THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 260, No. 11, Issue of June 10, pp. 7131-7136, 1985
Printed in U.S.A.

Predominance of Tyrosine Phosphorylation of Insulin Receptors during the Initial Response of Intact Cells to Insulin*

(Received for publication, September 24, 1984)

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Anti-phosphotyrosine antibody and anti-insulin receptor antibody were used to study insulin-stimulated phosphorylation of the β-subunit of the insulin receptor in [^{32}P]orthophosphate-labeled Fao hepatoma cells. Without insulin, the receptor contained both phosphoserine and phosphothreonine and could be immunoprecipitated with anti-receptor antibody but not with the anti-phosphotyrosine antibody. After incubation of these cells with insulin, both antibodies immunoprecipitated the phosphorylated receptor. The β-subunit of the receptor precipitated with anti-phosphotyrosine antibody from cells stimulated with insulin (100 nM) for 1 min contained predominantly phosphotyrosine, whereas, after 10 min with insulin, the amounts of phosphotyrosine and phosphoserine were nearly equal. These results suggest that insulin-stimulated tyrosine phosphorylation preceded insulin-stimulated serine phosphorylation of the β-subunit. Sequential immunoprecipitation of receptor with anti-phosphotyrosine antibody followed by precipitation of the remaining proteins with anti-receptor antibody suggests that insulin receptors which contain phosphoserine in the basal state are tyrosine phosphorylated more slowly than the dephosphorylated receptors or not at all after the addition of insulin. The β-subunit of the insulin receptor was the major phosphorylated protein precipitated by the anti-phosphotyrosine antibody from insulin-stimulated Fao cells. These results confirm our notion that insulin initially stimulated tyrosine autophosphorylation and subsequently serine phosphorylation of the insulin receptor in intact cells and suggests that this sequence of reactions occurs faster on receptors that are dephosphorylated before the incubation with insulin.

Insulin promotes tyrosine autophosphorylation of the β-subunit of its receptor in detergent extracts from several cell types (1-6). Studies with highly purified receptor (7-9),[1] double probe labeling studies (11), and studies using ATP affinity reagents (12-14) indicate that this is due to a kinase activity that is intrinsic to the receptor. The insulin receptor also catalyzes the phosphorylation of exogenous substrates on tyrosine residues (8) and this activity has been shown to be enhanced by tyrosine autophosphorylation of the β-subunit (15, 16).[1] Thus, it is attractive to view this insulin-promoted activation of the receptor kinase as a link in the chain of events responsible for the alterations in metabolism initiated by the binding of insulin to its receptor. However, in whole cells, phosphorylation of the β-subunit occurs predominantly on serine residues with a much smaller detected level of tyrosine phosphorylation after the addition of insulin (17, 18). These observations raise questions about the physiologic importance of tyrosine autophosphorylation and the sequence of phosphorylation reactions which occur on the β-subunit after insulin binding.

In this report, we have investigated insulin receptor phosphorylation in intact hepatoma cells (Fao) labeled with [^{32}P] orthophosphate making use of both anti-insulin receptor antibody (B-9) and an antibody which immunoprecipitates phosphotyrosine-containing proteins. Evidence presented here shows that at early times after the addition of insulin, receptor phosphorylation at tyrosine residues is the predominant insulin-promoted phosphorylation process as would be expected if activation of the tyrosine kinase activity were an early event in the reaction pathway for the intracellular transmission of the insulin signal. Interestingly, receptors that contain phosphoserine in the basal state are phosphorylated on tyrosine residues more slowly, or not at all, after addition of insulin. These results suggest that serine phosphorylation may inhibit insulin-stimulated tyrosine phosphorylation of the insulin receptor. Possibly, the two subsets of insulin receptors which differ in their state of phosphorylation after addition of insulin are located in different parts of the cell or are functionally distinct.

EXPERIMENTAL PROCEDURES

Materials—The following materials were obtained from the sources indicated: [^{32}P]orthophosphate and Triton X-100 were from New England Nuclear; phosphoamino acids and *N*-acetylglucosamine were from Sigma; porcine insulin (lot 1JM95AN) was from Elanco; reagents for SDS-PAGE[2] were purchased from Bio-Rad; Pansorbin was from Calbiochem and protein A-Sepharose was from Pharmacia; wheat germ agglutinin-agarose was from Vector. Cellulose thin-layer

* This work has been supported in part by Grants AM27659 to J. A. S., AM31036 and AM29770 to C. R. K., and Fellowship AM0716301 to M. F. W., from the Institute of Health and Human Development, National Institutes of Health, United States Public Health Service. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Part of this work is described in a Ph.D. dissertation to be submitted to the Graduate School of the University of Michigan.

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[1] D. T. Pang, B. R. Sharma, and J. A. Shafer (1985) *Arch. Biochem. Biophys.*, submitted for publication.

[2] The abbreviations used are: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; WGA, wheat germ agglutinin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TPA, 12-*O*-tetradecanyl phorbol 13-acetate.

plates (20 × 20 cm) were obtained from Analtech (G1140); RPMI 1640 tissue culture medium and fetal bovine serum were purchased from GIBCO.

Cell Culture and Insulin Receptor Phosphorylation—The experiments were performed with a highly differentiated and insulin-sensitive hepatoma cell line (Fao) originally provided by Dr. M. C. Weiss (Gif-sur-Yvette, France) (19). These cells possess a high concentration of insulin receptors and respond in many ways to insulin stimulation (20). Monolayer cultures of Fao cells were maintained at 37 °C in a humidified atmosphere composed of 95% air and 5% CO_2 and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum as described elsewhere (21). Twelve hours before each experiment, the medium was changed to serum-free RPMI 1640.

Labeling of the Fao cells with [^{32}P]orthophosphate was done as previously described (21). The phosphorylation reactions were quenched by rapid aspiration of the incubation medium from the tissue culture plates followed immediately by the addition of liquid nitrogen. The frozen cells were thawed in 2 ml of a solution containing HEPES (50 mM, pH 7.4), Triton X-100 (1%), sodium pyrophosphate (10 mM), sodium fluoride (100 mM), EDTA (4 mM), sodium vanadate (2 mM), phenylmethylsulfonyl fluoride (2 mM), and aprotinin (0.1 mg/ml, 14 trypsin inhibitor units/mg). In some experiments with the anti-phosphotyrosine antibody, proteins were immunoprecipitated directly from this crude detergent extract. In other experiments, the insulin receptor was partially purified by chromatography on 0.2 ml of wheat germ agglutinin agarose before immunoprecipitation (21). After passing the crude detergent extract over the column four times, the agarose was washed with 120 ml of 50 mM HEPES (pH 7.4) containing Triton X-100 (0.1%), sodium pyrophosphate (10 mM), sodium fluoride (100 mM), EDTA (4 mM), and sodium vanadate (2 mM). The bound glycoproteins were eluted with two 0.5-ml portions of this solution supplemented with *N*-acetylglucosamine (300 mM).

Immunoprecipitation with Anti-insulin Receptor and Anti-phosphotyrosine Antibodies—The insulin receptor purified by wheat germ agglutinin affinity chromatography was immunoprecipitated with anti-insulin receptor antibody (B-9) as previously described (21).

Immunoprecipitation of the tyrosine-phosphorylated receptor was performed with an anti-phosphotyrosine antibody prepared in rabbits by injection of *N*-bromoacetyl-*O*-phosphotyramine conjugated to keyhole limpet hemocyanin. The serum was purified before use on a phosphotyramine-Sepharose column as described elsewhere.[1] Crude extracts or wheat germ agglutinin agarose-purified extracts of Fao cells were incubated with 20 μg of anti-phosphotyrosine antibody in 1.5-ml microcentrifuge tubes at 4 °C for 2 h. A suspension of protein A-Sepharose (0.1 ml of 50% v/v in 50 mM HEPES, pH 7.4, 0.1% Triton X-100) was added to the reaction mixture, and the resulting gel suspension was shaken at 4 °C for 2 h. After this incubation, the gel was sedimented by a brief centrifugation. The gel pellet was washed at 4 °C three times with 0.5 ml of 50 mM HEPES containing Triton X-100 (0.1%) and NaCl (150 mM) and once with this solution lacking the NaCl. The phosphotyrosine-containing proteins were eluted from the Sepharose pellet by two incubations at 4 °C for 20 min with 0.15 ml of *p*-nitrophenyl phosphate (10 mM) in 50 mM HEPES containing Triton X-100 (0.1%). A second treatment of the cell extract with fresh antibody or extraction of adsorbed proteins remaining in the pellet after elution with *p*-nitrophenyl phosphate with Laemmli SDS-PAGE sample buffer did not yield significant amounts of phosphorylated β-subunit. These results indicate that the initial adsorption by the phosphotyrosine antibody was complete.

The phosphoproteins immunoprecipitated by each antibody and reduced by dithiothreitol were separated by SDS-PAGE as previously described (21). The following proteins were used to estimate molecular weight: myosin, M_r = 200,000; β-galactosidase, M_r = 116,250; phosphorylase *b*, M_r = 94,000; bovine serum albumin, M_r = 66,200; ovalbumin, M_r = 45,000. The [^{32}P]phosphoproteins were identified by autoradiography of the stained and dried gels using Kodak X-Omat film and an intensifying screen. The radioactivity in gel fragments was quantified by Cerenkov counting (22).

RESULTS

Immunoprecipitation of the Insulin Receptor from a Glycoprotein-enriched Cell Extract with Anti-phosphotyrosine Antibody—Insulin receptors purified from [^{32}P]orthophosphate-labeled Fao cells by affinity chromatography on wheat germ agglutinin agarose were immunoprecipitated with an anti-phosphotyrosine antibody. In the absence of insulin, no detectable phosphoproteins were bound to and specifically eluted from the phosphotyrosine antibody (Fig. 1*A*, 0 min). This result contrasts with previous experiments (17, 23) and experiments carried out in parallel (not shown) in which the β-subunit of the insulin-free receptor was immunoprecipitated with anti-receptor antibody and shown to contain both phosphoserine and phosphothreonine (see Fig. 4).

Incubation of Fao cells with 100 nM insulin during a 1- to 10-min interval resulted in the stimulation of a single [^{32}P] phosphoprotein in the WGA agarose-purified cell extract which was precipitated by the anti-phosphotyrosine antibody (Fig. 1*A*). Insulin-stimulated phosphorylation was nearly complete within 1 min. This phosphoprotein migrated as a M_r = 95,000 protein under reducing conditions which corresponds to the migration of the β-subunit of the insulin receptor. Two mM phosphotyrosine (Fig. 1*B*) completely blocked immunoprecipitation of radiochemical label whereas 2 mM DL-phosphoserine (Fig. 1*C*) and 2 mM DL-phosphothreonine (Fig. 1*D*) did not, suggesting that the M_r = 95,000 protein which was selectively precipitated from the WGA agarose-eluate by the antibody to phosphotyrosine contained phosphotyrosyl residues. Under nonreducing conditions, SDS-PAGE of one sample yielded a single radiochemically labeled protein similar in molecular weight to that observed for the intact insulin receptor from human placenta (24).[1]

Immunoprecipitation of Phosphotyrosine-containing Proteins from a Crude Cell Extract—To determine whether insulin stimulated phosphorylation of other proteins on tyrosine, the anti-phosphotyrosine antibody was used to precipitate labeled proteins from a detergent extract of [^{32}P]orthophosphate-labeled Fao cells prior to WGA chromatography. In the absence of insulin, SDS-PAGE separation under reducing conditions of the *p*-nitrophenyl phosphate eluate revealed on an autoradiogram two faint bands corresponding to phosphoproteins of M_r = 120,000 and 75,000 (Fig. 2, 0 min). Incubation of Fao cells with insulin for 1, 5, and 10 min promoted a dramatic increase in phosphorylation of a protein of M_r = 95,000, corresponding to the migration of the β-

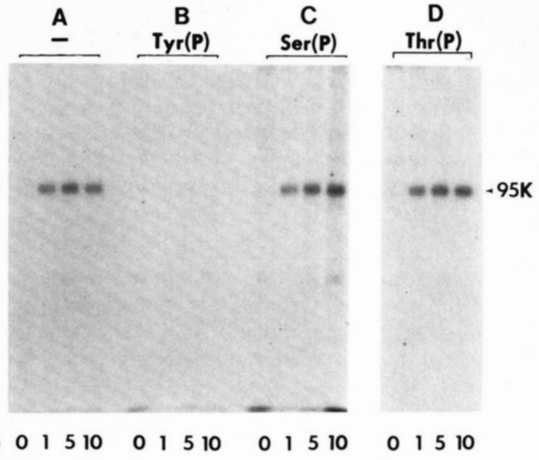

FIG. 1. **Adsorption of phosphorylated insulin receptor by anti-phosphotyrosine antibody.** Fao cells were labeled with [^{32}P] orthophosphate for 2 h, treated with 100 nM insulin for 0, 1, 5, and 10 min at 37 °C, and solubilized under conditions shown to inhibit dephosphorylation. After partial purification by WGA agarose chromatography, the cell extract was incubated with anti-phosphotyrosine antibody in the absence of phosphoamino acid (−) or in the presence of 2 mM phosphotyrosine (*Tyr(P)*), 2 mM phosphoserine (*Ser(P)*), or 2 mM phosphothreonine (*Thr(P)*). After precipitation of the complexes by protein A-Sepharose, specifically bound phosphoproteins were eluted with 10 mM *p*-nitrophenyl phosphate and subjected to SDS-PAGE. An autoradiogram of the dried gel is shown.

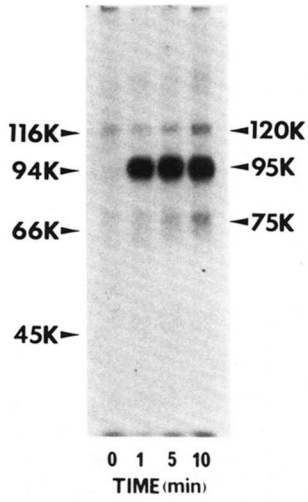

FIG. 2. **Anti-phosphotyrosine antibody immunoprecipitation of phosphotyrosine-containing proteins from a crude extract of Fao cells.** [^{32}P]Orthophosphate-labeled Fao cells were treated with 100 nM insulin for 0, 1, 5, and 10 min at 37 °C and solubilized with a Triton X-100 in the presence of phosphatase inhibitors. Phosphoproteins specifically bound and eluted from the anti-phosphotyrosine antibody were separated by SDS-PAGE. An autoradiogram of the dried gel is shown.

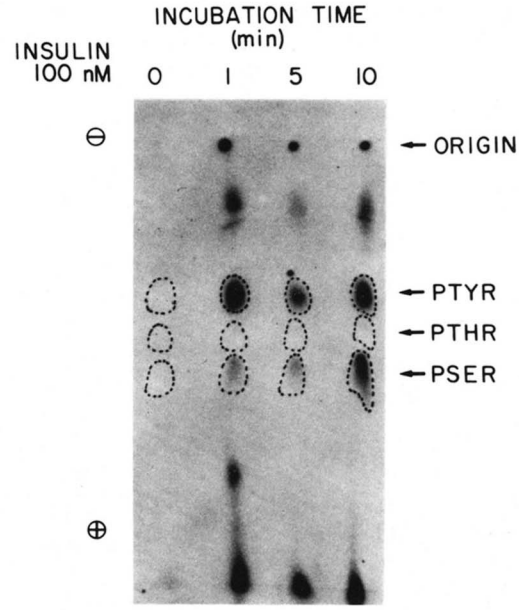

FIG. 3. **Identification of phosphoamino acids in the β-subunit precipitated by and eluted from the anti-phosphotyrosine antibody.** Fao cells labeled with [^{32}P]orthophosphate were treated with 100 nM insulin for 0, 1, 5, and 10 min at 37 °C and solubilized with Triton X-100 in the presence of phosphatase inhibitors. Phosphoproteins specifically bound and eluted from the anti-phosphotyrosine antibody were precipitated with anti-receptor antibody (B-9), and the precipitated proteins were separated by SDS-PAGE. The fragments of dried gel which contained the β-subunit were excised, washed in 10% methanol, and incubated with trypsin. The resulting peptides were hydrolyzed in 6 N HCl for 2 h at 110 °C. Phosphoamino acids were separated by high voltage electrophoresis on a thin layer cellulose plate. An autoradiogram of the plate is shown, and the migration of the standards is identified. The less intense spots in the 5-min sample are due to the use of 50% less sample in this analysis. *PTYR*, phosphotyrosine; *PTHR*, phosphothreonine; *PSER*, phosphoserine.

subunit of the insulin receptor (Fig. 2). The minor phosphoproteins of M_r = 120,000 and 75,000 were also observed to increase after incubation with insulin, but to a minimal extent. Antibody to the insulin receptor (B-9) precipitated almost entirely the M_r = 95,000 phosphoprotein which was purified from crude detergent extracts of insulin-stimulated cells by the phosphotyrosine antibody but not the proteins of M_r = 120,000 and M_r = 75,000 (data not shown). This result suggests that the predominant phosphotyrosine-containing protein in Fao cells after insulin stimulation is the β-subunit of the receptor. The significance of the M_r = 120,000 and the M_r = 75,000 proteins is not known.

Insulin Initially Stimulates Tyrosine Phosphorylation of Receptors That Contain Little or No Phosphoserine or Phosphothreonine—The nature of the phosphorylated forms of the insulin receptor was studied by extracting insulin receptor from Fao cells and subjecting the receptor to both separate and sequential immunoprecipitations with anti-phosphotyrosine antibody and anti-receptor antibody. The population of insulin receptor which was precipitated with anti-phosphotyrosine antibody exhibited a striking excess of phosphotyrosine relative to phosphoserine in the β-subunit 1 min after the addition of insulin (Fig. 3, 1 min). After a 10-minute incubation with insulin, however, the amounts of phosphotyrosine and phosphoserine were about equal (Fig. 3, 10 min). These results suggest that insulin initially promotes phosphorylation of its receptor at tyrosine residues.[3]

Prior to addition of insulin some of the receptor molecules are phosphorylated at residues other than tyrosine as judged by the precipitation of the ^{32}P-labeled β-subunit by anti-receptor antibody but not by anti-phosphotyrosine antibody (Fig. 4). This result is consistent with previous studies that the insulin receptor in the basal state contains mainly phosphoserine, some phosphothreonine, but no phosphotyrosine (17, 23, 25). After incubation of the Fao cells with insulin for 1, 5, and 10 min, the amount of phosphorylated β-subunit precipitated from separate cell extracts by anti-receptor or anti-phosphotyrosine antibodies rose in parallel suggesting that all of the newly phosphorylated receptors contained some phosphotyrosine (Fig. 4). However, the receptor precipitated with the anti-phosphotyrosine antibody was consistently less than the total precipitated with anti-receptor antibody suggesting that some receptors are phosphorylated on serine and threonine residues only.

The subset of receptor molecules that were not precipitated by anti-phosphotyrosine antibody was further characterized by sequential immunoprecipitation of a WGA agarose-purified cell extract with the anti-phosphotyrosine antibody followed by anti-receptor antibody. With increasing insulin concentrations, the anti-phosphotyrosine antibody precipitated more receptor (Fig. 5*A*), whereas the amount of phosphorylated receptor molecules remaining in solution and which were precipitated by anti-receptor antibody was independent of insulin (Fig. 5*B*). Phosphoamino acid analysis detected no phosphotyrosyl residues in the subset of receptor molecules which did not interact with the anti-phosphotyrosine antibody (data not shown). Thus, precipitation of the extract with anti-phosphotyrosine antibody followed by treatment of the

[3] The low level of phosphoserine observed in the population of tyrosine-phosphorylated receptor which was precipitated by anti-phosphotyrosine antibody after short exposure to insulin does not exclude the presence of some phosphoseryl residues which were not liberated after subjecting the denatured β-subunit of the insulin receptor to digestion with trypsin followed by hydrolysis in 6 N HCl for 2 h at 100 °C. It is unlikely, however, that receptor populations which liberate little or no phosphoserine upon acid hydrolysis contain more phosphoserine than receptor populations which liberate substantial phosphoserine upon acid hydrolysis.

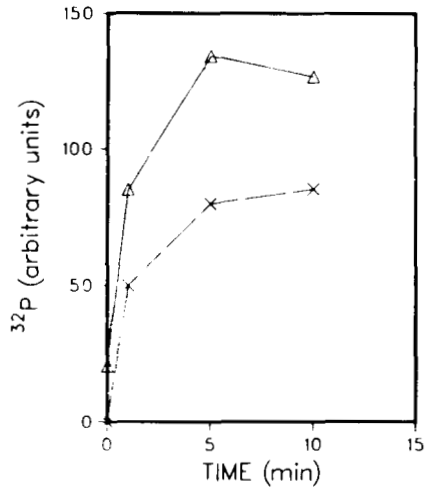

FIG. 4. **Time course of phosphorylation of the β-subunit precipitated with either anti-receptor antibody or anti-phosphotyrosine antibody.** Fao cells labeled with [^{32}P]orthophosphate were incubated with 100 nM insulin and solubilized after 0, 1, 5, and 10 min. After WGA agarose chromatography, insulin receptor was precipitated by anti-receptor (△) or anti-phosphotyrosine antibody (×), and the protein was separated by SDS-PAGE. An autoradiogram of the dried gel was prepared, and the relative labeling of the β-subunit was determined by densitometry.

unprecipitated material with anti-receptor antibody separated the receptor molecules which contained one or more phosphotyrosine residues from those which contained none. Insulin apparently stimulates tyrosine phosphorylation on receptors that initially are dephosphorylated which suggests the possibility that phosphoserine and phosphothreonine residues might inhibit insulin-stimulated tyrosine phosphorylation or cause receptors to be inaccessible to insulin (Fig. 5).

DISCUSSION

We have described the use of an anti-phosphotyrosine antibody to separate a subset of insulin receptor molecules that contain phosphotyrosine from the total set of molecules which bind to anti-receptor antibody (B-9). Our results show that addition of insulin to rat hepatoma cells labeled with [^{32}P]orthophosphate initially stimulates receptor phosphorylation almost entirely on tyrosine residues of the β-subunit. This insulin-stimulated reaction reaches steady state within 1 min. Based on recent findings with solubilized receptor preparations of various purity and origins, insulin-stimulated phosphorylation of its receptor in intact Fao cells probably occurs by an intramolecular autophosphorylation (11, 14, 26) initiated by the extracellular binding of insulin to the α-subunit (27) and catalyzed by the β-subunit through an interaction with cellular ATP and Mn^{2+} or Mg^{2+} (26, 28, 29). Although the rapid initial phosphorylation of tyrosyl residues of insulin receptor reported here was reproduced in all of the several preparations of Fao cells studied, additional studies will be required to determine whether this observation is unique to Fao cells.

The higher content of phosphotyrosine detected in the β-subunit extracted from Fao cells contrasts with earlier reports which suggested that phosphoserine was the major phosphoamino acid associated with the insulin receptor after insulin stimulation (17, 18). This difference is ascribed to the use of better conditions to quench the phosphorylation and dephosphorylation reactions during solubilization and purification. The addition of sodium vanadate during the preparation of the cell extracts and purification of the receptor resulted especially in a marked increase in the recovery of phosphotyrosine associated with the insulin-stimulated cell (23). Since vanadate does not promote phosphorylation during solubili-

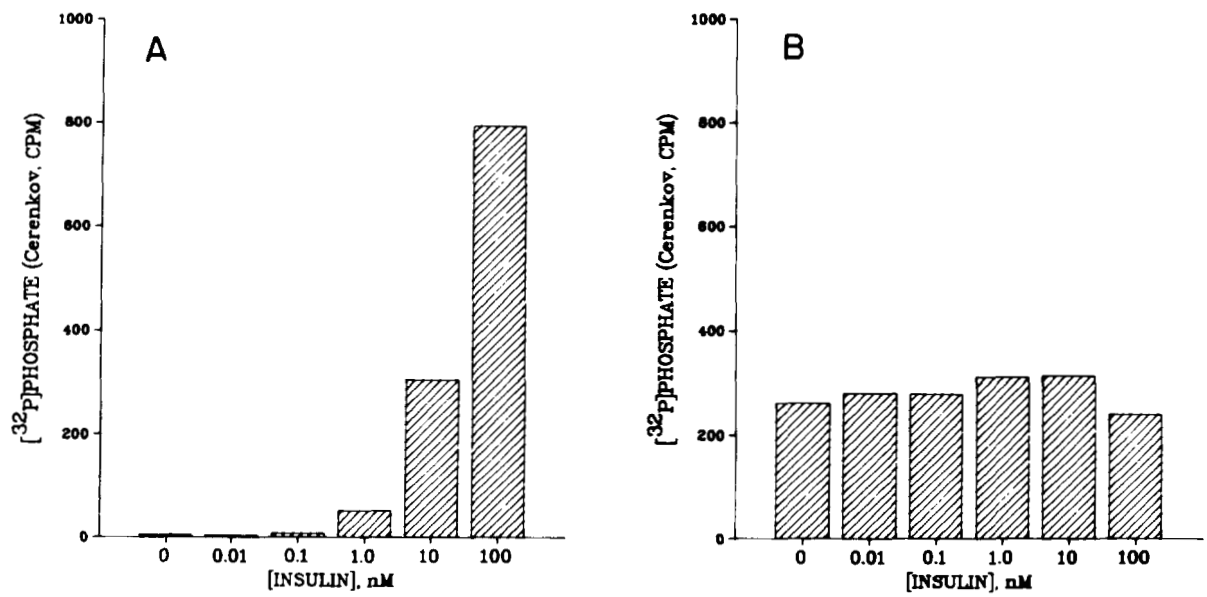

FIG. 5. **Insulin dose response of receptor phosphorylation.** Fao cells labeled with [^{32}P]orthophosphate were incubated for 10 min at 37 °C with 0 nM insulin or 0.01, 0.1, 1.0, 10, and 100 nM insulin. The Fao cells were solubilized, and the receptor was purified by WGA agarose chromatography. In *A*, the partially purified phosphotyrosine-containing receptor was precipitated by anti-phosphotyrosine antibody and protein A-Sepharose. The receptor was eluted from the antibody with 10 mM *p*-nitrophenyl phosphate. In *B*, the supernatant from *A* was treated with anti-receptor antibody and protein A-Sepharose. The immunoprecipitate was dissolved in Laemmli sample buffer. The proteins from the sequential immunoprecipitation were separated by SDS-PAGE. The β-subunit was localized by chromatography, and gel fragments containing the β-subunit were removed for determination of radioactivity by Cerenkov counting. *A* and *B*, respectively, indicate the effect of insulin of the phosphotyrosine-containing and phosphotyrosine-free β-subunit.

zation and purification of cell extracts,[4] this difference is most likely due to the more effective inhibition by vanadate of phosphotyrosine phosphatases (30) than the inhibition provided by fluoride, pyrophosphate, and EDTA combined.

Sequential immunoprecipitation of the insulin receptor with anti-phosphotyrosine followed by anti-receptor antibodies separated phosphotyrosine-containing receptor from phosphoserine- and phosphothreonine-containing receptors and allowed the identification of at least two distinct populations of receptors. Before the addition of insulin, only phosphoserine- and phosphothreonine-containing receptors were observed. As expected, these molecules did not cross-react with the anti-phosphotyrosine antibody. After a 1-min incubation with insulin, there was a selective increase in the receptors phosphorylated on tyrosine residues only. With continued incubation of the Fao cells with insulin (*e.g.* after 10 min as shown in Fig. 4), the amount of phosphoserine in the population of tyrosine-phosphorylated receptor increased substantially. This result suggests that tyrosine-phosphorylated receptors are subsequently phosphorylated on serine residues or that previously serine-phosphorylated receptors are tyrosine phosphorylated more slowly. Therefore, in the presence of insulin, it is possible that three forms of phosphorylated insulin receptors exist: (i) those which contain only phosphotyrosine; (ii) those containing only phosphoserine and phosphothreonine; or (iii) receptors that contain all three phosphoamino acids.

In light of the existence of phosphoserine in a subset of receptor molecules prior to addition of insulin (17), it was surprising that we observed little phosphoserine in receptors which were precipitated with the anti-phosphotyrosine antibody immediately after the addition of insulin. In fact, the phosphoserine-containing receptor molecules were in the supernatant and could be identified with anti-receptor antibody. Apparently, phosphoserine-containing receptors are either not accessible to insulin or unable to undergo tyrosine phosphorylation upon insulin binding at a rate comparable to dephosphorylated receptors.

Jacobs *et al.* showed that the phorbol ester TPA stimulates phosphorylation of the insulin receptor in IM-9 lymphocytes (31). TPA also stimulates phosphorylation of the epidermal growth factor receptor in A431 cells (32). Subsequently, it was shown in Fao cells that TPA stimulates serine phosphorylation of the β-subunit of the receptor and concurrently inhibits insulin-stimulated tyrosine autophosphorylation (23). Insulin binding was not altered in the Fao cell, although TPA has been shown to decrease insulin binding in other systems (33). These results together with our present findings suggest that serine-phosphorylated receptors in the basal state, or as a result of TPA stimulation, are tyrosine phosphorylated more slowly or not at all after the addition of insulin. Since TPA treatment of Fao cells apparently decreases both insulin stimulation of glycogen synthase activity and tyrosine amino acid transferase induction (23), it is possible that phosphorylation at serine and threonine residues of the β-subunit may antagonize the effects of insulin by inhibiting insulin-promoted autophosphorylation at tyrosine residues and the concomitant activation of the kinase activity of the receptor.

The β-subunit of the insulin receptor is the major phosphotyrosine-containing protein detected in crude detergent extracts of Fao cells by anti-phosphotyrosine antibody. Although other proteins were observed, they were very minor relative to the β-subunit and poorly stimulated by insulin. Interestingly, the M_r = 120,000 phosphoprotein precipitated by anti-phosphotyrosine antibody corresponds to the molecular weight of a protein contained in the WGA agarose-purified cell extract from rat liver membranes that was phosphorylated on tyrosine residues after the addition of [γ-^{32}P] ATP and insulin (34). Possibly, the anti-phosphotyrosine antibody does not bind to all phosphotyrosine-containing proteins because of inappropriate steric or electrostatic effects (35). It is also possible that the solution of inhibitors used during the extraction and purification of the receptors was not entirely effective in preventing dephosphorylation of phosphotyrosine residues. Furthermore, the concentration of substrates for the insulin receptor may be much lower than the receptor, a possibility which is consistent with the notion that maximum insulin effects are observed before insulin binding is saturated or receptor phosphorylation is maximal. Thus, phosphorylation of a small number of substrates which are not detectable by current assays could be all that is required to activate a target enzyme which catalyzes the rate-limiting step in an insulin-sensitive metabolic pathway. Interestingly, other studies using anti-phosphotyrosine antibodies suggest that the receptors for epidermal growth factor (35) and platelet-derived growth factor (10) are the primary phosphotyrosine-containing proteins immunoprecipitated from cells stimulated by the corresponding hormone.

The increase in phosphorylation of the receptor shown in Fig. 5*A* in response to 10-min incubations with increasing levels of insulin occurs at somewhat higher levels of insulin than that which might have been expected from the previously reported (20) value of 4.9 nM for the equilibrium constant for dissociation of insulin from its receptor in Fao cells. The 2.5-fold increase in receptor phosphorylation on going from 10 to 100 nM insulin would be more consistent with an equilibrium constant of 20 nM. This discrepancy might well reflect incomplete equilibration of the added insulin with its receptor during the 10-min incubation used to effect phosphorylation in the study depicted in Fig. 5.

The results presented in this study support the following conclusions: (i) insulin receptor autophosphorylation at tyrosine residues in the β-subunit is one of the earliest molecular events occurring after insulin binding; (ii) after insulin stimulation, tyrosine autophosphorylation precedes serine phosphorylation of the β-subunit; (iii) tyrosine autophosphorylation occurs on receptors which initially contain little or no phosphoserine and phosphothreonine suggesting that receptor activity may be regulated intracellularly by phosphorylation. These observations suggest a physiological role for the insulin receptor kinase activity; however, more evidence is necessary to prove a direct relation between tyrosine kinase activity and insulin action.

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