Biochem. J. (1988) 250, 509–519 (Printed in Great Britain)

Two systems *in vitro* that show insulin-stimulated serine kinase activity towards the insulin receptor

David M. SMITH, Martin J. KING and Graham J. SALE
Department of Biochemistry, School of Biochemical and Physiological Sciences, University of Southampton, Bassett Crescent East, Southampton SO9 3TU, U.K.

Two systems *in vitro* are described that show insulin-stimulated phosphorylation of the insulin receptor on serine residues. In the first system, insulin receptor was purified partially from Fao rat hepatoma cells by direct solubilization of the cells in Triton X-100 and chromatography on wheat-germ-agglutinin–agarose. Phosphorylation of these preparations with [γ-^{32}P]ATP in the presence or absence of insulin resulted in ^{32}P incorporation exclusively into phosphotyrosine residues. Serine kinase activity towards the insulin receptor was reconstituted by adding extracts of Fao cells. Prior exposure of the cells to insulin stimulated serine kinase activity towards the insulin receptor in extracts 7.2-fold. A receptor serine kinase activity enhanced by treatment of cells with cyclic AMP analogues was also retained in the reconstituted system. In the second system, insulin receptor and insulin-sensitive serine kinase activity towards the insulin receptor were co-purified from human placenta. The protocol involved preparation of membranes, before solubilization and chromatography on wheat-germ-agglutinin–agarose, by using gentle procedures designed not to disrupt a potentially labile association between the insulin receptor and the serine kinase. Serine kinase activity in these preparations towards the insulin receptor was stimulated up to 10-fold by insulin, and the stoicheiometry of serine phosphorylation was estimated to be approx 0.8 mol/mol of insulin receptor for phosphorylations performed in the presence of insulin. Thus a preparation of insulin receptor is described for the first time that is phosphorylated to high stoicheiometry on serine in an insulin-dependent manner. Conditions that facilitate recovery and assay of serine kinase activity are defined and discussed. These systems provide a basis for characterizing the nature of the insulin-sensitive serine kinase that phosphorylates the insulin receptor, and defining its role in insulin action and control of receptor function.

INTRODUCTION

The mechanism by which insulin binding to its receptor on the cell surface leads to the generation of an intracellular message that carries the insulin signal to metabolic targets is not known. Various hypotheses are at present under investigation. Several lines of evidence indicate that a tyrosine kinase activity intrinsic to the β-subunit of the insulin receptor, which is activated by insulin, is involved (see, e.g., Kasuga *et al.*, 1982; Morgan & Roth, 1987; Ebina *et al.*, 1987; Chou *et al.*, 1987). Although this may be an early step in the chain of events, the next link is not known. Physiological targets of the tyrosine kinase other than the β-subunit of the receptor have not been identified, although the tyrosine kinase does phosphorylate exogenous substrates *in vitro* (Stadtmauer & Rosen, 1983). A role of G-proteins in transduction of the insulin signal from the insulin receptor to cyclic AMP phosphodiesterase and adenylate cyclase (Houslay, 1985) and the generation of a mediator has been suggested (Saltiel *et al.*, 1986). Many of the effects of insulin ultimately involve altered phosphorylation of target proteins, usually on serine residues (occasionally threonine). For example, insulin induces dephosphorylation of pyruvate dehydrogenase and glycogen synthase, whereas the phosphorylation of acetyl-CoA carboxylase, ATP citrate lyase and ribosomal protein S6 is increased (Denton *et al.*, 1981; Cohen, 1985). Additionally, in intact cells phosphorylation of the insulin receptor on serine residues is increased by insulin (Gazzano *et al.*, 1983; Takayama *et al.*, 1984; White *et al.*, 1985; Stadtmauer & Rosen, 1986). Denton *et al.* (1981) have proposed that insulin actually causes the dissociation of a serine kinase from the plasma membrane. Thus the serine kinase that phosphorylates the insulin receptor could also act on other targets. Serine kinases activated in cells by insulin are poorly characterized.

The objective of the present work is to develop systems that will enable study of the insulin-sensitive serine kinase that phosphorylates the insulin receptor. This kinase has the potential not only to regulate insulin-receptor function but also to participate in a phosphorylation cascade that leads to the altered phosphorylation of metabolic targets, e.g. by phosphorylating and altering the activity of intermediary kinases/phosphatases or by direct phosphorylation of metabolic targets. To test this hypothesis, systems *in vitro* are required that show a high activity of this serine kinase towards the insulin receptor, and these have so far not been obtained. Thus, although purified or partially purified preparations of insulin receptor contain high tyrosine kinase activity, no preparations *in vitro* have been reported in the literature in which the insulin receptor is phosphorylated to a high

Abbreviation used: 8-CPT-cAMP, 8-(4-chlorophenylthio) cyclic AMP.

degree on serine in response to insulin. This suggests that the serine kinase activity involved is easily lost during purification of the insulin receptor, or inactivated. Most of the known serine kinases tested are unable to phosphorylate the insulin receptor (Haring *et al.*, 1985). However, phosphorylation of the insulin receptor by C-kinase may mediate the effects of phorbol esters on increasing receptor phosphorylation on serine in intact cells (Jacobs *et al.*, 1983; Takayama *et al.*, 1984; Bollag *et al.*, 1986). Raising the cyclic AMP concentration in intact cells also stimulates phosphorylation of the insulin receptor on serine (Stadtmauer & Rosen, 1986), although the kinase(s) involved has not been clearly identified (Van Obberghen *et al.*, 1983; Joost *et al.*, 1986; Roth & Beaudoin, 1987; Tanti *et al.*, 1987). The cyclic-AMP- and phorbol-ester-stimulated phosphorylations inhibit the tyrosine kinase activity of the insulin receptor, and this provides a molecular link for inhibition of insulin-receptor function by other hormones (Stadtmauer & Rosen, 1986; Haring *et al.*, 1986*a*,*b*). The effect of the insulin-stimulated serine phosphorylation on tyrosine kinase activity is not known.

In the present paper, two systems *in vitro* are described that show a high activity of the insulin-sensitive serine kinase that phosphorylates the insulin receptor.

EXPERIMENTAL

Materials

Triton X-100, *N*-acetylglucosamine, phosphoamino acids, aprotinin, histone II-A, phenylmethanesulphonyl fluoride, reagents for SDS/polyacrylamide-gel electrophoresis, Hepes, bovine serum albumin, human γ-globulin, pig insulin, trypsin (treated with tosylphenylalanylchloromethane) and bovine heart catalytic subunit of cyclic-AMP-dependent protein kinase were obtained from Sigma Chemical Co., Poole, Dorset, U.K. 8-(4-Chlorophenylthio) cyclic AMP (8-CPT-cAMP) was from BCL, Lewes, East Sussex, U.K. Pansorbin was purchased from Calbiochem, Cambridge, U.K. Serum from patient B9, containing autoantibodies to insulin receptors, was generously given by Dr. C. R. Kahn (Joslin Diabetes Center, Boston, MA, U.S.A.). Wheat-germ-agglutinin–agarose came from Vector Laboratories, Peterborough, Cambs., U.K. Tissue-culture media and flasks were obtained from GIBCO, Uxbridge, Middx., U.K. [γ-^{32}P]ATP was from Amersham International, Amersham, Bucks., U.K., and pig ^{125}I-insulin was from NEN (Du Pont), Stevenage, Herts., U.K. Kodak X-Omat AR film and Amersham Hyperfilm-MP were purchased from Kodak, Kirkby, Liverpool, U.K., and Amersham International, respectively. The Bradford protein assay kit was obtained from Bio-Rad, Watford, Herts., U.K. Cellulose thin-layer plates (20 cm × 20 cm; Merck), poly(ethylene glycol) 6000 and other chemicals were from BDH Chemicals, Poole, Dorset, U.K.

Cell culture

The highly differentiated and insulin-sensitive hepatoma cell (Fao) line was originally provided by Dr. M. C. Weiss (Gif-sur-Yvette, France) (Deschatrette *et al.*, 1979). These cells possess a high concentration of insulin receptors and many insulin-stimulated responses (Crettaz & Kahn, 1983). Monolayer cultures of Fao cells were grown to confluence in 175 cm^2 or 75 cm^2 plastic tissue-culture flasks containing 30 ml or 15 ml of RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum. The cell cultures were maintained at 37 °C in a humidified atmosphere of air/CO_2 (19:1) (Kasuga *et al.*, 1985).

Preparation of placental membranes

Human placental membranes were prepared by modification of published procedures (Fujita-Yamaguchi *et al.*, 1983; Pike *et al.*, 1984). Placentae were obtained within 1 h of delivery. All procedures were carried out at 4 °C. Placentae were trimmed of amnion and chorion, washed with 0.25 M-sucrose and cut into small pieces. The pieces were then homogenized for 3 × 1 min in a Waring blender in 1 vol. of 50 mM-Tris/HCl (pH 7.4) containing 0.25 M-sucrose and 1 mM-phenylmethanesulphonyl fluoride. The homogenate was centrifuged at 15000 *g* for 20 min, and the supernatants were collected and centrifuged at 100000 *g* for 1 h. The pellets were suspended in 10 vol. of 50 mM-Tris/HCl (pH 7.4) containing 1 mM-phenylmethanesulphonyl fluoride by homogenization by hand, and centrifuged at 100000 *g* for 1 h. The sedimented membranes were resuspended by homogenization by hand in 2 vol. of 50 mM-Tris/HCl (pH 7.4)/1 mM-phenylmethanesulphonyl fluoride, separated into 1.5 ml batches and stored at −70 °C. Typically 1–1.5 g of membranes was obtained per placenta.

Solubilization and purification of insulin receptor

(*a*) From placental membranes. All operations were performed at 4 °C. Membranes (700 mg) were solubilized in a final volume of 30 ml of 50 mM-Tris/HCl (pH 7.4) containing 2% Triton X-100, 0.1 mg of aprotinin/ml and 0.35 mg of phenylmethanesulphonyl fluoride/ml by homogenization by hand, followed by stirring for 2.5 h. The mixture was then centrifuged at 200000 *g* for 60 min. The supernatant was recycled three times through a 5 ml wheat-germ-agglutinin–agarose column, which had been equilibrated with 50 mM-Hepes (pH 7.4)/0.1% Triton X-100/0.1 mM-phenylmethanesulphonyl fluoride. The column was then washed with 200 ml of equilibration buffer and eluted with 15 ml of equilibration buffer containing 0.3 M-*N*-acetylglucosamine. The protein-containing fractions, determined with the Bradford assay (standardized with bovine serum albumin), were pooled, separated into 150 μl batches and stored at −70 °C. Yield of protein was typically 10 mg, and protein concentration in pooled fractions was typically 1 mg/ml.

(*b*) From hepatoma (Fao) cells. Twenty confluent flasks (175 cm^2) of cells were washed at 22 °C with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (Kasuga *et al.*, 1985). The cells were solubilized by adding to each flask at 22 °C 2 ml of 50 mM-Hepes (pH 7.4) containing 1% Triton X-100, 0.1 mg of aprotinin/ml and 0.3 mg of phenylmethanesulphonyl fluoride/ml and scraping, followed by vortex-mixing (Kasuga *et al.*, 1985). The material was then centrifuged and the insulin receptor purified on wheat-germ-agglutinin–agarose as described in (*a*), except that a 1 ml column was used and the three fractions containing the most protein were pooled. This gave a protein concentration typically of 0.4 mg/ml. The insulin receptor was stored at −70 °C in 250 μl batches.

Phosphorylation assays

Insulin receptors partially purified from hepatoma cells (approx. 0.4 μg of protein/μl) or from placental membranes (approx. 1 μg of protein/μl) were incubated for 15 min at 22 °C with or without 100 nM-insulin in 10–35 μl of 50 mM-Hepes (pH 7.4)/0.1 % Triton X-100 in the presence of $MnCl_2$, $MgCl_2$, dithiothreitol or sodium vanadate as indicated (Sale *et al.*, 1986). In the reconstitution experiments, extracts of hepatoma cells were then added. Phosphorylation reactions were initiated by addition of [γ-^{32}P]ATP (250 μM in reconstitution experiments and 100 μM in other experiments, except where stated otherwise; 5–30 c.p.m./fmol) and were for 5–30 min at the temperatures indicated. Incubations were terminated by one of two methods. In the first, 0.25 vol. of 5-fold-concentrated Laemmli (1970) sample buffer (100 mM-Tris/HCl, pH 7.4, 10 % SDS, 75 mg of dithiothreitol/ml, 0.1 % Bromophenol Blue) were added and the samples heated at 100 °C for 2 min. In the second method, 10–20 vol. of stop solution (50 mM-Hepes, pH 7.4, 0.1 % Triton X-100, 10 mM-sodium pyrophosphate, 100 mM-NaF, 5 mM-EDTA, 2 mM-sodium vanadate) were added at 0 °C and insulin receptors immunoprecipitated. Immunoprecipitation was achieved by incubation for 15 h at 4 °C with a 1:100 dilution of B9 serum containing autoantibodies against the insulin receptor. Then 0.1 vol. of Pansorbin (10 %, w/v) was added, and samples were incubated for 1 h at 4 °C and centrifuged for 5 min at 10000 ***g***. Immunoprecipitates were washed three times with 50 mM-Hepes (pH 7.4)/1 % Triton X-100/0.1 % SDS, once with 50 mM-Hepes (pH 7.4)/0.1 % Triton X-100, and then suspended in 40 μl of Laemmli (1970) sample buffer and boiled for 5 min (Kasuga *et al.*, 1985). Experiments in which control serum replaced B9 serum showed that no phosphorylated proteins were obtained in the vicinity of the β-subunit of the insulin receptor on SDS/polyacrylamide-gel electrophoresis.

Samples were analysed by SDS/polyacrylamide-gel electrophoresis (after adding sucrose, final concn. 200 mg/ml) on 4 %-acrylamide stacking and 7.5 %-acrylamide resolving gels (Laemmli, 1970). Electrophoresis was performed at 20 °C for about 3 h at 30–35 mA. Gels were stained for 20 min with 0.25 % Coomassie Brilliant Blue in 50 % (w/v) trichloroacetic acid and destained overnight in 5 % acetic acid/45 % methanol (both v/v). Gels were dried *in vacuo* for 1 h at 80 °C with a Bio-Rad Gel Dryer. Autoradiograms were obtained at −70 °C for 2–18 h within cassettes containing Dupont Cronex intensifier screens. ^{32}P associated with appropriate bands was determined by counting radioactivity of excised ^{32}P-labelled pieces directly in 5 ml of scintillation fluid [5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole (6 g/l) in toluene]. Alternatively, gel pieces were treated with H_2O_2 and counted for radioactivity as previously described (Sale & Denton, 1985). Both methods gave similar answers.

For phosphoamino acid analysis, a modification of the method of Cooper *et al.* (1983) was used. The region of the gel corresponding to the insulin receptor was excised, incubated with 10 ml of 20 % (v/v) methanol for 18 h at 37 °C and dried at 70 °C for 2 h in an oven. Then 2 ml of 50 mM-NH_4HCO_3 containing 100 μg of trypsin (treated with tosylphenylalanylchloromethane) was added. The mixture was incubated at 37 °C for 6 h, a further 100 μg of trypsin was added and incubation continued for a further 18 h. The samples were freeze-dried and hydrolysed in 6 M-HCl at 110 °C for 2 h. After addition of 1 ml of water and freeze-drying, the samples were dissolved in 10 μl of water containing phosphotyrosine, phosphoserine and phosphothreonine, each at 1 mg/ml. Phosphoamino acids were separated by electrophoresis on cellulose thin-layer plates at pH 3.5 for 1.5 h at 1 kV. Xylene cyanol was used as a tracking dye. The phosphoamino acid standards were identified by reaction with ninhydrin. Autoradiograms were obtained at −70 °C for 1–14 days as described above. ^{32}P associated with phosphoamino acids was quantified by densitometric scanning with a Joyce–Loebl Chromoscan 3 or by excision of the spots and liquid-scintillation counting of radioactivity. Both methods gave similar answers.

Insulin-binding assay

Insulin binding was carried out essentially as described by Taylor *et al.* (1982). Solubilized insulin receptor (20 μg) was incubated with pig ^{125}I-insulin (20000 c.p.m.) in 50 mM-Hepes buffer, pH 7.7, containing 0.1 % Triton X-100 and 0.1 % bovine serum albumin in the presence of different concentrations of unlabelled pig insulin (0–10 nM). The final assay volume was 0.2 ml. Non-specific insulin binding was determined in the presence of 5 μM unlabelled insulin. The receptor–^{125}I-insulin complex was separated from free ^{125}I-insulin by adding at 4 °C 0.3 ml of poly(ethylene glycol) (25 %, w/v) and 0.1 ml of human γ-globulin (3 mg/ml), vortex-mixing and centrifuging at 10000 ***g*** for 5 min. Pellets were washed at 4 °C with 0.3 ml of 12.5 % (w/v) poly(ethylene glycol) and counted for radioactivity.

RESULTS

Reconstitution system using Fao cells for studying kinases that phosphorylate the insulin receptor on serine

The basis of the system involves reconstituting insulin receptor with cell extracts. Insulin receptor was purified partially from a rat hepatoma-cell line (Fao) by direct solubilization of cells and affinity chromatography on wheat-germ-agglutinin–agarose. Phosphorylation of these preparations of insulin receptor in the presence or absence of insulin showed that > 98 % of ^{32}P incorporated was into tyrosine residues, in agreement with previous studies using this cell line and similar methods for isolating the insulin receptor (Haring *et al.*, 1984; Sale *et al.*, 1987). Thus these insulin-receptor preparations offer an excellent substrate that may be used to screen for the presence in cell extracts of serine kinases active towards the insulin receptor. Fao cells were also used to prepare cell extracts, as they are known to be rich in insulin-stimulated serine kinase activity towards the insulin receptor (Kasuga *et al.*, 1982; Haring *et al.*, 1984; Takayama *et al.*, 1984; White *et al.*, 1985). Cell extracts were rapidly prepared and reconstituted with insulin receptor as described below.

Confluent cultures of Fao cells in 75 cm^2 flasks were washed twice with serum-free RPMI 1640 and incubated in serum-free RPMI 1640 with no addition, or with insulin or 8-CPT-cAMP (100 μM) for 10 min. Cells were then washed twice at 0 °C with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline and extracted with 0.7 ml of ice-cold 0.1 % Triton X-100/50 mM-Hepes (pH 7.4)/

aprotinin (0.1 mg/ml)/phenylmethanesulphonyl fluoride (0.34 mg/ml). Extracts were immediately clarified (10000 *g*, 2 min) and used. Insulin receptor was preincubated in the presence or absence of insulin. Extracts (typically 6–12 μl) were combined with the preincubated insulin receptor (25 μl, 10 μg of total protein) and phosphorylated in the presence of 250 μM-[γ-^{32}P]ATP for 5 min at 30 °C. The insulin receptor was then immunoprecipitated and subjected to SDS/polyacrylamide-gel electrophoresis. ATPase activity limited the concentration of extract that could be added to the insulin receptor without inhibiting phosphorylation. Concentrations of extracts were chosen such that [γ-^{32}P]ATP concentration remained over 100 μM at the end of the phosphorylation. ATPase activity was assayed as described previously (Cooper *et al.*, 1974; Sale & Randle, 1982). No difference in ATPase activities in extracts from control, insulin- or 8-CPT-cAMP-treated cells was observed. Serine kinase assays were not linear with respect to time. Rates gradually declined, most likely owing to the fall in [γ-^{32}P]ATP concentration with time.

Phosphorylation of the insulin receptor in the presence of control cell extracts, 5 mM-$MgCl_2$, 250 μM-[γ-^{32}P]ATP and in the absence of insulin showed that ^{32}P was incorporated predominantly into tyrosine residues of the β-subunit (M_r 95000) (Fig. 1, lane *a*, and Table 1). In contrast, when extracts from insulin-treated cells were used, > 80% of ^{32}P was recovered in phosphoserine and total ^{32}P incorporation into the β-subunit was increased 5-fold (Fig. 1, lane *c*, and Table 1). Broadly similar incorporations of ^{32}P into phosphoserine were obtained in phosphorylations performed in the presence of insulin, although ^{32}P incorporations into phosphotyrosine were increased (7.93 ± 0.7-fold; mean ± S.E.M. for nine observations) owing to stimulation of insulin-receptor autophosphorylation. The mean effect of insulin treatment of cells on increasing serine kinase activity in extracts towards the insulin receptor was 7.18 ± 1.8-fold (mean ± S.E.M., six observations). Prior treatment of cells with 8-CPT-cAMP also increased the activity of a serine kinase toward the insulin receptor in extracts (6.7 ± 1.9-fold; mean ± S.E.M., six observations; Fig. 1, lanes *a*, *b*, *e*, *f* and Table 1). Including Mn^{2+} as well as Mg^{2+} in phosphorylation assays gave similar recoveries of phosphoserine to those obtained with Mg^{2+} alone, although tyrosine autophosphorylation increased approx. 3-fold. Mn^{2+} is known to be more effective than Mg^{2+} in supporting receptor tyrosine kinase activity (Pike *et al.*, 1984). On the basis of insulin binding (White *et al.*, 1984), the maximum stoicheiometry of serine

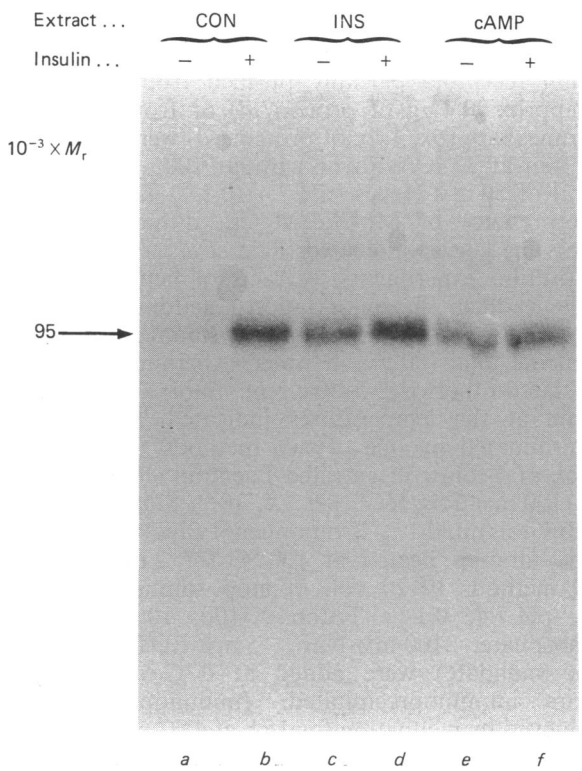

Fig. 1. Reconstitution of insulin receptor with cell extracts

Insulin receptor (Fao cells) was preincubated in the presence (+) or absence (−) of 100 nM-insulin for 10 min at 22 °C. Fao cells were incubated with no addition [control (CON)], insulin [100 nM, 5 min (INS)] or 8-CPT-cAMP [100 μM, 5 min (cAMP)]. Extracts of the cells were then prepared, combined with the preincubated insulin receptor and incubated in the presence of 250 μM-[γ-^{32}P]ATP/5 mM-$MgCl_2$ for 5 min at 30 °C. The insulin receptor was then immunoprecipitated and subjected to SDS/polyacrylamide-gel electrophoresis. An autoradiograph of the result is shown.

Table 1. Phosphoamino acid analysis of insulin receptor phosphorylated in the presence of cell extracts

Phosphorylation and SDS/polyacrylamide-gel electrophoresis were performed as in the legend to Fig. 1. The β-subunits of the insulin receptors were excised from gels and subjected to phosphoamino acid analysis. Relative ^{32}P incorporations into phosphotyrosine and phosphoserine were determined by densitometric scanning of autoradiographs and are given in arbitrary units (means ± S.E.M., $n = 3$). In the absence of added cell extract, > 95% of ^{32}P incorporated into the Fao-cell insulin-receptor preparations used was into phosphotyrosine, in both the presence and the absence of insulin.

Extract	Preincubation of insulin receptor with insulin	Relative amounts	
		Phosphotyrosine	Phosphoserine
Control	−	0.35 ± 0.03	0.20 ± 0.02
	+	3.42 ± 0.17	0.70 ± 0.03
Insulin	−	0.43 ± 0.05	2.22 ± 0.26
	+	2.43 ± 0.26	2.27 ± 0.24
Cyclic AMP	−	0.26 ± 0.01	2.16 ± 0.01
	+	2.07 ± 0.21	1.82 ± 0.18

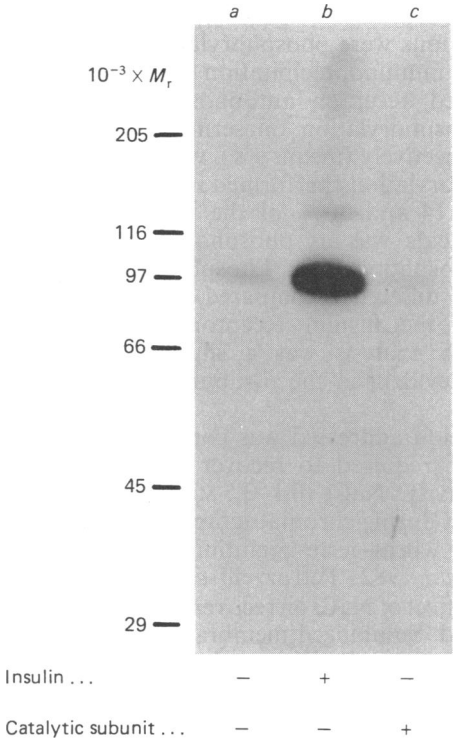

Fig. 2. Incubation of insulin receptor with the catalytic subunit of cyclic-AMP-dependent protein kinase

Insulin receptor was preincubated with (lane *b*) or without 100 nM-insulin (lanes *a* and *c*) for 10 min at 22 °C. Catalytic subunits (final concn. 1 μM) were then added in lane *c*. Samples were phosphorylated (50 μM-[γ-^{32}P]ATP, 10 mM-$MgCl_2$, 2 mM-$MnCl_2$, 20 min, 30 °C), immunoprecipitated, and subjected to SDS/polyacrylamide-gel electrophoresis followed by autoradiography.

phosphorylation was estimated to be of the order 0.1 mol of phosphate/mol of β-subunit. One difficulty encountered in performing reconstitutions was that autophosphorylation of the insulin receptor on tyrosine became progressively inhibited as increasing amounts of cell extract were added. For example, in a typical experiment (25 μl of insulin receptor, 10 min incubation at 30 °C in presence of 100 nM-insulin) tyrosine phosphorylation was decreased by 20 % and 66 % by 2 μl and 6 μl of extract respectively. The ATP concentration remained over 100 μM in each case at the end of the phosphorylation, indicating that this effect was not due to the increases in ATPase activity associated with using more extract. The reason for the inhibition is not known. At the extract concentrations used in the reconstitution experiments the degree of inhibition was 50–70 %. The ability of the catalytic subunit of cyclic-AMP-dependent protein kinase to mimic serine phosphorylation of the receptor observed with extracts from 8-CPT-cAMP-treated cells was studied. Phosphorylation of insulin receptors in the presence of physiological concentrations of catalytic subunit, followed by immunoprecipitation of insulin receptors, however, showed that under these conditions the insulin receptor was a poor substrate for this kinase (Fig. 2). We were unable to exclude the possibility that the small amount of labelling of the β-subunit evident after phosphorylation in the presence of the catalytic subunit was in part due to phosphorylation on serine residues. The incorporation of phosphate under these conditions was too low to perform phosphoamino acid analysis reliably. Control experiments with histone as substrate showed that the activity of the catalytic subunit was not appreciably decreased by 0.1 % Triton X-100 or the insulin-receptor preparation. Phosphorylations with catalytic subunit contained 16–17 units of catalytic-subunit activity (1 unit transfers 1 pmol of phosphate from [γ-^{32}P]ATP to histone per min at 30 °C) as assayed in the presence of the insulin-receptor preparation. Further controls showed that histone was phosphorylated by the catalytic subunit (even when mixed with the insulin receptor) at a histone concentration (20 nM) equivalent to the insulin-receptor concentration used in the insulin-receptor phosphorylations in Fig. 2 (results not shown).

Co-purification from human placenta of insulin receptor and insulin-sensitive serine kinase activity that phosphorylates the insulin receptor

Insulin receptor was partially purified from human placenta in a way designed to preserve a potentially fragile association between the insulin receptor and insulin-sensitive serine kinase. A crude microsomal membrane fraction was first rapidly prepared by using gentle homogenization techniques, with washing steps decreased to a minimum. The membranes were then solubilized in Triton X-100, and insulin receptor was purified by chromatography on wheat-germ-agglutinin–agarose. NaCl, which has often been included in the past during preparation of membranes and during lectin chromatography, was omitted.

These insulin-receptor preparations reproducibly retained kinase activity that phosphorylated the insulin receptor on serine residues in an insulin-dependent manner. In Fig. 3 insulin receptor was phosphorylated with [γ-^{32}P]ATP and then immunoprecipitated. As is

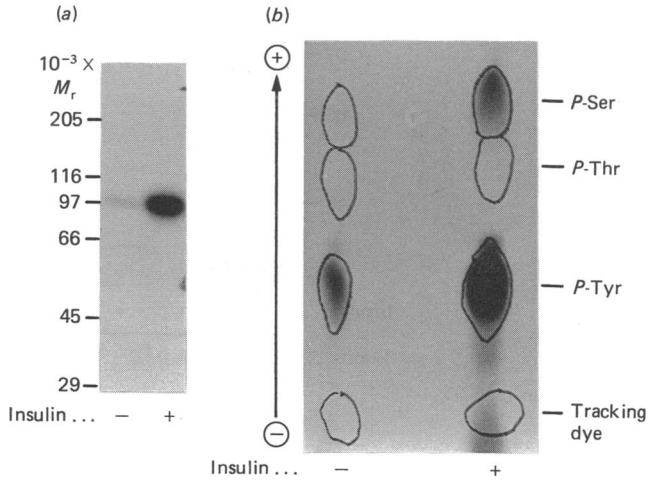

Fig. 3. Autoradiographs showing the phosphorylation of insulin receptor purified partially from human placenta

(*a*) Analysis by immunoprecipitation and SDS/polyacrylamide-gel electrophoresis. (*b*) Phosphoamino acid analysis of β-subunits excised from the gel. Insulin receptor was incubated with [γ-^{32}P]ATP, $MgCl_2$ (10 mM), $MnCl_2$ (2 mM), dithiothreitol (1 mM) and sodium vanadate (20 μM) for 10 min at 22 °C with or without preincubation with 100 nM-insulin.

evident from the autoradiogram of the phosphoamino acid analysis, insulin stimulated both receptor autophosphorylation on tyrosine (4-fold) and phosphorylation of insulin receptor on serine residues (> 10-fold). In the presence of insulin, 30% of the ^{32}P recovered in phosphoamino acids was present as phosphoserine in this experiment. Phosphorylation of the β-subunit on serine during insulin stimulation was rapid, and reached a steady state in 10–20 min. Identity of [^{32}P]-phosphoserine was confirmed by co-migration with authentic phosphoserine on two-dimensional electrophoresis at pH 3.5 and pH 1.9. The possibility that ^{32}P could be transferred as an artifact from tyrosine to serine residues during phosphoamino analysis was also considered, but is unlikely for the following reasons. (1) Insulin receptor from Fao cells, phosphorylated and subjected to phosphoamino acid analysis under identical conditions, gave only phosphotyrosine. (2) Phosphorylations of placental receptor at 0 °C or of receptor prepared in the presence of NaCl resulted in significantly decreased recoveries of phosphoserine relative to phosphotyrosine. (3) Phosphorylations of placental receptor in the absence of insulin and immunoprecipitation before phosphoamino acid analysis gave exclusively phosphotyrosine; no phosphoserine was obtained. (4) The phenomenon has never been previously observed in the literature, and there are many examples of phosphorylated proteins yielding only a single spot of phosphotyrosine or phosphoserine on phosphoamino acid analysis.

Similar results were obtained when placental insulin-receptor preparations were phosphorylated and directly analysed without immunoprecipitation (e.g., see Fig. 4). Insulin stimulated receptor autophosphorylation on tyrosine and phosphorylation on serine 3.8 ± 1.3- and 3.3 ± 0.6-fold respectively (means ± S.E.M., three observations). In phosphorylations performed in the presence of insulin, between 14 and 30% of the ^{32}P recovered in phosphoamino acids was as phosphoserine, with the remainder as phosphotyrosine. Phosphothreonine was absent. The only difference compared with the experiments in which the insulin receptor was immunoprecipated before analysis was a slight basal serine phosphorylation evident in the β-subunit region of the gel.

The next question addressed was concerned with the factors that were required to recover and assay high serine kinase activity. NaCl (0.1–0.5 M) has often been included by others during chromatography of solubilized membranes on wheat-germ-agglutinin–agarose (see, e.g., Kasuga *et al.*, 1982; Petruzzelli *et al.*, 1982; Pike *et al.*, 1984). The effect of NaCl on recovery of serine kinase activity was tested. Solubilized membranes were applied to wheat-germ-agglutinin–agarose columns, which were then washed with buffer containing various concentrations of NaCl. Insulin receptors were then eluted in the absence of NaCl and phosphorylated (Fig. 5). Inclusion of increasing concentrations of NaCl caused a striking decline in recovery of phosphoserine relative to either phosphotyrosine or total protein or per mg of protein.

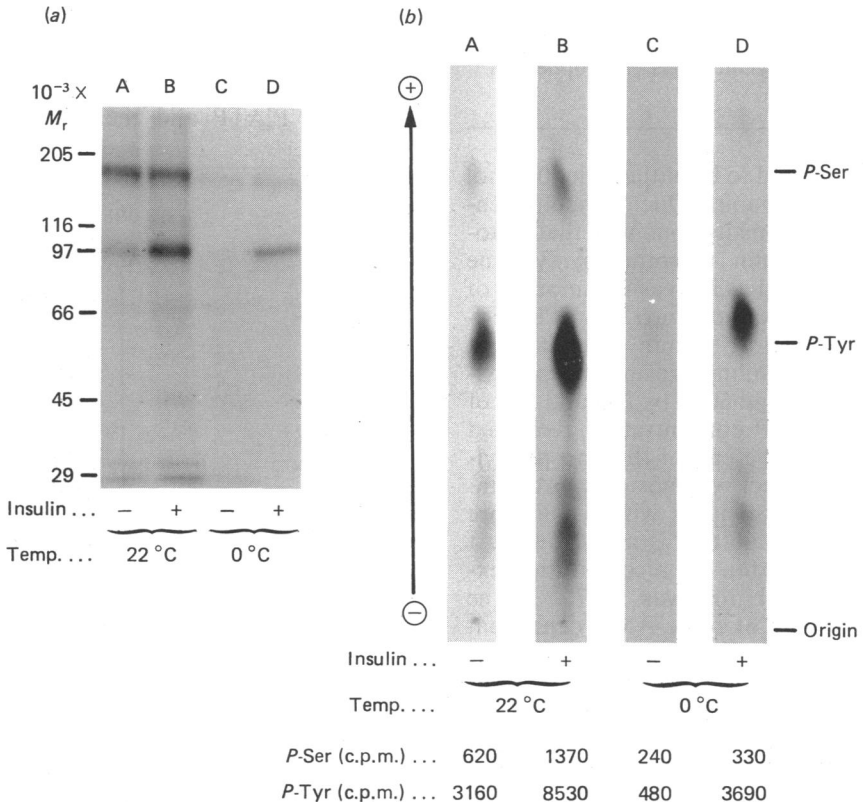

Fig. 4. Autoradiograms showing the effect of incubation temperature on phosphorylation of insulin receptor partially purified from human placenta

(*a*) SDS/polyacrylamide-gel electrophoresis. (*b*) Phosphoamino acid analysis. Insulin receptor was phosphorylated at 22 °C or 0 °C as in the legend to Fig. 3, but was directly analysed without immunoprecipitation.

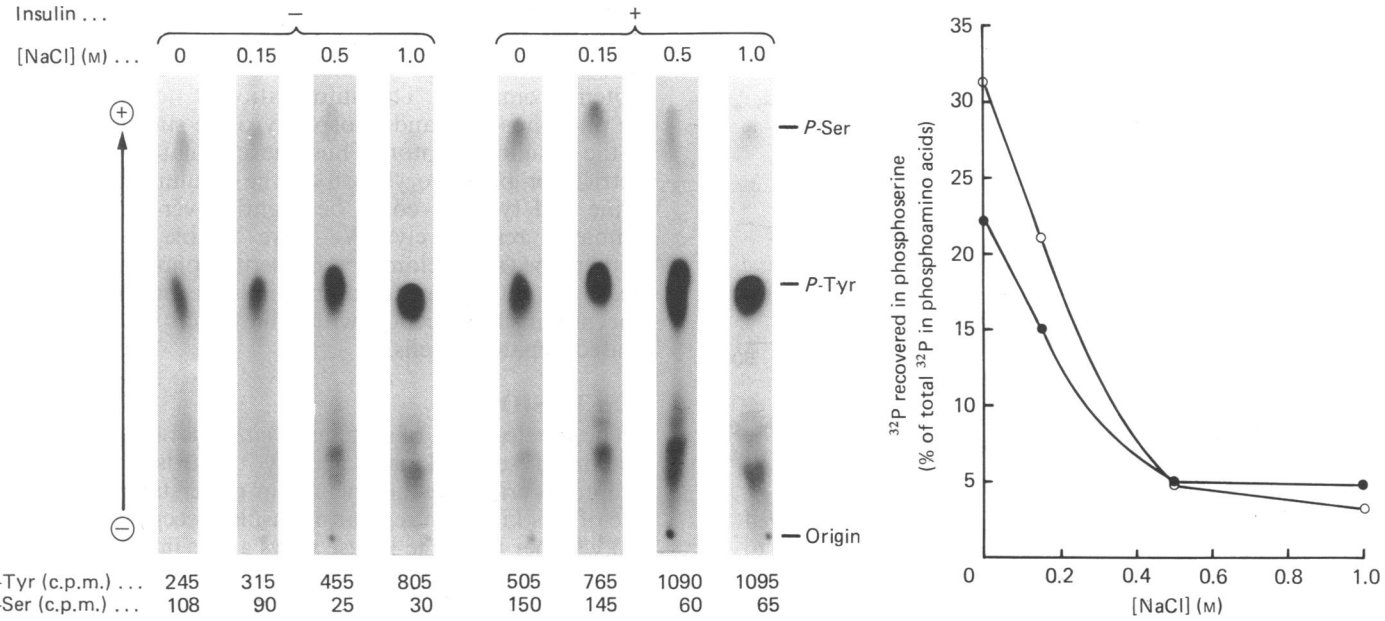

Fig. 5. Effect of washing wheat-germ-agglutinin–agarose columns with NaCl during chromatography of solubilized placental membranes on the recovery of insulin-sensitive receptor serine kinase activity

Solubilized membranes (6 ml) were loaded on to 1 ml lectin columns. Columns were washed with 40 ml of buffer containing 0, 0.15 M-, 0.5 M- or 1 M-NaCl and eluted with 0.3 M-*N*-acetylglucosamine in the absence of NaCl. Protein concentrations in eluted pooled samples (mg/ml) were 0.36, 0.51, 0.61 and 0.58, and yields of total protein (mg) were 0.93, 0.98, 1.16 and 1.35, for 0, 0.15 M-, 0.5 M- and 1 M-NaCl respectively. A 20 μl sample of each preparation was preincubated in the absence (○) or the presence (●) of 100 nM-insulin and then phosphorylated at room temperature for 10 min in the presence of 100 μM-[γ-^{32}P]ATP, 10 mM-$MgCl_2$, 2 mM-$MnCl_2$, 1 mM-dithiothreitol and 20 μM-sodium vanadate. After SDS/polyacrylamide-gel electrophoresis the β-subunit was subjected to phosphoamino acid analysis and autoradiography.

For example, in phosphorylations performed in the presence of insulin, the proportion of the ^{32}P recovered in phosphoamino acids as phosphoserine decreased from 23% (zero NaCl) to 5% (0.5 M-NaCl). Basal receptor phosphorylation on tyrosine increased somewhat as the concentration of NaCl used during chromatography increased. With regard to assay of serine kinase activity, the effect of temperature was examined (Fig. 4). Performing phosphorylations at 0 °C instead of 22 °C decreased both the total phosphorylation of the insulin receptor (3–4-fold) and the phosphoserine/phosphotyrosine ratio (approx. 2-fold). Phosphorylations at 30 °C gave phosphoserine/phosphotyrosine ratios similar to those for phosphorylations at 22 °C (results not shown). Mn^{2+} alone was found to be equally as effective as Mn^{2+} and Mg^{2+} together in supporting serine kinase activity (results not shown).

Stoicheiometry of serine phosphorylation

ATP (100 μM) was required to obtain maximal phosphorylation of the insulin receptor (Fig. 6). To estimate the stoicheiometry of phosphorylation of the insulin receptor, the concentration of receptors present was determined by insulin-binding assays and Scatchard analyses. Studies have shown that insulin receptor purified (Petruzzelli *et al.*, 1984) or partially purified (Pang & Shafer, 1984) from human placenta has a high affinity for only one molecule of insulin. In the present work, the molar concentration of insulin receptor was estimated from the concentration of high-affinity binding sites by the procedure recommended by Pang & Shafer (1984), with insulin concentrations < 20 nM. At these concentrations of insulin, binding to low-affinity sites is minimal and linear Scatchard plots were obtained (Fig. 7). Approx. 10 pmol of high-affinity sites was present per mg of protein. Assuming one high-affinity site for insulin per molecule of receptor, this corresponds to 10 pmol of insulin receptor/mg of protein. On this basis, total ^{32}P incorporation into the insulin receptor in the presence of insulin was calculated to be 4 ± 0.2 mol/mol of receptor. Between 14 and 30% (22 ± 4; mean ± S.E.M., four observations) of ^{32}P recovered in phosphoamino acids upon phosphoamino acid analysis was recovered in phosphoserine, with the remainder in phosphotyrosine. This gives stoicheiometries (mol/mol of insulin receptor) of 0.56–1.2 (0.88 ± 0.16; mean ± S.E.M., four observations) and 2.8–3.44 (3.12 ± 0.16; mean ± S.E.M., four observations) for ^{32}P incorporation into phosphoserine and phosphotyrosine respectively. This is equivalent to 0.44 ± 0.08 and 1.6 ± 0.08 mol of phosphoserine and phosphotyrosine respectively per mol of β-subunit. Values for phosphotyrosine are comparable with values of 2 mol/mol of β-subunit reported by White *et al.* (1984), who estimated receptor concentration in partially purified preparations from Fao cells by insulin binding, and of 2.2 mol/mol of β-subunit reported by Petruzzelli *et al.* (1984), who determined receptor molarity in pure preparations from human placenta by direct protein assay, assuming an M_r of 300000. The calculation in the present work assumes that the α- and β-subunits of the

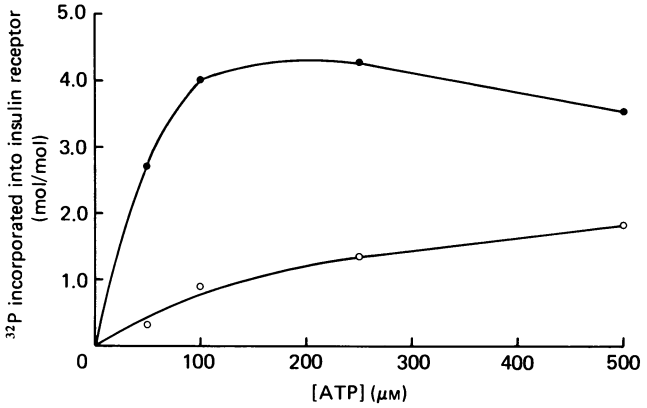

Fig. 6. Effect of ATP concentration on stoicheiometry of phosphorylation of insulin receptor partially purified from human placentae

Insulin receptor was phosphorylated with the indicated concentrations of [γ-^{32}P]ATP as described in the legend to Fig. 3 in the presence (●) or the absence (○) of insulin. Stoicheiometries were estimated from the ^{32}P incorporated into the β-subunits and by determining receptor molarity from insulin-binding assays as described in the text.

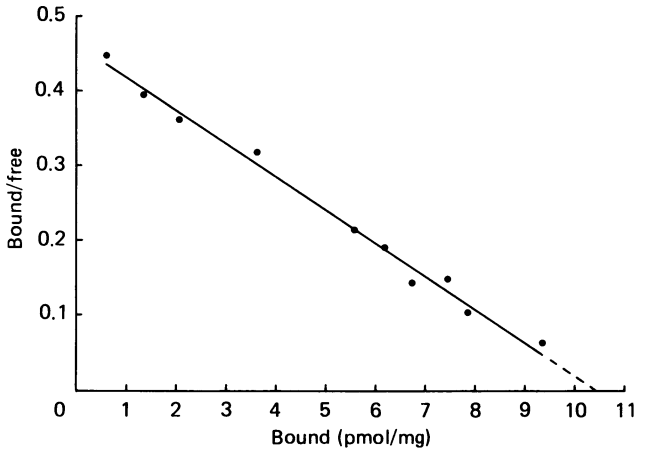

Fig. 7. Scatchard plot of insulin binding to insulin receptor partially purified from human placenta

insulin receptor are present in a 1:1 ratio and that no denaturation of the α- or β-subunits occurred during receptor isolation. The degree of endogenous receptor phosphorylation is not known. The calculation also assumes that relative recoveries of ^{32}P in phosphoserine and phosphotyrosine are similar. Phosphoamino acid analysis involves hydrolysis with 6 M-HCl at 110 °C for 2 h. Free phosphotyrosine has been reported to be somewhat more acid-labile than phosphoserine (Plimmer, 1941), but this may not be true for phosphotyrosine in a polypeptide. Both the rate of release of any particular amino acid residue and the stability of the phosphomonoester bond will be affected by its polypeptide environment. Hunter & Sefton (1980) have compared the recovery of phosphoserine and phosphotyrosine from pp60^{src} and a M_r-50000 protein, and found no major differences. In the present work, control experiments using various proteins phosphorylated on serine or tyrosine showed that, under our hydrolysis conditions, recovery of phosphotyrosine varied between 86% and 62% of that of phosphoserine, and depended on the proteins compared. This information is not known for the phosphoserine and phosphotyrosine sites of interest in the insulin receptor. Thus the calculated stoicheiometries for phosphorylation of the insulin receptor on serine and tyrosine could be slightly over- and underestimated respectively. We were unable to increase further the stoicheiometry of serine phosphorylation measured in the presence of insulin by reconstituting the placental insulin receptor with cell extracts from insulin-treated hepatoma cells.

DISCUSSION

The development of systems *in vitro* that show insulin-stimulated serine kinase activity towards the insulin receptor is critical to enhancing our understanding of the role of this kinase in regulating insulin-receptor function and whether the same kinase plays a role in transmitting the insulin signal to intracellular targets. In the present work, two systems *in vitro* are described which demonstrate insulin-sensitive serine kinase activity towards the insulin receptor.

The first system involves reconstituting cell extracts from Fao rat hepatoma cells with insulin receptor partially purified from Fao cells. Phosphorylation of these insulin-receptor preparations followed by phosphoamino acid analysis gave only phosphotyrosine (with or without insulin). Serine kinase activity towards the insulin receptor in cell extracts was increased approx. 7-fold by prior exposure of the cells to insulin. Thus, by judicious adjustment of the reconstitution conditions, problems of high ATPase activity and proteinase activity associated with use of extracts have been overcome. Similar approaches have been used to demonstrate in cell extracts S6 and acetyl-CoA carboxylase kinase(s) activated by prior exposure of cells to insulin (Brownsey *et al.*, 1984; Tabarini *et al.*, 1985; Cobb *et al.*, 1986), although these experiments are technically easier because higher concentrations of substrate can be used.

Reconstitutions between Fao-cell insulin receptor and extracts of Fao cells that had been treated with 8-CPT-cAMP showed the presence of an insulin-receptor serine kinase activated by this cyclic AMP analogue. Stadtmauer & Rosen (1986) have previously shown that 8-bromo-cAMP or forskolin stimulates phosphorylation of the insulin receptor on serine residues in intact IM9 cells. The results of the present work extend these observations to a different cell type and show that the kinase can be extracted from cells in an activated state, and that the activated kinase phosphorylates the insulin receptor *in vitro* on serine residues. As cyclic-AMP-dependent protein kinase is a candidate for being this kinase, the ability of the catalytic subunit of cyclic-AMP-dependent protein kinase to phosphorylate the insulin receptor under similar conditions was examined. The insulin receptor was a poor substrate for catalytic subunit under these conditions, in agreement with studies by Van Obberghen *et al.* (1983), Joost *et al.* (1986) and Tanti *et al.* (1987), and the fact that the insulin receptor does not contain a classic recognition sequence for cyclic-AMP-dependent protein kinase. These results support the notion (Czech, 1985; Stadtmauer & Rosen, 1986) that the effects of cyclic AMP are not directly mediated by cyclic-AMP-dependent protein kinase, and that activa-

tion of an intermediary kinase may underlie the phosphorylation and inhibition of receptor tyrosine kinase activity that occurs in response to cyclic AMP. The reconstitution system provides a means for the further study and identification of this kinase. In contrast, Roth & Beaudoin (1987) have reported phosphorylation of the insulin receptor by cyclic-AMP-dependent kinase. The reason for this discrepancy is unclear. However, the degree of inhibition of receptor tyrosine kinase produced in the Roth & Beaudoin (1987) experiments is significantly lower than that obtained by raising the cyclic AMP concentration in intact cells (Stadtmauer & Rosen, 1986), suggesting involvement of another kinase, in agreement with our results.

The second system for studying insulin-sensitive serine kinase activity that phosphorylates the insulin receptor involved co-purifying serine kinase activity with the insulin receptor. The rationale behind our preparation procedure was to isolate membranes from human placenta simply and rapidly, avoiding harsh homogenizations or extensive washes, which may disrupt a potentially delicate association between the insulin receptor and serine kinase. Insulin receptor was then purified by solubilization of the membranes at high concentrations in Triton X-100, followed by chromatography on wheat-germ-agglutinin–agarose. Serine kinase activity towards the insulin receptor was reproducibly recovered in these preparations, was stimulated many-fold by insulin and resulted in incorporation of approx. 0.8 mol of phosphate/mol of insulin receptor in the presence of insulin. This is the first description of a preparation of insulin receptor that is phosphorylated to high stoicheiometry on serine in an insulin-dependent manner. Phosphoserine/phosphotyrosine ratios obtained after phosphorylations of the insulin receptor in the presence of insulin were 0.28 ± 0.05 (mean $\pm$ S.E.M., four observations). Results of phosphoamino acid analysis obtained by others on phosphorylated preparations of insulin receptor can be divided into two categories. In the first category are those studies using partially purified insulin receptor (Kasuga *et al.*, 1982; Gazzano *et al.*, 1983; Haring *et al.*, 1984, 1986*a*; Sale *et al.*, 1987) and all studies using insulin receptor purified to apparent homogeneity (Kasuga *et al.*, 1983; Petruzzelli *et al.*, 1984; Bollag *et al.*, 1986) that have yielded exclusively phosphotyrosine. In the second category are those studies that have yielded small amounts of phosphoserine along with phosphotyrosine (Avruch *et al.*, 1982; Petruzzelli *et al.*, 1982; Shia & Pilch, 1983; Grigorescu *et al.*, 1983; Zick *et al.*, 1983; Blackshear *et al.*, 1984; Yu & Czech, 1984; Rees-Jones & Taylor, 1985; Haring *et al.*, 1985), although in approximately half of these studies the phosphoserine was not shown to be stimulated by insulin (it was either insensitive or not tested). Even in studies where insulin stimulation was evident, in every case except one (Zick *et al.*, 1983) either the phosphoserine spot present on autoradiograms has received no comment or no importance has been attached to the results. In the present work, we have obtained significantly higher phosphoserine/phosphotyrosine ratios than were measured in any previous studies, shown up to 10-fold stimulation of phosphoserine content by insulin, and for the first time estimated the stoicheiometry of the insulin-stimulated serine phosphorylation of the β-subunit. The stoicheiometries obtained (approx. 0.8 mol/mol of insulin receptor) are highly significant.

Differences in serine kinase activity towards the insulin receptor found in the various preparations of insulin receptor described in the literature and that found by us may be attributable to differences in recovery of kinase activity during receptor isolation, which may depend on the tissue and the protocol used, and/or to differences in phosphorylation assay conditions. In the present work specific factors affecting both recovery and assay of serine kinase activity have been identified. These are as follows.

(*a*) Assay conditions. In previous studies on insulin-receptor phosphorylation, phosphorylations were frequently performed at 0 °C (see, e.g., Avruch *et al.*, 1982; Blackshear *et al.*, 1984). In the present work, serine kinase activity towards the insulin receptor was shown to be markedly higher at 22 °C, relative to tyrosine kinase activity, than at 0 °C.

(*b*) Inclusion of NaCl during preparation of the insulin receptor. In a large number of previous procedures for isolating insulin receptor, NaCl (0.1–0.5 M) has been present either during preparation of membranes, including exhaustive washing steps, or during chromatography on wheat-germ-agglutinin–agarose (see, e.g., Kasuga *et al.*, 1982; Petruzzelli *et al.*, 1982; Avruch *et al.*, 1982; Blackshear *et al.*, 1984; Pike *et al.*, 1984). Washing with even higher concentrations of NaCl (0.5–1.0 M) has been used in purifying the insulin receptor to homogeneity on antibody columns (Bollag *et al.*, 1986) or on insulin–Sepharose (Kasuga *et al.*, 1983; Petruzzelli *et al.*, 1984). Phosphoserine has never been observed after phosphorylation of pure preparations of insulin receptor. In the present work, inclusion of NaCl (0.15–1.0 M) during chromatography of solubilized membranes on wheat-germ-agglutinin–agarose was shown to decrease significantly the amount of serine kinase activity recovered with the insulin receptor (Fig. 5). For example, washing columns with only 0.5 M-NaCl decreased by over 80% phosphoserine/phosphotyrosine ratios in the eluted insulin receptor after phosphorylation in the presence of insulin. Thus inclusion of NaCl during preparation of the insulin receptor is undoubtedly a factor that can decrease yields of serine kinase activity. The simplest explanation for this effect of NaCl is that it disrupts an association between the insulin receptor and serine kinase. If the serine was bound to wheat-germ-agglutinin–agarose columns because it is a glycoprotein, 0.5 M-NaCl would not be expected to disrupt that association.

(*c*) Preparation of membranes before solubilization as opposed to direct solubilization of cells. Insulin receptor isolated from Fao cells by direct solubilization in Triton X-100 gave exclusively phosphotyrosine. In contrast, insulin receptor prepared by isolating placental membranes before solubilization, which enables solubilization of insulin receptor at a much higher concentration, yielded significant amounts of phosphoserine after insulin treatment. Most studies reported in the literature that have used partially purified insulin receptors conform to this pattern. Thus, for example, studies that have yielded phosphoserine, albeit small amounts, have nearly always involved prior isolation of membranes, whereas, in studies in which phosphotyrosine has been the sole phosphoamino acid recovered, cells have been directly solubilized in Triton X-100. Exceptions to this are studies by Kasuga *et al.* (1982) and Haring *et al.* (1986*a*), in which membranes were isolated before solubilization

of insulin receptor, and phosphoserine was not found. In the procedure used by Kasuga *et al.* (1982), membranes were purified from rat liver on sucrose gradients and 0.15 M-NaCl was included during lectin chromatography. In the study by Haring *et al.* (1986*a*) with adipocytes, 100 mM-NaF, which may act like NaCl, was present through membrane solubilization and lectin chromatography. Thus these factors may have been responsible for low yields of serine kinase activity in those studies. It is possible, by using Fao cells, to co-purify insulin receptor and serine kinase activity by preparing membranes before solubilization and chromatography (results not shown), lending further support to the notion advanced in this section. Phosphoserine/phosphotyrosine ratios measured on Fao receptor isolated in this way were, however, several-fold lower than those for the placental receptor. This may be attributable to tissue or species differences, or to the fact that it was not possible to apply the placental method directly to Fao cells because of differences in scale and amount of protein. Thus membrane isolation and solubilization were of necessity carried out at markedly greater dilution with Fao cells than with placenta.

In studies by Gazzano *et al.* (1983), Machicao & Wieland (1985) and Ballotti *et al.* (1986), insulin-stimulated serine kinase activity has been co-purified with the insulin receptor that phosphorylates exogenous substrates such as histone and casein. This kinase did not, however, phosphorylate the insulin receptor itself. Thus its relationship to the insulin-stimulated receptor serine kinase activity described in the present work is unclear. Additionally, it is not known for certain whether the same insulin-stimulated receptor serine kinase is necessarily being studied in the two systems *in vitro* described in this paper.

Studies on insulin-receptor phosphorylation in intact cells have yielded wide variation in phosphoserine/phosphotyrosine ratios obtained after insulin stimulation. Ratios have varied from almost entirely phosphoserine (Gazzano *et al.*, 1983; Stadtmauer & Rosen, 1986) to predominantly phosphotyrosine, in other studies (Kohanski *et al.*, 1986; Ballotti *et al.*, 1987). Variation could be due to differences in cell type, incubation time with insulin or efficiency with which phosphoserine and tyrosine phosphatases have been inhibited. The degree of insulin stimulation of the serine kinase activity in intact cells has also shown considerable variation. In addition to the above reasons, this could also reflect differences in the extent of basal phosphorylation of the receptor, owing to other kinases acting on the insulin receptor. In our studies *in vitro* with the placental insulin-receptor system, basal phosphorylation was low and, importantly, serine kinase activity was stimulated up to 10-fold by insulin. Phosphoserine/phosphotyrosine ratios obtained in this system (0.28 ± 0.05; mean ± S.E.M., four observations) after insulin treatment are comparable with those reported in normal intact rat hepatocytes in which effective measures to prevent receptor dephosphorylation before analysis were used (Ballotti *et al.*, 1987). However, by using similar rapid cell-freezing techniques and phosphatase inhibitors, phosphoserine/phosphotyrosine ratios > 1 were obtained in a tumour-cell line derived from the same tissue (White *et al.*, 1985). Because the amount of insulin-sensitive serine kinase activity that phosphorylates the insulin receptor in intact human placenta is not known, the fraction of total cellular kinase activity that co-purifies with the receptor is not known.

The systems described for studying the insulin-sensitive serine kinase that phosphorylates the insulin receptor provide a basis for characterizing the nature of the kinase. In particular, it will be possible to address important questions about its mechanism of activation and its roles in insulin action and in regulating insulin-receptor function.

We thank Dr. C. R. Kahn for generously giving the anti-(insulin receptor) antibody. This work was supported by grants from the Medical Research Council, Wessex Medical School Trust and Nuffield Foundation. M. J. K. thanks the S.E.R.C. for a research studentship.

REFERENCES

Avruch, J., Nemenoff, R. A., Blackshear, P. J., Pierce, M. W. & Osathanondh, R. (1982) J. Biol. Chem. **257**, 15162–15166

Ballotti, R., Kowalski, A., Le Marchand-Brustel, Y. & Van Obberghen, E. (1986) Biochem. Biophys. Res. Commun. **139**, 179–185

Ballotti, R., Kowalski, A., White, M. F., Le Marchand-Brustel, Y. & Van Obberghen, E. (1987) Biochem. J. **241**, 99–104

Blackshear, P. J., Nemenoff, R. A. & Avruch, J. (1984) Endocrinology (Baltimore) **114**, 141–152

Bollag, G. E., Roth, R. A., Beaudoin, J., Mochly-Rosen, D. & Koshland, D. E. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 5822–5824

Brownsey, R. W., Edgell, N. J., Hopkirk, T. J. & Denton, R. M. (1984) Biochem. J. **218**, 733–743

Chou, C. K., Dull, T. J., Russell, D. S., Gherzi, R., Lebwohl, D., Ullrich, A. & Rosen, O. M. (1987) J. Biol. Chem. **262**, 1842–1847

Cobb, M. H., Burr, J. G., Linder, M. E., Gray, T. B. & Gregory, J. S. (1986) Biochem. Biophys. Res. Commun. **137**, 702–708

Cohen, P. (1985) Eur. J. Biochem. **151**, 439–448

Cooper, J. A., Sefton, B. M. & Hunter, T. (1983) Methods Enzymol. **99**, 387–402

Cooper, R. H., Randle, P. J. & Denton, R. M. (1974) Biochem. J. **143**, 625–641

Crettaz, M. & Kahn, C. R. (1983) Endocrinology (Baltimore) **113**, 1201–1209

Czech, M. P. (1985) Annu. Rev. Physiol. **47**, 357–381

Denton, R. M., Brownsey, R. W. & Belsham, G. J. (1981) Diabetologia **21**, 347–362

Deschatrette, J., Moore, E. E., Dubois, M., Cassio, D. & Weiss, M. C. (1979) Somatic Cell Genet. **5**, 697–718

Ebina, Y., Araki, E., Taira, M., Shimada, F., Mori, M., Craik, C. S., Siddle, K., Pierce, S. B., Roth, R. A. & Rutter, W. J. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 704–708

Fujita-Yamaguchi, Y., Choi, S., Sakamoto, Y. & Itakura, K. (1983) J. Biol. Chem. **258**, 5045–5049

Gazzano, H., Kowalski, A., Fehlman, M. & Van Obberghen, E. (1983) Biochem. J. **216**, 575–582

Grigorescu, F., White, M. F. & Kahn, C. R. (1983) J. Biol. Chem. **258**, 13708–13716

Haring, H.-U., Kasuga, M., White, M. F., Crettaz, M. & Kahn, C. R. (1984) Biochemistry **23**, 3298–3306

Haring, H.-U., White, M. F., Kahn, C. R., Ahmad, Z., DePaoli-Roach, A. A. & Roach, P. J. (1985) J. Cell. Biochem. **28**, 171–182

Haring, H., Kirsch, D., Obermaier, B., Ermel, B. & Machicao, F. (1986*a*) Biochem. J. **234**, 59–66

Haring, H., Kirsch, D., Obermaier, B., Ermel, B. & Machicao, F. (1986*b*) J. Biol. Chem. **261**, 3869–3875

Houslay, M. D. (1985) Mol. Aspects Cell. Regul. **4**, 279–333
Hunter, T. & Sefton, B. M. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 1311–1315
Jacobs, S., Sahyoun, N. E., Saltiel, A. R. & Cuatrecasas, P. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 6211–6213
Joost, H. G., Steinfelder, H. J. & Schmitz-Salue, C. (1986) Biochem. J. **233**, 677–681
Kasuga, M., Zick, Y., Blithe, D. L., Crettaz, M. & Kahn, C. R. (1982) Nature (London) **298**, 667–669
Kasuga, M., Fujita-Yamaguchi, Y., Blithe, D. & Kahn, C. R. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 2137–2141
Kasuga, M., White, M. F. & Kahn, C. R. (1985) Methods Enzymol. **109**, 609–621
Kohanski, R. A., Frost, S. C. & Lane, M. D. (1986) J. Biol. Chem. **261**, 12272–12282
Laemmli, U. K. (1970) Nature (London) **227**, 680–685
Machicao, F. & Wieland, O. H. (1985) Curr. Top. Cell. Regul. **27**, 95–105
Morgan, D. O. & Roth, R. A. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 41–45
Pang, D. T. & Shafer, J. A. (1984) J. Biol. Chem. **259**, 8389–8596
Petruzzelli, L. M., Ganguly, S., Smith, C. J., Cobb, M. H., Rubin, C. S. & Rosen, O. M. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 6792–6796
Petruzzelli, L., Herrera, R. & Rosen, O. M. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 3327–3331
Pike, L. J., Kuenzel, E. A., Casnellie, J. E. & Krebs, E. G. (1984) J. Biol. Chem. **259**, 9913–9921
Plimmer, R. H. A. (1941) Biochem. J. **35**, 461–469
Rees-Jones, R. W. & Taylor, S. I. (1985) J. Biol. Chem. **260**, 4461–4467
Roth, R. A. & Beaudoin, J. (1987) Diabetes **36**, 123–126
Sale, E. M. & Denton, R. M. (1985) Biochem. J. **232**, 897–904
Sale, E. M., White, M. F. & Kahn, C. R. (1987) J. Cell. Biochem. **33**, 15–26
Sale, G. J. & Randle, P. J. (1982) Biochem. J. **206**, 221–229
Sale, G. J., Fujita-Yamaguchi, Y. & Kahn, C. R. (1986) Eur. J. Biochem. **155**, 345–351
Saltiel, A. R., Fox, J. A., Sherline, P. & Cuatrecasas, P. (1986) Science **233**, 967–972
Shia, M. A. & Pilch, P. F. (1983) Biochemistry **22**, 717–721
Stadtmauer, L. A. & Rosen, O. M. (1983) J. Biol. Chem. **258**, 6682–6685
Stadtmauer, L. & Rosen, O. M. (1986) J. Biol. Chem. **261**, 3402–3407
Tabarini, D., Heinrich, J. & Rosen, O. M. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 4369–4373
Takayama, S., Morris, M. F., Lauris, V. & Kahn, C. R. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 7797–7801
Tanti, J.-F., Grémeaux, T., Rochet, N., Van Obberghen, E. & Le Marchand-Brustel, Y. (1987) Biochem. J. **245**, 19–26
Taylor, S., Hedo, J. H., Underhill, L. H., Kasuga, M., Eldus, M. J. & Roth, J. (1982) J. Clin. Endocrinol. Metab. **55**, 1108–1113
Van Obberghen, E., Rossi, B., Kowalski A., Gazzano, H. & Ponzio, G. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 945–949
White, M. F., Haring, H.-U., Kasuga, M. & Kahn, C. R. (1984) J. Biol. Chem. **259**, 255–264
White, M. F., Takayama, S. & Kahn, C. R. (1985) J. Biol. Chem. **260**, 9470–9478
Yu, K.-T. & Czech, M. P. (1984) J. Biol. Chem. **259**, 5277–5286
Zick, Y., Grunberger, G., Podskanly, J. M., Moncada, V., Taylor, S. I., Gordon, P. & Roth, J. (1983) Biochem. Biophys. Res. Commun. **116**, 1129–1135

Received 10 June 1987/18 September 1987; accepted 2 November 1987