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Phosphorylation of the Insulin Receptor by Casein Kinase I

Polygena T. Tuazon, Dennis T. Pang, Jules A. Shafer, and Jolinda A. Traugh

Department of Biochemistry, University of California, Riverside, California 92521 (P.T.T., J.A.T.) and Department of Biological Chemistry, The University of Michigan, Ann Arbor, Michigan 48109 (D.T.P., J.A.S.)

Insulin receptor was examined as a substrate for the multipotential protein kinase casein kinase I. Casein kinase I phosphorylated partially purified insulin receptor from human placenta as shown by immunoprecipitation of the complex with antiserum to the insulin receptor. Analysis of the phosphorylated complex by polyacrylamide gel electrophoresis under nonreducing conditions showed a major phosphorylated band at the position of the $\alpha_2\beta_2$ complex. When the phosphorylated receptor was analyzed on polyacrylamide gels under reducing conditions, two phosphorylated bands, M_r 95,000 and M_r 135,000, were observed which corresponded to the α and β subunits. The majority of the phosphate was associated with the β subunit with minor phosphorylation of the α subunit. Phosphoamino acid analysis revealed that casein kinase I phosphorylated only seryl residues. The autophosphorylated $\alpha_2\beta_2$ receptor purified by affinity chromatography on immobilized O-phosphotyrosyl binding antibody was also a substrate for casein kinase I. Reduction of the phosphorylated $\alpha_2\beta_2$ receptor indicated that casein kinase I incorporated phosphate into seryl residues only in the β subunit.

Key words: casein kinase, insulin receptor, phosphorylation

The insulin receptor is generally viewed as a glycoprotein complex consisting of two α (M_r 135,000) and two β (M_r 95,000) subunits linked together by disulfide bonds to form a symmetrical $\alpha_2\beta_2$ structure [1–9]. The β subunit can also exist in a proteolyzed form, M_r 45,000 (β_1); thus, two additional forms of the complex have been identified, $\alpha_2\beta\beta_1$ and $\alpha_2(\beta_1)_2$ [4]. Recent studies with intact cells as well as soluble cell-free systems have shown that the β subunit of the insulin receptor is phosphorylated and that this phosphorylation is stimulated by insulin [9–18]. Phosphorylation of the insulin receptor in human lymphocytes, rat hepatocytes, and hepatoma cells [9–11] is on the β subunit and occurs mainly on serine. After stimulation with insulin, there is an increase in the phosphorylation of seryl and threonyl residues and the appearance of phosphotyrosine [9–11]. Highly purified preparations of insulin receptor have been shown to incorporate phosphate into the β subunit on tyrosyl residues [15,19–24]. These observations, together with the identi-

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fication of an ATP binding site on the β subunit [8,13,20] and double-probe labeling [8], strongly suggest that the insulin receptor is a protein kinase with a high preference for tyrosyl residues. Partially purified preparations of the insulin receptor have been reported to contain serine protein kinase activity that phosphorylates the β subunit [11,25,26]. The identity and physiologic importance of the serine protein kinases that phosphorylate the insulin receptor have not been determined.

In these studies, the multipotential protein kinase casein kinase I has been utilized in an attempt to identify a serine/threonine protein kinase that is competent to phosphorylate the insulin receptor so that the possible regulatory roles of serine/threonine phosphorylation of the insulin receptor might be assessed. Casein kinase I appears to be ubiquitous in eukaryotes and has been shown to phosphorylate predominately seryl residues in a variety of acidic substrates (for review, see Hathaway and Traugh [27]). The enzyme has been purified to homogeneity and extensively characterized [28]. In this report we show that casein kinase I phosphorylates the insulin receptor from human placenta.

MATERIALS AND METHODS

Materials

Porcine insulin was purchased from Eli Lilly. Pansorbin was purchased from Calbiochem-Boehring Corporation. [γ-^{32}P]ATP was purchased from ICN or prepared as described previously [29]. Silica gel plates were from E. Merck; phosphoserine, phosphotyrosine, phosphothreonine, Triton X-100, and trypsin (DPCC-treated) were obtained from Sigma. Wheat germ agglutinin-agarose (WGA) was obtained from E-Y Laboratories. Antiserum to the insulin receptor was generously provided by Dr. C.R. Kahn, Harvard Medical School, and has been extensively characterized [7,10]. Casein kinase I was purified from rabbit reticulocytes as previously described [28] and stored in 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 1 mM EGTA, 1 mM EDTA, and 0.02% NaN_3.

Purification of Insulin Receptor

Insulin receptor was partially purified from human placental membranes through the WGA step as described previously [30,31]. The tyrosyl phosphorylated form of the insulin receptor was purified to near homogeneity by adsorption on O-phosphotyrosyl-binding antibody immobilized on Protein A-Sepharose followed by elution with 10 mM p-nitrophenylphosphate as described by Pang et al. [32] and then stored with 1 mg/ml bovine serum albumin.

Phosphorylation of Insulin Receptor

Phosphorylation was carried out in 0.10-ml reaction mixtures containing 25 mM Tris-HCl, pH 7.5, 5–10 mM $MgCl_2$, 0.1 mM Na_3VO_4, 0.05% Triton X-100, 0.10 mM [γ-^{32}P]ATP (5,000–10,000 cpm/pmol), and WGA-purified insulin receptor (10 μg) or purified autophosphorylated receptor ($\sim$ 0.02 μg), in the presence or absence of casein kinase I (10–300 units). In some autophosphorylation experiments, 2–10 mM $MnCl_2$ replaced $MgCl_2$, as noted in the figure legends. The insulin receptor was preincubated at room temperature for 45 min in the presence or absence of insulin (3.6 μg) before addition of the protein kinase; phosphorylation was initiated by addition of [γ-^{32}P]ATP. Reactions were incubated at 30°C for 30 min, and terminated

by addition of EDTA to 10 mM, unlabeled ATP to 10 mM, and gel electrophoresis sample buffer with or without 0.1 M 2-mercaptoethanol [29]. Samples were heated at 100°C for 5–10 min and subjected to polyacrylamide gel electrophoresis in sodium dodecyl sulfate [29]. Nonreduced samples were analyzed on 5% polyacrylamide gels (30% acrylamide: 0.3% bisacrylamide) and samples with reducing agent on 7.5% polyacrylamide gels (30% acrylamide: 0.8% bisacrylamide). Gels were stained, destained, and dried, and phosphorylated bands were detected by autoradiography as described previously [29]. Bands corresponding to the receptor complex or the individual substrates were excised from the gels using the autoradiogram as a guide, and $^{32}P_i$ was quantified by scintillation counting [29].

Immunoprecipitation of the Insulin Receptor

Insulin receptor was phosphorylated with and without casein kinase I in 0.10-ml reaction mixtures. Reactions were terminated by the addition of EDTA to 10 mM, then incubated with 0.001 ml of antireceptor antiserum (B-9) for 2 hr at 4°C. The solution was mixed with a Pansorbin precipitate obtained by centrifugation of 0.10 ml of a 10% suspension of Pansorbin that was prepared as described by Kasuga et al [31]. After 2 hr incubation at 4°C, Pansorbin was collected by centrifugation for 1 min in a Beckman microfuge B, washed three times with 0.20 ml of 25 mM Tris-HCl, pH 7.4, 0.1% Triton X-100, and suspended in gel electrophoresis sample buffer without 2-mercaptoethanol. After 5 min at 95°C, the Pansorbin was removed and the supernate was analyzed by gel electrophoresis as described above.

Phosphoamino Acid Analysis

Insulin receptor was phosphorylated in 0.10-ml reaction mixtures, and samples were subjected to electrophoresis on 7.5% polyacrylamide gels containing sodium dodecyl sulfate under reducing conditions. Protein bands corresponding to the α and β subunits were excised from the stained, dried gels, washed twice with 1 ml of distilled water for 15 min, and then with 1 ml of 0.2 M NH_4HCO_3 (pH 8.2). The slices were incubated overnight at 37°C in 0.5 ml of 0.2 M NH_4HCO_3 containing 0.1 mg trypsin. Incubation at 37°C was continued for another 6 hr after a second addition of 0.05 mg trypsin. The gel slices were removed and the solutions were lyophilized, then hydrolyzed for 2 hr at 100°C in 0.5 ml of 4.0 M HCl in evacuated tubes. The hydrolysates were rotary-evaporated to dryness and the residue was taken up in a minimum volume of distilled water. Samples with phosphoserine, phosphotyrosine and phosphothreonine added as internal standards were spotted on silica gel sheets. Thin-layer electrophoresis was carried out at pH 1.9 (78 ml acetic acid: 25 ml 88% formic acid: 897 ml water) at 800 V for 4–5 hr at 4°C on a Desaga-Brinkmann apparatus. Radioactive amino acids were located by autoradiography and compared with the positions of the standard phosphoamino acids.

RESULTS

Phosphorylation of the insulin receptor with casein kinase I was examined and compared to the insulin-stimulated autophosphorylation of the receptor. Insulin receptor from human placenta, partially purified by chromatography on WGA, was incubated with [γ-^{32}P]ATP in the presence or absence of added protein kinase and analyzed without prior reduction of disulfide bonds on polyacrylamide gels containing

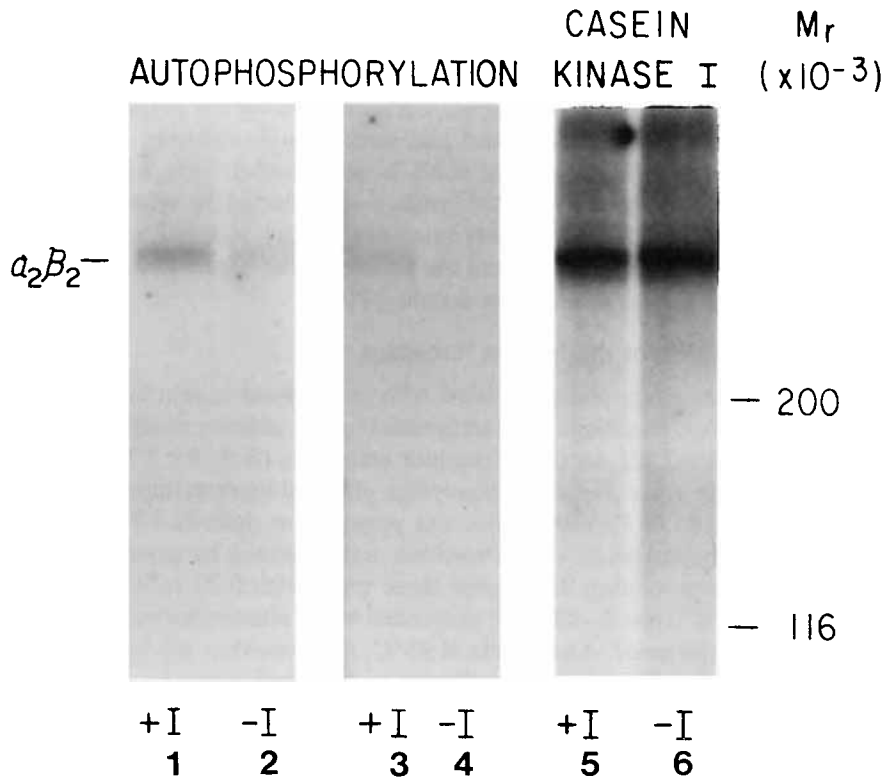

Fig. 1. Phosphorylation of WGA-purified insulin receptor by casein kinase I. Insulin receptor purified by chromatography of WGA was preincubated in the presence (+I) and absence (−I) of insulin. Phosphorylation was initiated with [γ-^{32}P]ATP in the presence and absence of casein kinase I (300 units), and the samples were analyzed under nonreducing conditions on polyacrylamide gels as described under Materials and Methods. Autoradiograms of the gels are shown. Autophosphorylation of the insulin receptor with 10 mM $MnCl_2$ (lanes 1,2) or $MgCl_2$ (lanes 3,4); phosphorylation by casein kinase I with 10 mM $MgCl_2$ (lanes 5,6). Lanes are numbered from left to right.

sodium dodecyl sulfate. The phosphorylated proteins were detected by autoradiography as shown in Figure 1. The extent of phosphorylation was quantified by scintillation counting of the radiolabeled bands following excision from the gel. When the insulin receptor was incubated with 10 mM $MnCl_2$, autophosphorylation of a single, high-molecular-weight band of ~ 370,000, as expected for phosphorylation of the $\alpha_2\beta_2$ receptor complex, was observed. Autophosphorylation was stimulated threefold to fivefold by preincubation of the receptor with insulin as has been observed in a number of laboratories [9,13–22]. A small amount of autophosphorylation of the same complex was identified when 10 mM $MgCl_2$ was substituted for $MnCl_2$. When the receptor was incubated with casein kinase I and $MgCl_2$, the main phosphoprotein comigrated with the $\alpha_2\beta_2$ complex. The extent of phosphorylation of the $\alpha_2\beta_2$ receptor complex was significantly higher than that obtained upon autophosphorylation, suggesting that casein kinase I modified the insulin receptor. Similar results were obtained at divalent cation concentrations of 2 and 5 mM.

To identify which subunit of the insulin receptor was phosphorylated, the band corresponding to the $\alpha_2\beta_2$ complex was excised from the 5% polyacrylamide gel and

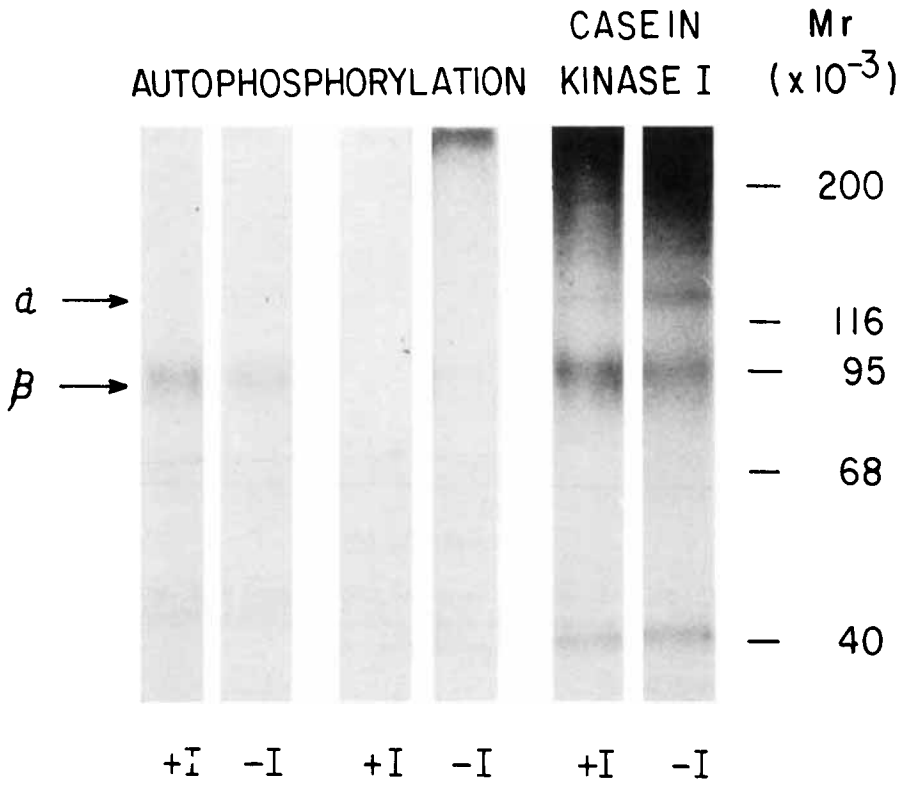

Fig. 2. Analysis of the $\alpha_2\beta_2$ receptor complex by gel electrophoresis under reducing conditions. The phosphorylated bands corresponding to the $\alpha_2\beta_2$ complex were excised from the 5% polyacrylamide gels presented in Figure 1, solubilized in 1% SDS overnight, and heated at 95° for 10 min in electrophoresis sample buffer containing 0.1 M 2-mercaptoethanol. The total volume of the reduced sample was applied to 7.5% polyacrylamide gels containing sodium dodecyl sulfate and run under reducing conditions in the same order as shown in Figure 1. Phosphorylated proteins were located by autoradiography; the autoradiograph is shown.

analyzed under reducing conditions on 7.5% polyacrylamide gels containing sodium dodecyl sulfate (Fig. 2). When insulin receptor incubated with either $MnCl_2$ or $MgCl_2$ was examined, one phosphorylated protein, M_r 95,000, which corresponded to the β subunit, was observed. This autophosphorylation was enhanced with insulin. As shown in Figure 1, $MnCl_2$ was better at promoting autophosphorylation than $MgCl_2$; little phosphorylation was observed with $MgCl_2$ under these conditions. When casein kinase I was present, the β subunit was phosphorylated to a greater extent than when the reaction was carried out in the absence of the enzyme. In addition, another band, M_r 135,000, which corresponded in electrophoretic mobility to the α subunit, was observed. The amount of phosphate incorporated into this band was variable and was minor at low concentrations of casein kinase I. Insulin specifically inhibited α phosphorylation (Fig. 2). Some phosphorylated material that comigrated with the intact $\alpha_2\beta_2$ receptor in the nonreducing gel did not comigrate with either subunit of the insulin receptor after reduction and remained near the top of the gel. This material

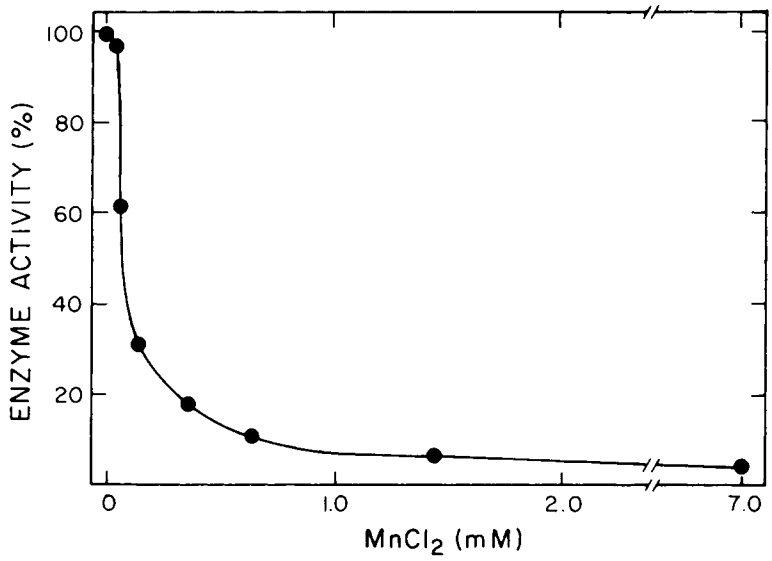

Fig. 3. Effect of $MnCl_2$ on casein kinase I. Assays for casein kinase I were carried out in 0.06-ml reaction mixtures with dephosphorylated casein as substrate, as described by Hathaway et al [29]. All assays contained 10 mM $MgCl_2$; $MnCl_2$ was varied as indicated.

has not been identified. Similar results were obtained when the entire reaction mixture (rather than the $\alpha_2\beta_2$ band) was analyzed directly under reducing conditions on 7.5% polyacrylamide gels.

Phosphorylation reactions with casein kinase I were carried out with $MgCl_2$, in the absence of $MnCl_2$, because of the observation that casein kinase I is inhibited by low concentrations of $MnCl_2$. As shown in Figure 3, phosphorylation of casein by casein kinase I was inhibited 50% with 0.1 mM $MnCl_2$ in the presence of 10 mM $MgCl_2$.

In order to confirm that the insulin receptor was phosphorylated by casein kinase I, B-9 antiserum to the insulin receptor [7,10] was used to examine the phosphorylated proteins. When immunoprecipitates of the autophosphorylated insulin receptor were examined, only the $\alpha_2\beta_2$ form of the receptor was detected and stimulation of phosphorylation by insulin was observed (Fig. 4). Phosphorylation of the insulin receptor by casein kinase I showed a major phosphorylated band at the position of the autophosphorylated $\alpha_2\beta_2$ complex. Some radiochemically labeled material, migrating just below the complex, appeared to correspond to the $\alpha_2\beta\beta_1$ and $\alpha_2(\beta_1)_2$ complexes. When the immunoprecipitates were analyzed under reducing conditions, phosphorylated proteins with molecular weights corresponding to those of the β and α subunits were observed. The insulin-stimulated autophosphorylated receptor contained phosphate only in the β subunit.

To identify the amino acids phosphorylated by the receptor-associated protein kinase and by casein kinase I, radiolabeled α and β subunits were excised from the polyacrylamide gel and subjected to acid hydrolysis, and the acid hydrolysates were analyzed by high-voltage, thin-layer electrophoresis at pH 1.9 (Fig. 5). When the receptor was autophosphorylated in the presence of $MgCl_2$, both phosphoserine and

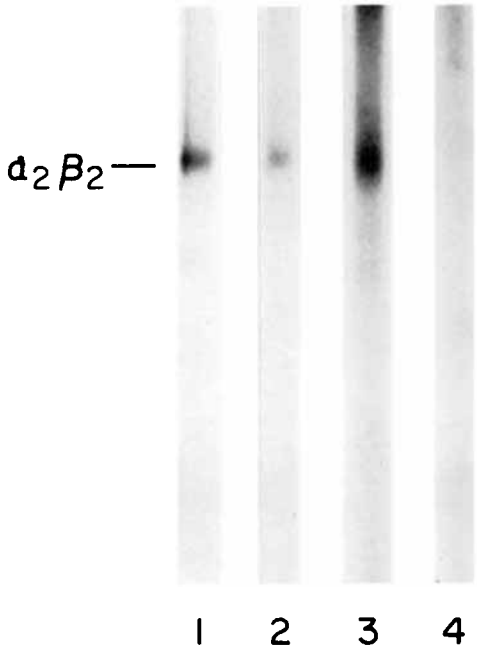

Fig. 4. Immunoprecipitation of the WGA-purified insulin receptor phosphorylated by casein kinase I. Insulin receptor was preincubated in the presence (lanes 1,3) and absence (lane 2) of insulin. Phosphorylation of the insulin receptor was conducted in the absence (lanes 1,2) and presence (lane 3) of casein kinase I with 10 mM $MgCl_2$ as described in Materials and Methods. Casein kinase I was also examined in the absence of insulin receptor and in the presence (lane 4) of insulin. Immunoprecipitates were analyzed on 5% polyacrylamide gels under nonreducing conditions. Autoradiograms of the stained gels are shown.

phosphotyrosine were detected in the β subunit. Insulin was shown to stimulate phosphorylation of tyrosyl residues, whereas no significant stimulation of phosphoserine formation by insulin was detected. When casein kinase I was added, an extensive increase in phosphoserine was observed in the β subunit; some phosphotyrosine was also present that appeared to be due to autophosphorylation. The phosphorylation of the α subunit observed with casein kinase I was shown to be exclusively on serine. Identical data were observed with immunoprecipitated insulin receptor.

Recently, insulin receptor autophosphorylated on tyrosyl residues has been purified to near homogeneity by affinity chromatography on O-phosphotyrosyl-binding antibodies [32]. The pure autophosphorylated insulin receptor was shown to be phosphorylated with casein kinase I upon gel electrophoresis under nonreducing conditions (Fig. 6). Upon incubation of the insulin receptor with $MgCl_2$, autophosphorylation occurred to a small extent. Casein kinase I stimulated phosphorylation of the complex threefold to fivefold, as determined by counting the radiolabeled bands. Electrophoresis of the reaction mixtures under reducing conditions indicated that casein kinase I catalyzed phosphorylation of the β subunit of the insulin receptor (Fig. 7). Also visible on the autoradiogram are autophosphorylated bands associated with casein kinase I and radioactivity migrating with bovine serum albumin (added to stabilize the purified complex). Slight autophosphorylation of the β subunit was

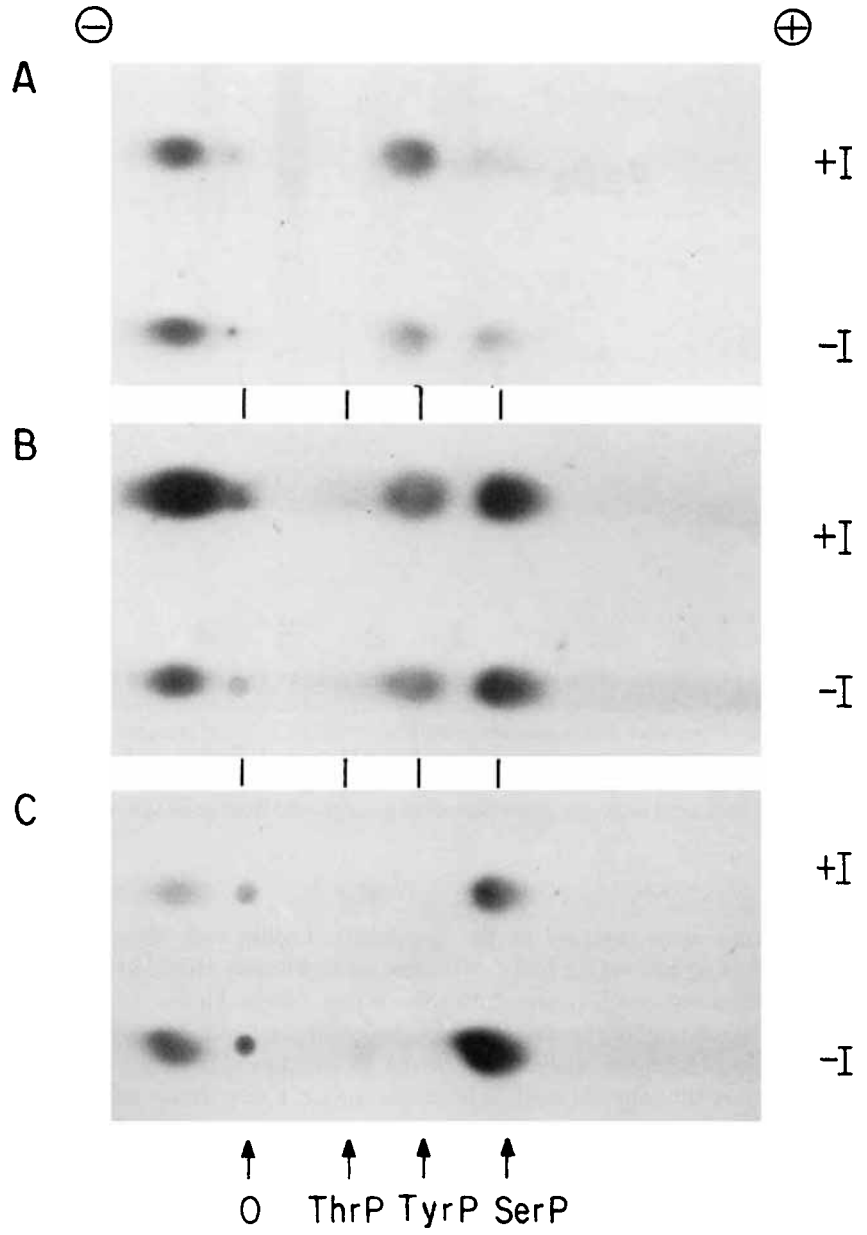

Fig. 5. Identification of phosphoamino acids in the α and β subunits of the insulin receptor. Insulin receptor was preincubated in the presence (+I) and absence (−I) of insulin. Phosphorylation was conducted in the presence and absence of casein kinase I with 10 mM $MgCl_2$. The reaction mixtures were analyzed by electrophoresis on 7.5% polyacrylamide gels under reducing conditions. Protein bands corresponding to the α and β subunits were excised from the gels and acid-hydrolyzed. Thin-layer electrophoresis was carried out at pH 1.9 with phosphoserine, phosphothreonine and phosphotyrosine as internal standards. The positions of the phosphoamino acid standards are indicated. Radioactive phosphoamino acids were detected by autoradiography. A) Autophosphorylation of the β subunit. B) Phosphorylation of the β subunit of casein kinase I. C) Phosphorylation of the α subunit by casein kinase I. Autoradiograms were exposed for 5 days at −70°C (A,B) or for 28 days (C).

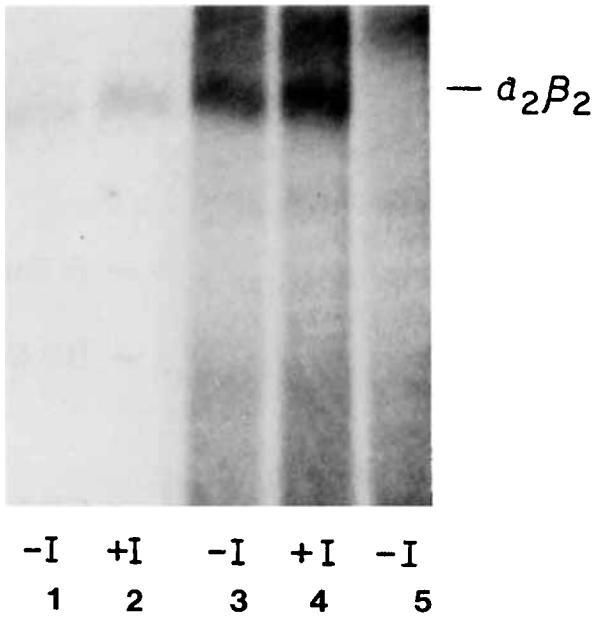

Fig. 6. Phosphorylation of purified insulin receptor by casein kinase I. Insulin receptor was preincubated with (+I) or without (−I) insulin. Phosphorylation was carried out in 0.050-ml reaction mixtures with 5 mM $MgCl_2$ with and without casein kinase I (125 units), as described under Materials and Methods. Samples were analyzed under nonreducing conditions on 5% polyacrylamide gels. Autoradiograms of the gels are shown. Lanes 1,2) insulin receptor alone; lanes 3,4) insulin receptor plus casein kinase I; lane 5) casein kinase I alone.

observed upon incubation with $MgCl_2$. With addition of casein kinase I, phosphorylation of the β subunit was increased threefold to fivefold. In contrast to results obtained with the partially purified preparation, no significant phosphorylation of the α subunit by casein kinase I was observed with the purified autophosphorylated form. Phosphoamino acid analysis of the β subunit of the insulin receptor indicated serine was phosphorylated by casein kinase I.

DISCUSSION

Insulin-stimulated phosphorylation of the insulin receptor on serine residues, originally found to occur only in whole cells [9–11], has been shown recently to occur also in partially purified preparations of insulin receptor [11,25,26]. Insulin-stimulated phosphorylation of exogenous substrates on both serine and tyrosine residues has likewise been shown with partially purified receptor [11,25,26]. The physiologic significance of these phosphorylations and the identity of the protein kinase catalyzing them remains to be established.

In this work we have examined the competence of the multipotential protein kinase, casein kinase I, to phosphorylate the insulin receptor. This protein kinase has been purified to apparent homogeneity from a number of eukaryotic tissues and is found in the cytosol and nucleus as well as in association with membranes [27].

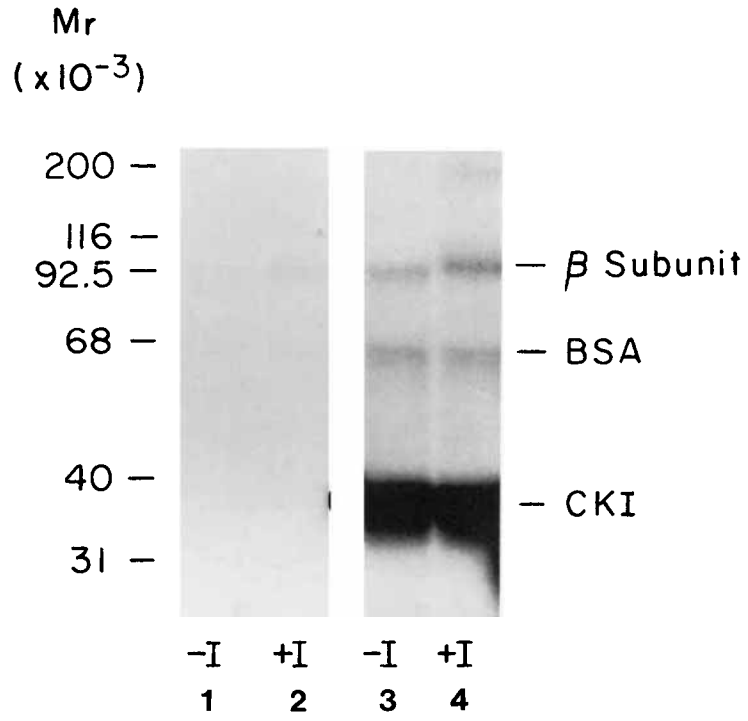

Fig. 7. Analysis of purified insulin receptor phosphorylated by casein kinase I under reducing conditions. Insulin receptor preincubated with (+I) or without (−I) insulin was phosphorylated using 5 mM $MgCl_2$ in the presence and absence of casein kinase I (250 units). Samples were reduced with 2-mercaptoethanol and analyzed by electrophoresis on 7.5% polyacrylamide gels. Autoradiograms are shown. Lanes 1 and 2) insulin receptor alone; lanes 3 and 4) insulin receptor plus casein kinase I. Phosphorylated bands corresponding to the β subunit of insulin receptor and casein kinase I (CK I) are indicated. Phosphate nonspecifically associated with the added bovine serum albumin (BSA) is also identified.

Casein kinase I consists of a single subunit (M_r 37,000) and utilizes only ATP as a phosphoryl donor [28,33]. The enzyme has a preference for acidic protein substrates and therefore might be expected to catalyze phosphorylation of the insulin receptor, which is an acidic protein with a pI of 3.9 [34].

In this report we demonstrate that casein kinase I phosphorylates the β subunit of insulin receptor partially purified by affinity chromatography on WGA and of insulin receptor purified to near homogeneity by chromatography on O-phosphotyrosyl-binding antibodies immobilized on Protein A-Sepharose. This phosphorylation occurs in the presence and absence of insulin and on the purified autophosphorylated form of the insulin receptor. It is important to note that in the phosphorylation of the insulin receptor with casein kinase I, $MgCl_2$, instead of $MnCl_2$, was used as the divalent cation, relatively low concentrations of $MnCl_2$ are inhibitory to the seryl protein kinase. This phosphorylation is the first identification of a seryl protein kinase that phosphorylates the insulin receptor. However, additional work is required before we can determine whether casein kinase I is responsible for the phosphorylation of

seryl residues of the receptor in vivo, and whether serine phosphorylation by casein kinase I affects the activity and function of the insulin receptor.

In partially purified preparations of insulin receptor, some phosphorylation of the α subunit by casein kinase I is observed. The degree of phosphorylation is less than that observed in the β subunit and is inhibited by the presence of insulin. Phosphorylation of the α subunit is greatly reduced or eliminated at low concentrations of casein kinase I and with pure receptor. The lack of phosphorylation of the pure receptor could be due to an alteration in the conformation of the α subunit by removal of contaminating protein(s) or by autophosphorylation of the β subunit. The inhibition of phosphorylation of the α subunit by casein kinase I, observed when insulin is bound to the receptor, suggests that the phosphorylation site(s) overlaps the insulin binding site on the α subunit or that the phosphorylation site(s) becomes inaccessible to the protein kinase as a result of an insulin-mediated conformational change of the receptor. The possibility also exists that phosphorylation of the α subunit, like the binding of insulin, may result in alteration of the tyrosine protein kinase activity or in the susceptibility of the β subunit to phosphorylation. Phosphorylation of the α subunit has also been observed in partially purified receptors from rat liver plasma membranes [11,15], although to our knowledge it has not yet been reported in experiments with whole cells. The significance of the phosphorylation of the α subunit by casein kinase I remains to be elucidated.

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