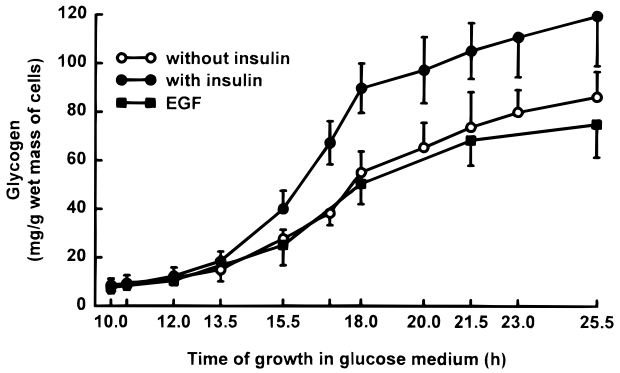

FIGURE 2: Effect of insulin on glycogen synthesis in glucose-deprived intact cells. Yeast cells were grown in YPD medium to saturation. Cells (0.2−1 mL) were inoculated in 750 mL of YPD medium containing 10 μM insulin (filled circles) or 10 μM EGF (squares) or lacking further additions (open circles) and allowed to continue growth. Samples of the culture were taken at different time points, cells collected by rapid filtration, acid extracts prepared, and the glycogen contents determined. The points represent means ± SEM of at least four independent incubations with determinations in duplicate each.

inhibition of GPH or both. Upon transfer of spheroplasts from succinate to glucose medium, total GSY activity was increased about 10-fold as soon as 2 min after incubation in glucose and remained at a high level for the next 30 min (Figure 3A). In some but not all experiments, a latency of a few minutes preceded synthase activation. On the other hand, total GPH activity rapidy decreased to less than 15% of the initial value within the first 2 min and then remained at that level (Figure 3B). Insulin added to the glucose medium led to a 25−45% increase of GSY (3.8 ± 0.4 vs 2.8 ± 0.2 basal) and a 30−50% decline of GPH (0.7 ± 0.1 vs 1.2 ± 0.2 basal) activities at the time points measured after 1 min. These changes in enzymic activity induced in spheroplasts by both glucose alone and glucose *plus* insulin were stable upon gel filtration (data not shown).

Since the intracellular concentration of glucose in yeast is known to be very low, it is unlikely that glucose is the trigger that causes inactivation of GPH and activation of GSY. Therefore, we studied the effect of several sugars or sugar analogues which can or cannot be phosphorylated to the corresponding 6-phosphoester by hexokinase. Fructose and mannose, which are good glycolytic substrates, were as effective as glucose in inducing inactivation of GPH and activation of GSY, both of which were more pronounced in the presence of insulin to a similar extent as in the presence of glucose (data not shown). Insulin also inactivated and activated GPH and GSY, respectively, if the spheroplasts were incubated with 2-deoxyglucose (Figure 3A) or, to a lesser degree, with 3-*O*-methylglucose (Figure 3B) which can be phosphorylated but not further metabolized (*50*). This ranking may be explained by the considerably lower affinities of the yeast glucose transporters and hexokinase for 3-*O*-methylglucose compared to those for 2-deoxyglucose as has been described for the mammalian counterparts (*51*−*53*).

A massive increase of total GSY and GPH activities was observed during late exponential growth of intact yeast cells in glucose medium, i.e., at a time point when the glucose concentration became limiting. Total GSY activity was maximal in medium and late exponential phase (10−12 h after glucose addition, 20-fold increase compared with the start of the culture) (Figure 4A, open circles). The rapid decline to about 50% of the maximal value observed when the cultures reached saturation (open circles at time points later than 12 h) was considerably delayed by the presence of insulin (Figure 4A, filled circles). On the other hand, total GPH activity rose continuously up to 25-fold during 22 h after glucose addition (Figure 5A, filled circles), and this increase was prevented in part by insulin (Figure 5A, open circles).

The insulin-dependent regulation of GSY or GPH is not restricted to the total activity, but occurs at the level of the activation state of these enzymes, in addition. After addition of glucose, the activity ratio of GSY (which represents the fraction of active enzyme at the time point of homogenization; see Experimental Procedures) was very high during early exponential growth (82 ± 13%). It declined to 26 ± 8% within the next 7 h after maximal activation when saturation was reached (Figure 4B). In the presence of insulin (10 μM), the activity ratio did not change significantly up to 18 h after glucose addition and was about 20−30% higher compared to the absence of insulin at each time point thereafter. Thus, insulin causes a considerable delay in the deactivation of GSY during and after exhaustion of glucose. The activity ratio of GPH decreased continuously during 7−22 h of incubation with glucose (Figure 5B). In the presence of insulin (open circles), the decline was faster (27 ± 6% residual activity after 22 h) compared to its absence (40 ± 8%) (filled circles). Importantly, the pronounced activation of GSY and inactivation of GPH and the additional effects exerted by insulin were seen only when the cells were isolated by the rapid filtration procedure. They were not observed when the cells were separated by centrifugation and washed once with water (data not shown). This difference is explained by the reversion of the enzymes' interconversion from an active to a less active form during washing (as demonstrated by others, see refs *25*, *30*). Interestingly, both the glucose and the insulin regulation of GSY and GPH was even more pronounced with a thermosensitive adenylate cyclase-deficient mutant (*cdc35*) at restrictive temperature irrespective of which isolation procedure had been used (data not shown). Therefore, we assume that in wild-type cells cAMP-dependent activation of GPH and inactivation of GSY occurring during centrifugation of the cells interfer with subsequent glucose and insulin regulation. Taken together, insulin makes GSY more refractory and GPH more sensitive to inactivation during the transition of cells from exponential growth to stationary phase and during deprivation of spheroplasts of glucose with respect to both the amount and the activation state of the enzymes.

Stimulation of Glucose Metabolism in Yeast by Insulin Is Specific. The following points strengthen the arguments in favor of specificity and selectivity of the observed insulin effects on oxidative and nonoxidative glucose metabolism in yeast: (1) A clear concentration−response relationship was observed for stimulation of glucose oxidation with human insulin (Table 1). (2) The ranking of different insulin analogues (I−IV) in stimulating lipogenesis in isolated rat adipocytes (the amino acid substitutions lead to higher EC_{50} values compared to human insulin but do not reduce the maximal response) correlated perfectly with their potency to activate glucose oxidation at 10 μM in yeast spheroplasts (Table 1). (3) Insulin analogue V (proinsulin) as well as

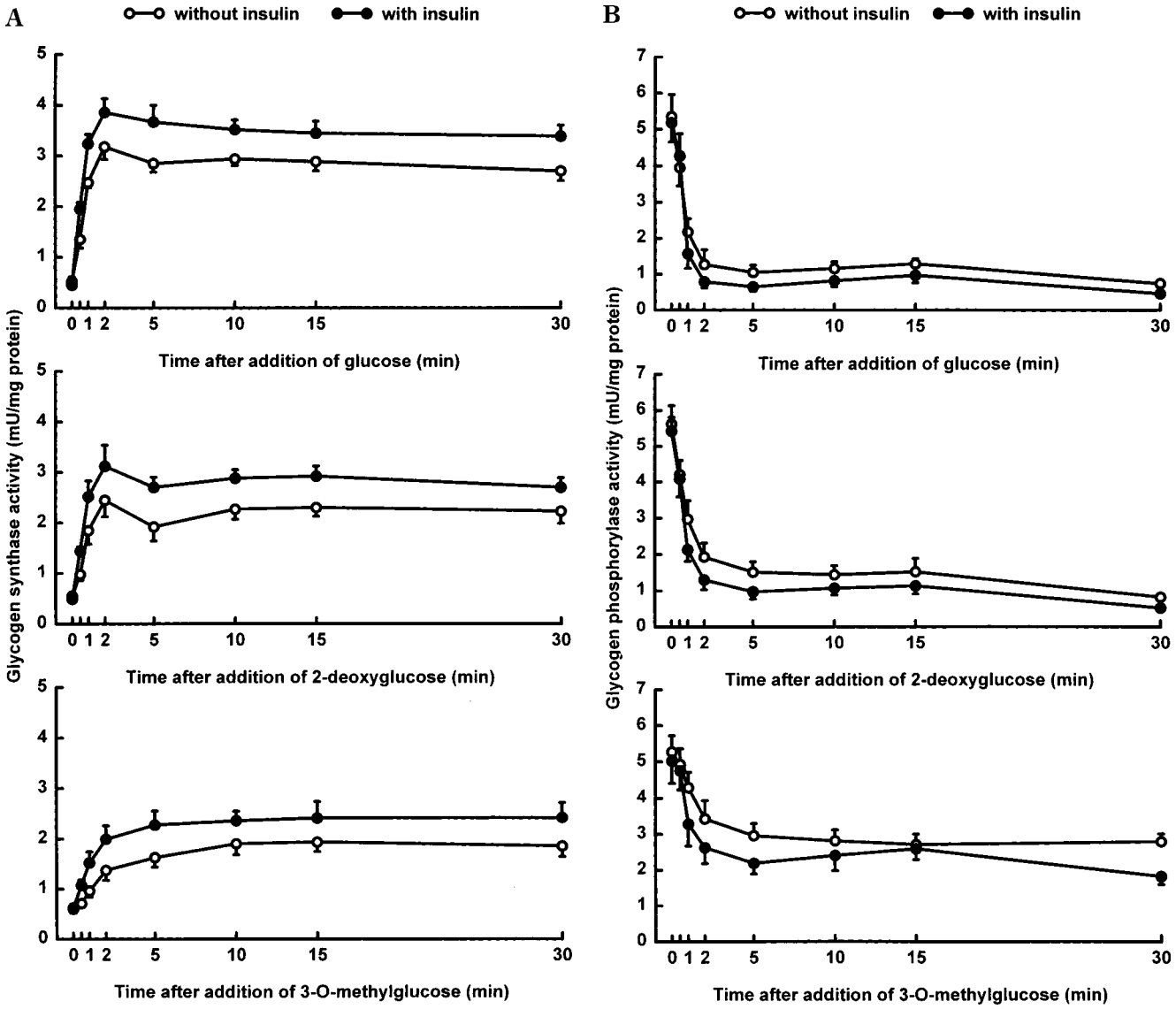

FIGURE 3: Effect of insulin on GSY and GPH activity in spheroplasts in the presence of glucose, 2-deoxyglucose, and 3-*O*-methylglucose. Spheroplasts derived from logarithmically grown yeast cells were incubated at a density of 10−15 mg wet mass/mL for 60 min in succinate medium (Experimental Procedures) prior to addition of glucose (top), 2-deoxyglucose (center), or 3-*O*-methylglucose (bottom) at a final concentration of 100 mM (open circles), or of glucose or its derivatives plus insulin (1 μM final concentration; filled circles). After the incubation periods indicated, aliquots of the suspension were removed and the spheroplasts collected by rapid centrifugation through Ficoll/sucrose for preparation of cell extracts and determination of GSY (panel A) and GPH (panel B) activities. The points represent means ± SEM of at least five independent incubations with activity determinations in quadruplicate each.

the separated α- and β-chains of insulin (obtained by reduction and alkylation of recombinant human insulin and subsequent chromatographic purification) was completely inactive in stimulating both lipogenesis in adipocytes and glucose oxidation in yeast spheroplasts (Table 1). (4) Human IGFI, IGFII, glucagon, and EGF exerted a rather modest increase (hIGFI) of glucose oxidation or no effect at all (Table 1). (5) The insulin effects on *Saccharomyces cerevisiae* seem to be restricted to activation of oxidative and nonoxidative glucose metabolism. No stimulation of lipid synthesis (assayed as incorporation of [3-^{3}H]glucose into total toluene-extractable acylglycerides) and growth or change in morphology of intact cells on either fermentable or nonfermentable carbon sources under growth-limiting or nonlimiting conditions was observed (E. Groβ and G. Müller, unpublished results). (6) The increase of glycogen accumulation critically depended on the limitation of nutrients available for growth, i.e., on conditions under which glycogen synthesis occurred (see above).

Insulin Does Not Affect Glucose Transport Activity. Part of the potent stimulation of glucose metabolism in insulin-sensitive mammalian cells by insulin (at least at low extracellular glucose concentrations) is assumed to rely on the activation of glucose transport. It is predominantly due to insulin-induced translocation of the glucose transporter molecules, GLUT4 (and to a minor degree GLUT1), from intracellular stores to the plasma membrane (for a review, see refs *54*, *55*). We therefore asked whether insulin increases glucose transport also in yeast spheroplasts during shift from succinate to glucose medium and in intact cells during exhaustion of glucose. It was observed that glucose transport (both the initial velocity and the time course in the considered interval of 30 min) by spheroplasts (as measured by the Ficoll centrifugation assay, Figure 6A) and by intact

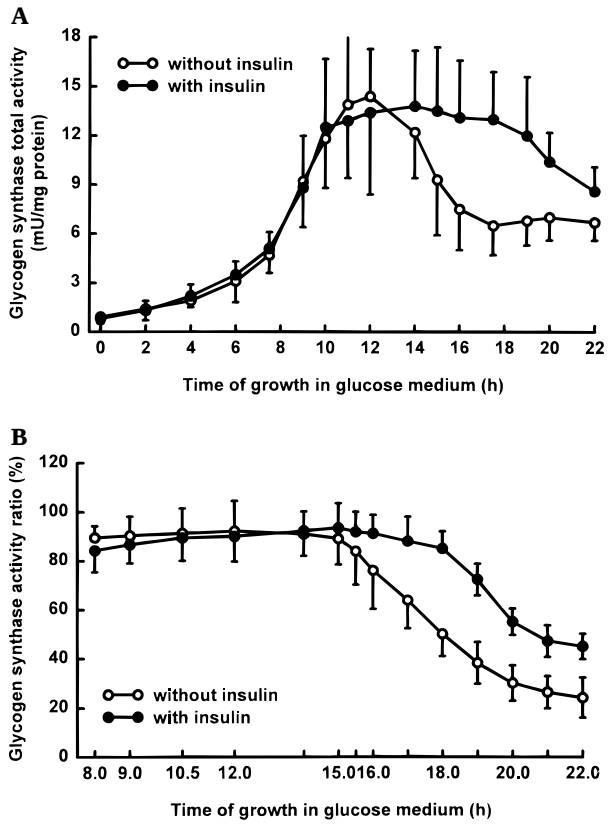

FIGURE 4: Effect of insulin on GSY activity in glucose-deprived intact cells. Growth conditions were as described for Figure 3 with YPD medium lacking (open circles) or containing 10 μM insulin (filled circles). Samples were taken at different time points, the cells collected by rapid filtration, acid extracts prepared, and the total activity (panel A) and activity ratio of GSY determined (panel B). The points represent means ± SD of at least six independent incubations with determinations in triplicate each.

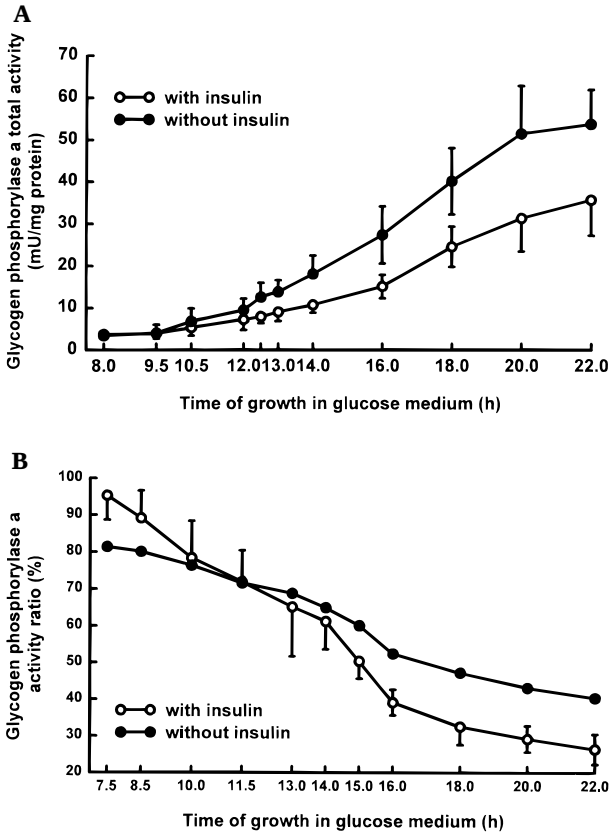

FIGURE 5: Effect of insulin on GPH activity in glucose-deprived intact cells. Growth conditions were as described for Figure 3 with YPD medium lacking (filled circles) or containing 10 μM insulin (open circles). Samples of the culture were taken at different time points. The total activity (panel A) and activity ratio (panel B) of GPH were determined. The points represent means ± SEM of at least six independent incubations with activity determinations in triplicate each.

cells (as measured by the filtration assay, Figure 6B) was similar in the absence and presence of insulin. This suggests that the Ficoll centrifugation assay is a valid method for determinating transport in fragile cells such as yeast spheroplasts and confirms previous findings obtained with *Neurospora crassa* (*56*). In spheroplasts, insulin (1 μM) did, neither at the beginning nor after 30 min of incubation with glucose (100 mM), affect the initial glucose transport velocity (Figure 6A). Likewise, in intact cells no activation by insulin (10 μM) of glucose transport was observed after either 10 or 20 h of growth in glucose medium (Figure 6B). The failure of insulin to activate glucose transport in *Saccharomyces cerevisiae* may be due to completely different modes of glucose transport regulation in mammalian cells and yeast. The latter apparently lacks the mechanism of glucose transporter translocation, as demonstrated by the recent finding that heterologous expression of GLUT1 or GLUT4 in *Saccharomyces cerevisiae* resulted in their accumulation in intracellular membranes but did not lead to increased glucose transport or transporter number at the plasma membrane (*57*).

Insulin Increases Snf1 Kinase Activity. The molecular mechanisms by which nutrients control yeast physiology in general and glycogen metabolism in particular are far from clear. The cAMP pathway is implicated in this nutritional response. For example, defects in *BCY1*, the gene encoding the regulatory subunit of PKA, lead to a constitutively active kinase and result in starvation sensitivity and the inability to sporulate (*58*). In contrast, mutants with attenuated cAMP synthesis (e.g., diploid cells in a *ras2/ras2* mutant genetic background) sporulate even in rich media (*59*). These strains also display aberrant glycogen accumulation, with *ras2* mutants showing hyperaccumulation and bcy1 mutants being unable to synthesize glycogen. Although it has been suggested that in cAMP pathway mutants glycogen accumulation reflects posttranslational controls exerted by PKA on GSY (*27*, *58*, *60*), this contention remains unproven. In any case, our observation that insulin does not alter cytosolic cAMP levels (see Figure 1E), under conditions which lead to insulin-induced glycogen accumulation (see Figure 1A), strongly suggests that cAMP-dependent protein phosphorylation is not involved in the insulin-dependent modulation of glycogen metabolism in yeast.

Based on genetic evidence, a central role of Snf1p in glucose repression has been established (*41*). Recently, the Snf1 serine/threonine-specific kinase has been shown to have sequence homology with the mammalian AMP-dependent protein kinase (*61*). Many of the phenotypes associated with loss of Snf1 function (*snf1*) are the same as those seen in strains with overactive PKA with respect to increased sensitivity toward nitrogen starvation and heat shock, inability to utilize alternative carbon sources, and failure to accumulate glycogen (*62*, *63*). Conversely, mutations that decrease cAMP signaling (e.g., *ras2*) reduce the sensitivity of *snf1*

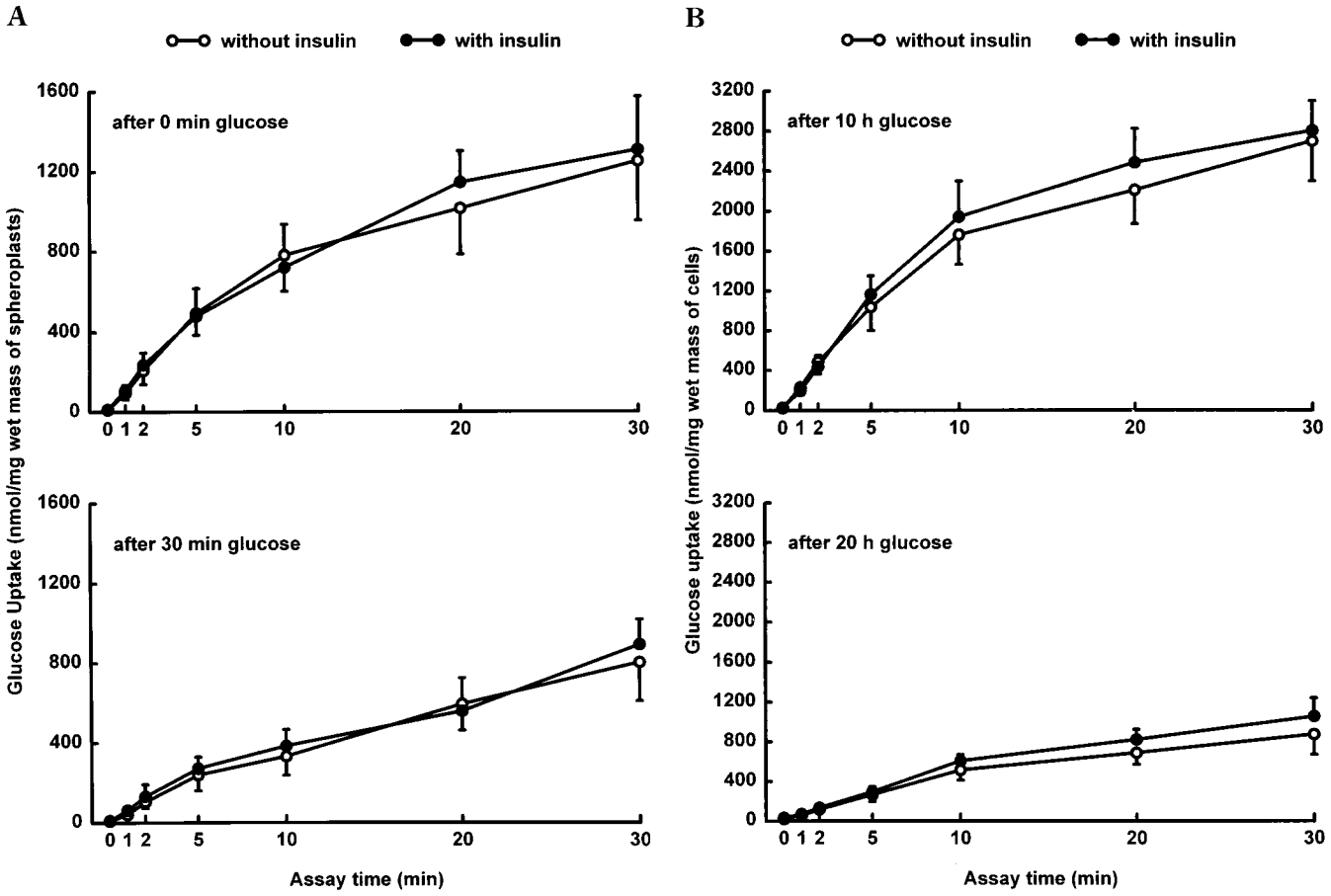

FIGURE 6: Effect of insulin on glucose transport in glucose-induced spheroplasts and glucose-deprived intact cells. Panel A: Spheroplasts derived from logarithmically grown yeast cells were incubated in succinate medium (see Figure 1) prior to the addition of glucose at a final concentration of 100 mM (open circles), or of glucose plus insulin (1 μM final concentration; filled circles). After 0 or 30 min incubation, spheroplasts were collected by rapid centrifugation of culture aliquots through Ficoll/sucrose. Panel B: Yeast cells were grown in YPD medium (see Figure 3) containing 10 μM insulin (filled circles) or lacking further additions (open circles). Samples of the cultures were taken at 10 and 20 h, and the cells were collected by rapid filtration. Aliquots of the resuspended spheroplasts (panel A) or cells (panel B) were assayed for glucose transport for the periods indicated. The points represent means ± SEM of at least four independent incubations with activity determinations in quadruplicate each.

cells to starvation. These observations led to the suggestion that the normal response to nutritional status may require the interplay between these regulatory networks, wherein the Snf1 pathway antagonizes the cAMP-mediated inhibition of glycogen synthesis (*64*). Although the present data demonstrate that insulin does not affect cAMP production/degradation (see Figure 1), hormone application may promote glycogen accumulation via the interference of Snf1p with the cAMP pathway at the level of cAMP signaling. Therefore, we studied the effect of insulin on Snf1 kinase activity in yeast spheroplasts and cells under conditions of insulin-induced glycogen synthesis.

Spheroplasts or intact cells were incubated in succinate or synthetic glucose medium (for 30 min) and in rich glucose medium (for 8 or 20 h), respectively, in the absence or presence of insulin (1 and 10 μM). Snf1p was immunoprecipitated from acid extracts by using affinity-purified polyclonal antibodies and assayed for (auto)phosphorylation activity by incubation with [γ-^{32}P]ATP (Figure 7A) and for the amount of Snf1p by immunoblotting with anti-Snf1p antiserum (Figure 7B). Analysis of total phosphoproteins by autoradiography (Figure 7A) revealed a 3.8-fold stimulation of the autophosphorylation of Snf1p (72 kDa) by insulin (1 μM) in spheroplasts incubated for 30 min in succinate medium (compare lane 1 with lane 3) which was increased to 7.5-fold in glucose-induced spheroplasts (compare lane 2 with lane 4) (the values have been corrected for the slightly differing amounts of immunoprecipitated Snf1p according to panel B). The stimulation of Snf1 phosphorylation by human insulin in spheroplasts was clearly dependent on its concentration (0.1−1 μM) (Table 2). In intact cells, insulin provoked a 2.9-fold and 4.8-fold increase of Snf1p autophosphorylation in immunoprecipitates after 8 h (compare lanes 5 with 7) and 20 h (compare lanes 6 and 8) incubation in glucose medium, respectively (again corrected for the actual amount of Snf1p). Under the conditions of the assay, glucose did not increase Snf1p autophosphorylation per se significantly (compare lanes 1 with 2 and 5 with 6) as has been reported previously for glucose repressed and derepressed cells (*41*, *65*). Rather, in spheroplasts, glucose induction for 30 min enhanced the stimulation of Snf1p autophosphorylation by insulin about 2-fold, whereas in intact cells exhaustion of glucose between 8 and 20 h incubation led to an 1.6-fold increase in insulin stimulation of Snf1 kinase activity.

This was also apparent from the analysis of the time course for insulin activation of Snf1 kinase in noninduced and glucose-induced spheroplasts (Table 2); 5−30 min after addition of glucose, insulin stimulation of Snf1p phosphorylation was 50−100% higher in glucose-induced vs control

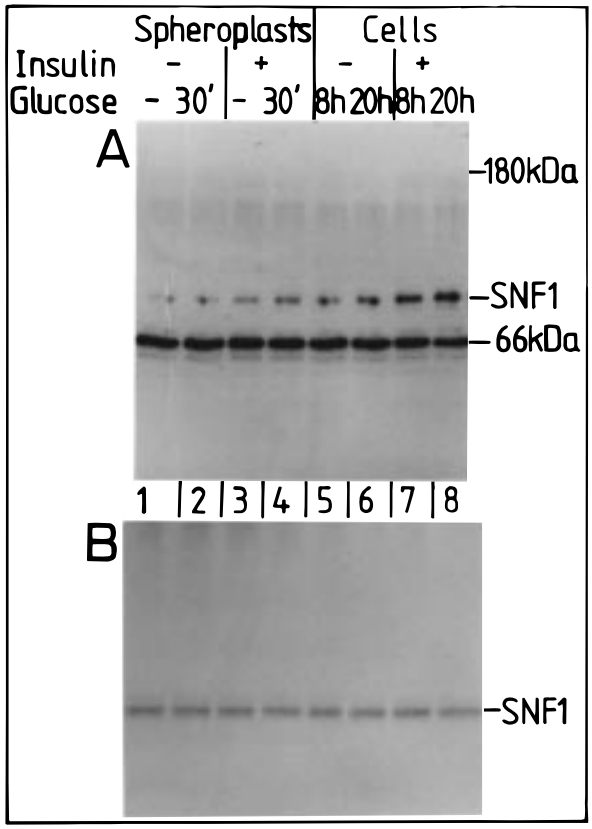

FIGURE 7: Effect of insulin on Snf1 kinase activity in glucose-induced spheroplasts and glucose-deprived intact cells. Spheroplasts derived from logarithmically grown yeast cells were incubated in the absence or presence of human insulin (1 μM final concentration) in succinate medium (see Figure 1) for 30 min. Half of each culture was supplemented with glucose (100 mM final concentration) and further incubated for 30 min; the other half was left. Subsequently, the spheroplasts were collected by rapid centrifugation through Ficoll/sucrose (lanes 1−4). Alternatively, yeast cells were grown in YPD medium (see Figure 3) containing 10 μM insulin or lacking further additions. Samples of the cultures were taken after 8 and 20 h and the cells collected by rapid filtration (lanes 5−8). Acid extracts were prepared from the spheroplasts and cells, respectively. Snf1p was immunoprecipiated with affinity-purified anti-Snf1 antibody (Experimental Procedures). Panel A: Half of each immunoprecipitate was incubated with [γ-^{32}P]ATP for 15 min at 30 °C and then subjected to SDS−PAGE. Phosphoproteins were visualized by autoradiography. The position of Snf1p is marked. Panel B: The other half of each immunoprecipitate was analyzed by immunoblotting with anti-Snf1 antiserum. The autoradiogram of a typical experiment is shown, repeated 2 times with similar results.

spheroplasts. Maximal insulin stimulation was rapidly reached at 5−10 min after addition of the hormone and remained constant thereafter for up to 30 min, irrespective of whether glucose had been added or not. This kinetics closely resemble those of shortOLINIT-term metabolic effects of insulin (e.g., stimulation of glucose transport) in isolated rat adipocytes. However, in contrast to the situation with adipocytes where the insulin effect persists during prolonged incubation with the hormone, the insulin stimulation for Snf1p autophosphorylation rapidly declined from 30 to 60 min and was virtually lost at 90 min after addition of insulin (Table 3). This was due to both a significant decrease of the insulin-induced Snf1p phosphorylation and a continuous and considerable elevation of its basal phosphorylation state in succinate- as well as glucose-grown spheroplasts. The early and transient insulin stimulation of Snf1 kinase

Table 2: Effect of Insulin and Insulin Analogues on Snf1 Kinase in Yeast Spheroplasts[a]

	phosphorylation of Snf1p (arbitrary units)
control cells	100 ± 27
0.1 μM insulin (human)	221 ± 55
0.3 μM insulin	456 ± 110
1 μM insulin	634 ± 166
10 μM insulin	577 ± 191
1 μM insulin analogue I	591 ± 144
1 μM insulin analogue II	402 ± 101
1 μM insulin analogue III	217± 60
1 μM insulin analogue IV	133 ± 40
1 μM insulin analogue V	84 ± 31
10 μM hIGFII	119 ± 27
10 μM hEGF	95 ± 18

[a] Spheroplasts derived from logarithmically grown yeast cells were incubated in the absence or presence of human insulin, various insulin analogues, or hormones (at the concentrations indicated) in succinate medium supplemented with glucose (100 mM final concentration) for 30 min. After collection of the spheroplasts by centrifugation through Ficoll/sucrose, acid extracts were prepared and Snf1p was immunoprecipitated with anti-Snf1 antiserum (Experimental Procedures). Half of each immunoprecipitate was incubated with [γ-^{32}P]ATP and then subjected to SDS−PAGE. The other half was analyzed by immunoblotting with anti-Snf1 antiserum. Phosphorylated and immunoreactive Snf1p were detected and quantitatively evaluated by phosphorimaging (Molecular Dynamics, Storm 840). The phosphorylation of Snf1p (given in arbitrary units) was corrected for the amount of immunoreactive Snf1p contained in each immunoprecipitate. The values represent the means ± SD of three independent incubations with activity determinations in triplicate each. For the structure of the insulin analogues, see legend to Table 1.

Table 3: Time Course for Activation of Snf1 Kinase by Insulin in Yeast Spheroplasts[a]

	phosphorylation of Snf1p (arbitrary units)				insulin stimulation (*x*-fold)	
	control		1 μM human insulin			
incubation time (min)	succinate	glucose	succinate	glucose	succinate	glucose
0	77 ± 19	68 ± 30	71 ± 30	80 ± 19	0.9	1.2
2	−	67 ± 42	−	196 ± 55	−	2.9
5	89 ± 29	75 ± 26	287 ± 90	341 ± 77	3.2	4.5
10	−	86 ± 39	−	487 ± 102	−	5.7
30	135 ± 31	100 ± 45	489 ± 78	594 ± 112	3.6	5.9
45	−	146 ± 31	−	634 ± 166	−	4.3
60	195 ± 55	213 ± 51	509 ± 98	577 ± 132	2.6	2.7
90	−	287 ± 77	−	302 ± 93	−	1.1
120	251 ± 64	233 ± 54	272 ± 88	210 ± 29	1.1	0.9

[a] The experiment was performed as described for Table 2 with the exception that either human insulin (1 μM final concentration) or no hormone was present during incubation of the spheroplasts in either succinate medium or succinate medium supplemented with glucose for the periods of time indicated. The values represent the means ± SD of four independent incubations with activity determinations in duplicate each.

may represent one of the signals required for initiation of glycogen synthesis. Future studies will address the molecular basis for the reduction of Snf1 kinase activity during prolonged insulin incubation of yeast spheroplasts. It is conceivable that in addition to direct insulin regulation of the Snf1 kinase, a putative (insulin-controlled) Snf1 phosphatase copurified with the anti-Snf1p immunoprecipitates is responsible for dephosphorylation of Snf1p in course of termination of the insulin signal to glycogen synthesis.

Remarkably, the phosphorylation states of two other phosphoproteins (66 and 180 kDa, the labeling of the latter was less consistent and weaker), recovered in the anti-Snf1p

immunoprecipitates, did not vary significantly between the different incubation conditions of spheroplasts and cells (Figure 7). These two proteins are frequently found associated with Snf1p by means of coimmunoprecipitation with anti-Snf1p antiserum (see ref *42*). This association obviously did not depend on the phosphorylation state of Snf1. When extracts from a haploid *snf1* deletion mutant (lacking the entire coding region of *SNF1*), which had been prepared from spheroplasts or intact cells (incubated in the absence or presence of insulin for 30 min or 20 h, respectively), were assayed, no labeled Snf1p was detected, and amounts of the other two prominent phosphorylated polypeptides were drastically reduced. Immunoblot analysis of the samples confirmed the absence of the Snf1p (data not shown). This result confirms the identity of Snf1p and shows that phosphorylation of the 66- and 180-kDa proteins depends on the presence of Snf1p. Moreover, immune complexes prepared from insulin-induced intact cells from the haploid substitution mutant *snf1-K84R* [with lysine 84 in the predicted ATP-binding site changed to arginine, resulting in catalytically inactive Snf1 protein kinase (*42*)] failed to label the mutant Snf1p and strongly reduced labeling of the other proteins. The amount of the *snf1-K84R* mutant protein present in the immunoprecipitate was, however, roughly the same as in the SNF1 wild type (data not shown). Taken together, we conclude that the Snf1 protein kinase activity is required for phosphorylation of Snf1p and that autophosphorylation of Snf1p is significantly stimulated by insulin in both spheroplasts and intact cells, irrespective of whether glucose is absent or present. Participation of Snf1 kinase in the insulin regulation of glucose metabolism is suggested by the observation that the relative ranking of the various insulin analogues with regard to their potency in stimulating Snf1 kinase (Table 2) and glucose oxidation (Table 1) was in perfect correlation, whereas hIGFII and hEGF were totally inactive in both cases. Furthermore, the insulin concentration−response relationship was comparable between glucose oxidation and Snf1 kinase (see Tables 1 and 2).

DISCUSSION

Human insulin has been found to exert effects on glucose transport and metabolism in several invertebrates (*14*, *15*) and on glucose metabolism in protists and microorganisms (*17*, *19*). The present study describes insulin actions in *Saccharomyces cerevisiae* and, for the first time, provides solid evidence that these effects are specific and selectively act on storage carbohydrate metabolism. Specificity was demonstrated in the first line by the parallel efficiency of action of human insulin and related hormones and insulin analogues on oxidative and nonoxidative glucose metabolism in insulin-responsive cells and tissues from mammals and in yeast (Table 1). The relatively high concentration of human insulin required to elicit these effects in yeast does not really argue against specificity. The concentrations of human insulin required for maximal stimulation of glycogen metabolism in glucose-induced yeast spheroplasts and glucose-deprived intact cells (about 10 and 100 μM, respectively) as well as the corresponding EC_{50} values for insulin (0.5−1 and 2−5 μM, respectively) are about 3−4 orders of magnitude higher (Table 1) than those required for stimulation of lipogenesis by insulin in isolated rat adipocytes (10 nM and 0.1−0.2 nM, respectively, see ref *47*) or the plasma insulin levels attained in man after an oral glucose load (1−5 nM). This cannot be explained by very rapid degradation of human insulin in the incubation medium of the spheroplasts or intact cells, respectively. In the presence of spheroplasts, the half-life of insulin was 40−50 min (as determined by radioimmunoassay, not shown) and thus in the range of the total incubation period, whereas the gradual loss of insulin during culturing of intact cells (half-life 2−2.5 h) was compensated for by repeated supplementation of identical amounts of insulin. The need for high concentrations of human insulin for efficacy in yeast may simply reflect the evolutionary divergence between a putative insulin-binding protein of the yeast plasma membrane and the mammalian insulin receptor. The 10-fold higher concentrations of insulin required for maximal activation of oxidative and nonoxidative glucose metabolism in intact yeast cells compared to those needed for spheroplasts (compare, e.g., Figures 3 and 4) may be accounted for by the limited penetration of the macromolecule across the yeast cell wall which allows efficient passage of molecules <1500 Da, only. Therefore, it is conceivable that insulin gains access to the plasma membrane of intact cells predominantly during the cell division process when the cell wall aquires a less tightly cross-linked structure. This would be compatible with the observed gradual decline of insulin sensitivity of intact yeast cells during the transition from very late exponential growth to stationary phase when the number of budding cells becomes greatly reduced. These considerations argue that the relatively high insulin concentrations required for maximal effects may be due to simple natural reasons and do not reflect the unspecific action of insulin.

The apparent specificity and selectivity of the insulin effects in yeast raise the possibility of the existence in this lower eucaryote of functionally and structurally related "insulin-like material". Another lower eucaryote, *Neurospora crassa*, has been demonstrated to produce and secrete insulin-like material in amounts detectable by radioimmunoassay. This material resembled mammalian insulin in its chromatographic behavior and was capable of inducing insulin-like effects in mammalian adipocytes (*8*, *66*). Controversially, several metabolic responses of *N. crassa* to added mammalian insulin point clearly to the existence of an insulin-induced signal transduction system: (1) growth enhancement (*67*); (2) increased rates of glycolysis, glucose oxidation, and alanine synthesis (*67*, *68*); and (3) increased glycogen synthesis, arising from the insulin-induced activation (presumably by dephosphorylation) of GSY (*69*) and modulated intracellular Na^+ levels (*70*).

In contrast to these findings and a number of additional reports on the synthesis of insulin-like peptides by diverse unicellular pro- and eucaryotes (see the introduction), the existence of insulin-like material of proteinaceous nature in yeast with resemblance in structure and immunological cross-reactivity to mammalian insulin has not been reported so far. Polyclonal antisera raised against diverse peptides derived from human insulin did not recognize a polypeptide of a size similar to mammalian insulin in a specific manner (E. Gro*β*, unpublished data). A "glucose tolerance factor" has been purified from yeast extract powder, which potently stimulates glucose uptake in isolated rat adipocytes (*71*) and lowers nonfasting plasma glucose levels in normal and genetically diabetic d*b*/d*b* mice (*72*, *73*). Its exact structure

has not yet been determined. However, partial characterization makes structural similarity with insulin very unlikely (*74*). The first demonstration in the present study of potent effects of human insulin on wild-type yeast (increase of glycogen accumulation, glucose oxidation, GSY activation, GPH inhibition, and Snf1 kinase activation) strongly implies the possibility for synthesis and release of an endogenous natural ligand. The presumptive protein (hormone) is likely to be recognized by a plasma membrane receptor protein and to trigger the apparant insulin-like signal transduction pathway mediating the effects on glucose metabolism mimicked here by exogenous human insulin. Such a system may regulate and coordinate glycogen accumulation under conditions of restricted growth (exhaustion of carbon, sulfur, or nitrogen) in an autocrinous manner and/or (less likely) between cells as part of an endocrine signaling circuit. It will be interesting to see whether the expression of elements of the mammalian insulin signal transduction cascade in the yeast *Saccharomyces cerevisiae* will increase the sensitivity of its glycogen storage pathway or change the pattern of its responses toward exogenous insulin or affect its signaling behavior toward nutrients present in the culture medium.

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