Insulin Differentially Regulates Protein Phosphotyrosine Phosphatase Activity in Rat Hepatoma Cells†

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ABSTRACT: We have studied the effect of insulin stimulation on phosphotyrosine phosphatase (PTPase) activity in the well-differentiated rat hepatoma cell line Fao. PTPase activity was measured using a ^{32}P-labeled peptide corresponding to the major site of insulin receptor autophosphorylation. Of the PTPase activity in Fao cells, 14% was in the cytosolic fraction, whereas 86% was in the particulate fraction; this latter fraction also had a 4-fold higher specific activity. Purification of the particulate fraction by lectin chromatography resulted in a 50% increase in specific activity, although this glycoprotein-rich fraction contained only 1.5% of the total activity. Both the cytosolic and particulate PTPase fractions were active toward the tyrosyl-phosphorylated insulin receptor in vitro. The activity of the particulate fraction but not the cytosolic fraction was inhibited by addition of a micromolar concentration of a phosphorylated peptide corresponding to residues 1142–1153 of the human insulin receptor sequence. By contrast, addition of the nonphosphorylated peptide even at millimolar concentration was without effect. Both PTPase fractions were inhibited by Zn^+ at similar concentrations, whereas the cytosolic PTPase activity was 10-fold more sensitive to vanadate inhibition. Treatment of cells with 100 nM insulin increased PTPase activity in the particulate fraction by 40% and decreased activity in the cytosolic fraction by 35%. These effects occurred within 15 min and were half-maximal at 3–4 nM insulin. When assessed as total activity, the magnitude of the changes in PTPase activity in the particulate and cytosolic fractions could not be explained on the basis of a translocation of PTPases between the two pools. We conclude that hepatoma cells possess discrete membrane and cytosolic PTPases with activity toward the tyrosyl-phosphorylated insulin receptor which can be regulated by physiological concentrations of insulin. PTPases may therefore modulate the tyrosyl phosphorylation of the insulin receptor and its substrates, and may be involved in the regulation of insulin-stimulated metabolic and growth-related effects.

Tyrosyl phosphorylation of cellular proteins has been implicated in the regulation of a variety of metabolic and growth-related processes. A number of oncogene products and growth-factor receptors, including the insulin receptor, possess intrinsic tyrosine kinase activity. The binding of insulin to its receptor induces rapid autophosphorylation of the receptor, activating its kinase activity toward both endogenous and exogenous substrates (Kasuga et al., 1981; White & Kahn, 1986). Several lines of evidence suggest that the tyrosine kinase activity of the insulin receptor is essential for insulin-stimulated signal transduction (White & Kahn, 1986; Rosen, 1987).

Tyrosyl autophosphorylation of the insulin receptor kinase activates it, whereas dephosphorylation by the action of protein tyrosine phosphatase(s) (PTPases)[1] inactivates it (Rosen et al., 1983; Yu & Czech, 1984; Haring et al., 1984; King & Sale, 1990); the activity of cellular PTPases thus forms an important regulatory component in the mechanism of action of the insulin receptor and other tyrosine kinases (Cicirelli et al., 1990; Hunter, 1989). PTPases have been detected in a variety of tissues and cell lines, and possess molecular masses ranging from 23 kDa to >200 kDa [reviewed in Lau et al. (1989), Fischer et al. (1991), and Saito and Streuli (1991)]. PTPases are distinct from phosphoserine phosphatases as evidenced by their substrate specificity, activity in the presence of EDTA and sodium fluoride, and selective inhibition by micromolar concentrations of zinc and vanadate. A significant advance in the understanding of cellular PTPases occurred when a cytosolic PTPase 1B from human placenta was sequenced and found to be homologous to the lymphocyte common antigen CD45 (Tonks et al., 1988a; Charbonneau et al., 1988, 1989; Chernoff et al., 1990). Recent cloning efforts by a number of laboratories have revealed a family of related PTPases arising from distinct genes as well as variable splicing events (Cool et al., 1989; Guan et al., 1990; Swarup et al., 1991; Matthews et al., 1990; Kaplan et al., 1990; Sap et al., 1990; Brown-Shimer et al., 1990). In general, the PTPases can be divided into two classes: one represents a group of lower molecular weight cytosolic enzymes and the other a group of higher molecular weight transmembrane molecules which have a receptor-like structure, although no ligands for these have yet been identified. Overexpression of a human T-cell PTPase in BHK cells diminished platelet-derived growth factor-stimulated tyrosyl phosphorylation, and microinjection of the major placental PTPase into xenopus oocytes inhibited meiotic cell division and insulin-stimulated maturation (Cicirelli et al., 1990; Tonks et al., 1990; Cool et al., 1990). Thus, PTPases may play multiple roles in the regulation of cell growth

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[1] Abbreviations: PTPase, phosphotyrosine phosphatase; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol.

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and metabolism. Although not extensively studied, there is evidence to suggest that PTPases are somewhat substrate-specific, and different enzymes may have the ability to dephosphorylate different phosphotyrosine-containing substrates (Sparks & Brautigan, 1985).

Thus far, little is known about the cellular regulation of PTPase activity. The extensive sequence homology between the cytosolic placental PTPase and the intracellular domain of some membrane PTPases raises the possibility that regulation of PTPase activity might occur through transmembrane interactions (Charbonneau et al., 1988, 1989), and the divergent extracellular domains of brain PTPases suggest that different PTPases may be regulated by different ligands. Alternatively, PTPases may be regulated through the action of other signal-transducing proteins. We have previously demonstrated that alterations in hepatic PTPase activity occur in insulin-resistant mice (Meyerovitch et al., 1991) and insulin-deficient rats (Meyerovitch et al., 1989) and in the latter group these changes are reversed by insulin treatment, suggesting that insulin might be a modulator of hepatic PTPase activity. In this study, we address the question of whether insulin can regulate PTPase activity in the well-differentiated, insulin-responsive rat hepatoma line Fao. Fao cells contain PTPase activities associated with both cytosolic and particulate fractions which are active toward the tyrosyl-phosphorylated insulin receptor. Using a synthetic peptide substrate corresponding to the major site of insulin receptor autophosphorylation (residues 1142–1153) (Ullrich et al., 1985), we show that insulin induces a rapid increase in membrane-associated PTPase activity, as well as a smaller decrease in cytosolic PTPase activity. These changes in PTPase activity occur at physiological concentrations of hormone and are consistent with a role for PTPases in the regulation of insulin-stimulated signal transduction.

EXPERIMENTAL PROCEDURES

Materials. [^{32}P]ATP (3000 Ci/mmol) was obtained from New England Nuclear; wheat germ agglutinin–agarose was from Vector Laboratories (Burlinghame, CA); sodium orthovanadate was from Aldrich Chemical Co., Inc. (Milwaukee, WI). HEPES, phenylmethanesulfonyl fluoride (PMSF), aprotinin, leupeptin, benzamidine, and *N*-acetyl-D-glucosamine were from Sigma Chemicals (St. Louis, MO); porcine insulin was from Calbiochem (La Jolla, CA). Dithiothreitol (DTT), Coomassie Blue G250, Triton X-100, and Dowex AG 1-X2 acetate ion-exchange resin were purchased from Bio-Rad Laboratories (Richmond, CA). Peptide corresponding to residues 1142–1153 of the human insulin receptor (Ullrich et al., 1985) was purchased from Dr. D. Coy, Tulane University. The anti-phosphotyrosine antibody was prepared as described (Pang et al., 1985). Protein A–agarose was purchased from Pierce (Rockford, IL).

Preparation of ^{32}P-Labeled Peptide 1142–1153. WGA-purified insulin receptor preparations, isolated from rat liver as previously described (Kasuga et al., 1984), were diluted in 50 mM HEPES, pH 7.0, and 0.1% Triton X-100 to a final protein concentration of 0.1–0.2 mg/mL. The receptor was incubated overnight with 2 mM 1142–1153 peptide at 4 °C in the presence of 100 nM insulin, 100 μM [^{32}P]ATP (specific activity 14.2 Ci/mmol), and 5 mM Mn^{2+}. ^{32}P-Labeled peptide was then separated from unincorporated [^{32}P]ATP by chromatography on Biored AG 1-X2 acetate (Sparks & Brautigan, 1985) and SEP-PAK cartridges (Waters); the purified peptide was lyophilized and stored at 4 °C until use.

Cell Culture and Fractionation. Fao cells (Deschatrette et al., 1979) were grown in monolayer culture on 15-cm dishes in RPMI-1640 medium (Gibco) containing 10% fetal bovine serum in a humidified atmosphere of 5% CO_2. Cells were used on the day of confluence, and were incubated in the absence of serum for 16 h prior to each experiment. The cells were incubated in the absence or presence of insulin at 37 °C. At varying times, the cells were rapidly chilled by immersion in ice-cold phosphate-buffered saline (PBS), pH 7.4, and washed twice in PBS and once in buffer A [10 mM HEPES, pH 7.0, 250 mM sucrose, 5 mM EDTA, 10 μg/mL aprotinin, 25 μg/mL leupeptin, 0.5 mM benzamidine, 0.0025 (w/v) PMSF, and 0.1% (v/v) 2 mercaptoethanol]. The cells were scraped into 5 mL of buffer A, sedimented by centrifugation at 600*g* for 10 min, and resuspended in 1 mL of buffer A. The resuspended cells were homogenized on ice with 15 strokes of a glass–Teflon homogenizer. Homogenates were centrifuged at 600*g* for 10 min, and the postnuclear supernatants were then centrifuged for 1 h at 100000*g*. The final supernatant was designated the cytosolic fraction. The resulting pellet was solubilized in buffer A containing 1% Triton X-100 and was designated the particulate fraction. When indicated, this fraction was further fractionated by chromatography on WGA–agarose in buffer A containing 0.1% Triton X-100 as previously described (Kasuga et al., 1984). These preparations were stored at −70 °C prior to use. Protein concentrations of cell fractions were determined by the method of Bradford (1976).

PTPase Assays. Purified ^{32}P-labeled 1142–1153 peptide was incubated with fractions from control or insulin-stimulated Fao cells at 30 °C for 5 min in 50 mM HEPES buffer, pH 7.0, containing 0.1% Triton X-100, 2 mM EDTA, and 1 mM DTT in a final volume of 50 μL. The phosphatase assay was terminated by the addition of 30 μL of 10% trichloroacetic acid and 20 μL of 1% (w/v) BSA, and the tubes were incubated at 4 °C for 10 min. After centrifugation to remove precipitated proteins, [^{32}P]P_i released from the ^{32}P-labeled peptide was measured by organic extraction (Shacter, 1984). Reaction rates were linear up to 20% dephosphorylation of the peptide; all phosphatase assays were kept within this limit. The concentration of phosphotyrosyl peptide was calculated from the ^{32}P content and the specific radioactivity of the [^{32}P]ATP. One unit of PTPase was defined as the amount of enzyme which hydrolyzed 1 pmol of phosphate per minute, assayed at a substrate concentration of 0.12 μM. K_m and V_{max} values were calculated using the Enzyme program (Luts et al., 1986). Data were presented as mean ± SEM. The Student's *t* test was used to compare groups.

Dephosphorylation of the Insulin Receptor β-Subunit. ^{32}P-labeled insulin receptors were prepared by incubating aliquots of wheat germ agglutinin-purified receptor (20 μg of protein) with insulin (1 μM) for 2 h at 4 °C in 200 μL of 50 mM HEPES buffer containing 0.1% Triton X-100. Receptor autophosphorylation was initiated by adding 100 μCi of [^{32}]-ATP (40 Ci/mmol) containing 5 mM Mn^{2+} (final). After 1 h at 4 °C, the reaction mixture was desalted by gel filtration chromatography using Bio-Gel P-6. Receptor dephosphorylation by Fao cell fractions was assayed by incubating cytosolic or particulate preparations (40 or 5 μg of protein, respectively) with ^{32}P-labeled receptors in the presence of 2 mM EDTA, 1 mM dithiothreitol, PMSF (0.0025% w/v), 0.5 mM benzamidine, 25 μg/mL leupeptin, and 5 μg/mL aprotinin. The mixtures containing Fao cell fractions or buffer were incubated for 20 min at 30 °C and then stopped by the addition of 0.5 mL of 50 mM HEPES, pH 7.4, containing 10 mM ATP, 10 mM sodium pyrophosphate, 4 mM EDTA, 100 mM NaF, 2 mM sodium vanadate, 2 mM sodium molybdate,

Table I [a]

fraction	sp act. (units/mg)	protein (mg)	total act. (units)	K_m (μM)	V_{max} (pmol min^{-1} mg^{-1})
particulate	11.6 ± 0.1	25.2 (61.3)	292.3 ± 3.0 (85)	1.3 ± 0.15	151 ± 26
cytosolic	2.8 ± 0.1	15.6 (37.9)	50.9 ± 0.9 (13.5)	1.0 ± 0.16	33 ± 50
glycoprotein	17.4 ± 0.5	0.3 (0.1)	5.9 ± 0.5 (1.5)		

[a] Particulate, cytosolic, and glycoprotein-enriched fractions were prepared from Fao cells as described under Experimental Procedures. Aliquots from each fraction were assayed for PTPase activity against ^{32}P-labeled peptide for 5 min at 30 °C in 50 mM HEPES, pH 7.0, 2 mM EDTA, and 1 mM DTT. Assays were terminated by the addition of 30 μL of 10% trichloroacetic acid/20 μL of 1% (w/v) BSA and centrifugation to remove precipitated proteins. One unit of PTPase was defined as the amount of enzyme which hydrolyzed 1 pmol of phosphate per minute. The specific activities are expressed as units per milligram of protein, and the total activities are expressed as units per 15-cm dish. Numbers in parentheses indicate percent of total. Kinetic parameters were determined as described using the Enzyme program (Luts et al., 1986).

0.1 mg/mL aprotinin, and 2 mM PMSF solution. Samples were immunoprecipitated with anti-phosphotyrosine antibody as previously described (White et al., 1988). Phosphoproteins were eluted in Laemmli sample buffer containing 100 mM DTT and separated by SDS–PAGE (7.5% resolving gels) (Laemmli, 1970). The stained gels were subjected to autoradiography, and the incorporation of ^{32}P into the 95-kDa β-subunit band was quantitated by scanning densitometry.

RESULTS

PTPase Distribution and Activity in Subcellular Fractions from Fao Cells. The distribution of phosphotyrosine phosphatase (PTPase) activity between subcellular fractions of Fao cells was examined in confluent cells under normal growth conditions (Table I). Cytosolic, particulate, and glycoprotein-rich fractions were prepared as described above and assayed for phosphatase activity against the ^{32}P-labeled peptide substrate in the presence of 0.1% Triton X-100 (v/v). PTPase specific activity was highest in particulate and glycoprotein fractions of the cell. When expressed as units per milligram of protein, the glycoprotein fraction was enriched about 1.5-fold as compared to the particulate fraction (17.4 versus 12 units/mg). The specific activity of the soluble fraction activity was significantly lower (3 units/mg). Correcting for protein recovery, the particulate fraction contained 85% of the total PTPase activity, versus 13.5% of the total activity in the cytosolic fraction. The glycoprotein-enriched fraction, while highest in specific activity, represented only 1.5% of the total PTPase activity. Lineweaver–Burk analysis of the velocity of peptide dephosphorylation by the cytosolic and particulate fractions revealed that the particulate activity was slightly more active with respect to K_m and significantly more active with respect to V_{max} (Figure 1). The K_m values for the particulate and cytosolic PTPase activities were 1.32 ± 0.15 and 1.0 ± 0.16 μM, respectively; the corresponding V_{max} values were 151 ± 26 (particulate) and 33.0 ± 5.5 (cytosolic) pmol of phosphate mg^{-1} min^{-1} ($p < 0.005$) (Table I). These values were obtained on crude extracts, not purified enzymes, and should be used for comparative purposes only.

In addition to their activity toward the synthetic peptide derived from the sequence of the human insulin receptor regulatory region, both the particulate and cytosolic PTPases were active toward the intact tyrosyl-phosphorylated insulin receptor in vitro (Figure 2). Incubation of ^{32}P-labeled insulin receptors with cytosolic and particulate fractions from Fao cells (lanes b and h) leads to a >90% decrease in the radioactivity in the insulin receptor β-subunit band. The dephosphorylation of insulin receptor by the particulate fraction was inhibited by addition of micromolar concentrations of the phosphorylated 1142–1153 peptide; the ^{32}P incorporation in the 95-kDa β-subunit band was 6%, 24%, 46%, and 100% of control in the presence of 0, 4, 8, and 16 μM phosphopeptide, respectively (Figure 2, lanes h–k). In

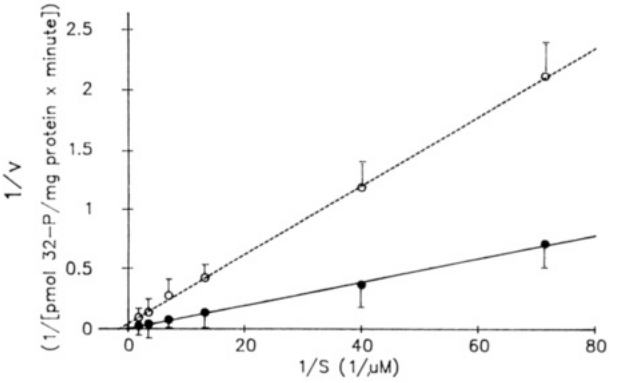

FIGURE 1: Double-reciprocal plot of the dephosphorylation rate of the 1142–1153 peptide. Cytosolic and particulate PTPase fractions were incubated with various concentrations of ^{32}P-labeled 1142–1153 peptide for 5 min at 30 °C. After precipitation of proteins in 10% TCA, free [^{32}P]P_i was extracted and counted. Open and filled circles represent the cytosolic and particulate PTPase activity, respectively. The data are expressed as the mean ± SEM of triplicates; similar results were obtained in three separate experiments.

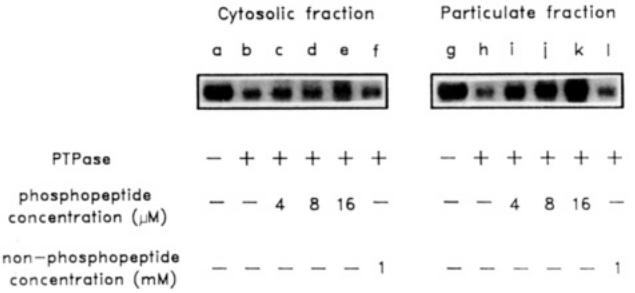

FIGURE 2: Dephosphorylation of the 95-kDa subunit of the insulin receptor by cytosolic and particulate PTPases of Fao cells. ^{32}P-Labeled insulin receptors, prepared as described under Experimental Procedures, were incubated either with buffer (lanes a, g) or with cytosolic (lanes e–f) or particulate (lanes h–l) fractions from Fao cells. Incubations also included varying concentrations of unphosphorylated (lanes f, l) or phosphorylated 1142–1153 peptide (lanes c–e, i–k). After 20 min at 30 °C, the reactions were stopped, and tyrosyl-phosphorylated proteins were immunoprecipitated with anti-phosphotyrosine antibody, eluted in Laemmli sample buffer, separated by SDS–PAGE, and visualized by autoradiography.

contrast, the nonphosphorylated 1142–1153 peptide did not inhibit insulin receptor dephosphorylation by the particulate PTPase even at 1 mM peptide (Figure 2, lane l). This result is in agreement with our observation that the addition of a 1000-fold excess of nonphosphorylated 1142–1153 peptide to the HPLC-purified phosphopeptide caused no significant change in the PTPase activity (data not shown). The inhibition of insulin receptor dephosphorylation by the phosphopeptide suggests that both are substrates of the same PTPase activity and validates our use of the receptor-derived peptide to study PTPases involved in the regulation of insulin receptor tyrosyl phosphorylation. Interestingly, neither the phosphorylated nor the unphosphorylated peptide inhibited insulin receptor

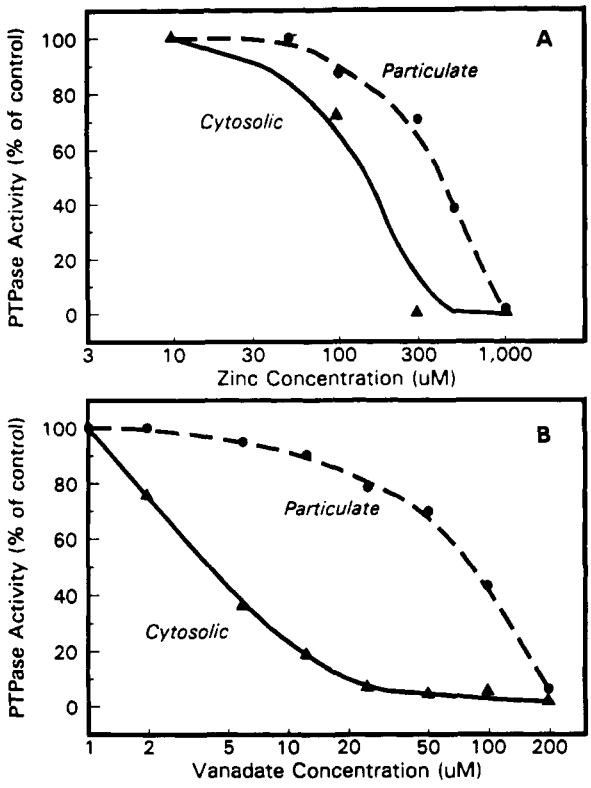

FIGURE 3: Inhibition of PTPase activity from Fao cells by phosphatase inhibitors. Particulate (dashed lines) and cytosolic (solid lines) PTPases from Fao cells were assayed for PTPase activity toward ^{32}P-labeled peptide as described in Figure 1 in the presence of varying concentrations of zinc (upper panel) or orthovanadate (lower panel). The data are expressed in terms of percent inhibition of maximal PTPase activity.

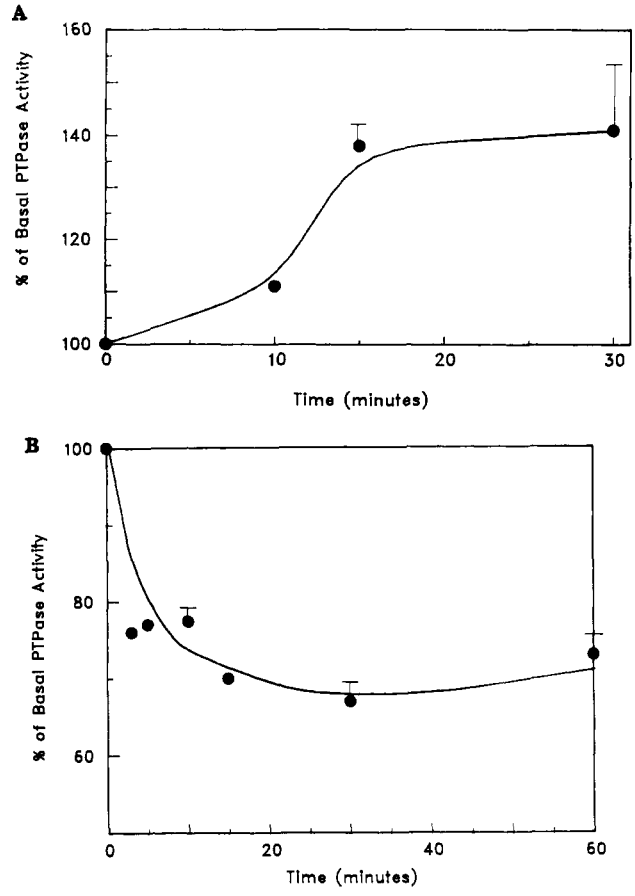

FIGURE 4: Insulin-stimulated changes in PTPase activity from Fao cells. Fao cells were stimulated with 100 nM insulin at 37 °C. At the indicated time, the cells were rapidly chilled and fractionated as described under Experimental Procedures. Aliquots from each fraction were assayed for PTPase activity toward ^{32}P-labeled peptide for 5 min at 30 °C. The reaction were stopped by precipitation with 10% TCA, and free [^{32}P]P_i was extracted and counted. The data are expressed as the mean ± SEM of triplicates in three independent experiments; error bars are omitted when the error is within the area of the point. (A) Particulate PTPase activity; (B) cytosolic PTPase activity.

dephosphorylation by the cytosolic PTPase (Figure 2, lanes b–f). This apparent difference in specificity suggests that the cytosolic and particulate PTPase activities represent different enzymes.

Effect of Inhibitors on PTPase Activity. To further characterize possible differences between PTPases present in the particulate and cytosolic fractions, we studied the effect of three known phosphatase inhibitors on the activity from each fraction: vanadate, zinc, and sodium fluoride. Both cytosolic and particulate PTPase activities were insensitive to sodium fluoride, an inhibitor of serine phosphatases, at concentrations up to 10 mM. In contrast, both Fao PTPase activities were inhibited by zinc and vanadate. With zinc, half-maximal inhibition was achieved at similar concentrations for the cytosolic and particulate activities (0.2 vs 0.5 mM) (Figure 3A). With vanadate, however, there were marked differences in sensitivity (Figure 3B); half-maximal inhibition of the cytosolic PTPase activity occurred at 4–5 μM vanadate, whereas half-maximal inhibition of the particulate activity was achieved only at 100 μM vanadate. Thus, the PTPase activities from the particulate and cytosolic fractions showed significantly different inhibitor profiles, maximal velocities, and substrate specificities.

Insulin-Stimulated Changes in Particulate and Cytosolic PTPase Activity. Insulin treatment of intact Fao cells caused significant and rapid changes in the specific activity of PTPases present in different subcellular fractions. Following stimulation with 100 nM insulin, the particulate PTPase specific activity increased by 40% as compared to unstimulated cells. This increase was maximal after 15 min of insulin stimulation, and remained elevated after 30 min (Figure 4A). In contrast, insulin decreased the specific activity of cytosolic PTPases by 30% (Figure 4B). This decrease, which was more rapid than the changes seen in the particulate PTPase activity, was half-maximal in less than 3 min and maximal before 15 min. The decrease in the cytosolic PTPase activity was sustained after 60 min of insulin stimulation. The changes in PTPase specific activity in both particulate and cytosolic fractions were highly reproducible and statistically significant ($p < 0.01$), and were similar in magnitude to those seen in a report of insulin-stimulated serine phosphatase activity (Chan et al., 1988).

The dose dependence of insulin-stimulated changes in PTPase activity, measured after 15 min, was similar in both fractions (Figure 5). The effects of insulin on the PTPase specific activity of both cytosolic and particulate fractions occurred within physiological ranges, with half-maximal effects detected at approximately 3 nM insulin in the particulate fraction and at approximately 4 nM insulin in the cytosolic fraction.

Insulin Effects on Total PTPase Activity in Cell Fractions. The contrasting directions of insulin's effects on particulate and cytosolic PTPase activity, as well as the similar dose reponse of the changes in each fraction, suggested that insulin might be inducing a translocation of PTPases from one compartment to the other. To test this hypothesis, we measured the effect of insulin stimulation on both the specific activity and the total PTPase activity in each fraction (Figure

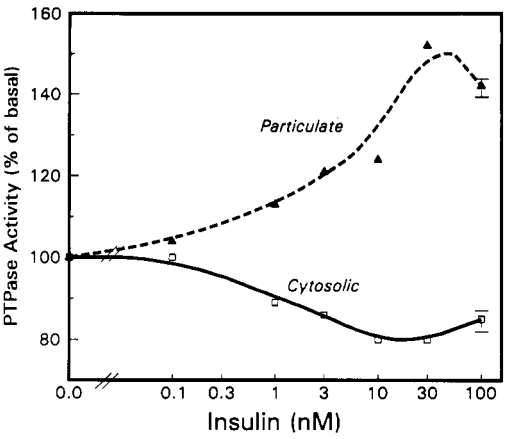

FIGURE 5: Dose response of insulin-stimulated changes in PTPase activity in Fao cells. Fao cells were stimulated with the indicated concentration of insulin at 37 °C for 15 min. Particulate fractions (dashed line) or cytosolic fractions (solid line) were prepared and assayed for PTPase activity as described in the legend to Figure 1. The results represent the mean ± SEM of duplicates; similar results were obtained in two separate experiments. The data are plotted as the percent of basal activity.

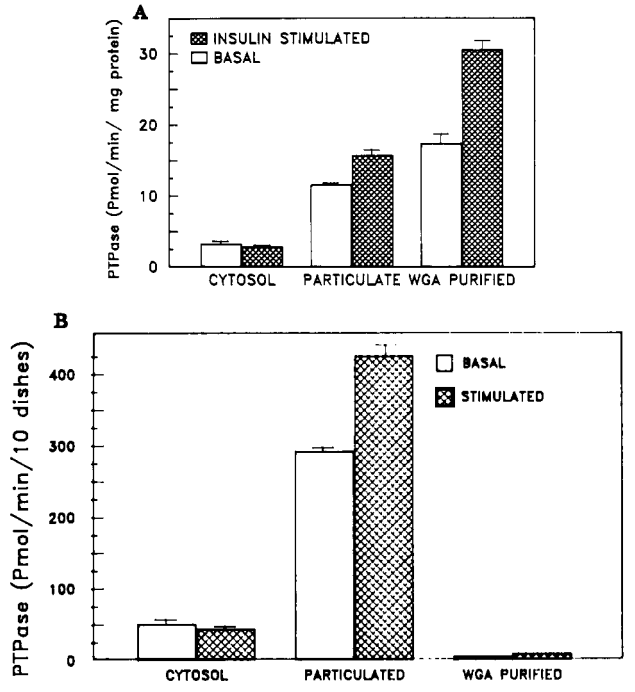

FIGURE 6: Magnitude of insulin-stimulated changes in PTPase activity in subcellular fractions from Fao cells. Fao cells were incubated in the absence (open bars) or presence (cross-hatched bars) of 100 nM insulin for 15 min at 37 °C. The cells were fractionated, and PTPase activity was measured as described in the legend to Figure 1. (A) Insulin-stimulated changes in specific PTPase activity. (B) Insulin-stimulated changes in total PTPase activity. The data are expressed as the mean ± SEM of triplicates; similar results were obtained in three separate experiments. Insulin stimulation of PTPase activity was significant at a level of $p < 0.001$ using the Student's t test.

6). Insulin (100 nM, 15 min at 37 °C) stimulated PTPase specific activity in the particulate fraction by 32%, and stimulated the glycoprotein-enriched fraction by 67% (Figure 6A); insulin decreased the PTPase specific activity in the cytosolic fraction by 15%.

When the data were recalculated to reflect the total activity, insulin stimulation of Fao cells increased total particulate PTPase activity from 292 ± 3 to 425 ± 17 units ($p < 0.001$), a change of 133 units (Figure 6B). In contrast, the insulin-stimulated decrease in total cytosolic PTPase was only 6.6 units, from 50.9 ± 0.9 to 44.3 ± 1.8 units ($p < 0.001$), a 20-fold smaller change. In the WGA-purified fraction, the total activity increased from 5.9 ± 0.5 to 9.5 ± 0.5 units ($p < 0.001$). Therefore, the magnitude of the insulin-stimulated increase in particulate PTPase activity was much greater than the changes seen in either the cytosolic or the glycoprotein-enriched fraction. The effects of insulin on PTPase activity in Fao cells cannot be explained solely on the basis of translocation between different pools, suggesting that insulin modulates the activity of the PTPases in each fraction.

DISCUSSION

The role of phosphotyrosyl phosphatases (PTPases) in the regulation of cell metabolism and growth has been a topic of much recent discussion (Hunter, 1989; Lau et al., 1989; Fischer et al., 1991; Saito & Streuli, 1991). Although PTPases have long been suggested as possible regulators of tyrosine kinase activity, recent studies have directly linked elevations of PTPase activity to regulation of the insulin receptor, the platelet-derived growth factor receptor, the acetylcholine receptor, and pp56^{lck} kinases (Cicirelli et al., 1990; Hunter, 1989; Lau et al., 1989; Fischer et al., 1991; Saito & Streuli, 1991; Cool et al., 1990; Chan et al., 1988; Mustelin & Altman, 1990; Mei & Huganir, 1991). Furthermore, the leukocyte common antigen CD45, whose intracellular domain possesses PTPase activity, has been shown to play an important role in antigen-induced regulation of B- and T-cell proliferation (Pingel & Thomas, 1989; Justement et al., 1991; Broxmeyer et al., 1991) and interleukin 3 receptor signaling (Machicao et al., 1982). The factors which control the activity of cellular PTPases are not well understood. In the present study, we have demonstrated that insulin regulates the activity of phosphotyrosyl phosphatases (PTPases) in an insulin-responsive cell line. Insulin-stimulated changes in PTPases activity are rapid in onset and occur at physiological levels of insulin. Thus, insulin may modulate the balance between phosphorylation and dephosphorylation of cellular tyrosyl phosphoproteins from both sides of the equation.

Insulin-stimulated tyrosyl phosphorylation of the insulin receptor activates the tyrosyl kinase in the β-subunit, whereas dephosphorylation of the insulin receptor inactivates it (Kasuga et al., 1982; Rosen et al., 1983; Yu & Czech, 1984; Haring et al., 1984; King & Sale, 1990). The insulin receptor does not possess intrinsic PTPase activity, and is therefore deactivated in vivo by cellular PTPases (Haring et al., 1984; King & Sale, 1990; Machicao et al., 1982; Kowalski et al., 1983). The PTPases described here are active toward a peptide substrate derived from the major site of insulin receptor autophosphorylation, and also dephosphorylate the autophosphorylated insulin receptor itself under the same assay conditions. Moreover, the insulin receptor dephosphorylation by the particulate PTPase can be inhibited by micromolar concentrations of the 1142–1153 phosphorylate peptide, suggesting that the same enzyme is responsible for the dephosphorylation of both substrates. Insulin-stimulated changes in the activity of the particulate PTPase(s) may therefore play a role in regulating signal transmission by the insulin receptor.

Studies using digitonin-permeabilized adipocytes have suggested that during insulin stimulation the insulin receptor cycles rapidly between phosphorylated and dephosphorylated states (Mooney & Anderson, 1989). During constant insulin stimulation, a change in PTPase activity might shift the steady-state level of receptor phosphorylation. In Fao cells, the total level of insulin-stimulated insulin receptor phosphorylation is maximal within seconds after insulin stimulation and does

not change during 60 min of stimulation (White & Kahn, 1986). However, subsets of receptors may be regulated by PTPase activity. We have previously observed that dephosphorylation of internalized tyrosyl-phosphorylated insulin receptors in Fao cells is modulated by insulin; dephosphorylation is enhanced after 30–60 min of insulin stimulation, consistent with the activation of a tyrosyl phosphatase (Backer et al., 1989). The activation of particulate PTPase activity in Fao cells requires 20 min of insulin stimulation to reach maximal levels, and may be responsible for the increased dephosphorylation of internalized insulin receptors.

An alternative level of regulation of insulin receptor kinase activity by PTPases could involve changes in specific sites of autophosphorylation within the β-subunit. White et al. (1988) suggested that full activation of the insulin receptor kinase requires phosphorylation of the "regulatory" domain of the receptor at tyrosines-1146, -1150, and -1151 (tris-phosphorylation). Furthermore, the number of tris-phosphorylated receptors present in vivo is markedly less than that seen in vitro; only 20% of the total number of tyrosyl-phosphorylated receptors in insulin-stimulated Fao cells are tris-phosphorylated (White et al., 1988; White & Kahn, 1989). The mechanism which limits the number of tris-phosphorylated receptors in vivo is not understood. The tris-phosphorylated receptor might be a preferential PTPase substrate, facilitating the modulation of insulin receptor kinase activity by transitions between bis- and tris-phosphorylated receptor states. Consistent with this hypothesis, in vitro dephosphorylation of the tris-phosphorylated receptor by rat liver PTPases is 3–10 times faster than that of the bis- or mono-phosphorylated receptor (King & Sale, 1990).

While changes in PTPase activity could regulate the insulin receptor kinase directly, they could also affect insulin signal transmission through the dephosphorylation of endogenous substrates of the insulin receptor. The activity of Fao PTPases toward tyrosyl phosphoproteins other than the insulin receptor itself has not been measured. However, the tyrosyl phosphorylation of a prominent substrate of the insulin receptor in Fao cells, pp185, begins to decrease after 10–15 min of insulin stimulation despite constant levels of insulin receptor autophosphorylation.[2] Thus, the dephosphorylation of at least one endogenous substrate of the insulin receptor in Fao cells occurs with a time course similar to the activation of the particulate PTPase activity described here.

The mechanism by which insulin regulates PTPase activity in Fao cells is unknown. Insulin may stimulate a cascade of tyrosine and serine phosphorylation which ultimately affects the phosphorylation state of PTPases, similar to that observed for other insulin-regulated enzymes. Insulin had been shown to activate type 1 phosphatase in Swiss 3T3 cells, and protein phosphatases of acetyl-CoA carboxylase (Chan et al., 1988; Witters et al., 1988; Oliver et al., 1988). Alternatively, a hormonally-stimulated translocation of the PTPase might provide a regulatory mechanism, in a manner analogous to the translocation of protein kinase C or the insulin-responsive glucose transporter (Graeme et al., 1986; Cooper et al., 1987; Cushman & Wardzala, 1980). An insulin-stimulated redistribution of the MgATP-dependent multifunctional protein phosphatase activator has in fact been demonstrated in human platelets (Yang et al., 1988). However, translocation alone cannot explain our data since the observed increase in the particulate fraction activity is 20-fold greater than the decrease seen in the cytosolic activity. It is possible, however, that the presence of phosphatase inhibitors in the cytosolic fraction (or particulate) could account for the disparate activities we observe in the two fractions and insulin-stimulated changes in the concentration or activity of PTPase inhibitors could provide a third possible mechanism (Graeme et al., 1986). Protein inhibitors of serine phosphoprotein phosphatases have been well documented, and some studies suggest the presence of PTPase inhibitors (Ingebritsen, 1989). A fourth potential mechanism might be suggested by the recent observation that the primary insulin receptor substrate IRS-1 has a repetitive YMXM motif capable of binding to SH_2 domains of proteins (Sun et al., 1991) and that at least one PTPase has been found to have SH_2 domains (Shen et al., 1991). Thus, a potential interaction between phosphorylated IRS-1 and one or more PTPase could provide a unique mechanism for insulin regulation of PTPase activity.

PTPase activities from a variety of tissues have been detected in both membrane-associated and cytosolic fractions (Lau et al., 1989; Fischer et al., 1991; Saito & Streuli, 1991; Rotenberg & Brautigen, 1987; Brautigen et al., 1981; Foulkes et al., 1981; Tonks et al., 1988b; Liao et al., 1991), but the relationship between these activities is not well understood. Recent cloning data would suggest that these are two classes of enzymes, although it is possible that some of the small PTPases lacking a transmembrane domain still associate with the membrane in some regulated fashion. In the present study, we demonstrate that 85% of the PTPase activity in Fao cells is present in the particulate fraction and that this fraction is stimulated by insulin. A glycoprotein-enriched fraction, representing less than 2% of the total activity, had a specific activity 1.5–2-fold higher than that of the particulate fraction and was also stimulated by insulin. This fraction may represent a distinct enzyme which either is glycosylated or copurifies with the glycoprotein fraction of the cell. The distribution of PTPase activities in Fao cells is similar to that observed by us (Meyerovitch et al., 1989) and others (King & Sale, 1988) in rat livers. It seems likely that the cytosolic and membrane PTPase activities represent different enzymes since they differ in their response to insulin, as well as to PTPase inhibitors. Furthermore, a phosphorylated peptide derived from the sequence of the insulin receptor regulatory region inhibits the activity of the particulate PTPase, but not the cytosolic PTPase, suggesting that the major PTPase activities in the two fractions possess different substrate specificities. Molecular cloning has revealed mRNAs which code for discrete membrane and cytosolic PTPases, although many of these have been identified in different cell types (Saito & Streuli, 1991; Tonks et al., 1988a; Charbonneau et al., 1988, 1989; Chernoff et al., 1990; Cool et al., 1989; Guan et al., 1990; Swarup et al., 1991; Mathews et al., 1990; Kaplan et al., 1990; Sap et al., 1990; Brown-Shimer et al., 1990; Streuli et al., 1988, 1989). If there are indeed several PTPases in the membrane and cytosolic fractions from Fao cells, then the insulin-stimulated changes in activity observed in this study could be considerably magnified when the individual enzymes are assessed. A more detailed characterization of the relationship between the PTPase activities present in hepatoma cells will clarify this issue. In any case, these data indicate the potential for hormonal regulation of both insulin receptor tyrosyl phosphorylation and insulin receptor tyrosyl dephosphorylation. Coupled with the observations that hepatic PTPase activity is altered in animal models of diabetes (Meyerovitch et al., 1989, 1991) and that there is abnormal regulation of PTPase in skeletal muscle of insulin-resistant humans (McGuire et al., 1991), it will be of great interest to determine the

[2] P. Rothenberg and C. R. Kahn, unpublished results.

mechanisms by which insulin modulates these two classes of PTPases.

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Registry No. PTPase, 79747-53-8; insulin, 9004-10-8; vanadate, 14333-18-7.