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Interaction of the Insulin Receptor Kinase With Serine/Threonine Kinases In Vitro

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Insulin causes rapid phosphorylation of the β subunit (M_r = 95,000) of its receptor in broken cell preparations. This occurs on tyrosine residues and is due to activation of a protein kinase which is contained in the receptor itself. In the intact cell, insulin also stimulates the phosphorylation of the receptor and other cellular proteins on serine and threonine residues. In an attempt to find a protein that might link the receptor tyrosine kinase to these serine/threonine phosphorylation reactions, we have studied the interaction of a partially purified preparation of insulin receptor with purified preparations of serine/threoine kinases known to phosphorylate glycogen synthase. No insulin-dependent phosphorylation was observed when casein kinases I and II, phosphorylase kinase, or glycogen synthase kinase 3 was incubated in vitro with the insulin receptor. These kinases also failed to phosphorylate the receptor. By contrast, the insulin receptor kinase catalyzed the phosphorylation of the calmodulin-dependent kinase and addition of insulin in vitro resulted in a 40% increase in this phosphorylation. In the presence of calmodulin-dependent kinase and the insulin receptor kinase, insulin also stimulated the phosphorylation of calmodulin. Phosphoamino acid analysis showed an increase of phosphotyrosine content in both calmodulin and calmodulin-dependent protein kinase. These data suggest that the insulin receptor kinase may interact directly and specifically with the calmodulin-dependent kinase and calmodulin. Further studies will be required to determine if these phosphorylations modify the action of these regulatory proteins.

Key words: insulin receptor, tyrosine phosphorylation, serine kinases

Insulin stimulates the phosphorylation of the β subunit (M_r = 95,000) of the insulin receptor in intact cells [1–3] and in several cell-free systems [4–13]. In the broken cell, this phosphorylation is due to the activation of a tyrosine-specific protein kinase which appears to be contained in the β subunit of the receptor. This kinase activity is retained in a highly purified preparation of receptor [6] and is active both in autophosphorylation of the receptor and on exogenous substrates [7,9]. Several

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groups have also shown that the β subunit can be labeled using ATP-affinity reagents [14–16]. In addition to the receptor, insulin stimulates the phosphorylation and dephosphorylation of a number of other cellular proteins, including a variety of enzymes involved in both carbohydrate and lipid metabolism [17–21]. Thus, phosphorylation may be an important part of a process by which the insulin signal is transmitted at the postreceptor level.

In intact cells, phosphorylation of the receptor occurs at serine and threonine residues in addition to tyrosine residues [2]. Since the receptor itself possesses only tyrosine kinase activity, this suggests that the receptor in vivo is also a substrate for a serine/threonine kinase that is independent of the receptor. The serine phosphorylation also appears to be insulin stimulated [2]. Thus, one of the substrates of the receptor kinase may be a serine kinase that is activated by tyrosine phosphorylation and that may then phosphorylate other cellular substrates, including the insulin receptor, at serine or threonine residues. In an attempt to find an enzyme that might be a physiological substrate of the receptor, we have studied a variety of serine kinases as potential substrates of the receptor kinase in vitro. These include casein kinases I and II [22,23], a calmodulin-dependent protein kinase [24], glycogen synthase kinase 3 [25,26], and phosphorylase kinase [27,28]. We find that the calmodulin-dependent protein kinase and calmodulin are both phosphorylated in an insulin-stimulated way, suggesting a possible interaction between these proteins and the insulin-receptor kinase.

MATERIALS AND METHODS

Materials

Porcine insulin (lot 1JM95AN) was purchased from Elanco (Indianapolis, IN); [^{32}P]-ATP and Triton X-100 were from New England Nuclear (Boston, MA). Aprotinin, phenylmethylsulfonyl fluoride, N-acetyl-D-glycosamine, phosphoserine, phosphothreonine, and phosphotyrosine were obtained from Sigma (St. Louis, MO); wheat germ agglutinin coupled to agarose was from Vector Miles. All reagents for $NaDodSO_4$-polyacrylamide gel electrophoresis were from Bio-Rad (Rockville Center, NY). Coon's modified Ham's F12 culture medium and fetal calf serum were purchased from Grand Island Biological Company (Grand Island, NY). All other reagents were of the best grade commercially available.

Protein Kinases

Casein kinase II was purified to homogeneity from rabbit skeletal muscle as described by DePaoli-Roach et al [23]. Phosphorylase kinase, also from rabbit skeletal muscle, was isolated by the method of Cohen [28,29]. Rabbit liver casein kinase I was purified, to approximately 50% purity, as described by Ahmad et al [26]. The calmodulin-dependent protein kinase, also from rabbit liver, was essentially homogeneous and was isolated according to Ahmad et al [25]. The glycogen synthase kinase 3/GSK-3 was partially purified from rabbit liver as described in Ahmad et al [26]. Calmodulin from rabbit skeletal muscle was isolated by a modification of the method of Dedman et al [30].

Cell and Incubation Procedures

Fao is a well-differentiated clonal line of rat hepatoma cells derived from the H4-II-EC_3 line [30,31] established from a Reuber H-35 minimal deviation hepatoma

[32]. This cell line, originally provided by Dr. M. Weiss (Gif-sur-Yvette, France), exhibits a number of liver-specific properties, including an inducible tyrosine aminotransferase activity, the secretion of albumin, and the synthesis of liver-specific isomers of alcohol dehydrogenase and aldolase [30]. The cell line has many insulin receptors and is very sensitive to insulin as measured by stimulation of glycogen synthase, tyrosine aminotransferase, and γ-amino-isobutyric-acid influx [30,33]. The cells were cultivated in a modified Ham's F12 medium [34] supplemented with 5% fetal calf serum in a humidified atmosphere of 5% CO_2, 95% O_2 at 37°C. For all experiments, cells grown attached to the surface of Corning plastic bottles were detached with a solution of 0.05% trypsin and 0.02% EDTA and replaced in Corning plastic dishes 3 to 5 days prior to an experiment. Cells were used at confluency. Confluent 150-mm dishes contained about 50×10^6 cells.

In Vitro Receptor and Substrate Phosphorylation

A partially purified preparation of receptor was prepared as previously described [4]. Fao cells from 20 to 30 dishes (150 mm) were solubilized in 2 ml/dish of 25 mM Hepes buffer (pH 7.4) containing PMSF (1 mM), Aprotinin (1 TIU/ml), and Triton X-100 (1%) at 4°C for 30 min. Insoluble material was removed by centrifugation at 200,000g for 60 min, and the supernatants were applied to columns containing wheat germ agglutinin coupled to agarose. After extensive washing with 25 mM Hepes buffer (pH 7.4) containing 0.1% Triton X-100, the bound material was eluted with buffer supplemented with 0.3M N-acetyl-glucosamine. Aliquots of the eluate from the lectin column containing 4–6 μg protein were incubated with or without insulin at room temperature for 1 hr. Test substrates were added at the concentrations indicated (Figures 1–5). Phosphorylation was studied by incubation with 40 μM [γ^{32}P]-ATP (specific activity, 2,900 Ci/mmole) in 25 mM Hepes buffer, pH 7.4, containing 10 mM $MnCl_2$ at 25°C for 10 min [2,13]. The incubation was stopped by boiling in Laemmli buffer. The proteins were then separated by $NaDodSO_4$-polyacrylamide gel electrophoresis and identified by autoradiography.

Identification of Phosphoamino Acids

Phosphoamino acids were analyzed by a modification of the method of Hunter and Sefton [36]. The [^{32}P]-labeled bands were localized by autoradiography and the corresponding portion of the dried gel was excised and washed for 12 hr at 37°C in 10% methanol. The gel fragment (about 1 cm^2) was dried at 100°C for 1 hr and rehydrated with 2 ml of 50 mM NH_4HCO_3 containing 50 mg/ml trypsin (Worthington crystalline trypsin-TPCK, 238 U/mg). The digestion was allowed to proceed for 16 hr at 37°C. The supernatant which contained about 90% of the total radioactivity was lyophilized and redissolved in 200 μl of 6N HCl. Acid hydrolysis was performed for 2 hr at 110°C. The samples were then diluted with 2 ml water, lyophilized, and redissolved in 10 μl water containing 1 mg/ml of phosphoserine, phosphothreonine, and phosphotyrosine as standards. The samples were applied onto cellulose-thin layer plates (Analtech Newark, DE, G1140, 20 × 20 cm). These plates were sprayed uniformly with pyridine/acetic acid (1%/10%) and electrophoresis was performed for 70 min at 15°C and 400 V. The phosphoamino acid standards were identified by reaction with ninhydrin. An autoradiogram was obtained by exposing Kodak X-Omat AR film with a Dupont Cromex Lightning Plus intensified screen to the dried chromatogram for 10 days at −80°C.

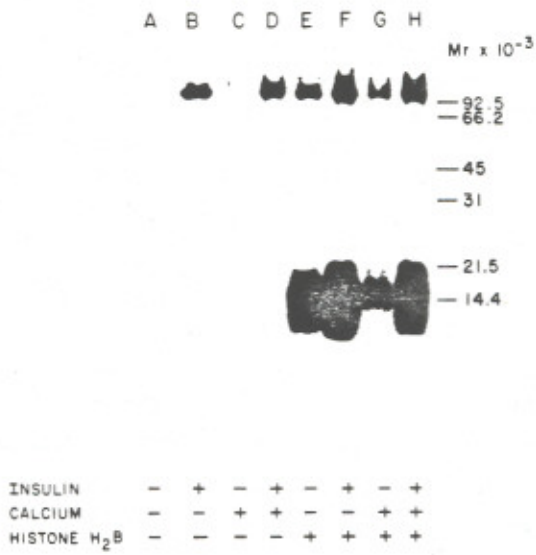


Fig. 1. Phosphorylation of histone H_2B by the insulin receptor. Partially purified insulin receptor (5 μg) was incubated at 22°C for 1 hr with $MnCl_2$ (5 mM) and in the absence of insulin (lane A) or with insulin (10^{-7}M, lanes B, D, F, H) and $CaCl_2$ (5 mM, lanes C, D, G, H). Then 10 μg of histone was added (lanes E–H) and phosphorylation was initiated by adding [γ^{32}P]-ATP (40 μM, 0.01 μCi). After 10 min, the reaction was stopped by adding Laemmli buffer and heating the samples to 100°C for 3 min. The phosphoproteins were separated by $NaDodSO_4$ polyacrylamide gel (15%) electrophoresis.

RESULTS

Phosphorylation of the Insulin Receptor and Histone

Incubation of wheat germ purified insulin receptor from Fao-hepatoma cells with [γ^{32}P]-ATP in the presence of 10 mM $MnCl_2$ revealed a [^{32}P]-labeled band with M_r = 95,000, as well as minor bands of higher molecular weight (Fig. 1, lanes A, B). Preincubation of the receptor with insulin (10^{-7}M) for 1 hr at room temperature increased the [^{32}P] incorporation in this band up to 20-fold. This phosphoprotein has been shown previously to correspond to the β-subunit of the insulin receptor based on electrophoretic mobility, recognition by various antiinsulin receptor sera, and the specificity of stimulation by insulin analogues [1,2].

To test the activity of the wheat germ-enriched insulin receptor on a known protein substrate [7,9], 10 μg histone H2B (Worthington Freehold, NJ) was incubated with the receptor and [γ^{32}P]-ATP in the presence or absence of insulin stimulated phosphorylation of both the β subunit of the receptor and of a diffuse band of M_r = 15,000–20,000 corresponding to the histone. In contrast to the finding of Shulman et al [37], neither the autophosphorylation nor the histone phosphorylation was affected by the addition of $MgCl_2$ (data not shown). Addition of 10 mM $CaCl_2$ (Fig. 1, lanes C, D, G, H) to the $MnCl_2$ had no effect on autophosphorylation, but caused a slight decrease in histone phosphorylation. Since we have previously shown

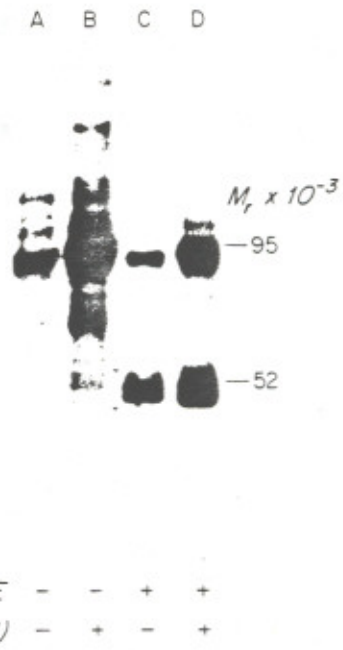


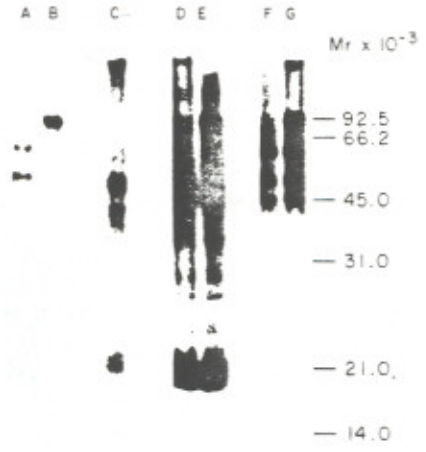
Fig. 2. Autoradiogram showing the incorporation of [^{32}P] from [^{32}P]-ATP into calmodulin-dependent (CDR) kinase using solubilized receptor from hepatoma cells. Experimental conditions were as described in Figure 1. Lanes A and B show samples where only receptor was present; in lanes C and D, calmodulin-dependent kinase (0.5 μg) and calmodulin were added. Lanes A and C show phosphorylation in the absence of insulin; lanes B and D show samples where the receptor was preincubated with insulin 10^{-7} M for 1 hr.

that Mn^{2+} most actively supports insulin receptor autophosphorylation [13], and since avoiding Mg^{2+} reduces the background phosphorylation owing to other kinases that might contaminate either the receptor or serine kinase preparation, as standard conditions to test the phosphorylation of various susbstrates by the receptor kinase we therefore used 5 μg of wheat germ-enriched receptor, 40 μM [γ^{32}P]-ATP and 5–10 mM Mn^{2+} and 2–10 μg of the various protein kinases to be tested.

Calmodulin-Dependent Protein Kinase and Calmodulin as Substrates for the Insulin Receptor

The effect of the insulin receptor was tested on five serine-directed protein kinases that are known to phosphorylate glycogen synthase in vitro [20,38]. These include phosphorylase kinase [27,28], glycogen synthase kinase 3 (also termed Fa/GSK-3) [25,26], casein kinases I and II (the latter is also referred to as $PC_{0.7}$, or glycogen synthase kinase 5) [22,23], and calmodulin-dependent protein kinase [24,25].

The effect of the insulin receptor kinase on the calmodulin-dependent kinase is illustrated in Figure 2. As shown above, insulin-stimulated phosphorylation of the insulin receptor was observed in the presence or absence of the calmodulin-dependent



INSULIN RECEPTOR	+	+		−		+	+		+	+
INSULIN	−	+		−		−	+		−	+
CALMODULIN KINASE	−	−		+		+	+		+	+
CALMODULIN	−	−		+		+	+		+	+
CALCIUM	−	−		+		+	+		−	−

Fig. 3. An autoradiograph showing autophosphorylation of the insulin receptor (β subunit M_r = 92.5), calmodulin-dependent kinase (M_r = 51,000 and M_r = 53,000), and calmodulin (M_r = 21,000). Insulin receptor (5 μg) was incubated for 1 hr with insulin (10^{-7}M) and $MnCl_2$ (10 mM) at 22°C and then calmodulin-dependent kinase (10 μg), calmodulin (10 μg), and $CaCl_2$ (5 mM) were added as indicated by the chart under the lanes. The phosphoproteins were separated by $NaDodSO_4$-PAGE under reducing conditions. This is a composite of several separate experiments and represents a 15% gel.

kinase (Fig. 2, lanes B and D vs lanes A and C). In the absence of insulin but in the presence of insulin receptor, Mn^{2+}, and [γ^{32}P]-ATP, the calmodulin-dependent kinase also exhibited phosphorylation (Fig. 2, lane C). This is seen as a doublet near M_r = 52,000 and is in agreement with previous work that has shown that this kinase undergoes an autophosphorylation reaction [25]. Preincubation of the insulin receptor kinase with insulin (10^{-7} M) increased the phosphorylation of the calmodulin-dependent kinase about twofold in this experiment. In eight separate experiments, the average kinase increase in [^{32}P] was 45% above basal.

Addition of calmodulin to the incubation mixture did not appear to alter the phosphorylation of the calmodulin-dependent kinase; however, it resulted in the appearance of a phosphorylated protein near the front of the 7.5% gel. To better explore better the possibility that calmodulin was phosphorylated, 15% gels were run. In these gels, a phosphorylated band of $M_r \simeq$ 21,000 was clearly apparent (Fig. 3, lanes D, E). This band occurred in the same position as calmodulin identified by Coomassie blue staining. Preincubation of the receptor kinase with insulin increased the phosphorylation of this protein by an average of 45% (N = 4). Similar results were obtained with a highly purified calmodulin preparation (kindly provided by Dr. A. Means, Houston, TX). Phosphorylation of calmodulin did not occur when the protein was incubated with the insulin receptor in the presence of calcium but the

absence of the calmodulin-dependent kinase (data not shown) or when the reaction was conducted in the absence of calcium but the presence of calmodulin-dependent kinase (Fig. 3, lanes F, G).

Phosphoamino Acid Analysis

The insulin receptor kinase has been shown to phosphorylate itself [4] and exogenous substrates at tyrosine residues [7,9]. To test if the increase in phosphorylation of the calmodulin-dependent kinase and of calmodulin was due to the activation of the receptor kinase by insulin, we performed a phosphoamino acid analysis of the phosphorylated proteins of M_r = 51–53,000 and of M_r = 21,000. Figure 4 shows one-dimensional electrophoresis of acid hydrolysates obtained from these phosphoproteins. In the absence of insulin, both peptides were phosphorylated primarily on threonine and serine, in agreement with the known specificity of the calmodulin-dependent kinase [24,25]. Addition of insulin did not influence the phosphothreonine and phosphoserine content; however, phosphorylation on tyrosine residues was increased, confirming the fact that the increased phosphorylation of substrates was due to the insulin receptor kinase.

Phosphoamino acid analysis of the insulin receptor was also conducted from these experiments, the rationale being that in vitro, the insulin receptor exhibits primarily tyrosine autophosphorylation and thus any increase in serine or threonine phosphorylation might represent the effect of the added serine kinase. However, the level of total phosphorylation and serine or threonine phosphorylation of the insulin receptor was unaffected by the addition of the calmodulin-dependent kinase tested (Fig. 4B).

Other Protein Kinses as Substrates for the Insulin Receptor Kinase

The ability of insulin-receptor kinase to phosphorylate the other serine kinases in vitro was tested using a similar protocol (Fig. 5). In all cases, autoradiograms of the $NaDodSO_4$ gels revealed an insulin-stimulated band of M_r = 95,000 which represents the β subunit of the insulin receptor. All of the serine kinases also exhibited autophosphorylation in vitro. In the case of casein kinase I, this was apparent as a band with M_r = 30,000, casein kinase II exhibited bands at M_r = 43,000 and 25,000, glycogen synthase kinase 3 had a faint band at $M_r \simeq$ 50,000, and phosphorylase kinase revealed multiple bands ranging in apparent molecular weight from 17,000 to 150,000. Although in occasional experiments insulin appeared to increase the phosphorylation of these proteins, this was not observed in a reproducible manner. Furthermore, phosphoamino acid anlaysis of these phosphorylated serine kinases in both the basal and insulin-stimulated states revealed only phosphoserine and phosphothreonine (Fig. 4). In no case was phosphotyrosine observed. Also, none of these kinases appeared to increase phosphorylation of the insulin receptor.

DISCUSSION

Although the exact mechanism of insulin action remains unknown, insulin has been shown to affect the state of phosphorylation for a variety of cellular proteins and enzymes [17–21]. This protein modification has been suggested to be at least part of the postreceptor transmission of the insulin signal. Recently, we and others presented evidence that the insulin receptor is a protein kinase, and we proposed that receptor-

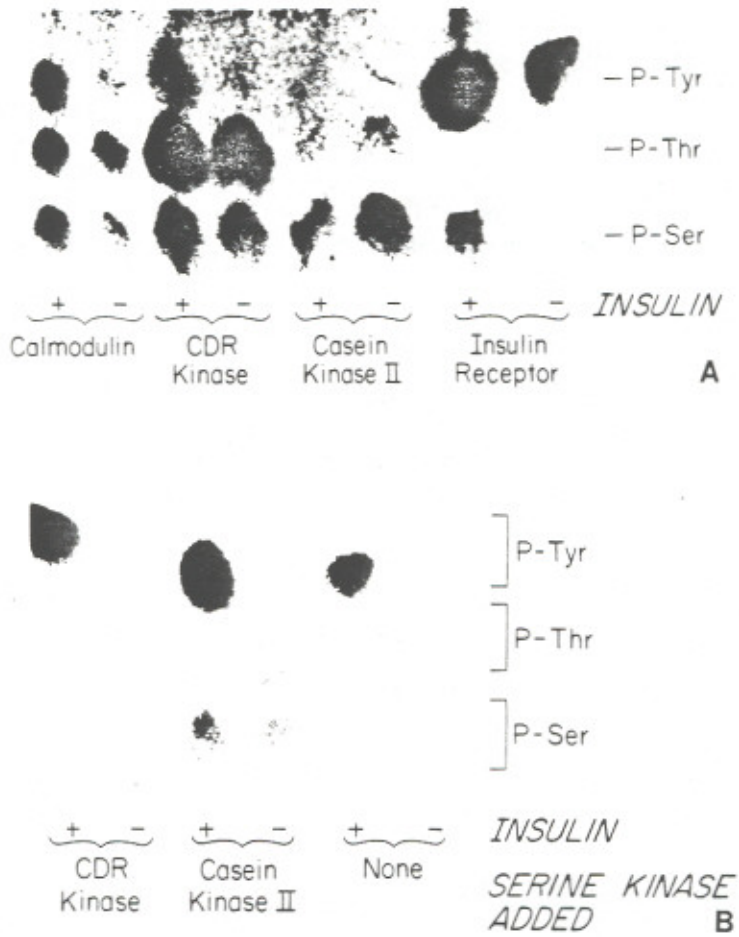


Fig. 4. Identification of phosphoamino acids in the β subunit of the insulin receptor, casein kinase II, the calmodulin-dependent (CDR) kinase, and calmodulin. A) The M_r = 51,000 and the M_r = 21,000 protein was phosphorylated with or without incubation with insulin in vitro as described in Figures 2 and 3. The bands were localized by autoradiography, were excised from the gel, and protein was digested by trypsin; for further details see Materials and Methods. After hydrolysis in 6N HCl at 110°C for 2 hr, phosphoamino acids were separated by cellulose-thin layer electrophoresis. Samples of authentic phosphoserine, phosphothreonine, and phosphotyrosine were added to all radioactive samples analyzed. The standards were located by ninhydrin and the radioactive material was located by autoradiography. In each case, the lane at the right was taken from the sample incubated in the absence of insulin while that at the left was taken from a sample where insulin 10^{-7} had been added to the receptor for 1 hr prior to the phosphorylation reaction. B) The M_r = 95,000 band representing the β subunit of the insulin receptor was excised, hydrolyzed, and analyzed as described above.

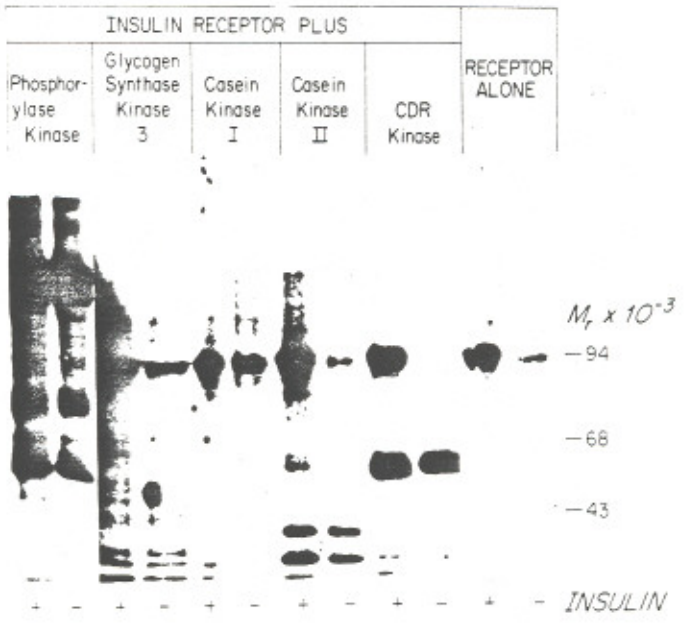


Fig. 5. The effect of insulin on phosphorylation of different serine kinases by the wheat germ purified insulin receptor kinase from Fao hepatoma cells. Wheat germ purified receptor kinase (5 μg) was used in each assay. Insulin 10^{-7} M was added to aliquots for 1 hr at room temperature before addition of substrates. Phosphorylation was studied for 10 min with 40 μM [γ^{32}P]-ATP and 10 mM MnCl. The reaction was stopped by boiling in Laemmli buffer. Samples were analyzed in $NaDodSO_4$ 7.5% and 10% polyacrylamide gels. The respective amounts of substrate were as follows: calmodulin-dependent (CDR) protein kinase, 1–2 μg, and calmodulin, 2–4 μg; glycogen-synthase kinase, 3.5–7 μg; casein-kinase II, 40–80 ng; casein-kinase I, 25–50 ng; and phosphorylase-kinase, 3 μg.

induced phosphorylation may be an early step in the signal-transmitting process [1–16]. Evidence that the insulin receptor is itself a protein kinase includes the following facts: 1) the kinase activity of the receptor is retained after purification of the receptor to near homogeneity [6]; 2) the kinase activity is retained in the receptor after immunoprecipitations by a serum containing antibodies against the insulin receptor [6]; 3) the partially purified receptor preparations from several tissues [7,9] and the highly purified receptor from human placenta membranes catalyze the phosphorylation of tyrosine residues of several artificial substrates; and 4) the β subunit of the insulin receptor is labeled with ATP affinity reagents, suggesting that the catalytic domain is located in this subunit [14–16]. Furthermore, autophosphorylation of the kinase appears to increase its activity for exogenous substrates [12].

The notion that the insulin receptor is a tyrosine-specific protein kinase parallels findings for the receptors of two other growth-regulating peptides, namely, epidermal growth factor [39–41] and platelet-derived growth factor [42,43]. These receptors, like the transforming gene products of the Rous sarcoma virus [44–46], the feline sarcoma virus [47], and the Abelson murine leukemia virus [48] are tyrosine kinases. In all these systems, phosphorylation of proteins at tyrosine residues is suspected to

have a signal-transmitting function, although identification of three natural substrates involved has been limited and proof of this hypothesis in most cases is still lacking.

Several proteins have been shown to be substrates of the partially or highly purified insulin receptor kinase in vitro. These proteins include histone H_2B, casein, actin, tubulin, angiotensin II, rabbit anti-src IgG, and an src-related peptide which resembles the sequence around tyrosine 419 in pp^{60src} [7,9,49]. Using the latter peptide as substrate of highly purified receptor from human placenta, Kasuga et al [7] found that insulin stimulated phosphorylation of these exogenous substrates by increasing the V_{max} of the receptor kinase with no major change of K_M [7]. Insulin also increases the V_{max} of the receptor kinase for autophosphorylation [13]. The insulin effect on autophosphorylation usually represents a 10–20–fold increase in [^{32}P] content, whereas with exogenous substrates, phosphorylation is usually increased only two- and fourfold. A functional alteration or a physiological role of the phosphorylation of these exogenous substrates has thus far not been found.

In the present study, we have investigated the effects of insulin receptor kinase on a number of proteins that are known to play a role in the cellular insulin effect. The serine kinases studied are all known to be capable of phosphorylating glycogen synthase. Regulation of glycogen synthase in vitro by phosphorylation/dephosphorylation is well characterized [20,38]. Insulin activates glycogen synthase from the glycose-6-P-dependent form to the glucose-6-P-independent form with a decrease in the level of phosphorylation of the enzyme. This insulin effect could occur through a decrease in activity of one of the glycogen synthase kinases or through an increase in activity of phosphatase. Therefore, glycogen synthase kinases are potential primary candidates for a direct effect of the receptor kinase.

Glycogen synthase is phosphorylated in vitro by at least seven protein kinases [20,28], and we tested five of the enzymes: 1) Phosphorylase kinase phosphorylates glycogen synthase at a serine residue at site 2 [27,38]. The enzyme consists of four subunits (α = 118,000; β = 107–136,000; γ = 40–45,000; δ = 17,000). 2) Glycogen synthase kinase 3 (M_r = 50,000) phosphorylates glycogen synthase on three sites, termed a, b, and c [25,26]. Phosphorylation of these sites produces a greater decrease of activity than phosphorylation of site 1a, 1b, and 2. 3) Glycogen synthase 5 is also referred to as $PC_{0.7}$, or as casein kinase II, and has an apparent M_r of 170,000–190,000 [22,23]. It consists of two α subunits (M_r = 43,000) and two β subunits (M_r = 25,000). It phosphorylates a single serine residue of glycogen synthase termed site 5. 4) Calmodulin-dependent protein kinase [24,25] requires Ca^{2+} and calmodulin for activity and phosphorylates glycogen synthase at serine (site 2). It has an M_r = 600,000. On $NaDodSO_4$-polyacrylamide gels under reducing conditions, two subunits of M_r = 51,000 and 53,000 are found. 5) Casein kinase I [22] (M_r = 30,000) phosphorylates glycogen synthase at at least two sites and reduces the activity of muscle glycogen synthase. Some differences between this enzyme and casein kinase II are that this enzyme phosphorylates serine residues only and will not utilize GTP as phosphate donor.

Interestingly, all kinases showed autophosphorylation in the presence of $MnCl_2$ and ATP. Of the five kinases tested, the only one that appeared to be a substrate of the insulin receptor kinase was the calmodulin-dependent kinase. Insulin induced a small, but reproducible, increase in phosphorylation of the calmodulin-dependent kinase of about 40%. In the presence of the calmodulin-dependent kinase, insulin also stimulated phosphorylation of calmodulin to the same extent. The increase in both

cases was on tyrosine residues, suggesting that this phosphorylation was indeed mediated by the insulin receptor kinas Phosphorylation on threonine and serine residues was unchanged. The latter is probably a reflection of activity of the calmodulin-dependent kinase, since under our experimental conditions, the insulin receptor phosphorylates proteins of tyrosine residues only [4,7,13].

Whether this insulin-induced phosphorylation on tyrosine alters the function of these two proteins is at present unknown. It is interesting to speculate, however, that the role of Ca^{2+} in insulin action might somehow be connected with the insulin receptor kinase through a modification of calmodulin by the receptor kinase [50]. Although calmodulin has not been regarded classically as a protein whose activity is regulated by phosphorylation [51] Plancke and Lazarides [52] recently presented evidence for a phosphorylated form of calmodulin in chicken brain and muscle. They also found that phosphorylase kinase would phosphorylate calmodulin in vitro. In both cases, however, this occurred primarily on serine residues. Very recently, McDonald et al [53] showed that the insulin receptor binds to calmodulin, possibly through its β subunit. Calcium and calcium ionophores have also been shown to modify insulin receptor phosphorylation [54], and evidence has been presented for a Ca^{2+} binding site on the receptor itself [55]. Although the exact site of tyrosine phosphorylation in calmodulin has not yet been determined, calmodulin contains only two tyrosine residues (residues 99 and 138) [51]. Both occur in calcium binding domains, but the latter is preceded by a sequence similar to that observed in other proteins that are tyrosine phosphorylated (Glu-Val-Asn-Tyr-Glu-Glu) [7,48,51]. Although further studies will be needed to clarify the relationship of these proteins to one another within the cell, these observations may provide a mechanism to link the insulin-receptor kinase to its postreceptor events.

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REFERENCES

1. Kasuga M, Karlsson FA, Kahn CR: Science 215:185, 1982.
2. Kasuga M, Zick Y, Blithe DL, Karlsson FA, Haring HU, Kahn CR: J Biol Chem 257:9891, 1982.
3. Haring HU, Kasuga M, Kahn CR: Biochem Biophys Res Commun 108:1538, 1982.
4. Kasuga M, Zick Y, Blithe DL, Crettaz M, Kahn CR: Nature 298:667, 1982.
5. Zick Y, Kasuga M, Kahn CR, Roth J: J Biol Chem 258:75, 1983.
6. Kasuga M, Fujita-Yamaguchi Y, Blithe DL, Kahn CR: Proc Natl Acad Sci USA 80:2137, 1983.
7. Kasuga M, Fujita-Yamaguchi Y, Blithe DL, White MF, Kahn CR: J Biol Chem 258:10973, 1983.
8. Van Obberghen E, Kowalski A: FEBS Lett 143:179, 1982.
9. Petruzzelli LM, Ganguly S, Smith CR, Cobb MH, Rubin CS, Rosen O: Proc Natl Acad Sci USA 79:6792, 1982.
10. Machicao F, Urumow T, Wieland OH: FEBS Lett 149:96, 1982.

11. Avruch J, Nemenoff RA, Blackshear PJ, Pierce MW, Osathanondh R: J Biol Chem 257:15162, 1982.
12. Zick Y, Wittaker J, Roth J: J Biol Chem 259:3432, 1982.
13. White MF, Haring HU, Kasuga M, Kahn CR: J Biol Chem 259:255, 1984.
14. Roth RA, Cassell DJ: Science 219:229, 1983.
15. Van Obberghen E, Rossi B, Kowalski A, Gazzano H, Ponzio G: Proc Natl Acad Sci USA 80:945, 1983.
16. Shia MA, Pilch PF: Biochemistry 22:717, 1983.
17. Avruch J, Alexander MC, Palmer JL, Pierce MW, Nemenoff RA, Blackshear PJ, Tipper JD, Witters LA: Fed Proc 41:2629, 1982.
18. Brownsey RW, Denton RM: Biochem J 202:77, 1982.
19. Krebs EG, Beauo J: Annu Rev Biochem 48:923, 1979.
20. Chock PB, Rhee SG, Stadtman ER: Annu Rev Biochem 49:813, 1980.
21. Sheorain VS, Khatra BS, Soderling TR: J Biol Chem 257:3462, 1982.
22. Itarte E, Huang KP: J Biol Chem 254:4052, 1979.
23. DePaoli-Roach AA, Ahmad Z, Roach PJ: J Biol Chem 256:8955, 1981.
24. Payne EM, Soderling TR: J Biol Chem 255:8054, 1980.
25. Ahmad Z, DePaoli-Roach AA, Roach PJ: J Biol Chem 257:8348, 1982.
26. Ahmad Z, Canuci M, DePaoli-Roach AA, Roach PJ: J Biol Chem 259:3420, 1984.
27. Cohen P: Eur J Biochem 34:1, 1973.
28. DePaoli-Roach AA, Roach PJ, Larner J: J Biol Chem 254:4212, 1979.
29. Dedman JR, Potter JD, Jackson RL, Johnson JD, Means AR: J Biol Chem 252:8415, 1977.
30. Descharette J, Weiss MC: Biochemie 11:1603, 1974.
31. Pitot JC, Peraino C, Moore DA, Potter VT: Natl Cancer Inst Monogr 12:229, 1964.
32. Reuber MD: JNCI 26:891, 1961.
33. Crettaz M, Kahn CR: Endocrinology 113:1201, 1983.
34. Coon HG, Weiss MC: Proc Natl Acad Sci USA 62:852, 1969.
35. Kahn CR, Bertolotti R, Ninio M, Weiss MC: Nature 290:717, 1981.
36. Hunter T, Sefton BM: Proc Natl Acad Sci USA 62:852, 1969.
37. Shulman GI, Nemenoff RA, Kwok YC, Blackshear PJ, Avruch J: Diabetes 32 (Suppl 1):56A, 1983.
38. Roach PJ: Curr Top Cell Regul 20:45, 1981.
39. Carpenter G, King L, Cohen S: J Biol Chem 255:4884, 1979.
40. Ushiro H, Cohen S: J Biol Chem 255:8353, 1980.
41. Pike LJ, Collis B, Casnellie JE, Bornstein P, Krebs EG: Proc Natl Acad Sci USA 79:1443, 1982.
42. Ek B, Westermark B, Wasteson A, Heldin CH: Nature 295:419, 1982.
43. Pike LJ, Bowen-Pope DF, Ross R, Krebs EG: J Biol Chem 258-9380, 1983.
44. Erikson RL, Collett MS, Erikson E, Purchio AF: Proc Natl Acad Sci USA 76:6260, 1979.
45. Levinson AD, Oppermann H, Varmus HE, Bishop JM: J Biol Chem 255:11973, 1980.
46. Hunter T, Sefton BM: Proc Natl Acad Sci USA 77:1311, 1980.
47. Reynolds FH, Van de Ven WJM, Stephenson JF: J Biol Chem 255:11040, 1980.
48. Bishop, JM: Ann Rev Biochem 52:301, 1983.
49. Stadtmauer LA, Rosen OM: J Biol Chem 258:6682, 1983.
50. Akhtar RA, Perry MC: Biochem Biophys Acta 585:107, 1979.
51. Means AR: Rec Prog Horm Res 37:333, 1979.
52. Plancke YD, Lazarides E: Mol Cell Biol 3:1412, 1983.
53. McDonald JM, Goewert RR, Graves CB: Diabetes 33:10A, 1974.
54. Plehwe WE, Williams PF, Caterson ID, Harrison LC, Turtle JR: Biochem J 214:361, 1983.
55. Williams PF: Diabetes 33:10A, 1984.