Regulation of phosphate (Pi) transport and NaPi-III transporter (Pit-1) mRNA in rat osteoblasts

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Abstract

In osteoblasts only the type III Na⁺-dependent phosphate (NaPi) transporter isoforms Pit-1 and Pit-2 have been identified. We tested the effects of extracellular Pi, Ca²⁺ and IGF-I on Na_dPi transport and Pit-1 or Pit-2 mRNA expression in rat osteoblastic (PyMS) cells. The $v_{\max}$ of Na_dPi transport was higher in cells kept in Pi-free, serum-free medium for 24 h than in controls at 1 mM Pi (2.47 ± 0.20 vs 1.83 ± 0.17 nmol/mg protein $\times$ 10 min). The apparent affinity constant (K_M) for Pi remained unchanged. Pi withdrawal for 24 h did not impair cell viability whereas increasing the extracellular Pi to 5 mM

resulted in cell death. Pit-1 (but not Pit-2) mRNA was upregulated following Pi deprivation, Ca²⁺ treatment or after treatment with 1 nM IGF-I, known to stimulate Na_dPi transport and cell proliferation. IGF-I also stimulated Na_dPi transport and Pit-1 mRNA in primary rat calvarial osteoblasts. Expression of Pit-1 mRNA *in vivo* and the coordinate regulation of Pit-1 mRNA and Pi transport in osteoblastic cells suggest that Pit-1 is a candidate transporter of physiological relevance in bone.

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Introduction

Phosphate (Pi) is essential for all living cells and plays a central role in bone mineralization where its local availability is a prerequisite for normal hydroxyapatite (crystal) formation. In this context, its facilitated transport across the kidney tubule is necessary to maintain adequate circulating Pi levels. Homeostasis of extracellular Pi is achieved primarily by its reabsorption in the proximal kidney tubule through the hormone- and phosphate-sensitive type II Na⁺-dependent phosphate (NaPi) transporter (Tenenhouse 1997). Inefficient Pi reabsorption by the kidney is associated with severe hypophosphatemia and defective mineralization of the skeleton, which manifests itself as rickets in children and in osteomalacia in adults (Beck *et al.* 1998).

The most recently described family of NaPi transporters (type III) was originally identified as retroviral receptors for gibbon ape leukemia virus Glvr-1 (O'Hara *et al.* 1990) and for rat amphotropic virus Ram-1 (O'Hara *et al.* 1990, Miller *et al.* 1994, Olah *et al.* 1994, van Zeijl *et al.* 1994). A slight sequence similarity with a putative phosphate permease from *Neurospora crassa* suggested that these membrane proteins might exhibit Pi transport activity (Kavanaugh *et al.* 1994). Indeed, both receptors, human Glvr-1 and rat Ram-1, were found to mediate Pi transport in a Na⁺-dependent manner after expression in *Xenopus*

laevis oocytes. Referring to the newly discovered functional characteristics of these viral receptors, Glvr-1 and Ram-1 were termed Pit-1 and Pit-2 respectively (Kavanaugh & Kabat 1996). Pit-1 and Pit-2 belong to the SLC20 family of solute carriers (Collins *et al.* 2004).

Several features distinguish type III NaPi transporters from their more extensively studied type I and II counterparts. In contrast to the type I liver/kidney- and the type II kidney-specific NaPi transporters, Pit-1 and Pit-2 are widely expressed in mammalian tissues (Boyer *et al.* 1998, Overbaugh *et al.* 2001). Even though Pit-1 and Pit-2 exhibit Pi affinities of the same order of magnitude as the kidney-specific transporters, they display no obvious sequence similarity to the latter (Murer & Biber 1996, Werner *et al.* 1998). Pit-1 and Pit-2 play a fundamental housekeeping role in Pi transport, such as absorption of Pi from interstitial fluid to maintain normal cellular functions (metabolism, signal transduction, and nucleic acid and lipid synthesis), whereas type II transporters are responsible for transepithelial Pi reabsorption in the kidney (Murer & Biber 1996). Another distinguishing feature is that type III transport is specifically blocked following infection with the appropriate virus. The loss of type III transporter-mediated Pi transport does not compromise the viability of the infected cell, suggesting that additional housekeeping Pi transporters may exist that are capable of compensating for the loss of Pit-1- and Pit-2-mediated Pi uptake

(Kavanaugh *et al.* 1994). In contrast, the loss of either type I or II Pi transporters in the kidney has severe metabolic consequences (Beck *et al.* 1998, Tenenhouse *et al.* 1998, Hoag *et al.* 1999, Gupta *et al.* 2001).

Insulin-like growth factor-I (IGF-I) has been shown to stimulate NaPi-II-mediated Pi transport in an opossum kidney cell line (Jehle *et al.* 1998). Moreover, IGF-I increases Pit-1 mRNA expression and Pi uptake in a human osteosarcoma cell line (Palmer *et al.* 1997, Veldman & Schmid 1998). In contrast to the NaPi transporter kidney isoforms, NaPi transporter species expressed in bone have not been extensively studied. Therefore, apart from assessing tissue-specific and age-dependent mRNA expression of NaPi isoforms in newborn and adult rats, we investigated their regulation by extracellular Pi deprivation and by Ca^{2+} and IGF-I in bone-derived rat cells *in vitro*.

Our data show upregulation of Pit-1 (but not Pit-2) mRNA expression and enhanced Pi transport in osteoblastic cells in response to Pi depletion or following treatment with Ca^{2+} or IGF-I.

Materials and Methods

Animals

All animal experiments were approved by the Institutional Animal Welfare Committee. Brain, calvarial bone, heart, lung, liver, kidney and intestine from newborn (1 day old) or adult male (150–160 g) rats (Wistar; Harlan, Horst, The Netherlands) were excised, blotted on filter paper, weighed, immediately frozen in liquid nitrogen and stored at -80°C until RNA was isolated.

Cell cultures

PyMS osteoblast-like cells were kindly provided by Dr A Lichtler (University of Connecticut) (Veldman *et al.* 1997, 1998). The cells were passaged in tissue culture flasks (Falcon, Meyland Cedex, France) in Dulbecco's modified Eagle's medium (DMEM) supplemented with gentamicin (50 µg/ml), glutamine (2 mmol/l), and fetal calf serum (FCS; 5%; all from Gibco) at 37°C in an atmosphere of 5% CO_2 in air. Cell cultures between passages 8 and 23 were used.

Calvaria of newborn rats were sequentially digested with bacterial collagenase (type CLS II, Worthington, Freehold, NJ, USA) in PBS and the released cells were grown as described previously (Veldman *et al.* 1997).

Both cell types were grown to confluence and detached from the dishes with 0.25% trypsin (Gibco) and replated in multiwell tissue culture plates (Falcon, 35 mm diameter) at a density of 2×10^5 cells/well. Confluent monolayer cultures were used 4 days after seeding. Twenty-four hours before studying Pi transport and Pi transporter

mRNA expression, cells were rinsed with serum-free medium, and the growth medium was replaced by serum-free F-12 medium containing gentamicin (50 µg/ml), glutamine (2 mmol/l) and charcoal-treated BSA (Serva, Heidelberg, Germany) at 1 g/l. For the Pi deprivation studies, Pi-free DMEM medium ($[\text{Ca}^{2+}]$ 1.8 mM) was used instead of F-12; for the Ca^{2+} -treatment experiments, F-12 medium ($[\text{Pi}]$ 1 mM, $[\text{Ca}^{2+}]$ 0.3 mM) was used. Aliquots of test agents (NaH_2PO_4 (1 mM), recombinant human (rh) IGF-I (1 nM; Ciba-Geigy, Basel, Switzerland), CaCl_2 and phosphonoformic acid (PFA; foscarnet 0.5 mM, a blocker of Pi transport through the plasma membrane; Sigma) were added directly to the media. Cells were incubated as specified in the Results.

For RNA analysis, cells were plated at a density of 10^6 cells/10 cm diameter dish (Falcon) in DMEM containing 5% FCS. Confluent monolayer cultures were used 4 days after seeding. Cells were rinsed with serum-free medium and exposed to test media ($\pm$ Pi, $\pm$ 1 nM IGF-I, $\pm$ 0.25 or 1 mM additional CaCl_2) for 24 h as described above, then rinsed with ice-cold PBS and lysed in ice-cold 4 M guanidine isothiocyanate containing 5 mM sodium citrate (pH 7.0), 0.1 M β -mercaptoethanol and 0.5% sarcosine.

RNA isolation

Calvarial bones were quickly removed and placed in Petri dishes containing SET solution (1% SDS, 5 mM EDTA, 10 mM Tris, pH 7.5). They were cleaned of adherent tissue including the periosteum, and the edges were cut (Zoidis *et al.* 2000). The pieces of bone and the other frozen tissues (0.5–1 g) were homogenized in a Polytron homogenizer (Brinkmann Instruments, Westbury, NY, USA) in 3 ml ice-cold 4 M guanidine isothiocyanate containing 5 mM sodium citrate (pH 7.0), 0.1 M β -mercaptoethanol and 0.5% sarcosine. The homogenates were centrifuged at 500 g for 5 min at 4°C and filtered through a Millex-HA filter unit (0.45 µm pore size, Millipore, Bedford, MA, USA) (Zoidis *et al.* 2000).

Total RNA from bone, osteoblast-like cells (as described above) and all examined tissues was obtained by high-speed sedimentation through a cesium chloride cushion. Concentrations were determined spectrophotometrically ($1 \text{ OD}_{260 \text{ nm}} = 40 \text{ mg/ml RNA}$), and stored at -80°C until assayed (Zoidis *et al.* 2002).

Northern-blot analysis

Denatured total RNA (20 µg) from tissues and cells was electrophoresed on a 1% agarose gel (stained with ethidium bromide) containing 2 M formaldehyde, transferred onto a nylon membrane (Hybond-N, Amersham) by capillary blotting, and fixed by u.v. cross-linking according to standard procedures (Sambrook *et al.* 1989). Prehybridization and hybridization were carried out at

Table 1 Oligonucleotide primers used in this study

Name	Strand	Primer sequence	Position ^a	Species ^b	GenBank acc. no.
NaPi-I-f	Sense	GGACCATTTATTGCCCTGCTTG	578	Rat	U28504
NaPi-I-r	Antisense	CTGACATCTGACCTGCTACG	1004	Rat	U28504
BNPI	Sense	CTCGCCGCTACATCATC	302	Rat	U07609
BNPI	Antisense	ACACTTCTCCTCGCTCATCT	1647	Rat	U07609
NaPi-IIa-f	Sense	CCGTTGATGCTAGGCTTCCTTAC	366	Rat	L13257
NaPi-IIa-r	Antisense	GATGACATTGGCCACTTGGCCC	1202	Rat	L13257
NaPi-IIb-f	Sense	TGTCCATGACTTCTTCAACT	624	Rat	AF157026
NaPi-IIb-r	Antisense	AATGGCAGTCGTGGTGGTGCG	1421	Rat	AF157026
Pit-2-f	Sense	TGCCCTGCTGTTCCGCTTCT	935	Rat	L19931
Pit-2-r	Antisense	CATTTCCACGCCGCCCTCTT	1646	Rat	L19931
Pit-1-1A	Sense	CCCTTATCGTCTGGTCTCTC	830	Rat	AB000489
Pit-1-2A	Sense	TGTTACTGCTTCTGCTCCAC	133	Rat	AB000489
Pit-1-2B	Antisense	ATTTCTGCTCCCTTTTGTC	1431	Rat	AB000489
Pit-1-SP1	Antisense	CACACAGCAGAACCAAC	450	Rat	AB000489
Pit-1-SP2	Antisense	TCCGGATGGTTTCACTAC	362	Rat	AB000489
Pit-1-SP3	Antisense	CACGGAGAATGCCAAGACAA	320	Rat	AB000489
Pit-1-SP5	Sense	GGCACTCTGCTCCCTGTAC	2597	Rat	AB000489
Pit-1-SP6	Sense	AAGCACATCATTCTGCCGGTGTG	2114	Rat	AB000489
Pit-1-SP7	Sense	AGCGTGCACAGGGATTAAACAAC	2530	Rat	AB000489
Pit-1-SP8	Sense	TAGAGGGCCGAGAATCTTTGGCAC	2651	Rat	AB000489
Pit-1-SP1B	Antisense	GGCTCTGCTTGGCTGT	2483	Rat	AB000489
GADPH U2	Sense	AAGGCTGAGAAATGGG	205	Rat	M17701
GADPH L1	Antisense	ACCCTGTTGCTGTAGCC	995	Rat	M17701

^aThe indicated numbers refer to the nucleotide position within the respective sequence where the 5' end of the primer will anneal.

^bSpecies from which the primers were designed.

f, forward; r, reverse.

42 °C in a hybridization incubator (Appligene Inc., CA, USA). Membranes were prehybridized in a solution containing 50% (v/v) deionized formamide, 5 × Denhardt's solution (0.02% (w/v) Ficoll and 0.02% (w/v) polyvinyl pyrrolidone), 5 × SSPE (20 × = 3.6 M NaCl, 0.2 M sodium phosphate, 0.02 M EDTA, pH 7.7), 0.2% SDS and 100 µg/ml heat-denatured salmon sperm DNA. Hybridizations were performed in the same solution with 20–25 ng (~2 × 10⁷ c.p.m.) labeled cDNA/membrane. The following cDNAs (made by reverse transcription (RT)-PCR in our laboratory) were used for hybridization (Table 1): rat type I sodium-dependent (Na_d) phosphate (Pi) transporter (NaPi-I) cDNA (nucleotide 578–1004, GenBank accession no. U28504); rat brain-specific NaPi-I (BNPI) cDNA (nucleotide 302–1647, GenBank accession no. U07609); rat NaPi-IIa cDNA (nucleotide 366–1202, GenBank accession no. L13257) (Magagnin *et al.* 1993) rat NaPi-IIb cDNA (nucleotide 624–1421, GenBank accession no. AF157026) (Hashimoto *et al.* 2000); three distinct rat Pit-1 cDNAs (nucleotides 133–1431, 133–450 and 2114–2483, GenBank accession nos AB000489 and L20852); rat Pit-2 cDNA (nucleotide 935–1646, GenBank accession no. L19931). The cDNA probes were labeled by random primer extension using a commercial kit (Boehringer Mannheim) and [α -³²P]deoxy-CTP (3000 Ci/mmol; Amersham) to specific activities of 2–4 × 10⁹ c.p.m./µg

DNA. After 48 h of incubation at 42 °C, the membranes were washed twice for 15 min at room temperature in 2 × SSPE–0.1% SDS and then twice for 10 min in 0.1% SSC–0.1% SDS at 49–55 °C. Membranes were then exposed at –80 °C to an X-Omat AR-5 film (Kodak, Rochester, NY, USA) in cassettes equipped with intensifying screens to visualize ³²P-labeled cDNA–mRNA hybrids. mRNA levels were quantitated by scanning densitometry using a BioRad video densitometer. Variations of gel loading were corrected against 18S ribosomal RNA values.

Transport studies

Confluent PyMS cells were kept in Pi-free or 1 mM Pi-containing, serum-free medium for 24 h before determination of the initial rate of Pi uptake (initiated by adding 1 ml of transport buffer, containing ³²PO₄) in buffer containing 0.02–10 mM KH₂PO₄. Except for the kinetic data presented in Table 2, transport studies were conducted at Pi concentrations of 1 mM, i.e. a Pi concentration close to the physiological extracellular Pi concentration. Before the start of the experiments, monolayers were washed (and left for 20 min) at room temperature with 1 ml transport buffer A or B containing (in mmol/l): 140 NaCl or choline chloride, 5 KCl, 1 MgCl₂, 1.5 CaCl₂, 15 N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid

Table 2 Protein content, alkaline phosphatase (ALP) activity, [³H]thymidine incorporation, Na⁺-dependent Pi transport, kinetic parameter and Pit-1 mRNA levels of PyMS cells exposed to 1 mM Pi-containing or Pi-free DMEM for 24 h

Parameter	Pi concentration of medium	
	1 mM	0 mM
Protein content (µg/dish) (n=6)	109.4 ± 6.7	108.3 ± 6.8
ALP activity (µmol/mg protein × h) (n=6)	2.54 ± 0.40	2.63 ± 0.43
[³ H]-thymidine incorporation (c.p.m./dish × 3 h) (n=4)	674 ± 84	611 ± 69
Na ⁺ -Pi transport (pmol Pi/mg protein × 10 min) (n=6)	699 ± 31	819 ± 30*
K _M (µmol/l) (n=5)	109 ± 12	117 ± 9
v _{max} (nmol Pi/mg protein × 10 min) (n=5)	1.83 ± 0.17	2.47 ± 0.20*
Pit-1 mRNA (relative expression levels) (n=4)	1 ± 0.05	1.7 ± 0.06*

Values represent the means ± S.E.M. from four to six separate experiments (n=4–6).

*P<0.05 vs 1 mM Pi.

(HEPES), adjusted to pH 7.4 with Tris-(hydroxymethyl)aminomethane HCl. Transport buffer B containing choline chloride instead of NaCl was used for measuring Na⁺-independent Pi uptake. The ³²Pi-uptake studies were performed at room temperature and initiated by adding 1 ml transport buffer, containing 0.1 mmol/l KH₂³²PO₄ (1 µCi/ml; 200 mCi/mmol; Amersham). Transport buffer contained, in addition, unlabeled KH₂PO₄ as indicated in the Results; 10 min later the buffer was removed, and the dishes were quickly rinsed three times with 1 ml ice-cold transport buffer. The cells were then solubilized with 1 ml of 2% SDS, and the radioactivity of a 0.5 ml aliquot was counted in a liquid scintillation counter (Betamatic, Kontron Instruments AG, Zurich, Switzerland).

Determination of protein content and alkaline phosphatase activity

After 4 days of culture, including 24 h in test medium as described above, cells were lysed from dishes into 2 ml of 0.1% Triton X-100 for the determination of protein content by the bicinchoninic acid (BCA) method (Smith *et al.* 1985) and of alkaline phosphatase activity by measuring the cleavage of *p*-nitrophenyl phosphate to *p*-nitrophenol at pH 10.2 using the Sigma Fast kit (Veldman *et al.* 1998).

[³H]thymidine incorporation into DNA

Cells were plated and grown as above, but exposed to DMEM (containing 3 µM thymidine, different Pi concentrations and/or PFA as indicated) for 18 h, then pulsed with methyl-[³H]thymidine (Amersham; 80 Ci/mmol; 1 µCi/dish) for 3 h at 37 °C, rinsed with cold PBS, and DNA was precipitated and washed three times *in situ* with

10% trichloroacetic acid; incorporated radioactivity was measured in a liquid scintillation counter.

Statistical analysis

Results are expressed as means ± S.E.M. or S.D. as indicated, and are representative of at least three independent experiments. Statistical analysis was performed by Student's *t*-test or ANOVA, with *P*<0.05 considered statistically significant.

Results

Age-dependent and tissue-specific Pit-1 and Pit-2 mRNA expression

Northern-blot analysis showed a wide but distinct tissue distribution of Pit-1 (4.3 kb) and Pit-2 (4.1 kb) mRNA (Figs 1 and 2). Pit-1 mRNA expression was highest in newborn brain followed by newborn heart, lung, calvaria and kidney (Fig. 1). Only in the intestine was Pit-1 mRNA expression higher in adult than in newborn rats (Fig. 1). Pit-2 mRNA expression was also higher in newborn than in adult animals (Fig. 2) and was most prominent in the heart. In contrast to Pit-1, Pit-2 mRNA expression was low in newborn and adult intestine. Adult liver showed 2-fold higher expression than newborn liver (Fig. 2).

We also performed hybridization analysis with rat cDNA probes (cDNA primer sequences and annealing position shown in Table 1) for the NaPi-I (specific for liver–kidney), NaPi-II (a, specific for kidney; b, specific for gut–lung) and for the rat BNPI isoforms. While specific transcripts of the expected size were detected in the corresponding tissues (1.8 and 2.3 kb NaPi-I transcripts in liver and kidney, 2.7 kb NaPi-II

Figure 1 Tissue-specific and age-dependent expression of Pit-1 mRNA. Upper panel: expression levels of Pit-1 mRNA (OD values, means $\pm$ S.E.M.) in newborn and adult rat tissues normalized for equal loading (18 S ribosomal RNA values) and quantified by scanning densitometry. Lower panel: 20 μ g total RNA extracted from various newborn and adult rat tissues was separated on a 1% agarose formaldehyde gel. The gel was stained with ethidium bromide to test for equal loading, blotted on a nylon membrane and hybridized with a [32 P], 1.3 kb (nucleotide 133–1431) rat Pit-1 probe. The 4.3 kb Pit-1 signals were detected by autoradiography as described in Materials and Methods. Exposure time was 3 days.

transcripts in kidney 4.4 kb NaPi-IIa transcripts in lung and intestine, and 2.8 kb BNPI transcripts in brain), none of these transcripts were detected in bone and osteoblastic cells (not shown).

An additional 6 kb transcript of Pit-1 was found in calvarial bones of newborn animals (Fig. 1, lower panel). After longer exposure (7–10 days), this transcript was also detectable in the lung of newborn rats and in PyMS cells. However, our attempts to obtain the full-length transcript (consistently detected with three different cDNA probes: nucleotides 133–1431, 133–450 and 2114–2483; see primer sequences and annealing position in Table 1) by using rapid amplification of cDNA ends (RACE) and a variety of 5'- and 3'-end primers (Table 1) were unsuccessful.

Effects of Pi deprivation and increased Pi concentrations on protein levels, alkaline phosphatase activity, [3 H]thymidine incorporation, Na_dPi transport and kinetic parameters

Pi deprivation for 24 h in serum-free medium did not affect protein content, alkaline phosphatase activity and [3 H]thymidine incorporation rates of PyMS cells (Table 2), suggesting no detrimental effects on cell viability under these conditions, i.e. when the proliferation rate of these cells is low.

As illustrated in Table 2, Na_dPi transport was significantly decreased when 1 mM Pi was added to the Pi-free medium for the last 24 h of culture, reflecting an increase in Na_dPi transport when cells are switched to and kept in Pi-free medium for the last 24 h of culture (Table 2).

The kinetic parameters of the Na_dPi transport system were obtained by Lineweaver–Burk analysis from experiments where Pi transport was studied over a range of Pi concentrations from 0.02 to 10 mM (not shown). Data from five pairs of curves are combined in Table 2 for calculation of the apparent affinity constant (K_M) and maximal transport rate (v_{max}). The stimulatory effect of Pi depletion was due to an increase in v_{max} from 1.83 ± 0.17 to 2.47 ± 0.20 nmol Pi/mg protein $\times$ 10 min ($P < 0.05$) without a significant change in the apparent K_M for Pi (109 ± 12 vs 117 ± 9 μ mol/l) (Table 2).

32 Pi uptake in cells kept for 24 h at 5 or 10 mM Pi was even higher in choline- than in sodium-containing buffer (not shown) and, thus, was no longer Na⁺-dependent probably due to leakage of damaged cell membranes. In contrast to Pi deprivation, 5 and 10 mM Pi resulted in a large number of detached cells and was obviously toxic. Consistent with the toxic effect of high Pi, protein content per dish was decreased and thymidine incorporation into DNA was nearly abolished. This was prevented by incubation with 0.5 mM PFA, a specific

Figure 2 Tissue-specific and age-dependent expression of Pit-2 mRNA. Upper panel: expression levels of Pit-2 mRNA (OD values, means $\pm$ S.E.M.) in newborn and adult rat tissues normalized for equal loading (18 S ribosomal RNA values) and quantified by scanning densitometry. Lower panel: 20 μ g of total RNA was processed as described in Fig. 1 and hybridized with a [32 P], 0.7 kb (nucleotide 935–1646) rat Pit-2 probe. The 4.1 kb Pit-2 signals were detected by autoradiography as described in Materials and Methods. Exposure time was 4 days.

competitive and reversible inhibitor of Na_dPi transport (not shown).

Effects of Pi deprivation, and of IGF-I and Ca^{2+} on Pit-1 mRNA expression in PyMS cells

PyMS cells express Pit-1 transcripts of the expected size of 4.3 kb (Fig. 3, lower panel). Quantitative evaluation of the 4.3 kb Pit-1 mRNA levels showed decreased Pit-1 mRNA when Pi concentration was increased (as compared with the Pi-free control). The experiments reflect an increase in Pit-1 mRNA levels after Pi withdrawal by a mean of 40 and 70% ($P < 0.05$) relative to the expression in cells kept at 0.5 and 1 mM Pi respectively (Fig. 3, upper panel, black columns).

IGF-I (1 nM) stimulated Pit-1 mRNA expression to a higher extent in Pi-free media than in media containing 1 mM Pi. Pit-2 mRNA expression levels were not affected (not shown). Stimulation of Pit-1 mRNA expression by IGF-I was 1.65 ± 0.2 , 1.8 ± 0.2 and 1.3 ± 0.1 -fold at 0, 0.5 and 1 mM Pi (Fig. 3, upper panel, gray columns) as compared with the corresponding IGF-I-free media (black columns).

Increasing the Ca^{2+} concentration by 1 mM to a final concentration of 1.3 mM stimulated Pit-1 (Fig. 4), but not Pit-2 mRNA levels (not shown). We have previously shown that increasing extracellular Ca^{2+} and

IGF-I stimulate Na_dPi transport and [^3H]thymidine incorporation in PyMS cells in a dose-dependent manner. Ca^{2+} and IGF-I have qualitatively similar effects in primary rat calvarial cells on DNA synthesis and Na_dPi transport, but higher concentrations of IGF-I than in PyMS cells are required for a significant effect (Veldman *et al.* 1997, Schmid *et al.* 1998). IGF-I (10 nM) also stimulated the expression of Pit-1 mRNA in calvarial cells (Fig. 5).

Discussion

In this study we demonstrate age-dependent and tissue-specific expression of Pit-1 and Pit-2 (type III NaPi) mRNA and upregulation of Pit-1 but not Pit-2 mRNA expression by Pi depletion, or by treatment with Ca^{2+} or IGF-I. The non-malignant rat cell line PyMS was used since these osteoblastic cells have been well characterized regarding IGF-I- and Ca^{2+} -stimulated Pi transport (Veldman *et al.* 1997, 1998, Schmid *et al.* 1998). For all tissues examined, except intestine, NaPi-III mRNA levels were higher in newborn than in adult rats. This is consistent with increased Pi requirement in the rapidly growing organism and suggests a role of type III Na^+ -dependent Pi transporters in Pi retention during this highly anabolic phase. In contrast to humans, rats keep

Figure 3 Pit-1 mRNA expression in PyMS cells exposed for 24 h to Pi-free, 0.5 mM Pi- or 1 mM Pi-containing medium, with or without addition of 1 nM IGF-I. Cells were switched to Pi-free DMEM (no Pi) supplemented with 0.5 and 1 mM Pi and 1 nM IGF-I as indicated. Upper panel: expression levels of Pit-1 mRNA (OD values) in PyMS cells, normalized for equal loading (^{18}S ribosomal RNA values) and quantified by scanning densitometry (means $\pm$ S.D., $n=4$ experiments). $^*P<0.05$ vs complete Pi deprivation (no Pi), $^{\#}P<0.05$ vs control (without IGF-I). Lower panel: 20 μg total RNA was processed as described in Fig. 1 and hybridized with a [^{32}P], 1.3 kb (nucleotide 133–1431) rat Pit-1 probe. The 4.3 kb Pit-1 signals were detected by autoradiography as described in Materials and Methods. Exposure time was 2 days.

growing throughout life, their epiphyses remain open, and their serum phosphate remains high after sexual maturation. In adult rats, Pit-1 mRNA expression is most abundant in tissues rich in highly polarized epithelial cells (intestine and lungs) which also express NaPi IIb (Hilfiker *et al.* 1998, Feild *et al.* 1999, Xu *et al.* 1999, Hashimoto *et al.* 2000, Zoidis *et al.* unpublished observations). In contrast, Pit-2 mRNA expression is highest in heart, which has a high Pi requirement for energy metabolism. Kavanaugh *et al.* (1994) also demonstrated a wide tissue distribution of Pit-1 (with highest mRNA levels in bone marrow) and Pit-2 (with highest mRNA levels in heart), consistent with their roles in mediating blood-borne targeting of viral infections to multiple tissues, but in their study neither bone nor osteoblasts were examined.

Investigations by Jono *et al.* (2000) in human aortic smooth muscle cells pointed to a role of Pit-1 in mediating matrix mineralization. This is also in line with the findings of Nielsen *et al.* (2001) that during differentiation of mouse osteoblastic MC3T3-E1 cells, which is accompanied by increased mineralization, Pit-1 is higher than Pit-2 mRNA expression. Nevertheless, bone and osteoblastic cells do not express Pit-1 and Pit-2 at higher levels than other tissues and cell types. Pit-1 mRNA has also been identified in human osteosarcoma cells (Chien *et al.* 1997, Palmer *et al.* 1997, Zoidis *et al.* unpublished observations). Kinetic analysis of Pi transport revealed a rather high K_M of 454 $\mu\text{mol/l}$ in the osteosarcoma cell study by Palmer

et al. (1997) and a K_M of 137 $\mu\text{mol/l}$ in the study by Veldman & Schmid (1998), i.e. a value which is close to that found in the current study for PyMS cells (Table 2). The K_M for Pi in PyMS cells (109 $\mu\text{mol/l}$) is close to the K_M reported for fibroblasts, 133 $\mu\text{mol/l}$ (O'Hara *et al.* 1990), but higher than the K_M for Pi when human Pit-1 is expressed in *Xenopus* oocytes, 24.1 $\mu\text{mol/l}$ (Kavanaugh *et al.* 1994). Pit-1 appears to be expressed at high levels also by most tumor cells and may therefore serve to cover the particular requirement for rapid growth, as also described for glucose transporter (GLUT)-1 mediated glucose uptake. In an *in situ* hybridization study in mice, Palmer *et al.* (1999) found Pit-1 transcripts in chondrocytes but failed to identify Pit-1 transcripts in osteoblasts.

The occurrence of a 6 kb Pit-1-related transcript in bone and osteoblastic cells, which is absent in fibroblasts (these express only the 4.3 kb transcript), may point to a novel 'bone-specific' Pi transporter-related mRNA. Unfortunately, we could not obtain full-length products of the observed transcript size in our RACE experiments. Therefore, we do not know whether the 6 kb transcript represents an alternative splice variant or an unspliced precursor RNA, but it may be of interest to note that the age dependence and the restriction of the 6 kb transcript expression to bone and lung resembles that of Phex, an enzyme which plays an important role in the regulation of extracellular Pi (HYP Consortium 1995, Tenenhouse 1999, Drezner 2000).

Figure 4 Pit-1 mRNA expression in PyMS cells after 24 h treatment with CaCl₂. Cells were incubated in F-12 medium (containing 0.3 mM calcium, control) to which 0.25 and 1 mM calcium were added to yield final concentrations of 0.55 and 1.3 mM respectively. Upper panel: 20 µg total RNA was processed as described in Fig. 1 and hybridized with a [³²P], 1.3 kb (nucleotide 133–1431) rat Pit-1 probe. The 4.3 kb Pit-1 signals were detected by autoradiography as described in Materials and Methods. Exposure time to visualize signals was 3 days. Lower panel: expression levels of Pit-1 mRNA (OD values) in PyMS cells after treatment with CaCl₂, normalized for equal loading (¹⁸S ribosomal RNA signals) and quantified by scanning densitometry (means ± S.D., n=4 experiments). *P<0.05 vs control.

Increased Na₄Pi transport due to Pi deprivation was accompanied by increased Pit-1 (but not Pit-2) mRNA levels; this is in line with findings by Chien *et al.* (1997) in two human osteosarcoma cell lines. Obviously, Pi deprivation used for *in vitro* studies is not a physiological condition, and its *in vivo* significance is unclear. In the artificial setting of cultured cells, the Pi requirement may be different from that in osteoblasts *in vivo*. Moreover, there is no circulating Pi supplying extracellular milieu, and the contact time with the extracellular medium is markedly prolonged in the cultured cells. However, it is of note that our cells remained viable after the 24 h exposure to Pi-free medium as apparent from unchanged DNA synthesis, protein content and alkaline phosphatase activity. Pi uptake in the absence of extracellular Na⁺ was unaffected, suggesting that the change in Na₄Pi uptake cannot be accounted for by a non-specific increase in Pi diffusion. Enhanced Na₄Pi transport was due to an increase in the apparent $v_{\max}$ with no change in the apparent K_M for Pi. Raising the extracellular Pi

Figure 5 Pit-1 mRNA expression in calvaria and PyMS cells after 24 h treatment with IGF-I. Cells were exposed to F-12 medium to which IGF-I was added as indicated. Left panel: ethidium bromide staining. Right panel: hybridization with Pit-1 probe. Both calvaria and PyMS cells were treated with 10 nM IGF-I for 24 h; total RNA was prepared and loaded on a gel (18 µg in the case of calvaria cells, 20 µg in the case of PyMS cells) and processed as described in Fig. 1. One of two experiments (with comparable outcomes) is shown.

concentration to 5 and 10 mM induced cell death; this was prevented by incubation with 0.5 mM PFA (not shown), in agreement with observations in osteoblast-like cells isolated from human bone (Meleti *et al.* 2000).

Higher Pit-1 mRNA levels after Pi withdrawal were observed irrespective of additional treatment with IGF-I. This anabolic hormone stimulates Pi uptake rapidly in osteoblasts by increasing $v_{\max}$ (Veldman *et al.* 1997). The acute component of this stimulation is blocked by colchicine but not by actinomycin and cycloheximide (Campbell *et al.* 1992, Veldman & Schmid 1996). Longer-term stimulation of Na_dPi transport by IGF-I may be attributed to an increase in Pit-1 mRNA as also reported for osteosarcoma cells (Palmer *et al.* 1997, Zoidis *et al.* unpublished observations). Similar to IGF-I, increasing extracellular Ca^{2+} concentrations (starting from a basal medium with a low calcium concentration, lower than usually met *in vivo*) stimulated Pit-1 but not Pit-2 mRNA levels. Since Pit-1 expression was highest at the high (more physiological) Ca^{2+} concentration, one could also argue that lowering Ca^{2+} decreases Pit-1 expression.

IGF-I not only increases Na_dPi transport (Veldman *et al.* 1997), but also Pit-1 mRNA (Fig. 5) in 'normal' primary rat calvarial osteoblasts although these cells are less responsive to exogenous IGF-I, due to auto-/paracrine IGF-I production (Veldman *et al.* 1997, 1998, Schmid *et al.* 1998). Many active Pi transporters including some that have no role in transcellular Pi movement are found in several types of animal cells (Wehrle & Pedersen 1989). The tissue-specific Pi handling by osteoblasts is determined by the high Pi requirement of skeletal tissue at the time of mineralization. Pit-1 may well serve this function. Thus far, there is no evidence for an additional osteoblast-restricted NaPi isoform. IGF-I stimulates growth together with Phex and osteocalcin mRNA expression in calvarial bones of hypophysectomized rats (Zoidis *et al.* 2002) at a time when net transfer from gut to bone is increased, but serum Pi and renal NaPi-II mRNA expression remain unaffected. Therefore, we performed preliminary RT-PCR analysis of RNA samples isolated from calvarial bone of the vehicle- and the IGF-I-treated hypophysectomized rats of the study of Zoidis *et al.* (2002). We found that IGF-I treatment resulted in a 5-fold increase of relative Pit-1 mRNA expression (not shown). In conclusion, our data show regulation of Pit-1 expression in bone and suggest that Pit-1 is a candidate transporter of physiological relevance in bone.

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