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Insulin activates a tyrosine-specific protein kinase in extracts of 3T3-L1 adipocytes and human placenta

(insulin receptor/tyrosine phosphorylation/cyclic AMP/ribosomal protein S6/histone phosphorylation)

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ABSTRACT **Insulin activates a tyrosine-specific cAMP-independent protein kinase when added directly to detergent extracts of differentiated 3T3-L1 adipocytes and humal placental membranes. The kinase is also activated by antibody to the insulin receptor and, to a lesser extent, by proinsulin. It catalyzes the phosphorylation of the 92,000-dalton component of the insulin receptor, histone, and casein; in each case, tyrosine is the principal amino acid modified. Under the conditions used to activate the kinase, insulin does not affect the rate of dephosphorylation of the receptor or of histone. The insulin-activated kinase is copurified with the human placental insulin receptor until the final elution from insulin-Sepharose. It remains to be established whether the kinase and the insulin receptor are separate molecules.**

The initial step in insulin action is the interaction of insulin with its high-affinity cell surface receptor. The transduction of the signal from the insulin–receptor complex ultimately involves phosphorylation and dephosphorylation of specific cellular proteins. In 3T3-L1 and other cells, insulin stimulates the phosphorylation of both ribosomal protein S6 (1–3) and the enzyme, ATP citrate (*pro*-3*S*)-lyase (4–9). In each case, phosphorylation occurs on serine residues and is cAMP independent. Insulin also stimulates the cAMP-dependent phosphorylation of a cyclic nucleotide phosphodiesterase resident in rat liver plasma membranes (10). Recently, Kasuga *et al.* (11) reported that the addition of insulin to intact lymphocytes and hepatoma cells brought about the incorporation of ^{32}P into the 94,000-dalton subunit of the insulin receptor. Kasuga *et al.* also have shown that a detergent-solubilized extract of rat liver plasma membranes phosphorylates tyrosine residues on the insulin receptor (12).

We present evidence for the insulin-activated phosphorylation of the insulin receptor in extracts of insulin-sensitive 3T3-L1 adipocytes and in partially purified preparations of the human placental insulin receptor. The kinase from both sources phosphorylates tyrosine residues in both the receptor and, in exogenous substrates, is activated specifically by molecules that interact with the insulin receptor and is cAMP independent.

MATERIALS AND METHODS

[γ-^{32}P]ATP (3,000 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) was from Amersham. Purified cAMP-dependent protein kinase inhibitor was a gift from J. Demaille; human antibody to the human insulin receptor (B-9) was provided by C. R. Kahn, and rabbit antibody to rat liver membrane insulin receptor was from S. Jacobs. Insulin and proinsulin were from Eli Lilly, and epidermal growth factor (EGF) was from Collaborative Research (Waltham, MA). Histone H2B was from Worthington, and casein α-S was provided by E. Bingham. Agarose-bound wheat germ agglutinin and formalin-fixed protein A-bearing *Staphylococcus aureus* (Pansorbin) were from Vector and Calbiochem, respectively. Bovine brain calmodulin (13) and rat liver ATP citrate lyase (9) were prepared as described, and ribosomal 40S subunits were prepared from *Artemia salina* (14).

3T3-L1 cells were grown and differentiated as described (15). Cells were disrupted by sonication (10^7 cells per ml) in 100 mM sodium phosphate, pH 7.4/5 mM EDTA/0.25 M sucrose/25 mM benzamidine/50 mM NaF, followed by centrifugation first at 550 × *g* for 5 min and then 400,000 × *g* for 50 min. The final pellet was resuspended in 1 ml of 10 mM Hepes, pH 7.4/0.25 M sucrose/0.5 mM $MgCl_2$/2% Triton X-100 and was incubated for 60 min at 4°C. The suspension was then centrifuged for 60 min at 150,000 × *g*. The supernatant was adsorbed and eluted from agarose-linked wheat germ agglutinin (16): ≈500 fmol of insulin-binding activity was applied to 0.4 ml of resin, and typically 50% of the insulin-binding activity was recovered in 500 μl of eluate. The eluted glycoprotein fraction (0.3–1.3 mg of protein per ml) was either used directly or stored frozen at −20°C.

Purification of the placental insulin receptor and measurement of insulin-binding activity were performed as described (17). Prior to assay for protein kinase activity, all fractions containing receptor were applied to and eluted from the wheat germ agglutinin-agarose as described above. Recovery was monitored by assaying insulin-binding activity. Immunoprecipitation was performed as described (17). The receptor–antibody complex was precipitated by the addition of Pansorbin. Electrophoresis was carried out by the methods of Laemmli (18) and Smith *et al.* (2). Bands were cut out of the dried gels and assayed for radioactivity. Another area of each lane was used to estimate background radioactivity. Proteins were determined by the method of Lowry *et al.* (19).

Unless otherwise indicated, protein kinase activity was assayed in the following manner. Wheat germ eluates containing 10–20 fmol of insulin-binding activity in 6–26 μg of protein (20 μl) were incubated for 1 hr at 23°C in the presence or absence of hormone in a final volume of 55 μl of 20 mM Hepes (pH 7.4) containing 10% (vol/vol) glycerol, 30 mM NaCl, 12 mM $MgCl_2$, 2 mM $MnCl_2$, 20 mM *p*-nitrophenyl phosphate, and 10 μg of bovine serum albumin per ml. The reaction was initiated by the addition of 25 μM [γ-^{32}P]ATP (15,000 cpm/pmol) and allowed to proceed for 10 min at 23°C. The reaction was stopped by the addition of 20 μl of 10% $NaDodSO_4$/0.3 M Tris, pH 6.8/ 1 M mercaptoethanol/0.11 M dithiothreitol. After being heated at 100°C for 10 min, samples were subjected to electrophoresis. Phosphoamino acid analysis was performed as described (20) and by electrophoresis in 6.9% formic acid. With the exception

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Abbreviation: EGF, epidermal growth factor.

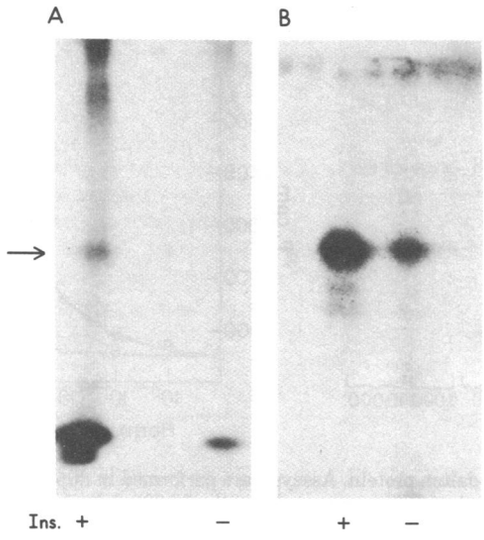

FIG. 1. Immunoprecipitation of the 92,000-dalton protein phosphorylated in intact 3T3-L1 cells (*A*) and in extracts derived from 3T3-L1 cells (*B*). Lane designations show insulin (Ins.) content: +, with; −, without. (*A*) Cells (10^8) were treated with $^{32}P_i$ with or without insulin and extracted with Triton X-100. Rabbit anti-rat receptor IgG (2 μg) was added to each fraction, and immunoprecipitation was performed. Nonimmune IgG did not precipitate the 92,000-dalton protein indicated with an arrow. (*B*) The kinase assay was carried out with 38 fmol of insulin-binding activity with or without insulin (5 μg/ml). Immunoprecipitation was with 3 μl of human anti-receptor antiserum. Radioautogram *A* was developed for 24 hr and radioautogram *B*, for 16 hr—both at −70°C with Quanta III intensifying screens.

of the data presented on purification of the insulin receptor, the source of the protein kinase was the partially purified glycoprotein fraction of 3T3-L1 cells.

RESULTS

Assay Requirements. The ability of insulin to stimulate phosphorylation of a 92,000-dalton protein was assessed (see Fig. 5*B*), and there was a requirement for either Mg^{2+} or Mn^{2+}. Protein kinase activity could be detected with 50 mM $MgCl_2$, but the optimal condition was a combination of 10–12 mM $MgCl_2$ and 2 mM $MnCl_2$. There was no effect of 1 mM Ca^{2+} or 1 mM Ca^{2+} with 500 nM calmodulin.

Protein kinase activity was proportional to the amount of eluate added and the time of incubation (1–15 min) at 23°C. The addition of 1.0 mM GTP to the assay did not diminish the phosphorylation of the 92,000-dalton protein, suggesting that GTP could not serve as a phosphoryl donor in the reaction. Phosphorylation of both the 92,000-dalton band and histone (see below) were unaffected by the addition of 10 μM cAMP or sufficient protein kinase inhibitor (21) to completely inhibit equivalent phosphorylation of histone by cAMP-dependent protein kinase. *p*-Nitrophenyl phosphate was added to inhibit phosphatases in the extract. Protein phosphorylation was not seen in assays with wheat germ eluate that had been heated at 95°C for 5 min prior to assay.

Identification of the 92,000-Dalton Protein. The 92,000-dalton protein whose phosphorylation *in vitro* was stimulated by the direct addition of insulin was immunoprecipitated by human antibody to the insulin receptor (Fig. 1). The ^{32}P-labeled band was not visible in precipitates with nonimmune serum and, as predicted from the structure of the insulin receptor in most tissues (22–24) including 3T3-L1 cells (25) and placenta (17, 26), was not seen without conditions that can reduce the disulfide bonds. Without reduction prior to electrophoresis, ^{32}P was retained in a high molecular mass species (>200,000 daltons). The position of the immunoprecipitated ^{32}P-labeled band was the same as that seen when intact 3T3-L1 cells were incubated with $^{32}P_i$ for 1 hr and with insulin (5 μg/ml) for 15 min prior to cell lysis and immunoprecipitation (Fig. 1). The observations that (*i*) the 92,000-dalton protein could be immunoprecipitated by antiserum to the insulin receptor, (*ii*) conditions for disulfide bond reduction were required to release the protein from a high molecular mass complex, and (*iii*) the insulin-treated intact cell phosphorylated a 92,000-dalton protein with similar antigenic properties led to the conclusion that the 92,000-dalton protein is a subunit of the insulin receptor. In addition, this ^{32}P-labeled protein could be bound to and eluted from insulin-Sepharose (data not shown). The incorporation of ^{32}P into the insulin receptor depicted in Fig. 2 suggests that a significant proportion of the receptor population is phosphorylated. The precise stoichiometry cannot be calculated because insulin-binding assays and kinase assays were performed with different concentrations of insulin.

Requirement for Insulin. Although an effect of insulin was detectable at 1–10 ng/ml, the amount of ^{32}P incorporated into the 92,000-dalton band continued to increase as the insulin concentration was raised to 1–10 μg/ml (Fig. 3). Proinsulin, which has <10% of the affinity of insulin for the insulin receptor (17, 27, 28), had little effect at 10–100 ng/ml but induced substantial phosphorylation at higher concentrations (Fig. 3). EGF (1.6 μg/ml) had no effect. This was also true in placental extracts in which this concentration of EGF (as previously described in ref. 29) stimulated the phosphorylation of its own receptor. Antibody to the insulin receptor, an insulin agonist in most insulin receptor-mediated events (1, 30, 31), activated the kinase. In

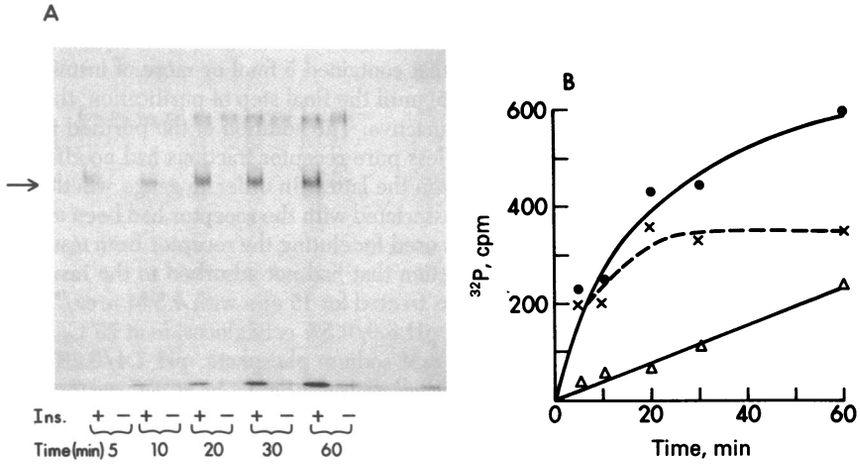

FIG. 2. Effect of time of incubation on phosphorylation of the 92,000-dalton protein. (*A*) Radioautogram of assays containing 10.2 fmol of insulin-binding activity incubated for the indicated times after preincubation with (lanes designated +) or without (lanes designated −) 5 μg of insulin (Ins.) per ml. (*B*) Graph of the data for the 92,000-dalton protein obtained from *A*. ●, With insulin; △, without insulin; ×, with insulin (●) minus the control (△).

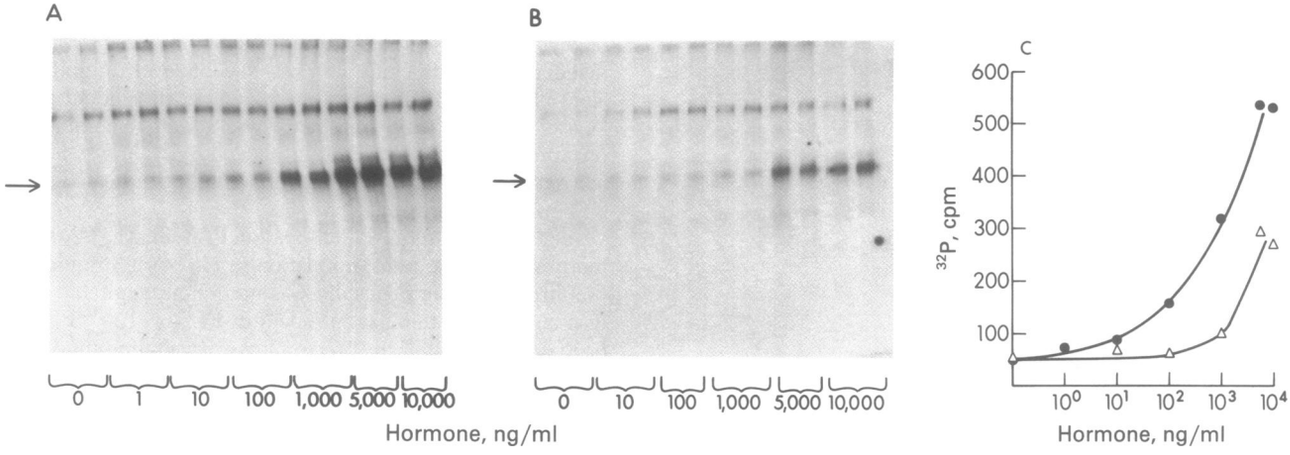

FIG. 3. Effect of insulin (*A*) and proinsulin (*B*) on phosphorylation of the 92,000-dalton protein. Assays were performed in duplicate with the concentrations of hormone indicated and 19.6 fmol of insulin-binding activity. (*C*) Graph of ^{32}P incorporated into the 92,000-dalton protein derived from *A* and *B*: ●, Insulin; △, proinsulin.

a typical experiment, 0.6 μg of rabbit anti-receptor IgG elicited the incorporation of 633 cpm into the 92,000-dalton band compared with 167 cpm for an equivalent amount of nonimmune IgG. In the same experiment, insulin elicited the incorporation of 522 cpm.

Other Substrates for Insulin-Stimulated Phosphorylation. Histone H2B, 40S ribosomal subunits, casein α-S, and homogeneous rat liver ATP citrate lyase were tested for their ability to serve as exogenous substrates for the insulin-activated kinase (Table 1). Histone was an excellent substrate (Fig. 4). Casein α-S and the 31,000-dalton (S6) component of the 40S ribosomal subunit were phosphorylated to a lesser extent. ATP citrate lyase was not a substrate.

Phosphoamino Acid Analysis. The results of a phosphoamino acid analysis of the product formed in the *in vitro* reaction are shown in Fig. 5. [^{32}P]Tyrosine was the only amino acid formed in an insulin-dependent manner. Because the 92,000-dalton protein was the only protein phosphorylated in an insulin-dependent manner in the material used for the hydrolysis (Fig. 5*B*), it can be concluded that tyrosine is the principal amino acid modified in the receptor. Similarly, when more than 97% of the insulin-stimulated phosphorylation occurred in histone, the only ^{32}P-labeled amino acid detected whose presence was dependent upon insulin was [^{32}P]tyrosine (Fig. 4). The same was true for casein.

Phosphoprotein Phosphatase Activity. Because insulin promotes dephosphorylation of some proteins in intact cells and can stimulate dephosphorylation *in vitro* by generation of a phosphatase activator (32–34), it was important to assess phosphatase activity in the tyrosine-specific kinase preparation. In the presence of either 20 mM *p*-nitrophenyl phosphate or 20 mM EDTA, the ^{32}P incorporated into the receptor by either 3T3-L1 or placental protein kinase was stable in a subsequent 30-min incubation at 23°C. To assess whether insulin activated a phosphatase during the 1-hr preincubation, histone was phosphorylated *in vitro* in the presence of insulin (Fig. 4 legend), isolated by precipitation with 10% trichloroacetic acid, and then added to a 3T3-L1 protein kinase preparation that had been incubated for 1 hr with or without 5 μg of insulin (in the absence of *p*-nitrophenyl phosphate) per ml. In both conditions, there was a 30–35% decrement in ^{32}P-labeled histone after 90 min and a similar decrease in the ^{32}P associated with the 92,000-dalton protein. Thus, the protein phosphorylation enhanced by insulin cannot be attributed to activation of a phosphatase providing new substrate sites for an unaltered kinase.

Table 1. Phosphorylation of exogenous substrates

Exp.	Substrate	Receptor, cpm With Ins.	Receptor, cpm Without Ins.	Substrate, cpm With Ins.	Substrate, cpm Without Ins.
1	Histone H2B	653	174	19,540	1,742
	Casein-αS	168	17	1,700	814
2	ATP-citrate lyase	211	66	0	110
3	S6	264	66	73	18

Ins., insulin.

Substrates were added 10 min prior to the [γ^{32}P]ATP, and incubations were for 30 min. Assays of casein phosphorylation contained no $MnCl_2$. The final concentrations of added substrates were histone (0.38 mg/ml), ATP-citrate lyase (0.1 mg/ml), 40S ribosomal subunit (the 31,000-dalton component of which is taken as S6) (0.18 mg/ml), and casein (0.42 mg/ml). The radioactivity presented is that found in the 92,000-dalton protein (receptor) and in the substrate bands. In experiments 1, 2, and 3, the insulin-binding activity was 8.6, 10.5, and 10.2 fmol, respectively.

Fractionation of Protein Kinase Activity During Purification of the Placental Insulin Receptor. All of the properties described for the insulin-activated kinase in 3T3-L1 cell extracts were demonstrable in comparable extracts of human placental membrane. To determine whether the insulin-activated kinase could be resolved from the insulin receptor, placental membranes were extracted with Triton X-100 and subjected to DEAE-cellulose chromatography, followed by affinity chromatography on insulin-Sepharose. The receptor in each of these fractions was adsorbed to and eluted from wheat germ agglutinin-agarose and assayed. Kinase activity was present in all of the fractions that contained 5 fmol or more of insulin-binding activity (Fig. 6) until the final step of purification; the purified receptor was inactive. The addition of the purified insulin receptor to the less pure receptor fractions had no effect on the kinase activity in the latter. In order to gauge whether the kinase activity associated with the receptor had been affected by the conditions used for eluting the receptor from insulin-Sepharose, the fraction that had not adsorbed to the insulin-Sepharose resin was treated for 15 min with 4.5 M urea/50 mM sodium acetate, pH 6.0/0.5% octylglucoside at 23°C, diluted 2-fold with 100 mM sodium phosphate, pH 7.4/0.2% octylglucoside at 4°C, and dialyzed for 13 hr at 4°C against 100 mM sodium phosphate, pH 7.4/0.2% octylglucoside/50% sucrose. This fraction, now treated as the purified receptor had been

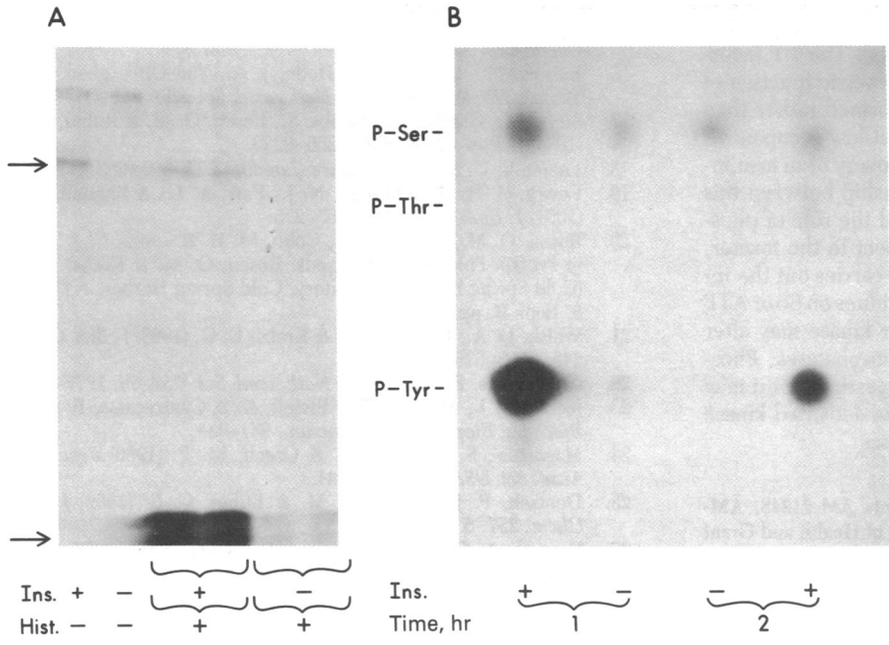

FIG. 4. Insulin (Ins.)-stimulated phosphorylation of histone (Hist.) and phosphoamino acid analysis of the product. Lane designations show insulin (Ins.) and histone (Hist.) content: +, with; −, without. (*A*) Radiogram used for Table 1. (*B*) Assays containing histone (0.213 mg) and 167 fmol of insulin-binding activity were stopped by the addition of 10% trichloroacetic acid. The precipitate was washed with ether and hydrolyzed. The positions of the phosphoamino acid markers are indicated, as is the duration of hydrolysis. At 1 hr, the radioactivities in the serine and tyrosine spots were 5,334 and 37,782 cpm in the assay with insulin (5 μg/ml) and 2,971 and 2,237 cpm in the control (without insulin), respectively. At 2 hr, the corresponding numbers were 2,684 and 10,250 cpm in the assay with insulin and 3,291 and 1,187 in the control.

(17)*, was purified through wheat germ agglutinin and assayed. Although 8.3 fmol of insulin binding was present in the assay, no kinase activity was detected. Thus, either the kinase is selectively inactivated by the procedure used to elute the receptor from insulin-Sepharose or the kinase and receptor (or some component necessary for the kinase activity of the receptor) are separable entities, resolved after exposure to urea and octylglucoside by either insulin-Sepharose or wheat germ agglutinin-agarose chromatography.

DISCUSSION

The insulin receptor consists of at least two, possibly three, kinds of subunits with molecular masses of 135,000, 90,000, and 45,000 daltons. The 135,000-dalton subunit is the insulin-binding component of the receptor (35–39). The 90,000-dalton subunit serves as the substrate for the insulin-activated kinase. The evidence that insulin mediates tyrosine-specific phosphorylation of its receptor *in vitro* is reminiscent of the EGF-mediated phosphorylation of the EGF receptor (40, 41). However, EGF and insulin do not stimulate the phosphorylation of each other's receptors, and it is unclear whether the insulin-activated kinase is an intrinsic component of the insulin receptor, as is the case for EGF.

Although the ability of insulin to promote receptor phosphorylation is mediated by insulin-specific receptors, the insulin dose–response relationship for receptor phosphorylation is vastly different from that observed for the acute physiological effects of insulin. High concentrations of insulin were also re-

* The use of octylglucoside (in place of Triton X-100) during elution from insulin-Sepharose gives the same purification as that in ref. 17.

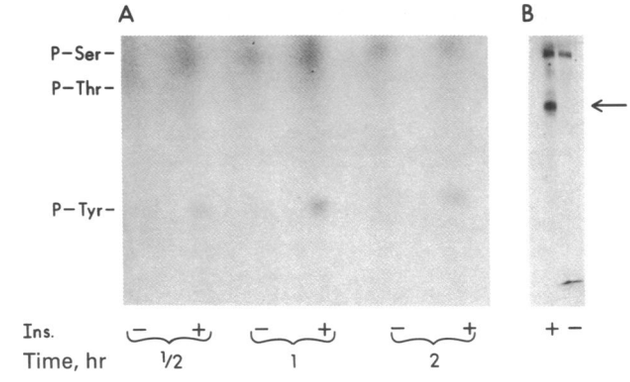

FIG. 5. Phosphoamino acid analysis of the ^{32}P-labeled 92,000-dalton protein. (*A*) Radioautogram of the chromatogram derived from the ^{32}P-labeled 92,000-dalton protein depicted in *B*. For hydrolysis, 313 fmol of insulin-binding activity was assayed in the presence (lanes designated +) or absence (lanes designated −) of insulin (Ins.; 5 μg/ml). At 0.5, 1, and 2 hr, the radioactivities in serine spots were 1,517, 2,698, and 1,862 cpm for insulin-treated samples and 1,591, 1,890, and 1,889 cpm for controls without insulin; the corresponding data for tyrosine were 744, 1,533, and 1,673 cpm compared to control levels of 80, 728 and 664 cpm.

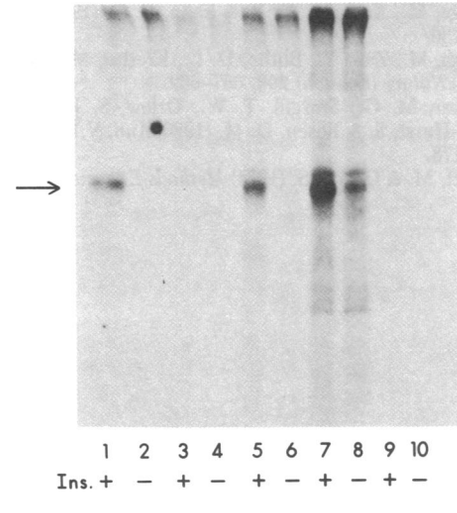

FIG. 6. Insulin-activated kinase activity during purification of the placental insulin receptor. Assays were performed with (+) or without (−) insulin (Ins.) (5 μg/ml) in wheat germ eluates derived from crude extracts (lanes 1 and 2), material not adsorbed to DEAE-cellulose (lanes 3 and 4), DEAE-cellulose eluate (lanes 5 and 6), material not adsorbed to the insulin-Sepharose (lanes 7 and 8), and purified receptor (lanes 9 and 10). The insulin-binding activities in the assays of these fractions were (in order) 11.6, 0.9, 17.3, 23.0, and 20.5 fmol. The radioactivities in the 92,000-dalton band in the assays with insulin were (from left to right) 362, 15, 272, 261, and 0 cpm and in the control assays were 12, 0, 26, 63, and 0 cpm.

quired for maximal receptor phosphorylation in intact lymphocytes (11) and 3T3-L1 cells (unpublished data). The ^{32}P incorporated into the receptor may be a stoichiometric function of the amount of receptor hormone complex formed rather than an amplified function that can be saturated at low occupancy.

The important questions raised by the discovery of an insulin-activated protein kinase include the relationship between this kinase and the other activities of insulin and the role of phosphorylation in receptor function. With respect to the former, it is unlikely that the insulin receptor kinase carries out the insulin-promoted phosphorylation of serine residues on S6 or ATP citrate lyase. However, the insulin receptor kinase may alter the activities of other protein kinases or phosphatases. Phosphorylation of the receptor is reversible, suggesting that it may be regulated by the net activity of the insulin-activated kinase and one or more phosphoprotein phosphatases.

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