RESEARCH COMMUNICATION

Regulation of an hepatic low- M_r membrane-associated protein-tyrosine phosphatase

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Protein-tyrosine phosphatases (PTPases), active against autophosphorylated insulin and epidermal growth factor (EGF) receptors in rat liver, are predominantly membrane associated. Fasting of rats for 48 h decreased hepatic particulate PTPase activity by 15.0–26.9%. This reduction in particulate PTPase activity was due to a rather specific decrease in activity of > 85% of a single species of PTPase, termed PTPase I. Disappearance of PTPase I activity from the particulate fraction was not accounted for by its translocation to the cytosol. PTPase I displayed the highest activity against autophosphory-

lated insulin and EGF receptors, relative to activity against a ^{32}P -labelled peptide substrate, of three PTPases resolved from the liver particulate fraction. The M_r value of PTPase I, as determined by gel filtration on a Superose 12 column was approx. 42000, indicating that PTPase I belongs to the low- M_r class of PTPases. An antibody raised against PTPase 1B, the prototype of this class of PTPases, did not react with PTPase I in Western blots. The potential importance of the novel change in activity of PTPase I in the regulation of insulin-receptor signal transduction is discussed.

INTRODUCTION

Binding of insulin to the α -subunit of the insulin receptor stimulates the autophosphorylation of the β -subunit of the receptor on tyrosine residues (see Sale, 1988 for review). Attainment of the trisphosphorylated form in the tyrosine-1150 or regulatory domain of the β -subunit correlates with a marked activation of the ability of the tyrosine kinase to phosphorylate other substrates (White et al., 1988; Flores-Riveros et al., 1989; King et al., 1991). The tyrosine-1150 domain in the trisphosphorylated form is also, relative to other phosphorylated forms of the insulin receptor, exquisitely sensitive to dephosphorylation by protein-tyrosine phosphatases (PTPases) present in rat liver particulate and soluble extracts and also to certain isolated PTPases, including PTPase 1B and 1A purified from human placenta (King and Sale, 1990; King et al., 1991; Tappia et al., 1991; Sale, 1991, 1992; Hashimoto et al., 1992a). The rapid dephosphorylation of the trisphosphorylated tyrosine-1150-domain species of the insulin receptor, and the accompanying deactivation of tyrosine kinase activity by PTPases, may offer a sensitive mechanism for regulating or terminating insulin-receptor tyrosine kinase action. This pivotal position of the PTPases makes them an ideal target for regulation.

PTPases exist as two structurally distinct types (Fischer et al., 1991). Transmembrane PTPases, such as CD45, LAR and PTP α usually consist of two conserved phosphatase domains, a transmembrane region and a non-conserved extracellular domain. In many cases the extracellular domains are receptor-like and thought to transduce as-yet-undefined signals. Non-transmembrane PTPases, like PTPase 1B and T-cell PTPase, possess a single catalytic domain.

In this work the regulation of PTPases in rat liver by fasting and streptozotocin-induced diabetes is studied. The results show that the activity of a specific species of particulate PTPase is

markedly reduced in response to fasting. The implications of this to the control of insulin signalling are discussed.

EXPERIMENTAL

Materials

Poly(L-lysine)-agarose and a glucose assay kit were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Superose 12 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Anti-(PTPase 1B) antibody, prepared by immunizing rabbits with a recombinant glutathione S-transferase fusion protein corresponding to the N-terminal region of human PTPase 1B (amino-acid residues 1–158; Frangioni et al., 1992), was from TCS Biologicals Ltd., Botolph Claydon, Bucks., U.K. The enhanced chemiluminescence (ECL) kit was purchased from Amersham International, Amersham, Bucks., U.K. Other biochemicals and chemicals were obtained from sources described in King and Sale (1990) or Smith et al. (1988).

Preparation of rat liver particulate fractions

Livers were obtained from male albino Wistar rats (250 g body wt.). Rats were allowed free access to food and water, except for fasted animals, which were deprived of food for 48 h before use. Rats were on a normal light/dark cycle, and in the case of the fasted animals food was removed at 10:00 h. Diabetes was induced by the intravenous injection of streptozotocin (60 mg/kg) into a femoral vein under diethyl ether anaesthesia and confirmed 48–140 h later by monitoring the concentration of blood glucose at the time of removal of the liver. Preparation of particulate fractions was performed at 4 °C as described by King and Sale (1988a), except that 10 mM imidazole/HCl buffer (pH 7.2) containing 10 mM EDTA, 0.1% (v/v) 2-mercaptoethanol, 0.02% (w/v) phenylmethanesulphonyl fluoride, 1 mM

Abbreviations used: EGF, epidermal growth factor; PTPase, protein-tyrosine phosphatase (EC 3.1.3.48); MBP, myelin basic protein; RCM, reduced, carboxyamidomethylated and maleylated.

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benzamidine, 0.01 % (v/v) Brij 35, 1 $\mu\text{g/ml}$ leupeptin and 20 mM NaF was used and the particulate pellet was solubilized before use. Solubilization was achieved by manual homogenization in the above buffer containing 1 % (v/v) Triton X-100 using a Teflon/glass homogenizer, stirring for 1 h, centrifugation at 100000 g for 1 h and filtration through glass wool.

Poly(L-lysine)-agarose chromatography and f.p.l.c.

A poly(L-lysine)-agarose column (10 ml bed volume) was equilibrated with the above Triton X-100-containing buffer and the solubilized particulate fraction (45 mg of protein) recycled three times through the column at 4 °C. After washing with 2 bed vol. of the Triton X-100-containing buffer the column was eluted with a linear 0–1 M NaCl gradient (30 ml) at 4 °C in the same buffer, at a flow rate of 0.04 ml/min. A Superose 12 column (20 ml bed volume) was equilibrated with 3 bed vol. of 20 mM Tris/HCl, pH 7.4, containing 0.1 mM EDTA, 140 mM NaCl and 1 mM dithiothreitol at a flow rate of 0.4 ml/min. PTPase peaks (200 μl ; approx. 60 μg of protein) eluted from the poly(L-lysine)-agarose column were then applied and the Superose 12 chromatography was performed at 0.5 ml/min.

Western blotting

Samples were separated by SDS/PAGE on 4 % (w/v) acrylamide/0.27 % bisacrylamide stacking and 10 % (w/v) acrylamide resolving gels (Laemmli, 1970) using a Bio-Rad Mini Gel apparatus with Pyronin Y as the dye-front marker and transferred (30 V, 30–60 min, 22 °C) to nitrocellulose membranes (0.45 μm pore size) using 20 % (v/v) methanol/25 mM Tris base/192 mM glycine, pH 8.3, as the transfer buffer. Protein marker lanes were removed, stained with Amido Black [0.1 % (w/v) in 45 % (v/v) methanol/10 % (v/v) acetic acid] and destained with several changes of 45 % (v/v) methanol/5 % (v/v) acetic acid. The remainder of the nitrocellulose membranes were then incubated for 60 min at 22 °C with shaking in blocking buffer [10 mM Tris/HCl, pH 7.5 (4 °C), containing 1.88 mM EDTA, 133 mM NaCl, 0.5 % (w/v) BSA and 0.5 % (v/v) Triton X-100] followed by incubation (2 h, 22 °C, with shaking) with 0.5 $\mu\text{g/ml}$ anti-(PTPase 1B) antibody diluted in blocking buffer. After three 10-min-long washes (22 °C, with shaking) with blocking buffer membranes were incubated (22 °C, 1 h, with shaking) with a 1:5000 dilution of sheep anti-(rabbit IgG) antibody conjugated to horseradish peroxidase in blocking buffer followed by three 10-min-long washes (22 °C, with shaking) with 50 mM Tris/HCl, pH 7.5 (4 °C), containing 100 mM NaCl and 0.1 % (v/v) Tween 20. Blots were then visualized by the enhanced chemiluminescence technique, which was used according to the manufacturer's instructions.

Assays

PTPase activity was assayed against ^{32}P -labelled phosphotyrosyl peptide 1142–1153 (0.1–0.6 μM) of the insulin receptor and ^{32}P -labelled phosphotyrosyl reduced, carboxyamidomethylated and maleylated (RCM) lysozyme (7 μM) as described previously (Tappia et al., 1991). Assays with [^{32}P]phosphotyrosyl myelin basic protein (MBP) (5 μM) were as described by Gruppiso et al. (1991). Dephosphorylation of ^{32}P -labelled autophosphorylated insulin and epidermal growth factor (EGF) receptors with the indicated amounts of PTPase activity were performed as de-

scribed by King and Sale (1990). ^{32}P , which was associated with the receptors, was determined by SDS/PAGE, autoradiography and densitometric scanning of autoradiographs with a Joyce-Loebl Chromoscan 3 instrument (King and Sale, 1990). Protein was assayed by the Bradford assay using BSA as standard. DNA was assayed as described by Richards (1974).

RESULTS AND DISCUSSION

The majority (70–80 %) of hepatic PTPase activity, as assayed with ^{32}P -labelled phosphotyrosyl peptide 1142–1153 derived from the regulatory region of the insulin receptor or autophosphorylated insulin receptor as substrate, is known to be located in the particulate fraction (King and Sale, 1988a,b). Thus in the present work studies into the regulation of hepatic PTPases have focused principally on the particulate PTPases. Multiple PTPases have been identified in rat liver, based on isolation of cDNAs (Hashimoto et al., 1992b), and at least three species have been separated from the particulate fraction by chromatography on poly(L-lysine)-agarose using a combination of phosphotyrosyl RCM lysozyme and phosphotyrosyl MBP as substrates (Gruppuso et al., 1991). In the present work the insulin-receptor-related ^{32}P -labelled phosphotyrosyl peptide 1142–1153 was used as a substrate to study the regulation of the various species of PTPases, as it was found to overcome the selectivity associated with the above artificial substrates. Thus chromatography of the solubilized particulate fraction on a poly(L-lysine)-agarose column and assays with ^{32}P -labelled peptide 1142–1153 revealed the presence of three major PTPase peaks (I, II and III) and several minor peaks of activity (Figures 1 and 2). In contrast, phosphotyrosyl RCM lysozyme only revealed PTPase I and II as

Figure 1 Separation of rat liver membrane PTPases by chromatography on a poly(L-lysine)-agarose column and assay with ^{32}P -labelled peptide 1142–1153 (○), RCM lysozyme (●) and MBP (■)

PTPase activity was assayed with phosphotyrosyl (P-Tyr)-peptide 1142–1153 (0.3 μM), -RCM lysozyme (7 μM) or -MBP (5 μM). Fractions of 1 ml were collected.

Figure 2 Poly(L-lysine)-agarose chromatography of liver particulate fractions prepared from normal (a) and fasted (b) rats

PTPase activity was assayed with ^{32}P -labelled peptide 1142–1153. Fractions of 1 ml were collected.

major peaks of PTPase activity and phosphotyrosyl MBP yielded PTPase III as the dominant peak (Figure 1).

Gel filtration of the poly(L-lysine)-agarose-chromatographed PTPase peak I on a Superose 12 column yielded only a single peak of PTPase activity which was of M_r approx. 42000 (Figure 3), indicating that PTPase I belongs to the low- M_r class of PTPases. PTPase II was also of the low- M_r -type (results not shown). PTPase III was of high M_r (> 150000 ; results not shown) and has been proposed to contain LAR-like activities (Boylan et al., 1992).

Figure 4 shows the results of typical Western blots of poly(L-lysine)-agarose-separated PTPase peaks I, II and III using an antibody raised against PTPase 1B, the prototype of the low- M_r class of PTPases. The anti-(PTPase 1B) antibody was prepared by immunizing rabbits with a recombinant glutathione S-transferase fusion protein corresponding to the N-terminal region of human PTPase 1B (amino-acid residues 1–158; Frangioni et al., 1992). Only PTPase peak II reacted with the anti-(PTPase 1B) antibody and yielded a band of M_r approx. 37000. Western blots of the particulate fraction of rat liver with the anti-(PTPase 1B) antibody showed two bands of M_r values 50000 and 37000, the proportions of which varied between preparations (Figure 4). Full-length PTPase 1B has an M_r value of 50000 (Frangioni et al., 1992), but PTPase 1B is commonly isolated in a truncated form of M_r approx. 37000 that lacks some C-terminal residues (Tonks et al., 1988); this is consistent with results in Figure 4. Gruppiso et al. (1991) have previously reported, during resolution of rat liver particulate PTPases, that the first PTPase peak to elute from poly(L-lysine)-agarose displayed reactivity

Figure 3 Purification of PTPase I using Superose 12

PTPase activity was assayed using ^{32}P -labelled peptide 1142–1153. Fractions of 0.5 ml were collected.

Figure 4 Western blotting of rat liver particulate fractions and poly(L-lysine)-agarose-chromatography PTPase peaks I, II and III with anti-(PTPase 1B) antibodies

Lanes 1–3, analysis of 60 μg of three different preparations of the particulate fraction of rat liver. Lanes 4, 5 and 6, analysis of 20 μg of PTPase peaks I, II and III eluted from a poly(L-lysine)-agarose column respectively. Western blots representative of several experiments are shown.

with an antibody raised against residues 161–178 of human placental PTPase 1B in Western blots. The difference between the results of the present work and those of Gruppiso et al. (1991) could be explained if the full-length form of PTPase 1B, which may elute in a different position from the 37000- M_r form, was recovered by Gruppiso et al. (1991).

PTPase activities in poly(L-lysine)-agarose-separated peaks I, II and III were each able to dephosphorylate autophosphorylated insulin and EGF receptors (Figure 5a). The activity of PTPase

Figure 5 Autoradiographs showing the dephosphorylation of ^{32}P -labelled insulin and EGF receptors

(a) Autophosphorylated receptors were incubated for 30 min at 30 °C with PTPase peaks from poly(L-lysine)-agarose chromatography. Incubations contained 454 (PTPase I), 750 (PTPase II) and 918 (PTPase III) fmol/min per ml of PTPase activities measured against ^{32}P -labelled peptide 1142–1153. Abbreviation: CON, control incubated without added PTPase activity. (b) Time course of dephosphorylation by rat liver particulate PTPases from normal and fasted rats. Autophosphorylated receptors were incubated with 0.25 mg/ml of rat liver particulate fraction from normal or fasted rats for the indicated times. Samples were subjected to SDS/PAGE. Results are from typical experiments which were repeated at least three times.

peak III against the autophosphorylated receptors, relative to its activity against ^{32}P -labelled peptide 1142–1153, was the lowest of the three PTPases. Relative to PTPase peak III, peaks I and II displayed 5.21 and 2.31 times the activity measured against the autophosphorylated EGF receptor and 3.51 and 1.49 times the activity measured against the autophosphorylated insulin receptor when the same ^{32}P -labelled peptide PTPase activity was used in the incubations. These activity ratios are from a representative experiment which was repeated three times. Studies by Boylan et al. (1992) have also shown that PTPase III has a relatively low activity against the phosphotyrosyl insulin-receptor-kinase domain.

Regulation of rat liver PTPases

Fasting for 48 h caused a modest inhibition of rat liver particulate PTPase activity as assayed against ^{32}P -labelled peptide 1142–1153 ($15.0 \pm 8.1\%$ inhibition; results not shown), autophosphorylated insulin receptor ($26.9 \pm 8.5\%$ inhibition; Figure 5b) or autophosphorylated EGF receptor ($21.6 \pm 17.8\%$ inhibition; Figure 5b) (mean $\pm$ S.E.M., four observations). Resolution of PTPases on a poly(L-lysine)-agarose column and assays with ^{32}P -labelled peptide 1142–1153 revealed that the effect of fasting was to cause a rather specific and marked reduction ($89.4 \pm 4\%$, mean $\pm$ S.E.M., four experiments) in the activity of one species of PTPase, PTPase I (Figure 2). PTPase peak II may have been slightly inhibited ($15.2 \pm 9.1\%$ inhibition, mean $\pm$ S.E.M., four experiments), whereas PTPase peak III was not significantly altered by fasting for 48 h.

Fasting could change PTPase I in a manner causing it not to bind to poly(L-lysine)-agarose, or it could cause translocation to the soluble fraction. To eliminate the first possibility, PTPase activity that did not bind to the column was assayed with ^{32}P -labelled peptide 1142–1153. Non-binding activity amounted to

Figure 6 Dephosphorylation of autophosphorylated insulin receptor by hepatic particulate PTPase I from normal (○) and fasted (●) rats

Autophosphorylated receptors (250 μl) were incubated with 50 μl of poly(L-lysine)-agarose-separated PTPase peak I at 30 °C for the indicated times and insulin-receptor dephosphorylation determined by SDS/PAGE. The graph shows a typical result which was repeated three times.

$20.8 \pm 1.03\%$ and $21.7 \pm 1.3\%$ (mean $\pm$ S.E.M., three observations) of recovered PTPase activity for liver particulate fractions from control and fasted rats respectively (equivalent to a non-binding activity of 4.5 and 3.6 pmol/min out of a total recovered PTPase activity of 20.5 and 18.6 pmol/min for control and fasted rats respectively, in a typical experiment). Thus loss of the PTPase peak I during fasting did not appear to be accounted for by the PTPase no longer binding to the column. To eliminate the second possibility, the soluble fraction prepared from livers of normal and fasted rats was subjected to poly(L-lysine)-agarose chromatography. There was no increase in the peak corresponding in position to PTPase I upon fasting (results not shown), indicating that translocation of the particulate PTPase I to the soluble fraction was not the cause of the reduction in particulate PTPase I activity induced by fasting.

Assays of the activity of poly(L-lysine)-agarose-separated peak I from the particulate fraction against autophosphorylated insulin receptor showed that receptor dephosphorylation was reduced by $> 85\%$ by fasting (Figure 6); in contrast, fasting did not appreciably alter activities of PTPase peaks II and III against autophosphorylated insulin receptor (results not shown). Induction of diabetes with streptozotocin did not significantly alter the liver particulate PTPase activity assayed against autophosphorylated insulin receptor or EGF receptor, or activity in PTPase peaks I, II and III after separation on a poly(L-lysine)-agarose column and assay with ^{32}P -labelled peptide 1142–1153. In the above experiments, the same amount of particulate-fraction protein was applied to the poly(L-lysine)-agarose columns or used in direct assays of particulate-fraction PTPase activity for control and fasted or diabetic animals. DNA assays showed that the protein content of fasted liver fell 18.7%. Thus relating effects of fasting to DNA rather than protein increases the magnitude of the effects of fasting by decreasing fasted activities by 18.7%.

The decrease in PTPase I activity reported in the present work appears to be the largest regulatory response in PTPase activity

hitherto reported in animals or mammalian cell lines. In agreement with our results, Begum et al. (1991) have previously reported a significant reduction in PTPase activity in adipocytes upon fasting of rats, although individual species of PTPase were not analysed. Consistent with the effects of fasting, insulin is known to increase particulate PTPase activity in Fao cells (Meyerovitch et al., 1992). Moreover, in animal models of non-insulin-dependent diabetes PTPase activities are reduced (Meyerovitch et al., 1991). Results of investigations into the effects of insulin deficiency on PTPase activities in animals have been rather variable, and dependent on the substrate employed, duration and method of induction. Boylan et al. (1992) observed that 4 days of alloxan-induced diabetes caused an 86 % increase in rat liver membrane PTPase activity over normal animals when assayed with phosphotyrosyl MBP, but no effect when phosphotyrosyl RCM lysozyme or autophosphorylated insulin-receptor tyrosine kinase domain were employed as substrates. The diabetes-induced activation seen with phosphotyrosyl MBP as substrate was due to activation of the high- M_r (> 150000) PTPase III. Goldstein et al. (1991) observed, using a phosphorylated insulin-receptor-related peptide as substrate, that the particulate fraction of liver exhibited an increase in PTPase activity of 30 % after 2 days of streptozotocin-induced diabetes, but after 30 days and in the chronically diabetic BB rat PTPase activity was reduced to about 70 % of control levels. In the present work no consistent effects of streptozotocin-induced diabetes were found using the autophosphorylated receptors of ^{32}P -labelled peptide as substrate. In CV1 kidney cells a 150000- M_r PTPase is activated approx. 2-fold by phorbol esters and by increasing the intracellular cyclic AMP concentration (Brautigan and Pinault, 1991).

Exposure of cells to elevated insulin concentrations results in down-regulation of the insulin receptor. Receptor down-regulation occurs in both obesity and type-II diabetes mellitus, and in both cases is associated with an insulin-resistant state. A decrease in receptor number decreases sensitivity to insulin. In an analogous way the reduction in PTPase I activity during fasting may provide a mechanism to promote receptor phosphorylation and increase sensitivity to insulin at low circulating insulin concentrations. Validation of this hypothesis will require the

demonstration that endogenous PTPase I does act on auto-phosphorylated insulin receptor *in vivo* and determination of the role of PTPase I *in vivo* versus other PTPases. Further studies are required to delineate the mechanism for the change in PTPase I activity.

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