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Assay of phosphotyrosyl protein phosphatase using synthetic peptide 1142–1153 of the insulin receptor

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Synthetic peptide 1142–1153 of the insulin receptor was phosphorylated on tyrosine by the insulin receptor and found to be a potent substrate for dephosphorylation by rat liver particulate and soluble phosphotyrosyl protein phosphatases. Apparent K_m values were ~5 μM. V_m values (nmol phosphate removed/min per mg protein) were 0.62 (particulate) and 0.2 (soluble). This corresponds to 80% of total activity being membrane-associated, indicating that membrane-bound phosphatases are important receptor phosphatases. The phosphatase activities were distinct from acid and alkaline phosphatase. In conclusion peptide 1142–1153 provides a useful tool for the further study and characterization of phosphotyrosyl protein phosphatases.

Insulin; Receptor; Protein phosphorylation; Phosphopeptide; Tyrosine kinase; Protein phosphatase

1. INTRODUCTION

The insulin receptor is an insulin-activated, tyrosine-specific protein kinase which catalyzes the autophosphorylation of tyrosine residues in its own β-subunit [1–3]. Autophosphorylation activates the tyrosine kinase to phosphorylate other proteins [4,5]. This activated state is maintained even after dissociation of bound insulin and consequently dephosphorylation is required to terminate tyrosine kinase activity. Thus, the phosphotyrosyl protein phosphatase(s) which catalyzes dephosphorylation of the insulin receptor plays an important function in terminating the insulin signal and in the overall scheme of insulin action.

Study of phosphotyrosyl protein phosphatase(s) with activity against the insulin receptor has been hampered by the low amounts of receptor routinely available and the lack of a suitable alternate specific substrate. Studies in other systems on phosphotyrosyl protein phosphatases have utilized artificial substrates like casein or bovine serum albumin that have been phosphorylated on tyrosine [6–13]. However, phosphotyrosine in these substrates may not be present within protein sequences representative of the insulin receptor raising the possibility that phosphatases with the highest activity and specificity towards the insulin receptor may be missed. Most ideal would be to use as substrate a synthetic peptide corresponding to the phosphorylation sites of the insulin receptor. However, a previous study showed that a phosphotyrosyl peptide derived from $pp60^{src}$ was not dephosphorylated by two species of cytosolic phosphotyrosyl protein phosphatase and it was concluded that more extensive tertiary structure than afforded by short peptides was required for recognition by phosphotyrosyl protein phosphatase [14].

In the present work we show that peptide 1142–1153 of the insulin receptor which contains three of the tyrosine residues autophosphorylated in the insulin receptor (Tyr 1146, 1150, 1151) is after phosphorylation a highly effective substrate for phosphotyrosyl protein phosphatase. The use

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of phosphotyrosyl peptide in phosphatase assays and properties of rat liver particulate and soluble phosphatase activity against the phosphotyrosyl peptide are described.

2. MATERIALS AND METHODS

Sources of materials and methods were as described in [16,17] except where detailed below.

2.1. *Preparation of ^{32}P-labelled peptide 1142–1153*

Peptide 1142–1153, 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 using a solid-phase method in collaboration with Dr R. Sharma of this department. The peptide (200 μM) was monophosphorylated as described in [15] by incubation for 90 min at 22°C with human placental insulin receptor [16], 150 nM insulin, 200 μM [γ-^{32}P]ATP (2–4 cpm/fmol), 2 mM $MnCl_2$, 10 mM $MgCl_2$, 0.3 M NaCl in 30 mM Hepes (pH 7.4)/0.07% Triton X-100. Under these conditions approx. 90% of ^{32}P incorporated into the peptide was located at Tyr 1150 and the stoichiometry of phosphorylation was approx. 0.1 mol/mol peptide. ^{32}P-peptide 1142–1153 was separated from non-peptide-bound ^{32}P by chromatography on AG 1-X2 acetate (Bio-Rad) columns [14] using 1 M acetic acid and lyophilized.

2.2. *Dephosphorylation assay*

Particulate and soluble fractions were prepared from a rat liver homogenate by centrifugation at 100 000 × *g* for 1 h as in [17]. ^{32}P-peptide 1142–1153 at the concentrations indicated was incubated at 30°C in 50 mM Hepes (pH 7.4)/0.2 mM dithiothreitol with particulate or soluble extract (0.25–1 mg/ml) in a final volume of 30–100 μl. Other additions of metal ions, EDTA, calmodulin or sodium vanadate were present where indicated. Loss of $^{32}P_i$ from the peptide was measured by mixing aliquots of the reaction mixture with an equal volume of 10% (w/v) trichloroacetic acid, centrifuging 10 000 × *g*, 5 min) and spotting 15-μl aliquots of the supernatant onto 1-cm squares of Whatman P81 phosphocellulose paper [18]. The papers were washed in 0.42 mM acetic acid (pH 3.5) at 70–75°C for 4 min and rinsed in distilled water. ^{32}P-peptide 1142–1153 bound to the papers was measured by placing the papers in 5 ml scintillation fluid [5-(4-biphenylyl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole (6 g/l) in toluene] and counting. Alternatively, $^{32}P_i$ released from ^{32}P-peptide 1142–1153 was measured using a procedure [19] which measured ^{32}P-labelled P_i as opposed to peptide-bound ^{32}P. With this method, incubations were terminated by adding 3 vols of 10% (w/v) trichloroacetic acid and centrifuging (10 000 × *g*, 5 min). A 70 μl aliquot of the supernatant was mixed with 0.14 ml of 1.25 mM KH_2PO_4 in 1.0 N H_2SO_4 and 0.35 ml isobutanol/benzene (1:1) was added. The solutions were mixed and 70 μl of 5% ammonium molybdate was added. After further mixing the organic and aqueous phases were allowed to separate and 0.1 ml of the organic phase counted. The procedure was performed at 0–4°C. Both methods gave similar results. Reaction rates were linear up to 15% phosphate released from the peptide, and the extent of dephosphorylation was therefore kept within this limit in quantitative assays. Concentrations of phosphotyrosyl peptide were calculated from ^{32}P content and specific activity of [γ-^{32}P]ATP.

3. RESULTS AND DISCUSSION

The ability of phosphotyrosyl protein phosphatase(s) to dephosphorylate [^{32}P]phosphotyrosyl peptide 1142–1153 was studied using rat liver particulate and soluble fractions. Recently, we have shown that both these subcellular fractions contain a high activity of phosphatase towards phosphotyrosyl insulin receptor [17]. Additionally, properties of the phosphatase activities against phosphotyrosyl insulin recpetor were characterized. Thus, rat liver particulate and soluble fractions provide an excellent source of phosphatase activity for analysing the viability of ^{32}P-peptide 1142–1153 as a suitable substrate.

Fig.1 shows that ^{32}P-peptide 1142–1153 was dephosphorylated rapidly by both particulate and soluble fractions from rat liver. For comparison, dephosphorylation of the peptide by calmodulin-dependent protein phosphatase (also called protein phosphatase 2B or calcineurin), an enzyme reported to dephosphorylate tyrosyl residues in autophosphorylated epidermal growth factor receptor and other proteins [20,21] was assessed. ^{32}P-peptide 1142–1153 was a poor substrate for calmodulin-dependent protein phosphatase even at high concentrations of the phosphatase (0.1 mg/ml). This observation supports the idea that calmodulin-dependent protein phosphatase is not a major phosphotyrosyl insulin receptor phosphatase [17]. Plots of initial rates of ^{32}P removal from the peptide vs amount of phosphatase activity added were linear (not shown).

The properties of phosphotyrosyl protein phosphatase activity against ^{32}P-peptide 1142–1153 were examined (table 1). Both particulate and soluble phosphatase activities were inhibited >90% by Zn^{2+} (500 μM) or vanadate (20 μM) but were not inhibited by EDTA (1 mM). Activity in the presence of EDTA [22] and inhibition by vanadate [23–25] show that these phosphotyrosyl protein phosphatases are distinct from alkaline phosphatase and acid phosphatase. Mn^{2+}, Ca^{2+} and Mg^{2+} at 1 mM did not profoundly alter phosphatase activity. Ni^{2+} (1 mM) inhibited phosphatase activity 76–52%. These characteristics of rat liver particulate and soluble phosphotyrosyl

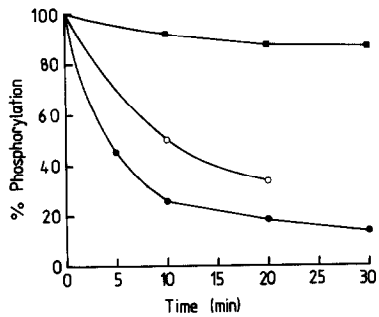

Fig.1. Time courses for dephosphorylation of ^{32}P-peptide 1142–1153. Incubations contained phosphotyrosyl peptide (1 μM) and 1.0 mg/ml of rat liver particulate (●) fraction, soluble fraction (○) or 0.1 mg/ml of purified bovine brain calmodulin-dependent protein phosphatase (■). At the indicated times peptide-bound ^{32}P was measured using the phosphocellulose paper square method. Calmodulin-dependent protein phosphatase was activated by preincubation at 30°C for 20 min with 1 mM Mn^{2+}/1 mM Ca^{2+}/1 μM calmodulin prior to use. The same mixture of metal ions and calmodulin was present in the incubations with calmodulin-dependent protein phosphatase. Incubations with rat liver fractions contained 0.8 mM EDTA.

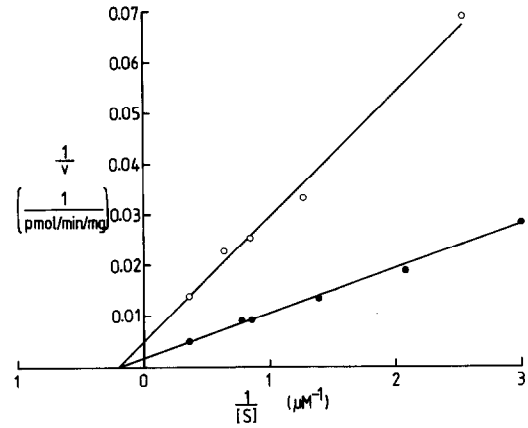

Fig.2. Lineweaver-Burk plot for dephosphorylation of ^{32}P-peptide 1142–1153 by rat liver particulate (●) and soluble phosphatases (○). Various concentrations of phosphotyrosyl peptide (0.3–2.8 μM) were incubated with 0.25 mg/ml of particulate or soluble fraction in the presence of 0.5 mM EDTA. After 1, 2, 5 and 10 min of incubation aliquots were assayed for $^{32}P_i$ released using the molybdate method and initial rates of $^{32}P_i$ release calculated.

protein phosphatase activity against ^{32}P-peptide 1142–1153 parallel those found using autophosphorylated insulin receptor in 0.1% Triton X-100 as substrate [17].

Apparent K_m values of particulate and soluble phosphatase activity for ^{32}P-peptide 1142–1153 were not significantly different (5.4 ± 0.8 and 4.8 ± 0.6 μM, respectively; means ± SE, 3 observations; fig.2). In Lineweaver-Burk plots there was no evidence of curvature, indicating the absence of phosphatases of markedly differing affinities in either fraction. Additionally, it is noteworthy that several species of phosphotyrosyl protein phosphatase resolved from both particulate and soluble phases of human placenta all had identical K_m values for phosphotyrosyl lysozyme [13]. V_m values of particulate and soluble phosphatase activity towards ^{32}P-peptide 1142–1153 were 0.62 ± 0.13 and 0.2 ± 0.05 nmol ^{32}P removed/min per mg protein, respectively (means ± SE, 3 observations). After correcting for yields of particulate and soluble fractions this corresponds to 80 ± 5% (means ± SE, 3 observations) of phosphatase activity being membrane-associated. This proportion is similar to that measured directly using either autophosphorylated insulin or EGF receptor as substrate [17], but significantly higher than that reported in other studies which have utilized artificial substrates like phosphotyrosyl bovine serum albumin [11,26].

Table 1

Effect of various agents on rat liver particulate and soluble phosphatase activity against ^{32}P-peptide 1142–1153

Conditions	pmol $^{32}P_i$ released/min per mg	
	Soluble	Particulate
Control	71.3	208
Mg^{2+} (1 mM)	92.2	225
Ca^{2+} (1 mM)	87.0	230
Mn^{2+} (1 mM)	68.6	204
Ni^{2+} (1 mM)	22.0	111
Na_3VO_4 (20 μM)	0.0	23
Zn^{2+} (500 μM)	0.6	11
EDTA (1 mM)	67.5	176

Phosphotyrosyl peptide (2.5 μM) was incubated with 0.25 mg/ml of particulate or soluble fraction in the presence of the indicated additions for 5 min. $^{32}P_i$ released from the peptide was determined by the molybdate method

In conclusion, phosphotyrosyl peptide 1142–1153 has been shown to be an efficient substrate for dephosphorylation by phosphotyrosyl protein phosphatase(s). Thus, extensive tertiary structure is not a prerequisite for interaction of all

phosphotyrosyl protein phosphatases with their substrates. Properties of rat liver phosphatases active against the peptide paralleled those active against autophosphorylated insulin receptor. Thus, phosphotyrosyl peptide 1142–1153 is an excellent substrate for the further study and characterization of phosphotyrosyl protein phosphatases. The phosphatases exhibited high affinities for the peptide. This may be of significance in facilitating dephosphorylation of receptors present at low concentrations in cells. Potent phosphatase activity against phosphotyrosyl peptide 1142–1153 was found in both cytosolic and membrane fractions of rat liver with the majority (80%) in the latter. This places the phosphatase in the correct location to interact with the insulin receptor.

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