INTRODUCTION

Protein tyrosine phosphorylation is recognized as an important reversible reaction controlling many aspects of cellular function, including growth and differentiation, cell cycle control, and cytoskeletal integrity (Tonks et al., 1992; Mourey and Dixon, 1994). The role of protein tyrosine kinases (PTKs) in these processes and the induction of their activity have been extensively studied, but the recent identification and characterization of many protein tyrosine phosphatases (PTPases) suggest that dephosphorylation of signal transduction proteins is also a crucial event in signal transduction (Fischer et al., 1991; Brady-Kalnay and Tonks, 1994). To date more than 30 PTPases have been identified (Mourey and Dixon, 1994). Sequence comparisons have revealed that there are two major classes of PTPases (for review see Tonks et al., 1992; Mourey and Dixon, 1994), namely (i) those found in the cytoplasm or nucleus that contain only one tyrosine phosphatase domain and (ii) membrane-bound, receptor-like PTPases (RPTPases) that have two tandemly repeated catalytic domains. There also exist intermediate forms such as HPTPβ (Krueger et al., 1990) and PTP-SL (Hendriks et al., 1995) that are transmembrane proteins with only one catalytic domain.

The RPTPases can be subdivided into five types based on the structure of their extracellular segments (Mourey and Dixon, 1994). Type I represents the CD45 family, which comprises multiple isoforms arising from differential splicing of the RNA of a single gene. Type II members (e.g., LAR, HPTPδ, RPTPσ, RPTPμ, and RPTPκ) are characterized by the presence of multiple immunoglobulin (Ig) and fibronectin (FN) type III-like domains. Type III members carry multiple FN-III domains (e.g., HPTPβ), while type IV members (e.g., HPTPα and HPTPε) possess small glycosylated segments. Finally, type V members (e.g., HPTPζ and HPTPγ) have an amino-terminal motif with homology to carbonic anhydrase.

The extracellular region of the type II RPTPases resembles that of cell adhesion molecules (CAMs) such as N-CAM (Edelman and Crossin, 1991). Recently, homophilic adhesive properties have been attributed to RPTPμ and RPTPκ (Gebbink et al., 1993; Brady-Kalnay et al., 1993; Sap et al., 1994). In combining cell adhesion motifs at the cell surface with intracellular
phosphatase activity, these RPTPases may be involved in the control of cell growth, motility, and differentiation in a direct response to cell-cell interaction.

The cytoplasmic region of most type II RPTPases contains two tandem phosphatase domains of approximately 260 amino acid residues each, but it is not clear at present whether both domains are enzymatically active in vivo or exhibit different substrate specificities. Structure-function relationships have been established only for phosphatase domains expressed in bacteria and therefore outside their natural context. Although site-directed mutagenesis studies have shown the significance of the "signature sequence" (I/V)HCXAGXXR(S/T)G for tyrosine phosphatase activity of the first, transmembrane proximal phosphatase domain (Moore and Dixon, 1994), it remains to be determined whether the distal phosphatase domain also exhibits catalytic activity (Wang and Fallen, 1991; Tan et al., 1993) or plays only a regulatory role in modulating the activity of the first domain (Streuli et al., 1990; Pot et al., 1991; Krueger and Saito, 1992). However, in vitro studies are inadequate to address such questions because specific inhibitors of PTPases are not yet at hand and substrates and ligands are unknown.

To initiate studies of the role of LAR domains in an organismal context, we set out to isolate structural and genomic mouse LAR (mLAR) DNAs. Here we describe the organization of the Ptpzf gene region encoding the cytoplasmic part of mLAR. Compared with known exon-intron structures of other mouse PTPase genes, such as Ptpra (Wong et al., 1993) and Ptprc (Sage et al., 1988), the Ptpzf gene exhibits a more compact structure.

Comparison of both phosphatase domains of mLAR with closely homologous sequences of MPTPγ (Mizuno et al., 1993) and mRPTPα (Ogata et al., 1994) revealed a higher evolutionary conservation of the second, C-terminal domain compared to the proximal "catalytically active" domain.

Finally, we report the chromosomal localization of the Ptpzf gene to region C6-D1 of mouse chromosome 4.

MATERIALS AND METHODS

Isolation of mouse LAR genomic and cDNA clones. Cosmid clones were isolated from a mouse 129/SvEv genomic DNA cosmid library (kindly provided by Dr. M. Hofker, University of Leiden, Leiden) using a random-primed (Feinberg and Vogelstein, 1983) 1.6-kb EcoRI-SalI human LAR (hLAR) cDNA fragment (positions 4444-5933, Streuli et al., 1988) as a probe. Hybridizations were carried out according to Church and Gilbert (1984), and filters were washed for 5, 10, and 30 min at 65°C in 0.25 M sodium phosphate/1% SDS. Positive clones were purified by subsequent rounds of low-density screening.

The same mLAR cDNA probe was used to screen an oligo(dT)-primed mouse brain cDNA library in Lambda ZAPII (Stratagene). Filters were washed three times for 30 min at 65°C in 2x SSC/0.1% SDS. Positive phages were plaque-purified, and inserts were rescued as pBluescript SK plasmids according to the manufacturer's protocol.

Sequence analysis. Nested deletion mutants of cDNA clone mLAR#9 were generated using Exonuclease III (Promega), and nucleotide sequences were determined using the double-stranded DNA dyeexosequencing method (Hattori and Sakaki, 1986). A 6.5-kb SalI-BamHI Ptpzf genomic fragment was used for exon-intron determination according to the shotgun sequencing strategy of Deininger (1988). Briefly, fragments of around 500 bp were generated by sonication, blunt-ended using Klenow (Boehringer), and subcloned into the Smal site of vector pGEM4 (Promega). Clones, grown in microtiter plates, were replica plated onto nitrocellulose filters and screened with the mLAR cDNA#9 insert. Thirty positive clones were used for sequence determination (Hattori and Sakaki, 1986). DNA sequence gel readings were recorded, compared, edited, and assembled using the IGSUITE 5.35 package (Intelligenetics Inc., Mountain View, CA). Sequences were aligned using the GCG Wisconsin programs PILEUP and LINEUP (Devereux et al., 1984). Evolutionary reconstruction was performed using the program FITCH supplied in the phylogeny inference package PHYLIP (distributed by Dr. J. Felsenstein, University of Washington, Seattle). FITCH uses distance matrices to construct trees without allowing negative branch length (Fitch and Margoliash, 1967).

Fluorescence in situ hybridization. Fluorescence in situ hybridization was performed on mouse strain 129-derived E14 embryonic stem cell (Handyside et al., 1989) metaphase spreads for the regional localization of the Ptpzf gene, using mouse cosmid 5 as a probe. Biotinylated cosmid DNA was dissolved in 10 μl hybridization solution containing 2x SSC, 10% dextran sulfate, 1% Tween-20, and 50% formamide. The probe mixture was heat-denatured at 80°C for 10 min, followed by incubation at 37°C to allow reannealing of highly repetitive sequences. Hybridization of this probe to heat-denatured chromosome spreads, under a cover slip, was carried out overnight at 37°C. Hybridizing probe was detected immunohistochemically using fluorescein isothiocyanate conjugated to avidin-D (Suijverbuijk et al., 1991). Images of metaphase preparations were captured by a cooled high-performance CCD camera (Photometrics) coupled to a Macintosh II computer. Separate images of both mLAR hybridizing signals and DAPI-counterstained chromosomes were transformed into pseudocolored images using image analysis software.
RESULTS AND DISCUSSION

Isolation and Characterization of the mLAR-Encoding Ptprf Gene

We are currently investigating the biological role of LAR and the functions of the tyrosine phosphatase domains in the LAR signal transduction pathway using reverse genetic approaches. As a first step we isolated and characterized mouse LAR genomic and cDNA clones, with special emphasis on the region encoding the phosphatase domains.

A 1.5-kb EcoRI–SalI mLAR cDNA probe encoding...
both phosphatase domains (Streuli et al., 1988) was used to isolate cosm id clones, two of which, 5 and 11, repeatedly showed stronger hybridization signals under more stringent washing conditions. These clones were characterized further. Southern blot and restriction enzyme analyses revealed that these two clones overlap and span some 50 kb of the mouse Ptprf locus, as shown in Fig. 1. We assume that the other, more weakly hybridizing cosm id clones contained either Ptprf pseudogenes or genes with homology to Ptprf, such as Ptprd (encoding MPTPδ, Mizuno et al., 1993) or Ptprs (coding for mRPTPσ, Ogata et al., 1994).

We compared genomic and cDNA sequences to position exon—intron boundaries within the Ptprf gene. To this end, 10 cDNA clones were isolated from a Lambda ZAPII mouse brain cDNA library using the hLAR cDNA probe mentioned above. End-in sequencing and comparison with the cognate positions in hLAR (Streuli et al., 1988), rat LAR (rLAR, Pot et al., 1991), and fragments of mLAR (Hendriks et al., 1995) revealed that three clones contained genuine mLAR cDNAs. The other cDNA clones contained mLAR homologous sequences. In Fig. 2, the sequence of the largest mLAR cDNA clone, mLAR#9, is depicted alongside the deduced amino acid sequence.

All three mLAR cDNAs ended in an A-rich stretch in the 3' untranslated region (3' UTR) also found in the hLAR mRNA message (position 6900, Streuli et al., 1988). Screening of the mouse cDNA library with a more 3' UTR hLAR probe (SalI—EcoRI, positions 5933—7700, Streuli et al., 1988) yielded additional clones that terminated at the genuine polyadenylation

**FIG. 3.** Comparison of exon segmentation as observed in the Ptpra, Ptprc, and Ptprf genes within the region encoding the phosphatase domains of RPTPases LRP (Wong et al., 1993), Ly-5 (Saga et al., 1988), and mLAR, respectively. Dashed lines indicate identical boundaries. Downward arrows indicate introns present in Ptpra and Ptprc but missing in the Ptprf gene. The upward arrow points to an intron present in Ptprf but absent in Ptpra and Ptprc. Exon numbers for LRP and Ly-5 are according to the literature.

**FIG. 4.** Chromosomal localization of the Ptprf gene. Unique hybridizing signals of cosm id mLAR#5 were recorded in more than 30 metaphase spreads (right). Positively imaged chromosomes were counterstained with DAPI for the identification of the individual chromosomal subbands (left).
We therefore conclude that clone mLAR#9 represents a mLAR cDNA in which the reverse transcription reaction started at the A-rich stretch within the 3′ UTR.

Southern blot analysis using cDNA clone mLAR#9 as a probe revealed that the sequence encoding the mLAR cytoplasmic region resided in a 6.5-kb SstI-BamHI genomic fragment. Sequence analysis showed both phosphatase domains to be encoded by 11 exons contained within a 4.5-kb EcoRI-BamHI fragment (Fig. 1). The most 3′ exon contains the last 22 codons for the protein and the entire 3′ UTR of the mRNA. The intron lengths in the gene segment shown in Fig. 1 vary from only 80 bp to 0.5 kb. Comparison of the deduced organization of the Ptprf gene with that of the Ptpra and Ptprc RPTPase genes (encoding for LRP and Ly-5, respectively, Wong et al., 1993; Saga et al., 1988) revealed conspicuous differences (Fig. 3). Whereas the genomic organization of the first phosphatase domain is similar but not identical to the second domain in all three PTPase family members, both mLAR phosphatase domains together are encoded by only 11 exons, in contrast to the 17 (spanning ~34 kb) and 16 (spanning ~27 kb) exons for LRP and Ly-5, respectively. This finding is at variance with the conclusions of Wong et al. (1993), who postulated that all RPTPases exhibit a similar genomic organization of the region encoding the phosphatase domains. Our results show clear differences in the gene structure of RPTPases. We propose that an ancestral gene originally contained a single tyrosine phosphatase domain encoded by at least nine exons. This may have been duplicated later to produce RPTPases with two phosphatase domains. Subsequently, several intron losses may have occurred for both of the phosphatase domains as well as for the various types of RPTPases. One intron (between exons P and Q) still present in the Ptprf gene has been removed from the Ptpra/Ptprc ancestor. In the Ptprf gene, five introns that are still present in the other two RPTPase genes have been removed (Fig. 3). The reason why the gene structure of the Ptprf gene is so much more compact than other RPTPase genes remains, however, obscure for the moment.

Chromosomal Localization of the Ptprf Gene

Mouse cosmid clone mLAR#5 was used as a probe for fluorescence in situ hybridization to determine the chromosomal localization of the Ptprflocus. Alignment to G-banded chromosome images showed that the Ptprf gene localizes within the region C6–D1 of mouse chromosome 4 (Fig. 4). Based on data on evolutionary conservation of chromosome segments in human and mouse (Lyon and Kirby, 1993), this is in agreement with the localization of the Ptprf gene within the p32–p33 region of human chromosome 1 (Streuli et al., 1992). This localization is distinct from those of the Ptprd and Ptprsloci, which have been assigned to the region around the 6 locus on mouse chromosome 4 (Mizuno et al., 1993) and distal mouse chromosome 17 (Yan et al., 1993), respectively. Together with RNA in situ hybridization data, which reveal different expression patterns for mLAR, MPTPδ, and mRPTPσ (R. Q. J. Schaapveld, J. T. G. Schepens, and W. J. A. J. Hendriks, unpublished results), these mapping data are in agreement with Mizuno et al. (1993) and demonstrate that mLAR, MPTPδ, and mRPTPσ are indeed separate entities.

Homology to Other Receptor-like PTPases

The mLAR cDNA sequence presented here is nearly identical to that of rat (Pot et al., 1991) and human LAR (Streuli et al., 1988); the overall homology at the amino acid level is 99.6 and 98.2%, respectively. The homology (up to 90% at the amino acid level) to other type II RPTPases, MPTPδ and mRPTPσ (Mizuno et
al., 1993; Ogata et al., 1994), which also contain two catalytic domains, a single transmembrane domain, and, extracellularly, eight FN-III and three Ig-like domains, is also striking. Indeed, we also isolated clones representing MPTPδ and mRPTPσ by screening both the genomic and the cDNA libraries at low stringency (not shown). Nucleotide sequence alignment of the phosphatase domains was used to gain insight into their evolutionary history. A phylogenetic tree, as depicted in Fig. 5, suggests that a common ancestor for PTPδ and RPTPσ diverged from the LAR lineage most probably before the time of mammalian radiation. These results suggest that the mutation rate for LAR is lower than those for both PTPδ and RPTPσ, reflecting the maintenance of selective pressure on the LAR lineage and the adaptation to new roles in growth and development for PTPδ and RPTPσ.

In addition, the homology among these three proteins is more conserved in phosphatase domain 2 than in domain 1. This is not seen for the other, more distant members of the type II RPTPases (i.e., RPTPκ and RPTPk) or among type IV (e.g., HPTPa and HPTPe) and type V (e.g., HPTPa and HPTPy) RPTPases (Jiang et al., 1993; Krueger et al., 1990; Krueger and Saito, 1992; Kaplan et al., 1990). Further studies of the biological significance of each individual phosphatase domain in RPTPases are now in progress.

ACKNOWLEDGMENTS

We thank Drs. Michel Streuli and Haruo Saito for providing the hLAR cDNA, Dr. Marten Hokker for the mouse 129/SvEv cosmid library, Dr. A. Berns for the E14 embryonic stem cells, Dr. David Brady-Kalnay, S. M., Flint, A. J., and Tonks, N. K. (1993). Homophilic binding of PTPμ, a receptor-type protein tyrosine phosphatase, can mediate cell-cell aggregation. J. Cell Biol. 122: 961–972.


