Adv. Prot. Phosphatases 6, 1-17 (1991)

HEPATIC PROTEIN-TYROSINE PHOSPHATASES AND THEIR REGULATION IN DIABETES

Barry J. GOLDSTEIN, Joseph MEYEROVITCH, Wei-Ren ZHANG, Jonathan M. BACKER, Peter CSERMELY, Naotake HASHIMOTO, and C. Ronald KAHN

Research Division, Joslin Diabetes Center and Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02215 USA

INTRODUCTION

Insulin-stimulated tyrosine phosphorylation of the insulin receptor plays a key role in the molecular mechanism of insulin action (1,2). The phosphotransferase activity of the insulin receptor kinase is known to be increased and decreased by tyrosine phosphorylation and dephosphorylation of the receptor. Phosphorylation on tyrosines is due to the intrinsic activity of the receptor itself, whereas dephosphorylation requires the action of one or more protein-tyrosine phosphatase (PTPase) enzymes present in target cells for the hormone. Recently, these enzymes have been shown to comprise a family of distinct membrane and cytoplasmic proteins that appear to play an important physiologic role in the regulation of insulin action (3).

Our laboratory has been interested in characterizing and ultimately cloning the specific PTPase(s) that regulate the phosphorylation state of the insulin receptor in order to evaluate their potential role in insulin action and states of insulin resistance. In these studies, we have taken two parallel experimental approaches to answer these questions. These include an evaluation of the biochemistry and physiology of hepatic cytosolic and membrane PTPases as well as identification of PTPases at a molecular level by cDNA cloning in rat liver.

INSULIN RECEPTOR ACTIVATION AND DEACTIVATION

Insulin Action and Tyrosine Phosphorylation

Although the exact molecular mechanism of insulin action in target cells remains uncertain, it is clear that insulin elicits its pleiotropic effects on cellular growth and metabolism by binding to its plasma membrane receptor and that this receptor is a protein tyrosine kinase (1,2). Thus, insulin binds to the α-subunit of the receptor and this leads to a rapid autophosphorylation of specific tyrosine residues in the β-subunit with activation of the receptor kinase towards exogenous protein substrates in target cells (2,4,5). Several lines of evidence have shown that the tyrosine kinase activity of the insulin receptor plays a key role in cellular insulin action. These data include the observation that several patients with genetic disorders associated with severe insulin resistance have been found to have mutations in the receptor which alter kinase activity (6), transfection of cells with normal insulin receptors enhances cellular insulin sensitivity while expression of kinase-defective receptor mutants does not (7-9), and evidence that insulin action is blocked by introducing antibodies to phosphotyrosine or to the receptor kinase domain into cells (10,11).

Tyrosine phosphorylation of cellular substrates for the insulin receptor kinase is also highly regulated. One of the best characterized substrates is a protein of Mr = 185 kDa termed pp185 (12). Phosphorylation of pp185 occurs rapidly following stimulation of the receptor by insulin, however, dephosphorylation of pp185 also occurs within minutes despite the continued presence of insulin and receptor activation (12,13). The phosphorylation state of the receptor and also of these putative substrates in the insulin action pathway, therefore, depend on the activity of specific PTPases whose identification is of critical importance for understanding the overall regulation of insulin action.

Deactivation of the Insulin Receptor by PTPase Activity

When the partially purified insulin receptor is autophosphorylated *in vitro* in response to insulin stimulation, the receptor retains its phosphorylation state and the tyrosine

kinase remains activated even if insulin is removed from the ligand binding site (14). Although one early study reported that PTPase activity was associated with the receptor itself (15), several others demonstrated that immunoprecipitated insulin receptors do not have intrinsic PTPase activity (14,16). Furthermore, an active liver membrane PTPase may co-purify with the insulin receptor by lectin affinity chromatography and contaminate a purified receptor preparation (see below).

In vivo, in hepatoma cells (14) as well as a permeabilized adipocyte model (17,18), dissociation of insulin from the receptor is followed by a rapid dephosphorylation of the beta subunit and a concomitant deactivation of insulin receptor function. Therefore, just as the activity of many other metabolic enzymes is affected by specific phosphorylation/dephosphorylation reactions (19), the intrinsic activity of the insulin receptor is enhanced by autophosphorylation and attenuated by cellular enzymes that dephosphorylate phosphotyrosine residues (20).

Regulation of the Receptor Kinase by Multisite Phosphorylation:

Detailed studies of the tyrosine residues in the insulin receptor that become phosphorylated with insulin stimulation have shown that five residues are subject to autophosphorylation (1,2). Several laboratories have provided evidence that full activation of the receptor kinase correlates with phosphorylation of a cluster of only three closely spaced tyrosines at positions 1146, 1150, 1151 in the so-called "regulatory domain" of the receptor (4,21). The receptor phosphorylated on two residues (typically 1146 and either the 1150 or 1151 tyrosine) is only activated to approximately 10% of its full activity (21).

When the phosphorylation state of the receptor is directly compared *in vivo* and *in vitro*, the number of insulin receptors fully (tris-) phosphorylated in the regulatory domain present after insulin stimulation of intact cells is markedly less than that seen for insulin activation of solubilized, partially purified insulin receptors (Table 1). This has been shown for a variety of cell types, suggesting that in intact cells the fully

phosphorylated insulin receptor might be a preferential PTPase substrate (22). These data have lead to the speculation that specific PTPase enzymes might modulate the receptor kinase activity by transitions between bis- and tris-phosphorylated receptor states in cells. A recent study was consistent with this hypothesis showing that the tris-phosphorylated (activated) insulin receptor was much more rapidly dephosphorylated than the bis-phosphorylated form of the receptor *in vitro* using subcellular fractions of rat liver as a source of PTPase activity (23).

REGULATION OF PTPASE ENZYME ACTIVITIES

PTPase activities in subcellular fractions of rat liver

To better understand the PTPase activities in liver tissue that might be acting on the autophosphorylated insulin receptor, we have performed some preliminary studies on the localization of these enzymes and their potential regulation using as substrates both the intact insulin receptor that had been autophosphorylated *in vitro* and a [^{32}P]-phosphopeptide substrate containing sequence

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Table 1: Phosphorylation of the insulin receptor regulatory region in intact cells and in purified receptors after insulin stimulation

	Relative abundance of Tris-phosphorylated insulin receptor regulatory domain	
Cell Line	Intact Cells	Isolated Receptors
Fao	12%	72%
CHO/HIRC	23	78
3T3/HIRC	23	74

Insulin receptors were phosphorylated after insulin stimulation of intact FAO rat hepatoma cells, or two cell lines, chinese hamster ovary (CHO) or 3T3 fibroblasts expressing the human insulin receptor cDNA (HIRC). Partially purified insulin receptors were also isolated from these tissues and phosphorylated *in vitro*. The phosphorylation state of the receptor regulatory domain (tyrosine-1150 region) was analyzed and the relative abundance of Tris-phosphorylated receptors (Tris/Tris+Bis phosphorylated receptors) is shown. Adapted from reference 22.

identity to the insulin receptor regulatory region (residues 1142-1153; ref. 4). This peptide was phosphorylated with an activated insulin receptor preparation and γ-[^{32}P]-ATP, isolated by Sep-Pak chromatography and/or HPLC and used as a substrate for *in vitro* dephosphorylation. Dephosphorylation was assessed as the amount of radioactivity in inorganic phosphate released and extracted with an organic solvent (24).

PTPase activity was detected in both cytosolic and particulate fractions with a similar distribution in both rat liver and Fao hepatoma cells (Table 2). Approximately 14% of the total PTPase activity was in the cytosolic fraction, whereas 85% was in the particulate fraction; this latter fraction also had a 4-fold higher specific activity. Further purification of the solubilized particulate fraction by affinity chromatography on a wheat germ agglutinin lectin-agarose affinity column resulted in a 50% increase in specific activity of the PTPase, although this glycoprotein-rich fraction contained only 1.5% of the total activity. This finding suggested that at least one of the insulin receptor-related PTPases may be a glycosylated cell surface protein, or perhaps is tightly associated with one.

When the inhibition profile of these enzymes was examined, both the cytosolic and particulate PTPase fractions were inhibited by zinc at similar concentrations. The cytosolic PTPase activity, however, was 10-fold more sensitive to inhibition by vanadate and may therefore be different from that in the membrane (25).

Alteration in Hepatic PTPase Activities in Diabetes:

We have obtained some evidence that changes in PTPase activity may occur in states of altered insulin action. For example, defective receptor autophosphorylation and kinase activity occur in several animal models of diabetes with insulin resistance (26-28). To investigate the potential role of changes in PTPase activity in these disease states, we measured PTPase activities in rats made insulin-deficient diabetic by streptozotocin treatment (24). In this model, cytosolic PTPase activity increased to 180% ($p<0.02$) of the control values after two days of diabetes and remained elevated

for at least 30 days (Figure 1, upper panel). Gel filtration on Sephadex-75 revealed a single peak of activity in the cytosol in both control and diabetic animals at Mr ~30 kDa and confirmed the increased levels. The increase in cytosolic PTPase activity could also be detected using phosphorylated intact insulin receptor. In BB diabetic rats, another model of insulin deficiency, the PTPase activity in the cytosolic fraction was similarly increased to about 230% of control values.

The particulate fraction of liver also exhibited an increase in PTPase activity by 30% and 80% after two and eight days of STZ diabetes, respectively. However, this increase was not sustained, and after 30 days of STZ diabetes, and in the chronically diabetic BB rat, PTPase activity associated with the particulate fraction was reduced to about 70% of the control levels (Figure 1, lower panel). Treatment of STZ-diabetic rats with subcutaneous insulin or vanadate in their drinking water for three days reduced PTPase activity in the particulate, but not in the cytosolic fraction. This was associated with a change in blood glucose toward normal. These data indicate insulin deficient diabetes is accompanied by significant changes in hepatic PTPase activity affecting both the cytosolic and particulate compartments (24). An increase in specific PTPase activity affecting the state of insulin receptor phosphorylation may be an important factor in the physiological resistance to insulin associated with these diabetic states.

Table 2: Protein-tyrosine phosphatase activities in subcellular fractions of Fao rat hepatoma cells

Fraction	Specific Activity (pmol PO_4/min/mg)	Total Activity in Fraction (pmol PO_4/min)
Cytosol	2.8 ± 0.1	50.9 ± 0.9 (13.5%)
Particulate	11.6 ± 0.1	292.3 ± 3.0 (85%)
Glycoprotein	17.4 ± 0.5	5.9 ± 0.5 (1.5%)

Protein-tyrosine phosphatase activities in Fao cell fractions were assayed using a labeled peptide derived from the insulin receptor regulatory domain. Adapted from data in reference 25.

Effects of Insulin on PTPase Activity in Hepatoma Cells

To complement the studies in diabetic animals, we were interested in whether physiological levels of insulin itself might be an important factor in the regulation of insulin receptor PTPase activity. For these studies, we used the well-differentiated rat hepatoma cell line Fao which has been used as a model for insulin action in normal liver (29). As in the animal studies, PTPase activity was measured using the ^{32}P-labeled peptide corresponding to the insulin receptor regulatory domain (Figure 2).

Insulin (100 nM) increased PTPase activity in the particulate fraction by 40% and decreased the activity in the cytosolic fraction by 35%. These effects occurred within 15 min and were half maximal at 3-4 nM insulin (25). When assessed as total activity, the magnitude of the changes in PTPase activity in the particulate and cytosolic fractions could not be explained solely on the basis of a

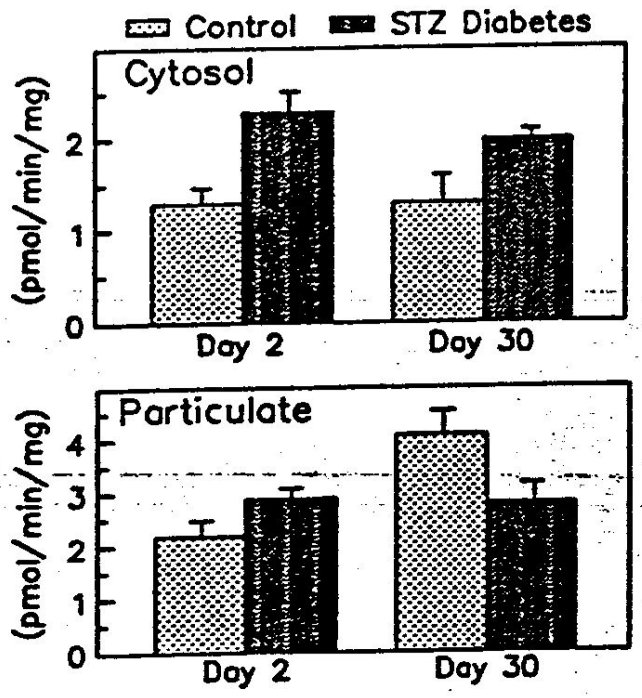

Figure 1: Alterations in cytosol (upper panel) and particulate (lower panel) PTPase specific activities in rat liver after 2 or 30 days of streptozotocin (STZ)-induced diabetes. PTPase activity was measured using a phosphorylated peptide from the insulin receptor regulatory domain. Adapted from reference 24.

translocation of PTPases between the two pools. Thus, in hepatoma cells, both membrane and cytosolic PTPases can be regulated by physiological concentrations of insulin, suggesting that modulation of PTPase activity may secondarily affect insulin action in states of diabetes with insulin resistance.

WHICH CELLULAR PTPases ARE POTENTIALLY RELEVANT TO INSULIN ACTION?

Recent biochemical and molecular cloning studies have revealed that there is a discrete family of membrane and cytoplasmic enzymes that possess PTPase specificity (3). To date, these include single-domain PTPase enzymes that lack transmembrane domains and are localized to an intracellular compartment. The cloned enzymes in this category include the placental PTPase IB and an isolate from a T cell library with a similar structure (30,31).

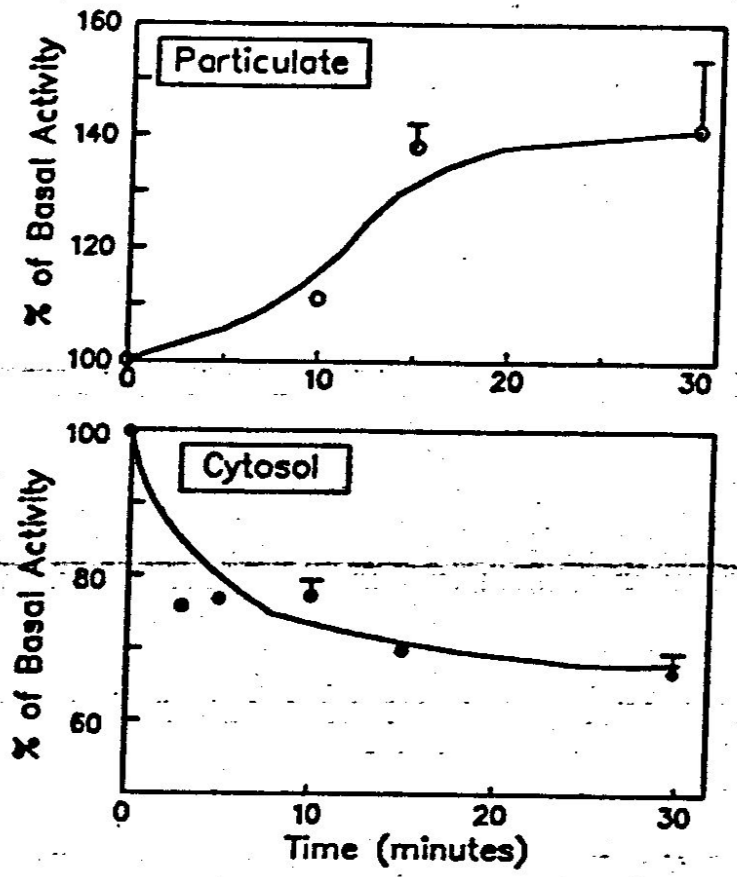

Figure 2: Effect of insulin (100 nM) on PTPase activities in Fao rat hepatoma cells. The insulin receptor peptide was used as substrate. Adapted from reference 25.

A second broad class of PTPases have an overall structure like that of a membrane receptor with an extracellular glycosylated domain, a single transmembrane domain and an intracellular enzymatic component. The cytoplasmic domain of these enzymes which accounts for the PTPase activity consists of tandemly duplicated segments of about 300 amino acids each that have close homology to the PTPase domain of the single domain enzymes described above. The enzymes in this group include CD45 (LCA), which is restricted to hematopoietic cells, and LAR which has a broader tissue distribution (3,32). Several other transmembrane PTPases have recently been cloned and are in the early stages of characterization (33).

Characterization of PTPase Specificity and Activity

Besides subcellular localization, the insulin receptor PTPase might be expected to have some specificity towards the phosphorylated insulin receptor regulatory domain as a substrate. To begin to analyze whether catalytic domains of two PTPases might differ in their specificity towards phosphorylated growth factor receptors as substrates we compared the activity of CD45, LAR and a crude rat hepatic membrane preparation against the intact insulin and EGF receptors. In collaboration with Dr. Haruo Saito, we obtained a crude bacterial lysate containing the CD45 and LAR intracellular PTPase domains induced with IPTG from a plasmid vector in an E. coli strain deficient in alkaline phosphatase activity. These recombinantly-generated proteins were about 1% pure (34). Phosphorylated receptor substrates were prepared from a glycoprotein-rich WGA-purified fraction of hepatic membranes by phosphorylation with γ-[^{32}P]-ATP in the presence of insulin for 60 min at 4°C to stimulate the phosphorylation of the insulin receptor. Under these conditions the EGF receptor also undergoes constitutive autophosphorylation. The labeled receptors were subjected to dephosphorylation using each of the PTPase preparations for 0 to 80 min. At each time point, the reaction mixture was sampled and subjected to SDS gel electrophoresis and the resulting autoradiogram was scanned to quantitate the dephosphorylation.

All three PTPase preparations were active but there were several striking differences (Figure 3). First, the hepatic membrane preparation contained the most active PTPase. Secondly, both LAR and CD45 produced only about a 50% dephosphorylation of the insulin receptor suggesting that specific phosphorylation sites may be resistant to dephosphorylation by certain PTPase catalytic domains. Finally, there was also a clear difference between the potencies of the recombinant PTPases toward the EGF receptor with LAR being more potent than CD45.

These preliminary data suggest that the hepatic membrane contains a PTPase (or a combination of PTPases) which is more active than the recombinant catalytic domains of LAR or CD45 towards two of the membrane receptors present in this tissue. Furthermore, these findings also suggest that various PTPases exhibit differences in

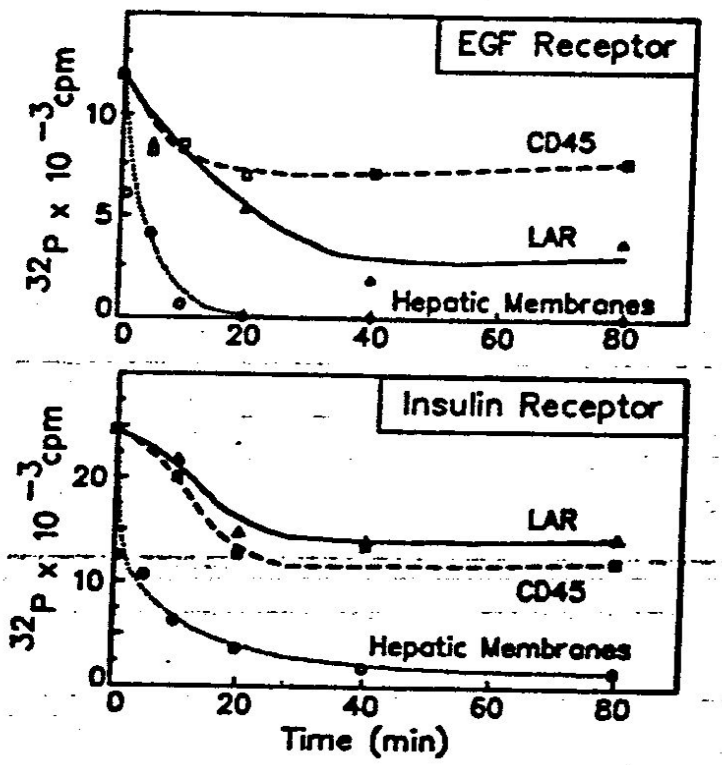

Figure 3: Dephosphorylation of partially purified epidermal growth factor (EGF) receptors (upper panel) and insulin receptors (lower panel) by catalytic domains of LAR and CD45 PTPases expressed in a bacterial system. Results with a solubilized rat liver membrane fraction are also shown.

potency and specificity, possibly even to different phosphorylation sites within a single protein. This result raises the interesting possibility that multiple PTPases may interact with membrane receptors to regulate their state of phosphorylation on specific tyrosine residues and ultimately affect their activity in signal transduction.

Identification of the "Insulin Receptor" PTPase

In addition to the CD45 and LAR preparation described above, other PTPases have been shown to have activity against the IR *in vitro*. In a series of elegant experiments by Tonks et al. (35,36), the cytosolic placental PTPase 1B has been shown to be active not only *in vitro*, but also *in vivo*, against the phosphorylated insulin receptor β-subunit. When injected into Xenopus oocytes, this enzyme blocked insulin-stimulated S6 peptide phosphorylation, and retarded insulin-induced maturation of the oocytes indicating that insulin action in these cells can be modulated by PTPases. However, given the lack of specificity of PTPase catalytic domains in *in vitro* experiments and the large number of these enzymes in various compartments *in vivo*, it is safe to say that the PTPase(s) relevant to the cellular regulation of the insulin receptor remain unknown.

As discussed above, PTPase activities against the phosphorylated insulin receptor are found in both the cytosol and membrane fractions of liver. Two pieces of evidence have suggested that a membrane PTPase might possibly be more important in the physiological dephosphorylation of the insulin receptor *in vivo*. One comes from studies with permeabilized adipocytes in which the rapid dephosphorylation of the receptor persists despite a virtually complete exchange of the cellular cytoplasmic compartment (17). In addition, as shown above, the bulk of the insulin receptor PTPase activity in liver is found in solubilized membranes and the highest specific activity in a glycoprotein fraction. PTPase at the cell surface may thus serve to regulate the phosphorylation state of the insulin receptor in the membrane. It is possible that additional PTPase activities come into play as the receptor is

internalized to other sites in the cell. Other PTPases may also function in cells to dephosphorylate protein substrates of the insulin receptor such as pp185, which appears to be even more susceptible to dephosphorylation than the receptor itself (12,13).

MOLECULAR CLONING OF RAT LIVER PTPases

While biochemical studies have provided some insights into tissue PTPase activities using particular substrates, these studies do not allow one to isolate and characterize the molecular origin of the various cytosolic and membrane enzymes which may be present. To better characterize PTPases relevant to insulin receptor regulation, we have begun an effort to identify, clone and sequence PTPases from rat liver.

In order to determine which of the recently cloned PTPases might be useful to screen for related PTPases in insulin-sensitive tissues, we used a segment corresponding to the PTPase domain of the cDNA for human LAR (32) and the T-cell PTPase (31) (kindly provided by Drs. H. Saito and E. Fischer, respectively) as probes on a Northern blot with several rat tissues to explore their distribution (Figure 4). Messenger RNA for the T-cell enzyme was restricted in its distribution to rat spleen. In contrast, LAR mRNA, or that corresponding to a related enzyme, was expressed primarily in liver, but also found in kidney, fat and brain. LAR, therefore, appeared to be an appropriate candidate for the initial cloning of PTPase enzymes in rat liver because of its tissue expression, its transmembrane localization, and the demonstration that its catalytic domain has activity against the insulin receptor (Figure 3).

We initially used 4 cDNA fragments encoding most of the human LAR coding region, to screen 1 million phage plaques of a rat liver cDNA library at reduced stringency (40% formamide, 35°C). Twenty-four positive clones were obtained after secondary screening with the LAR cDNA. Insert-bearing pBluescript phagemids were excised from the lambda ZAPII clones using the helper phage R408 and plasmids were prepared by infection of XL-1 Blue *E. coli* host cells. Plasmid slot-blots were used to identify 15 cDNA inserts

which hybridized strongly with a LAR segment restricted to the PTPase domain in a tertiary screening.

Sequence and RNA blot analysis of these multiple clones showed that at least two distinct PTPases were represented. One set of cDNAs encoded the rat homolog of LAR, or rLAR, which was identical in the tissue distribution and the size of its mRNA transcripts as that observed with the human LAR cDNA probe (Figure 4). Two of the cDNAs corresponded to a PTPase sequence that was somewhat different from LAR, and on Northern analysis a single mRNA transcript of 7.1 kb was observed in liver for these cDNAs that migrated between the major LAR mRNAs of 7.5 and 5.2 kb. We have called this cDNA rPTP-2. Genomic Southern blot analysis confirmed that the LAR and PTP-2 sequences corresponded to distinct, single copy genes in the

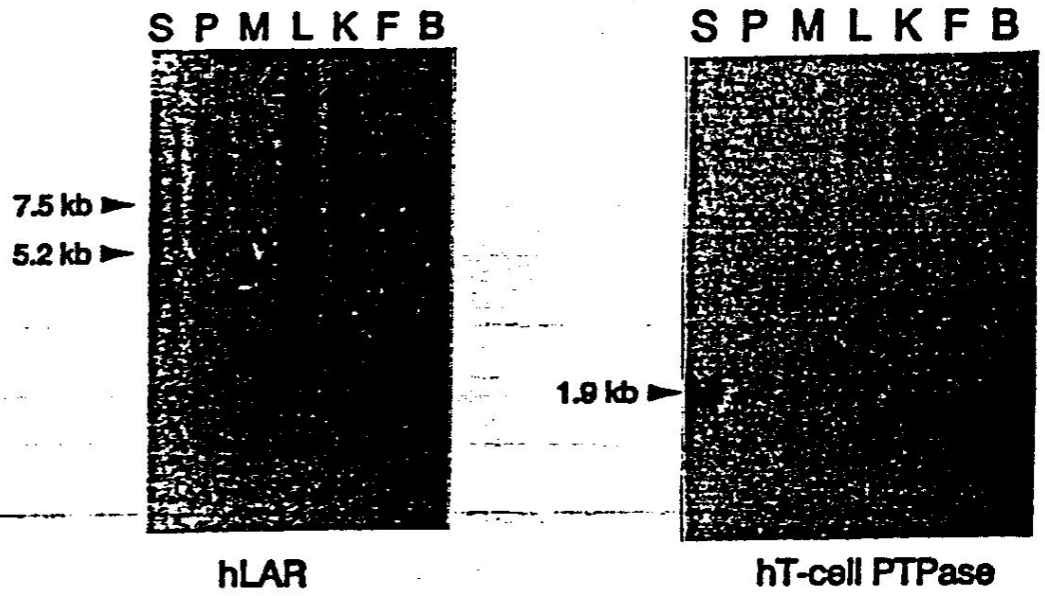

Figure 4: Northern blot analysis of mRNA from several rat tissues using the human LAR and human T-cell PTPase cDNAs as probes. Each lane was loaded with poly(A)+ RNA as follows: spleen (S) 24 ug, placenta (P) 45 ug, muscle (M) 25 ug, liver (L) 30 ug, kidney (K) 22 ug, fat (F) 5 ug and brain (B) 14 ug.

rat (37). A single cDNA for a third type of sequence with PTPase homology has been isolated and is currently in the process of being sequenced. Using this approach, therefore, we have isolated several candidate rat liver PTPase homologs that exhibit tissue specificity and occur in Northern analysis in insulin-sensitive tissues such as liver.

The relative abundance of the isolated clones suggests they are quite rare. Based on the independent clones isolated from the library, rat LAR mRNA apparently represents 7 copies per million, which is similar in magnitude to the abundance of rat insulin receptor mRNA which we had cloned from the same library. The abundance of PTP-2 appears to be more rare in liver, on the order of 1 to 2 copies per million.

Sequence Homology of Rat Liver PTPase Clones

We have obtained partial sequence data for the coding region of rat LAR and rat PTP-2. The deduced amino acid sequence for each clone has two tandemly repeated intracellular domains with close homology to known PTPases and nearly full conservation of the specific residues found in all PTPase sequences to date (33). Each enzyme has a single transmembrane region and a large extracellular domain for which we have obtained a partial sequence of approximately 700 amino acids for each clone. When the available sequences are compared to human LAR (32), the rat homolog is identical in the transmembrane domain and 98% and 93% identical in the intracellular and extracellular segments, respectively (Figure 5). Rat PTP-2, is quite similar to LAR in the second cytoplasmic PTPase domain, but has less than 80% identity in the proximal PTPase domain and the transmembrane region. The extracellular portion of the sequence is markedly different from LAR with approximately 50% sequence identity.

Thus we have isolated a series of cDNAs that are likely to encode members of a family of PTPases in rat liver. Variations in the PTPase catalytic sequence as well as the adjacent polypeptide structure of these enzymes is likely to influence their intracellular location as well as their specificity towards various

protein-tyrosine substrates in the cell. Association with other cell surface proteins may also regulate their activity or substrate specificity. Since these are transmembrane receptor-like proteins, further work should be aimed at discovering potential ligands that may affect the activity of the intracellular catalytic domain of these PTPases in an analogous fashion to hormone receptor activation.

SUMMARY

In summary, there is growing evidence that mammalian cells and tissues, including liver, possess a family of related cytoplasmic and membrane proteins with PTPase activity. There is some evidence suggesting that there are differences in the specificity of these PTPases for certain substrates. The transmembrane structure of some PTPases has also suggested that they may serve as receptors for some undefined ligand molecules. Since tyrosine phosphorylation plays a central role in the action of several hormones and in the metabolic regulation of cells by several growth factors and oncogenes, elucidation of the biochemistry and regulation of PTPases will be critical for a full understanding of the cellular role of these

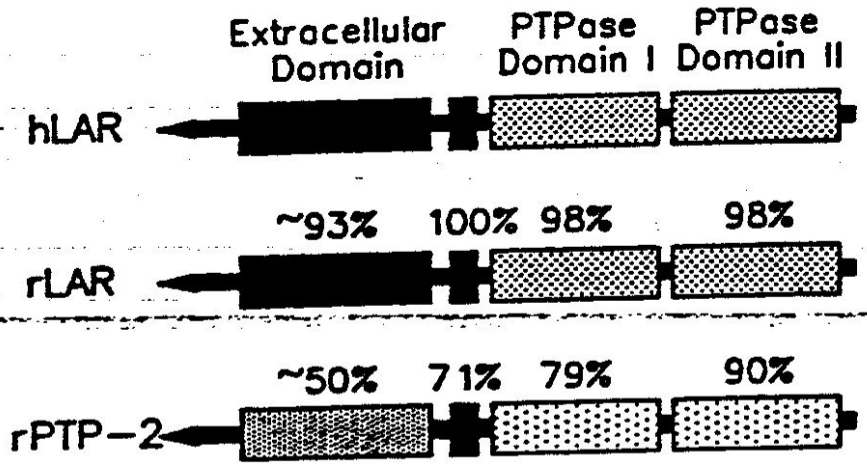

Figure 5: Schematic diagram of sequence identity between human LAR (hLAR), rat LAR (rLAR) and rat PTP-2 in the tandem PTPase domains, the transmembrane segment and the available extracellular sequence (approximately 700 residues for each of the rat enzymes).

important regulatory systems. Furthermore, characterization of the specific PTPase(s) that regulate the phosphorylation state of the insulin receptor may lead to the discovery of agents which, by modulating PTPase activity, may ultimately provide a novel therapeutic approach to enhancing insulin action in insulin-resistant human diabetes.

ACKNOWLEDGEMENTS

We are grateful to Dr. Haruo Saito (Dana Farber Cancer Institute) and Dr. Edmond Fischer (University of Washington) for providing us with PTPase cDNA clones. These studies were supported by the Joslin Diabetes Center DERC grant DK36836 and the following individual research grants: BJG, a Research and Development Award from the American Diabetes Association; JMB, a Postdoctoral Fellowship from the Juvenile Diabetes Foundation (JDF 390549); PC, a Fogarty International Research Fellowship Award (TW04319-01); CRK, NIH grant DK33201, JDF grant 188182 and a Pfizer Biomedical Research Award.

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