Regulation of membrane-associated tyrosine phosphatases in UMR 106.06 osteoblast-like cells

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Protein tyrosine phosphatases play an important role in cell metabolism. Three distinct protein tyrosine phosphatase activities have been identified in an osteoblast-like cell line, UMR 106.06. These activities comprised two membrane-associated phosphatases and one cytosolic phosphatase of apparent molecular mass > 153 kDa, 80 kDa and 40 kDa respectively, estimated by gel filtration. On the basis of differences in apparent molecular mass, proteolytic-digestion profiles, substrate specificities and responses to a range of extracellular influences and inhibitor molecules, the two membrane-associated tyrosine phosphatases are distinct proteins. Tyrosine phosphatase activity in UMR 106.06 cells was sensitive to cell density. Cells at confluence contained membrane protein tyrosine phosphatase with specific activity 9-fold higher than cells at medium or low cell density. This elevation in

membrane tyrosine phosphatase activity was due specifically to an increase in the high-molecular-mass enzyme. This phosphatase was also responsive to extracellular matrix components. This activity was elevated in cells grown on a collagen type-I matrix independently of cell density. Membrane and cytosolic protein tyrosine phosphatases were differentially regulated by a variety of agents including phorbol 12-myristate 13-acetate, parathyroid hormone, epidermal growth factor, okadaic acid and transforming growth factor β . These observations suggest that regulatory influences control tyrosine phosphorylation in UMR 106.06 cells including cell–cell contact, cell–matrix contact and signal transduction involving tyrosine and serine/threonine phosphorylation events.

INTRODUCTION

Tyrosine phosphorylation of proteins has been identified as an essential element in the control of both normal and neoplastic cell growth (Yarden and Ullrich 1988). As it is a dynamic and reversible process, an understanding of its regulation of cell function requires a knowledge of both the tyrosine kinases and tyrosine phosphatase involved in a given system. To date, there is much more information available on phosphorylation by tyrosine kinases and their regulation than the corresponding dephosphorylation by the protein tyrosine phosphatases (PTPases). This study therefore focused on the PTPases responsible for co-regulation of the tyrosine phosphorylation pathways of the rat osteoblast-like UMR 106.06 cell line.

The family of tyrosine phosphatases is clearly diverse and multimembered. PTPases of a number of cell types have been purified and characterized and exist in multiple molecular forms which may reside in either the cytosol (Tonks et al., 1988a,b) or the cell membrane (Carpenter et al., 1979; Brautigan et al., 1981; Tonks et al., 1988c). In several cases these distinct multienzyme forms can be separated chromatographically. For example, the rat hepatic membrane fraction has been shown to contain at least three distinct PTPases using poly-L-lysine chromatography (Gruppiso et al., 1991), and at least three distinct forms of brain cytosol PTPase activity can be separated using DEAE-cellulose (Foulkes et al., 1983). These enzymes exhibit inherent differences in substrate specificity (Krueger et al., 1990), molecular mass, antigenicity (Gruppiso et al., 1991), effector compounds (Pallen et al., 1988) and ion-exchange-binding affinity (Foulkes et al., 1983; Tung and Reed, 1987).

The recent cloning of multiple PTPases has confirmed the diversity of this family and shown that its members can be

grouped into three classes. Class I are cytoplasmic and contain one PTPase domain. Class II and III are transmembrane proteins that contain one or two intracellular PTPase domains respectively and extracellular domains that share sequence homology with immunoglobulin, fibronectin type III and the neural cell-adhesion molecule. The receptor-like PTPase μ (RPTP μ), which contains an immunoglobulin-like and four fibronectin type-III-like domains, has been demonstrated to promote cell-to-cell adhesion when expressed in insect cells (Gebbinck et al., 1993).

UMR 106.06 cells, a rat osteoblast-like cell line, have been widely used to explore regulation of cell growth (Ng et al., 1983a,b), hormone receptor signal transduction (Schneider et al., 1992), cell–matrix interaction (Ikeda et al., 1993) and regulation of gene expression of osteoblast products (Zhou et al., 1991). Although some of these cell functions may involve tyrosine phosphorylation or dephosphorylation, these two processes have not been adequately studied in UMR 106.06 cells. As regulation of PTPases has not been well studied, it was of particular interest to investigate the regulation of membrane-associated PTPases in UMR 106.06 cells. Other studies have suggested that cell–cell contact and/or cell adhesion to extracellular matrix may directly influence the activity of PTPases that have extracellular ‘receptor-like’ domains (Mustelin et al., 1989; Koretzky et al., 1991; Ostergaard and Trowbridge, 1991). Pallen and Tong (1991) have reported that solubilized membranes from 3T3 cells contain a 37 kDa PTPase the activity of which is elevated 8-fold in contact-inhibited cells. In the present study it was found that UMR 106.06 cells contain a high-molecular-mass membrane-associated PTPase that is increased approximately 9-fold at high cell density. This response was mimicked by cells grown on a collagen type-I matrix, in this case independently of cell density.

Abbreviations used: PTPase, protein tyrosine phosphatase; PMA, phorbol 12-myristate 13-acetate; PTH, parathyroid hormone; EGF, epidermal growth factor; OA, okadaic acid; TGF β , transforming growth factor β ; RCM-lysozyme, reduced carboxamidomethylated and maleylated lysozyme; MBP, myelin basic protein; RPTP μ , receptor-like PTPase μ .

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EXPERIMENTAL

Materials

Myelin basic protein (MBP; from bovine brain), trypsin (diphenyl-carbamyl chloride treated type XI from bovine pancreas) and trypsin inhibitor (type I-S from soyabean) were obtained from Sigma, St. Louis, MO, U.S.A. [γ - 32 P]ATP was from Dupont, New Research Products (Boston, MA, U.S.A.). Minimal essential medium was obtained from Gibco-BRL Laboratories, Grand Island, NY, U.S.A. and fetal calf serum from the Commonwealth Serum Laboratories (Melbourne, Australia). The DC protein assay kit was supplied by Bio-Rad Laboratories, Richmond, CA, U.S.A. Agarose was obtained from Kodak International Biotechnologies and collagen type I from Collaborative Research, Waltham, MA, U.S.A. Other reagents were of ultrapure grade and supplied by Sigma, AJAX Chemicals or BDH. The S-300 Sephacryl (superfine) resin was obtained from Pharmacia LKB (Uppsala, Sweden).

Cell culture

The establishment of the clonal cell line UMR 106 (rat osteosarcoma cells enriched in osteoblast-like properties) has been described previously by Partridge et al. (1980, 1981). A subclone, UMR 106.06 (Forrest et al., 1985) was grown in minimal essential medium, buffered in Hepes (20 mM), with 10% fetal calf serum, supplemented with gentamicin (80 mg/l). Cells were grown at 37 °C in an atmosphere of 5% CO₂. Cell treatments were performed by removing the culture medium and adding fresh medium containing the appropriate agent. For cell growth on collagen, plates were coated with collagen type I by diluting the collagen into culture medium to a final concentration of 50 μ g/cm², pouring the mixture into cell culture dishes (90 mm) and allowing the matrix to set at 37 °C for 1 h. UMR 106.06 cells were then subcultured on to the collagen matrix or on to plastic as a control.

A431 cell membranes

Epidermal carcinoma cell (A431) membranes were prepared as described previously by House et al. (1984), using the method of Carpenter et al. (1979) as modified by Brautigan et al. (1981) in the absence of Ca²⁺ as recommended by Cassel and Glasser (1982).

Preparation of tyrosine-phosphorylated substrates

Reduced, carboxamidomethylated and maleylated (RCM) lysozyme was prepared by the procedure of Crestfield et al. (1963) as modified by Tonks et al. (1988a). MBP and RCM-lysozyme were phosphorylated on tyrosine at 0.5 mg/ml in 35 mM imidazole, pH 7.2, containing 50 mM NaCl, 12 mM magnesium acetate, 0.2 mM EGTA, 3% (v/v) glycerol, 0.1 mM NaVO₃, 2 mM dithiothreitol, 4 mM MnSO₄, 2.5 μ g/ml epidermal growth factor (EGF), 2 mM [γ - 32 P]ATP (20 c.p.m./pmol) and solubilized A431 cell membranes containing EGF receptor tyrosine kinase. The phosphorylation reaction was carried out at 37 °C and 32 P incorporation monitored by taking 5 μ l portions from the reaction mixture at time intervals and spotting on to phosphocellulose ion-exchange paper (Whatman P81). The papers were washed three times in 75 mM orthophosphoric acid followed by 95% (v/v) ethanol, dried and counted with 10 ml of organic scintillant. The reaction mixture was incubated overnight before terminating with trichloroacetic acid added to 20% (v/v). Precipitated [32 P]phosphoprotein was washed three times in 20% trichloroacetic acid, then with ethanol/ether (1:4, v/v). Air-

dried [32 P]phosphoprotein was dissolved in 100 mM Tris/HCl, pH 7.5, to 10000 c.p.m./ μ l and stored at -20 °C.

Synthetic peptides (0.05 mg/ml) were phosphorylated on tyrosine essentially as described for protein substrates except that [γ - 32 P]ATP was removed by sequential anion-exchange chromatography over two AG1X8 columns (1 cm $\times$ 10 cm) in 30% acetic acid. The [32 P]phosphopeptide-containing eluate was concentrated, purified by rotary evaporation and dissolved in 100 mM Tris/HCl, pH 7.5, to 10000 c.p.m./ μ l (equivalent to 50 μ M phosphotyrosine-containing peptide).

Cell fractionation

Both solubilized membrane and cytosolic fractions of cells were prepared by a modification of the method of Brautigan and Pinault (1991) in which confluent 106.06 cells were washed twice with PBS and resuspended in buffer A [25 mM Hepes, pH 7, 150 mM NaCl, 2 mM EDTA, 20 μ g/ml leupeptin, 20 μ g/ml aprotinin and 0.14 mM phenylmethanesulphonyl fluoride]. Cells were scraped into 1 ml of solution A and sonicated for 2 s using a Branson Sonifier 450. Sonicated suspensions were centrifuged at 100000 *g* for 30 min at 4 °C in a Beckman TL-100 ultracentrifuge with a TLA-100.2 fixed-angle rotor. The supernatant was used as the cytosolic source of tyrosine phosphatases. The pellet was solubilized in buffer B (25 mM Hepes, pH 7, 2 mM EDTA, 20 μ g/ml leupeptin, 20 μ g/ml aprotinin, 0.14 mM phenylmethanesulphonyl fluoride, 1% Triton X-100 and 15 mM 2-mercaptoethanol). This suspension was again centrifuged at 100000 *g* for 30 min at 4 °C and the supernatant was the source of solubilized membrane tyrosine phosphatase.

Protein determination

Protein concentration was determined using the DC protein assay (Bio-Rad).

Protein separation by gel chromatography

Gel filtration was performed using an S-300 Sephacryl (superfine) column (1.5 cm $\times$ 40 cm). The gel-filtration procedure was that of Brautigan and Pinault (1991) with some modifications. The column was equilibrated in buffer C [25 mM Hepes, pH 7, 1 mM EDTA, 10% (v/v) glycerol, 150 mM NaCl, 10 μ g/ml gelatin, 45 mM 2-mercaptoethanol and 2 mM dithiothreitol]. PTPase fractions (solubilized membrane and cytosolic fractions) were chromatographed in buffer C. The S-300 Sephacryl column was run under gravity at 4 °C, and 7-drop fractions were collected and assayed for PTPase activity. The column was calibrated using ovalbumin (43 kDa), aldose (158 kDa) and Dextran Blue (2000 kDa).

Partial trypsin digestion of PTPase

Protein samples (mg/ml) were digested with trypsin at 30 °C in 10 mM Hepes, pH 7.4. Trypsin solutions were prepared fresh. Reactions were initiated by the addition of trypsin to 20 μ g/ml. Portions were removed at the indicated times and added to trypsin inhibitor (40 μ g/ml), and stored on ice. A *t*₀ sample was taken before trypsin addition. The samples were then assayed for PTPase activity.

PTPase assay

This was based on the measurement of 32 P released from a phosphotyrosine-labelled substrate. For the protein substrates MBP and RCM-lysozyme, samples of solution to be assayed

were added directly to the assay buffer [25 mM imidazole/HCl, pH 7.2, 1 mg/ml BSA and 0.1% (v/v) 2-mercaptoethanol]. Reaction mixtures were incubated at 37 °C for 2 min before the reactions were initiated by the addition of phosphorylated protein (approx. 1×10^6 c.p.m./ml). Portions were removed at the time intervals indicated in the text. Reactions were terminated by the addition of trichloroacetic acid to 10% (w/v) and BSA was added as a carrier protein to 2 mg/ml. The suspension was vortexed, allowed to stand on ice for 10 min and centrifuged at 12 500 *g* for 3 min.

Assays of dephosphorylation of peptide substrates were performed as described by Streuli et al. (1990), in 25 mM Hepes, pH 7.3, containing 5 mM EDTA and 10 mM dithiothreitol. Peptide substrates were added to 40 µg/ml (approx. 1×10^6 c.p.m./ml) to initiate the dephosphorylation reaction at 37 °C. Samples were removed at time intervals and added to 15-fold (v/v) excess of charcoal solution [4% (w/v) Norit A in 0.9 M HCl/90 mM Na₄P₂O₇/2 mM NaH₂PO₄]. This suspension was then vortexed for 1 min followed by centrifugation for 5 min at 12 000 *g* to pellet the charcoal phase. Supernatants containing released ³²P were withdrawn and quantified by liquid-scintillation counting in a β-counter.

RESULTS

Gel chromatography of UMR 106.06 PTPases

Three distinct PTPase species were identified by gel filtration of the cellular fractions of UMR 106.06 cells. Two were found associated with the membrane. Activity in solubilized membranes was eluted in two broad peaks designated peak A (apparent molecular mass greater than 153 kDa) and peak B (apparent molecular mass 80 kDa) (Figure 1). The cytosolic fraction contained one detectable peak of PTPase with apparent molecular mass 40 kDa designated peak C (Figure 1). All three PTPase activities identified were inhibited by 10 µM NaVO₃, a potent PTPase inhibitor (results for cytosolic fraction not shown).

Substrate specificity

The PTPase activities measured in peak-A and peak-B material had different substrate specificities. The ratio of activities of peak

Figure 1 Gel-filtration profile of PTPase activity of UMR 106.06 cell fractions

Cell fractions were prepared as outlined in the Experimental section. Phosphorylated MBP was used as substrate. PTPase-gel-filtration profiles of the cytosolic (○) and membrane fraction in the absence (□) and presence (●) of NaVO₃ are shown. The peaks of PTPase activity are designated A, B and C as indicated. The elution positions of the molecular-mass protein standards are indicated.

Table 1 Effector compounds of the PTPase activity of peak A and peak B

Pooled PTPase activity of peak-A and peak-B material was collected by gel filtration and assayed in the presence of multiple effector compounds as listed. The PTPase substrate used in the assay was MBP and the reaction was allowed to continue for 2 min to ensure that dephosphorylation of the substrate was still proceeding in a linear fashion. In each case activities are expressed as the percentage of control values and represent means ± S.E.M. of triplicate determinations. The significant difference between the values for peak A and those for peak B was determined for each effector compound using Student's *t* test (**P* < 0.02, ***P* < 0.01, ****P* < 0.005 and *****P* < 0.00001).

Effector compound	Percentage of control PTPase activity	
	Peak A	Peak B
Control	100 ± 4.9	100 ± 1.1
ZnCl ₂ (100 µM)	112 ± 2.9	86 ± 1.9*
CaCl ₂ (1 mM)	100 ± 2.4	61 ± 0.5***
NaF (1 mM)	96 ± 3.0	91 ± 8.9
EDTA (2 mM)	90 ± 1.5	83(± < 0.1)
EGTA (2 mM)	86 ± 3.6	67 ± 4.1
MnSO ₄ (2 mM)	67 ± 0.6	17 ± 3.6**
Hg(CH ₃ COO) ₂ (20 mM)	50 ± 0.6	22 ± 0.2****
PP _i (1 mM)	40 ± 1.2	31 ± 0.6
MgCl ₂ (10 mM)	30 ± 1.8	6 ± 0.5**
ATP (1 mM)	13(± < 0.1)	11 ± 1.0
NaVO ₃ (10 µM)	10 ± 3.0	3(± < 0.1)
ADP (1 mM)	9 ± 1.4	9(± < 0.1)

A to peak B were for angiotensin II 1.0:1.6, for band-III peptide 1.4:1.0, for MBP 1.0:1.1 and for RCM-lysozyme 1.0:1.4. Thus angiotensin and RCM-lysozyme were the best substrates tested for peak B and band III-peptide was the best substrate for peak A.

Effect of PTPase inhibitors and stimulators

Compounds previously shown to exert inhibitory or stimulatory influences on PTPases, including a number of bivalent metal ions, phosphonucleotides, metal chelators and pyrophosphate, were screened for their ability to modify peak-A and peak-B activities. ATP, ADP and sodium vanadate were found to inhibit both activities by at least 85% whereas ZnCl₂, CaCl₂, Hg(CH₃COO)₂, MnSO₄, NaVO₃ and MgCl₂ differentially affected the PTPase activity of peak A compared with peak B (Table 1). In most cases peak-B PTPase activity was more sensitive to the inhibitors than that of peak A. These results again suggest that the two peaks contain distinct PTPases.

Trypsin activation of PTPases

It was found that the PTPase activity of both peak A and peak B was enhanced by partial trypsin digestion (Figure 2). This was determined by first fractionating solubilized membrane material and then treating peak-A and peak-B material with trypsin. The enhancement of peak-A activity was transient with respect to the time of trypsin treatment, the activity of the digested material falling below that of the undigested starting material by 10 min of treatment. Trypsin digestion rapidly (within 30 s) enhanced the activity of peak-B material and the activity remained elevated after 10 min digestion. The total membrane preparation was treated with trypsin and then fractionated by gel filtration, and the PTPase activity profile determined. Digestion of solubilized membrane material for 10 min at 30 °C greatly reduced the PTPase activity eluted at the positions of peak A and peak B and gave rise to an active PTPase fragment eluted with the 43 kDa

Figure 2 Activation of PTPase activity by trypsin digestion

Solubilized membranes were fractionated by gel filtration as described in the Experimental section. Pooled peak-A and peak-B material as described in Figure 1 was digested with trypsin and portions of the reaction mixture were removed at the times indicated. The samples were assayed for PTPase activity using phosphorylated RCM-lysozyme as substrate. The assay was performed for 30 min at 37 °C. PTPase activity of peak A (○) and peak B (●) after trypsin digestion is shown.

Table 2 Effect of cell density on membrane and cytosolic PTPase activity

UMR 106.06 cells were harvested at low, medium or high cell density as described in the text. PTPase activity contained in the cytosolic and membrane fraction at each cell density was determined using MBP as the substrate. The values represent means $\pm$ S.E.M. ($n = 4$). Significant difference was determined by Student's t test ($*P < 0.001$). This experiment was repeated several times with similar results.

Cell density	PTPase activity (pmol/min per mg of cell protein)	
	Membrane	Cytosolic
Low	2410 $\pm$ 895	2610 $\pm$ 357
Medium	3020 $\pm$ 273	2960 $\pm$ 290
High	22470 $\pm$ 471*	5760 $\pm$ 279

molecular-mass standard that was not observed in the undigested material.

Effect of cell density on PTPases

Some transmembrane PTPases have extracellular structures that may be involved in cell-cell or cell-matrix contacts. It was therefore of interest to test whether the PTPase identified in UMR 106.06 cells was modulated by cell-cell contact at different cell densities. Cells were therefore subcultured at three cell densities, defined as low cell density (separate cells), medium cell density (separate cells and small clumps) and high cell density (confluence). The membrane and cytosolic fraction of each culture was prepared and PTPase activity determined. Membrane-associated PTPase activity in cells grown at high cell density was 9-fold higher than that in cells grown at low- or medium-cell density (Table 2). There was a small increase in

Figure 3 Gel-filtration profile of membrane PTPase activity of UMR 106.06 cells harvested at low and high cell density

The membrane fractions of UMR 106.06 cells harvested at high (●) and low (□) cell density were separated by gel filtration as described in the Experimental section. PTPase activity was determined using MBP as the substrate in a 15 min assay. The arrows indicate the elution positions of peaks A, B and C as described in Figure 1.

Table 3 Comparison of PTPase activities of UMR 106.06 cells grown on plastic or collagen matrix

UMR 106.06 cells were passaged on to type-I collagen matrix as described in the Experimental section and incubated for 2 days at low or high cell density. The cells were then harvested and the membrane fraction prepared as described in the Experimental section. PTPase activity of each membrane sample was determined using phosphorylated MBP as substrate. The PTPase assay was terminated after 2 min at a point at which dephosphorylation was linear with respect to time. The values represent means $\pm$ S.E.M. ($n = 3$). The significant difference between the cells plated on plastic and those plated on a collagen type-I matrix at the same cell density was determined by Student's t test ($*P < 0.01$). This experiment was repeated several times with similar results.

Cell density	PTPase activity (pmol/min per mg of cell protein)	
	Plastic	Collagen
Low	2410 $\pm$ 471	12000 $\pm$ 1730*
High	22470 $\pm$ 895	21400 $\pm$ 1100

PTPase specific activity of the cytosolic fraction prepared from cells grown at high cell density.

Gel-filtration profiles of PTPases prepared from UMR 106.06 cells harvested at low and high densities were compared. Consistent with the results shown in Figure 1, the gel-filtration profile of cells harvested at high cell density displayed both peak-A and peak-B PTPase activities (Figure 3). This was strikingly different from the gel-filtration profile of PTPase activity in cells harvested at low cell density; there was no significant PTPase activity at the elution position of peak A.

Effect of a type-I collagen substrate on PTPase activity

In view of the effect of cell density on PTPase activity, it was of interest to investigate possible effects of cell-matrix contacts on UMR 106.06 cell PTPase activity. As growth on type-I collagen had already been shown to modify the behaviour of these cells (Ikeda et al., 1993), this substrate was chosen for the studies described here. Cells were cultured at low or high cell density on an exogenous type-I collagen matrix. Membrane-associated PTPase activity in cells harvested from the collagen matrix at low

Figure 4 Gel chromatography of UMR 106.06 cells harvested from type-I collagen matrix

UMR 106.06 cells were passaged on to a collagen type-I matrix and incubated for 2 days at low or high cell density. The cells were then harvested and the membrane fraction prepared as described in the Experimental section. The solubilized membrane fraction from cells harvested from collagen (○) or plastic (■) at low cell density was fractionated by gel filtration and the PTPase activity profile determined using phosphorylated MBP as the substrate in a 10 min assay. The arrows indicate elution of peak A and peak B as designated in Figure 1.

cell density was 4-fold higher than that in cells cultured at the same density on plastic (Table 3). However, this enzyme activity in cells grown on collagen type I at high cell density did not differ significantly from that in cells harvested from plastic at high cell density. Cytosolic PTPase activity was not affected by growing the cells on a collagen type-I matrix.

The gel-filtration profile of membrane-associated PTPase activity from cells harvested at low cell density from a collagen type I matrix showed a dramatic increase in activity eluted in peak A (Figure 4). This suggested that the increased PTPase activity of cells grown on collagen could be largely accounted for by an increase in peak-A material.

Regulation of cellular PTPases

To investigate the regulation of cell-membrane-associated PTPases of UMR 106.06 cells, compounds that had either previously been shown to affect PTPases of other cell types or that were known to exert effects in UMR 106.06 cells were selected. Brief treatment with the protein kinase C activator, phorbol 12-myristate 13-acetate (PMA), was found to either increase or decrease PTPase activity in the membrane fraction depending on whether the cells were treated at low or high cell density (Table 4). In contrast, the potent phosphatase 1 and phosphatase 2B inhibitor, okadaic acid (OA), increased the membrane-associated PTPase activity of cells treated at low and high cell density. Transforming growth factor β (TGF β), NaF and ZnCl₂ were all found to diminish PTPase activity in the membrane fraction when cells were treated for 24 h, the latter two regardless of cell density. Interestingly, the decrease in membrane-associated PTPase activity after treatment with NaF, ZnCl₂ or TGF β was found to be due to a decrease in the abundance of both peak-A and peak-B PTPase activities (not shown). EGF was found to elevate specifically the membrane-associated PTPase activity of cells treated at low cell density. In contrast, parathyroid hormone (PTH) had an inhibitory effect on the membrane-associated PTPase activity of cells treated at low cell density only.

Table 4 Modulation of membrane-associated PTPase activity

Membrane-associated PTPase activity is expressed as percentage of control activity (mean $\pm$ S.E.M.; $n = 3$) and measured as described in the Experimental section. The concentration and time of each treatment (under cell culture conditions) is listed in the treatment column. The significant difference between the values for high and low cell density was determined using Student's *t* test (* $P < 0.05$ and ** $P < 0.001$).

Treatment	PTPase activity (% of control)	
	Low density	High density
PMA (100 nM; 10 min)	203 $\pm$ 7.6	45 $\pm$ 4.6**
OA (100 nM; 5 min)	216 $\pm$ 3.6	190 $\pm$ 6.7*
NaF (100 nM; 24 h)	39 $\pm$ 7.6	37 $\pm$ 2.1
ZnCl ₂ (100 nM; 24 h)	44 $\pm$ 3.7	31 $\pm$ 5.2
TGF β (1 ng/ml; 24 h)	87 $\pm$ 4.3	63 $\pm$ 6.4
PTH (300 nM; 30 min)	32 $\pm$ 9.0	108 $\pm$ 12.5**
EGF (300 nM; 30 min)	162 $\pm$ 16.0	106 $\pm$ 15.5*

DISCUSSION

UMR 106.06 cells are a well-characterized rat osteogenic sarcoma cell line (Forrest et al., 1985), with phenotypic features of both transformation and osteoblast lineage. In order better to understand the processes of these cells with respect to both aspects of their phenotype it was of interest to explore regulation of tyrosine phosphorylation.

There is growing evidence that tyrosine phosphorylation is involved in the regulation of a number of important cellular processes such as growth, movement, adhesion and oncogenic transformation (Hanks et al., 1992; Makgoba et al., 1992; Guan and Shalaway, 1992). The steady-state level of cellular tyrosine phosphorylation is obviously dependent on the balance of PTPase and tyrosine kinase activity. There has been considerable recent progress in the characterization of cellular PTPases. These molecules are now known to belong to a multimembered family comprised of non-receptor (cytosolic) and receptor-like (cell-membrane-spanning) forms (Fischer et al., 1991). PTPases share regions of homology including a well-conserved active domain. Comparatively little is known, however, about their regulation. In this study we have obtained evidence that UMR 106.06 cells express at least three PTPase activities, including two membrane-associated and one cytosolic form of apparent molecular mass > 153 kDa, 80 kDa and 40 kDa respectively, estimated by gel filtration. The cytosolic PTPase may correspond to a 53 kDa species described recently in neonatal rat osteoblasts and another transformed osteoblast-like cell line, ROS 17/2-8 (Titus et al., 1993). The two membrane-associated PTPases were characterized as distinct molecules by several criteria: they displayed different chromatographic behaviour on S-300 Sephacryl, had different substrate specificities and were differentially sensitive to inhibition by a number of compounds. In addition, peak-A and peak-B activities were differentially sensitive to partial tryptic proteolysis. The activity of peak-A material was enhanced by short exposure to trypsin but reduced thereafter; peak-B activity was enhanced in a sustained manner by tryptic digestion. Trypsin treatment may remove regulatory sequences from both species but prolonged tryptic digestion appears to disrupt peak-A activity, suggesting that the active site of this enzyme is vulnerable to tryptic cleavage whereas peak-B activity is not.

The studies presented here also show that integral membrane PTPases are responsive to a range of extracellular signals

produced by molecules such as TGF β , EGF and PTH. Since some of these molecules have been shown to modulate growth (Ng et al., 1983b), gene expression (Zhou et al., 1991) and signal transduction (Schneider et al., 1992) in UMR 106.06 cells, it is possible that some of these earlier findings may be mechanistically linked to PTPase activation or inhibition. For the present it remains a matter for speculation as to how agonists such as EGF or PTH might modulate PTPase activity, although in each case it is likely that phosphorylation pathways are involved. Sequence analysis of CD45 and other membrane-associated PTPases reveals that these proteins contain multiple potential phosphorylation sites (Thomas et al., 1985). Ostergaard and Trowbridge (1991) have presented evidence that CD45 is negatively regulated by serine dephosphorylation. Table 2 shows that treatment of UMR 106.06 cells with the serine/threonine phosphatase inhibitor OA elevated the PTPase activity of the membrane fraction, indicating that serine/threonine phosphorylation does play a role in the regulation of membrane PTPases in these cells.

Extracellular matrix molecules have been shown to influence cell migration, proliferation and differentiation (Hay, 1984; McDonald, 1989; Bixby, 1989; Ingber, 1991). There is emerging evidence that cells of the osteoblast lineage can respond to the extracellular matrix proteins of bone. Recent studies from this laboratory have found that UMR 201 rat osteoblastic cells grown on type-I collagen display significantly different basal and retinoic acid-stimulated expression of typical osteoblast gene products (Traianedes et al., 1993). UMR 106.06 cells grown on collagen display different calcitonin-receptor-mediated signal transduction from cells grown on plastic (Ikeda et al., 1993). The experiments shown here extend these findings and show modulation of the activity of membrane PTPases by growth of UMR 106.06 cells on type-I collagen. The observed increase in PTPase activity was possibly due to direct activation of membrane PTPases via the extracellular domain of these molecules with extracellular matrix although such an interaction has not previously been documented. As osteoblasts express among others the $\alpha_2\beta_1$ integrin which binds to type-I collagen (Hynes, 1992), it is also possible that PTPase activation is integrin-mediated. The increase in PTPase activity seen in cells grown on collagen was largely accounted for by an increase in activity eluted at the position of peak A. There was also a specific activation of peak-B activity when cells were grown at high cell density compared with low cell density. UMR 106.06 cells growing at high cell density had 9-fold more PTPase activity than cells at low cell density. This is consistent with work with A431 cells in which high cell density reduced the net amount of cellular tyrosine phosphorylation; this reduction appeared to be due to an increase in PTPase activity in cells rather than a decrease in tyrosine kinase activity (Mansbridge et al., 1992). Pallen and Tong (1991) also observed elevated PTPase activity in density-dependent growth-arrested fibroblasts. There is recent evidence that the extracellular domain of membrane-spanning PTPases is capable of mediating cell-cell contact. Transfection of cells with the RPTP μ produces cell clumping in otherwise non-interacting cells (Gebbinck et al., 1993). As yet the implications of this for RPTP μ activity are not known.

The present study therefore shows convergence of signals of various types, including hormonal, cell-substrate and cell-cell interaction on PTPase activity and underlines the importance of this class of PTPases in cell function. Determination of the mechanisms of PTPase regulation and the significance for cells of the osteoblast lineage are the basis of ongoing studies.

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