6-1-1992

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Biochem. J. (1992) 284, 569–576 (Printed in Great Britain)

Insulin receptor and epidermal growth factor receptor dephosphorylation by three major rat liver protein-tyrosine phosphatases expressed in a recombinant bacterial system

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Protein-tyrosine phosphatases (PTPases) play an essential role in the regulation of signal transduction mediated by reversible protein-tyrosine phosphorylation. In order to characterize individual rat hepatic PTPases that might have specificity for autophosphorylated receptor tyrosine kinases, we isolated cDNA segments encoding three PTPases (PTPase 1B, LAR and LRP) that are expressed in insulin-sensitive liver and skeletal muscle tissue, and evaluated their catalytic activity *in vitro*. The intrinsic PTPase activities of the full-length PTPase 1B protein and the cytoplasmic domains of LAR and LRP were studied by expression of recombinant cDNA constructs in the inducible bacterial vector pKK233-2 using extracts of a host strain of *Escherichia coli* that lacks endogenous PTPase activity. Each of the cloned cDNAs dephosphorylated a cognate phosphopeptide derived from the regulatory region of the insulin receptor. Despite having only 30–39% sequence identity in their catalytic domains, LAR and PTPase 1B had similar relative activities between the peptide substrate and intact insulin receptors, and also displayed similar initial rates of simultaneous dephosphorylation of insulin and epidermal growth factor (EGF) receptors. In contrast, LRP exhibited a higher rate of dephosphorylation of both intact receptors relative to the peptide substrate, and also dephosphorylated EGF receptors more rapidly than insulin receptors. These studies indicate that three PTPases with markedly divergent structures have the catalytic potential to dephosphorylate both insulin and EGF receptors in intact cells and that redundant PTPase activity may occur *in vivo*. For these PTPases to have specific physiological actions in intact cells, they must be influenced by steric effects of the additional protein segments of the native transmembrane enzymes, cellular compartmentalization and/or interactions with regulatory proteins.

INTRODUCTION

The reversible phosphorylation of protein-tyrosine residues plays an essential role in the control of a variety of specialized cellular functions, including signal transduction by cell surface receptors with tyrosine kinase activity, such as those for insulin and epidermal growth factor (EGF) (Yarden & Ullrich, 1988). The tyrosine phosphorylation state of cellular proteins in these pathways is regulated by a balance between the action of tyrosine kinases, which may be the autophosphorylated receptors themselves, and specific enzymes that contain protein-tyrosine phosphatase (PTPase) activity (Hunter, 1989).

While much has been learned about the cellular and molecular physiology of tyrosine kinases over the past decade, only recently have a number of full-length and partial PTPase sequences been determined by several laboratories, revealing that they comprise a family of related proteins (reviewed by Fischer *et al.*, 1991). The catalytic domain of these PTPases consists of single or tandemly duplicated segments of approx. 260 amino acids that contain a series of residues that are highly conserved. Two distinct classes of PTPase have also been distinguished by their overall structure. One class includes the single-domain PTPase 1B and a related enzyme isolated from a T-cell cDNA library, both of which lack transmembrane domains (Cool *et al.*, 1989; Guan *et al.*, 1990; Chernoff *et al.*, 1990). The other class of PTPases consists of those with a receptor-like transmembrane structure and includes the tandem-domain enzymes leucocyte common antigen (LCA; CD45), whose expression is limited to haematopoietic cells (Tonks *et al.*, 1988*a*; Thomas, 1989), and two structurally similar PTPases, LAR (for LCA-related) and LRP (for LCA-related phosphatase; also called PTP-α) which have wider tissue distributions (Streuli *et al.*, 1988; Matthews *et al.*, 1990; Sap *et al.*, 1990; Krueger *et al.*, 1990; Jirik *et al.*, 1990; Kaplan *et al.*, 1990). Although the cloning of PTPase homologues has progressed rapidly, little is known about their potential physiological roles, especially in non-haematopoietic tissues. Only recently have data been reported on the biochemical characteristics of individual PTPases and, importantly, the substrates of these enzymes in intact cells are not known.

One of the fundamental physiological roles of PTPases is their action on hormone and growth factor receptors with tyrosine kinase activity and their potential modulation of signal transduction through these pathways (Cicirelli *et al.*, 1990; Tonks *et al.*, 1990). We are particularly interested in the regulation of insulin signalling by reversible tyrosine phosphorylation under physiological conditions in tissues involved in normal metabolic homeostasis (Goldstein, 1992). Since the main target tissues of insulin action in the control of glucose metabolism are skeletal muscle and liver, we have begun to identify specific PTPases that are expressed in these tissues in order to evaluate their physiological role in the regulation of insulin receptor signalling, and ultimately their possible involvement in states of insulin resistance (Goldstein *et al.*, 1991, 1992; McGuire *et al.*, 1991).

Recently we reported that LAR and LRP are major PTPase homologues expressed in rat liver and muscle (Zhang &

Abbreviations used: PTPase, protein-tyrosine phosphatase; EGF, epidermal growth factor; LCA, leucocyte common antigen; LAR, LCA-related; LRP, LCA-related phosphatase; IPTG, isopropylthio-β-D-galactoside; DTT, dithiothreitol; PMSF, phenylmethanesulphonyl fluoride.

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Goldstein, 1991). These results are extended in the present study, where we show that PTPase 1B is also abundantly expressed in normal liver and skeletal muscle tissue. We further investigated the catalytic activity of these three major PTPase enzymes, as expressed in a recombinant bacterial system, to assess their relative activity against intact, autophosphorylated insulin and EGF receptors, which represent potential physiological substrates for these enzymes in intact cells.

MATERIALS AND METHODS

Materials

A mixed oligo(dT) and random hexamer-primed cDNA library in λZAPII prepared from a male Sprague–Dawley rat liver, *Escherichia coli* XL-1 blue host cells and the helper phage R408 were obtained from Stratagene (La Jolla, CA, U.S.A.). DNA restriction endonucleases, modifying enzymes and size markers were purchased from New England Biolabs (Beverly, MA, U.S.A.). Oligonucleotides were obtained from Oligos Etc. (Guilford, CT, U.S.A.), and the Joslin DERC Molecular Core facility. *Thermus aquaticus* DNA polymerase was obtained from Perkin–Elmer Cetus (Norwalk, CT, U.S.A.). Modified T7 DNA polymerase (Sequenase) was obtained from U.S. Biochemical (Cleveland, OH, U.S.A.). [α-^{35}S]dATP and [γ-^{32}P]ATP were from New England Nuclear (Beverly, MA, U.S.A.). The pKK233-2 expression vector was obtained from Pharmacia (Piscataway, NJ, U.S.A.). Wheat-germ-agglutinin–agarose was from Vector Laboratories (Burlingame, CA, U.S.A.). Aprotinin, lysozyme and *N*-acetyl-D-glucosamine were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). EGF was from Collaborative Research (Bedford, MA, U.S.A.). Dowex AG 1-X2 acetate and Bio-Gel P-6 were from Bio-Rad Laboratories (Richmond, CA, U.S.A.). The insulin receptor peptide substrate (residues 1154–1165; numbered according to Ebina *et al.*, 1985) was synthesized by Dr. Steven Shoelson (Joslin Diabetes Center, Boston, MA, U.S.A.).

Animals

Male Sprague–Dawley rats weighing 150–175 g were obtained from Taconic Farms (Germantown, NY, U.S.A.), and had free access to food and water.

Preparation of tissue RNA

Total RNA was prepared from the indicated tissues by homogenization in guanidinium thiocyanate, extraction with phenol/chloroform and precipitation from propan-2-ol (Chomczynski & Sacchi, 1987). In some experiments the polyadenylated mRNA fraction was enriched by chromatography over oligo-(dT)–cellulose (Aviv & Leder, 1972).

cDNA cloning

To obtain a cDNA insert encoding the rat homologue of the LAR PTPase, 1×10^6 phage plaques of the rat liver cDNA library were hybridized in a solution containing 40% (v/v) formamide, 0.1% (w/v) Ficoll, 0.1% (w/v) polyvinylpyrrolidone, 0.1% (w/v) BSA, 5 mM-EDTA, 0.1% (w/v) SDS, 100 μg of denatured salmon sperm DNA/ml, 750 mM-NaCl and 50 mM-NaH_2PO_4, pH 7.4, at 35 °C with a labelled cDNA encoding all but the extreme 5′ end of the human LAR sequence (kindly provided by Dr. Haruo Saito, Dana Farber Cancer Institute, Boston, MA, U.S.A.). Filters were washed at 50 °C in buffer containing 75 mM-NaCl and 0.1% (w/v) SDS in 7.5 mM-sodium citrate, pH 7.0. Insert-bearing pBluescript phagemids were excised from the λZAPII clones using the helper phage R408 and plasmids were prepared by infection of XL-1 Blue *E. coli* host cells. Double-stranded sequencing was performed using Sequenase, as described by the manufacturer, using T3 and T7 promoter sequencing primers, or synthetic 17-mer oligonucleotides derived from known sequences (Sanger *et al.*, 1977). For large (> 2 kb) inserts, nested deletions were prepared for sequencing each strand, as described previously (Goldstein & Dudley, 1990).

cDNA inserts encoding the rat PTPase 1B and LRP enzymes were obtained by a coupled reverse transcription/PCR technique. For PTPase 1B, a specific antisense oligonucleotide primer derived from amino acid residues at the 3′ end of the rat PTPase 1B coding region (Guan *et al.*, 1990) was used in a reverse transcription reaction on rat liver total RNA as described previously (Goldstein & Dudley, 1990). This was followed by cDNA amplification with the addition of an oligonucleotide primer that flanks the extreme 5′ end of the 1.3 kb cDNA coding region. The amplification primers also incorporated *Nco*I and *Hin*dIII restriction sites for subcloning of the cDNA products in frame into the bacterial expression vector pKK233-2 (Sambrook *et al.*, 1989). For the LRP PTPase, primers derived from the published murine LRP cDNA sequence (Matthews *et al.*, 1990) were used for reverse transcription of rat liver RNA and subsequent cDNA amplification. The antisense primer extended from just beyond the translation stop codon and covered the 5 amino acid residues that lie at the immediate 3′ end of the coding region. In order to express the intact cytoplasmic domain of LRP, the 5′ end amplification primer was identical to the cDNA encoding the 6 amino acid residues that immediately follow the transmembrane domain, with the addition of an *Nco*I site for translation initiation and subcloning into pKK233-2. The subcloned cDNA inserts for PTPase 1B and LRP were then sequenced as described above.

Sequence analysis

PTPase sequence identities and alignments were identified using programs from the Molecular Engineering Research Center at Boston University (Boston, MA, U.S.A.).

Northern blot analysis

Tissue poly(A)$^+$ RNA (20 μg) was fractionated by electrophoresis in 1% agarose gels containing 0.66 M-formaldehyde and transferred to a nitrocellulose filter (Davis *et al.*, 1986). The filter was hybridized and washed under high-stringency conditions with a cDNA probe labelled by random hexamer priming (Feinberg & Vogelstein, 1983).

cDNA expression in a bacterial system

As described above, the cDNA inserts for PTPase 1B and rat LRP obtained by cDNA amplification were directly subcloned into the isopropylthio-β-D-galactoside (IPTG)-inducible expression vector pKK233-2 (Amann & Brosius, 1985). For LAR, one cDNA insert isolated from the rat liver library that spanned the entire cytoplasmic domain of LAR was used as a template in a modified PCR to subclone this region in frame into the pKK233-2 vector. For this reaction, a 5′ end oligonucleotide primer (AAAGAGGAAGAGAACACA) was designed for DNA amplification of the intracellular segment of rat LAR that would allow this insert to be expressed in frame after the addition of *Pst*I linkers and ligation to the *Pst*I site of pKK233-2. In order to minimize the possibility of error incorporation during cDNA amplification, the 5′ end primer and a Te promoter primer were used with 3 μg of the plasmid cDNA template, and reaction conditions were limited to 10 cycles that consisted of 55 °C for 90 s, 72 °C for 150 s and 96 °C for 60 s (Goldstein & Dudley, 1990). The amplification products were gel-purified and treated with *Pst*I methylase to block the internal *Pst*I sites, and

phosphorylated *Pst*I linkers (New England Biolabs) were added (Sambrook *et al.*, 1989). After digestion with *Pst*I, the insert was symmetrically subcloned into the unique *Pst*I site of pKK233-2. This construct was completely sequenced and found to contain no amplification errors.

For protein expression, the plasmid was transformed into *E. coli* strain DHB4 [ΔphoA phoR (F′ lacI^q pro)] which carries a defect in the genes encoding alkaline phosphatase activity (Boyd *et al.*, 1987). Cultures were induced with 2 mM-IPTG and the bacteria were sedimented, lysed with lysozyme and subjected to three cycles of freeze–thawing in a solid CO_2/ethanol bath. Supernatants were prepared by treating the lysed cells with DNAase I and removing insoluble material by centrifugation at 15000 *g* for 15 min at 4 °C (Streuli *et al.*, 1989).

Assay of recombinant PTPase activities in bacterial extracts

A synthetic peptide derived from the autophosphorylated regulatory region of the insulin receptor β-subunit (residues 1154–1165) was used as a phosphatase substrate. The peptide was labelled with insulin receptors that were solubilized from a membrane fraction of Chinese hamster ovary cells transfected with the cloned human insulin receptor (White *et al.*, 1987) and partially purified by wheat-germ-agglutinin–agarose chromatography (Kasuga *et al.*, 1985). Aliquots of the lectin column eluate were incubated with 1 μM-insulin for 30 min at 4 °C, after which 500 μCi of [γ-^{32}P]ATP was added at a final concentration of 25 μM, followed by $MnCl_2$ to 5 mM and 1 mg of the 1154–1165 peptide in a volume of 350 μl. After 16 h at 4 °C, 1 ml of 5% (w/v) trichloroacetic acid was added and the ^{32}P-labelled peptide in the supernatant was purified on AG1-X2 acetate resin followed by passage through a C_{18} Sep-Pak cartridge (Waters Associates, Milford, MA, U.S.A.) and lyophilized (Sparks & Brautigan, 1985). When prepared in this way, the peptide is monophosphorylated on the tyrosine-1162 residue (S. Shoelson, unpublished work).

PTPase activity was assayed by incubating portions of the bacterial extract with labelled substrate in a reaction buffer containing 1 mM-dithiothreitol (DTT) and 2 mM-EDTA in 50 mM-Hepes, pH 7.0, at 30 °C. Reactions were terminated by the addition of 1 mM-H_2SO_4 and 5 mM-silicotungstic acid, and [^{32}P]P_i released from the labelled peptide was measured by organic extraction of the supernatant (Shacter, 1984). Specific activity is presented as pmol of PO_4 released/min per mg of protein.

Dephosphorylation of insulin and EGF receptors

Growth factor receptors were obtained from a solubilized membrane fraction of rat liver by wheat-germ-agglutinin–agarose chromatography (Kasuga *et al.*, 1985). Insulin and EGF receptors (4 μg of protein from the lectin column eluate) were labelled in a 0.6 ml reaction containing 1 μM-insulin, 5 μg of EGF/ml, 5 mM-$MnCl_2$, 25 μM-ATP and 375 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) in 50 mM-Hepes, pH 7.6, at 4 °C for 60 min. Unincorporated ATP was removed using a Bio-Gel P6 spin column, and 30 μl aliquots of the labelled receptors were incubated with 31.5 μg of bacterial lysate protein in a 150 μl reaction containing 1 mM-DTT and 2 mM-EDTA in 50 mM-Hepes, pH 7.0, at 30 °C. After incubation for the indicated period of time, 0.5 ml of a chilled stop solution containing 10 mM-ATP, 10 mM-sodium pyrophosphate, 4 mM-EDTA, 100 mM-NaF, 5 mM-sodium vanadate, 0.1 mg of aprotinin/ml and 2 mM-phenylmethanesulphonyl fluoride (PMSF) in 50 mM-Hepes buffer, pH 7.6, was added. Samples were immunoprecipitated with anti-phosphotyrosine antibody, absorbed to Trisacryl–Protein A and washed. After boiling in gel sample buffer containing 100 mM-DTT, samples were subjected to electrophoresis in gels containing SDS and 7.5% polyacrylamide (Laemmli, 1970). The incorporation of ^{32}P into the 95 kDa insulin receptor β-subunit and the 170 kDa EGF receptor polypeptide was quantified by scanning densitometry of gel autoradiograms (Molecular Dynamics).

Statistical analysis

GraphPAD software (San Diego, CA, U.S.A.) was used to calculate *t* test and analysis of variance results. Bonferroni's correction was employed for calculation of *P* values in the event of multiple comparisons.

RESULTS

cDNA cloning of PTPase homologues in rat liver and skeletal muscle

A total of 10^6 plaques of the rat liver cDNA library were screened with probes from human LAR cDNA. After secondary screenings and sequence analysis, seven overlapping cDNA inserts were identified that had 98% overall sequence identity with human LAR in the deduced 623 amino acids of its cytoplasmic domain and thus encoded the rat homologue of LAR (Pot *et al.*, 1991; Goldstein *et al.*, 1992).

A cDNA segment encoding the cytoplasmic domain of the rat homologue of the PTPase designated LRP was obtained by cDNA amplification. After subcloning and sequence analysis, the deduced 629-amino-acid sequence of this clone had over 97% identity with the corresponding region of the alternatively spliced murine LRP cDNA that lacked the 108-nucleotide exon insertion in the PTPase domain (Matthews *et al.*, 1990; Sap *et al.*, 1990). The cDNA sequence of the entire 432-amino-acid protein-coding region for the rat homologue of PTPase 1B, also obtained by cDNA amplification, was identical to that described by Guan *et al.* (1990).

In previous work we demonstrated by amplification of cDNA, library screening and Northern blot hybridization that LAR and LRP are major PTPases expressed in normal rat liver and

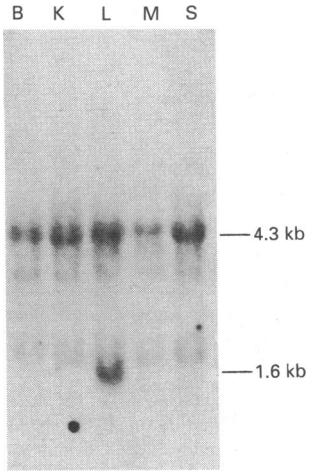

Fig 1. Northern blot analysis of PTPase 1B mRNA expression in several rat tissues

Tissues were brain (B), kidney (K), liver (L), muscle (M) and spleen (S). A 20 μg portion of poly(A)$^+$ RNA from each of the indicated tissues was denatured and subjected to electrophoresis in 1% agarose/0.66 M-formaldehyde gels. The RNA was then transferred to a nitrocellulose filter and hybridized under high-stringency conditions with a ^{32}P-labelled cDNA probe encoding the entire coding region of PTPase 1B as described in the Materials and methods section. A representative autoradiogram is shown.

(*a*)

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PTPase1B: AKLPKNKNRNRYRDVSPFDHSRIKL....HQED....NDYINASLIKMEEAQRSYILTQG
 rLAR D1: SNSEV--PK---AN-IAY----VL-....TSI-gvpgS-----NY-DGYRK-NA--A---
 rLRP D1: -SKEE--EK---VNIL-Y----VH-tpveGVP-....S------F-NGYQEKNKF-AA--

PTPase1B: PLPNTCGHFWEMVWEQKSRGVVMLNRIMEKGSLKCAQYWPQKEEKEMVFDDTNLKLTLIS
 rLAR D1: ---E-M-D--R-----RIAT---MT-LE--SRV--D----ARGTETYGL....IQV--VD
 rLRP D1: -KEE-VND--R-I---NTATI--VTNLK-RKEC-------DQGCWTYGNVRVSVEDVTVL

PTPase1B: EDVKSYYTVRQLELENLATQEAREILHFHYTTWPDFGVPESPASFLNFLFKVRESGSLSP
 rLAR D1: TVELAT--M-TFA-HKSGSS-K--LRQ-QFMA---H----Y-TPI-A--RR-KACNP-..
 rLRP D1: V-YTVRKFCI-QVGDVTNRKPQ-L-TQ--F-S-------FT-IGM-K--K--KACN..PQ

PTPase1B: EHGPIVVHCSAGIGRSGTFCLADTCLLLMDKRKDPSSVDIKKVLLEMRRFRMGLIQTADQ
 rLAR D1: DA--M--------V--T-C-IVI-...AMLERM-HEKT---YGHVTC--SQ-NYMV--E--
 rLRP D1: YA-A---------V--T---VVI-...AML-MMHSERK--VYGFVSRI-AQ-CQMV--DM-

PTPase1B: LRFSYLAVIEGAKFIMG
 rLAR D1: YV-IHE-LL-A-MCGHT
 rLRP D1: YV-I-Q-LL-HYLYGDT
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(*b*)

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PTPase1B: .AKLPKNKNRNRYRDVSPFDHSRIKL....H....QEDNDYINASLIKMEEAQRSYILTQ
 rLAR D2: s-N--C--FK--LVNIM-YELT-VC-....QpirgV-GS------FLDGYRQ-KA--A--
 rLRP D2: tGN--A-MKK--VLQII-YEFN-VIIpvkrG....E-NT--V---F-DGYRQKD---AS-

PTPase1B: GPLPNTCGHFWEMVWEQKSRGVVMLNRIMEKGSLKCAQYWPQKEEKEMVFDDTNLKLTLI
 rLAR D2: ---AESTED--R-L--HN-TII---TKLR-M-RE--H----AERSARYQY....FVVDPM
 rLRP D2: ---LH-TED--R-I--W--CSI---TELE-R-QE-------....SDGLVSYGDITVE-K

PTPase1B: SEDVKSYYTVRQLELENLATQEAREILHFHYTTWPDFGVPESPASFLNFLFKVRESGSLS
 rLAR D2: A-YNMPQ-IL-EFKVTDARDGQS-T-RQ-QF-D--EQ---KTGEG-ID-IGQ-HKTKEQF
 rLRP D2: K-EECES----D-LVT-TRENKS-Q-RQ--FHG--EV-I-SDGKGMI-IIAA-QKQQQQ-

PTPase1B: PEHGPIVVHCSAGIGRSGTFCLADTCLLLMDKRKDPSSVDIKKVLLEMRRFRMGLIQTAD
 rLAR D2: GQD---T------V--T-V-ITLSIV-ER-RYEGVVDMFQTV-T-RTQ-PAMVQTEDQYQ
 rLRP D2: GN-.--T------A--T----ALS-...VLERV-AEGVL-VFQTVKSL-LQ-PHMV--LE

PTPase1B: QLRFSYLAVIEGAKFIMG
 rLAR D2: LCYRAA-EYLGSFDHYAT
 rLRP D2: -YE-C-KV-Q-YIDAFSD
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Fig 2. Sequence alignment of PTPases

(*a*) Optimal alignment of the deduced amino acid sequences for the single core PTPase catalytic domain of PTPase 1B with the proximal PTPase domains (D1) of rat LAR (rLAR) and rat LRP (rLRP). Aligned non-identical amino acids are shown by upper case single-letter code symbols; unaligned amino acids are shown in lower case; ---, aligned identical amino acids; ....., a gap introduced for alignment. (*b*) Alignment of the core catalytic domain of PTPase 1B with the distal PTPase domains (D2) of rat LAR and rat LRP.

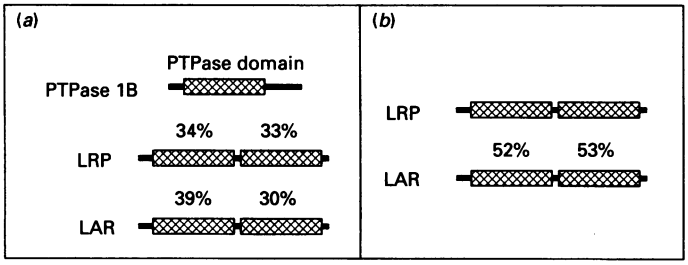

Fig 3. Quantification of the conservation of identical amino acid residues in the core PTPase domains of rat PTPase 1B, LRP and LAR derived from the data presented in Fig. 2

The schematic drawings show either the entire coding region (PTPase 1B) or the cytoplasmic domain (LRP and LAR) with the conserved PTPase region highlighted (⊠). (*a*) Comparison of the PTPase domains of LRP and LAR with the single domain of PTPase 1B. (*b*) Comparison between the proximal and distal domains of LRP and LAR.

skeletal muscle tissue (Zhang & Goldstein, 1991; Goldstein *et al.*, 1991). To examine the distribution of PTPase 1B in normal rat tissues, the cDNA encoding its full-length sequence was used to probe a Northern blot containing mRNA from rat brain, kidney, liver, muscle and spleen (Fig. 1). Expression of PTPase 1B mRNA was found in each tissue as a 4.3 kb transcript, and an additional mRNA of 1.6 kb was found in liver. These results confirm the expression of this PTPase homologue in rat liver and in skeletal muscle, which has not previously been noted. Thus the three PTPase homologues, LAR, LRP and PTPase 1B, are expressed in insulin-sensitive tissues and have a potential role in the regulation of insulin receptor autophosphorylation.

Sequence similarities of the recombinant PTPases

The alignment of the proximal (D1) and distal (D2) core PTPase domains of rat LAR and LRP with the single PTPase region of PTPase 1B is presented in Fig. 2. Quantification of the level of conservation of identical amino acids among the PTPase domains revealed that LAR and LRP have from 30% to 39% identity with the PTPase 1B sequence (Fig. 3). Comparing LRP with LAR, the proximal and distal PTPase domains had sequence identities of 52% and 53% respectively.

Bacterial system for PTPase expression

To initiate studies into the biochemical characteristics of each of the cloned PTPases, the intact cytoplasmic domains of rat LAR and LRP, and the full-length coding region of PTPase 1B, were subcloned in the proper reading frame into the inducible expression vector pKK233-2. For protein expression, the plasmids were transformed into *E. coli* strain DHB4, which lacks

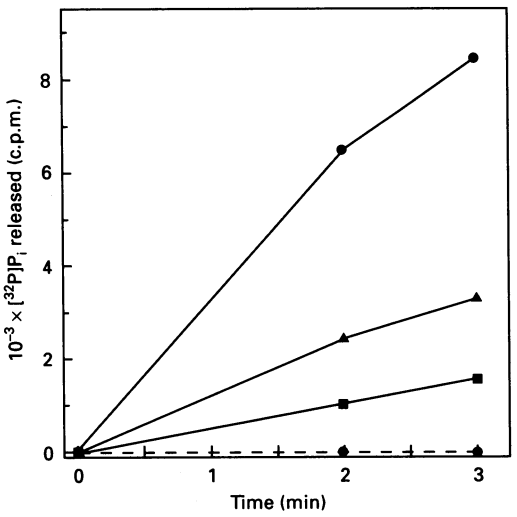

Fig 4. Dephosphorylation of the cognate insulin receptor phosphopeptide by recombinant PTPases expressed in a bacterial system

Complementary DNAs encoding intact PTPase 1B and the cytoplasmic domains of LAR and LRP were subcloned into the expression vector pKK233-2 and transformed into *E. coli* DHB4 host cells. Cultures were induced with IPTG and cell lysates were used to assay PTPase activity against the phosphorylated insulin receptor peptide as described in the Materials and methods section. The cultures included DHB4 cells carrying the native pKK233-2 vector (----), LRP (■), LAR (▲) and PTPase 1B (●).

endogenous alkaline phosphatase activity. Cultures of logarithmically growing cells were induced with IPTG, sedimented, and lysates were prepared. Aliquots of equivalent amounts of bacterial extracts from control cells (transformed with the native pKK233-2) and those expressing the PTPase cDNAs were analysed by gel electrophoresis. The appearance of the expected full-length recombinant protein products, approx. 72 kDa for LAR and LRP and 50 kDa for PTPase 1B, was confirmed for each strain that was absent in the control cells.

Assay of PTPase activity

The bacterially expressed recombinant PTPases were tested for their catalytic activity towards insulin-receptor-related sequences, first using a tyrosyl-phosphorylated peptide substrate derived from the insulin receptor regulatory domain (Tyr-1162 region). With the phosphopeptide substrate, the induced DHB4 cells carrying the native pKK233-2 plasmid without a cDNA insert had no detectable phosphatase activity (Fig. 4). Each of the bacterial lysates containing the recombinant PTPase domains, however, was active in dephosphorylating the labelled insulin-receptor-related peptide. This reaction was essentially linear throughout the time course of the incubation, and was also dependent on the amount of enzyme extract present (results not shown). For the extracts used in these studies, the enzymic specific activities (pmol of ^{32}P released/min per mg bacterial extract protein; means $\pm$ S.E.M., $n = 3$) were 32.5 ± 4.2, 52.7 ± 6.0 and 9.5 ± 1.1 for LAR, PTPase 1B and LRP respectively.

Inhibitory effects of vanadate and NaF on PTPase activities

Using the peptide substrate assay, each of the expressed recombinant PTPases was insensitive to NaF, an inhibitor of serine phosphatases, at concentrations up to 100 mM. Sodium vanadate, a widely used PTPase inhibitor, blocked the activity of each of the three PTPases in a dose-dependent fashion. Half-maximal inhibitory concentrations (IC_{50}) in three replicate experiments were determined to be 1.50 ± 0.12 mM (mean $\pm$ S.E.M.) for LAR ($P < 0.05$ versus PTPase 1B by analysis of variance), 1.07 ± 0.09 mM for LRP and 0.58 ± 0.13 mM for PTPase 1B.

Dephosphorylation of insulin receptors by recombinant PTPases

The activity of the recombinant PTPase domains was further assessed *in vitro* towards the dephosphorylation of intact, autophosphorylated insulin receptors as a potential physiological substrate from rat liver membranes (Figs. 5 and 6). In these studies, the dephosphorylation of the 95 kDa insulin receptor β-subunit was assessed by gel electrophoresis, autoradiography and densitometric scanning of the labelled receptor protein after incubation with each of the bacterial lysates from induced DHB4 cells. With the control extract of induced DHB4 cells carrying the native pKK233-2 vector, minimal dephosphorylation of the insulin receptors was observed during the incubation period up to 30 min. Each of the recombinant PTPase preparations was active in dephosphorylating the autophosphorylated insulin receptors, although some differences were noted in the initial rate and the apparent overall extent of receptor dephosphorylation. Specifically, LAR and PTPase 1B displayed similar profiles of receptor dephosphorylation, whereas LRP had a lower rate of receptor dephosphorylation over the initial 10 min of the reaction which was also maintained throughout the incubation.

These differences in insulin receptor dephosphorylation could result either from variations in the amount of active PTPase expressed from each of the bacterial vector constructs or from actual variation in the substrate specificity of the individual enzymes. In order to distinguish between these two possibilities, we calculated the initial rate of dephosphorylation of the insulin receptor peptide by each of the PTPases, and compared these data to the initial rate of intact insulin receptor dephosphorylation in four replicate experiments similar to those shown in Figs. 5 and 6. As shown in Table 1, PTPase 1B had the highest relative activity towards the peptide substrate, and this was not significantly different from that of LAR. LRP, in contrast, exhibited a 2–3-fold higher relative activity towards the intact insulin receptor compared with the other recombinant PTPases.

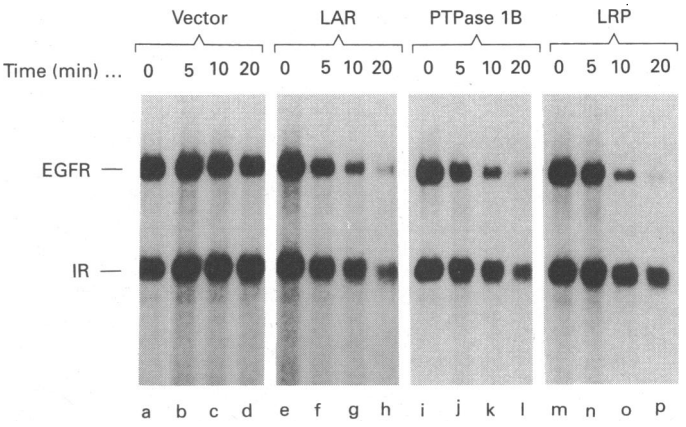

Fig 5. Dephosphorylation of insulin and EGF receptors by recombinant PTPases

The intact coding region of PTPase 1B and the cytoplasmic domains of LAR and LRP were expressed by induction of transformed DHB4 cells as described in the legend to Fig. 4. Aliquots of bacterial lysate protein (31.5 μg) were incubated with autophosphorylated rat liver insulin receptors (IR) and EGF receptors (EGFR) and after the indicated period of time the receptors were purified by immunoprecipitation with anti-phosphotyrosine antibodies and subjected to gel electrophoresis and autoradiography as described in the Materials and methods section.

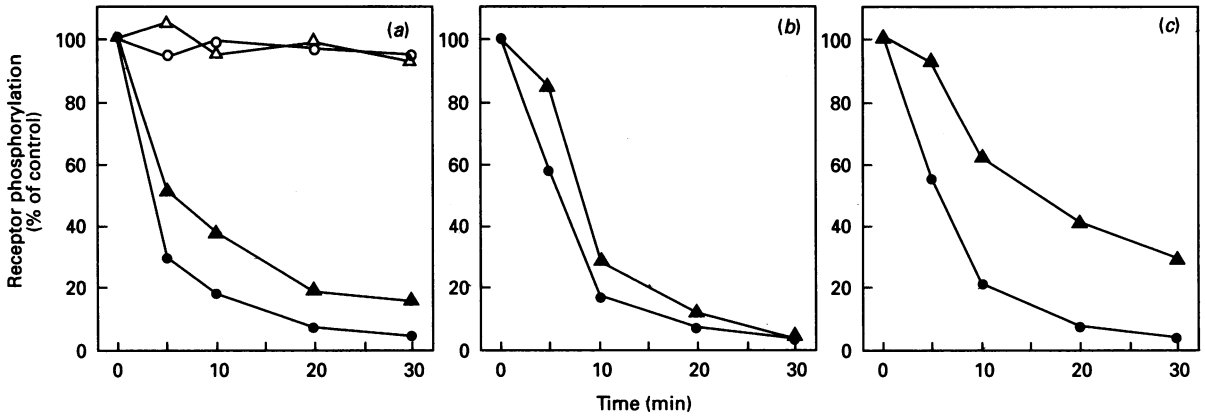

Fig 6. Quantification of insulin and EGF receptor dephosphorylation by recombinant PTPases

Gel autoradiograms from experiments performed as described in Fig. 5 were quantified by densitometric scanning. The radioactivity remaining in the 95 kDa insulin receptor β-subunit (▲, △) and the 170 kDa EGF receptor protein (●, ○) was quantified by scanning densitometry of gel autoradiograms. (*a*) Control lysate (vector alone; △, ○), and LAR (▲, ●); (*b*) PTPase 1B; (*c*) LRP.

Table 1. Relative initial rates of dephosphorylation of the insulin receptor peptide and intact insulin receptors by recombinant PTPases

Results are means ± S.E.M. ($n = 4$). * $P < 0.05$ versus PTPase 1B by analysis of variance with correction applied for three comparisons.

Recombinant PTPase	Relative activity (peptide/intact insulin receptor)
LAR	5.33 ± 1.03
PTPase 1B	8.76 ± 1.63
LRP	2.57 ± 0.24*

Table 2. Relative initial rate of simultaneous dephosphorylation of insulin and EGF receptors by recombinant PTPases

Results are means ± S.E.M. of numbers of determinations given in parentheses. * $P < 0.05$ versus LAR and PTPase 1B by analysis of variance with correction applied for three comparisons. EGFR, EGF receptor; IR, insulin receptor.

Recombinant PTPase	Relative activity (peptide/intact insulin receptor)
LAR	1.29 ± 0.10
PTPase 1B	1.28 ± 0.15
LRP	1.88 ± 0.08*

Simultaneous dephosphorylation of insulin and EGF receptors by recombinant PTPases

One of the major goals of the present study was to determine whether the catalytic domains of three PTPases which are expressed in liver tissue demonstrate a substrate preference between two autophosphorylated plasma membrane receptors to which the PTPases might have access *in vivo* in hepatocytes. To address this possibility, the relative specificity of the recombinant PTPase enzymes was assessed *in vitro* towards the simultaneous dephosphorylation of intact autophosphorylated insulin and EGF receptors which were partially purified from rat liver membranes (Figs. 5 and 6). In these studies the dephosphorylation of the 95 kDa insulin receptor β-subunit and the 170 kDa EGF receptor protein was assessed by gel electrophoresis and autoradiography of the receptor polypeptides as described above. As noted for the insulin receptor, only minimal dephosphorylation of the EGF receptor was observed during incubation with the extract of induced cells carrying only the pKK233-2 vector. Each of the recombinant PTPase preparations, however, actively dephosphorylated the labelled EGF receptors with a similar profile, even though the extracts differed in their total content of PTPase activity when assayed with the insulin-receptor-related peptide substrate (see above).

When the simultaneous dephosphorylations of the two plasma membrane receptors were compared directly (Fig. 6), a similar profile of time-dependent insulin and EGF receptor dephosphorylation was observed with the LAR and PTPase 1B enzymes. In contrast, the recombinant LRP PTPase produced a much slower overall dephosphorylation of insulin receptors compared with EGF receptors present in the same reaction. This is also demonstrated in the representative experiment shown in Fig. 5. For a more quantitative assessment of these data, additional experiments were performed and the initial rate of dephosphorylation for each receptor was calculated at the earliest time point, and a ratio of these values was calculated (Table 2). In accordance with the representative profiles shown in Fig. 5, these results show that LAR and PTPase 1B dephosphorylated EGF receptors only slightly more rapidly than insulin receptors (activity ratio 1.3). However, by *t* test analysis, the EGF/insulin receptor ratios for LAR and PTPase 1B were not statistically significantly different from 1.0 (*P* values of 0.058 and 0.13 respectively), indicating that these enzymes do not markedly differentiate between the two protein-tyrosine substrates during the initial stages of the assay. In contrast, the mean initial rate of EGF receptor dephosphorylation by the recombinant LRP enzyme was almost twice that for dephosphorylation of the insulin receptor. This unique substrate preference for the EGF receptor is substantiated by the significant difference in the relative activity ratio for LRP compared with the two other PTPases by analysis of variance after applying a correction for three comparisons ($P < 0.05$).

DISCUSSION

PTPases play an essential role in a number of signal transduction pathways regulated by reversible protein-tyrosine phosphorylation (Hunter, 1989; Fischer *et al.*, 1991). Previous work on PTPase enzymes in tissue extracts has demonstrated the presence of multiple enzymes in both soluble and particulate cell fractions that have generally been difficult to purify by bio-

chemical techniques (Rotenberg & Brautigan, 1987: Tonks *et al.*, 1988*c*; Roome *et al.*, 1988; Lau *et al.*, 1989; Jones *et al.*, 1989; Meyerovitch *et al.*, 1989; Gruppuso *et al.*, 1991). These factors have hindered the isolation and characterization of individual PTPase homologues from various sources. In the present work we have taken advantage of the recent molecular cloning of a number of PTPases to begin to examine the tissue expression and catalytic properties of individual PTPase enzymes *in vitro*. These studies will enable us to gain some insight into whether these enzymes have generalized or more specific physiological roles in intact cells, with particular attention to the regulation of signalling by tyrosine kinase receptors in normal tissues.

First, we have identified several cloned PTPase enzymes which are expressed in tissues such as liver and skeletal muscle, where one of the critical roles of PTPases is in the regulation of insulin action and metabolic homeostasis *in vivo*. Our previous work and the data reported herein show that LAR, LRP and PTPase 1B are major PTPase homologues in these insulin-sensitive tissues and may thus play a role in the regulation of signalling by the insulin receptor. Although similar in their conservation of specific residues found in core PTPase sequences (Krueger *et al.*, 1990), the catalytic domains of these enzymes retained a substantial degree of structural divergence that might be expected to produce differences in their catalytic activity towards potentially relevant substrates for the enzymes *in vivo*, and suggest a mechanistic role for the enzymes in intact cells. In order to examine this possibility, we assayed the relative activities of their catalytic domains towards a phosphopeptide substrate and two autophosphorylated plasma membrane receptors that are also found in liver cells.

Interestingly, the relative activities of LAR and PTPase 1B with the phosphopeptide and the intact autophosphorylated insulin receptor were not significantly different (Table 1). In addition, the rate of simultaneous dephosphorylation of insulin and EGF receptors was not significantly different between LAR and PTPase 1B. Thus these two enzymes have similar catalytic activities towards a variety of protein-tyrosine substrates and recognize autophosphorylation sites, e.g. on both insulin and EGF receptors, which contain multiple phosphorylated protein-tyrosine moieties. These findings were somewhat unexpected, since these recombinant enzymes have significantly different overall structures, with LAR having two tandem PTPase domains and PTPase 1B only a single domain (Fig. 3). It is of interest that most of the catalytic activity of LCA (CD45) and LAR is contained in the proximal PTPase domain in studies using site-directed mutagenesis and bacterial expression of truncated recombinant cDNA constructs (Streuli *et al.*, 1990; Pot *et al.*, 1991). Thus, although LAR has tandemly conserved PTPase domains, it appears that the active proximal domain alone exhibits a similar catalytic activity to PTPase 1B. While the second domain of LAR may not be catalytically active, it is highly conserved between species, suggesting that it may possibly play an important role in regulation of the PTPase domain by its interaction with potential substrates or associated proteins *in vivo*.

The recombinant cytoplasmic domain of LRP exhibited markedly different properties when compared with the activities of LAR and PTPase 1B, including a significantly higher preference for the dephosphorylation of both the intact insulin receptor and EGF receptor protein compared with the receptor peptide substrate. These results suggest that, for the recombinant LRP enzyme, assays using the insulin receptor peptide, or perhaps other peptide substrates, may not reflect its potential activity with other larger proteins or specific substrates with possible physiological relevance. In addition, LRP produced a much slower overall dephosphorylation of insulin receptors compared with EGF receptors in a simultaneous reaction, raising the possibility that, in intact cells, the EGF receptor may be a preferential substrate for LRP.

A number of biochemical properties of purified placental PTPase 1B (Tonks *et al.*, 1988*b*) or recombinant forms of LAR and LRP (Krueger *et al.*, 1990; Cho *et al.*, 1991; Daum *et al.*, 1991; Streuli *et al.*, 1990; Pot *et al.*, 1991) have been examined in separate studies using several peptide and protein substrates. In general, while maintaining specificity for protein-tyrosine moieties, these PTPases have many properties in common, including the ability to dephosphorylate a variety of substrates with only relative preferences and sensitivities to certain inhibitors and activating agents. The results of our present work are in agreement with previous data that the human homologue of PTPase 1B (Tonks *et al.*, 1988*b*) will dephosphorylate insulin receptors, and that recombinant human LAR (Goldstein *et al.*, 1991) is active against both insulin and EGF receptors. However, the present work represents the first assessment of receptor dephosphorylation by LRP and, furthermore, our studies allow for a direct comparison of relative PTPase activities towards the two autophosphorylated receptor substrates.

The potential role of PTPase 1B, LAR and LRP in the physiological dephosphorylation of insulin receptors is also further supported by their presumed subcellular localization, since the bulk of insulin receptor dephosphorylating activity is found in the particulate fraction of liver and hepatoma cells (Sale, 1991; Goldstein *et al.*, 1991). LAR and LRP are transmembrane enzymes, and recent work has shown that PTPase 1B, while structurally lacking a transmembrane domain, is also associated with a particulate fraction of cells by virtue of its interaction with a non-catalytic subunit protein or through hydrophobic interactions (Cool *et al.*, 1990; Woodford *et al.*, 1991; Pallen *et al.*, 1991; Brautigan & Pinault, 1991).

An additional factor which remains to be studied is whether these PTPases differ not only in their overall dephosphorylation of the insulin receptor, but also in their action on specific phosphotyrosine sites in the insulin receptor regulatory domain that are critical to the regulation of the intrinsic kinase activity of the receptor. Insulin-stimulated autophosphorylation of its receptor occurs as a sequential cascade involving both the receptor kinase domain and the *C*-terminus (Tornqvist *et al.*, 1987; White *et al.*, 1988; Tavare & Denton, 1988; Flores-Riveros *et al.*, 1989). Recent work has shown that autophosphorylation of three tyrosine residues in the so-called receptor 'regulatory domain' is required for activation of the insulin receptor kinase towards exogenous substrates (White *et al.*, 1988; Flores-Riveros *et al.*, 1989). Other related studies have shown that the triply-phosphorylated form of the regulatory region of isolated insulin receptors was rapidly dephosphorylated by the PTPase activity present in extracts of rat liver, and that the initial dephosphorylation of this region was also closely correlated with deactivation of the receptor kinase activity (King & Sale, 1990; King *et al.*, 1991). Thus the action of one or more cellular PTPases might be to limit progression of specific events in the receptor autophosphorylation cascade and attenuate the activation of the receptor kinase.

In conclusion, these studies have demonstrated a number of similarities and differences in the catalytic activities of three major hepatic PTPases towards plasma membrane receptors that are also expressed in liver. While each PTPase had activity towards both autophosphorylated receptors, LRP exhibited a significantly accelerated initial dephosphorylation of the EGF receptor over the insulin receptor protein. Overall, these data provide evidence that each of these PTPases may act physiologically to dephosphorylate insulin and EGF receptors. Our results further suggest that, rather than having specificity for certain cellular substrates, PTPases may serve a more generalized

function in intact cells and act on a number of phosphotyrosine-containing substrates. Redundant PTPase activities may exist in cells to provide a buffer of control over the steady-state level of critical protein-tyrosine phosphorylations and keep metabolic and other cellular signalling pathways in balance. An alternative hypothesis is that the cellular action of the intact forms of these and other PTPases *in vivo* is potentially modulated by a number of factors, including steric considerations, compartmentalization within the cell, and potential interactions with other regulatory proteins that may strongly influence substrate specificity and catalytic activity. Further studies involving expression of these PTPases in insulin-sensitive cultured cells will begin to answer some of these questions.

Note added in proof (received 16 March 1992)

The dephosphorylation of both insulin and EGF receptors by PTPase 1B has also been recently reported by Tappia *et al.* (1991).

We thank Dr. Haruo Saito for providing us with a segment of the human LAR cDNA and a culture of DHB4 cells. Anti-phosphotyrosine antibodies were kindly provided by Dr. Montse Miralpeix and Dr. Morris White. We are also grateful to Eric Britton for his contribution to this work during a 1991 Juvenile Diabetes Foundation Summer Student Fellowship, Alana Dudley for technical assistance and Dr. C. Ronald Kahn for his support and helpful discussion. This project was supported by a Research and Development Award from the American Diabetes Association and NIH grant DK43396, both to B. J. G. Molecular Core laboratory services were provided by the Joslin Diabetes and Endocrinology Research Center Grant DK36836.

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Received 4 November 1991; accepted 7 January 1992

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