Increase in receptor-like protein tyrosine phosphatase activity and expression level on density-dependent growth arrest of endothelial cells

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Protein tyrosine phosphatase (PTPase) activity was examined in two cell lines: human umbilical vein endothelial (HUVE) cells, which display contact inhibition of cell growth, and A427 human adenocarcinoma cells, which have lost this ability. HUVE cells harvested at high density displayed a 10-fold increase in membrane-associated PTPase activity. A427 cells exhibited no such phenomenon. Moreover, modification of HUVE cell growth rate by a stimulating agent such as basic fibroblast growth factor or by blocking compounds such as thymidine or suramin resulted in no change in PTPase activity, suggesting that the observed increase in membrane-associated activity at confluency was specific for cell–cell-contact-directed growth arrest. The expression of various PTPase mRNAs was examined in HUVE and A427 cells. Of the receptor-like PTPases tested, two were exclusively expressed in HUVE cells (PTP γ and HPTP β). Only HPTP β, which is structurally similar in its extracellular region to cell-adhesion receptors of the immunoglobulin superfamily, displayed a pattern of expression related to the increase in PTPase activity. Competitive PCR was used to quantify its expression during cell culture. A 12-fold increase in HPTP β mRNA expression was detected and it parallelled the time course of PTPase activity. This observation strongly implicates receptor-like PTPases in density-dependent growth arrest.

INTRODUCTION

Cycles of protein phosphorylation and dephosphorylation are important for the regulation of cellular proliferation, differentiation and malignant transformation [1–4]. Protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPases) are involved in these events as a result of their opposing actions on protein tyrosine residues. PTPases may counterbalance PTK activity or alternatively may assist tyrosine kinase-mediated signal transduction by activating PTKs via dephosphorylation of key tyrosine residues [3,5,6].

PTPases form a growing family of enzymes that display a high degree of homology in their catalytic domain, whereas their non-catalytic parts, like members of the PTK family, show considerable heterogeneity, which suggests the possibility that multiple interactions between the two groups of proteins modulate specific cellular events. They can be categorized according to structure into two groups: non-receptor PTPases and receptor-like PTPases. Non-receptor PTPases (class I) correspond to intracellular proteins with a single catalytic domain. Several structural features in the different regions surrounding the catalytic domain provide clues to their regulation and function [4] and they have been implicated in many signal-transduction pathways such as platelet activation [7,8] and growth factor signalling [9,10].

Receptor-like PTPases exist as two classes. Class-II PTPases have a single catalytic domain, and only five members have been described: DPTP 10D [11,12], DPTP 4E [13] in *Drosophila*, HPTP β [14], SAP-1 [15], DEP-1 [16] in humans and GLEPP1 [17] in rabbit. Class-III PTPases have two tandemly arranged catalytic domains, one proposed to regulate the activity of the other as observed in leucocyte common antigen-related molecule (LAR) [18]. These PTPases have variable extracellular domains which may confer their specificity and probably allow the regulation of their activity by interaction with specific ligands. Most of them display multiple fibronectin type-III (FN-III) repeats in combination with immunoglobulin-like domains. These types of structure have been described in many neural adhesion molecules (e.g. N-CAM, L1 [19]). Interestingly, various studies have demonstrated that four receptor-like PTPases from *Drosophila* are specifically expressed in developing axons of the central nervous system, raising the possibility that they may act as signal-transducing cell adhesion receptors [11–13].

However, the ligands of receptor-like PTPases are still not known. Recently, one of the PTPases, PTP μ, was shown to mediate cell–cell aggregation via a homophilic binding mechanism [20–22]. There is no evidence that these interactions alter PTPase activity directly but they suggest that they could be involved in signal-transduction pathways directed by cell contact. It is becoming clear that PTPases play a role in the contact inhibition of cell growth and then counteract the effects of tyrosine kinases. An increased cellular non-receptor PTPase activity has indeed been observed in response to density-dependent contact inhibition [23]. Moreover, inhibition of PTPase activity by orthovanadate can confer a reversibly transformed phenotype on cultured cells [24,25]. In addition, overexpression of several PTPases inhibits normal and transformed cell growth [26–28].

Of the receptor-like class-II PTPases, HPTP β has a single catalytic domain, which implies that intercatalytic domain effects on activity are not responsible for regulation [14,29]. Its activity is probably mainly regulated by its large extracellular region composed of 16 FN-III repeats. Several studies on the recombinant catalytic domain of HPTP β have given clues to its biochemical activity, but nothing is known about its physiological role and regulation [29,30]. Very recently, a receptor-like PTPase

Abbreviations used: PTPase, protein tyrosine phosphatase; PTK, protein tyrosine kinase; HUVE cells, human umbilical vein endothelial cells; GPDH, glyceraldehyde-3-phosphate dehydrogenase; LAR, leucocyte common antigen-related molecule; bFGF, basic fibroblast growth factor; FN-III, fibronectin type III.

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sharing structural homology with HPTP β, DEP-1 was shown to display enhanced expression and activity with increasing cell density [16]. Moreover, during the preparation of this paper, it was reported that the activity of a high-molecular-mass membrane-associated PTPase increased in relation to cell density and extracellular matrix adhesion in UMR 106.06 osteoblast-like cells [31].

We have previously reported that expression of HPTP β is dramatically decreased in lung cancer and tumour cell lines such as A427 human lung adenocarcinoma cells, which have lost contact inhibition. Furthermore, inhibition of PTPase activity by orthovanadate was found to overcome contact inhibition in confluent human umbilical vein endothelial (HUVE) cells [32]. This prompted us to investigate in more detail whether density-dependent growth arrest could affect the level of PTPase activity in HUVE cells, which display strict contact inhibition. They were compared with A427 cells used as a negative control. We demonstrate that contact-inhibited HUVE cells exhibit a significant increase in membrane-associated PTPase activity which can be correlated with an increase in HPTP β messenger, suggesting that this enzyme could be directly involved in density-dependent growth arrest.

EXPERIMENTAL

Cell culture

HUVE cells were isolated from umbilical cord veins and cultured as previously described [33] on fibronectin-coated supports (1 μg/cm^2; Sigma). HUVE and A427 cells (American Type Culture Collection CCL 92) were grown in RPMI 1640/M199 medium (1:1) supplemented with 20% AB$^+$ human serum (Jacques Boy, Reims, France). Cells were seeded at low density (6×10^3 cells/cm^2) in tissue culture flasks (Nunc) and grown to confluency, with a change of medium every 2 days. For experiments on modification of growth rate, exponentially growing cells were cultured in medium supplemented with 5% human serum. After 24 h, basic fibroblast growth factor (bFGF) was added at 10 ng/ml and suramin (Calbiochem) at 100 μM, and cells were cultured for a further 36 h more before being harvested. For thymidine-dependent growth arrest, sparse cells (6×10^3 cells/cm^2) were grown in the presence of 10 mM thymidine (Sigma), with a change of medium every day.

Preparation of cytosolic, membrane and cytoskeletal extracts from HUVE and A427 cells

Cells were washed three times with PBS and harvested after incubation in PBS/2mM EDTA for 30 min. After centrifugation (1000 *g*; 10 min; 4 °C), the cells were suspended at 2.5×10^6 cells/ml in buffer E [50 mM Hepes (pH 7.0), 2 mM EDTA, 2 mM EGTA, 10 mM 2-mercaptoethanol, 1 mM PMSF, 2 mM benzamidine, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM *o*-phenanthroline]. The cells were lysed by sonication (6×10 s) and centrifuged (100000 *g*; 30 min; 4 °C) to obtain the cytosolic fraction. The pellet was resuspended in the same volume of buffer E with 2% (v/v) Triton X-100, incubated at 4 °C for 1 h, and centrifuged (100000 *g*; 30 min; 4 °C) to obtain the solubilized membrane fraction. The pellet contained the Triton X-100-insoluble fraction corresponding to the cytoskeleton-associated proteins.

Measurement of PTPase activity

Assay of tyrosine phosphatase activity was performed on subcellular fractions of A427 and HUVE cells using the ^{32}P-labelled synthetic peptide Raytide (Oncogene Science, Uniondale, NY, U.S.A.), which has been used as a common substrate for various PTPases with no evidence for a strong preference for any of these enzymes [15,17,18,34–38].

Table 1 Designation and nucleotide sequence of the oligonucleotides used in PCR experiments

Name	5′→3′	Amplified molecules
Incel 5	GCCATGGTCGAGAAAGACCCTCTG	
*Ava*II-race	CTCGCTCGCCCATGGAGGATTCGGTCCAATGC	HPTP β
β-Stop	GGGAAGCTTATGCCTTGAATAGACTGGATC	
GPDH (G5)	GCAAATTCCATGGCACCGTCA	GPDH
GPDH (G3)	TCTAGACGGCAGGTCAGGTC	
P9-5PTPU	AAATTGCTGGAGTCATCGCGGG	PTP μ
M4-3PTPU	CATTCTGTTCTCATCTTTCTTAGCCG	
P10-5LAR	TCCGCCTCTAAGGATGAGCAGTCG	LAR
M6-3LAR	TCCAACGGGTCACCTGAATAAGGC	
HPTPGOLMBa	GGCCAAGTCGAGAAGGATCCATGAACTG	PTP γ
HPTPGHinIC	GTCTTCAGAAGCTTACAAATTAAGTGCC	

For the radiolabelling of the substrate, 30 μg of Raytide dissolved in 30 μl of assay buffer [50 mM Hepes (pH 7.5), 0.1 mM EDTA] was incubated with 27 μl of kinase buffer [assay buffer with 0.1 mg/ml BSA and 0.2% (v/v) 2-mercaptoethanol], 12 units of pp60$^{c\text{-}src}$ tyrosine kinase (Oncogene Science) and 30 μl of ATP mix (kinase buffer with 0.3 mM ATP and 30 mM $MgCl_2$) containing 30 μCi of [γ-^{32}P]ATP (Amersham). The reaction was performed at 37 °C overnight and terminated by the addition of 150 μl of 10 mg/ml BSA and 1 ml of 20% (w/v) trichloroacetic acid/20 mM NaH_2PO_4. After 20 min on ice, the precipitate was centrifuged (10000 *g*; 10 min; 4 °C) and washed three times in 20% (w/v) trichloroacetic acid/20 mM NaH_2PO_4 solution. The radioactive substrate was dissolved in 500 μl of 1 M Tris/HCl, pH 8.0.

The reaction mixture (100 μl) contained 50 mM Mes, pH 6.0, 1 mg/ml BSA, 0.5 mM dithiothreitol, 0.01% (w/v) CHAPS and 6.2 μM [^{32}P]Tyr-labelled Raytide (10000 c.p.m.). The phosphatase assay was performed for 15 min at 30 °C and the reaction was determined by the addition of 1.1 ml of acidic charcoal mixture [0.9 M HCl, 0.1 M sodium pyrophosphate, 2 mM NaH_2PO_4, 10% (w/v) Norit A]. After centrifugation in a Microfuge, the amount of radioactivity in 0.6 ml of supernatant was measured in a scintillation counter. Enzyme activity was expressed as pmol of [^{32}P]P_i released/min per 1×10^7 cells.

RNA purification

Total RNA was purified by means of the RNAzol B method (Bioprobe systems, Montreuil, France) [39]. After a second precipitation with 0.1 vol. of 3 M sodium acetate and 2.5 vol. of ethanol overnight at −20 °C, RNA was collected by centrifugation (10000 *g*; 15 min; 4 °C), washed in 75% ethanol and the pellet was resuspended in 50 μl of diethyl pyrocarbonate-treated water. The quantity of RNA in each sample was deduced from the absorbance at 260 nm, assuming that 40 μg/ml RNA corresponds to 1 absorbance unit.

Oligonucleotide synthesis

Oligonucleotides were synthesized in a PCR-mate DNA synthesizer (Applied Biosystems, Les Ulis, France) using cyanoethyl phosphoramidite chemistry. The oligonucleotides synthesized for this study are listed in Table 1.

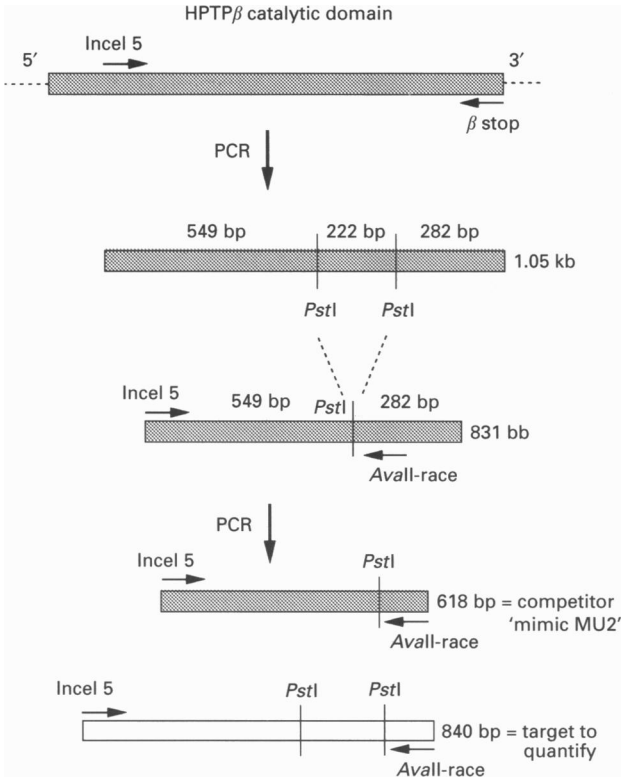

Figure 1 Construction of 'mimic MU2' DNA for HPTPβ competitive PCR experiments

The whole procedure used to obtain the 618 bp fragment of competitor is summarized. A 1.05 kb DNA fragment specific for the catalytic domain of HPTP β was amplified with primers Incel 5–β-stop and a deletion was obtained by restriction enzyme digestion (*PstI*). After ligation of the 549 bp and 282 bp products, amplification with primers Incel 5–*Ava*II-race gave rise to the 618 bp competitor 'mimic MU2'.

Reverse transcription and PCR

Total RNA (15 μg) was converted into single-strand cDNA using oligo(dT) priming and Moloney murine leukaemia virus reverse transcriptase (Superscript Preamplification System; Gibco–BRL, Cergy, France), in a final volume of 100 μl. The cDNA from 0.75–3 μg of total RNA was used as a template in *in vitro* amplification in a DNA thermal cycler (Cetus-Perkin–Elmer, Paris, France), with 2.5 units of *Taq* polymerase (Beckman, Palo Alto, CA, U.S.A.) and 100 pmol of each primer. The 30-cycle program consisted of 1 min at 94 °C (denaturation), 1 min at 58 °C (annealing) and 1 min at 72 °C (extension). Amplified fragments were separated at 1% agarose gel and visualized by ethidium bromide staining.

Competitive PCR experiments were performed with a mixed amount of cDNA (equivalent to 0.75 μg of total RNA) and a variable amount of a competitor DNA template referred to as 'mimic MU2' corresponding to 1–10000 fg of DNA; 10 μCi of [α-^{32}P]dCTP was added in each reaction. A program of 30 cycles of amplification was required and the products were separated on to 1% Nusieve/1% agarose (FMC Bioproducts, Paris, France) and visualized by ethidium bromide staining after addition of non-radioactive co-migrating DNA. Fragments were cut out from the gels and the incorporated radioactivity was counted.

Construction of a PCR internal standard: 'mimic MU2'

To construct a competitive template, a 222 bp deletion was created using restriction enzyme digestion in the sequence corresponding to the catalytic domain of HPTP β (Figure 1). The cDNA of HUVE cells was used as a template to amplify a 1053 bp fragment with primers Incel 5 and β-stop (Table 1). This PCR product was purified on 1% agarose gel using the Magic-prep-PCR kit (Promega, Charbonnières, France). Digestion with *Pst*I was then performed, and the 5′ fragment (549 bp) and 3′ fragment (282 bp) were purified by electrophoresis and ligated using T4 DNA ligase (New England Biolabs) overnight at 16 °C. The ligation product was submitted to another PCR amplification using primers Incel 5 and *Ava*II-race, giving a fragment of 618 bp identical with the Incel 5–*Ava*II-race fragment from the native template (840 bp) except for the 222 bp *Pst*I–*Pst*I deletion. This 618 bp fragment was purified by electrophoresis and quantified by measuring absorbance at 260 nm (50 μg/ml DNA corresponds to 1 absorbance unit). This fragment was used as the PCR internal standard in all quantitative experiments.

RESULTS

Evaluation of protein tyrosine phosphatase activity in HUVE and A427 cells in relation to cell density

In order to compare the distribution of PTPase activity in normal HUVE cells and in A427 adenocarcinoma cells, cells were grown and harvested at low (6×10^3 cells/cm^2) or high (2×10^4 for confluent HUVE or 5×10^4 cells/cm^2 for A427) density. In each experiment, cells were brought to the same concentration (2.5×10^6 cells/ml) and fractionated into cytosolic, membrane and cytoskeletal extracts. PTPase activity towards the synthetic peptide, Raytide, in each subcellular fraction was then assayed [15,17,18,34–38].

PTPase activity in cytosolic and cytoskeletal fractions from HUVE cells increased about 2.5-fold ($P < 0.01$), whereas it remained unchanged in these two fractions from A427 cells (Table 2). In contrast, the PTPase activity of membrane-associ-

Table 2 Measurement of PTPase activity in subcellular fractions of HUVE and A427 cells

HUVE and A427 adenocarcinoma cells were harvested at low (6×10^3 cells/cm^2) or high (2×10^4 for HUVE and 5×10^4 for A427) density. A427 cells were also harvested at very high density (15×10^4 cells/cm^2). Synchronized cells were obtained by serum deprivation for 24 h before the addition of 20% human serum for a further 24 h. They were then fractionated into cytosolic, Triton X-100-solubilized membrane and cytoskeletal-associated proteins as detailed in the Experimental section. PTPase activity towards [^{32}P]Raytide was assayed in each fraction, and is expressed as pmol of [^{32}P]P_i release/min per 1×10^7 cells. The values represent means ± S.D. from three independent experiments. Significant difference was determined by Student's *t* test (***$P < 0.001$, **$P < 0.01$).

	PTPase activity (pmol/min per 1×10^7 cells)		
Cell type	Membrane	Cytosol	Cytoskeleton
Huve			
Low density	3.6 ± 0.5	8.9 ± 2.5	2.0 ± 0.07
High density	32.2 ± 4.3***	21.4 ± 1.2**	5.6 ± 0.8**
A427			
Low density	4.8 ± 0.5	3.6 ± 0.3	0.7 ± 0.2
High density	6.2 ± 0.5	3.2 ± 0.4	1.0 ± 0.05
Very high density	5.8 ± 0.8	3.0 ± 0.3	0.9 ± 0.1
Synchronous	6.4 ± 0.9	3.0 ± 0.2	0.9 ± 0.02
Without serum	3.6 ± 0.3	1.0 ± 0.04**	0.7 ± 0.04

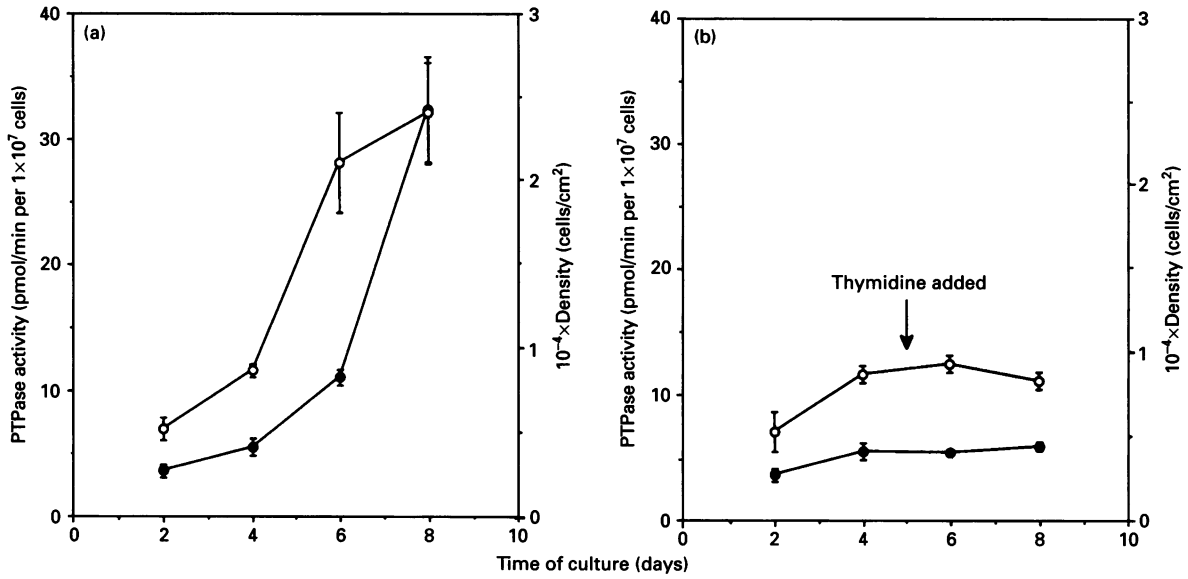

Figure 2 Relationship of membrane-associated PTPase activity to cell density and growth rate in HUVE cells

HUVE cells were seeded at low-density and grown to confluency. Every 2 days, cells were harvested and counted (○), and membrane-associated PTPase activity (●) towards [^{32}P]Raytide was assayed. (**a**) Cells were cultured in RPMI1640/M199 medium supplemented with 20% human serum. (**b**) After 5 days of culture in the conditions described above, 10 mM thymidine was added to the culture medium to block cell proliferation. Results are expressed as pmol of [^{32}P]P_i released/min per 1×10^7 cells, and are the means ± S.D. of three to five independent experiments.

ated proteins from high-density HUVE cells was about 10-fold higher ($P < 0.001$) than the activity measured for low-density HUVE cells, whereas the PTPase activity in every subcellular fraction from A427 remained essentially unchanged, both when A427 cells were in synchronous growth and at very high density (15×10^4 cells/cm^2). Serum deprivation of low-seeded A427 cells produced no increase in PTPase activity; instead, cytosolic PTPase activity was decreased compared with exponentially growing cells.

Therefore an increase in membrane-associated PTPase activity was observed only in HUVE cells, the growth of which was contact-inhibited, and not in A427 cells, the proliferation of which was not modified by cell density.

Relationship of PTPase activity to growth rate in HUVE cells

The observed modification of PTPase activity in HUVE cells could be a direct effect of cell density or reflect the rate of cell growth. In order to discriminate between these two possibilities, modification of PTPase activity during cellular multiplication was examined. HUVE cells were seeded at low density (6×10^3 cells per cm^2) and grown to confluency (2×10^4 cells per cm^2) with harvest, fractionation and determination of PTPase activity every 2 days until 8 days after plating. Figure 2(a) shows that little change occurred in the first days of culture when cells proliferated rapidly (2–6 days after plating). However, when the rate of division decreased (after 6 days), membrane-associated PTPase activity increased rapidly (about 10-fold), and this was totally inhibited by the addition of 200 μM sodium orthovanadate (a PTPase inhibitor), showing that the activity measured was really tyrosine phosphatase activity (results not shown).

In order to modify the growth rate, HUVE cell proliferation was either stimulated by the addition to the culture medium of 10 ng/ml bFGF or stopped by the addition of 100 μM suramin over a period of 36 h. These treatments resulted in no significant modification of membrane-associated PTPase activity. The cytosolic PTPase activity decreased about 2–3-fold when cells were treated with suramin, probably reflecting blocking of signals triggered by the growth factor receptors (Table 3). Cell-growth arrest by the addition of 10 mM thymidine to the medium produced no change compared with parameters observed for sparsely growing cells (Figure 2b). Thus these data strongly suggest that membrane-associated PTPases were specifically activated when HUVE cell proliferation was arrested by cell contact and that this phenomenon was not associated with a modification of the multiplication index.

Table 3 Modulation of PTPase activity in relation to growth rate

HUVE cells were cultured with 5% human serum over 24 h before 36 h of treatment with 10 ng/ml bFGF (growth factor for endothelial cells), 100 μM suramin (antiproliferative drug) or nothing (control cells). Cells were then harvested, fractionated and PTPase activity towards [^{32}P]Raytide was assayed as described in the Experimental section. PTPase activity was expressed as [^{32}P]P_i released/min per 1×10^7 cells. Results are the means ± S.D. of three independent experiments. The significant difference between control and treated cells was determined using Student's t test (**$P < 0.01$).

	PTPase activity (pmol/min per 1×10^7 cells)		
Treatment	Membrane	Cytosol	Cytoskeleton
Control	0.6 ± 0.1	2.8 ± 0.2	0.9 ± 0.3
bFGF (10 ng/ml)	1.0 ± 0.3	2.7 ± 0.3	1.5 ± 0.4
Suramin (100 μM)	0.5 ± 0.1	1.0 ± 0.4**	1.2 ± 0.4

Modification of receptor-like PTPase expression in relation to proliferation

We examined the expression of various receptor-like PTPases that were structurally similar to adhesion molecules in HUVE and A427 cells during cell culture. Total RNA was extracted and

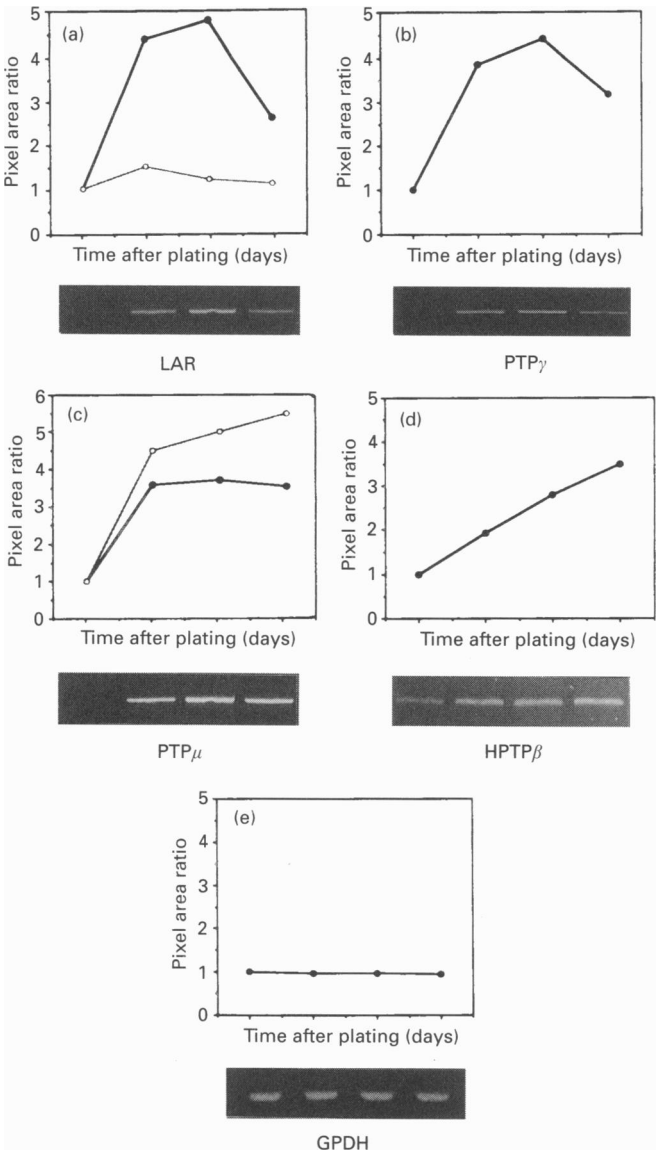

Figure 3 Expression of receptor-like PTPases in HUVE and A427 cells during culture

Electrophoresis of reverse-transcribed PCR products amplified from 0.75 μg of total RNA of sparse to confluent or dense cells (2–8 days after plating), in the linear range of amplification, with primers specific for (**a**) LAR, (**b**) PTP γ, (**c**) PTP μ, (**d**) HPTP β and (**e**) GPDH. The amplification products were visualized by ethidium bromide staining after migration on agarose gel. The results for HUVE cells are shown under the curves. Densitometric analysis was used to quantify the signals obtained and allowed measurement of pixel area. Curves represent the ratio of the pixel area of each band/the pixel area of the band from cells 2 days after plating (i.e. level of expression in sparse cells) in HUVE cells (●) and A427 cells (○). Results are representative of two experiments.

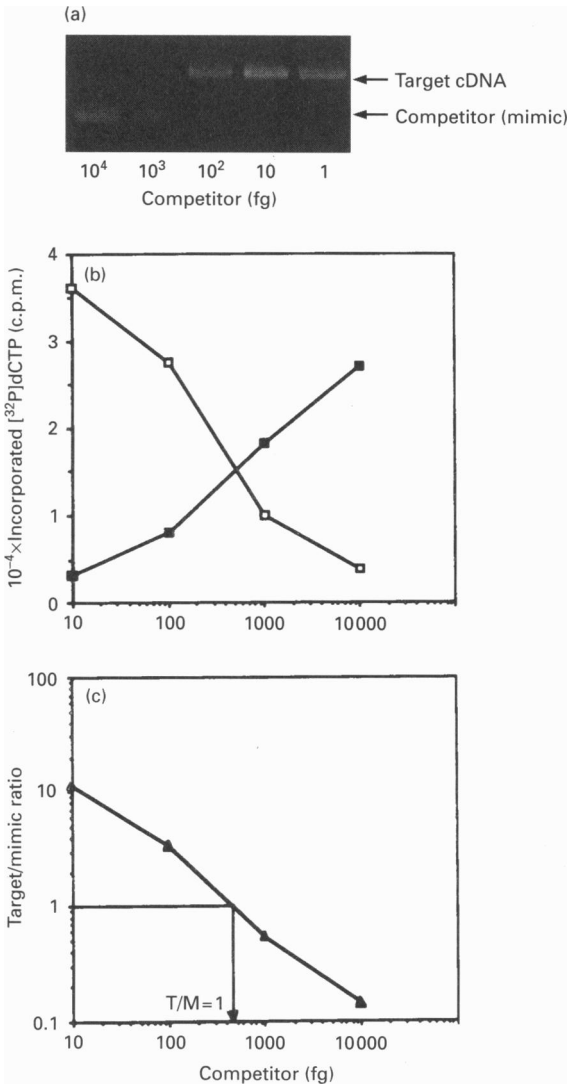

Figure 4 Competitive PCR between HPTP β target cDNA (840 bp) and 'mimic MU2' (618 bp)

HUVE-cell cDNA corresponding to 0.75 μg of reverse-transcribed total RNA was co-amplified with increasing amounts of 'mimic MU2' DNA (from 1 to 1×10^4 fg) in the presence of 10 μCi of [α-^{32}P]dCTP using the Incel 5–*Ava*II race set of primers. Amplification products were separated and visualized as described in the Experimental section. (**a**) Ethidium bromide-stained gel from a typical competitive experiment; (**b**) incorporation of [^{32}P]dCTP into target (□) and mimic (■) DNA; (**c**) linear representation of the data using the ratio of recovered radioactivities in target HPTP β over mimic DNA.

submitted to reverse transcription and PCR using the appropriate primers (Table 1). PCR was performed with two different cDNA concentrations corresponding to 0.75 and 1.5 μg of total RNA to ensure that reactions were in the linear range of amplification, which enabled comparison of the levels of amplified products although it was not a quantitative procedure. The GPDH gene transcript was taken as an internal standard; it remained constant in each sample (Figure 3e). In HUVE cells, we noticed that all the tested PTPases were poorly expressed 2 days after plating (Figures 3a–3d). Increased expression of LAR and PTP γ was observed up to 6 days after plating, followed by a decrease when the culture reached saturating density (Figures 3 (closed circles) and 3b). LAR was also expressed in A427 cells but the pattern of expression was only slightly modified during the culture period (Figure 3a, open circles). PTP μ was expressed in the two cell types as soon as 4 days after plating and the level of expression remained constant over the period examined (Figure 3c). Interestingly, PTP γ and HPTP β are exclusively expressed in HUVE cells but only the HPTP β transcript increased continuously until the cells reached confluency, i.e. 8 days after plating.

Quantification of HPTP β mRNA expression during HUVE cell culture

To measure precisely the range of modification of HPTP β gene expression in contact-inhibited HUVE cells, we used a method of quantification based on a competitive PCR. The competitor was a truncated DNA lacking a 222 bp fragment (Figure 1). Target HPTP β cDNA (840 bp) was co-amplified with a dilution of competitor DNA (618 bp) of known concentration. The amplification products were then separated by electrophoresis and visualized by direct ethidium bromide staining (Figure 4a). Then the labelled bands were cut out from the agarose gel and ^{32}P incorporation from [α-^{32}P]dCTP was measured. The radioactivity of the internal standard band was then corrected for increased radiolabelling of the largest fragment (840 bp for the target and 618 bp for the competitor). Figure 4(b) shows the correlation between the decrease in target DNA amplification and the increase in competitor amplification. The ratio target/mimic was then plotted as a function of the amount of competitor added to the PCR (Figure 4c). At the equivalence point (i.e. when target/mimic ratio equals 1), equimolar quantities of competitor and HPTP β cDNA are amplified so that the concentration of HPTP β cDNA corresponding to the specific mRNA present in total HUVE-cell RNA can be estimated from the abscissa (Figures 4b and 4c).

This method was applied to cDNA obtained from HUVE cells at various times of culture from sparse cells to confluency. HPTP β mRNA was poorly expressed in sparse HUVE cells (0.1 pg of specific mRNA/μg of total RNA). It slowly increased during exponential growth (0.4 and 0.6 pg of specific mRNA/μg of total RNA respectively, 4 and 6 days after plating) and had increased significantly when cells reached confluency (1.25 pg of specific mRNA/μg of total RNA, 8 days after plating). It is interesting to note that the increase in HPTP β mRNA expression was in the same range (about 12-fold) and followed the same time course as the observed increase in membrane-associated PTPase activity (Figure 2a).

DISCUSSION

In the present study, we have examined the modification of PTPase activity in HUVE cells when cellular growth is affected by conditions such as cell density or compounds known to modulate proliferation rate. We compared these changes with those observed in the A427 adenocarcinoma cell line, which does not display contact inhibition. We noticed that membrane-associated tyrosine phosphatase activity is significantly elevated in density-arrested HUVE cells (about 10-fold over that in sparse cells), whereas A427 PTPase activity changes little whatever the culture conditions (very high density resulting in the emergence of cell clumps on the first cell layer or synchronization). Serum deprivation of A427 cells induces a 2–3-fold decrease in cytosolic PTPase activity which probably reflects loss of stimulation of the growth factor-signalling pathways which is known to involve activation of cytosolic PTPases [11–13]. The membrane-associated PTPase activity in HUVE cells remains relatively low during the exponential phase of growth, increases suddenly when the rate of cell proliferation decreases, and reaches its highest level when cell growth is arrested by confluency. However, such an increase is not detected when exponentially growing sparse cells are induced to cease proliferation by the addition of thymidine to the culture medium, resulting in blocking of cells in the S-phase. In addition, significant change was observed when HUVE cells were treated with bFGF, a growth factor for endothelial cells *in vitro* [40], or suramin, which inhibits the binding of growth factors to their cell-surface receptors [41]. Interestingly, suramin treatment induces a 2–3-fold decrease in cytosolic PTPase activity, an effect similar to that obtained with A427 cells on removal of serum. Therefore elevated PTPase activity is not associated with a decrease in proliferation rate. It appears that some regulatory mechanisms maintain the membrane-associated PTPase activity at basal level during cell proliferation, whereas density-dependent growth arrest increases it. Thus the balance in tyrosine phosphorylation could be altered in favour of PTPases, which may contribute to the negative regulation of cell growth by as yet unknown regulatory pathways.

In order to identify the PTPase(s) implicated in this increase in activity, we focused our attention on transmembrane PTPases. Some receptor-like PTPases such as LAR [18,34], PTP μ (which has been shown to mediate cell–cell aggregation by homophilic binding [20–22]) and HPTP β display structural similarity to cell-adhesion receptors. PTP γ was also interesting because of loss of expression in various tumours [42,43]. Of the PTPases tested, LAR and PTP μ are the only ones expressed in both cell types. In A427 cells, LAR expression remains essentially constant during cell culture whereas PTP μ expression increases, as it does in endothelial cells. Expression of LAR, PTP γ and PTP μ increases during HUVE cell culture, but the time course of expression appears to be different from the increase in activity, as the maximum is reached during exponential cell growth. PTP γ and HPTP β are expressed in HUVE cells only, but only HPTP β exhibits an increase in expression that parallels the variation in membrane-associated PTPase activity. These observations make HPTP β a very good candidate for a role in cell–cell interactions.

To quantify the variation in the expression of the mRNA encoding this protein, we have used a method based on a competitive PCR [44]. This technique provides a rapid and reliable way of quantifying the amount of cDNA from very poorly expressed mRNA, such as that encoding cell-surface receptors. Using this experimental approach, we confirmed previous data obtained with semiquantitative PCR on HPTP β expression. In addition, it allowed us to determine more accurately the increase in HPTP β mRNA (12-fold), which is essentially identical with that determined for membrane-associated PTPase activity. This observation strongly suggests that HPTP β could be directly involved in this increase in activity. It would not be surprising if the PTPase activity of HPTP β is regulated mainly at the transcriptional level, assuming that this protein has a single catalytic domain. It has been suggested that receptor-like PTPases with two catalytic domains are regulated by one of them, the other one being responsible for the detected activity, as observed for LAR [18]. Having only a single tyrosine phosphatase domain, HPTP β cannot be regulated in this way. Moreover, HPTP β does not contain a potential tyrosine-phosphorylation site able to interfere with its activity, as observed for SH-PTP1 [45–47] and SH-PTP2 [9,48–51]. However, HPTP β can be phosphorylated *in vitro* by protein kinase C on Ser-1632 and/or Ser-1636, but these modifications do not seem to affect its activity [30].

The mechanism by which the enhanced expression and activity of HPTP β are regulated, probably involving ligand binding, remains unclear and nothing is known about the ligands or substrates of this enzyme. Work is in progress in our laboratory to investigate HPTP β at the protein level. However, a rabbit polyclonal antibody raised against a fusion protein was not sensitive enough to detect the native protein in endothelial cells. Curiously, although HPTP β cDNA was cloned 4 years ago [14], no information about the biological role of this large but scarce protein is yet available.

In conclusion, we have shown that the density-dependent growth arrest of HUVE cells is accompanied by an increase in

membrane-associated PTPase activity and an increase in expression of receptor-like PTPases, HPTP β expression being the most closely related to the increase in PTPase activity. Of course, a complex mechanism such as contact inhibition of cell growth probably involves several PTPases. The increase in tyrosine phosphatase activity described here may alter the balance between tyrosine phosphorylation and dephosphorylation in favour of the latter and contribute to negative regulation of cell growth. This places PTPases at a crucial position in the regulatory mechanisms of normal cell physiology and transformation.

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