Evidence that a novel serine kinase catalyses phosphorylation of the insulin receptor in an insulin-dependent and tyrosine kinase-dependent manner

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Insulin receptor was co-purified from human placenta together with insulin-stimulated kinase activity that phosphorylates the insulin receptor on serine residues. By using this 'in vitro' system, the mechanism of activation of the serine kinase by insulin was explored. Peptide 1150, histone, poly(Glu-Tyr), eliminating Mn^{2+} (Mg^{2+} only), treatment at 37 °C (1 h), *N*-ethylmaleimide, phosphate, β -glycerol phosphate and anti-phosphotyrosine antibody all inhibited insulin-receptor tyrosine kinase activity and the ability of insulin to stimulate phosphorylation of the insulin receptor on serine. Additionally, direct stimulation of the receptor tyrosine kinase by vanadate increased serine phosphorylation of the insulin receptor. Insulin-stimulated tyrosine phosphorylation preceded insulin-stimulated serine phosphorylation of the insulin receptor. The activity of the insulin-sensitive receptor serine kinase was not augmented by cyclic AMP, cyclic GMP, Ca^{2+} , Ca^{2+} + calmodulin, Ca^{2+} + phosphatidylserine + diolein or spermine, or inhibited appreciably by heparin. Additionally, the serine kinase phosphorylated casein or phosvitin poorly and was active with Mn^{2+} . This indicates that it is distinct from Ca^{2+} , Ca^{2+} /phospholipid, Ca^{2+} /calmodulin, cyclic AMP- and cyclic GMP-dependent protein kinases, casein kinases I and II and insulin-activated ribosomal S6 kinase. Taken together, these data indicate that a novel species of serine kinase catalyses the insulin-dependent phosphorylation of the insulin receptor and that activation of this receptor serine kinase by insulin requires an active insulin-receptor tyrosine kinase.

INTRODUCTION

The insulin receptor is an insulin-activated tyrosine-specific protein kinase which catalyses the autophosphorylation of tyrosine residues in its own β -subunit and subsequently the phosphorylation of other proteins (Kasuga *et al.*, 1982; Rosen *et al.*, 1983; Ullrich *et al.*, 1985; for review see Sale, 1988). Interference in receptor kinase function by site-directed mutagenesis or micro-injection of monoclonal antibodies prevents many of the biological responses of insulin (Morgan & Roth, 1987; Ebina *et al.*, 1987; Chou *et al.*, 1987). The potential role of the insulin-receptor kinase in mediating in transmission of the insulin signal raises the possibility that the tyrosine kinase initiates a cascade of protein-phosphorylation reactions. For example insulin is known to stimulate the serine (occasionally threonine) phosphorylation of a whole range of cellular substrates, including acetyl-CoA carboxylase, ATP citrate lyase, ribosomal protein S6, cyclic AMP phosphodiesterase and several unidentified proteins (Denton *et al.*, 1981; Marchmont & Houslay, 1981; Avruch *et al.*, 1985). These effects of insulin involve activation of the serine kinases (Brownsey *et al.*, 1984), although the actual kinases are poorly characterized. Interestingly, in intact cells phosphorylation on serine residues of the β -subunit of the insulin receptor itself is increased by insulin (Gazzano *et al.*, 1983; Takayama *et al.*, 1984). The serine kinase activity responsible appears to be distinct from the insulin receptor and has the potential to phosphorylate the other targets. The problem with attempting to study this serine kinase has been the lack of a system *in vitro* in which the serine

kinase activity can be demonstrated. Most preparations of purified or partially purified insulin receptor contain zero or little of the serine kinase activity. Recently we have refined the protocol for isolation of the insulin receptor and defined conditions that enable co-purification of the insulin receptor from human placenta with the insulin-stimulated receptor serine kinase activity (Smith *et al.*, 1988). With this preparation, insulin stimulates phosphorylation of the insulin receptor to high stoichiometry on serine residues (~ 0.9 mol/mol).

In the present work we have investigated the identity of the insulin-stimulated receptor serine kinase and explored its mechanism of activation in response to insulin. Evidence has been obtained that an active tyrosine kinase is necessary to observe insulin-stimulated serine phosphorylation of the insulin receptor. The properties of the serine kinase distinguish it from many of the well-known serine kinases, suggesting that it is a novel species.

EXPERIMENTAL

Anti-phosphotyrosine antibody was generously given by Dr. Morris F. White (Joslin Diabetes Center, Boston, MA, U.S.A.). Peptide 1150 (*N*-Thr-Arg-Asp-Ile-Tyr-Glu-Thr-Asp-Tyr-Tyr-Arg-Lys-C), corresponding to amino acids 1142–1153 in the precursor of the human insulin receptor, was synthesized manually by a solid-phase method by Martin J. King and Dr. Ram P. Sharma of this Department. Other chemicals and biochemicals were obtained from Sigma Chemical Co.,

Poole, Dorset, U.K., except where indicated otherwise in Smith *et al.* (1988). Insulin receptor was co-purified from human placenta with insulin-stimulated receptor serine kinase activity, phosphorylated, analysed on 4%-acrylamide stacking/7.5%-acrylamide resolving gels and subjected to phosphoamino acid analysis as described previously (Smith *et al.*, 1988). Before use, lipids were dispersed by sonication (3 × 30 s burst) in 50 mM-Hepes (pH 7.4) by using an MSE Soni-Prep 150 sonicator. In experiments using histone (Sigma type VII-S) as an exogenous substrate, 15%-acrylamide resolving gels were employed.

RESULTS

Comparison of time courses of insulin-receptor autophosphorylation on tyrosine and phosphorylation on serine

Insulin receptor was co-purified together with insulin-stimulated insulin-receptor serine kinase activity by solubilization of placental membranes in Triton X-100, followed by affinity chromatography on wheat-germ-agglutinin-agarose (Smith *et al.*, 1988). Maximal phosphorylation (in the presence of insulin) of the preparations used in the present study followed by phosphoamino acid analysis gave phosphoserine/phosphotyrosine ratios of 0.2 ± 0.01 (mean $\pm$ S.E.M., 17 observations). The time courses in the presence of insulin of phosphorylation of the insulin receptor by the tyrosine kinase intrinsic to the β subunit and the insulin-sensitive serine kinase are compared in Fig. 1. Autophosphorylation of the insulin receptor on tyrosine was rapid and 50% complete in less than 1 min, whereas phosphorylation on serine was slower and took 5 min to reach 50% completion. Additionally, there was a lag in the phosphorylation of the serine residues, but not of the tyrosine residues. For example, after 15 s incubation tyrosine phosphorylation was 21% of maximal, whereas phosphoserine was barely detectable ($< 1\%$ of ^{32}P recovered in phosphoamino acids). The phosphoserine/phosphotyrosine ratio increased from almost zero at 15 s to 0.25 after 20 min. These results show that initial stimulation by insulin of insulin-receptor tyrosine kinase precedes serine phosphorylation of the insulin receptor. This temporal relationship is consistent with the notion advanced below that insulin-stimulated serine phosphorylation of the insulin receptor depends on tyrosine kinase activity.

The possibility was considered that depletion of ATP by endogenous ATPases could be responsible for activation of the serine kinase if the serine kinase was inhibited by high concentrations of ATP and had a relatively low K_m for ATP. This was excluded by demonstrating activation of the serine kinase at starting ATP concentrations ranging from 50 to 500 μM (results not shown). Additionally, with 100 μM - $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, ATPase assays (Smith *et al.*, 1988) showed that the concentration of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ remained over 50 μM after 10 min.

Effects of inhibitors and activators of insulin-receptor tyrosine kinase on insulin stimulated serine phosphorylation of the insulin receptor

To test whether triggering of serine phosphorylation of the insulin receptor by insulin required the tyrosine kinase activity of the insulin receptor, the effects of a wide range of inhibitors and activators of the tyrosine

Fig. 1. Time course of insulin-stimulated tyrosine and serine phosphorylation of the placental insulin receptor

Insulin receptor was incubated for 15 min at 22 °C in the presence of 10 mM- Mg^{2+} and 2 mM- Mn^{2+} with or without 100 nM-insulin. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (100 μM) was then added, and incubations continued at 22 °C for the times indicated. After SDS/polyacrylamide-electrophoresis the β subunit was excised from the gel, counted for radioactivity and subjected to phosphoamino acid analysis/auto-radiography (a). Phosphorylation (% of maximum) of serine (●) and tyrosine (○) residues, for incubations performed in the presence of insulin, was calculated from the ^{32}P incorporated into the β subunit and the phosphoserine/phosphotyrosine ratio (b). The inset shows the early time points in detail.

kinase were studied. The agents chosen featured a range of different mechanisms of action.

Competing substrates. Exogenous substrates of the insulin-receptor tyrosine kinase are known to inhibit receptor autophosphorylation on tyrosine (Sale *et al.*, 1986). Thus, exogenous substrates decrease the use of the receptor as substrate. Similarly, if serine phosphorylation of the insulin receptor depends on insulin-receptor tyrosine kinase activity, exogenous substrates may be expected to inhibit serine phosphorylation of the insulin receptor. In the present work, poly(Glu-Tyr) (Glu/Tyr 4:1), histone (Sigma, type VII-S) and the short synthetic peptide, peptide 1150, were employed. Peptide 1150 contains amino acid residues 1142–1153 of the insulin-receptor precursor and three of the tyrosine residues

Table 1. Effect of various agents on insulin-receptor autophosphorylation on tyrosine and phosphorylation on serine

Insulin receptor was incubated for 15 min at 22 °C in the presence of 100 nM-insulin, 10 mM-MgCl₂, 2 mM-MnCl₂ (except for 'Mg²⁺ only') and additions where indicated. [γ -³²P]ATP (100 μ M) was then added and incubations were continued for a further 10 min at 22 °C. In the heat-inactivation experiments, insulin receptor was pre-incubated at 37 °C for 1 h before incubation. Samples were subjected to SDS/polyacrylamide-gel electrophoresis and phosphoamino acid analysis. Phosphorylation of serine and tyrosine residues was calculated from the ³²P incorporated into the β -subunit and the phosphoserine/phosphotyrosine ratio. Data representative of several experiments are shown. In the controls (means $\pm$ S.E.M. for 14 observations), the phosphoserine/phosphotyrosine ratio after phosphorylation in the presence of insulin was 0.2 ± 0.01 , and phosphorylation on serine and tyrosine was stimulated 6.0 ± 0.51 and 4.4 ± 0.53 fold, respectively, by insulin.

Condition	Phosphorylation (% of control)	
	Phosphoserine	Phosphotyrosine
Control	100	100
Peptide 1150 (0.1 mM)	58.8	85.7
Peptide 1150 (0.5 mM)	28.7	69.6
Peptide 1150 (2 mM)	9.5	36.7
Peptide 1150 (3 mM)	0.0	3.7
Peptide 1150 (5 mM)	0.0	0.0
Poly(Glu-Tyr) (1 mg/ml)	28.1	33.0
Histone (1 mg/ml)	12.0	48.8
Preincubation at 37 °C (1 h)	19.8	23.8
Phosphate (7.5 mM)	24.1	30.3
β -Glycerol phosphate (40 mM)	42.6	56.2
Mg ²⁺ only (12 mM)	35.8	23.3
Anti-phosphotyrosine antibody (0.12 μ g/ μ l)	22.8	54.3
Anti-phosphotyrosine antibody (0.24 μ g/ μ l)	22.1	57.3
N-Ethylmaleimide (10 mM)	29.4	58.0
Iodoacetamide (10 mM)	94.9	110.7
Sodium vanadate (27 μ M)	141.0	151.0
Dithiothreitol (1 mM)	287.0	145.0
Sodium vanadate (27 μ M) + dithiothreitol (1 mM)	391.0	278.0
GTP (100 μ M)	118.0	106.7
Cyclic GMP (6 μ M)	114.9	91.5
Cyclic AMP (5 μ M)	111.0	91.5
Heparin (2.6 μ g/ml)	108.7	94.6
Heparin (100 μ g/ml)	79.7	89.3
Spermine (7 mM)	90.8	107.1
Ca ²⁺ (1 mM)	91.1	98.1
Ca ²⁺ (1 mM) + calmodulin (2 μ M)	86.7	103.5
Ca ²⁺ (1 mM) + phosphatidylserine (80 μ g/ml) + diolein (8 μ g/ml)	77.3	92.0

autophosphorylated in the insulin receptor (tyrosine-1146, -1150 and -1151). Insulin receptor was incubated with [γ -³²P]ATP, Mg²⁺, Mn²⁺ and insulin in the presence or the absence of the exogenous substrates. Each of the

three exogenous substrates reproducibly inhibited both autophosphorylation of the insulin receptor on tyrosine and phosphorylation on serine (Table 1). Peptide 1150 was the most effective inhibitor. At 2 mM, peptide 1150 inhibited autophosphorylation on tyrosine by approx. 65% and phosphorylation on serine by approx. 90% (Fig. 2). The extent of inhibition was dependent on the concentration of the peptide (Table 1); at 5 mM, phosphorylation of both tyrosine and serine was completely inhibited (Fig. 2). These results provide strong evidence that insulin-stimulated serine phosphorylation of the insulin receptor requires tyrosine kinase activity. As expected, peptide 1150 also inhibited insulin-stimulated phosphorylation on tyrosine of minor proteolytic degradation products of the insulin receptor (Fig. 2). Additionally, peptide 1150 inhibited autophosphorylation of the EGF receptor on tyrosine. This is not unexpected, given the similar substrate specificities of the insulin- and epidermal-growth-factor-receptor tyrosine kinases.

A lower concentration of peptide 1150 was required to inhibit insulin-receptor phosphorylation on serine than autophosphorylation on tyrosine. This could be because the peptide specifically inhibits the phosphorylation of one (or a subset) of tyrosine residues that are particularly important in affecting activation. Alternatively, it could be because measurements of phosphorylation after incubation for 10 min do not represent initial rates. Given the time course in Fig. 1, a considerable decrease in tyrosine kinase activity might have relatively little effect on tyrosine autophosphorylation after 10 min but a large effect on serine phosphorylation.

Phosphate and β -glycerol phosphate. Many serine-specific protein kinases are known to be fully active in phosphate-containing buffers, and indeed are often assayed in phosphate buffers. Common examples include cyclic AMP-dependent protein kinase (Corbin & Reimann, 1974) and pyruvate dehydrogenase kinase (Sale & Randle, 1982). The phosphate derivative, β -glycerol phosphate, stabilizes during purification and assay the activities of insulin-stimulated serine-specific protein kinases that phosphorylate ribosomal protein S6 (Tabarini *et al.*, 1985). In the present work phosphate (7.5 mM) and β -glycerol phosphate (40 mM) were found to inhibit reproducibly insulin-receptor tyrosine kinase activity (by 70 and 44% respectively; Table 1) and the ability of insulin to stimulate phosphorylation of the receptor on serine (by 76 and 57% respectively). These data support a role of the tyrosine kinase activity of the insulin receptor in mediating in insulin-stimulated serine phosphorylation of the insulin receptor.

Anti-phosphotyrosine antibody and histone as an exogenous substrate. Anti-phosphotyrosine antibody (0.12 μ g/ μ l) inhibited insulin-stimulated tyrosine kinase activity towards the insulin receptor by approx. 45% and towards histone (Sigma, type VII-S) by 50–60% (Tables 1 and 2). The antibody is known to inhibit tyrosine kinase activity by binding to sites in the tyrosine-1150 domain of the β -subunit as they become phosphorylated. This inhibits subsequent autophosphorylation of sites responsible for stimulating phosphotransferase activity towards histone (Sigma, type VII-S) by 50–60% (Tables 1 and 2). The activation of the serine kinase in response to insulin requires the tyrosine kinase activity of the insulin receptor, anti-phosphotyrosine antibody may be expected

Fig. 2. Effect of peptide 1150 and anti-phosphotyrosine antibody on insulin-receptor autophosphorylation on tyrosine and phosphorylation on serine

Insulin receptor was incubated for 15 min at 22 °C in the presence of 10 mM-Mg²⁺ and 2 mM-Mn²⁺ with or without 100 nM-insulin, peptide 1150 and anti-phosphotyrosine antibody, as indicated. [γ -³²P]ATP (100 μM) was then added, and incubations were continued for a further 10 min at 22 °C. Samples were subjected to SDS/polyacrylamide-gel electrophoresis and autoradiography (a). The bands of M_r 95000 and 170000 represent the β -subunit of the insulin receptor and epidermal growth factor (EGF) receptor respectively. The β subunits were excised, counted for radioactivity and subjected to phosphoamino acid analysis/autoradiography (b). Phosphorylation (%) was calculated from the ratio of ³²P recovered in phosphoserine/phosphotyrosine and from the ³²P incorporated into the β subunit.

to inhibit serine phosphorylation of the insulin receptor. This expectation was realized in the experiment shown in Fig. 2. In the presence of insulin, anti-phosphotyrosine antibody (0.12 $\mu\text{g}/\mu\text{l}$) inhibited serine phosphorylation by approx. 75%. In addition to the antibody working by inhibiting the tyrosine kinase activity of the insulin receptor, the antibody might also work by directly inactivating the serine kinase through binding to putative tyrosine phosphorylation sites in the serine kinase.

Histone was also found to be phosphorylated on serine by the placental insulin receptor in an insulin-stimulated manner (Table 2). The magnitude of insulin-stimulated serine phosphorylation was modest (1.5-fold) compared with insulin-stimulated tyrosine phosphorylation of histone (3.7-fold). Anti-phosphotyrosine antibody (0.12 $\mu\text{g}/\mu\text{l}$) inhibited the ability of insulin to stimulate phosphorylation of histone on serine (Table 2). Assuming that the same insulin-stimulated serine kinase is phos-

phorylating histone and the insulin receptor, this indicates that the anti-phosphotyrosine antibody was not inhibiting serine phosphorylation of the insulin receptor merely by masking the site(s) of serine phosphorylation in the insulin receptor.

Heat inactivation and Mg²⁺. The tyrosine kinase of the insulin receptor is exquisitely sensitive to heat inactivation. Incubation of the insulin receptor at 37 °C for 1 h inhibited autophosphorylation of the insulin receptor on tyrosine in the presence of insulin by 76% (Table 1). Serine phosphorylation of the insulin receptor was inhibited in parallel (80% inhibition). Similarly, performing phosphorylations in the presence of Mg²⁺ (12 mM) only, as opposed to Mn²⁺ (2 mM) + Mg²⁺ (10 mM), resulted in a parallel decrease in tyrosine autophosphorylation and serine phosphorylation of the insulin receptor by 77% and 64%, respectively, in the

Table 2. Phosphorylation of histone by placental insulin receptor: effect of anti-phosphotyrosine antibody

Insulin receptor was incubated for 15 min at 22 °C with histone (0.5 mg/ml), 10 mM-Mg²⁺, 2mM-Mn²⁺, with or without 100 nM-insulin, and with or without anti-phosphotyrosine antibody (0.12 µg/µl). [γ -³²P]ATP (100 µM) was then added, and incubations were continued for a further 10 min at 22 °C. After SDS/polyacrylamide-gel electrophoresis the histone was subjected to phosphoamino acid analysis. Phosphorylation of serine and tyrosine residues was calculated from the ³²P recovered in histone and the phosphoserine/phosphotyrosine ratio. Values are means of three to seven observations; S.E.M. values are given for the ratio.

	Phosphorylation (c.p.m.)				Ratio of ³² P	
	Phosphotyrosine		Phosphoserine		Phosphotyrosine	Phosphoserine
	No insulin	+ Insulin	No insulin	+ Insulin		
Control	3778	14163	768	1114	3.74 ± 0.47	1.45 ± 0.10
Anti-phosphotyrosine	1576	7532	721	757	4.78 ± 0.76	1.05 ± 0.18

presence of insulin (Table 1). Unlike the majority of serine kinases (which are fully active in the presence of Mg²⁺), the activity of the insulin-receptor tyrosine kinase is known to be supported more effectively by Mn²⁺ than by Mg²⁺ (Pike *et al.*, 1984). Thus these two further methods of inhibiting receptor tyrosine kinase activity strongly add to the case that stimulation by insulin of serine phosphorylation of the insulin receptor requires an active insulin-receptor tyrosine kinase.

Dithiothreitol and thiol-modifying reagents. Phosphorylation of freshly prepared insulin receptor on either tyrosine or serine was not usually stimulated by 1 mM-dithiothreitol. However, with certain preparations of insulin receptor, e.g. those stored at -70 °C for several months, dithiothreitol stimulated both tyrosine kinase and serine kinase activities (Table 1). With preparations of receptor aged for 3 months at -70 °C, 1 mM-dithiothreitol increased receptor autophosphorylation on tyrosine and phosphorylation on serine by approx. 1.5- and 2.9-fold respectively in the presence of insulin (mean values for two preparations). In the absence of insulin, effects of dithiothreitol were more pronounced; autophosphorylation on tyrosine was stimulated 2-fold and phosphorylation on serine 4-fold (results not shown). With freshly prepared insulin receptor the thiol-modifying reagent *N*-ethylmaleimide inhibited both tyrosine kinase (42 %) and serine kinase (70 %) activities towards the insulin receptor. Iodoacetamide was without effect (Table 1). These results, although not conclusive by themselves, are consistent with a requirement for tyrosine kinase activity for insulin-stimulated serine phosphorylation of the receptor.

Sodium vanadate. Sodium vanadate (27 µM) was observed to increase autophosphorylation of the insulin receptor on tyrosine in the presence of insulin by approx. 50 % (Table 1). This effect was due in a small part to inhibition of contaminating insulin-receptor phosphotyrosyl-protein phosphatase activity. Assays of phosphatase activity (King & Sale, 1988) showed that the phosphatase present was capable of dephosphorylating approx. 2 % of fully autophosphorylated insulin receptor during a 10 min incubation at 22 °C. This suggests that the effect of vanadate on increasing receptor autophosphorylation was largely mediated by a different

mechanism. Tamura *et al.* (1984) have proposed that vanadate can directly activate highly purified insulin receptor which is devoid of phosphatase. Our data support this proposal. Interestingly, in the presence of insulin vanadate reproducibly stimulated an approx. 41 % increase in serine phosphorylation of the insulin receptor. In the absence of insulin, the effects of vanadate were even greater; autophosphorylation on tyrosine was stimulated 2.2-fold and phosphorylation on serine 2.7-fold (results not shown). This provides strong evidence that the tyrosine kinase of the insulin receptor mediates in the insulin-stimulated serine phosphorylation of the receptor.

Studies into the identity of the insulin-stimulated serine kinase that phosphorylates the insulin receptor

In addition to insulin, cyclic AMP analogues and phorbol esters promote phosphorylation of the insulin receptor on serine residues in intact cells (Jacobs *et al.*, 1983; Takayama *et al.*, 1984; Stadtmauer & Rosen, 1986; Smith *et al.*, 1988). Serine phosphorylation of the partially purified placental insulin receptor was not significantly affected by 5 µM-cyclic AMP and was slightly inhibited (23 % inhibition) by 1 mM-Ca²⁺ + phosphatidylserine (80 µg/ml) + diolein (8 µg/ml) (Table 1). Similar results were obtained in the absence of insulin (not shown). These results indicate that the insulin-stimulated insulin-receptor serine kinase is distinct from cyclic AMP- and Ca²⁺/phospholipid-dependent protein kinases. Additionally, the cyclic AMP-dependent protein kinase inhibitor did not alter the phosphoserine/phosphotyrosine ratio measured in the insulin receptor after phosphorylation in the presence or the absence of insulin (results not shown). Ca²⁺ (1 mM), Ca²⁺ (1 mM) + calmodulin (2 µM), cyclic GMP (6 µM), heparin (up to 100 µg/ml), spermine (7 mM) or GTP (100 µM) had either no effect or only small effects on the incorporation of ³²P into phosphoserine or phosphotyrosine in either the presence (Table 1) or the absence (results not shown) of insulin. The ability of the placental insulin receptor to catalyse insulin-stimulated serine phosphorylation of casein (1.73 mg/ml) and phosphovitin (0.5 mg/ml) was examined; both were poor substrates under our assay conditions (results not shown). These results indicate that the insulin-stimulated

receptor serine kinase is distinct from casein kinase I (phosphorylates casein and phosvitin), casein kinase II (activated by spermine; inhibited by heparin), and cyclic GMP-, Ca^{2+} - and calmodulin-dependent protein kinases. The insulin-stimulated receptor serine kinase is fully active with Mn^{2+} (Smith *et al.*, 1988), indicating that it is different from the insulin-stimulated ribosomal S6 kinase described by Tabarini *et al.* (1985), which is inhibited by 0.05 mM- Mn^{2+} . Additionally, S6 kinase is usually completely inhibited by heparin at concentrations below 100 $\mu\text{g}/\text{ml}$ (Erikson & Maller, 1986) and 40 % inhibited by 2 mM-spermine (Cobb, 1986), whereas the insulin-stimulated receptor serine kinase was not appreciably inhibited by heparin at 100 $\mu\text{g}/\text{ml}$ or by 7 mM-spermine. Properties of other insulin-stimulated serine kinases are ill-defined. The lack of effect of GTP suggests that a G-protein does not participate in the activation *in vitro* of the serine kinase by insulin.

DISCUSSION

Serine phosphorylation of cellular substrates triggered by insulin is an important part of the action of this hormone. A whole range of cellular substrates undergoing this modification have been identified, ranging from enzymes involved in glycolysis (phosphofructokinase: Sale & Denton, 1985), fatty acid metabolism (acetyl-CoA carboxylase, ATP citrate lyase: Denton *et al.*, 1981; Avruch *et al.*, 1985) and cyclic AMP metabolism (cyclic AMP phosphodiesterase: Marchmont & Houslay, 1981) to proteins involved in the control of protein synthesis (ribosomal protein S6: Tabarini *et al.*, 1985) and to the insulin receptor itself (Gazzano *et al.*, 1983). Therefore it is of rather general importance to identify the mechanisms by which activation of these protein serine kinases in cells is initiated by insulin. In the present work we have studied this by using a system in which the insulin receptor has been co-purified with insulin-sensitive serine kinase activity that phosphorylates the insulin receptor (Smith *et al.*, 1988). Such a system *in vitro* is ideal for exploring the dependency of triggering of serine kinase activity on the tyrosine kinase activity of the insulin receptor. The activity of the tyrosine kinase was manipulated with a range of inhibitors and activators, and effects on insulin-stimulated serine phosphorylation of the insulin receptor were examined. Nine different inhibitors of the tyrosine kinase, which acted by five different mechanisms, were all shown to inhibit insulin-stimulated phosphorylation of the insulin receptor on tyrosine and serine in parallel (Table 1). A short synthetic peptide (peptide 1150, containing tyrosine-autophosphorylation sites 1146, 1150, 1151), which acts as a competing substrate, was the best inhibitor. This peptide is devoid of serine residues and would be expected to be a highly specific inhibitor of tyrosine kinases. No agents were discovered that inhibited the tyrosine kinase activity without also inhibiting insulin-stimulated serine phosphorylation of the receptor. Additionally, vanadate, which directly stimulates the receptor tyrosine kinase (Tamura *et al.*, 1984), stimulated serine phosphorylation of the receptor. Taken together, these results provide strong evidence that insulin-stimulated serine phosphorylation of the insulin receptor requires an active tyrosine kinase. Consistent with this conclusion, there was a pronounced lag in the phosphorylation of serine residues in the insulin receptor

compared with tyrosine residues after insulin stimulation (Fig. 1). The conclusion assumes that the agents used to manipulate tyrosine kinase activity did not directly affect the serine kinase activity in the same way. This possibility is highly unlikely, given the diversity of agents used and that the serine kinase would need to show similar sensitivity to inhibition and activation by each of the > 10 agents used to the tyrosine kinase displayed. Additionally, as a control, the activity of an insulin-insensitive serine kinase present in the insulin-receptor preparation was monitored. None of the methods used to manipulate tyrosine kinase activity significantly altered the activity of this kinase (results not shown).

Previous studies linking a role of insulin-receptor tyrosine kinase to bio-effects of insulin have mainly relied either on site-directed mutagenesis or inhibitory monoclonal antibodies. Mutation of the insulin receptor at the presumed ATP-binding site results in decreased insulin-stimulated tyrosine kinase activity and failure of insulin to stimulate fully glucose uptake, ribosomal S6 serine-protein kinase activity, glycogen deposition and thymidine incorporation into DNA (Ebina *et al.*, 1987; Chou *et al.*, 1987). Similar results have been obtained by introducing into intact cells monoclonal antibodies that inhibit receptor tyrosine kinase activity (Morgan & Roth, 1987). Neither of these methods is necessarily without disadvantage. Mutation induced in the receptor may modify properties in addition to the receptor tyrosine kinase, for example by causing non-specific changes in the conformation of the β subunit. A large monoclonal antibody, almost twice the size of the β subunit, bound at the tyrosine kinase active site could also act non-specifically. Thus the results of the present work, providing evidence that a further insulin bio-effect depends on tyrosine kinase activity, by using a range of different methods, complements these results and adds weight to the evidence that insulin-receptor autophosphorylation/tyrosine kinase mediates in insulin signalling.

The site-directed-mutagenesis and antibody studies do not distinguish between the importance in insulin signalling of tyrosine autophosphorylation of the insulin receptor itself and phosphorylation of other substrates on tyrosine. Similarly, two main types of mechanisms can be envisaged for how the tyrosine kinase of the insulin receptor activates serine phosphorylation of the receptor. Firstly, the tyrosine kinase may phosphorylate the serine kinase on tyrosine, causing direct activation. Secondly, tyrosine autophosphorylation of the insulin receptor itself may make the β subunit a better substrate for the kinase. In this second hypothesis the actual activity of the serine kinase itself would not be altered by insulin. The experiment in Table 2, showing that histone is phosphorylated in an insulin-dependent manner on serine by the placental insulin-receptor preparation, favours the idea that direct activation of the serine kinase occurs. This conclusion assumes that the same insulin-stimulated serine kinase phosphorylates both the insulin receptor and histone. These results are supported by those of Yu *et al.* (1987) showing that 22 % of an insulin-stimulated histone serine kinase activity in adipocytes can be adsorbed by anti-phosphotyrosine antibody, and may be phosphorylated on tyrosine. However, the relationship of this histone serine kinase activity studied by Yu *et al.* (1987) to the insulin-stimulated receptor serine kinase activity is not known.

To investigate the identity of the insulin-stimulated

receptor serine kinase, its properties were compared with those of known serine kinases. Of importance was the possibility that the kinase may be a Ca^{2+} /phospholipid-dependent protein kinase. This possibility arises because: (i) phorbol esters, which activate Ca^{2+} /phospholipid-dependent kinases, stimulate serine phosphorylation of the insulin receptor in intact cells (Jacobs *et al.*, 1983; Takayama *et al.*, 1984); (ii) purified insulin receptor may be phosphorylated on serine by Ca^{2+} /phospholipid-dependent protein kinase (Bollag *et al.*, 1986); (iii) insulin stimulates the breakdown of a membrane glycolipid to give a phospho-oligosaccharide, which may act as a second messenger for some of the actions of the hormone, and a novel species of diacylglycerol, which might activate Ca^{2+} /phospholipid-dependent kinase (Saltiel *et al.*, 1987; Mato *et al.*, 1987). Thus the possibility arises that insulin might increase serine phosphorylation of the insulin receptor through this pathway. Phosphatidylserine + Ca^{2+} + diolein did not augment the serine kinase activity against either the insulin receptor (Table I) or histone (results not shown), indicating that the insulin-stimulated receptor serine kinase is not a Ca^{2+} /phospholipid-activated kinase. This conclusion is consistent with the observation by Spach *et al.* (1986) that insulin is unable to activate Ca^{2+} /phospholipid-dependent kinase in BC_3H_1 myocytes. Similarly, evidence was obtained that the insulin-stimulated receptor serine kinase was distinct from casein kinase I and II, and cyclic AMP-, cyclic GMP- and Ca^{2+} /calmodulin-dependent protein kinases and insulin-activated ribosomal S6 protein kinase. Thus the insulin-stimulated receptor serine kinase appears to be a new kinase whose activation *in vitro* by insulin requires an active insulin-receptor tyrosine kinase.

We thank Dr. M. F. White for generously giving the anti-phosphotyrosine antibody. This work was supported by grants from the Medical Research Council, Wessex Medical School Trust and Nuffield Foundation.

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Received 13 June 1988/16 August 1988; accepted 22 August 1988