Expression of protein-tyrosine phosphatases in the major insulin target tissues

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Abstract Protein-tyrosine phosphatases (PTPs) are key regulators of the insulin receptor signal transduction pathway. We have performed a detailed analysis of PTP expression in the major human insulin target tissues or cells (liver, adipose tissue, skeletal muscle and endothelial cells). To obtain a representative picture, all tissues were analyzed by PCR using three different primer sets corresponding to conserved regions of known PTPs. A total of 24 different PTPs were identified. A multiprobe RNase protection assay was developed to obtain a semi-quantitative measure of the expression levels of selected PTPs. Surprisingly, PTP-LAR, previously suggested to be a major regulator of the insulin receptor tyrosine kinase, was expressed in extremely low levels in skeletal muscle, whereas the related receptor-type PTP-σ and PTP-α were expressed in relatively high levels in all four tissues. The low levels of LAR PTP mRNA in skeletal muscle were further confirmed by Northern blot analysis.

Key words: Protein-tyrosine phosphatase; Insulin receptor; Tissue distribution; RNase protection; PCR

1. Introduction

Receptor tyrosine kinases, including the insulin receptor, are tightly regulated by counter-acting protein-tyrosine phosphatases (PTPs) [1]. Thus, several studies have shown that the insulin receptor is dephosphorylated by PTPs during internalization following insulin binding with concomitant activation and autophosphorylation [2–4]. While the insulin receptor tyrosine kinase is negatively regulated by PTP-mediated dephosphorylation, other insulin signalling processes seem to be positively regulated by members of the PTP family, e.g. SHP-2 [5].

The intimate relationship between the insulin receptor and PTPs is further evidenced by in vitro studies. Thus, insulin treatment of the well-differentiated rat hepatoma cell line Fao leads to increased PTP activity in the particulate fraction, but a decrease in the cytosolic fraction [6]. In another rat hepatoma cell line, McA-RH7777, the insulin signalling is augmented by antisense inhibition of PTP-LAR [7].

Little is known about the identity of the PTPs involved in insulin receptor (IR) regulation. We hypothesize that: (a) the same PTPs will be involved in IR regulation in the major insulin target tissues, (b) these PTPs are significant contributors to the overall PTP activity in the tissues, and (c) these PTPs belong to the class of 'classical' PTPs which can be identified with degenerate PCR primers corresponding to conserved regions of known PTPs. Thus, a detailed analysis of PTP expression in these tissues should provide essential information for identification of the insulin receptor associated PTPs. In the present study, using PCR we have analyzed the expression pattern of PTPs in the major insulin target tissues: skeletal muscle, liver, adipose tissue and endothelial cells. This analysis was followed by semi-quantitative assessment of the expression levels in normal tissue using the multiprobe RNAse protection assay approach.

2. Materials and methods

2.1. PCR

Five degenerate PTP specific PCR primers were constructed. The two sense primers: F1: 5′-CGGGATCCA(C/T)TT(C/T)TGG(A/C/G)(A/G)(A/G)ATG(A/G) T(T/C/G/A)TGG-3′ and F2: 5′-CGGGATCCA(C/T)TT(C/T)TGG(C/A)(T/G/A)(T/G)ATG(A/G)T(T/C/A)TGG-(G/C)A-3′ correspond to the PTP amino acid consensus sequence **FWXMXW**.

The three antisense primers: B1: 5′-CGGAATTCC(T/C/G/A)A-(C/T)(A/G/T)CC(A/T/C)GC(A/G)CT(G/A)CA(G/A)TG-3′, B2: 5′-CGGAATTCGCCC(A/G)A(C/T)(T/C/G/A)GC(T/C/G/A)GC(T/C/G/A)CT(G/A)CA-(G/A)TG-3′, and B3: 5′-GGTGTTCTAGACC(T/C/G/A)GC(A/G)CT(A/G)CA(G/A)TG-3′ correspond to the PTP amino acid consensus sequence **HCSAG**. PCR was performed with the primer sets F1+B1, F2+B2, and F1+B3. QUICK-Clone cDNA preparations (Clontech) from human skeletal muscle, endothelial cells, liver and fat were used as templates.

Three nanograms of cDNA were used in PCR reactions with 100 pmoles of each of the above primers for each primer set. The PCR reactions were performed in a 100 μl volume using GeneAmp (Perkin Elmer Cetus). The reaction mixture was heated to 95°C for 4 min, and then subjected to 3 cycles under the following conditions: 95°C for 1 min, 37°C for 2 min, and 72°C for 3 min, 5 cycles under the following conditions: 95°C for 1 min, 42°C for 2 min, and 72°C for 3 min, 5 cycles under the following conditions: 95°C for 1 min, 47°C for 2 min, and 72°C for 3 min, and finally 27 cycles under the following conditions: 95°C for 1 min, 53°C for 2 min, and 72°C for 3 min.

The reaction mixture was subjected to gel electrophoresis on a 1.5% agarose gel and 0.4 kb DNA fragments isolated. After digestion with *Bam*HI and *Eco*RI for primer sets (F1+B1) and (F2+B2) or with *Bam*HI and *Xba*I for primer set (F1+B3), ligation to the 2.8 kb *Bam*HI-*Eco*RI or *Bam*HI-*Xba*I fragment of plasmid pTZ19R (Pharmacia) was performed, followed by transformation of a competent *E. coli* strain (r^-, m^+). Plasmids from the resulting colonies were sequenced using Sequenase (U.S. Biochemicals). The results are presented in Table 1.

2.2. Multiprobe RNase protection assay

Total RNA from human skeletal muscle, liver and placenta were commercial preparations (Clontech). Total RNA from cultured human vascular endothelial cells (HUVEC) was prepared using

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RNAgents Total Isolation System (Promega). Human adipose tissue total RNA was a kind gift from Per B. Jensen, Novo Nordisk.

Template DNAs were prepared as previously described in detail [8]. In short, templates from the various PTPs were isolated from cDNA in which the PTPs had been identified by PCR as described above, using specific primers designed from the known DNA sequences and standard PCR techniques. The template DNA was cloned into the plasmid pGEM-7Zf(+) (Promega) as *Hin*dIII-*Xba*I fragments, such that transcription from the T7 RNA polymerase promoter would produce the corresponding PTP antisense riboprobe containing an additional 36–38 ribonucleotides at the ends to be removed during the ribonuclease protection step. The template plasmids were linearized by *Hin*dIII digestion, purified and then mixed at a final concentration of 4 ng/μl of each template in two sets as indicated in Table 2.

Three μl of each of the two template sets were labelled for 60 min at 37°C with ^{32}P-UTP in a total volume of 15 μl transcription mixture (40 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 0.47 mM each of GTP, ATP, and CTP, 9.4 μM UTP, 4.7 μM [α-^{32}P]-UTP (3000 Ci/mmol, 10 mCi/ml, DuPont NEN), 10 mM DTT, 14 u RNasin ribonuclease inhibitor (Promega) and 24 u T7 RQ1 RNA polymerase (Promega). The DNA templates were removed by addition of 3 u of RQ1 RNase-free DNase (Promega) followed by incubation for 30 min at 37°C. After extraction with phenol and chloroform, the labelled riboprobe sets were precipitated with ammonium acetate and ethanol and dissolved in hybridization buffer (80% formamide, 40 mM PIPES, pH 6.7, 0.4 M NaCl, 1 mM EDTA) at a concentration of 2×10^6 cpm/μl.

As an internal control for expression levels, a ribosomal protein L32 riboprobe was similarly prepared. Due to the relatively high concentration of L32 mRNA in all the tissues and cells examined the L32 riboprobe was prepared with 12 times less specific activity of α-^{32}P-UTP than the PTP riboprobe set. The L32 probe was dissolved at a concentration of 1×10^5 cpm/μl and mixed with equal volumes of each of the two PTP riboprobe sets.

Two μl of labelled probe set were added to 3 μl hybridization buffer containing 5 μg total RNA from human skeletal muscle, endothelial cells, adipose tissue, liver, or placenta. Hybridization was at 56°C overnight. The mixture was treated with 0.25 μg RNase A (Boehringer) and 0.25 u RNase T1 (Life Technologies) at 30°C for 60 min in 50 μl RNase buffer (10 mM Tris-HCl, pH 7.5, 300 mM NaCl, 5 mM EDTA). Then 5 μl of 6% SDS containing 12 μg proteinase K (Boehringer) was added, and the mixture incubated at 37°C for 30 min. After extraction with phenol and chloroform, 5 μg of yeast tRNA was added as a carrier, and the protected RNA precipitated from ammonium acetate with ethanol and then subjected to electrophoresis on a standard sequencing polyacrylamide gel. The dried gel was placed on a PhosphorImager screen (Molecular Dynamics) for 3 days. The screen was scanned and bands analyzed by the ImageQuant software (Molecular Dynamics) by rectangle mode and volume integration. Semi-quantification in relation to the L32 band was performed after normalization for uridine content and correction for the lower specific activity of the L32 probe.

2.3. Northern blot analysis

The 211 bp *Hin*dIII-*Xba*I DNA fragment from the LAR ± exon 13 riboprobe template plasmid described above was labelled with α-^{32}P-dCTP (Megaprime Labelling System, Amersham). The probe will hybridize to LAR mRNA independently of the presence or absence of exon 13.

10^7 cpm of labelled probe was hybridized to a Northern blot of mRNA from human tissues (Human MTN Blot, Clontech) in 5 ml of ExpressHyb hybridization solution for 1 h at 68°C. The blot was washed three times 15 min at room temperature in 2×SSC, 0.05% SDS and finally twice for 20 min at 50°C in 0.1×SSC, 0.1% SDS. Autoradiography was performed with intensifying screen at −70°C for 5 h and 10 h. As a control the MTN blot was stripped for the LAR probe and reprobed with a beta-actin probe under similar conditions as above.

3. Results

3.1. PTPs identified by PCR in the major insulin target tissues

The expression of PTPs in the major insulin target tissues was analyzed with PCR. To obtain a representative picture, we attempted to minimize the bias commonly observed with PCR by using three different primer sets corresponding to conserved regions of known PTPs. As seen in Table 2, significant differences were observed between the four tissues. A

Table 1
Number of specific PTP clones obtained by PCR on cDNA from the indicated human tissues

PTP	Endothel. cells	Liver	Skeletal muscle	Fat
Receptor-type PTPases				
PTP-α	8	27	18	62
PTP-ε	21			1
LAR	36	42		15
PTP-σ	2	2	2	5
PTP-δ	12	3	1	8
PTP-κ	7	3	4	5
PTP-μ	2		2	
PCP-2	1	3	29	4
CD45		8	3	20
PTP-γ	1	4	2	3
PTP-β	32	21	33	29
DEP-1		1		3
SAP-1				1
S31				28
Intracellular PTPases				
PTP-1B	3	7	10	2
T-cell PTP		2		1
BAS	2	1	1	4
MEG1		1	4	1
PTP-H1		3	4	
PTP-D1	7	20	84	38
PTP-D2	2	2	4	4
SHP-1		3	2	1
PEST	38	2	5	6
MEG2		3	3	5

The PTPs are grouped in families based on sequence homology. The primer sets do not allow detection of SHP-2. The total number of PTP clones were: 174 from endothelial cells, 158 from liver, 211 from skeletal muscle and 246 from fat cDNA.

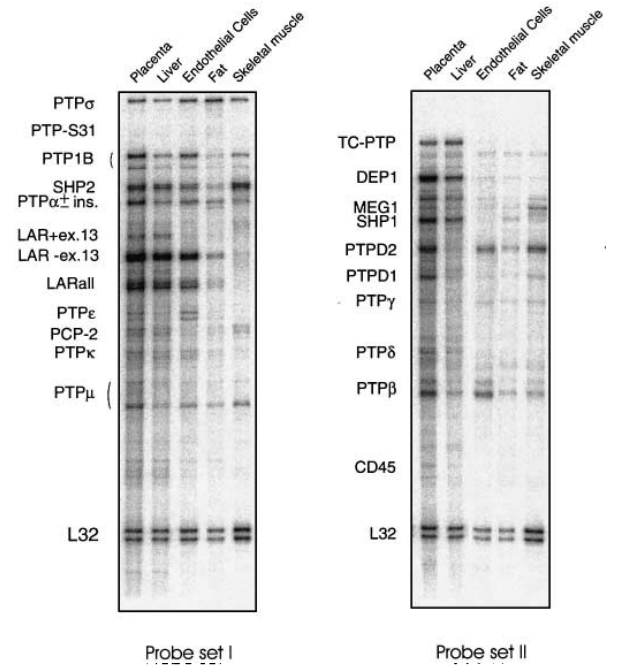

Fig. 1. Multiprobe ribonuclease protection assay (MRPA) using probe set I and II (see Table 2) with the L32 ribosomal protein probe as an internal reference.

total of 24 different PTPs were identified. Twelve of these were found in all four tissues. Two PTPs, S31 and SAP-1, were identified in fat only. It should be noted that the primer sets used do not allow identification of SHP-2 which is thought to be a positive regulator of the insulin signal. SHP-2 was found to be expressed in all four tissues using specific primers (data not shown) and with RNase protection assay (see below).

Results obtained with the conventional PCR method are not quantitative. Therefore, the number of clones do not indicate the relative expression levels of PTPs within a particular tissue. However, we believe that our approach with three different primer sets allows identification of a specific, 'classical' PTP in a particular tissue unless the expression level is extremely low. In this context, it appears significant that PTP-ε is almost exclusively expressed in endothelial cells, and S31 in fat only, whereas PTP-LAR is not observed in skeletal muscle. In a similar PCR experiment, using human skeletal muscle cDNA from an independent source, the lack of LAR was also observed (data not shown).

3.2. Multiprobe ribonuclease protection assay (MRPA)

To obtain semi-quantitative measures we employed our multiprobe ribonuclease protection assay protocol [8] on total RNA from the major insulin target tissues (Figs. 1 and 2). Probes were prepared for 20 of the PTPs identified with PCR (Table 1). Special attention was given to PTPs claimed in the literature to be involved in regulation of the insulin signal: PTP-1B (and the related TC-PTP); PTP-α (and the related PTP-ε); LAR (and the related PTP-δ and PTP-σ); SHP-2 (and the related SHP-1). Placenta is included as a reference tissue representing several different cell types.

3.2.1. Receptor-type PTPs

3.2.1.1. PTP-α and PTP-ε. These two PTPs are characterized by very short, highly glycosylated extracellular domains. PTP-α is expressed in all four tissues at similar levels, whereas PTP-ε is mainly expressed in endothelial cells. A

Table 2
Riboprobes included in probe sets I and II

Riboprobe set	Size, protect., bases	GenBank Acc. no.	Residues number
I			
PTP-σ	407	U35234	3501–3907
PTP-S31	361 TM form, 325 IC form		
PTP-1B	295	M31724	1101–1395
SHP-2	250	X70766	1662–1911
PTP-α	235−insert, 220+insert	M34668	1094–1110/1138–1355
LAR exon 13	205+exon 13, 190−exon 13	Y00815	2666–2870
LAR all	171	Y000815	5349–5519
PTP-ε	154	X54134	1668–1822
PCP-2	146	X97198	3021–3166
PTP-κ	133	Z70660	2998–3130
PTP-μ	119	X58288	3898–4016
II			
TC-PTP	320	M25393	964–1283
DEP1	265	U10886	2941–3205
MEG1	240	M68941	1745–1984
SHP-1	220	X62055	1821–2040
PTP-D2	200	X82676	2871–3070
PTP-D1	180	X79510	2939–3118
PTP-γ	160	X54132	1633–1792
PTP-δ	140	L38929	5611–5750
PTP-β	120	X54131	5034–5153
CD45	100	Y00062	2850–2949
L32	78	X03342	42–119

Before hybridization to total RNA the labelled L32 reference probe was added to each of the two labelled probe sets. Prior to RNase protection all riboprobes contained an additional 36–38 ribonucleotides at the ends. The LAR exon 13 probe contains the exon 13 sequence, residues no. 2654–2680. The LAR all probe is directed to the second PTP domain and should detect all splice variants of LAR. The PTP-α riboprobe is able to distinguish between the two forms of PTP-α with and without the 9 amino acid insert 3 residues upstream from the TM segment. PTP-S31 is a PTP [22] containing a single PTP domain which exists in a receptor type, transmembrane (TM) spanning form as well as in an intracellular (IC) form.

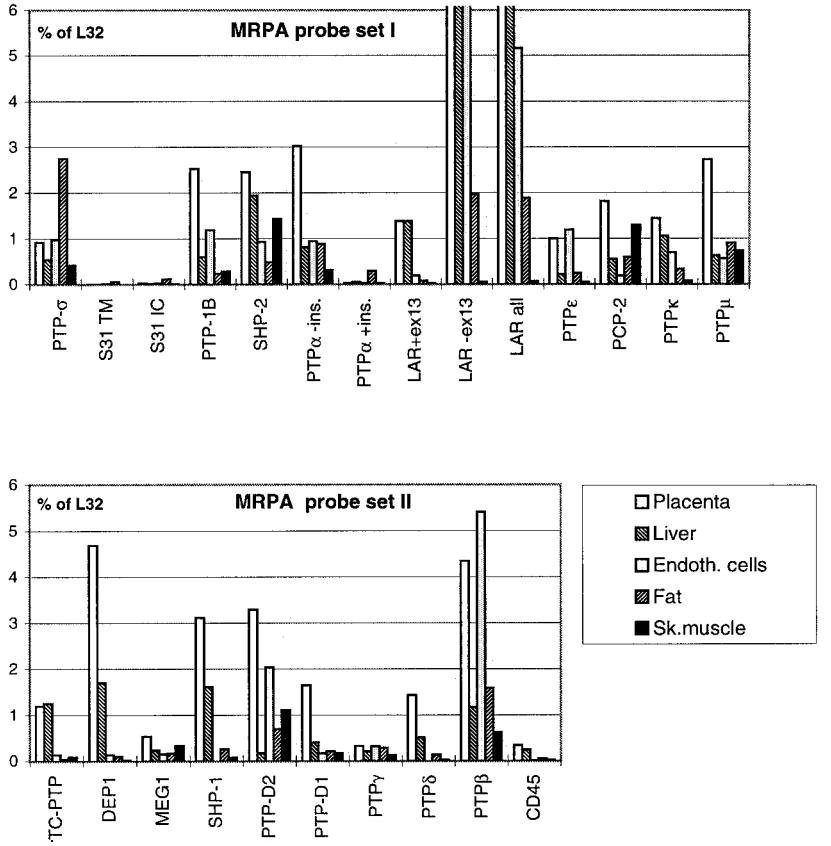

Fig. 2. Semi-quantification of PTPs in insulin target tissues from MRPA. The values in % of L32 for LAR−exon 13 are 9.1 for endothelial cells, 13.9 for liver, and 15.1 for placenta. For LAR all, the values are 10.2 for liver and 12.7 for placenta.

splice variant of PTP-α containing a nine amino acid insert [9] was found to be expressed in fat.

3.2.1.2. PTP-LAR family. In liver and endothelial cells, LAR is the most abundantly expressed PTP of the 20 investigated in the present study. Surprisingly, but in accordance with the findings with PCR described above, and the results from the Northern blot experiment (Fig. 3), extremely low levels of LAR expression are observed in skeletal muscle. The same result was observed in separate MRPAs with probe set I on total RNA from skeletal muscle from two normal individuals (data not shown). The splice variant of LAR (+exon 13) is mainly expressed in the liver. PTP-δ shows very low levels of expression in skeletal muscle and endothelial cells. In contrast, PTP-σ is expressed in all four tissues, and, in fat, it is the most abundantly expressed of the PTPs investigated.

3.2.1.3. PTP-μ family. The three members analyzed from this PTP subfamily have overlapping but distinct tissue distribution patterns. Thus, the recently discovered PCP-2 shows relatively high expression levels in skeletal muscle, whereas PTP-κ is mainly found in liver. PTP-μ is expressed at similar levels in all four tissues.

3.2.1.4. CD45. CD45 is supposed to be exclusively expressed in hematopoietic cells. Therefore, the low levels of expression of CD45 are likely to be caused by contaminating blood cells.

3.2.1.5. PTP-γ family. These PTPs are characterized by a large extracellular domain containing a carbonic anhydrase-like domain in the N-terminal region. With PCR we observed one member of the PTP-γ family. Low expression levels of this member were observed in all four tissues.

3.2.1.6. PTP-β family. In contrast to other receptor-type PTPs, this class contains only one intracellular enzyme domain [10]. Four members were observed with PCR: PTP-β, DEP-1, SAP-1 and S31. Three of these were analyzed with MRPA. Extremely low levels of S31 were detected in all four tissues, whereas DEP-1 was a major PTP in liver. PTP-β was abundantly expressed in all tissues analyzed.

3.2.2. Intracellular PTPs

3.2.2.1. PTP-1B family. PTP-1B, which is considered to

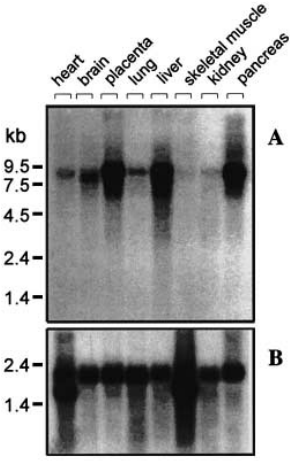

Fig. 3. A: Northern blot analysis of LAR expression in various human tissues. The blot is overexposed in order to show the very faint LAR band in skeletal muscle. B: Beta-actin control.

be ubiquitously expressed, was found in all tissues with the highest levels in endothelial cells. TC-PTP is mainly expressed in liver.

3.2.2.2. Ezrin domain containing PTPs. Five members from this family of PTPs were identified with PCR. Three of these were analyzed with MRPA. MEG1 and PTP-D1 were expressed at relatively low levels in all tissues, whereas PTP-D2 was a major PTP in endothelial cells and skeletal muscle.

3.2.2.3. SH2 domain containing PTPs. SHP-2 is expressed ubiquitously [11]. Relatively high expression levels were observed in liver for SHP-1 which is otherwise believed to be expressed in hematopoietic cells only [12].

3.3. Northern blot analysis

The expression of LAR in various human tissues was further investigated by Northern blot analysis (Fig. 3). High expression levels were found in placenta, liver and pancreas, medium levels were observed in heart, brain and lung, while low levels were present in skeletal muscle and kidney.

4. Discussion

The present study was undertaken with the aim of: (a) identifying the most abundantly expressed PTPs in the major insulin target tissues (using PCR); (b) providing a semi-quantitative evaluation of PTP expression in normal tissues.

Twenty-four different PTPs were identified by PCR and significant differences were observed between the different tissues. Most of the differences observed with PCR were confirmed with the RNase protection assay.

We hypothesize that the PTPs involved in the regulation of the insulin receptor must be expressed in all major insulin target tissues. Previous studies have indicated that PTP-1B [13–15], PTP-LAR [16] and PTP-α [17] are negative regulators of the insulin receptor tyrosine kinase, and that SHP-2 is a downstream positive regulator of the insulin signal. In the present study PTP-α, PTP-1B and SHP-2 were found to be expressed at fair levels in all of the main insulin target tissues thus supporting earlier studies on the role of these PTPs as modulators of the insulin receptor kinase activity. The expression levels of LAR in skeletal muscle were found to be extremely low, whereas the highly homologous PTP-σ was well expressed in all tissues. These results do not seem to support the implication of LAR as the main direct modulator of the insulin receptor kinase activity in skeletal muscle.

A series of publications have strongly suggested that PTP-LAR is a key regulator of the insulin receptor. First, Xhang and Goldstein using PCR on rat skeletal muscle cDNA identified a total of 3 PTPs (PTP-LAR, PTP-α and CD45) [18]. It was confirmed by Northern blotting that PTP-α is expressed in rat skeletal muscle. The authors conclude that LAR and PTP-α are major PTPs in skeletal muscle, although it was noted that skeletal muscle has a relatively low content of PTPs compared to liver. Next, by comparing the rate of dephosphorylation/inactivation of purified IR by recombinant PTP-1B and the cytoplasmic domains of PTP-LAR and PTP-α, it was proposed that LAR might play a role in the physiological regulation of insulin receptors in intact cells [19]. In an attempt to directly identify the PTP(s) involved in regulation of the IR, Ahmad and Goldstein purified PTPs from rat skeletal muscle using the insulin receptor for detection during the purification procedures [20]. Using standardized immunoblots PTP-LAR was found to be a predominant PTP in skeletal muscle in addition to SHP-2 and PTP-1B, although both LAR and PTP-1B were relatively rare proteins in skeletal muscle. In contrast to the earlier studies, PTP-α was not identified via this approach. To investigate whether the obvious discrepancies between these studies and the results presented here might be explained by species differences, we performed a Northern analysis of rat tissues using a rat LAR probe (data not shown). Similar to Northern analysis of human RNA (Fig. 3), rat skeletal muscle poly A^+ RNA gave an extremely weak hybridization signal indicating very low expression levels of LAR. The fact that RNA levels investigated in the present work do not necessarily reflect protein levels, could explain the discrepancies regarding LAR to some of the above mentioned studies. We suggest that a more likely explanation for these discrepancies regarding LAR is that the skeletal muscle samples used in the different studies have been contaminated to a varying degree with fat tissue, which expresses high levels of LAR (Fig. 2). Further, it has recently been found that an increased abundance of PTP-LAR accounts for the elevated insulin receptor dephosphorylating activity in adipose tissue from obese human subjects [16].

By reducing the level of LAR expression in a rat hepatoma cell line through antisense-mediated suppression [21] it was found that the insulin-dependent insulin receptor tyrosine kinase activation is increased about 3-fold. However, the epidermal growth factor receptor and hepatocyte growth factor-dependent receptor autophosphorylation were similarly increased. The present study has shown that LAR is the most abundantly expressed PTP among the PTPs studied in liver. It is thus not inconceivable that an antisense-mediated reduction in the expression level of LAR will result in pronounced effects in a hepatoma cell line.

Studies on mice deficient in LAR tyrosine phosphatase activity have shown defective mammary gland development at late pregnancy as the only appreciable phenotype. The mice grow and develop otherwise normally, and blood glucose levels are within the normal range (R.Q.J. Schaapveld and W. Hendriks, personal communication). These results do not support the role of LAR as the key regulator of the insulin receptor kinase (IRTK).

We believe that the present study has provided a basic framework for further studies of PTPs involved in the insulin receptor signal transduction pathway.

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