Biochem. J. (1991) 275, 413–418 (Printed in Great Britain)

Site-specific dephosphorylation and deactivation of the human insulin receptor tyrosine kinase by particulate and soluble phosphotyrosyl protein phosphatases

Martin J. KING,* Ram P. SHARMA and Graham J. SALE†
Department of Biochemistry, University of Southampton, Bassett Crescent East, Southampton SO9 3TU, U.K.

Insulin receptor tyrosine kinase activation, induced by insulin-stimulated autophosphorylation, was measured using a synthetic peptide containing residues 1142–1153 of the insulin receptor and shown to be reversed by both particulate and soluble phosphotyrosyl protein phosphatases from rat liver. Deactivation of the tyrosine kinase was highly sensitive to phosphatase action and was correlated best with disappearance of insulin receptors triphosphorylated in the tyrosine-1150 domain. Dephosphorylation of the di- and mono-phosphorylated forms of the tyrosine-1150 domain generated during dephosphorylation or of phosphorylation sites in the *C*-terminal or putative juxtamembrane domains occurred 3– > 10-fold more slowly than deactivation of the tyrosine kinase, and these phosphorylated species did not appear to appreciably (< 20%) contribute to tyrosine kinase activation. These results indicate that the transition from the triply to the doubly phosphorylated form of the tyrosine-1150 domain acts as an important switch for deactivation of the insulin receptor tyrosine kinase during dephosphorylation. The exquisite sensitivity of this dephosphorylation/deactivation event to phosphotyrosyl protein phosphatase action, combined with the high affinities of the phosphatases for substrates and the high activities of the phosphatases in cells, suggests that the tyrosine kinase activity expressed by insulin-stimulated insulin receptors is likely to be stringently regulated.

INTRODUCTION

The insulin receptor is a transmembranous heterotetrameric protein composed of two types of subunit linked by disulphide bonds to give a β-α-α-β structure (Rosen, 1987; Sale, 1988). The α-subunit, which is entirely extracellular, contains the insulin-binding site, while the β-subunit spans the membrane and possesses a tyrosine-specific protein kinase activity in a cytoplasmic domain. The tyrosine kinase is activated by insulin binding to the α-subunit. Mutant insulin receptors, in which lysine 1018 in the ATP-binding domain of the β-subunit has been changed to one of several amino acids, which are expressed in cells, bind insulin normally but are totally inactive as kinases and are totally defective in mediating stimulation of metabolism by insulin (Ebina *et al.*, 1987; Chou *et al.*, 1987). This suggests that the tyrosine kinase activity of the insulin receptor is critical for insulin signalling.

The tyrosine kinase catalyses autophosphorylation of the β-subunit. At least seven tyrosine residues in the β-subunit have been reported to undergo autophosphorylation (Tornqvist *et al.*, 1987, 1988; White *et al.*, 1988; Tavaré & Denton, 1988; Tavaré *et al.*, 1988; King & Sale, 1990). These sites of autophosphorylation appear to be clustered in three domains. Two sites (tyrosine residues 1316 and 1322) are located in the *C*-terminal domain. Three sites (tyrosines 1146, 1150 and 1151) reside in the tyrosine-1150 domain. Two sites (tyrosines 953 and 960 or 972) are believed to reside in another domain which has putatively been identified as the juxtamembrane domain. All of these sites can be phosphorylated both *in vivo* and *in vitro*, although phosphorylation of the putative juxtamembrane domain sites has not always been observed.

Insulin-stimulated autophosphorylation functions to dramatically increase insulin receptor tyrosine kinase activity towards other proteins (Rosen *et al.*, 1983; Yu & Czech, 1984). This is well illustrated with competing substrates which, when added prior to ATP and insulin, totally block insulin-stimulated autophosphorylation and as a consequence block insulin-stimulated substrate phosphorylation. Autophosphorylation maintains the tyrosine kinase in the activated state even in the absence of bound insulin. Thus dephosphorylation, and not simply dissociation of bound insulin, is required to terminate tyrosine kinase activity.

Analysis of the state of autophosphorylation of the β-subunit and the ability of the tyrosine kinase to catalyse phosphorylation shows that activation of the tyrosine kinase of the rat and mouse insulin receptor correlates best with phosphorylation of all three tyrosines in the tyrosine-1150 domain (White *et al.*, 1988; Flores-Riveros *et al.*, 1989). Thus the tyrosine-1150 domain may function as a regulatory domain.

The majority (approx. 70%) of phosphotyrosyl protein phosphatase activity against autophosphorylated insulin receptor appears to be located in the particulate fraction, at least in rat liver (King & Sale, 1988*b*). Recently we have characterized the dephosphorylation of human insulin receptor autophosphorylation sites by both rat liver particulate and soluble phosphotyrosyl protein phosphatases (King & Sale, 1990). Both phosphatase preparations behaved similarly and yielded marked differences in the rate of dephosphorylation of individual insulin receptor autophosphorylation sites, providing a good model system with which to analyse the role of the various autophosphorylation sites in tyrosine kinase activation. Moreover, the tyrosine-1150 domain of the insulin receptor in the triphosphorylated state was found to be exquisitely sensitive to dephosphorylation by the phosphatases (King & Sale, 1990), and it was suggested that this may provide a sensitive mechanism for regulating insulin receptor tyrosine kinase action. To test this proposal further, the relationship between dephosphorylation of insulin receptor autophosphorylation sites and deactivation of the tyrosine kinase is examined in this study.

* Present address: Department of Medical Biochemistry, University of Calgary, Calgary, Canada T2N 4N1.
† To whom correspondence should be addressed.

EXPERIMENTAL

Materials

The peptide Thr-Arg-Asp-Ile-Tyr-Glu-Thr-Asp-Tyr-Tyr-Arg-Lys, corresponding to amino acids 1142–1153 in the human insulin receptor precursor, was synthesized manually using a solid-phase method (Merrifield, 1986). Other biochemicals and chemicals were obtained from sources described in King & Sale (1990) or Smith *et al.* (1988).

Insulin receptor autophosphorylation, dephosphorylation and tyrosine kinase assay

Insulin receptors were purified partially from solubilized human placental membranes by affinity chromatography on wheat-germ-agglutinin–agarose as previously described (Smith *et al.*, 1988). The receptor was preincubated (approx. 1.5 mg of protein/ml) for 10 min at 22 °C in 0.1–1 ml of a solution containing 50 mM-Hepes (pH 7.4), 4.8 mM-$MnCl_2$, 180 nM-insulin and 0.1 % Triton X-100 (Sale *et al.*, 1986). At this insulin concentration occupancy of the insulin receptor by insulin was fast and was > 90 % complete in 5 min and > 95 % complete in 10 min.

Two protocols for autophosphorylation, dephosphorylation and tyrosine kinase assay were employed. In the first protocol (Figs. 1 and 2), the preincubated insulin receptor was divided into three parts, which were incubated in parallel for 30 min at 22 °C with 0.19 vol. of 1.6 mM-[γ-^{32}P]ATP (8–12 c.p.m./fmol), 0.19 vol. of 1.6 mM-ATP or 0.19 vol. of water. The final concentration of [γ-^{32}P]ATP or ATP was 250 μM. Incubations were terminated by adding 0.053 vol. of 200 mM-EDTA (final concentration 10 mM). The [γ-^{32}P]ATP and insulin concentrations and the incubation time were optimal for obtaining maximum insulin receptor autophosphorylation on tyrosine. Omission of both sodium vanadate and dithiothreitol minimized insulin receptor phosphorylation on serine residues relative to autophosphorylation on tyrosine (Smith & Sale, 1988; King & Sale, 1990). Phosphoamino acid analysis of β-subunits from preparations of insulin receptors phosphorylated in this study showed that 6.1 ± 1.7 % (mean ± S.E.M., 4 observations) of the ^{32}P recovered in phosphoamino acids was attached to serine, with the remainder attached to tyrosine. Samples of autophosphorylated insulin receptors were further incubated in parallel with 0.10–0.18 vol. of a 22–13 mg/ml solution of rat liver particulate or soluble fraction (final concentration 2 mg/ml) for the indicated times at 30 °C. The presence of EDTA during these incubations, in addition to inhibiting insulin receptor autophosphorylation, will inhibit serine kinase activity against the insulin receptor that could be present in the liver fractions. Furthermore, dephosphorylation of the small amount of [^{32}P]phosphoserine attached to the insulin receptor by the rat liver fractions was slow in the presence of EDTA (< 15 % dephosphorylation of [^{32}P]phosphoserine in 10 min; King & Sale, 1988*b*). For measurement of dephosphorylation, incubations with ^{32}P-labelled insulin receptors were terminated by adding Laemmli (1970) sample buffer followed by boiling for 2 min, and samples were then subjected to SDS/PAGE and autoradiography (King & Sale, 1990). ^{32}P-labelled β-subunits were then excised and analysed by peptide mapping. The non-radioactive parallel assays were used to assay tyrosine kinase activity. These dephosphorylation assays were simultaneously terminated and tyrosine kinase assays were initiated by mixing 15 μl portions with 25 μl of a cocktail comprising 12.8 mM-$MnCl_2$, 16 mM-$MgCl_2$, 160 μM sodium vanadate, 4 mM-[γ-^{32}P]ATP (40–250 c.p.m./pmol) and 1.6–4.8 mM-insulin receptor-(1142–1153)-peptide. In this way the contribution of the non-radioactive ATP did not appreciably (< 4 %) alter the specific radioactivity of the [γ-^{32}P]ATP in the tyrosine kinase assays. The concentration of vanadate employed inhibited particulate and soluble phosphotyrosyl protein phosphatase activity against autophosphorylated insulin receptors by 101.5 ± 8.4 % (mean ± S.E.M., 26 observations). The concentration of peptide used was assessed for individual preparations of insulin receptor, such that it was sufficient to inhibit reactivation of the tyrosine kinase by > 90 %. The concentration of insulin was approx. 50 nM; the tyrosine kinase activity of the insulin receptor was > 90 % of maximum at this insulin concentration. Under the assay conditions used, the contribution of epidermal growth factor receptor, which is also present, to peptide phosphorylation was estimated to be < 10 % of that due to autophosphorylated insulin receptor. Control experiments established that the insulin receptor was not significantly phosphorylated on serine by liver extracts. This was not unexpected as there was no pretreatment, before preparation of extracts, with agents that activate the serine kinases. Tyrosine kinase assays were terminated after 10 min at 30 °C by adding trichloroacetic acid to a concentration of 4 % (w/v). After centrifugation (10000 *g*, 5 min), 10 μl portions were spotted on to Whatman P81 phosphocellulose paper and ^{32}P incorporated into the peptide was determined as described previously (King & Sale, 1988*a*). ^{32}P incorporated into the peptide is expressed in terms of pmol/min for the 10 μl samples spotted onto the paper.

In the second protocol (Fig. 3), insulin receptors were autophosphorylated with 250 μM-[γ-^{32}P]ATP as above and then separated from [γ-^{32}P]ATP by rapid gel filtration at 4 °C (King & Sale, 1988*b*). The autophosphorylated insulin receptor was incubated with 0.22 vol. of rat liver particulate or soluble fraction (5.5 mg/ml; final concentration 1 mg/ml). At the indicated times three samples were removed. One sample was mixed with Laemmli sample buffer, followed by boiling for 2 min, SDS/PAGE and autoradiography (King & Sale, 1990). The β-subunits were excised from the gels and analysed by peptide mapping. Duplicate samples were assayed for tyrosine kinase activity as above, except that the final [γ-^{32}P]ATP concentration was 1 mM. Although the concentration of insulin in these tyrosine kinase assays was likely to be low, the tyrosine kinase activity of insulin receptors activated by autophosphorylation is independent of the insulin concentration.

The K_m of non-phosphorylated or phosphorylated insulin receptors for ATP was 10–25 μM, in agreement with previous studies (Kwok *et al.*, 1986). Thus ATP concentrations during autophosphorylation or tyrosine kinase assay were at least 10-fold greater than the K_m of the receptor for ATP.

Preparation of particulate and soluble fractions

Particulate and soluble fractions containing phosphotyrosyl protein phosphatase activity were prepared from a rat liver homogenate by centrifugation at 100000 *g* for 1 h as previously described (King & Sale, 1988*b*) by using 50 mM-Hepes buffer (pH 7.4) containing 1 mM-dithiothreitol, 5 mM-EDTA, 0.5 mM-EGTA, 1 mM-benzamidine, 1 μg of leupeptin/ml, 1 μg of soyabean trypsin inhibitor/ml and a 1:1000 dilution of 2.5 % (v/v) phenylmethanesulphonyl fluoride dissolved in propan-2-ol.

Peptide mapping

Digestions with trypsin (treated with Tos-Phe-CH_2Cl) and two-dimensional tryptic peptide mapping on cellulose thin layer plates followed by autoradiography were performed as described previously (King & Sale, 1990). The tyrosine-1150 autophosphorylation domain, which contains tyrosines 1146, 1150 and 1151, was recovered as peptides that were monophosphorylated (C1), diphosphorylated (B2 and B3) and triphosphorylated (A1 and A2). A1/A2 and B2/B3 represent

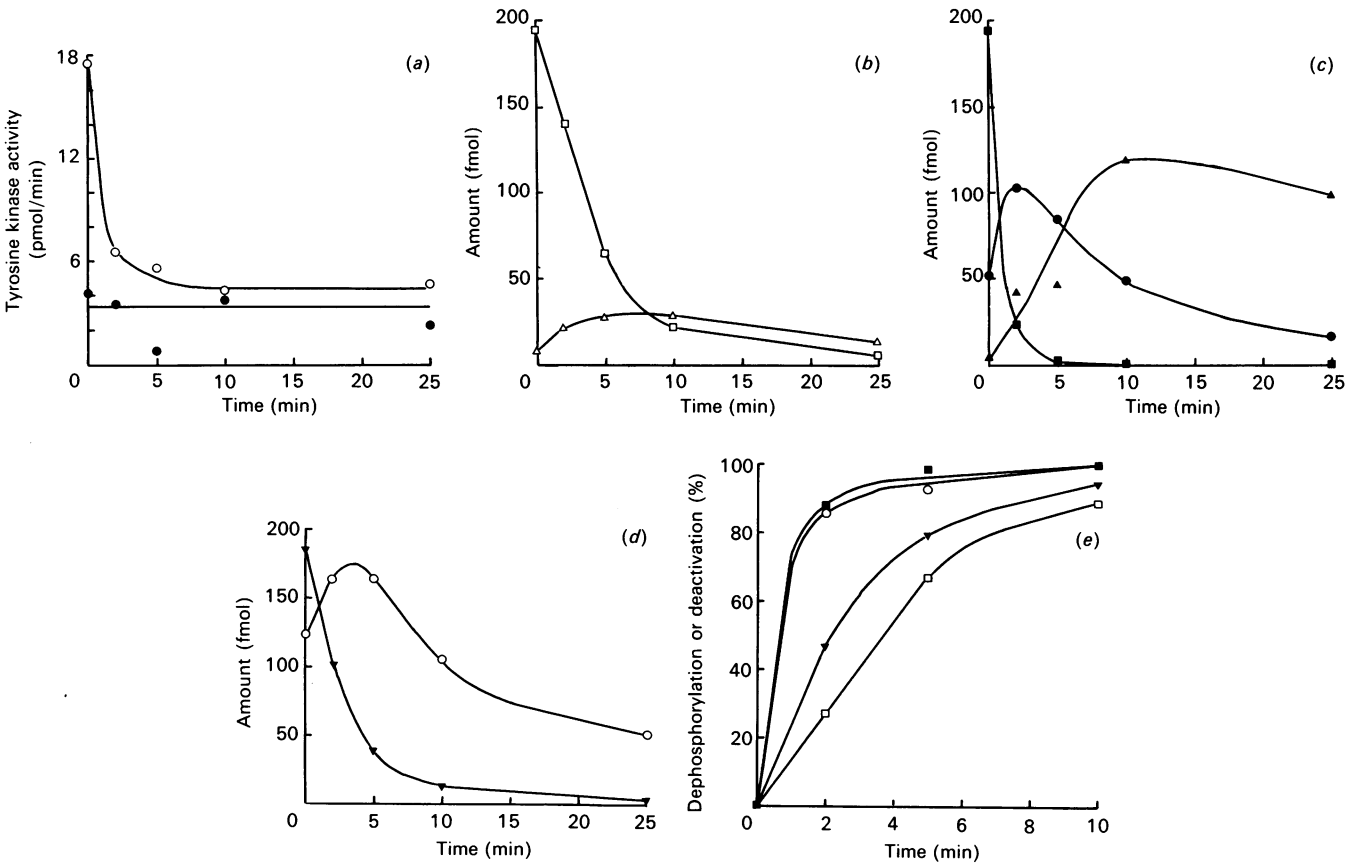

Fig. 1. Time courses of dephosphorylation of insulin receptor autophosphorylation sites and deactivation of tyrosine kinase by soluble phosphatase

Insulin receptors were incubated in the presence of insulin without ATP to generate non-phosphorylated insulin-stimulated insulin receptors, or with 250 μM-ATP or 250 μM-[γ-^{32}P]ATP to generate insulin-stimulated autophosphorylated insulin receptors. After 30 min at 22 °C, incubations were terminated by addition of EDTA and samples were treated with soluble phosphatase extract (2 mg/ml) at 30 °C for the indicated times. (*a*) Tyrosine kinase activity against insulin receptor-(1142–1153)-peptide assayed using the non-radioactive samples. ○, Autophosphorylated insulin receptors in the presence of insulin; ●, non-phosphorylated insulin receptors in the presence of insulin. (*b*), (*c*) and (*d*) Dephosphorylation of autophosphorylation sites measured using the ^{32}P-labelled insulin receptor and tryptic peptide mapping. (*b*) *C*-Terminal domain. □, diphosphorylated; △, monophosphorylated. (*c*) Tyrosine-1150 domain: ■, triphosphorylated; ●, diphosphorylated; ▲, monophosphorylated. (*d*) Putative juxtamembrane domain (King & Sale, 1990) assuming diphosphorylated (▼) and monophosphorylated (○) forms. (*e*) Dephosphorylation [%; ■, triphosphorylated tyrosine-1150 domain; □, diphosphorylated *C*-terminal domain; ▼, diphosphorylated putative juxtamembrane domain] and deactivation of tyrosine kinase [% of maximum (○)] during the initial 10 min of incubation with the phosphatase.

different digestion products of the peptide. The *C*-terminal autophosphorylation domain, which contains tyrosines 1316 and 1322, was recovered as peptides that were diphosphorylated (B1) and monophosphorylated (NP1). The putative juxtamembrane autophosphorylation domain yielded peptides C3 and C1′, which may represent diphosphorylated and monophosphorylated peptides respectively. The terminology and assignments of identity are based on previous work (Tavaré & Denton, 1988; King & Sale, 1990). Although the origin of most of the above peptides is known with certainty, proof that C3 and C1′ originate from the juxtamembrane domain is lacking. The evidence that C3 and C1′ are derived from the juxtamembrane domain is based on their size ($M_r \sim 3500$), charge and the cleavage patterns obtained upon digestion with *Staphylococcus aureus* V8 proteinase (Tavaré & Denton, 1988). Evidence that C3 and C1′ represent diphosphorylated and monophosphorylated forms respectively, is based on the time course of dephosphorylation and charge considerations (King & Sale, 1990). Thus C3 appears to be phosphorylated on tyrosines 953 and 960 or 972 and C1′ appears to be phosphorylated on just one of these tyrosines. Phosphoamino acid analysis was used to correct for the contribution of [^{32}P]phosphoserine to peptides C1 and C1′; phosphopeptides A1, A2, B1, B3, C3 and NP1 yielded only [^{32}P]phosphotyrosine. Phosphoamino acid analysis was performed as described by Smith *et al.* (1988).

Autoradiographs of peptide maps were quantified by scanning as previously described (King & Sale, 1990). The total yield of phosphopeptides from the β-subunit was calculated from the ^{32}P in the β-subunit, determined by liquid scintillation counting as described in King & Sale (1990), and from the ratio of ^{32}P recovered in phosphopeptides. The amount (fmol) of each phosphorylated species was then calculated by dividing the amount of ^{32}P (fmol) in the phosphopeptides by the number of sites phosphorylated. The numbering of insulin receptor amino acids used in this paper is based on the sequence of the precursor of the human insulin receptor described by Ullrich *et al.* (1985).

RESULTS

The insulin receptor was autophosphorylated by incubation in the presence of 250 μM-ATP and insulin for 30 min at 22 °C (King & Sale, 1990). Autophosphorylation was terminated by adding EDTA and the autophosphorylated insulin receptor was incubated with rat liver preparations of particulate and soluble phosphotyrosyl protein phosphatase (Figs. 1 and 2). Insulin-receptor phosphotyrosyl protein phosphatases are known to be

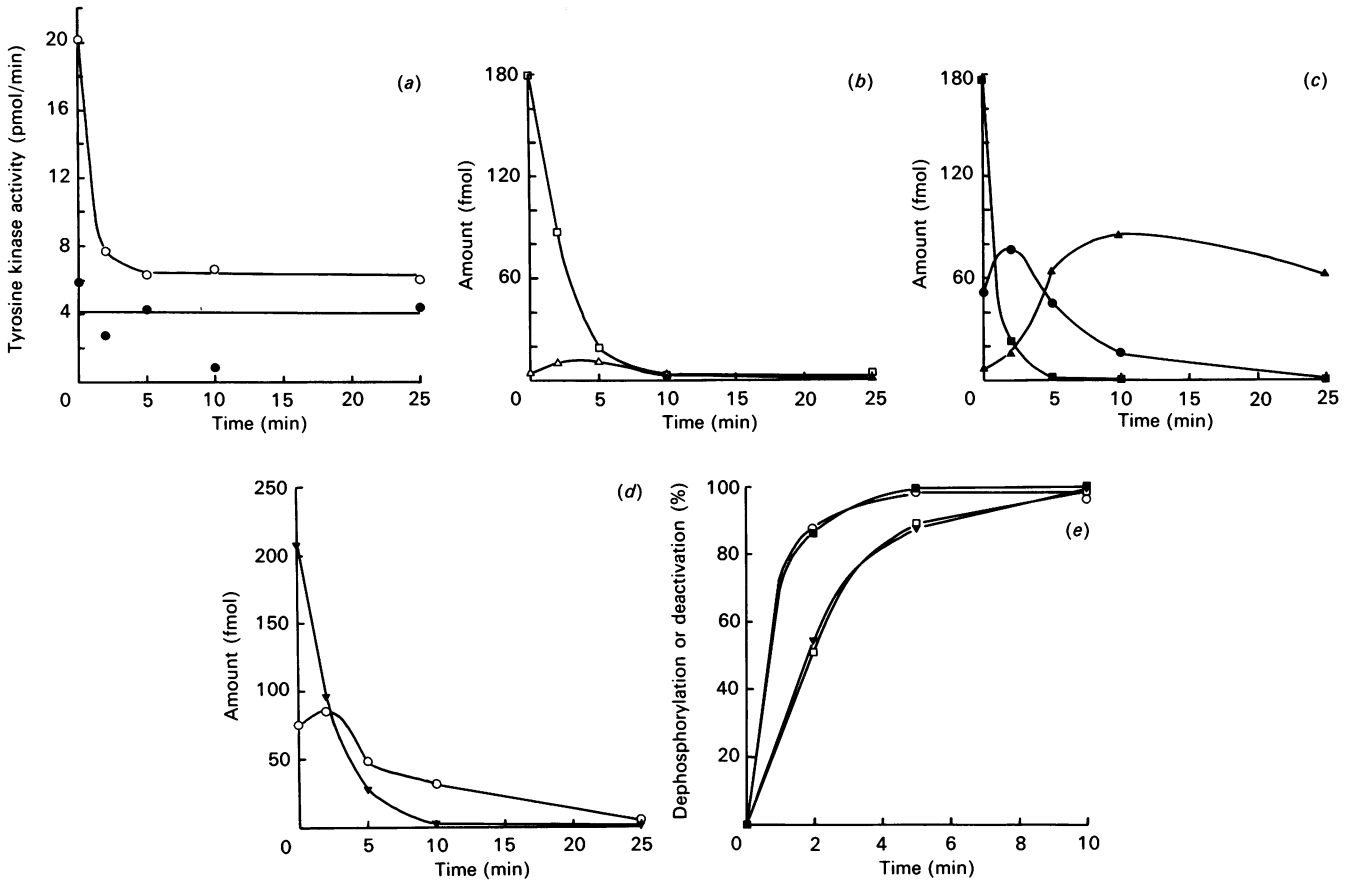

Fig. 2. Time courses of dephosphorylation of insulin receptor autophosphorylation sites and deactivation of tyrosine kinase by particulate phosphatase

(*a*) Tyrosine kinase activity. ○, Autophosphorylated insulin receptor in the presence of insulin; ●, non-phosphorylated insulin receptor in the presence of insulin. (*b*), (*c*) and (*d*) Dephosphorylation of autophosphorylation sites. (*b*) *C*-Terminal domain: □, diphosphorylated; △, monophosphorylated. (*c*) Tyrosine-1150 domain: ■, triphosphorylated; ●, diphosphorylated; ▲, monophosphorylated. (*d*) Putative juxtamembrane domain assuming diphosphorylated (▼) and monophosphorylated (○) forms. (*e*) Dephosphorylation [%; ■, triphosphorylated tyrosine-1150 domain; □, diphosphorylated *C*-terminal domain; ▼, diphosphorylated putative juxtamembrane domain] and deactivation of tyrosine kinase [% of maximum (○)] during the initial 10 min of incubation with the phosphatase. Other details are as described in the legend to Fig. 1, except that particulate phosphatase extract (2 mg/ml) was employed.

active in the presence of EDTA (King & Sale, 1988*b*). The distribution of ^{32}P amongst autophosphorylation sites was determined by two-dimensional peptide mapping of tryptic phosphopeptides. The tyrosine kinase activity of the insulin receptor was assayed with the synthetic peptide containing residues 1142–1153 of the insulin receptor; this contains three of the tyrosine residues autophosphorylated in the insulin receptor (tyrosines 1146, 1150 and 1151). At concentrations in the 1–5 mM range this peptide also inhibits insulin receptor autophosphorylation and consequently reactivation of tyrosine kinase activity which may otherwise occur during the assay of tyrosine kinase activity (White *et al.*, 1988; Smith & Sale, 1988; Wilden *et al.*, 1990).

Figs. 1 and 2 show the time courses of dephosphorylation of insulin receptor autophosphorylation sites and deactivation of the tyrosine kinase by rat liver preparations of soluble and particulate phosphotyrosyl protein phosphatase. As a control the tyrosine kinase activity of non-phosphorylated insulin receptors in the presence of insulin was also measured. Wilden *et al.* (1990) have resolved activation of the tyrosine kinase into two components. The first was dependent on insulin binding. The second and larger component was due to insulin-stimulated autophosphorylation. In the present work the tyrosine kinase activity of non-phosphorylated insulin receptors in the presence of insulin was approx. 20% of the tyrosine kinase activity of the autophosphorylated insulin receptors in the presence of insulin. This value is comparable with that reported by Wilden *et al.* (1990). Incubation of the insulin-stimulated autophosphorylated insulin receptor with either particulate or soluble phosphotyrosyl protein phosphatase resulted in a decrease in the tyrosine kinase activity to a level that plateaued at approx. 25–31% (Figs. 1*a* and 2*a*). In contrast, the tyrosine kinase activity of the insulin-stimulated non-phosphorylated insulin receptor remained stable at ~ 18–21% during incubation with either phosphatase. Thus the activation of substrate phosphorylation induced by autophosphorylation was largely reversed by the phosphatases. This phosphatase-catalysed deactivation of the tyrosine kinase of the autophosphorylated insulin receptor occurred rapidly with both particulate and soluble activities and correlated best with the disappearance of insulin receptors that were triphosphorylated in the tyrosine-1150 domain (Figs. 1*e* and 2*e*). For example, after incubation with the soluble phosphatase for 2 min the tyrosine kinase activity was, as a percentage of the maximum deactivation obtained, 85% deactivated, and dephosphorylation of the triphosphorylated tyrosine-1150 domain was 88.8% complete (Fig. 1*e*). Dephosphorylation of the diphosphorylated *C*-terminal domain species or the putative diphosphorylated juxtamembrane domain species catalysed by either the particulate or the soluble phosphatase preparation occurred approximately three times more slowly than the rate of deactivation (Figs. 1*e* and 2*e*).

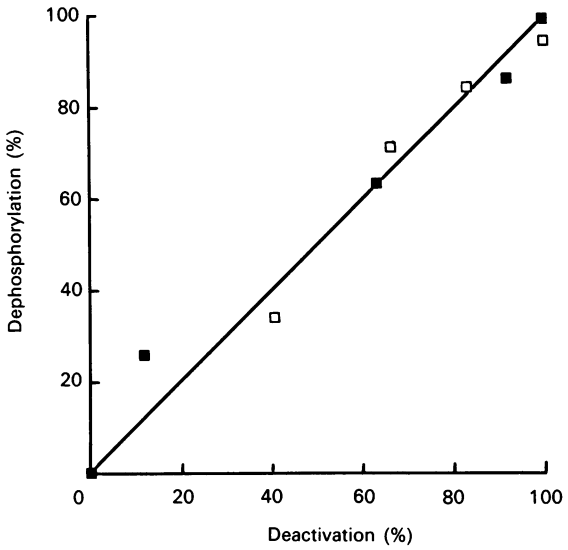

Fig. 3. Relationship between the amount of the triphosphorylated tyrosine-1150 domain species and tyrosine kinase activity

Insulin receptor was autophosphorylated in the presence of insulin and 250 μM-[γ-^{32}P]ATP, then subjected to rapid gel filtration to remove [γ-^{32}P]ATP. The gel-filtered autophosphorylated insulin receptor was incubated with particulate (■) or soluble (□) phosphatase extract (1 mg/ml) at 30 °C. At 0, 2, 5, 10 and 25 min duplicate samples were assayed for tyrosine kinase activity against insulin receptor-(1142–1153)-peptide and for the amount of the triphosphorylated form of the tyrosine-1150 domain by tryptic peptide mapping. In the gel-filtered autophosphorylated insulin receptor used as substrate, phosphorylated domains contained ^{32}P in the proportions 1:0.29:0.17:0.83:0.05:0.37:0.38 (tri-:di-:mono-phosphorylated tyrosine-1150 domain:di-:mono-phosphorylated *C*-terminal domain: di-: mono-phosphorylated putative juxtamembrane domain).

During the first 2 min of incubation with either phosphatase, the amounts of insulin receptor diphosphorylated in the tyrosine-1150 domain increased significantly, in the opposite direction to the change in tyrosine kinase activity (Figs. 1*a*, 1*c*, 2*a* and 2*c*). After a 2 min incubation with either phosphatase, approx. 40% of insulin receptor β-subunits appeared to be diphosphorylated in the tyrosine-1150 domain. Similarly, the amount of insulin receptors monophosphorylated in the tyrosine-1150 domain increased during incubation with either phosphatase, reaching a peak after around 10 min of incubation, at which time approx. 50% of insulin receptor β-subunits appeared to contain this phosphorylated species (Figs. 1*c* and 2*c*). Thus substantial amounts of the partially phosphorylated forms of the tyrosine-1150 domain persisted after the tyrosine kinase activity was largely deactivated. Additionally, as the di- and mono-phosphorylated forms of the tyrosine-1150 domain and the putative monophosphorylated form of the juxtamembrane domain underwent net dephosphorylation in the latter stages of incubation, there was little change in tyrosine kinase activity (Figs. 1*a*, 1*c*, 1*d*, 2*a*, 2*c* and 2*d*). Changes in the level of phosphorylation of the monophosphorylated form of the *C*-terminal domain did not correlate with deactivation (Figs. 1*a*, 1*b*, 2*a* and 2*b*). These results indicate that, during phosphorylation, tyrosine kinase activation was primarily a function of the amount of receptor phosphorylated on all three tyrosines (1146, 1150 and 1151) in the tyrosine-1150 domain, with other species making at most a small (< 20%) contribution.

In the above experiments (Figs. 1 and 2) it was not practical to directly perform tyrosine kinase assays on the radioactive incubations because of the presence of high specific radioactivity [γ-^{32}P]ATP from the initial phosphorylation in variable amounts due to ATPase activity in the liver fractions. Instead, tyrosine kinase assays were performed on samples incubated identically and in parallel using non-radioactive ATP in the initial autophosphorylation. A 33-fold higher ATP concentration was used in the tyrosine kinase assays than the maximum that could be carried over from the autophosphorylation in order to minimize the effect of the ATPase activity on the specific activity of the [γ-^{32}P]ATP in the tyrosine kinase assays to < 4%. To validate the relationship between the disappearance of insulin receptors triphosphorylated in the tyrosine-1150 domain and deactivation of the tyrosine kinase obtained by this method, a different protocol was also used. In this procedure insulin receptors were autophosphorylated in the presence of insulin and [γ-^{32}P]ATP and then separated from [γ-^{32}P]ATP by rapid gel filtration before use in phosphatase assays. The advantage of this procedure was that the same incubations could be assayed both for tyrosine kinase activity and for occupancy of phosphorylation sites; however, a disadvantage was that the autophosphorylated insulin receptors prepared by gel filtration were somewhat less fully phosphorylated. Deactivation of the tyrosine kinase activity of the autophosphorylated/gel-filtered insulin receptor upon incubation with particulate or soluble phosphatase correlated well with the disappearance of the triphosphorylated tyrosine-1150 domain species (Fig. 3), and occurred faster than dephosphorylation of the other species (results not shown).

DISCUSSION

Results presented here indicate that conversion of the triphosphorylated tyrosine-1150 domain species to its diphosphorylated derivative acts as an important switch for deactivation of the insulin receptor tyrosine kinase during dephosphorylation. This dephosphorylation/deactivation event was exquisitely sensitive to phosphatase action and occurred many-fold faster than dephosphorylation of other autophosphorylation sites. Thus substantial amounts of the partially phosphorylated forms of the tyrosine-1150 domain and the receptor diphosphorylated in the *C*-terminal and putative juxtamembrane domains remained after the deactivation event was largely complete. Previously there had been no information on whether phosphorylation of the putative juxtamembrane domain could be activating, although its phosphorylation was reported to correlate with the extent of kinase activation (Tornqvist & Avruch, 1988). The key activating role of the triphosphorylated tyrosine-1150 domain species of the human insulin receptor found in this study during dephosphorylation supports correlations made between the tyrosine kinase activity of the rat and mouse insulin receptor and site occupancy during phosphorylation (White *et al.*, 1988; Flores-Riveros *et al.*, 1989) and the observation that proteolytic removal of the *C*-terminal domain does not affect the tyrosine kinase activity of the rat insulin receptor (Goren *et al.*, 1987). Evidence for a requirement of phosphorylation of all three tyrosines in the tyrosine-1150 domain for full activation of the tyrosine kinase is provided by studies with mutant insulin receptor molecules in which tyrosine residues 1146 or 1150 or 1150 and 1151 have been replaced by phenylalanine (Ellis *et al.*, 1986; Wilden *et al.*, 1990). These mutants exhibit a dramatic impairment of the ability of insulin to stimulate substrate phosphorylation. The results herein also provide evidence that the di- or mono-phosphorylated forms of the tyrosine-1150 domain and phosphorylation sites in the *C*-terminal domain and putative juxtamembrane domain do not act synergistically to induce appreciable (> 20%) tyrosine kinase activation.

The predominant (> 80%) form of the diphosphorylated

tyrosine-1150 domain species generated by the action of the rat liver particulate or soluble phosphatases is known to be phosphorylated on tyrosines 1146 and 1150 or 1151 (King & Sale, 1990), indicating that this species was deactivated. Additionally, this species has been trapped during phosphorylation by including anti-phosphotyrosine antibody and shown to have a tyrosine kinase activity comparable with that of the non-phosphorylated insulin receptor (White *et al.*, 1988). The remainder (15–20%) of the diphosphorylated tyrosine-1150 domain species present during dephosphorylation are known to be phosphorylated on both tyrosines 1150 and 1151 (King & Sale, 1990), but because of their small proportion, it was not possible to ascertain from this study whether this species played a role in tyrosine kinase activation. Flores-Riveros *et al.* (1989) have, however, argued that the tyrosine kinase of mouse insulin receptor, which is diphosphorylated on the corresponding vicinal tyrosine residues 1152 and 1153, is not activated. In the study of Flores-Riveros *et al.* (1989) the appearance of the diphosphorylated tyrosine-1150 domain species, of which approx. 37.5% were phosphorylated on the vicinal tyrosines, and the appearance of the diphosphorylated *C*-terminal domain species preceded tyrosine kinase activation during phosphorylation.

Phosphotyrosyl protein phosphatases purified using various artificial phosphotyrosyl protein substrates have been shown to be specific for dephosphorylating phosphotyrosyl residues in artificial proteins, and showed little activity against phosphoseryl/phosphothreonyl proteins (Roome *et al.*, 1988; Tonks *et al.*, 1988*a*; Swarup & Subrahmanyam, 1989). These results, findings that purified phosphotyrosyl protein phosphatases were not inhibited by inhibitor 2 (Tonks *et al.*, 1988*a*) and sequencing/peptide mapping studies (Tonks *et al.*, 1988*a*; Charbonneau *et al.*, 1989) indicate that phosphotyrosyl protein phosphatases comprise a unique family of enzymes distinct from the protein serine/threonine phosphatases. The phosphotyrosyl protein phosphatases have submicromolar K_m values for model substrates and specific activities equivalent to or greater than those of the type 1 or 2 serine/threonine phosphatases (Roome *et al.*, 1988; Tonks *et al.*, 1988*a*; Swarup & Subrahmanyam, 1989). The phosphotyrosyl protein phosphatases, therefore, exhibit a 20–50-fold higher affinity for protein substrates than do the serine/threonine phosphatases.

The particulate and soluble fractions containing phosphotyrosyl protein phosphatases used in this study will contain the native spectrum of phosphatases found *in vivo*. The rapid dephosphorylation of the triphosphorylated tyrosine-1150 domain species and deactivation of tyrosine kinase activity by phosphotyrosyl protein phosphatases may offer a sensitive mechanism for terminating or regulating insulin receptor tyrosine kinase action and insulin signalling. Moreover, the phosphotyrosyl protein phosphatase activity in cells is high [e.g. 10000 units/g in the particulate fraction of human placenta with phosphotyrosyl lysozyme as substrate under V_m conditions, compared with levels of 2600 units/g and 1300 units/g for protein phosphatases 1 and 2A respectively in skeletal muscle (Tonks *et al.*, 1988*b*)], and the phosphotyrosyl protein phosphatases have very high affinities for model substrates (see above). Thus the tyrosine kinase activity expressed by insulin-stimulated receptors is likely to be under tight control. This is supported by the low levels of the triphosphorylated form of the tyrosine-1150 domain found in insulin-stimulated Fao or H_4 rat hepatoma cells (White *et al.*, 1988; Tornqvist *et al.*, 1988), and the low activation state of the tyrosine kinase of the insulin receptor isolated from the insulin-stimulated Fao cells.

This work was supported by grants from the British Diabetic Association and the Wessex Medical School Trust. M. J. K. thanks the S.E.R.C. for a research studentship.

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Received 15 August 1990/19 November 1990; accepted 29 November 1990