Insulin Signaling in the Yeast *Saccharomyces cerevisiae*. 3. Induction of Protein Phosphorylation by Human Insulin

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Received January 7, 1998; Revised Manuscript Received January 8, 1998

ABSTRACT: A low affinity insulin-binding protein in the plasma membrane of *Saccharomyces cerevisiae* has been identified recently (Müller, G., Rouveyre, N., Upshon, C., Gross, E., and Bandlow, W., preceding paper in this issue). Since the mammalian insulin receptor functions as a tyrosine kinase with autophosphorylation capacity, kinase studies were performed with the partially purified insulin-binding protein preparation. Incubation with [γ-^{32}P]ATP in vitro led to phosphorylation of the 53-kDa insulin-binding protein on serine but not on tyrosine residues. In addition, a 70-kDa polypeptide, copurified with the insulin-binding protein preparation, was tyrosine-phosphorylated under the same conditions. Phosphorylation of both proteins was enhanced by human insulin. These results obtained by immunoprecipitation and immunoblotting using specific anti-phosphoserine/threonine/tyrosine antibodies were confirmed by phosphoamino acid analysis of the individual immunoprecipitated and gel-purified ^{32}P-labeled phosphoproteins. During gel filtration, the 53-kDa protein coeluted as a 300-kDa complex together with the 70-kDa phosphotyrosine-containing protein and was coimmunoprecipitated with the latter using an anti-phosphotyrosine antibody, strongly arguing for complex formation between the two proteins. The data presented raise the possibility that stimulation of glycogen synthesis by insulin in yeast is mediated by a 53-kDa insulin-binding protein and a 70-kDa phosphotyrosine-containing protein which are organized in a large plasma membrane-bound signaling complex. Elucidation of the function and molecular mode of interaction of these components in yeast may help to understand metabolic insulin signaling in mammalian cells.

Human insulin has been shown to exert some effects on glucose and glycogen metabolism in *Saccharomyces cerevisiae* (*1* and first of three papers in this issue). The signal transduction pathway for insulin in mammalian cells involves the following key elements, operating at five hierarchic levels (for reviews, see refs *2*−*4*): (Ia) a plasma membrane receptor protein and (Ib) associated tyrosine kinase, (II) tyrosine phosphorylation of adaptor proteins (IRS-1/2/3, Shc), (III) protein/protein interactions between signaling proteins having or lacking enzymatic function (e.g., Grb-2, Ras, Raf-1, PI 3-kinase), (IV) serine/threonine phosphorylation cascades (e.g., MAPK[1] pathway, Rsk, p70S6 kinase), and finally (V) activation/inhibition of terminal effector enzymes by phosphorylation/dephosphorylation (e.g., glycogen synthase, Phas-1).

With regard to level "Ia" in yeast, a 53-kDa plasma membrane protein has been identified, which specifically binds human insulin with K_d values at 0.3−0.7 μM (see preceding paper in this issue). With regard to the downstream elements of signaling cascades, in general, striking functional and structural similarities have been recognized between yeast and higher eucaryotes. At level "IV", serine/threonine kinase signaling pathways have been identified in the yeasts *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* that contain components analogous to those of higher eucaryotes. The kinases of the mammalian MAPK pathway (MEKK, MEK, ERK-1/2) have counterparts in *Saccharomyces cerevisiae* (i) in the FUS3/KSS1 pathway (STE11, STE7, FUS3/KSS1) mediating the response to mating pheromones (*5*, *6*), (ii) in the MPK1 pathway (BCK1, MPK1/2, MPK1) regulating cell shape, and (iii) in the HOG1 pathway (MAPKKK, PBS2, HOG1) controlling cell integrity and osmoregulation (*7*). FUS3/KSS1 appear to be elements of the MAPK pathway of yeast that most closely resembles the receptor tyrosine kinase linked signaling cascades in higher eucaryotes (*8*, *9*; for a review, see ref *10*). Compatible with this view is the striking sequence similarity between the serine/threonine-specific kinases, FUS3 and KSS1, from yeast and ERK-1 from rat (*11*). The demonstration that some of these yeast kinases can be replaced functionally by vertebrate enzymes shows that there is conservation of function as well as structural homology among these signaling components (*12*, *13*). With regard to level "V", glycogen synthase in both higher and lower eucaryotes is regulated by its phosphorylation state. The extent of phosphorylation is determined in part by serine/threonine-specific protein phosphatases, i.e.,

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[1] Abbreviations: EGF, epidermal growth factor; IGF-1, insulin-like growth factor; MAPK(K), mitogen-activated protein kinase (kinase); PEG, poly(ethylene glycol); SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TCA, trichloroacetic acid.

S0006-2960(98)00102-0 CCC: $15.00
Published on Web 05/27/1998

PP1G in mammalian cells (*14*) and PP1 as well as PP2A in yeast (*15, 16*). Recent evidence suggests that in glucose-induced spheroplasts of *Saccharomyces cerevisiae* activation of PP2A by human insulin correlates well with increased glycogen synthesis (Müller, G., Rouveyre, N., Jung, C., and Bandlow, W., manuscript in preparation).

In the present study, we asked whether nutritional signaling in yeast involves similar mechanisms and components as insulin action in mammals, in particular at levels "Ib−III" defined above for the mammalian situation. Consequently, we looked for insulin-induced tyrosine and serine/threonine phosphorylation as well as for specific interactions between phosphoproteins, since these mechanisms represent key elements of insulin signal transduction in mammalian cells. The 53-kDa insulin-binding protein was found to undergo serine/threonine-specific phosphorylation in vitro in response to insulin and to be associated with a 70-kDa phosphotyrosine-containing protein. The identified complex may constitute the initial component of a signaling cascade mediating metabolic insulin action in *Saccharomyces cerevisiae*.

EXPERIMENTAL PROCEDURES

Materials. [γ-^{32}P]ATP (6000 Ci/mmol) was bought from NEN/DuPont (Bad Homburg, Germany). Okadaic acid, PEG 6000, and myelin basic protein were obtained from Sigma (Deisenhofen, Germany). Protein A−Sepharose was provided by Pharmacia/LKB (Freiburg, Germany). Protease inhibitors, phosphatase inhibitors, and detergents were purchased from Boehringer Mannheim (Mannheim, Germany). All other materials were obtained as described previously (*17*−*19*; see preceding two papers in this issue).

Protein Kinase Assay. Two to five micrograms of solubilized and partially purified insulin-binding protein in storage buffer (see preceding paper) was supplemented with kinase buffer (50 mM Hepes, pH 7.2, 0.1% TX-100, 5 mM $MnCl_2$, 0.2 mM Na_3VO_4, 100 mM NaF, 5 μM okadaic acid, 25 mM glycerol 3-phosphate) to a total volume of 180 μL and incubated in the absence or presence of hormone as indicated for 20 min at 22 °C. The kinase reaction was initiated by the addition of 20 μL of 0.2 mM ATP containing 50 μCi of [γ-^{32}P]ATP. After incubation for 10 min at 10 °C, the reaction was terminated by addition of 800 μL of 50 mM Hepes/KOH (pH 7.2), 0.1% TX-100, 0.1% SDS, 0.2 mM Na_3VO_4, 10 mM $Na_2P_2O_7$, 4 mM EDTA. The mixture was precleared by addition of 100 μL of 100 mM Hepes/KOH (pH 7.2), 100 mg protein A−Sepharose/mL, 1.5 M NaCl, 4 mM EDTA, 200 mM NaF, 100 mM $Na_2P_2O_7$, 1% TX-100 and centrifugation (12000*g*, 2 min, 4 °C). The supernatant was supplemented with 10 μg of monoclonal anti-phosphotyrosine antibody (Biomol, Hamburg, Germany; clone 3B12) or 15 μg of a kit containing four different monoclonal anti-phosphoserine antibodies (Biomol; clones 1C8, 4A3, 4A9, 4H4) and the monoclonal anti-phosphothreonine antibody (clone 1E11). After incubation for 4 h at 4 °C, the antibody complexes were precipitated during a 2-h incubation with 50 μL of 50 mg of protein A−Sepharose per milliliter of 50 mM Hepes (pH 7.4), 0.1% TX-100. The precipitates were collected by centrifugation (12000*g*, 2 min, 4 °C), and washed 3 times with 1 mL each of 50 mM Hepes/KOH (pH 7.4), 150 mM NaCl, 100 mM NaF, 0.2 mM Na_3VO_4, 0.1% SDS, 1% TX-100 by centrifugation and resuspension in the same buffer and finally twice with buffer lacking TX-100. Phosphoproteins were eluted from the washed precipitates by incubation with 250 μL of 50 mM Hepes/KOH (pH 7.4), 50 mM *p*-nitrophenyl phosphate for 30 min at 4 °C. The supernatants obtained by centrifugation were precipitated. The pellet were suspended in Laemmli sample buffer and subjected to SDS−PAGE. The dried gels were analyzed by autoradiography (Kodak X-Omat AR film).

Gel Filtration Chromatography. Up to 1 mL of binding protein sample (obtained after termination of the cross-linking reaction with insulin before final precipitation or after elution of the phosphoproteins generated during the kinase assay before final precipitation) was immediately applied to a Sephacryl S-300HR column (1.5 × 60 cm) equilibrated with 50 mM Hepes/KOH (pH 7.5), 150 mM NaCl, 1% glycerol, 0.1% octyl glucoside, 0.2 mM PMSF, 5 μM leupeptin, 10 μM pepstatin, 25 μM antipain, 0.5 mM benzamidine. Alternatively, the samples were precipitated prior to gel filtration by addition of TCA (10% final concentration), incubation on ice (1 h), and centrifugation (12000*g*, 10 min, 4 °C), washed 2 times with acetone, dried, and finally dissolved in the same volume of the above buffer. Fractions (1.2 mL) were collected at 4 °C and counted for radioactivity (γ-counter for ^{125}I-labeled samples and liquid scintillation counter for ^{32}P-labeled samples). The fractional elution positions (K_{av}) of the various standard proteins, used to calibrate the column, were determined by their optical densities at 280 nm. Standard proteins were apoferritin (443 kDa), β-amylase (200 kDa), yeast alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), α-chymotrypsinogen (25 kDa), and horse heart cytochrome *c* (12.4 kDa). Blue dextran (2000 kDa) was used to determine the void volume.

Phosphoamino Acid Analysis. Phosphoamino acid analysis was performed by the method of Cooper et al. (20) with some modifications. Briefly, following SDS−PAGE, the gels were fixed for 30 min in 30% methanol, 2% glycerol and dried. The radiolabeled bands were excised and homogenized in 1 mL of 0.1 M NH_4HCO_3, 0.1% TX-100, 5% 2-mercaptoethanol plus human γ-globulin (5 μg/mL) as a carrier. The mixture was then precipitated with 10% TCA, and the precipitate was washed with ethanol/ether (1:1, by volume) at −20 °C. The protein was acid-hydrolyzed (100 °C, 2 h, 6 N HCl), and the liberated [^{32}P]phosphoamino acids were purified from the hydrolysate by chromatography on Dowex AG1-X8 before resolution by thin-layer electrophoresis using precoated cellulose plastic sheets (20 × 20 cm) for 60 min (50 V/cm). Radioactive amino acids were visualized by autoradiography and identified by comparison with radiolabeled standard phosphoamino acids.

Miscellaneous. Published procedures were used for cell growth and preparation of spheroplasts, measurement of glycogen synthase and SNF1 kinase activities, partial purification of the insulin-binding protein, and cross-linking with [^{125}I]monoiodo[B_{26}]insulin (see two preceding papers), isoelectric focusing including sample preparation (*19*), SDS−PAGE and fluorography (*21*), protein determination (*22*), protein precipitation (*23*), and silver staining (*24*). Immunoblotting using chemiluminescence detection with the ECL kit from Amersham-Buchler (Braunschweig, Germany) was performed according to the instructions of the manufacturer. All data analysis was performed using SigmaPlot for Windows 4.0 statistical software.

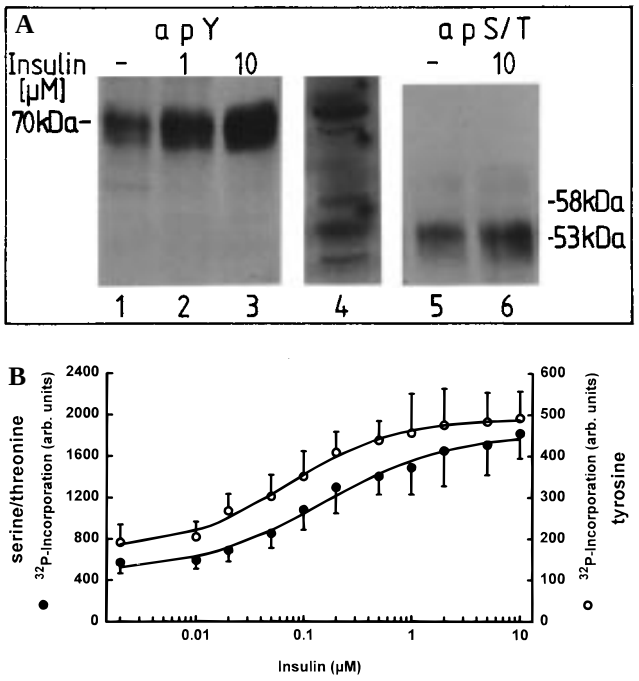

FIGURE 1: Kinase activity of the insulin-binding protein preparation. Five micrograms of partially purified insulin-binding protein from glucose-induced spheroplasts was incubated in the absence (panel A, lanes 1 and 5) or presence of 1 μM (lane 2), 10 μM (lanes 3 and 6), and various concentrations of human insulin (panel B) with [γ-^{32}P]ATP (see Experimental Procedures). Subsequently, the mixtures were incubated with either anti-phosphotyrosine (panel A, lanes 1−3, apY; panel B, open circles) or anti-phosphoserine/threonine antibodies (panel A, lanes 5 and 6, apS/T; panel B, filled circles). The immunoprecipitates were analyzed by SDS−PAGE and autoradiography (panels A) or phosphorimaging (panel B). Panel A: Autoradiograms; lane 4 of Panel A shows the partially purified insulin-binding protein preparation cross-linked to [^{125}I]monoiodo-[B_{26}]insulin run in parallel on the same gel together with the anti-phosphoserine/threonine and anti-phosphotyrosine immunoprecipitates. The positions of the 70- and 53-kDa phosphoprotein species are indicated. The anti-phosphoserine/threonine and anti-phosphotyrosine immunoprecipitates as well as the cross-link products were run in parallel on the same gel, but the corresponding segments of the gel had to be exposed for different periods of time due to great differences in the amount of radioactivity incorporated into the differently labeled proteins and therefore have been separated from one another. The experiment was repeated 3 times with similar results. Panel B: Quantitative evaluation by phosphorimaging analysis of the insulin concentration−response curves. Each point represents the mean ± SD of five different preparations of the insulin-binding protein with kinase assays performed in triplicate.

RESULTS

Saccharomcyes cerevisiae Contains Insulin-Inducible Protein Kinases. The B-subunit of the mammalian insulin receptor is a tyrosine-specific protein kinase with the capacity of autophosphorylation (for reviews, see refs *4*, *25*). Therefore, we studied whether the 53-kDa insulin-binding protein from yeast is phosphorylated in response to insulin. For this, the solubilized and partially purified insulin-binding protein preparation was incubated with human insulin and [γ-^{32}P]-ATP and immunoprecipitated with anti-phosphotyrosine as well as anti-phosphoserine/threonine antibodies. The autoradiographic comparison of the SDS gel patterns (Figure 1A) of the ^{32}P-labeled phosphotyrosine (lanes 1−3) or phosphoserine/threonine (lanes 5, 6) containing proteins (after immunoprecipitation with anti-phosphotyrosine and anti-phosphoserine/threonine antibodies, respectively) with the

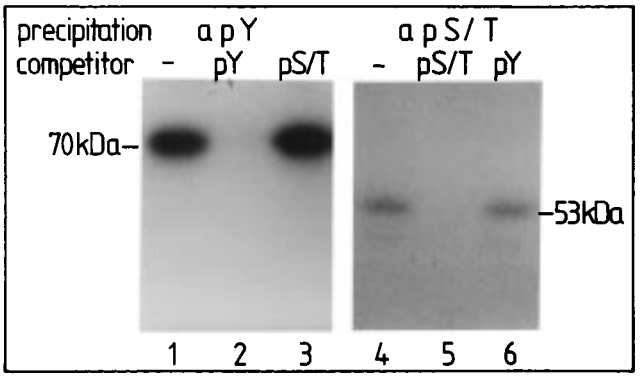

FIGURE 2: Specificity of the immunoprecipitation using anti-phosphoamino acid antibodies. Five micrograms of partially purified insulin-binding protein from glucose-induced spheroplasts was incubated with [γ-^{32}P]ATP in the presence of 1 μM human insulin (see Experimental Procedures). Subsequently, the mixtures were incubated with anti-phosphotyrosine (lanes 1−3, apY) or anti-phosphoserine/threonine antibodies (lanes 4−6, apS/T) in the absence or presence of 1 mM phosphotyrosine (pY) or phosphoserine/threonine (pS/T). The immunoprecipitates were analyzed by SDS−PAGE and autoradiography. The positions of the 70- and 53-kDa phosphoprotein species are indicated. The anti-phosphoserine/threonine and anti-phosphotyrosine immunoprecipitates were run in parallel on the same gel (see Figure 1 for the need of separating the gel segments).

^{125}I-labeled polypeptides (after affinity cross-linking with [^{125}I]monoiodo[B_{26}]insulin, lane 4) revealed that the partially purified insulin-binding protein preparation contained polypeptides in the range of 53−50 kDa which were radiolabeled at serine/threonine residues to a significant extent ("53-kDa species") even in the absence of insulin (lane 5), but were not phosphorylated at tyrosine residues even in the presence of insulin (lanes 2 and 3). The 53-kDa polypeptide may correspond to the 58-kDa cross-linked insulin-binding protein (lane 4). Human insulin (10 μM) stimulated serine/threonine phosphorylation of this protein 1.7−2.2-fold (lane 6). Serine/threonine phosphorylation of other polypeptides could not be detected with this preparation, whereas several proteins in the size range from 72 to 65 kDa ("70-kDa species") were phosphorylated on tyrosine in an insulin-dependent manner (lanes 1−3). Phosphorylation of both the 53-kDa polypeptide at serine/threonine and the 70-kDa polypeptide at tyrosine residues was dependent on the concentration of human insulin added to the in vitro kinase assay (Figure 1B, filled and open circles, respectively). These findings indicate that the protein preparation containing the insulin-binding protein harbors both serine/threonine- and tyrosine-specific protein kinase activities which are stimulated by insulin.

The specificity of the immunoprecipitation using anti-phosphotyrosine and anti-phosphoserine/threonine antibodies was controlled in an experiment by inclusion of an excess of the corresponding phosphoamino acid in the incubation mixture together with the antiserum prior to addition of protein A−Sepharose (Figure 2). The immunoprecipitation by anti-phosphotyrosine antibodies of the 70-kDa protein contained in the insulin-binding protein preparation and phosphorylated in response to insulin (lane 1) was completely blocked by anti-phosphotyrosine (lane 2) but not anti-phosphoserine/threonine (lane 3), whereas phosphoserine/threonine (lane 5) but not phosphotyrosine (lane 6) inhibited the immunoprecipitation of the 53-kDa phosphoprotein by anti-phosphoserine/threonine antibodies (lane 4).

Remarkably, we completely failed to recognize tyrosine phosphorylation of proteins using immunoprecipitation and subsequent immunoblotting with anti-phosphotyrosine antibodies in both total cellular membranes and isolated plasma membranes from intact yeast cells or spheroplasts under conditions of insulin-stimulated glycogen synthesis (data not shown). This may be explained by both a rather limited number and a very low abundance of phosphotyrosine-containing proteins in *Saccharomyces cerevisiae* even in the insulin-stimulated state. In addition, tyrosine phosphorylation may be lost during the procedures of homogenization, membrane preparation, and detection despite the inclusion of a mix of typical inhibitors of protein tyrosine phosphatases (e.g., Na_3VO_4, phenylarsine oxide) in the crude samples. Thus, the detection of tyrosine-specific protein kinase activity in yeast might necessitate high enrichment and partial purification of the kinases and their substrates (as performed in the present study by sequential lectin and insulin affinity chromatographies) as well as subsequent phosphorylation in vitro.

The Insulin-Binding Protein Forms a High-Molecular-Mass Complex with Insulin-Stimulated Protein Kinase(s) and Phosphoproteins. The 53-kDa insulin-binding protein as identified by affinity cross-linking may constitute a serine/threonine-specific protein kinase with the capacity of autophosphorylation. Alternatively, it may represent a substrate for serine/threonine-specific phosphorylation by a distinct insulin-dependent kinase contained in the partially purified insulin-binding protein preparation. The 70-kDa polypeptide species apparently is a substrate for an insulin-dependent tyrosine-specific kinase. All these proteins may have been recovered with the insulin-binding protein preparation used for the in vitro kinase assay by specific association with the insulin-binding protein or by mere fortuitous copurification along the wheat germ lectin and insulin affinity chromatographies. Consequently, we tested the possibility for the existence of a noncovalent "signaling" complex which consists of the insulin-binding protein and the (insulin-controlled) phosphoproteins and resists solubilization of the plasma membranes with 1% TX-100. For this, partially purified insulin-binding protein was either cross-linked to [^{125}I]-monoiodo[B_{26}]insulin or incubated with [γ-^{32}P]ATP (in the absence or presence of insulin) and then size-fractionated by gel filtration (Figure 3).

The elution profiles revealed two peaks of both ^{125}I and ^{32}P radioactivity (panel A). With respect to the first peak, the elution position (I) of the cross-linked insulin-binding protein/complex (triangles) coincided with that of the phosphoprotein/complex (squares, presence of insulin) and corresponded to about 300 kDa between that of the calibrating molecular weight markers, apoferritin (443 kDa) and β-amylase (200 kDa)(panel B, arrow I). In the absence of insulin, the amount of phosphorylated protein eluted at position I was reduced to about 25% (panel A, circles), demonstrating the insulin dependence of the phosphorylation. The ^{125}I radiolabel eluting at position II (triangles), roughly coincident with BSA (panel B), accounted for about the same total radioactivity as eluting at 300 kDa and slightly preceded that of the ^{32}P radiolabel (position III, squares). It most likely corresponded to the cross-linked insulin-binding protein.

The phosphorylated proteins contained in the second peak (in the presence of insulin, squares; in the absence of insulin, circles) accounted for about 35% of the total amount of phosphoproteins at position I and eluted as a broad peak (covering 70−45 kDa) with a maximum at about 55 kDa (position III) and considerable overlap with the cross-linked insulin-binding protein (triangles). Insulin did not affect the relative elution pattern, but increased the amount of phosphoprotein at position III by 2−3-fold. Apparently, gel filtration on Sephacryl S-300HR allows efficient separation

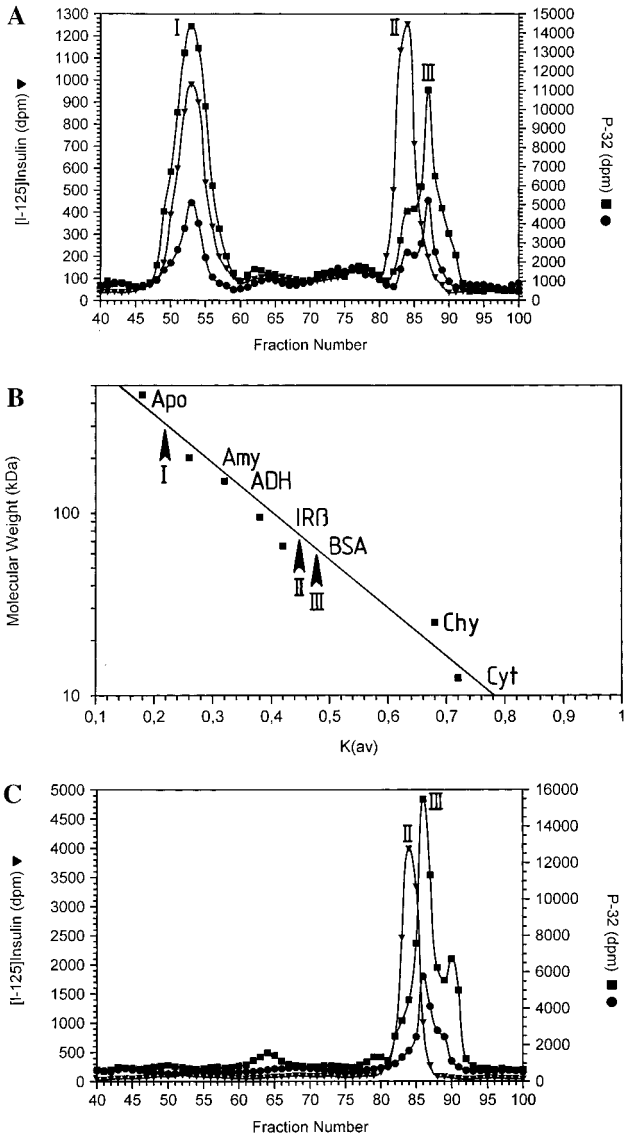

FIGURE 3: Gel filtration chromatography of the complex. One to two micrograms of partially purified insulin-binding protein from glucose-induced spheroplasts was incubated with [^{125}I]monoiodo-[B_{26}]insulin and cross-linked using disuccinimidyl suberate (triangles). Another 5-μg portion of insulin-binding protein was incubated in the absence (circles) or presence of 1 μM human insulin (squares) together with [γ-^{32}P]ATP (see Experimental Procedures). After termination of the cross-linking and protein kinase assays, halves of each mixture were precipitated with TCA and the precipitates then dissolved (panel C); the other halves were left untreated (panel A). Each mixture was subjected to gel filtration (see Experimental Procedures). Fractions were collected and counted for radioactivity. The three peaks are designated by "I, II, and III". The fractional elution volumes (K_{av}) of various standard proteins used to calibrate the column are given in panel B. The arrows correspond to the fractional elution positions I, II, and III of the complex and its constituents. The elution profiles of a representative experiment (repeated 5 times with similar results) are shown.

of a large 300-kDa complex consisting of the 53-kDa insulin-binding protein and associated phosphoproteins from the constituent monomeric components. The observation that only 30−50% of the insulin-binding protein and phosphoproteins were recovered as complex may be explained by incomplete recruitment (e.g., in case of regulated assembly from the monomeric subunits) or dissociation of the complex during partial purification and analysis. The noncovalent nature of the complex was demonstrated by TCA precipitation of the partially purified insulin-binding protein, which had been cross-linked to [^{125}I]monoiodo[B_{26}]insulin or incubated with [^{32}P]ATP, prior to gel filtration analysis (panel C). The ^{125}I and ^{32}P radiolabels were eluted at positions II (triangles) and III (squares, circles), respectively, corresponding to the monomeric insulin-binding protein and phosphoproteins. Strikingly, no radiolabel was detected at position I, indicating the absence of significant amounts of the 300-kDa complex after TCA precipitation. (The total recovery of ^{125}I- and ^{32}P-labeled proteins after gel filtration was comparable between the precipitated and control samples.)

The presence of phosphoproteins in the complex was confirmed by analysis using SDS−PAGE and autoradiography of the proteins contained in selected fractions after gel filtration of native (−TCA) or denatured (+TCA) material (Figure 4A). Phosphoproteins (predominantly in the range of 70 and 50 kDa) were recovered in fraction 53 (corresponding to position I of the complex) under native conditions, only, whereas fractions 85−91 (corresponding to positions II and III of the monomers) contained phosphoproteins under both native and denatured conditions. The nature of the phosphoproteins eluting as monomers in fraction 86 and as a complex in fraction 53 (under native conditions) was tested by immunoprecipitation with anti-phosphoserine/threonine or anti-phosphotyrosine antibodies followed by SDS−PAGE and autoradiography. It can be seen from Figure 4B that the predominant 70- and 53-kDa phosphoprotein species, contained in fractions 53 and 86, were phosphorylated on tyrosine (lanes 1−4) and serine/threonine (lanes 5−8), respectively. Incubation of the partially purified insulin-binding protein with human insulin prior to gel filtration significantly increased the phosphorylation of both proteins. Taken together, these data argue that the insulin-binding protein forms a noncovalent 300-kDa complex consisting of the insulin-sensitive 70-kDa phosphotyrosine- and 53-kDa phosphoserine/threonine-containing proteins. This complex is stable in 1% TX-100 and 150 mM NaCl, but dissociates during TCA precipitation.

The nature of the 53-kDa and 70-kDa phosphoproteins assembled in the 300-kDa complex was confirmed by phosphoamino acid analysis (Figure 5A) of the individual polypeptides isolated from the gel after SDS−PAGE separation of the immunoprecipitated total (lanes 1−6) or size-fractionated (lanes 7−10) ^{32}P-labeled insulin-binding protein preparation. The 53-kDa and 70-kDa proteins prepared from the total complex (lanes 1−3 and 4−6, respectively) or from fractions 53 and 86 of the gel-filtrated complex (lanes 7, 8 and 9, 10, respectively) contained exclusively phosphoserine and phosphotyrosine, respectively. Insulin present during the kinase reaction increased the amount of phosphoserine and phosphotyrosine recovered with the immunoprecipitated 53-kDa and 70-kDa proteins about 5.5-fold and 2.9-fold, respectively, above basal. Thus, the phosphoamino acid

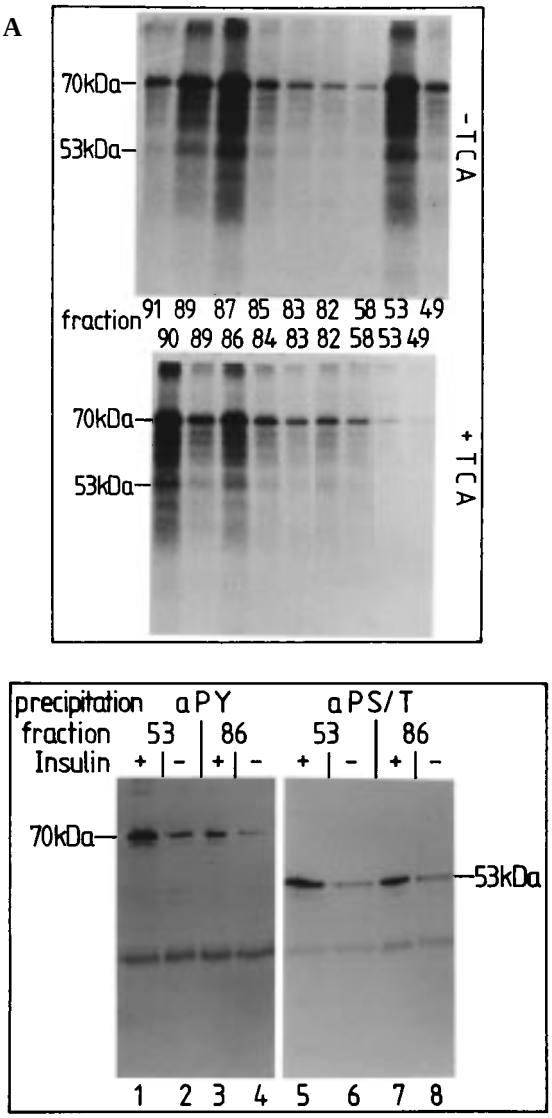

FIGURE 4: SDS−PAGE analysis of the phosphoproteins contained in the complex. Panel A: Partially purified insulin-binding protein phosphorylated with [γ-^{32}P]ATP in the presence of human insulin (1 μM) was subjected to gel filtration (half directly, upper portion; the other half after TCA precipitation, lower portion) as described for Figure 3. Equivalent volumes of selected fractions (as indicated) were precipitated and analyzed by SDS−PAGE and autoradiography. Panel B: Partially purified insulin-binding protein phosphorylated with [γ-^{32}P]ATP in the absence or presence of human insulin (1 μM) was subjected directly to gel filtration as described for Figure 3. Halves of fractions 53 and 86 were subjected to immunoprecipitation with anti-phosphotyrosine (apY) and anti-phosphoserine/threonine (apS/T) antibodies. The immunoprecipitates were analyzed by SDS−PAGE and autoradiography. The autoradiograms of a typical experiment (repeated 3 times with comparable results) are shown.

analysis confirmed the specificity of the anti-phosphoserine/threonine and anti-phosphotyrosine antibodies used for immunoprecipitation and immunoblotting of the phosphoproteins constituting the 300-kDa insulin-binding protein complex.

In addition, we analyzed the nature of the phosphoproteins in the size range of 58−68 and 35−49 kDa which were obtained by direct gel filtration of the phosphorylated insulin-binding protein omitting prior to immunoprecipitation and SDS−PAGE separation (see Figure 4A). Phosphoamino acid analysis of the 58−68-kDa proteins contained in gel

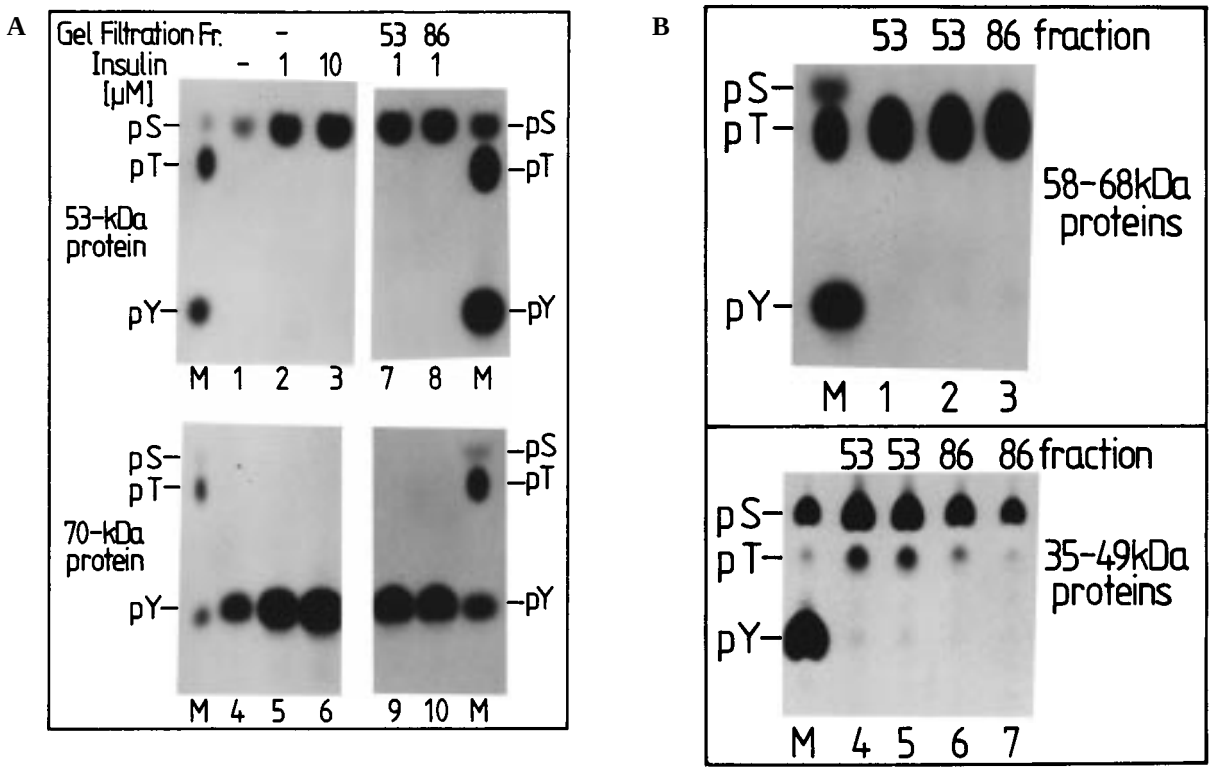

FIGURE 5: Phosphoamino acid analysis of ^{32}P-phosphorylated polypeptides contained in the insulin-binding protein preparation. Panel A, lanes 1−6: 10 μg of partially purified insulin-binding protein from glucose-induced spheroplasts was incubated with [γ-^{32}P]ATP in the absence or presence of human insulin at the concentrations indicated and subsequently immunoprecipitated with anti-phosphoserine/threonine antibodies or anti-phosphotyrosine antibodies. Panel A, lanes 7−10: 5 μg of partially purified insulin-binding protein phosphorylated with [γ-^{32}P]ATP in the presence of human insulin was subjected to gel filtration as described for Figure 3. Halves of fractions 53 and 86 were subjected to immunoprecipitation with anti-phosphoserine/threonine or anti-phosphotyrosine antibodies. All lanes: The immunoprecipitates were analyzed by SDS−PAGE and autoradiography. Gel pieces containing the radiolabeled 53-kDa (lanes 1−3, 7, 8) and 70-kDa (lanes 4−6, 9, 10) proteins were excised, and the polypeptides were extracted and hydrolyzed (as described under Experimental Procedures). Phosphoamino acids were resolved by thin-layer electrophoresis. The positions of radiolabeled phosphoserine (pS), phosphothreonine (pT), and phosphotyrosine (pY) run in parallel on the same thin-layer plate are indicated (M). Panel B: 5 μg of partially purified insulin-binding protein phosphorylated with [γ-^{32}P]ATP in the presence of human insulin was subjected to gel filtration. Fractions 53 and 86 were analyzed by SDS−PAGE and autoradiography as described for Figure 3. Gel pieces containing some radiolabeled polypeptides in the molecular mass range of 58−68 kDa (lanes 1−3) and 35−49 kDa (lanes 4−7) from each fraction were excised, and the polypeptides were extracted and subjected to phosphoamino acid analysis as described for panel A. Several fractions obtained from gel filtrations of different insulin-binding protein preparations and phosphorylation reactions were analyzed in parallel.

filtration fractions 53 as well as 86 from several independent insulin-binding protein preparations revealed the presence of phosphothreonine, exclusively (Figure 5B, lanes 1−3). Thus, these polypeptides are apparently not proteolytically derived from the 70-kDa phosphotyrosine-containing protein. The failure to detect these proteins by immunoprecipitation of the total (see Figure 1A) or size-fractionated (see Figure 4B) phosphorylated insulin-binding protein complex indicates a rather low affinity of the anti-phosphothreonine antibody used. The 35−49-kDa proteins recovered with gel filtration fractions 53 and 86 from several independent insulin-binding protein preparations habored predominantly phosphoserine and, to a minor degree, phosphothreonine (Figure 5B, lanes 4−7). Therefore, it cannot be excluded that (a portion of) these polypeptides represent(s) proteolytic degradation products of the 53-kDa phosphoserine-containing protein. However, these polypeptides comprised a rather minor fraction of the total phosphoproteins, only, so that they could not be recognized by immunoprecipitation with anti-phosphoserine antibodies (see Figures 1A and 4B).

Next we tested whether the 300-kDa complex is capable of insulin-dependent tyrosine phosphorylation in the absence of monomeric insulin-binding and 70-kDa phosphotyrosine-containing proteins. For this, the 300-kDa complex was recovered by gel filtration (see above) prior to immunoprecipitation with anti-phosphotyrosine antibodies. The immunoprecipitates were assayed for protein kinase activity by simultaneous incubation with human insulin (various concentrations, Figure 6, left panel) and [γ-^{32}P]ATP (various periods of time, Figure 6, right panel). The analysis of the immunoprecipitates by isoelectric focusing revealed the presence of three phosphotyrosine-containing proteins (p*I* 5.1, 5.2, and 5.4). Phosphorylation of the p*I* 5.1 and 5.4 species was stimulated by human insulin in a concentration-dependent manner (EC_{50} values about 0.1 μM; maximally 5−7-fold increase), whereas the amount of the pI-5.2 phosphotyrosine-containing protein did not change significantly with the insulin concentration (left panel). The time course (right panel) revealed that the insulin-stimulated tyrosine phosphorylation remained about constant with time for the p*I* 5.2 species, but drastically increased for the p*I* 5.4 species and decreased for the p*I* 5.1 species (possibly being due to dephosphorylation by an associated phosphatase). On SDS−PAGE, these three components could

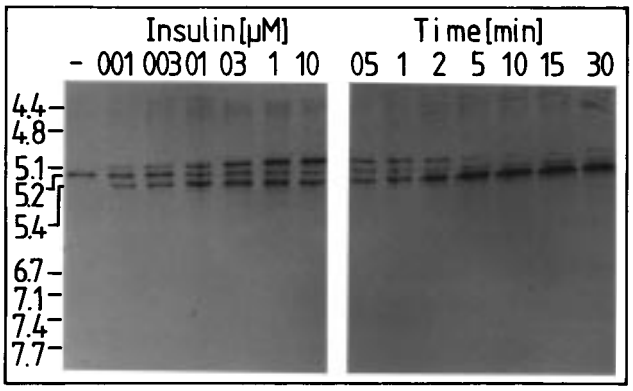

FIGURE 6: Analysis of tyrosine phosphorylation of the complex by isoelectric focusing. Partially purified insulin-binding protein was directly subjected to gel filtration. Pooled fractions 51−55 (see Figure 3) were immunoprecipitated with anti-phosphotyrosine antibodies. The collected beads were suspended in kinase buffer and assayed for protein kinase activity in the presence of [γ-^{32}P]-ATP and human insulin at various final concentrations (left panel, vanadate present in the kinase buffer) for 5 min or at 1 μM for various periods of time (right panel, vanadate has been omitted from the kinase buffer and added for termination, only). After termination of the kinase assay, the immunoprecipitates were collected and analyzed by isoelectric focusing and autoradiography. The autoradiograms of a typical experiment (repeated twice with similar results) are shown.

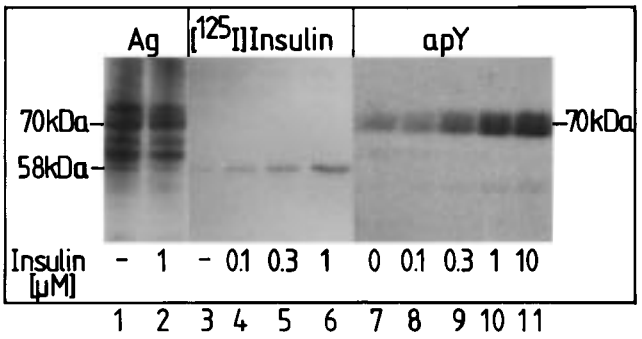

FIGURE 7: Coimmunoprecipitation of the insulin-binding protein and the 70-kDa phosphotyrosine-containing protein. Partially purified insulin-binding protein was subjected to protein kinase assay (vanadate present in the kinase buffer) with unlabeled ATP in the absence or presence of the indicated concentrations of human insulin and then size-fractionated by gel filtration. Pooled fractions 51−55 [see Figure 3A, portions of which were analyzed directly by SDS−PAGE and silver staining (lanes 1 and 2; Ag)], were immunoprecipitated with anti-phosphotyrosine antibodies. Half of each immunoprecipitate was analyzed by immunoblotting with anti-phosphotyrosine antibodies and subsequent detection by chemiluminescence (lanes 7−11; apY). The other half was cross-linked to [^{125}I]monoiodo[B_{26}]insulin using disuccinimidyl suberate after suspension of the collected immunoprecipitates in cross-linking buffer (as described under Experimental Procedures) and then analyzed by SDS−PAGE and autoradiography (lanes 3−6; [^{125}I]-Insulin). The molecular masses indicated were derived from marker proteins run in parallel on the same gel. The figure shows a typical experiment repeated twice with similar results.

not be separated from one another and migrated at about 70 kDa (see below).

The interaction of the insulin-binding protein with phosphotyrosine-containing proteins was confirmed by coimmunoprecipitation with anti-phosphotyrosine antibodies. For this, the partially purified and size-fractionated (300-kDa) complex was phosphorylated with unlabeled ATP in vitro in the presence of increasing concentrations of human insulin and then subjected to immunoprecipitation with anti-phosphotyrosine antibodies (Figure 7). The presence of the insulin-binding protein in the immunoprecipitates was demonstrated by subsequent cross-linking with [^{125}I]monoiodo-[B_{26}]insulin (lanes 3−6). The insulin-dependent increase in the amount of cross-linked 58-kDa insulin-binding protein (lanes 3−6) correlated well with the increment of tyrosine phosphorylation of the 70-kDa protein as detected by immunoblotting with anti-phosphotyrosine antibodies (lanes 7−11). The silver-stained protein patterns of the 300-kDa complex (lanes 1 and 2) displayed five major polypeptides (with no difference between the absence and presence of insulin during the preceding phosphorylation), among them candidates for the insulin-binding and 70-kDa phosphotyrosine-containing proteins. Therefore, it remains open whether these two proteins interact directly with one another or via additional components contained in the complex. Taken together, we conclude that the plasma membrane of *Saccharomyces cerevisiae* harbors a 300-kDa complex consisting of insulin-regulated tyrosine-specific protein kinase(s) (and possibly phosphatase(s)) along with the 70-kDa substrate protein and the 53-kDa insulin-binding protein. Since the 53-kDa phosphoserine-containing protein was coeluted during gel filtration as well as coimmunoprecipitated with anti-phosphotyrosine antibodies together with the 300-kDa complex, it seems likely that it is identical with the insulin-binding protein. However, it cannot be ruled out that a phosphoserine-containing protein of similar size as the insulin-binding protein is a distinct component of the complex.

The Affinity of the Binding Protein to Insulin Analogues Is Correlated to Their Potency in Inducing Protein Phosphorylation and Biological Effects. Next we tried to obtain evidence for the involvement of the 53-kDa insulin-binding protein and the associated phosphoproteins in mediating insulin stimulation of glycogen synthase and SNF1 kinase in *Saccharomyces cerevisiae* (*1*; Müller, G., Rouveyre, N., Crecelius, A., and Bandlow, W., first of three papers in this issue). For this, the efficiency of insulin was compared with regard to (i) binding to the partially purified insulin-binding protein and stimulation of (ii) serine phosphorylation of the 53-kDa polypeptide, (iii) tyrosine phosphorylation of the 70-kDa polypeptide, (iv) glycogen synthase, and (v) SNF1 kinase (Table 1). Four mutant analogues of insulin with multiple amino acid substitutions (which vary in the EC_{50} values for activation of lipogenesis in isolated rat adipocytes) as well as IGF1 and EGF were used in this experiment. The biological activities were determined in glucose-induced spheroplasts incubated in the presence of 1 μM hormone (in the range of the K_d of the insulin-binding protein; see second of three papers in this issue). The kinase activities were assayed in vitro after incubation of the partially purified insulin-binding protein (from glucose-induced spheroplasts) in the presence of 1 μM hormone. Binding was measured as displacement of [^{125}I]monoiodo[B_{26}]insulin from partially purified insulin-binding protein (from glucose-induced spheroplasts) by 10 μM unlabeled hormone (which enables differentiation between the various analogues). The relative efficacies of the insulin analogues in competing binding of human insulin (ranking: human insulin > analogue I > analogue II > analogue III > analogue IV) correlated well with their potential to activate glycogen synthase, SNF1

Table 1: Correlation between the Affinity to the Insulin-Binding Protein and Biological Effects of Human Insulin and Insulin Analogues[a]

	serine/threonine kinase activity	tyrosine kinase activity	binding
human insulin	100 (0.2)	100 (0.1)	100
analogue I	93 ± 11	112 ± 17	81 ± 7
analogue II	64 ± 13	82 ± 10	49 ± 5
analogue III	19 ± 9	47 ± 14	6 ± 2
analogue IV	4 ± 2	11 ± 6	0
IGFI	71 ± 9	57 ± 18	33 ± 8
EGF	3 ± 2	7 ± 5	2 ± 2

[a] Serine/threonine- and tyrosine-specific kinase activities were determined with the partially purified insulin-binding protein incubated in the absence or presence of insulin and analogues at 1 μM final concentration for 20 min by immunoprecipitation of the 53-kDa and 70-kDa phosphoproteins, respectively. The difference between the activities measured for hormone-treated and basal spheroplasts is given as the percentage of the maximal insulin effect (set at 100%). Binding was assayed as displacement of [^{125}I]monoiodo[B_{26}]insulin by 10 μM unlabeled competing hormone (see Figure 2). The amount of radiolabeled insulin displaced by human insulin (difference between total and unspecifically associated insulin) was set at 100%. Each value represents the mean ± SEM of at least three different binding protein preparations with assays performed in triplicate. The EC_{50} values (μM) for human insulin are given in parentheses. 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).

kinase, serine phosphorylation of the 53-kDa protein, and tyrosine phosphorylation of the 70-kDa protein. IGF1 exerted medium effects comparable to those of analogue II. Analogue IV and EGF were almost inactive in each parameter studied in line with their very low insulin-mimetic activity in isolated rat adipocytes. The identical ranking of the insulin variants with respect to their relative potencies in increasing glycogen synthase and SNF1 activities as well as protein phosphorylation of components of the 300-kDa complex and their relative affinities to the insulin-binding protein strongly argues that the complex formed by the insulin-binding protein and phosphoproteins participates in mediating the effects of insulin on glycogen and glucose metabolism in *Saccharomyces cerevisiae*.

DISCUSSION

In the present study we demonstrated that the partially purified insulin-binding protein from yeast undergoes phosphorylation in response to insulin on serine residues, exclusively, as demonstrated by both immunoprecipitation/immunoblotting with anti-phosphoserine antibodies and phosphoamino acid analysis. This modification may be due to autophosphorylation or, alternatively, to a distinct insulin-responsive serine-specific kinase activity contained in the binding protein preparation. These properties clearly differ from those of the mammalian insulin receptor, which is a heterotetramer consisting of two A- and two B-chains (125 and 95 kDa, respectively). The B-chain functions as tyrosine kinase which is regulated by the disulfide-linked insulin-binding A-chain (for reviews, see refs *25*−*27*). It also differs from the putative insulin receptor from *Neurospora crassa* which migrates as a single band of 66 kDa on reducing SDS−PAGE but as two polypeptides of about 55 and 110 kDa on native PAGE (*28*−*30*). The latter species has been suggested to represent the dimeric form of the functional receptor which possesses no autophosphorylation activity or protein kinase activity toward exogenous substrates, and which contains no detectable phosphorylated amino acids (*29*).

Despite these obvious differences from both the mammalian and *Neurospora* insulin receptors, the following findings strongly argue that the 53-kDa insulin-binding protein functions as the "receptor" that mediates insulin-induced metabolic effects in *Saccharomyces cerevisiae*: (i) the K_d value of human insulin for binding to the 53-kDa protein as well as the insulin concentration required for induction of serine phosphorylation of the 53-kDa polypeptide is similar to the insulin concentrations exerting insulin-mimetic activity in glucose-induced spheroplasts. (ii) The affinity of various insulin analogues in binding to the 53-kDa protein as demonstrated by competition of both binding and affinity cross-linking of iodinated insulin strictly correlates with their efficacy in inducing insulin-mimetic effects (see preceding two papers). In view of the evolutionary distance, it is surprising to find identical relative affinities of insulin analogues (which are characterized by rather subtle structural differences from one another) to the insulin receptor from rat adipocytes and the insulin-binding protein from *Saccharomyces cerevisiae*. This suggests that in evolution insulin did not arise in the intestinal or neuroendocrinous tissues of primitive vertebrates or complex invertebrates but rather has its functional origins at least as far back as the simplest unicellular eucaryotes.

The apparent monomeric nature of the yeast insulin-binding protein and the missing intrinsic tyrosine kinase activity raises the possibility that a kinase domain may have been inactivated or proteolytically removed during its purification. Alternatively, ligand binding to the insulin-binding subunit may activate a tyrosine kinase which is constituted by a noncovalently associated subunit of a heterooligomeric receptor kinase complex in a manner similar to the system found with the cytokine receptors and JAK kinases (for a review, see ref *31*). The following lines of evidence hint to the latter possibility and argue that the signaling complex isolated is functional in vivo: (i) a 70-kDa polypeptide contained in the partially purified preparation of the insulin-binding protein was phosphorylated on tyrosine in vitro in an insulin-inducible fashion; (ii) the insulin-binding protein and the 70-kDa phosphotyrosine-containing protein coeluted during gel filtration as a 300-kDa complex being sensitive to denaturation; (iii) the insulin-binding protein and the 70-kDa phosphotyrosine-containing protein were coimmunoprecipitated by anti-phosphotyrosine antibodies; (iv) the relative efficacies of insulin variants in inducing serine-specific phosphorylation of the insulin-binding protein and tyrosine-specific phosphorylation of the 70-kDa phosphoprotein were identical. These data hint to a specific interaction of the two proteins either directly or via additional (bridging) proteins in a multimeric complex. Interestingly, in intact *Neurospora crassa* cells, insulin-stimulated tyrosine phosphorylation of a 38-kDa protein has been detected by both immunoblotting and immunoprecipitation with an antibody raised against a peptide from the human insulin receptor that contains an autophosphorylated tyrosine residue but itself does not bind insulin (32). One may speculate that simple unicellular eucaryotes contain an insulin receptor tyrosine kinase consisting of distinct insulin-

binding and tyrosine kinase subunits which in the case of *Saccharomyces cerevisiae* are noncovalently linked.

Strikingly, sequence-based searches gave no hints that budding yeast harbors members of the true protein-tyrosine kinase family present in mammalian cells (e.g., insulin receptor B-subunit; for a review, see ref *33*). Therefore, it may be anticipated that the predominant driving force for the evolution of protein-tyrosine kinases was the requirement for a signaling mechanism devoted exclusively to cell−cell communication within multicellular organisms. However, despite the absence of true protein-tyrosine kinases, yeast, based on sequence analysis, contains several so-called dedicated protein-tyrosine kinases of both single specificity (e.g., Swe1; see ref *34*) and dual specificy (e.g., Spk1 and Mps1; see ref *35*), among the latter are members of the MAPKK family. These dual-specificity kinases have been demonstrated to (auto)phosphorylate on serine, threonine, and tyrosine residues in vitro, some of them within the typical TXY motif in the activation loop of MAPK. Furthermore, several genes of *Saccharomyces cerevisiae*, among them YGR080W, are homologous to the sequence of the mammalian protein-tyrosine kinase A6 (33). This kinase is totally unrelated in structure to the true protein-tyrosine kinase family. Taken together, the existence of these putative protein-tyrosine kinases in yeast underscores the physiological importance of tyrosine phosphorylation also in unicellular eucaryotes.

Interestingly, serine-specific phosphorylation of the partially purified insulin-binding protein and insulin receptor in response to insulin has been demonstrated for both *Saccharomyces cerevisiae* (this study) and rat liver (36−39) and, in the latter case, attributed to a noncovalently associated serine/threonine-specific insulin receptor kinase or to a kinase activity intrinsic to the B-subunit. The functional significance of these kinetically delayed and potentially "secondary" phosphorylation events remains unclear so far but may be related to termination of the insulin signal or to down-regulation/desensitization in response to permanent signaling via the receptor.

The existence of apparently similar key elements and their analogous arrangement with regard to levels I and II as found in the present and preceding paper (see second of three papers) as well as levels "III−V" as described in previous reports (see the introduction) and the accompanying paper (see first of three papers) suggests that insulin signaling is part of an evolutionarily conserved signal transduction pathway for the control of glucose and glycogen metabolism.

ACKNOWLEDGMENT

We thank Dr. J. Ertl for providing the insulin analogues and Mrs. A. Unkelbach for expert assistance in preparing the figures (all Hoechst Marion Roussel Deutschland GmbH, Frankfurt, Germany) and Dr. R. Schulte-Brinkmann (University of Düsseldorf, Germany) for helpful advice on the phosphoamino acid analysis.

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BI980102Q