Regulation of Protein Tyrosine Phosphatases by Insulin and Insulin-like Growth Factor I

(Received for publication, May 25, 1993)

Kathleen A. Kenner, David E. Hill, Jerrold M. Olefsky, and Jyotirmoy Kusari

From the Department of Medicine, Division of Endocrinology/Metabolism, University of California, San Diego, La Jolla, California 92039, the Medical Research Service, Veterans Administration Medical Center, San Diego, California 92161, and Oncogene Science, Cambridge, Massachusetts 02142

In this study, we have examined the effects of insulin and insulin-like growth factor I (IGF-I) on protein tyrosine phosphatase (PTPase) activity in rat L6 skeletal muscle cells. Under basal conditions, about 85% of total cellular PTPase activity was associated with the particulate (Triton X-100-soluble) fraction. Incubation of the cells with 100 nM insulin or IGF-I significantly increased particulate PTPase activity (p < 0.005) without altering activity in the supernatant or Triton X-100-insoluble fractions. Dose response studies suggested that the effect of each hormone was mediated through its own receptor. PTPase activity was regulated by both acute and chronic insulin and IGF-I treatment. Maximal stimulation by both ligands occurred at 30 h and then declined. By using an antibody and a cDNA specific for PTPase1B, we found that the chronic stimulation of PTPase activity was accompanied by enhanced expression of PTPase1B mRNA and protein. Maximal induction of PTPase1B mRNA and protein by insulin and IGF-I occurred at 12 and 24 h, respectively. Based on these data, it can be suggested that ligand-stimulated PTPase activity might oppose tyrosine kinase-mediated insulin or IGF-I signal transmission and thus desensitize cells to long-term action by insulin and IGF-I. However, it is also possible that PTPases act as positive mediators of insulin and IGF-I action.

Insulin and insulin-like growth factor I (IGF-I) are homologous polypeptide hormones that generate similar biological responses in most cell types, inducing a wide variety of events in target tissues (1, 2). As a first step in initiating these responses, insulin and IGF-I bind to their specific plasma membrane receptors. Almost immediately after binding, the receptors for insulin and IGF-I undergo autophosphorylation on tyrosine residues. Autophosphorylation increases the tyrosine kinase activity of the receptor, which in turn phosphorylates one or more cellular substrates leading to a cascade of secondary phosphorylation and dephosphorylation reactions (3–6).

The regulation of tyrosine phosphorylation represents a balance of tyrosine kinase and phosphatase activities. Previous studies have focused on the role of protein tyrosine kinases in controlling phosphorylation. However, with increased numbers of reports documenting the cloning of protein tyrosine phosphatase (PTPase) genes (7, 8), the converse side of phosphorylation regulation has begun to receive more attention. Much progress has been made in the identification of PTPase isoforms; however, little is known about their physiological role or regulation. It is also not known whether any one of the PTPases so far isolated is involved in the dephosphorylation events of insulin or IGF-I action. Based on activity against autophosphorylated insulin receptors and expression in insulin-responsive tissues, PTPase1B is considered to be the most likely candidate for involvement in insulin and IGF-I action (9). In addition, direct effects of excessive PTPase1B activity on insulin signaling in intact cells have been demonstrated by microinjection of purified placental PTPase1B into Xenopus oocytes, which blocked insulin-stimulated S6 peptide phosphorylation and retarded insulin-induced oocyte maturation (10, 11). Recent studies also indicate that insulin and IGF-I may influence the tyrosine dephosphorylation mechanism, which is an essential component of the regulation of insulin/IGF-I signaling (9). Moreover, altered PTPase activity has been detected in experimental diabetes in rats (12, 13) and in insulin-resistant non-diabetic (14) and non-insulin-dependent diabetes mellitus subjects (15). Thus, it appears that insulin and IGF-I play an important role in the regulation of PTPase activity.

To further understand the relationship between PTPases and insulin/IGF-I action, we have studied the insulin- and IGF-I-specific regulation of PTPase activity in subcellular fractions of differentiated rat L6 muscle cells. L6 myotubes have been well characterized in terms of insulin and IGF-I action (16, 17). We have also examined the effects of insulin and IGF-I on the activity and expression of PTPase1B in L6 myotubes. The results described in this report indicate that insulin and IGF-I induce a time- and concentration-dependent increase in particulate PTPase activity. Stimulated PTPase activity is associated with increased PTPase1B activity, following increases in PTPase1B mRNA and protein.

EXPERIMENTAL PROCEDURES

Materials—[γ-32P]ATP (6,000 Ci/mmol) and [ε-32P]dCTP (3,000 Ci/mmol) were obtained from DuPont NEN. Wheat germ agglutinin coupled to agarose was from Vector Laboratories, Inc. (Burlingame, CA). Silicotungstic acid was from J. T. Baker Chemical Co. (Phillipsburg, NJ). Triton X-100 and AG 1-X2 acetate were purchased from Bio-Rad. Sep-Pak C18 cartridges were from Waters Associates

25455
from Myotubes-L6 myotubes were incubated at 37 °C in the absence and presence of insulin or IGF-I for different periods of time in media containing 0.05% FCS. For the preparation of whole cell homogenates, cell culture dishes and flasks were washed with LSB buffer (Life Technologies) and media replaced with fresh FCS-free media and left at 4 °C. Both increased cell number and presence of insulin or IGF-I for different periods of time and then solubilized in LSB buffer containing 2% Triton X-100 as described above. Supernatants collected after Triton X-100 treatment were used for the determination of PTPase activity and PTPaseB protein levels.

Differentiated L6 cells were incubated at 37 °C in the absence and presence of insulin or IGF-I (100 nM, unless otherwise stated) for different periods of time and then solubilized in LSB buffer containing 200 μl of 5% trichloroacetic acid and 20 μl of 1% bovine serum albumin. Samples were set at 4 °C for a minimum of 10 min before centrifugation at 14,000 x g for 10 min. Supernatants were collected and the pellets were resuspended in LSB buffer containing 10% bovine serum albumin. Samples were set at 4 °C for a minimum of 10 min before centrifugation at 14,000 x g for 10 min. Supernatants were collected and the pellets were resuspended in LSB buffer containing 10% bovine serum albumin. Supernatants were collected and the pellets were resuspended in LSB buffer containing 10% bovine serum albumin. Supernatants were collected and the pellets were resuspended in LSB buffer containing 10% bovine serum albumin.

Preparation of Whole Cell Homogenates and Subcellular Fractions from Myotubes—L6 myotubes were incubated at 37 °C in the absence and presence of insulin or IGF-I for different periods of time in media containing 0.05% FCS. For the preparation of whole cell homogenates, cells were rinsed with phosphate-buffered saline, mechanically homogenized in a Potter-Elvejem homogenizer in 10 volumes of low salt buffer (LSB = 25 mM imidazole, pH 7.2, 2 mM EGTA, 2 mM EDTA, 0.1% Triton X-100, 2 mM MgCl2, and 2 mg/ml benzamidine, 0.025% phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 20 μg/ml aprotinin, 250 mM sucrose, and 10 mM dithiothreitol) containing 2% Triton X-100, and set on ice for 30 min. Insoluble proteins were removed by centrifugation at 14,000 x g for 10 min. Supernatants were used for the determination of PTPase activity. For cell lysates, cells were rinsed with phosphate-buffered saline and homogenized in LSB. Homogenates were centrifuged at 14,000 x g for 10 min, and primary supernatants were retained as the "supernant fraction." Pellets were resuspended in the same volume of LSB containing 2% Triton X-100, set on ice for 30 min, then re-centrifuged 10 min at 14,000 x g. Supernatants collected at this point were retained as the "particulate-1 (P1) fraction." Triton X-100-insoluble proteins were rehomogenized in LSB and retained as the particulate-2 (P2) fraction. All manipulations were carried out at 4 °C.

Determination of PTPase Activity—PTPase activity was measured using a synthetic receptor peptide substrate homologous to the major site of tyrosine autophosphorylation of the human insulin receptor as follows: amino acids 1142-1153 (Thr-Arg-Asp-Thr-Arg-Lys)1153 (16). The substrate contains three potential sites of tyrosine phosphorylation. It was phosphorylated in vitro by purified activated human insulin receptor in the presence of [γ-32P]ATP or non-radioactive ATP and purified as described (22). The specific activity of the radiolabeled substrate was 6-8 x 106 cpm/μg IRP. The dephosphorylation reaction was carried out at 30 °C, in a final volume of 25 μl containing whole cell homogenates (250 μg/ml) or subcellular protein fractions (100 μg/ml) with 1-5 μg [γ-32P]ATP-labeled IRP and 20-25 μM non-radioactive I-IRP in LSB, unless otherwise stated (an average of 44,000 cpm/reaction). After 6 min, the reaction was terminated by adding 3-fold excess volume of ice-cold 10% trichloroacetic acid. [γ-32P]ATP-labeled from the released substrate was measured after organic extraction of [32P] by the method of Schacter (23). The specific activity of the enzyme was expressed as pmol of [32P] released per min/mg of protein. The reaction was linear with respect to time and amount of PTPase activity present until at least 40% of the [32P] had been released. All determinations were performed within the linear range.

Protein concentrations were determined by the method of Bradford (24).
Insulin/IGF-I and PTPases in Myotubes

RESULTS AND DISCUSSION

Although considerable progress has been made toward defining the molecular mechanism of insulin/IGF-I action, this still remains a controversial area of research, particularly with regard to the role played by protein tyrosine phosphorylation. To date, most attempts to assess the role of protein tyrosine phosphorylation in signal transduction have focused on the action of kinases and thus furnish an incomplete picture of this dynamic process. PTPases can be used as probes to approach this problem from another angle and will complement studies performed on the protein tyrosine kinases. With this objective, the present study focused on the regulation of protein tyrosine phosphatases by insulin and IGF-I and the identification of phosphatase(s) involved in hormone action.

The Effects of Insulin and IGF-I on PTPase Activities Associated with Three Subcellular Fractions of L6 Myotubes—PTPase activities associated with the P1, P2, and supernatant cell fractions were measured in L6 myotubes after incubation in the absence and presence of 100 nM insulin or IGF-I for 32 h. PTPase activities were assessed using the synthetic phosphopeptide substrate (IRP), which contains sequence identity with the major site of insulin receptor autophosphorylation (18). As shown in Fig. 1, under basal conditions, approximately 85% of total cellular PTPase activity was associated with the P1 fraction. Similar observations have been reported in a variety of tissues and cell lines (15, 31-33). Incubation of cells with insulin stimulated PTPase activity within the P1 fraction to 181% basal levels (p < 0.005). Stimulation of L6 cells with IGF-I resulted in an increase to 306% basal enzyme activity (p < 0.005). The greater stimulation of PTPase activity by IGF-I is consistent with the greater number of IGF-I receptors in differentiated L6 cells. In myotubes, IGF-I receptors are at least five times more abundant than insulin receptors (17). The maximal effects of insulin and IGF-I were not additive; thus, stimulation of PTPase activity in cells co-incubated with 100 nM insulin and 100 nM IGF-I was the same as in cells incubated with IGF-I alone (data not shown). Basal PTPase activity in the supernatant and P2 fractions accounted for only 7 and 8% of the total cellular content of PTPase activity, respectively. PTPase activity in both of these fractions remained unaltered in the presence of either insulin or IGF-I (Fig. 1). Since no alteration in PTPase activity associated with either the P2 or supernatant fractions was observed in the presence of either hormone, all subsequent studies were carried out using whole cell homogenates.

The mechanism through which the hormones stimulate PTPase activity in the P1 fraction remains unclear, in part because the specific factors regulating enzyme activity and their mechanisms of action are unknown. Our data indicate that no alteration occurs in PTPase activity associated with the supernatant or P2 fractions in response to hormone treatment. Therefore, increased particulate PTPase activity does not result from a redistribution of PTPases from the supernatant or P2 fractions to the P1 fraction. A number of other factors could be responsible for increased particulate (P1) PTPase activity. Hormone treatment could decrease the proteolytic degradation or increase the synthesis of particulate (P1) PTPases. As another possibility, hormone treatment could decrease the activation state of an endogenous PTPase inhibitor (34, 35). Protein inhibitors of serine phosphoprotein phosphatases have been well documented (36), and preliminary studies suggest the presence of PTPase inhibitors as well (33). As a final possibility, hormone treatment may lead to covalent modification of some PTPases or their association with or dissociation from other cellular components, resulting in increased particulate PTPase activity.

To confirm the specificity of our assay and to rule out the possible dephosphorylation of phosphorylated IRP by nonspecific enzymes contaminating the preparation, we measured PTPase activity in the presence of 100 nM sodium orthovanadate. The presence of vanadate (an inhibitor of PTPases) during exposure of the substrate to cellular PTPases resulted in the inhibition of phosphate release from the tyrosyl residues of IRP by more than 90% (not shown).

Time Course for Induction of PTPase Activity by Insulin and IGF-I—The time course for the stimulation of PTPase activity in whole cell homogenates by insulin or IGF-I is shown in Fig. 2. Differentiated L6 cells were incubated at 37 °C in the absence and presence of 100 nM insulin or IGF-

---

**Fig. 1.** The effects of insulin and IGF-I on tyrosine phosphatase activity in particulate (P1 and P2) and supernatant fractions of differentiated L6 cells. Cells were grown and differentiated for 6 days in culture and then incubated at 37 °C for 32 h in the absence and presence of 100 nM insulin or IGF-I. Cells were rinsed, homogenized, and fractionated as described under "Experimental Procedures." PTPase activity was determined by incubating particulate or supernatant proteins (100 μg/ml) with 5 μM [32P]IRP at 30 °C for 6 min. Liberated [32P] was extracted as described and quantified in a β counter. Results are shown as the percentage of the activity associated with the P1 fraction under basal conditions. Each point represents the mean ± S.E. (n = 3) of a representative experiment. Basal PTPase activity associated with the P1 fraction was 484 ± 0.27 pmol of [32P], released per min. mg of protein.

---

**Fig. 2.** The time course for insulin- and IGF-I-stimulated tyrosine phosphatase activity in differentiated L6 cells. Cells were differentiated as described in Fig. 1 and then incubated in the absence or presence of insulin or IGF-I (100 nM) at 37 °C for the indicated periods of time. After each incubation period, cells were rinsed and homogenized. Total cellular PTPase activity was measured as described in Fig. 1. Results are shown in relation to the activity measured under basal conditions. Each point represents the mean ± S.E. (n = 3) of a representative experiment, which was independently performed three times.
I for the indicated periods of time. Cells maintained in the presence of insulin or IGF-I displayed a complex time-dependent alteration in whole cell PTPase activity relative to control cultures maintained in the absence of either ligand. The initial phase of insulin stimulation was characterized by a small increase in PTPase activity (112% control, p < 0.05) observed at 30 min, followed by a decline to the basal PTPase activity level within 2 h. Subsequently, a second phase of insulin-stimulated PTPase activity was initiated after 4 h and gradually increased to a maximum (192% control, p < 0.0005) at 32 h. IGF-I treatment also stimulated a rapid increase in PTPase activity, which was detectable at 30 min (117% control, p < 0.05), but in contrast to insulin, IGF-I-stimulated activity remained elevated. IGF-I incubation for periods longer than 2 h stimulated a further gradual increase in PTPase activity to a maximum (281% control, p < 0.0005) at 32 h, similar to that detected in insulin-treated cells.

The magnitude of the acute stimulation of PTPase activity by insulin or IGF-I is small (12-17% of the basal activity). However, similar observations have been reported for insulin-stimulated serine and tyrosine phosphatase activities in various cell lines (37, 38). The small size of the change in PTPase activity in response to acute insulin or IGF-I treatment could be due to the substrate. It is not known how specific or general the insulin receptor peptide is as a substrate for PTPases; it might not measure the hormone-stimulated PTPase activity with perfect efficiency. It is also possible that multiple PTPases contribute to the total measured activity and that insulin or IGF-I stimulates only one of these enzymes. Therefore, the insulin/IGF-I-stimulated changes in activity observed here could be considerably amplified when the individual enzymes are assessed. This possibility is supported by the results (see Fig. 6) of insulin- and IGF-I-stimulated PTPase activity after immunoprecipitation with the monoclonal PTPase1B antibody, FG6-1G. In two independent determinations, PTPase1B-specific activity increased by an average of 23 (p < 0.0005) and 38% (p < 0.001) over basal with 30 min insulin or IGF-I treatment (100 nM), respectively.

Thus, it appears that PTPase activity is regulated in L6 cells by both acute and chronic insulin or IGF-I treatment, reminiscent of the pattern of hormone-induced changes in glucose transport (39). Acute induction of PTPase activity probably occurs by a post-translational mechanism such as covalent modification, while chronic stimulation with insulin and IGF-I might primarily influence PTPase gene expression.

Degradation of Insulin and IGF-I in L6 Muscle Cell Cultures—Since maximal hormone stimulation occurred at 32 h, we determined the rates of degradation of insulin and IGF-I in the incubating media at 37 °C over that time period. Hormone degradation was measured by trichloroacetic acid precipitation of insulin and IGF-I. When L6 cells were incubated with 30 pm [125I]IGF-I in the presence of 0.1 nM cold IGF-I, degradation was negligible (Fig. 3). This could be due to the absence of an IGF-I-specific protease in the media or to a slow rate of internalization and intracellular degradation of IGF-I. Alternatively, the presence of IGF-I-binding proteins in the media (40) could protect IGF-I from nonspecific proteases. On the other hand, degradation of 30 pm [125I]IGF-I labeled insulin in the presence of 0.1 nM unlabeled insulin occurred with a t1/2 of 18 h. This is possibly due to degradation by an insulin-degrading enzyme present in L6 cells (41), rapid internalization and intracellular degradation (42), or both. As a result, we modified ligand incubations to include the replacement of media with media containing fresh hormone each 12 h during extended hormone incubations, even though we did not observe any difference in hormone-stimulated PTPase activity with and without media replacement.

The Dose Dependence of the Stimulation of PTPase Activity by Insulin and IGF-I—The receptors for insulin and insulin-like growth factors have been characterized on L6 myotubes. However, the results from several reports are conflicting. Ballard et al. (43) demonstrated high affinity binding sites for both IGF-I and -II and suggested that the biological effects of these ligands occur primarily through binding to the IGF-I receptor. Burant et al. (44) showed that the receptor species that bound insulin was a high affinity IGF-I receptor. Recent studies of Maher et al. (45) described an insulin receptor species with an unusually low affinity for insulin and insulin bioeffects that appear to occur via IGF-I receptors. In contrast, Beguinot et al. (16) reported high affinity binding sites for insulin on L6 myoblasts, which increased in number after fusion and had properties consistent with insulin receptors, e.g., high affinity for insulin with lower affinity for proinsulin and IGF-II, down-regulation, and competition for binding by sera from a patient with anti-insulin receptor antibodies. The reason for the apparent discrepancies in previous reports as to the presence of high affinity insulin receptors in L6 cells is unknown but could be due to clonal variation. The L6 cells used here displayed both high affinity IGF-I and insulin receptors. At 33 pm [125I]IGF-I, 8% of the added ligand was specifically bound per 10⁶ cells. In the presence of increasing concentrations of unlabeled Des-Gly-Pro-Glu-IGF-I (an analog with low affinity for IGF-I-binding proteins and high affinity for IGF-I receptors) the half-maximal inhibitory concentration was 1–2 nM. Specific insulin binding was only 0.3% in the presence of 33 pm [125I]-labeled insulin with half-maximal displacement at a concentration of 2–3 nM, indicating the presence of a smaller number of high affinity insulin binding sites (not shown).

In order to determine the receptor through which ligand-stimulated PTPase activity occurred in our L6 myotubes, we compared the dose-response curves for the stimulation of PTPase activity by insulin and IGF-I. As shown in Fig. 4, insulin and IGF-I stimulated PTPase activity in whole cell homogenates in a comparable, dose-dependent manner at concentrations between 0.01 and 50 nM. The EC₅₀ values were
the absence or presence of increasing concentrations of insulin or phosphorylated IRP (1-5 um [32P]IRP and 20-25 um non-radioactive P-IRP) at 30 °C for 6 min. Liberated [32P], was extracted as described and quantified in a β counter. Results are depicted as the percent maximal activity (measured after incubation with 50 nM IGF-1). Inset, an enlargement of the graph from 0 to 1.0 nM. Each point represents the mean ± S.E. of four independent determinations, each performed in duplicate.

5 and 8 nM for insulin and IGF-I, respectively. The low concentrations required to generate the effects suggest that both hormones stimulate PTPase activity through interaction with their respective receptors. A similar conclusion was also drawn previously by Beguinot et al. (16, 17) and Walker et al. (39) for glucose and amino acid transport in L6 cells.

Effects of Insulin and IGF-I on the Expression of PTPase1B Protein—Recently, several experiments have been described that indicate that PTPase1B might be the phosphatase involved in insulin and IGF-I action (9-11). Studies in our laboratory also indicate that the expression of PTPase1B protein is decreased in skeletal muscle from Type II diabetic subjects, suggesting a link between alterations in PTPase1B and impaired insulin action. To determine whether hormone-stimulated PTPase1B activity involves the increased expression of PTPase1B, we measured PTPase1B protein levels by immunoblotting from differentiated L6 cells that had been incubated without and with 100 nM insulin or IGF-I for various periods of time. After cell solubilization, 50 μg of total cell protein were fractionated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. PTPase1B was detected using a monoclonal antibody (FG6-IG) (2 pg/ml). PTPase1B protein was visualized using alkaline phosphatase conjugated to goat anti-mouse heavy and light chain IgG, with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium for color development (A). PTPase1B protein levels were quantitated densitometrically from Western blots. The results of the densitometric scans are expressed relative to the quantity of PTPase1B in the range directly proportional to the quantity of PTPase1B in the range used for these experiments.

The increases in PTPase1B protein levels in cells stimu-
lulated by insulin and IGF-I followed similar time courses. Increased PTPase1B expression was first detected after 2 h and reached maximal levels (4.3-fold basal by insulin and 6.5-fold basal by IGF-I) after 24 h. These data suggest that the increased expression of PTPase1B protein is responsible, at least in part, for the chronic stimulation of PTPase activity by insulin and IGF-I. The mechanism by which PTPase1B protein expression is enhanced is not clear; it could be accounted for either by an increase in PTPase1B biosynthesis and/or by decreased PTPase1B degradation.

**Time Course of Insulin- and IGF-I-stimulated PTPase1B Activity**—To test whether the hormone-induced stimulation of total cellular PTPase activity is due to the enhanced activity of PTPase1B, FG6-1G was used to immunoprecipitate PTPase1B from L6 cells incubated in the absence and presence of 100 nM insulin or IGF-I for various periods of time, and PTPase activity was measured in the immune complex.

In control experiments, PTPase activity was measured in whole cell homogenates containing varying concentrations of FG6-1G. No inhibition was observed, confirming that the antibody has no intrinsic inhibitory effect on PTPase activity (not shown). FG6-1G immunoprecipitated a 56-kDa protein from L6 cells incubated in the absence and presence of insulin or IGF-I. The immunoprecipitation was specific, since no protein band was detected in the absence of the antibody. The immunoprecipitated protein co-migrated with the 56-kDa protein detected in whole cell homogenates after immunoblotting with FG6-1G (not shown).

In order to determine the contribution of PTPase1B activity to total cellular PTPase activity in L6 myotubes, PTPase activity and PTPase1B protein levels were measured under basal conditions in the FG6-1G immunoprecipitates and supernatant fractions in comparison with whole cell homogenates. Analysis of enzyme activity in the various fractions indicated that approximately 37% of the total cellular content of PTPase activity was immunoprecipitated by FG6-1G. Parallel immunoblot analysis of PTPase1B protein levels in the different fractions indicated that approximately 60% of the total cellular content of PTPase1B protein was immunoprecipitated by FG6-1G (not shown). These data suggest that a considerable portion of the total PTPase activity in L6 cells is due to PTPase1B. We do not know the reason for the incomplete precipitation of PTPase1B, even after incubation of the cell extracts with higher concentrations of the antibody. However, similar proportions (53–59%) of the total cellular content of PTPase1B were immunoprecipitated from both control and ligand-treated cells.

Analysis of the time course of hormone-stimulated PTPase1B activity associated with the anti-PTPase1B immunoprecipitates showed an increase within 30 min after addition of 100 nM insulin (23%, p < 0.005) or IGF-I (38%, p < 0.001) (Fig. 6). Peak activity (186% of basal) was observed at 32 h, after which activity gradually decreased. Since PTPase1B protein levels were not detectably altered within 30 min of hormone treatment (refer to Fig. 5), the increase in PTPase1B activity within this time period could be due to a post-translational modification of the protein. Post-translational activation of PTPase1B could, in turn, be responsible for the acute stimulation of total PTPase activity. This does not, however, exclude the possible regulation by insulin of other, as yet unidentified, PTPases as well.

The time course of PTPase1B activity during chronic hormone treatment is similar to that for total PTPase activity. These data suggest that the chronic induction of total PTPase activity is due, at least in part, to the stimulation of PTPase1B activity. Thus, the time course for hormone-stimulated phosphatase activity specific for PTPase1B contains both acute and chronic components, comparable with the time course of total PTPase activity measured in whole cell homogenates (shown in Fig. 2).

It was interesting that the relative chronic hormone-induced increase in PTPase1B specific phosphatase activity (Fig. 6) was somewhat less than the increase in PTPase1B protein content (Fig. 5). The reason for this is unclear, however, such disparities have been reported previously for other proteins (48–50). It is possible that PTPase1B activation depends on protein modifications that occur subsequent to protein synthesis. Hence, some PTPase1B protein molecules synthesized in response to insulin and IGF-I might fail to become post-translationally activated. As a result, hormone-induced increases in enzyme activity would not equal the increases in PTPase1B protein content.

**Insulin- and IGF-I-stimulated Expression of PTPase1B mRNA**—To examine whether enhanced PTPase1B protein expression is associated with a corresponding increase in mRNA, PTPase1B transcript levels were measured in L6 myotubes that had been incubated in the absence and presence of 100 nM insulin or IGF-I for 12 h. Total RNA was isolated, electrophoresed under denaturing conditions, and transferred to nitrocellulose filters for Northern blotting. The filters were probed with radiolabeled human placental PTPase1B cDNA. As a control, the same filters were reprobed with human PDH cDNA, since PDH mRNA levels are not altered by insulin or IGF-I treatment in L6 cell cultures. A representative Northern blot is shown in Fig. 7. The cDNA corresponding to PTPase1B recognized an mRNA of approximately 4.0 kilobases (Fig. 7A), corresponding to the size of the human placental PTPase1B mRNA (29). A second minor band of approximately 3.6 kilobases was also apparent. The minor band could represent the transcript of a related gene, or an alternatively spliced version of a single primary PTPase message. Both insulin (lane 2) and IGF-I (lane 3) increased the content of these mRNAs over the basal level (lane 1). Rehybridization of the same filter with PDH cDNA showed equal amounts of PDH mRNA in all lanes (Fig. 7B).

To determine the kinetics of induction, cells were incubated at 37 °C without and with 100 nM insulin or IGF-I for the indicated periods of time, total cytoplasmic RNA was isolated, and the amounts of the PTPase1B and PDH transcripts were determined. The mRNA levels were quantitated densitometrically from autoradiograms of the Northern blots. The relative amount of PTPase1B mRNA at each time point was normalized to the amount of PDH mRNA. The results of the densitometric scans are shown in Fig. 8, expressed relative to the amount of PTPase1B mRNA present in cells in the absence of insulin or IGF-I. The stimulated expression of PTPase1B mRNA with insulin and IGF-I followed similar time courses. 100 nM insulin or IGF-I stimulated increases in PTPase1B mRNA levels within 6 h, but the effects were not maximal until 12 h of ligand incubation. During longer incubation with the hormones, the mRNA accumulation gradually approached basal levels. Maximal induction by IGF-I (12.5-fold basal) was greater than by insulin (5.5-fold basal). This is consistent with insulin- and IGF-I-stimulated PTPase1B activity and protein accumulation and with the relative numbers of insulin and IGF-I receptors on L6 cells. The accumulation of PTPase1B mRNA in response to insulin and IGF-I may be due to increased transcription of the PTPase1B gene and/or stabilization of the mRNA.

---

3 W. H. Dillmann, personal communication.
Insulin/IGF-I and PTPases in Myotubes

Fig. 6. Time course of insulin- and IGF-I-induced PTPase1B activity. Cells were differentiated as described in Fig. 1 and incubated with 100 nM insulin or IGF-I for various periods of time at 37°C. Whole cell homogenates were immunoprecipitated with FG6-IG, and PTPase1B activity in the immune complex was measured as described under “Experimental Procedures.” The enzyme activity at each time point is depicted in relation to activity measured under basal conditions. Each point represents the mean ± S.E. of two independent determinations, each performed in triplicate.

Fig. 7. Insulin- and IGF-I-stimulated expression of PTPase1B mRNA in L6 myotubes. Differentiated L6 cells were incubated at 37°C in the absence and presence of 100 nM insulin or IGF-I for 12 h, and the total cellular RNA was isolated as described under “Experimental Procedures.” Each lane contains 20 μg of total RNA analyzed by Northern blotting. The same filter was probed sequentially with human placental PTPase1B cDNA (A) and human PDH cDNA (B). The filter was stripped of radioactivity after each hybridization. The locations of 28S and 18S RNA are indicated. Lane 1, control; 2, insulin; and 3, IGF-I. This is a representative experiment, independently performed three times.

In summary, insulin and IGF-I induce a time- and concentration-dependent increase in particulate PTPase activity in L6 myotubes. Increased activity is detected by enhanced in vitro dephosphorylation of phosphorylated insulin receptor peptide. The effect of each hormone is mediated through its own receptor. Insulin and IGF-I display no additive effect when administered in tandem. PTPase activity is regulated both acutely and chronically by insulin and IGF-I treatment. The acute effects of the hormones to stimulate PTPase activity probably occur by a post-translational modification of PTPase1B. The respective time courses for insulin- and IGF-I-stimulated expression of PTPase1B mRNA, protein, and enzyme activity suggest that the long-term effects on PTPase activity result from an increase in PTPase1B protein following the accumulation of PTPase1B mRNA.

Much work is needed to clarify our understanding of the functions of PTPases in insulin and IGF-I signal transduction. Based on our observations, we propose the following hypothesis to explain the relationship between PTPases and insulin/IGF-I action. After binding to its receptors, insulin or IGF-I stimulates PTPase activity, either by post-translational mechanisms and/or increased gene expression. Hormone-induced PTPase activity may attenuate responses to insulin and IGF-I by dephosphorylating the activated receptors and/or specific cellular substrates of the insulin/IGF-I receptor tyrosine kinases. Alternatively, activated PTPases could exert a positive influence on insulin and IGF-I signal transduction events. In support of this mechanism, recent studies have indicated that the activation of PTPase activity is essential for insulin-stimulated glucose uptake (51). In unrelated studies, a number of PTPases have been implicated as signal transduction molecules. For example, the PTPase CD45 catalyzes antigen-induced T-cell proliferative responses (52, 53), and CDC25 triggers the initiation of mitosis through dephosphorylation of CDC2 (54). Finally, it has been shown that some of the intracellular effects caused by insulin are me-
Acknowledgment—We thank Elizabeth Martinez for expert assistance in the preparation of this manuscript.

REFERENCES