Dephosphorylation of insulin-receptor autophosphorylation sites by particulate and soluble phosphotyrosyl-protein phosphatases

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Insulin stimulates autophosphorylation of the insulin receptor on multiple tyrosines in three domains: tyrosines 1316 and 1322 in the C-terminal tail, 1146, 1150 and 1151 in the tyrosine-1150 domain, and possibly 953, 960 or 972 in the juxtamembrane domain. In the present work the sequence of dephosphorylation of the various autophosphorylation sites by particulate and cytosolic preparations of phosphotyrosyl-protein phosphatase from rat liver was studied with autophosphorylated human placental insulin receptor as substrate. Both phosphatase preparations elicited a broadly similar pattern of dephosphorylation. The tyrosine-1150 domain in triphosphorylated form was found to be exquisitely sensitive to dephosphorylation, and was dephosphorylated 3–10-fold faster than the di- and monophosphorylated forms of the tyrosine-1150 domain or phosphorylation sites in other domains. The major route for dephosphorylation of the triphosphorylated tyrosine-1150 domain involved dephosphorylation of one of the phosphotyrosyl pair, 1150/1151, followed by phosphotyrosyl 1146 to generate a species monophosphorylated mainly (> 80%) at tyrosine 1150 or 1151. Insulin receptors monophosphorylated in the tyrosine-1150 domain disappeared slowly, and overall the other domains were completely dephosphorylated faster than the tyrosine-1150 domain. Dephosphorylation of the diphosphorylated C-terminal domain yielded insulin receptor in which the domain was singly phosphorylated at tyrosine 1322. Triphosphorylation of the insulin receptor in the tyrosine-1150 domain appears important in activating the receptor tyrosine kinase to phosphorylate other proteins. The extreme sensitivity of the triphosphorylated form of the tyrosine-1150 domain to dephosphorylation may thus be important in terminating or regulating insulin-receptor tyrosine kinase action and insulin signalling.

INTRODUCTION

The insulin receptor is an insulin-activated tyrosine-specific protein kinase (Kasuga *et al.*, 1982; Ullrich *et al.*, 1985; for review see Sale, 1988). Interference in the receptor kinase function either by site-directed mutagenesis or by microinjection of monoclonal antibodies prevents many of the biological responses to insulin, suggesting that the tyrosine kinase plays an important role in insulin signalling (Morgan & Roth, 1987; Ebina *et al.*, 1987; Chou *et al.*, 1987). Additionally, competing substrates and various inhibitors of the tyrosine kinase inhibit the ability of insulin to activate the insulin-receptor-associated serine kinase (Smith & Sale, 1988; Sale & Smith, 1989). The tyrosine kinase catalyses autophosphorylation of the β -subunit of the insulin receptor both *in vitro* and *in vivo* on at least six tyrosines clustered in three domains: tyrosines 1316 and 1322 near the C-terminus; tyrosines 1146, 1150 and 1151 in the tyrosine-1150 domain; and tyrosine(s) residing in a third domain, possibly 953, 960 or 972 located near the transmembrane domain (Tornqvist *et al.*, 1987, 1988; White *et al.*, 1988; Tavaré & Denton, 1988; Tavaré *et al.*, 1988). A further site or sites of tyrosine phosphorylation representing ~15% of the phosphate incorporated during autophosphorylation may be present (Tornqvist *et al.*, 1987). Autophosphorylation activates the tyrosine kinase to phosphorylate exogenous proteins and renders the tyrosine kinase constitutively active even when insulin is subsequently removed from the binding site (Rosen

et al., 1983; Yu & Czech, 1984; Tornqvist & Avruch, 1988). Consequently dephosphorylation, and not merely dissociation of insulin, is required to terminate tyrosine kinase activity. Autophosphorylation of the insulin receptor on all three tyrosines in the tyrosine-1150 domain appears to be necessary to activate the tyrosine kinase. In contrast, insulin receptor diphosphorylated in the tyrosine-1150 domain at tyrosines 1146 and 1150 or 1151 had the same activity as non-phosphorylated insulin receptor (White *et al.*, 1988). Thus the tyrosine-1150 domain seems to function as a regulatory domain. These observations are consistent with results of Tornqvist & Avruch (1988) showing that activation of the tyrosine kinase correlates with the formation of multiply phosphorylated insulin-receptor species.

The state of tyrosine autophosphorylation of the insulin receptor and its degree of activation *in vivo* will depend on the relative activities and phosphorylation-site specificities of the receptor kinase and the phosphotyrosyl-protein phosphatase(s) involved in dephosphorylation. The tyrosine kinase phosphorylates tyrosines in the tyrosine-1150 domain most rapidly. Phosphorylation of tyrosines in the C-terminal domain may proceed more slowly, and the tyrosines in the putative juxtamembrane domain may exhibit the slowest rate of phosphorylation (Tavaré & Denton, 1988; White *et al.*, 1988). The sequence of dephosphorylation will be a determinate of the timing of deactivation of the tyrosine kinase relative to the dephosphorylation of sites which may have other functions. Recently we have begun to characterize

phosphotyrosyl-protein phosphatases active against autophosphorylated insulin receptor (King & Sale, 1988a). Insulin-receptor phosphotyrosyl-protein phosphatase activity was found to be distributed among both particulate and soluble fractions of the cell, with the majority in the former. A similar distribution was obtained with ^{32}P -peptide 1142–1153 of the insulin receptor as substrate (King & Sale, 1988b). The major physiologically important phosphotyrosyl-protein phosphatases appear to belong to a novel class of phosphatases distinct from the enzymes which dephosphorylate phospho-seryl and -threonyl residues (King & Sale, 1988a; Tonks *et al.*, 1988b). In the present work phosphotyrosyl-protein phosphatase activity present in both particulate and soluble fractions of rat liver has been used to study the sequence of dephosphorylation of insulin-receptor autophosphorylation sites. This is important because these fractions contain the native spectrum of phosphotyrosyl-protein phosphatases found *in vivo*.

EXPERIMENTAL

Materials

Leupeptin and benzamidine were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Soya-bean trypsin inhibitor came from BDH Chemicals, Poole, Dorset, U.K. Cellulose thin-layer plates (20 cm $\times$ 20 cm) were from Kodak, Kirkby, Liverpool, U.K. Other chemicals and biochemicals were obtained from sources described in Smith *et al.* (1988).

Preparation of autophosphorylated insulin receptor

Insulin receptor was purified 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 15 min at 22 °C in 0.1–1 ml of a solution containing 50 mM-Hepes (pH 7.4), 2 mM-MnCl₂, 10 mM-MgCl₂, 100 nM-insulin and 0.1% Triton X-100 (Sale *et al.*, 1986). Phosphorylation reactions were initiated by addition of [γ - ^{32}P]ATP (250 μM ; 8–12 c.p.m./fmol) and were allowed to proceed at 22 °C for 30 min. Vanadate was omitted during phosphorylation, as it is a potent inhibitor of insulin-receptor phosphotyrosyl-protein phosphatases (King & Sale, 1988a). Additionally omission of both sodium vanadate and dithiothreitol decreased insulin-receptor phosphorylation on serine relative to autophosphorylation on tyrosine. Phosphoamino acid analysis of β subunits from preparations of insulin receptor phosphorylated in this study showed that $94 \pm 2\%$ (mean $\pm$ S.E.M., 5 observations) of ^{32}P recovered in phosphoamino acids was attached to tyrosine. Phosphorylation reactions were terminated by adding EDTA (16 mM), and samples were then used in dephosphorylations.

Dephosphorylation of autophosphorylated insulin receptor and preparation of tryptic phosphopeptides

Particulate and soluble fractions containing phosphotyrosyl-protein phosphatase activity were prepared from a rat liver homogenate by centrifugation at 100 000 *g* for 1 h as previously described (King & Sale, 1988a), 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 soya-bean trypsin

inhibitor/ml and a 1:1000 dilution of 2.5% (v/v) phenylmethanesulphonyl fluoride dissolved in propan-2-ol. The total phosphotyrosyl-protein phosphatase activity recovered in the particulate fractions was approx. 3 times that recovered in the soluble fractions for preparations used in this study. Autophosphorylated insulin receptors (1 mg of protein/ml) were incubated at 30 °C in 50 mM-Hepes (pH 7.4) with particulate or soluble extract (2 mg/ml) and the further addition of 2 μg of leupeptin/ml for the indicated times. A small degree of dephosphorylation of autophosphorylated insulin receptor was obtained in the absence of added phosphatase (King & Sale, 1988a). This endogenous phosphatase activity amounted to < 10% of the added particulate or soluble phosphatase activity. Dephosphorylation reactions were terminated by mixing 40 μl portions with 10 μl of SDS sample buffer [312.5 mM-Tris/HCl buffer (pH 7.4) containing 5% SDS, 75 mg of dithiothreitol/ml and 0.01% (w/v) Bromophenol Blue (Laemmli, 1970)], followed by boiling for 2 min. Sucrose was added to a final concentration of 200 mg/ml, and samples were analysed by SDS/polyacrylamide-gel electrophoresis on 4%-acrylamide stacking and 7.5%-acrylamide resolving gels (Laemmli, 1970). Electrophoresis was performed at 20 °C for approx. 3 h at 30–35 mA. Gels were stained for 20 min with 0.25% Coomassie Brilliant Blue in 50% (w/v) trichloroacetic acid, destained overnight in 5% acetic acid/45% methanol (both v/v) and dried *in vacuo* for 1 h at 80 °C. The region of the gel containing the β subunit of the insulin receptor was located by autoradiography, counted for radioactivity in 5 ml of scintillation fluid [5-(4-biphenyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole (6 g/l) in toluene], incubated with 10 ml of 20% methanol for 18 h at 37 °C and dried at 70 °C for 2 h in an oven. Then 2 ml of 50 mM-NH₄HCO₃ containing 100 μg of trypsin (treated with tosyl-phenylalanylchloromethane) 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 then freeze-dried.

Peptide mapping

The ^{32}P -labelled tryptic phosphopeptides were dissolved in 20 μl of water and separated on a cellulose thin-layer plate by electrophoresis at 400 V for 4 h at pH 3.5 (pyridine/acetic acid/water, 1:10:89, by vol.) in the first dimension and ascending chromatography (pyridine/acetic acid/butanol/water, 10:3:15:12, by vol.) in the second dimension (Tavaré & Denton, 1988). For phosphoamino acid analysis and protease-V8 cleavage of tryptic phosphopeptides purified by two-dimensional thin-layer mapping, the peptides were scraped off the plates and eluted by resuspension in either 1 ml of water (for peptides A1, A2, B1 and B3) or 1 ml of 33% (v/v) acetonitrile (for other peptides), followed by centrifugation (10 000 *g*, 2 min) and freeze-drying of the supernatants. Phosphoamino acid analysis was performed by hydrolysing the peptides in 6 M-HCl at 110 °C for 2 h. Phosphoamino acids were separated by electrophoresis on cellulose thin-layer plates at pH 3.5 for 1.5 h at 1 kV (Smith *et al.*, 1988). 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. Digestion of tryptic peptides with *Staphylococcus aureus* protease V8 was performed by

dissolving the peptides in 50 μ l of 50 mM-NH₄HCO₃, pH 7.8, containing 10 μ g of V8 protease/ml and incubating for 16 h at 30 °C. A further 50 μ l of buffer containing 10 μ g of V8 protease/ml was then added and incubation continued for a further 5 h. The samples were freeze-dried, and V8 peptides were separated by electrophoresis on cellulose thin-layer plates at 400 V for 2–3 h at pH 3.5 (pyridine/acetic acid/water, 1:10:89, by vol.). Autoradiography of thin-layer plates was performed at –70 °C for 2–7 days, by using Amersham Hyperfilm-MP within cassettes containing Dupont Cronex intensifier screens. ³²P associated with phosphopeptides and phosphoamino acids was determined by densitometric scanning of autoradiographs with a Joyce–Loebl Chromoscan 3 instrument, or by scraping the phosphopeptides or phosphoamino acids off the plates and counting for radioactivity in 5 ml of the toluene-based scintillant. The total yield of phosphopeptides from the β subunit was calculated from the ³²P present in the β -subunit and ratio of ³²P recovered in phosphopeptides. The amount (fmol) of each phosphopeptide derived from the C-terminal and tyrosine-1150 domains was then calculated by dividing the fmol of ³²P by the number of sites phosphorylated. For peptides C3 and C1' the numbers of sites phosphorylated are not known with certainty, and results are expressed in terms of fmol of ³²P. 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 AND DISCUSSION

Resolution of tryptic phosphopeptides by two-dimensional mapping

To analyse the state of phosphorylation of the insulin receptor, β subunits were isolated by SDS/polyacrylamide-gel electrophoresis and subjected to digestion with trypsin followed by two-dimensional thin-layer peptide mapping. A pattern of phosphopeptides broadly similar to that reported by Tavaré & Denton (1988) was obtained (Fig. 1). The identities of most of the major phosphotyrosyl tryptic peptides resolved by two-dimensional thin-layer mapping have been determined by Tavaré & Denton (1988), and these assignments are used in this paper (Table 1). The tyrosine-1150 domain, which contains tyrosines 1146, 1150 and 1151, was recovered as peptides monophosphorylated (C1), diphosphorylated (B2 and B3) and triphosphorylated (A1 and A2). The C-terminal domain, which contains tyrosines 1316 and 1322, was recovered as peptides diphosphorylated (B1) and monophosphorylated (NP1). Phosphotyrosyl peptides were also resolved which appeared to correspond to C3 and C1' described by Tavaré *et al.* (1988) and Tavaré & Denton (1988). The position of these phosphopeptides in the β subunit has not clearly been demonstrated. In dephosphorylation studies (see below) disappearance of C3 correlated with appearance of C1'. This observation and results of Tavaré & Denton (1988) are consistent with C3 and C1' being related and the possibility that C3 and C1' contain two and one tyrosines phosphorylated, respectively. Tavaré & Denton (1988) favour the possibility (Tornqvist & Avruch, 1988) that C3 and C1' both represent residues ~944–981 of the juxtamembrane

domain, which contains tyrosines 953, 960 and 972. A phosphopeptide corresponding to the position of C2 detected by Tavaré & Denton (1988) was barely evident. Digestion of tryptic phosphopeptides with *S. aureus* protease V8 and electrophoresis at pH 3.5 was used to confirm the identification of C1, B3, A1, A2, B1 as detailed by Tavaré & Denton (1988) (e.g. see Figs. 2 and 5 of the present paper) and that C3 and C1' were not derived from the tyrosine-1150 or C-terminal domains (results not shown). In previous work (Smith & Sale, 1989; Sale & Smith, 1989) the identities of phosphotyrosyl tryptic peptides were merely assumed on the basis of comparisons of the two-dimensional maps with those obtained by Tavaré & Denton (1988).

In the present work we have also detected a tryptic phosphopeptide that appears to be the monophosphorylated form of the C-terminal peptide (NP1); this phosphopeptide has not previously been detected. The net charge of this peptide at pH 3.5 would be expected to be +1.6, which is consistent with the mobility of NP1. NP1 produced by using particulate phosphatase was digested with protease V8 and subjected to electrophoresis at pH 3.5. This yielded a positively charged product (NP1b) that migrated identically with the V8 phosphopeptide, B1b, derived from the C-terminus of B1 (calculated net charge at pH 3.5 = +2.0); mobilities of NP1b and B1b relative to dinitrophenyl-lysine were 4.39 and 4.40 respectively (Fig. 2). A V8 phosphopeptide corresponding to the position of B1a (expected net charge at pH 3.5 = –1.4), which is derived from the N-terminal portion of B1, was not detected on digestion of NP1 with V8. This indicated that NP1 was predominantly phosphorylated on tyrosine 1322. NP1 produced by using soluble phosphatase gave similar results. NP1 was most evident after partial dephosphorylation, which may account for the failure to detect NP1 in previous studies during receptor autophosphorylation (Tornqvist *et al.*, 1987; White *et al.*, 1988; Tavaré & Denton, 1988).

Time course of dephosphorylation of insulin-receptor autophosphorylation sites

For optimum study of the sequence of dephosphorylation of autophosphorylated insulin receptor, receptor as near as possible equally phosphorylated in all phosphorylation sites was required as substrate. To facilitate maximum phosphorylation of the insulin receptor, a relatively high concentration of [γ -³²P]ATP was used (250 μ M) during phosphorylation, and the incubation time was extended to 30 min at 22 °C. Tryptic digestion of β -subunits isolated from autophosphorylated insulin receptor yielded an approximately equal molar ratio of phosphopeptides A1+A2:B1. The amount of B2+B3 was low (5–15% of the ³²P incorporated into the tyrosine-1150 domain). Only small amounts of the monophosphotyrosyl peptides, C1 and NP1, were present (<2% of the ³²P incorporated into the respective domains). This indicates that the insulin receptor was largely, but not 100%, phosphorylated in the C-terminal and tyrosine-1150 domains. As the numbers of sites phosphorylated in C3 and C1' are not known with certainty, the relative state of phosphorylation of this domain cannot be compared. This domain has been found to be more difficult to phosphorylate than the other domains in some previous studies (e.g. see White *et al.*, 1988).

Figs. 1, 3 and 4 show the time courses of de-

Fig. 1. Autoradiographs showing the time course of dephosphorylation of autophosphorylated insulin receptor by particulate phosphatase as analysed by two-dimensional mapping of tryptic phosphopeptides

Insulin receptor was autophosphorylated by preincubation with 100 nM-insulin, followed by incubation in the presence of 250 μ M-[γ - 32 P]ATP at 22 °C for 30 min. After termination of autophosphorylation by addition of EDTA, the autophosphorylated insulin receptor was incubated with particulate phosphatase extract (2 mg/ml) at 30 °C. At the indicated times, reactions were terminated by mixing samples with SDS sample buffer. The β -subunits of the insulin receptor were isolated by SDS/polyacrylamide-gel electrophoresis, digested with trypsin, and 32 P-labelled tryptic phosphopeptides were separated on cellulose t.l.c. plates by electrophoresis at pH 3.5 and ascending chromatography. The bottom right panel is a key to the identification of the 32 P-labelled tryptic phosphopeptides. The origin of sample application is marked by $\searrow$.

phosphorylation of the various phosphorylation sites in autophosphorylated insulin receptor by rat liver preparations of particulate and soluble phosphotyrosyl-protein phosphatase as analysed by two-dimensional mapping of tryptic phosphopeptides. In these experiments insulin-receptor autophosphorylations were terminated

by adding EDTA. Insulin-receptor phosphotyrosyl-protein phosphatases are known to be active in the presence of EDTA (King & Sale, 1988a). Both particulate and soluble phosphotyrosyl-protein phosphatase preparations were found to elicit a broadly similar pattern of dephosphorylation.

Table 1. Nomenclature of tryptic phosphopeptides separated by two-dimensional thin-layer peptide mapping and their V8 cleavage products

The nomenclature used and assignments of identity of phosphopeptides are based on work of Tavaré & Denton (1988). Possible sites of phosphorylation are underlined. Sites of V8 cleavage are indicated by the arrows.

Domain	Tryptic phosphopeptide	Number of phosphotyrosines	Sequence	Corresponding N-terminal protease-V8 phosphopeptide	Corresponding C-terminal protease-V8 phosphopeptide(s)
Tyrosine 1150 (contains tyrosines 1146, 1150 and 1151)	C1	1	↓ DI <u>Y</u> ETDYYRK (mixture of monophosphorylated forms)	C1a	C1b
	B2	2	↓ DI <u>Y</u> ETDYYRKGGK (mixture of diphosphorylated forms)	B2a	B2b (monophosphorylated) B2c (diphosphorylated)
	B3	2	↓ DI <u>Y</u> ETDYYRK (mixture of diphosphorylated forms)	B3a	B3b (monophosphorylated) B3c (diphosphorylated)
	A1	3	↓ DI <u>Y</u> ETDYYRKGGK	A1a	A1b
	A2	3	↓ DI <u>Y</u> ETDYYRK	A2a	A2b
	B1	2	↓ SYEEHIPYTHMNGGK	B1a	B1b
C-Terminal (contains tyrosines 1316 and 1322)	NP1	1	↓ SYEEHIPYTHMNGGK	Not detected	NP1b
Not known (perhaps juxta-membrane domain, which contains tyrosines 953, 960 and 972)	C3	Not known	Not known	Not known	Not known
	C1'	Not known	Not known	Not known	Not known

Fig. 2. Identification of NP1 by digestion with *S. aureus* protease V8 and electrophoresis

NP1 was obtained by incubating autophosphorylated insulin receptor (lacking NP1 on trypsinolysis) with particulate phosphatase extract for 2–25 min, followed by trypsinolysis of isolated β subunits and two-dimensional mapping as described in the legend to Fig. 1. NP1 phosphopeptides were scraped off the plates and pooled. B1 was isolated from autophosphorylated insulin receptor. The NP1 and B1 were digested with V8, electrophoresed on a thin-layer plate at pH 3.5 and autoradiographed. Arrows mark the position of the original undigested tryptic phosphopeptides, and the open circles mark the position of dinitrophenyl-lysine. Peptides: (a) B1 digested with protease V8, (b) NP1 digested with protease V8. The V8 phosphopeptides derived from B1 were present in a ratio of 1:0.6 (B1a: B1b), which is similar to that obtained by Tavaré & Denton (1988).

Dephosphorylation of triphosphorylated tyrosine-1150 domain. The most striking feature of the time courses of dephosphorylation was the rapid disappearance of tryptic phosphopeptides A1 and A2, which were derived from insulin receptor triphosphorylated in the tyrosine-1150 domain. For example, in Fig. 3, with the particulate phosphatase preparations, only 15% of insulin receptors triphosphorylated in the tyrosine-1150 domain remained after 2 min incubation, whereas 70–80% of insulin receptors diphosphorylated in the C-terminal domain or yielding C3 remained. Based on a total of five experiments with particulate and soluble phosphatase, the rate of disappearance of A1 + A2 was (mean $\pm$ S.E.M.) 4.64 ± 0.99 and 3.54 ± 0.45 times that of B1 and C3 respectively.

Analysis of diphosphorylated tyrosine 1150-domain. Disappearance of phosphopeptides A1 and A2 was associated with increases in B2 and B3, which were the products of insulin receptor diphosphorylated in the tyrosine-1150 domain. B2 and B3 disappeared significantly more slowly than A1 + A2. The maximum rate of disappearance of B2 + B3 was 6.95 ± 2.0 -fold lower than for A1 + A2 (mean $\pm$ S.E.M. for a total of five experiments with particulate and soluble phosphatase). B3 and B2 were present in a ratio of $\sim 25:1$.

B3 isolated from autophosphorylated insulin receptor incubated with phosphatase extracts was digested with *S. aureus* V8 proteinase, which cleaves B3 between residues 1146 and 1150/1151, presumably at the C-terminal side of glutamate 1147 (Tavaré & Denton, 1988). Cleavage of

Fig. 3. Graphs showing the time course of dephosphorylation of autophosphorylated insulin receptor by particulate phosphatase as analysed by two-dimensional mapping of tryptic phosphopeptides

(a) Tryptic phosphopeptides derived from the C-terminal domain. (b) Tryptic phosphopeptides derived from the tyrosine-1150 domain. (c) Tryptic phosphopeptides C3 and C1'. Details are as described in the legend to Fig. 1. The data are representative of three experiments on two preparations of insulin receptor.

Fig. 4. Graphs showing the time course of dephosphorylation of autophosphorylated insulin receptor by soluble phosphatase as analysed by two-dimensional mapping of tryptic phosphopeptides

(a) Tryptic phosphopeptides derived from the C-terminal domain. (b) Tryptic phosphopeptides derived from the tyrosine-1150 domain. (c) Tryptic phosphopeptides C3 and C1'. Autophosphorylated insulin receptor was incubated with soluble phosphatase extract (2 mg/ml) and analysed as described in the legend to Fig. 1. The data are representative of two experiments on different preparations of insulin receptor.

the B3 samples with V8 and electrophoresis at pH 3.5 yielded three new phosphopeptides, B3a, B3b and B3c (Fig. 5), of mobilities identical with those of the V8 phosphopeptides A2a/C1a (net charge at pH 3.5 approx. -1.7), C1b (net charge at pH 3.5 approx. +0.5), and A2b (net charge at pH 3.5 approx. -0.5) respectively. B3a,

B3b and B3c thus appear to be phosphorylated on tyrosine 1146, tyrosine 1150 or 1151, and tyrosines 1150 and 1151, respectively. B3 obtained from autophosphorylated insulin receptor incubated with particulate phosphatase for various times yielded in each case a near-identical ratio of the V8 phosphopeptides; the mean proportions (for seven samples) of ³²P were 1:0.91:0.36 (B3a:B3b:B3c). Similarly B3 obtained from autophosphorylated insulin receptor incubated with soluble phosphatase for various times yielded a mean ³²P distribution proportion of 1:0.92:0.49 (mean of seven samples). Thus between approx. 15.2 and 19.7% of diphosphorylated tyrosine-1150 domain species were phosphorylated on both tyrosines 1150 and 1151. This proportion is similar to that detected during the phosphorylation of human placental insulin receptor in a study by Tavaré & Denton (1988), although the species was undetected during phosphorylation in studies by Tornqvist *et al.* (1987) using the human placental insulin receptor, and by White *et al.* (1988) using insulin receptor partially purified from Fao rat hepatoma cells. The detection during dephosphorylation of diphosphorylated tyrosine-1150 domain species containing phosphorylated and non-phosphorylated tyrosine 1146 suggests that dephosphorylation of phosphotyrosyl 1146 with respect to the phosphotyrosyl pair, 1150 and 1151, is not strictly ordered. Whether dephosphorylation of phosphotyrosyl residues 1150 and 1151 with respect to each other is ordered or random cannot be deduced, because we cannot discriminate between phosphorylation of tyrosine 1150 and 1151.

Analysis of monophosphorylated tyrosine-1150 domain.

In the latter stages of dephosphorylation, appreciable amounts of insulin receptor in which the tyrosine-1150 domain was monophosphorylated were produced. C1 obtained from autophosphorylated insulin receptor incubated with particulate phosphatase yielded on V8 digestion and electrophoresis at pH 3.5 two products (Fig. 5) containing ³²P in a ratio of 1:0.18 (C1b:C1a; mean of four samples). Similarly, analysis of C1 obtained from autophosphorylated insulin receptor incubated with soluble phosphatase yielded a ³²P distribution ratio of 1:0.14 (C1b:C1a; mean of eight samples). This indicates that the insulin receptor containing monophosphorylated tyrosine-1150 domain produced by dephosphorylation by particulate or soluble phosphatase was phosphorylated predominantly on tyrosine 1150 or 1151, and that conversion of the diphosphorylated tyrosine 1150 species into the monophosphorylated form predominantly involved dephosphorylation of phosphotyrosyl 1146. Thus, although the phosphatases very rapidly removed the first phosphate from the phosphotyrosyl pair 1150/1151 present in the triphosphorylated tyrosine-1150 domain species, removal of the second phosphate from the 1150/1151 pair occurred rather slowly, and more slowly than dephosphorylation of phosphotyrosyl 1146. Insulin receptor containing monophosphorylated tyrosine-1150 domain disappeared rather slowly (Figs. 1, 3 and 4). This was significant, because it means that the C-terminal domain was completely dephosphorylated more rapidly than the tyrosine-1150 domain. For example, in Fig. 3, after 25 min incubation with the particulate phosphatase 93% of the C-terminal domain was completely dephosphorylated, compared with only 54% of the tyrosine-1150 domain. Similarly, C3 was largely

Fig. 5. Protease-V8 digestion of B3 and C1 derived from autophosphorylated insulin receptor incubated with particulate or soluble phosphatase

Insulin receptor was autophosphorylated and then incubated with particulate or soluble phosphatase extract (2 mg/ml) for times that maximized yields of B3 and C1, as described in the legend to Fig. 1. β subunits were digested with trypsin, followed by isolation of B3 and C1 by two-dimensional peptide mapping. The B3 and C1 along with A2 from autophosphorylated insulin receptor as a control were digested with protease V8, electrophoresed on a thin-layer plate at pH 3.5 and autoradiographed. Densitometric scans at 625 nm of the results are shown. Arrows mark the positions of the original undigested tryptic phosphopeptides. Dinitrophenyl-lysine marker migrated approx. 1 cm towards the cathode. Protease-V8 digests of: (a) C1, particulate phosphatase; (b) B3, particulate phosphatase; (c) A2; (d) C1, soluble phosphatase; (e) B3, soluble phosphatase.

absent after 25 min incubation of autophosphorylated insulin receptor with either the particulate or soluble phosphatase fraction. Disappearance of C3 correlated with the appearance of C1'. This was consistent with the possibility (Tavare & Denton, 1988) that C3 and C1' represented di- and mono-phosphorylated forms of the same peptide. After 25 min incubation with the particulate phosphatase, approx. 5 times as much ^{32}P remained in B2 + B3 + C1 compared with C3 + C1'. Thus the domain yielding C3 and C1' also appears to be completely dephosphorylated more rapidly than the tyrosine-1150 domain. Incubation of autophosphorylated insulin receptor separated from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$

by gel filtration (King & Sale, 1988a) with particulate or soluble extracts also resulted in a slow rate of disappearance of C1 (not shown). Therefore the slow rate of disappearance of C1 was not due to incomplete inhibition of autophosphorylation by EDTA.

Analysis of C-terminal domain. Dephosphorylation of the diphosphorylated C-terminal domain by the particulate or soluble phosphatase fractions appeared to occur specifically at tyrosine 1316, and thus may be ordered, resulting in production of receptor containing this domain singly phosphorylated at tyrosine 1322 (see above). However, as the amount of NP1 only reached a

maximum of ~30% of the initial amount of B1, this indicates that phosphotyrosyl 1322 is also quite susceptible to dephosphorylation.

General discussion

Phosphotyrosyl-protein phosphatase activity in both particulate and soluble fractions from rat liver was shown to elicit a broadly similar pattern of dephosphorylation of insulin-receptor autophosphorylation sites whether analysed by two-dimensional tryptic-peptide mapping or further cleavage of tyrosine-1150 and C-terminal domain tryptic phosphopeptides with V8 protease. Although the soluble and particulate fractions may each contain more than one species of phosphotyrosyl-protein phosphatase (Tonks *et al.*, 1988a), the overall phosphatase specificity of the two fractions towards insulin-receptor autophosphorylation sites was thus similar. This is consistent with the proposal that cytosolic phosphotyrosyl-protein phosphatases have similar membrane-bound counterparts (Tonks *et al.*, 1988b).

The triphosphorylated form of the tyrosine 1150 domain was found to be 3–10-fold more sensitive to dephosphorylation compared with the di- and monophosphorylated forms of the tyrosine-1150 domain or phosphorylation sites in other domains. This may account for the low levels of the triphosphorylated tyrosine-1150 domain detected in insulin-stimulated Fao or H₄ rat hepatoma cells (White *et al.*, 1988; Tornqvist & Avruch, 1988), although in two cell lines transfected with insulin-receptor cDNA (CHO.T and NIH 3T3 HIR3.5 cells) insulin stimulated the formation of larger amounts of the triphosphorylated species (Tavaré *et al.*, 1988).

The exquisite sensitivity of the triphosphorylated tyrosine-1150 domain species to dephosphorylation is of particular significance, as evidence has been obtained that this species plays a critical role in insulin-receptor tyrosine kinase activation (White *et al.*, 1988). In contrast, insulin receptor diphosphorylated in the tyrosine-1150 domain at tyrosines 1146 and 1150 or 1151 had the same activity as non-phosphorylated insulin receptor. Of the diphosphorylated tyrosine-1150 domain species detected during dephosphorylation, 80–85% were of this type. Only 15–20% of the diphosphorylated tyrosine-1150 domain species detected during dephosphorylation were phosphorylated at both the vicinal pair of tyrosines, 1150 and 1151; whether this phosphorylated species is active or inactive has not been studied. Removal of the C-terminal phosphorylation sites by mild trypsinolysis does not affect the phosphotransferase activity of the β subunit, and phosphorylation of sites residing in the putative juxtamembrane domain is not necessary to achieve activation of the insulin-receptor tyrosine kinase (White *et al.*, 1988). Thus the rapid dephosphorylation of the triphosphorylated tyrosine-1150 domain species by phosphotyrosyl-protein phosphatase may offer a sensitive mechanism for terminating or regulating insulin-receptor tyrosine kinase action and insulin signalling. Attenuation of insulin-receptor tyrosine kinase action may decrease the phosphorylation both of other sites on the insulin receptor as well as other proteins (White *et al.*, 1988). Moreover, the activities of the phosphotyrosyl-protein phosphatases may themselves be controlled.

The possibility arises that non-tyrosine-kinase-activated but phosphorylated insulin-receptor species may

be formed *in vivo* by phosphotyrosyl-protein phosphatase action and may persist after deactivation of the tyrosine kinase. Insulin receptors with a C-terminal truncation that lack autophosphorylation sites 1316 and 1322 show intact tyrosine kinase activity and normal internalization, but are defective in signalling metabolic effects (2-deoxy-D-glucose uptake or glycogen synthase activation) (McClain *et al.*, 1988; Maegawa *et al.*, 1988). Thus phosphorylated insulin receptors in which the tyrosine kinase is deactivated may continue to transmit insulin signals.

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