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Type II Na^+-P_i Cotransporters in Osteoblast Mineral Formation: Regulation by Inorganic Phosphate

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Key Words
Osteoblast • Na^+-Pi • Cotransporter • Phosphate • Bone • Mineralization • NaPi-IIa • NaPi-IIb

Abstract
During calcification of bone, large amounts of phosphate (P_i) must be transported from the circulation to the osteoid. Likely candidates for osteoblast P_i transport are the type II sodium-phosphate cotransporters NaPi-IIa and NaPi-IIb that facilitate transcellular P_i flux in kidney and intestine, respectively. We have therefore determined the cotransporters' expression in osteoblast-like cells. We have also studied the cotransporters' regulation by P_i and during mineralization *in vitro*. Phosphate uptake and cotransporter protein expression was investigated at early, late and mineralizing culture stages of mouse (MC3T3-E1) and rat (UMR-106) osteoblast-like cells. Both NaPi-IIa and NaPi-IIb were expressed by both osteoblast-like cell lines. NaPi-IIa was upregulated in both cell lines one week after confluency. After 7 days in 3mM P_i NaPi-IIa was strongly upregulated in both cell lines. NaPi-IIb expression was unaffected by both culture stage and P_i supplementation. The expression of both cotransporters was unaffected by P_i deprivation. *In vitro* mineralization at 1.5mM P_i was preceded by a three-fold increase in osteoblast sodium-dependent P_i uptake and a corresponding upregulation of both NaPi-IIa and NaPi-IIb. Their expression thus seem regulated by phosphate in a manner consistent with their playing a role in transcellular P_i flux during mineralization.

Introduction

During bone calcification the constituent ions of hydroxyapatite, calcium and phosphate (P_i), must be translocated from the circulation to sites of mineral formation. In order for osteoblasts to regulate the rate of mineralization acccording to physiological need, it would be advantageous for this process to be under cellular control as opposed to free diffusion. Ion concentrations in the osteoid, the mineralizing space between osteoblasts and calcified bone, are elevated compared to the

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extracellular fluid, suggesting that ion transport is directional, energy-dependent and at least partly transcellular [1-4]. The transport of calcium to mineralizing bone is most likely performed by uptake through L-type calcium channels and efflux via sodium-calcium exchangers facing the osteoid space [3], and inhibition of both L-type calcium channels and Na^+-Ca^{2+} exchangers has been shown to diminish bone formation [2-3]. Additionally, both odontoblast-like cells and bone cells *in vivo* have been demonstrated to perform directional calcium transport [4, 5].

The pathway of phosphate to the osteoid has been much less studied than calcium transport. The osteoblasts have so far been shown to express the two type III cotransporters Pit-1 and Pit-2 [6-9]. Pit-1 has been more investigated and have been shown to be upregulated as osteoblast differentiation progresses and to be regulated by certain osteogenic factors such as BMP-2 [10]. However the expression of Pit-1 in native bone is weak and expression in osteoblasts *in vivo* could not be detected by in-situ hybridization [9]. In addition, characterization of osteoblast P_i uptake have shown that the Km for phosphate uptake is closer to what would be expected from the type II Na^+-P_i cotransporters and that osteoblast P_i uptake can be inhibited by phosphonoformic acid (PFA) [11-12]. PFA is a commonly used inhibitor of type II Na^+-P_i cotransporters but only inhibits type III cotransporters weakly (Pit-2 [13], Pit-1 [14] and unpublished, personal communication Dr Ian Forster). The type III cotransporters are ubiquitously expressed and thought to perform a housekeeping function in cellular P_i metabolism, but have not been implicated in transcellular P_i flux [11]. It is likely that they perform the same role in osteoblasts.

In other tissues performing transcellular phosphate transport, such as kidney and intestine, cellular P_i uptake is mediated by type II Na^+-P_i cotransporters of the SLC34 family [11-12]. NaPi-IIa and NaPi-IIc are found in the proximal tubule of the nephron, while P_i absorption in the small intestine is performed by NaPi-IIb [11-12]. NaPi-IIb is also involved in transcellular P_i flux in liver and salivary glands [15-16]. These cotransporters catalyze uptake of P_i into cells utilizing energy provided by the cotransport of two to three sodium ions [11-12]. The cotransporters in the kidney and intestine are up-regulated in hypophosphatemia and down-regulated by excess serum P_i (the latter leading to reduced P_i uptake in the intestine and increased P_i wasting in the kidney) permitting the organism to maintain P_i homeostasis [11-12, 17]. Their role in transcellular P_i flux make members of the type II Na^+-P_i cotransporter family prime candidates for osteoblastic transcellular P_i transport. These cotransporters (NaPi-IIa and NaPi-IIb) have also been found in mineral-forming odontoblasts, MRPC-1 odontoblast-like cells and ameloblasts and their coexpression seems to be a common feature of some mineral-forming cell types [18-19]. Chondrocytes and osteoclasts have also been shown to express NaPi-IIa while NaPi-IIb has not been studied in those cell types [20-21]. This contrasts with kidney and intestine, where either NaPi-IIa or NaPi-IIb is found alone [11-12].

In MRPC-1 odontoblast-like cells, P_i uptake and NaPi-IIa cotransporter protein are found to be upregulated prior to the formation of mineral *in vitro* [18]. We hypothesized that a similar mechanism would be employed by osteoblasts, the cotransporters being upregulated to facilitate mineral formation. An important role of the osteoblasts is to help maintain ion homeostasis by removing excess Ca^{2+} and P_i from the circulation to storage in bone. If type II cotransporters are involved in osteoblast P_i transport they may therefore be up-regulated in response to elevated serum P_i to increase P_i flux into bone and thus help to reduce serum P_i concentrations to its normal level. Equally, P_i starvation may have an opposite effect, and precipitate a down-regulation of osteoblast sodium-phosphate cotransporters involved in transcellular P_i flux to limit bone formation when the ions are scarce.

Our goal was to determine the expression of NaPi-IIa and NaPi-IIb in osteoblast-like cells from mouse (MC3T3-E1) and rat (UMR-106). Two independent cell lines from different species were used to ascertain that the findings were of general importance and not limited to a certain cell line. We examined the effects of increased and decreased phosphate levels on expression of type II Na^+-P_i cotransporters and P_i uptake activity of osteoblast-like cells. Expression of cotransporters before and during mineral formation *in vitro* was also assessed.

Materials and Methods

Cell culture

UMR-106.01 and MC3T3-E1.14 cells (ATCC, Manassas, VA, USA) were seeded in 12 well plates for P_i uptake experiments and von Kossa staining, or in 25cm^2 flasks for protein preparations (cell culture vessels from Corning Costar, Acton, MA, USA). Cells were grown in alpha Minimal Essential Medium (αMEM, Gibco, Invitrogen, Carlsbad, CA, USA) with the addition of 10% (v/v) fetal calf serum, 100U/ml penicillin, 100μg/ml streptomycin, 2mM L-glutamine and 50μg/ml ascorbic acid (Gibco). Medium was exchanged every second day of the

culture period and always the day before uptake measurements or protein preparation. In the case of mineralizing cultures, inorganic phosphate was initially supplemented to 3mM final concentration. As this gave abnormal P_i uptakes, phosphate was later kept at 1.5mM to promote mineralization of UMR-106 cultures while this experiment could not be performed with MC3T3-E1 cells, see discussion.

For P_i starvation cells were first grown to confluency and then switched to a chemically defined, P_i-free, DMEM-like, serum free medium with 1% (w/v) BSA [22]. Cells were allowed to adapt for two days to the new medium, supplemented with 1mM P_i, after which the cultures were changed to a P_i-free medium for 72h. Control cultures were maintained in the same medium supplemented with 1mM P_i. Opposum kidney cells (OK), the most studied system of NaPi-IIa regulation by P_i, were cultured as described previously and used as controls in high P_i, P_i starvation experiments and pyrophosphate (PP_i) inhibition studies [22-23].

Western blotting

Membrane fractions were isolated from cultures of osteoblast-like cells grown in 25cm^2 flasks using the method described by Pfister et. al. [24]. Isolations were done from at least three independent cultures when quantifying NaPi-IIa or NaPi-IIb expression in osteoblast-like cell lines. Protein concentration was determined using the DC Protein Assay (Bio-Rad, Hercules, CA, USA). Membranes were stored at -80°C. Mouse kidney brush border membranes and small intestinal membranes were used as positive controls for NaPi-IIa and NaPi-IIb, respectively [25-26]. 30µg of membrane protein (2µg of kidney brush border membranes, or 10µg of intestinal brush border membranes) were denatured at 97°C for 2min under non-reducing conditions, separated on 10% SDS-PAGE gels and transferred to nitrocellulose membranes. Na^+-P_i cotransporter proteins were detected by antibodies specific for N-terminal peptides of either NaPi-IIa or NaPi-IIb as described previously [27-28]. To verify the identity of bands detected, two independent antibodies directed against the C-termini of the cotransporters were also used in control experiments [27-28]. Bound antibody was visualized by chemiluminescence (Super Signal west Pico, Pierce, Rockford, IL, USA). Band intensities were normalized against β-actin detected by a mouse monoclonal anti β-actin antibody (Sigma, St. Louis, MO, USA, Cat. No. A-5316). For quantification, 6 independent protein isolates were used in each experiment. Peptide protection was performed for N-terminal specific antibodies by incubating the blots in the presence of 100µg/ml immunogenic peptide to block antibody binding to the transferred protein.

Immunostainings

The subcellular localization of NaPi-IIa and NaPi-IIb in cultured cells grown on coverslips were determined by immunofluorescence. Cells were washed in PBS, fixed in 3% (w/v) paraformaldehyde for 10min, washed again and then blocked with 20mM glycine in PBS for 10min. Cells were then permeabilized for 30min with 0.1% (w/v) Saponin in PBS. Primary antibody (against NaPi-IIa or NaPi-IIb, as described above) was added for 2h at a dilution of 1:100 in PBS-Saponin. For negative controls rabbit pre-immune serum was used at the same duilution. After washing, secondary antibody, FITC-conjugated goat anti-rabbit IgG (Molecular Probes, Invitrogen, Carlsbad, CA, USA) diluted 1:50 in PBS-Saponin, together with Phalloidin Alexa Fluor (Molecular Probes) diluted 1:20, was added for 30min. Cover slips were washed, mounted and then analyzed by confocal microscopy (Leica, Wetzlar, Germany, Imaris imaging software v. 3.04, Bitplane AG, Zürich, Switzerland).

Phosphate uptake

Cells in 12 well culture plates were washed twice in Hank's balanced salt solution with Na^+ adjusted to 100mM, P_i at 1 mM (added as potassium salt) and10mM Hepes-Tris, pH 7.2. Sodium-independent uptake was determined by substituting sodium in the uptake buffer with equimolar choline. Cells were then incubated in the same buffer containing 1µCi /ml $^{32}P_i$ (New England Nuclear, PerkinElmer, Wellesley, MA, USA). In time course experiments uptake was stopped after 2, 4, 8 or 16min by the removal of uptake buffer and three consecutive washes with ice-cold uptake buffer without radionuclide. The cell layer was then lysed by addition of 0.5ml 0.2M NaOH, homogenized by pipetting and centrifuged to remove cell debris and collagenous extracellular matrix. Protein concentration of lysates was determined using the DC protein assay (Bio-Rad) according to the manufacturer's instructions. Aliquots of lysates and uptake buffer were counted by scintillation. Phosphate uptake was expressed as nmol P_i/mg protein.

To determine the apparent affinity for P_i, P_i concentration in the uptake buffer was varied between 0.01 and 1mM. pH dependency of P_i uptake was investigated by varying the pH of Hepes-Tris in uptake buffers between 5.6 and 7.4. The ability of pyrophosphate (PP_i) (Sigma) to inhibit sodium-dependent phosphate uptake in UMR-106 and OK cells was assessed in a concentration range of 0 to 3mM pyrophosphate. Phosphonoformic acid (PFA) inhibition of P_i uptake was studied at concentrations between 0 and 3mM PFA. PFA is a commonly used inhibitor of sodium-phosphate cotransporters, specific for the type II cotransporters but only weakly inhibiting the type III cotransporters,[11-13, Pit-1: [14] and unpublished, Dr. Ian Forster, personal communication]. For these experiments the uptake was allowed to proceed for 6min, within the linear phase of P_i uptake, and also when single time point measurements were done. For pH and inhibition measurements, P_i was kept at 0.5mM. For time-course and P_i affinity experiments data were fitted to the Michaelis-Menten equation by non-linear regression , in the case of P_i affinity after subtraction of the sodium-independent component of uptake. (Prism, Graphpad, San Diego, CA, USA).

P_i uptake was characterized in cells at confluency, at 1 week post-confluency, after culture in 3mM P_i for 24h or 7 days and in cells starved of P_i for 72h. Uptake experiments were also performed every second day in cells cultured under mineralizing conditions at 1.5mM P_i.

All P_i uptake experiments were performed at 30°C. All experiments were repeated at least 3 times.

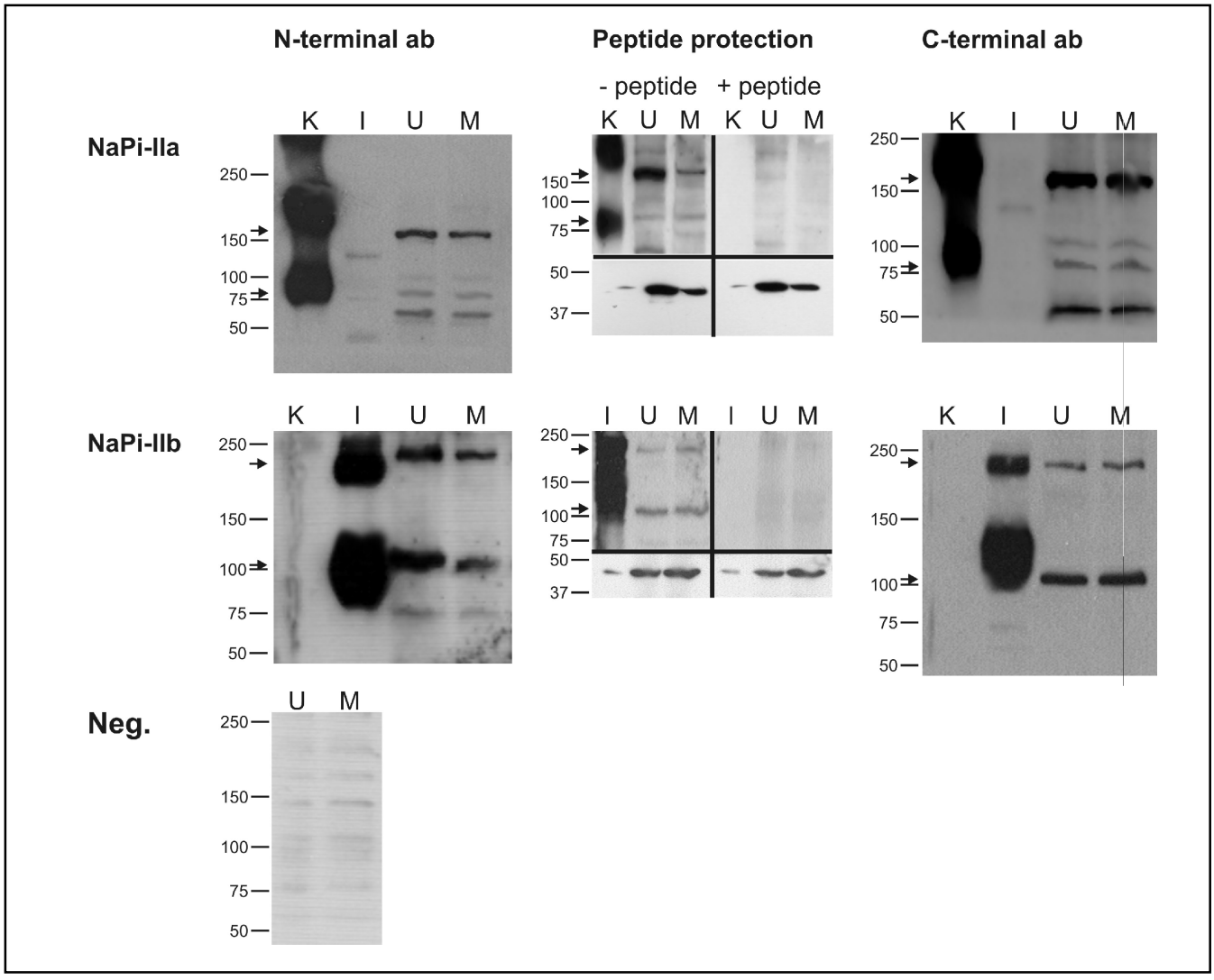


Fig. 1. Expression of NaPi-IIa and NaPi-IIb proteins in osteoblast-like cells. Western blot of NaPi-IIa and NaPi-IIb in osteoblast-like cells. With antibodies specific for N-terminal peptides of the cotransporters, NaPi-IIa was seen at 80 and 160kDa while NaPi-IIb could be detected at 105 and 210kDa. The higher molecular weight bands representing dimers of the cotransporters. The signals could be abolished by preincunbation of the primary antibodies with the corresponding immunogenic peptides. When repeating the experiment with antibodies directed against C-terminal peptides of the cotranporters gave rise to the same bands, again confirming their identity. Negative control experiments using pre-immune rabbit serum detected no bands corresponding to the cotransporters (Neg.). M: MC3T3-E1 membrane protein, U: UMR-106 membrane protein. K: Kidney brush border membrane. I: Intestinal brush border membrane.

von Kossa-staining

Mineralized nodules produced by cultured osteoblast-like cells were stained according to a protocol by von Kossa as modified by Krutsay [29]. Cells were fixed in ethanol, washed and incubated under normal lighting for 45min in 5% (w/v) $AgNO_3$. Cells were then rinsed and the staining stabilized in 16% (w/v) paraformaldehyde, 800mM Na_2CO_3 for 2min. After rinsing in water, unspecific silver stain was removed by reduction for 30s in a freshly prepared mixture of 400mM $Na_2S_2O_3$ and 300mM $K_3(Fe(CN)_6$ (20:1). Cells were rinsed again and photographed. Counterstaining was not performed.

All statistical tests and non linear curve-fittings were performed using Prism software (Graphpad), statistical tests utilized ANOVA with a $p< 0.05$ considered significant. Unless otherwise stated all chemicals were from Sigma and of the highest purity available.

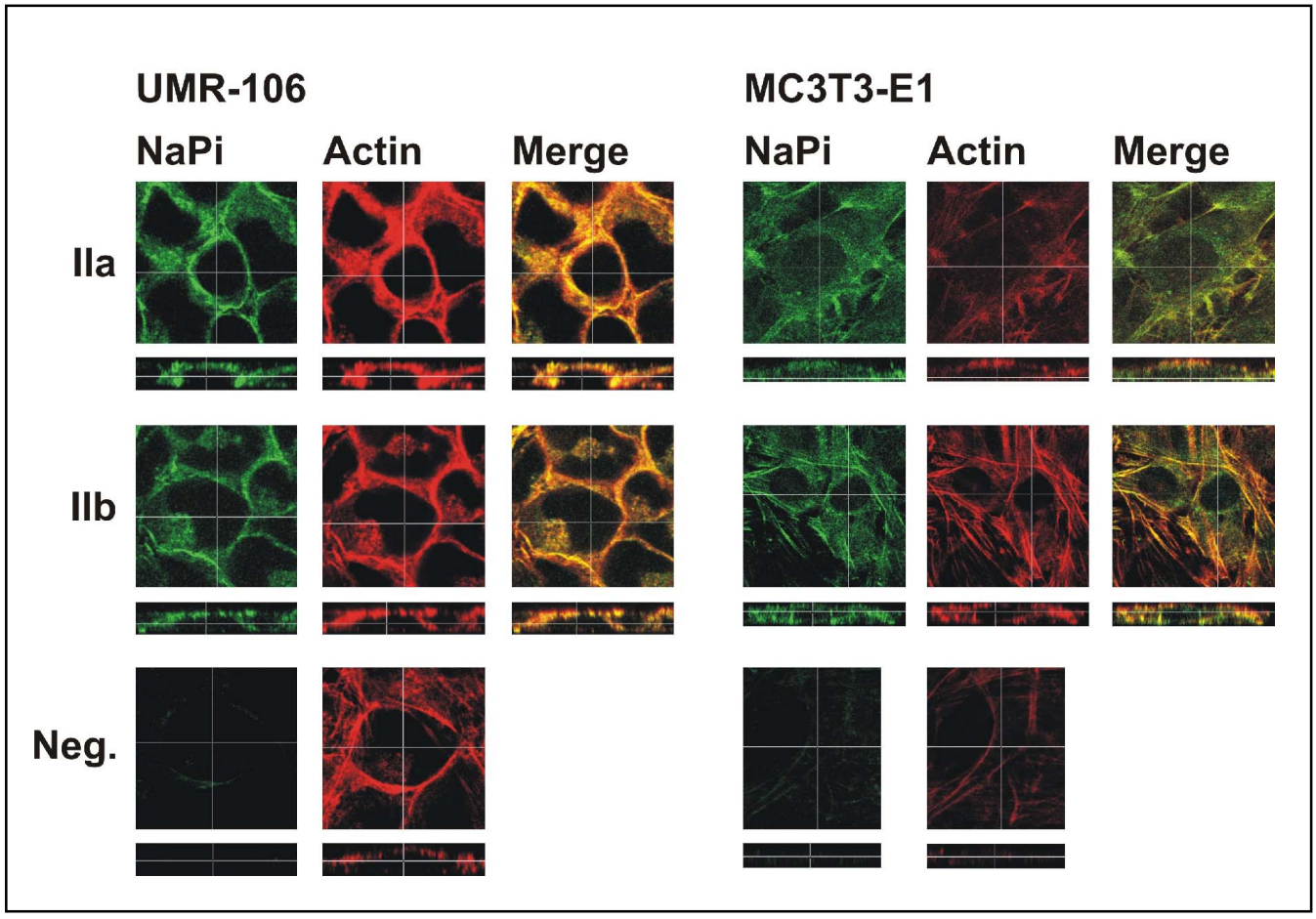


Fig. 2. Confocal immunofluorescent detection of NaPi-IIa and NaPi-IIb in osteoblast-like cells. Both Na^+-P_i cotransporters were found in the osteoblasts cell membrane and colocalized with β-actin as can be seen by the predominantly yellow merged signal. Some intracellular staining for NaPi-IIb was evident. Membrane localization was generally more pronounced in UMR-106 than in MC3T3-E1 cells. Negative controls showed weak unspecific staining. Green: NaPi-IIa or NaPi-IIb. Red: β-actin. Yellow: Merge. Neg: Negative control. Lower panels shows a cross section (z-axis) through the center line, apical side upwards.

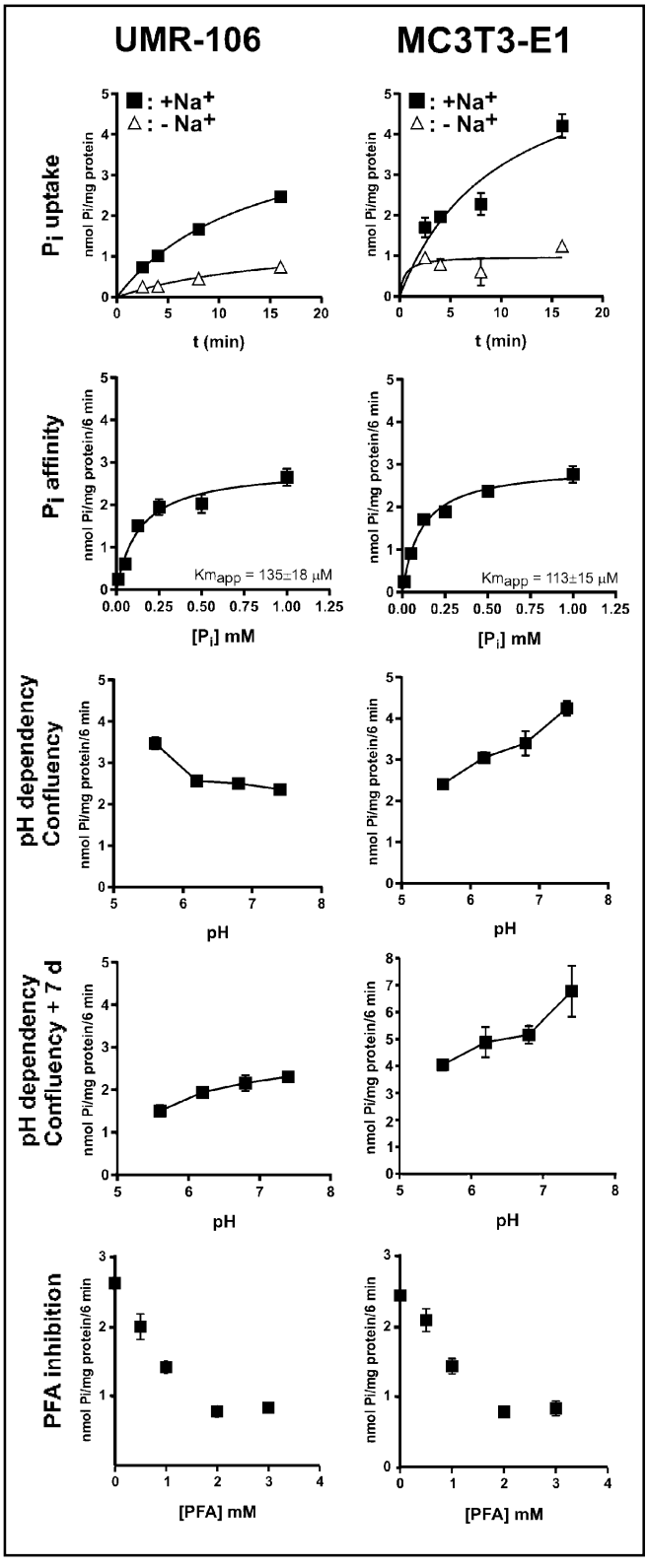
Results

Detection of type II Na^+-P_i cotransporters in osteoblast-like cells

Using antibodies directed against N-terminal peptides of NaPi-IIa and NaPi-IIb, both cotransporters could be detected in membrane fractions derived from UMR-106 and MC3T3-E1 cells (Fig. 1, left). NaPi-IIa was seen as bands of 80 and 160kDa, while NaPi-IIb was detected as bands of 105 and 210kDa; in each case the higher molecular weight bands likely represented a dimer of the cotransporter. In the case of NaPi-IIa, the 160kDa band was much stronger than the band at 80kDa, while for NaPi-IIb the 105kDa band dominated. In kidney (NaPi-IIa) and intestine (NaPi-IIb) positive controls the same relationship between the weaker and stronger band of each cotransporter was seen. As the dominant bands under all tested conditions amounted to a large majority of the total signal, and as the stronger and weaker bands always showed the same changes, in later experiments only the dominant band of each respective cotransporter is shown and quantified. Both NaPi-IIa and NaPi-IIb signals could be blocked by preincubation of the primary antibody with immunogenic peptide (Fig 1, middle panels). To verify the identity of the bands, Western blots were repeated with antibodies directed against C-terminal peptides of the cotransporters, again detecting the same bands in both cell lines (Fig. 1, right panels). Using rabbit pre-immune serum no corresponding bands were visible (Fig. 1, bottom).

Immunofluorescent detection of cotransporters in cultured cells revealed expression of both NaPi-IIa and

Fig. 3. Characterization of P_i uptake in osteoblast-like cells. Both cell lines showed a pronounced sodium-dependent P_i uptake, while sodium-independent uptake amounted to approximately 20% of total uptakes. Apparent P_i affinity, after subtraction of the sodium independent component, was for UMR-106 135±18μM and for MC3T3-E1 113±15μM (non-linear regression curve fits to the Michaelis-Menten equation). Km_{app} for P_i was not significantly different between the cell lines. MC3T3-E1 cells P_i uptake was positively correlated with pH at both culture stages. In contrast UMR-106 P_i uptake at confluency was pH-independent over most of the range tested but showed an inverse pH dependence for the lowest pH values. One week later UMR-106 P_i uptake showed the same positive pH dependence as seen in MC3T3-E1 cells. PFA at 1 mM inhibited 65-75% of the sodium-dependent P_i uptake in both cell lines. Higher concentrations of PFA left an uninhibitable component of P_i uptake amounting to approximately 30%. Curves for P_i affinity, PFA inhibition and pH dependency of uptakes are shown after subtraction of sodium-independent P_i uptake.



NaPi-IIb in both cell lines used (Fig. 2). Both cotransporters were detected by confocal microscopy in the cell membrane and colocalized with β-actin. Cotransporters were predominantly, but not exclusively, found at the apical face of osteoblastic cells. Some staining was also found on lateral sides and in some cases a weak staining was seen at the basolateral membrane. The polarized distribution of Na^+-P_i cotransporters was more pronounced for UMR-106 than for MC3T3-E1 cells. At this stage MC3T3-E1 cells showed a distended, pre-osteoblastic morphology. In later culture stages the cells showed a more pronounced polarization and columnar morphology. Pre-immune serum yielded no or weak staining of cultured cells. As the forming collagenous matrix led to a large autofluorescence in samples from late culture stages, immunofluorescence analyses were only performed on cells at early confluency.

Characteristics of P_i uptake in UMR-106 and MC3T3-E1 cells

At confluency both cell lines showed a pronounced sodium-dependent P_i uptake. Uptake was linear during the first eight minutes (Fig. 3). Km_{app}, obtained from fitting the Michaelis-Menten equation to the data in Fig. 3, for P_i were 135±18 μM in UMR-106, and 113±15μM in MC3T3-E1 cells, showing no significant difference between the two cell lines (Fig. 3). At confluency, MC3T3-E1 P_i uptake increased with increasing pH, while pH had little effect on P_i uptake in UMR-106 cells except for an increase in uptake at the most acidic condition (pH 5.6) (Fig. 3). P_i affinity, PFA inhibition and pH dependency curves in figure 4 show sodium dependent components of P_i uptakes after subtraction of sodium-independent P_i uptake.

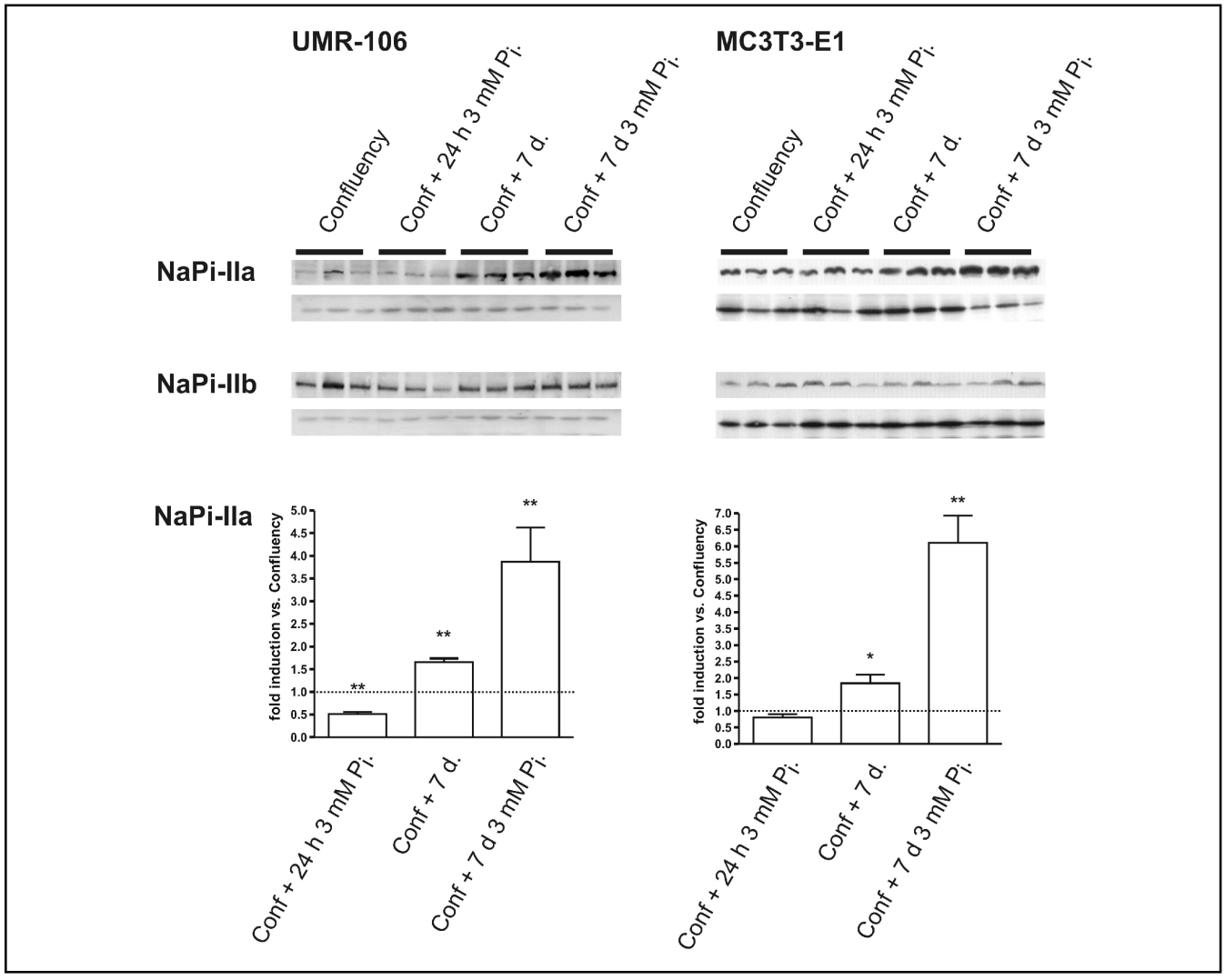


Fig. 4. Regulation of NaPi-IIa and NaPi-IIb expression in osteoblast-like cells by culture stage and inorganic phosphate. Expression of NaPI-IIa, shown at 160kDa, was influenced by both time in culture (confluency or 7 days post-confluency) and supplementation of P_i to 3mM (24h or 7 days) in both cell lines. Levels of NaPi-IIb, shown at 105kDa, were not affected by these conditions, quantification showed no significant differences between groups. Quantification showed that NaPi-IIa was decreased after 24 h in 3mM P_i in UMR-106 but not in MC3T3-E1 cells (UMR-106: -49±6%, **: $p<0.01$; MC3T3-E1: -20±12%, NS, SEM, N=6) and that the cotransporter was upregulated 7 days post-confluency (UMR-106: +66±8%, **: $p<0.01$; MC3T3-E1: +84±26%, *: $p<0.05$, SEM, N=6) and after long-term treatment with 3mM P_i for 7 days (UMR-106: +287±75%, **: $p<0.01$; MC3T3-E1: +510±83%, **: $p<0.01$, SEM, N=6) all as compared with the expression level at confluency (expression level at confluency set to 1, dotted line). For each condition three lanes of three parallel membrane protein isolations are shown. Lower panels show β-actin loading controls. Conf: confluency.

PFA at 1 mM inhibited 65-75% of the sodium-dependent P_i uptake in both cell lines (Fig. 3). Higher concentrations of PFA left an uninhibitable component of P_i uptake amounting to approximately 30%. This suggests that the type II cotransporters are the major catalysts of osteoblast P_i uptake while the type III cotransporters are responsible for approximately 30% of the total sodium-dependent P_i uptake. Pyrophosphate in concentrations up to 3mM did not inhibit sodium-dependent P_i uptake in either osteoblastic cells or in OK cells. This was not changed by addition of levamizole to the uptake solutions to inhibit alkaline phosphatase hydrolysis of PP_i (data not shown).

The effects of culture stage on expression and activity of Na^+-P_i cotransporters in osteoblast-like cells

Sodium-dependent P_i uptake in UMR-106 cells showed differences in pH dependency a week after confluency, compared with data from an early confluent stage. Neither the magnitude nor the Km_{app} of P_i measured at pH 7.2 uptake one week post-confluency was significantly different from the early confluent stage, in either cell line. In UMR-106 cells, pH dependency changed from being essentially pH independent in the 6.2-7.4 range at early confluency, into showing a clear pH dependency, uptake increasing with increasing pH over the entire pH range 7 days after confluency (Fig. 3). This behaviour is now similar to that seen in MC3T3-E1 cells at both early and late confluency (Fig. 3). The pH dependency of MC3T3-E1 P_i uptake was identical at both culture stages (Fig. 3).

As shown in Figure 4, NaPi-IIa membrane protein was increased in both cell lines from confluency to a culture stage one week later (UMR-106: +66±8%, **: $p<0.01$; MC3T3-E1: +84±26%, *: $p<0.05$, SEM, N=6). The expression of NaPi-IIb was not changed by time in culture, in either cell line.

The effects of P_i supplementation and starvation on expression and activity of Na^+-P_i cotransporters in osteoblast-like cells

When performing P_i uptake experiments on osteoblastic cells cultured in 3 mM P_i (added as phosphate or β-glycerophosphate) to promote mineralization, an approximately ten-fold increase in retained P_i in the wells was seen within one day of P_i supplementation (at which time mineral had started to form). This retention was not sodium-dependent (data not shown). The retention was also seen after first fixing cells in paraformaldehyde and removing cell membranes, showing that it was not a cell-dependent process. Neither was it due to binding to the mineralized matrix, as returning cultures to normal medium normalized uptake characteristics within 24h, while the mineralized ECM remained. Also, in MC3T3-E1 cells sodium-independent P_i retention was induced at 2mM P_i while mineralization only took place above 3mM P_i. As the magnitude of P_i binding far exceeded sodium-dependent P_i uptake, characterization of P_i uptake could not be performed for cells cultured in 3mM P_i. In contrast, OK cell controls responded to 3mM phosphate with a down regulation of sodium-dependent P_i uptake without changes in sodium-independent uptake, as has been described earlier (data not shown) [6].

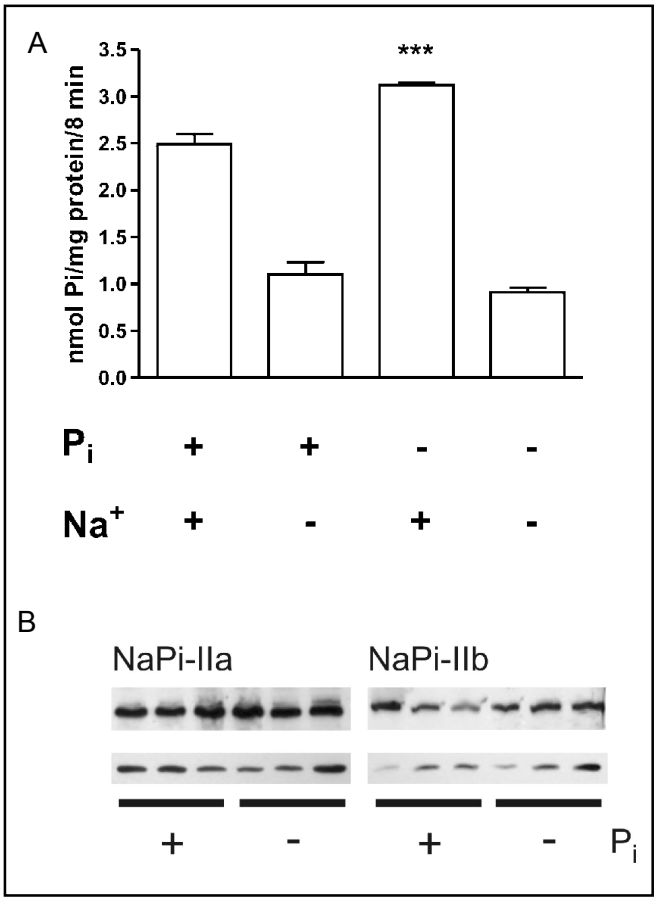


Fig. 5. Effects of P_i starvation on UMR-106 P_i uptake, NaPi-IIa and NaPi-IIb expression. A. P_i uptake: Phosphate starvation for 72h led to a small increase (approximately 25%) in UMR-106 sodium-dependent P_i uptake. (**, $p<0.01$) Sodium-independent P_i uptake was unaffected by P_i starvation. P_i: Culture in the precense or absence of P_i. Na^+: Uptake performed in the presence or absence of Na^+. B. Expression of NaPi-IIa (160kDa) and NaPi-IIb (105kDa). Levels of neither cotransporter changed in membrane protein fractions from UMR-106 cells in response to P_i starvation. For each condition three lanes of three parallel membrane protein isolations are shown. Lower panels show β-actin loading controls.

P_i supplementation to 3mM caused a decrease in the membrane expression of NaPi-IIa in UMR-106 cells within 24h while MC3T3 NaPi-IIa expression remained unchanged (UMR-106: -49±6%, **: $p<0.01$; MC3T3-E1: -20±12%, NS, SEM, N=6) (Fig. 4). However, after 7 days in 3mM P_i this phenomenon was reversed and membrane expression of NaPi-IIa was strongly increased in both cell lines as compared to the levels at confluency (UMR-106: +287±75%, **: $p<0.01$; MC3T3-E1: +510±83%, **: $p<0.01$, SEM, N=6), a stronger

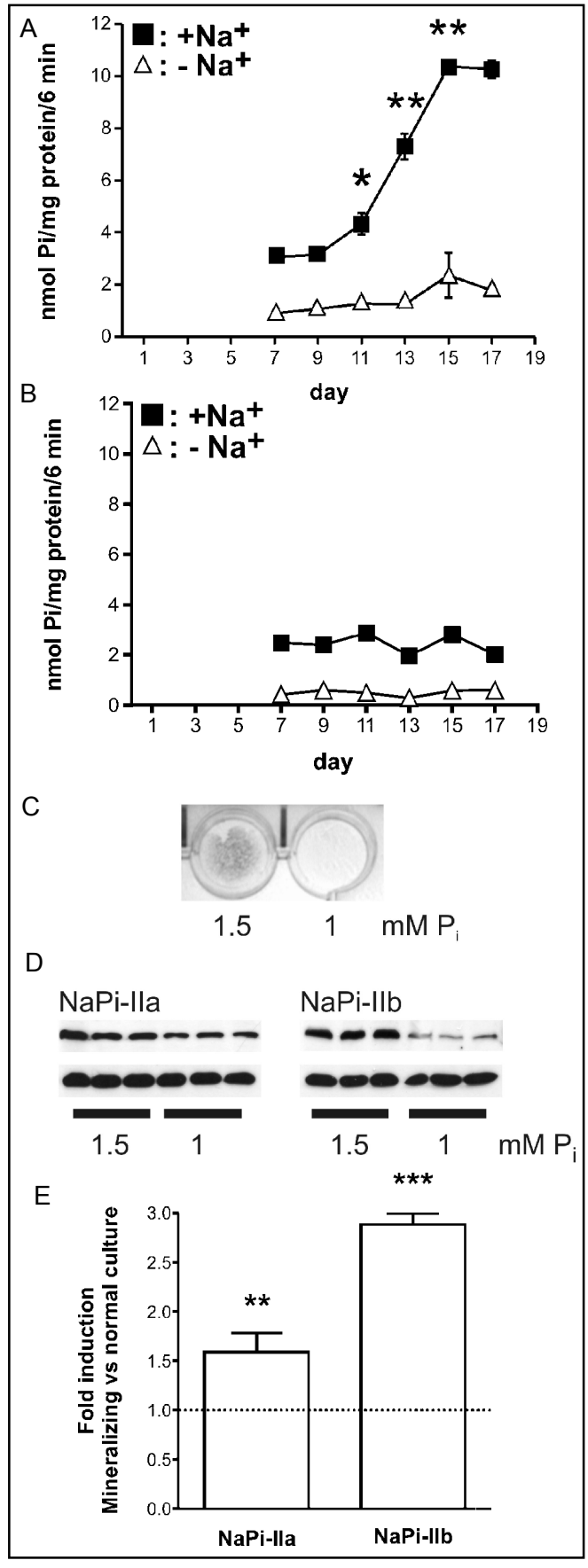


Fig. 6. P_i uptake and NaPi-IIa and NaPi-IIb expression in UMR-106 cells during formation of mineral *in vitro*. A. Development of UMR-106 P_i uptake 7-17 days after P_i supplementation to 1.5mM. Day 0 denotes confluency of cultures. Sodium-dependent uptake increases from day 9-15. Stars denote significant changes as compared with the previous time point, (*: $p<0.05$; **: $p<0.01$, N=3). B. Development of UMR-106P_i uptake during culture in normal medium at a P_i concentration of 1mM. No changes in P_i uptake seen with time. C. Mineral starts forming at day 16 after P_i supplementation to 1.5mM as visualized by von Kossa staining. No mineral could be detected at day15 or earlier. No mineral forms in normal medium. D. Western blot of NaPi-IIa (160kDa) and NaPi-IIb (105kDa) in UMR-106 cells. Both NaPi-IIa and NaPi-IIb are upregulated at mineralization after 16 days in 1.5mM P_i. For each condition three lanes of three parallel membrane protein isolations are shown. Lower panels show β-actin loading controls. E. Quantification of NaPi-IIa and IIb expression shown in panel D, NaPi-IIa increased by 59±18% (SEM, **: $p<0.01$, N=6) after 16 days of treatment with 1.5mM P_i, while NaPi-IIb increased by 188±11% (SEM, ***: $p<0.001$, N=6), as compared to untreated controls in 1mM P_i medium (expression level set to 1, shown by dotted line).

upregulation of NaPi-IIa than extended culture time alone. All data as compared to the expression level at confluency. In contrast, in both cell lines expression of NaPi-IIb was unaffected by P_i supplementation to 3mM. (Fig. 4).

In the P_i starvation experiment, culturing of UMR-106 in P_i free medium for 72h led to a small but significant (**: $p<0.01$) increase of 25-30% in cellular sodium-dependent phosphate uptake (Fig. 5A) while other P_i uptake characteristics were unchanged (data not shown). No significant changes in NaPi-IIa or NaPi-IIb expression were detected (Fig. 5B). Repeating the experiment with MC3T3-E1 cells gave the same results (data not shown). OK cell controls strongly upregulated their sodium-dependent P_i uptake by approximately 200% under the same culture conditions, as has been described previously (data not shown) [11].

P_i uptake and Na^+-P_i cotransporter expression in mineralizing UMR-106 cells

We found that sodium-independent P_i retention in osteoblast cultures was rapidly induced at medium P_i concentrations above 2mM. A phosphate concentration of 1.5mM in the culture medium was therefore selected to study P_i uptake and cotransporter expression under mineralizing conditions in UMR-106 cells. The experiment was not performed with MC3T3-E1 cells, see discussion.

Increasing the concentration of P_i in cell culture medium from 1 to 1.5mM led to no short term change in cellular P_i uptake. In UMR-106 cells sodium-dependent phosphate uptake began increasing at day 9 after P_i supplementation to 1.5mM (Fig 6A). Sodium-dependent uptake peaked at a level approximately 3 times higher than basal uptake on day 15-17 (+239±11%, ***: $p<0.0=1$, SEM, N=3). Mineral could be clearly seen in the cultures from day 16 (Fig. 6C). In UMR-106 cells cultured in standard medium, only small changes in P_i uptake were seen over time (Fig. 6B) and the cultures showed no mineral formation (Fig. 6C). In mineralizing cultures, as well as control cultures, around 70% of P_i uptake could be inhibited by PFA showing that the majority of P_i uptake under all conditions was catalyzed by type II cotransporters. The smaller PFA uninhibitable component catalyzed by type III cotransporters did increase in absolute magnitude during mineral formation, probably due to an upregulation of Pit-1 as has been shown previously [10].

In membrane samples from UMR-106 cells cultured for 16 days in 1.5mM P_i, at which time P_i uptake had increased and mineral started forming, a clear upregulation of NaPi-IIb as compared to the expression level in normal culture (+188±11%, ***: $p<0.001$, SEM, N=6) could be detected. NaPi-IIa expression was also increased (+59±18%, **: $p<0.01$, SEM, N=6) but to a lesser extent than NaPi-IIb (Fig. 6D, E). The magnitude of Na^+-P_i cotransporter upregulation was thus well correlated with the increase seen in sodium-dependent P_i uptake.

Discussion

We have shown that rat and mouse osteoblast-like cells express two type II Na^+-P_i cotransporters, NaPi-IIa and NaPi-IIb. As these cotransporters are respectively associated with epithelial transcellular P_i fluxes in kidney and intestine, and co-express in several cell types producing calcified tissues [18-19], we hypothesized that they would play a role in the flux of phosphate from the circulation to mineralizing calcified tissues. In this study we have demonstrated that NaPi-IIa but not NaPi-IIb is upregulated in osteoblast-like cells by relatively short-term increases in P_i concentration. We have also established that neither cotransporter is regulated by P_i starvation, whereas both cotransporters are upregulated concomitantly with increased cellular sodium-dependent P_i uptake prior to formation of mineral *in vitro*. This is consistent with the osteoblasts' role in bone calcification and in maintaining phosphate homeostasis, and supports a role for type II Na^+-P_i cotransporters in mediating regulated P_i transport in osteoblasts.

Both cotransporters could be detected in membrane fractions from both osteoblastic cell lines by western blotting, using two independent antibodies for each cotransporter. Both cotransporters could be detected both as monomers and what is likely dimers of the cotransporters, the dimer being the most abundant form of NaPi-IIa, while NaPi-IIb was primarily found as a monomer. It has been shown that NaPi-IIa to a large extent dimerizes when expressed in *Xenopus laevis* oocytes [30 and unpublished, Dr S Gisler, personal communication], while NaPi-IIb has not been studied in this respect.

Expression of both NaPi-IIa and NaPi-IIb was seen in the cell membranes of cultured osteoblast-like cells from rat and mouse. It was recently shown that both cotransporters are also expressed in rat and mouse odontoblasts and mouse ameloblasts [18-19]. It thus seems that co-expression of the cotransporters is a distinguishing feature of mineral-producing cell types, whereas in other tissues the cotransporters are expressed separately. NaPi-IIa has also been found in chondrocytes and osteoclasts, however NaPi-IIb has not been studied in these cells [20-21]. Despite the lack of one of these cotransporters NaPi-IIa knockout mice form a fully developed skeleton, exhibiting disturbances in bone formation previously attributed to their hypophosphatemic condition [20, 31]. Our finding of NaPi-IIa expression in osteoblasts suggests that these disturbances might also be due to a direct effect on osteoblast phosphate transport. That the skeletal phenotype is not more severe may be due to a compensatory role of NaPi-IIb.

Studies of Na^+-P_i cotransporters expressed in osteoblasts have thus far been limited to the ubiquitously expressed type III cotransporters Pit-1 and Pit-2 [6-9]. However, the level of Pit-1 seems low in osteoblasts and the transcript could not be detected by in situ hybridization on bone [9]. In a recent paper, Zoidis and co-workers made a comprehensive study of Na^+-P_i cotransporters in rat PyMS cells (osteoblast-like) and tissues [8]. They detected both Pit-1 and Pit-2 in osteoblasts but did not find expression of the type II cotransporters. The reason for this is unclear but could be due to a phenotypic difference betweeen PyMS and UMR-106 cells.

Previous studies of osteoblast P_i uptake have been extensive and our data on uptake levels, and pH dependency correlate well with earlier work with the exception of Km values. Earlier work has often yielded

$Km_{app, Pi}$ values around 200μM, significantly higher than our data. However, in these studies the sodium independent component of uptake had not been subtracted before Km determination. Repeating our calculations with the sodium-independent component included yielded Km values in the low 200μM range, in good agreement with earlier work [32-34]. Km values in this range are to be expected for P_i uptake catalyzed by type II sodium-phosphate cotransporters, while Km:s for type III cotransporters are significantly lower [35]. The apparent Km of sodium-dependent P_i uptake was unchanged between early and late confluency for both UMR-106 and MC3T3-E1 cells. However, after showing an increase in uptake at very acidic pH (5.6) at early confluency, phosphate uptake in UMR-106 was altered at late confluency, so that P_i uptake increased with rising pH and mirrored the situation in MC3T3-E1 cells. Caverzasio and co-workers [34] demonstrated that P_i uptake in UMR-106 at early confluency decreased with increasing pH, in agreement with our findings. This was taken as an indication of type III Na^+-P_i cotransporter activity in osteoblast-like cells [29]. Not only the type III cotransporters, however, but also NaPi-IIb show a pH dependency where uptake decreases with increasing pH [27], suggesting that NaPi-IIb activity is most significant in UMR-106 at early confluency. In contrast, the activity of NaPi-IIa, whose activity increases with rising pH, increases with time in culture in UMR-106 cells but is readily detectable at an early stage of growth in MC3T3-E1 cells. Measurements of PFA inhibition of osteoblast P_i uptake showed that the type II cotransporters were responsible for approximately 75% of the osteoblast P_i uptake, the remaining uninhibitable component deriving from type III cotransporter activity that would only be slightly inhibited (approximately 10%) (Pit-2: [13], Pit-1: [14] and unpublished, Dr. Ian Forster, personal communication] at the P_i and PFA concentrations used.

Western blotting of membrane proteins from these two culture stages showed an upregulation of NaPi-IIa, but not NaPi-IIb, one week post-confluency, mirroring the changes seen in pH dependency of P_i uptakes. That the phenotype of cultured osteoblast-like cells changes with culture stage has been described previously (for a review see ref. [36]).

Sodium-independent P_i background binding precluded direct measurements of cellular P_i uptake in high phosphate conditions, and to the best of our knowledge, osteoblast phosphate uptake experiments at elevated P_i concentrations have never been published. We could however detect a down-regulation of NaPi-IIa membrane protein in UMR-106, but not MC3T3-E1 cells, after a 24 h treatment with 3mM P_i while long-term culture in 3mM P_i medium strongly upregulated the cotransporter in both cell lines. Expression of NaPi-IIb was unaffected by both short and long-term treatments with 3mM P_i in our study. This contrasted with studies showing that in kidney and intestine, NaPi-IIa and NaPi-IIb are down-regulated in response to elevated P_i, both acutely and chronically, to limit phosphate influx to the circulation and help return serum P_i to its normal concentration [11, 17]. In hyperphosphatemia bone formation is increased, also to remove P_i from the circulation [37]. Upregulation of NaPi-IIa in osteoblasts, in response to elevated concentrations of P_i would therefore make physiological sense enabling the cells to remove a larger amount of P_i from the circulation to mineralizing bone. The initial down-regulation of NaPi-IIa seen in UMR-106 cells, if not an artifact, could possibly be a short-term response stabilizing P_i transport during short-term fluctuations in serum P_i levels to stabilize the rate of bone formation while the rapid response in kidney P_i reabsorption corrects the serum P_i concentration. As the response differed between the cell lines, definite conclusions are hard to draw.

P_i starvation of osteoblastic cells led to a small increase in cellular sodium-dependent phosphate uptake. As no change in type II Na^+-P_i cotransporter expression was induced by P_i starvation, the increased uptake is most likely due to an induction of Pit-1 that has earlier been shown to be induced in osteoblasts by low P_i conditions [8]. The absence of change in NaPi-IIa and NaPi-IIb expression again suggests that the cotransporters' role is not in providing P_i for cellular metabolism, that would need to be increased under P_i starvation, but in P_i transport to forming bone, a process that would be down-regulated as bone formation is diminished under hypophosphatemia.

It has previously been shown that the commonly used additions of 10mM P_i or β-glycerophosphate (from which all phosphate is liberated within minutes after contact with the osteoblasts), to osteoblast culture media to promote mineralization leads to changes in osteoblast phenotype, and to cell-independent mineralization as well as mineral formation in fibroblast cultures [38-41]. Under certain culture conditions elevated P_i concentrations have also been shown to induce apoptosis in primary osteoblasts and osteoblast-like cells [42]. No sign of P_i induced cell death could however be detected in this study. Phosphate is not normally considered a signalling substance but it is now known that P_i concentration influences the expression

osteopontin and a host of other genes in osteoblasts [43-45]. To study physiologically relevant behaviour of osteoblast cells it is thus necessary to keep P_i in a physiological range. Ten mM phosphate is a supraphysiological concentration, normal total plasma phosphate amounting to around 1.5mM, and data in this and previous studies suggest that a high concentration of phosphate influences the osteoblast phenotype in a way that might be inconsistent with normal mineralization [42-46]. As we saw in this study, even the use of a lower concentration of 3mM P_i to induce mineralization led to dramatic changes in the phenotype of osteoblast cultures. To counteract this, a concentration of 1.5mM P_i was chosen; this both promoted mineralization in UMR-106 cultures and allowed measurements of osteoblastic P_i uptake.

The model system of choice for studying osteoblast mineralization is usually considered to be MC3T3-E1 cells. However, after testing three different batches of MC3T3-E1 cells from ATCC it was found that they did not mineralize at P_i concentrations below 3mM, and above that very rapidly, within 1 to 2 days, contrary to their original characterization [47]. The above mentioned induction of sodium-independent P_i retention was induced in cultures already at 2mM P_i, making P_i uptake measurements in mineralizing MC3T3-E1 cultures impossible. Expression data for MC3T3-E1 type II Na^+-P_i cotransporters in 3mM P_i conditions have been presented above. A possible explanation for this observation is that ATCC propagate the cell line but do not select it for mineralization under low P_i conditions. The original phenotype might thus have been lost. We therefore chose to study the development of Na^+-P_i cotransporter expression and activity in mineralizing cultures of UMR-106 cells that formed mineral already at 1.5mM P_i without induction of sodium-independent P_i retention. While UMR-106 cells sometimes have been described as forming ectopic mineral, it has ben shown that the cell line exhibits normal osteoblastic mineralization at P_i concentrations of 5mM or lower [48]. As described by Bellows *et. al.* formation of ectopic mineral in cultures is also greatly reduced by keeping P_i concentrations below 4mM and by allowing cultures a lag time of ECM deposition before P_i supplementation, both conditions that were met in the present study [38].

We have shown that, using 1.5 mM P_i supplementation, UMR-106 cellular sodium-dependent P_i uptake increased threefold prior to formation of mineral *in vitro;* the increase in uptake starting approximately nine days after P_i supplementation. At the same time both NaPi-IIa and NaPi-IIb were upregulated in UMR-106 cells and upregulation correlated in magnitude with the increased phosphate uptake. It is noteworthy that 3mM P_i supplementation leads to a rapid increase in NaPi-IIa expression and mineralization, while the response to 1.5mM P_i involves an upregulation of both cotransporters and a long lag-phase prior to increased uptake and mineralization. This suggests an ordered progression and maturation of osteoblast cultures and extracellular matrix prior to mineral deposition under more physiological concentrations of P_i [38]. This closely resembles the situation in mineralizing cultures of odontoblast-like cells where increased sodium-dependent P_i uptake and NaPi-IIa upregulation was seen prior to mineral formation [18]. However, NaPi-IIb was down-regulated at this stage, pointing to differences in phosphate handling by odontoblasts and osteoblasts. It was recently shown that BMP-2 enhanced mineralization and P_i uptake by MC3T3-E1 cells through an induction of Pit-1 expression [10]. This stimulatory effect, but not basal mineralization, was blocked by RNAi of Pit-1 showing that this cotransporter is also of importance for osteoblast mineralization. However the stimulation could also be blocked by PFA at a concentration that would have no inhibitory effect on Pit-1, suggesting that the increase in mineralization also involved a type II cotransporter even if this was not studied directly [10]. Our data suggests that the type II cotransporters are responsible for most P_i transport in mineralization, but stimulation with osteogenic factors might lead to the requirement for a larger contribution from the type III cotransporters.

In osteoblasts, P_i uptake has been shown to be regulated by several calciotropic hormones, including PTH and vitamin D, which also influence bone formation and mineral homeostasis [32, 49, 50]. NaPi-IIa expression is regulated in the kidney by PTH and vitamin D, and NaPi-IIb is influenced by vitamin D in the intestine [11, 12, 24]. We consider it likely that these factors also influence NaPi-IIa and NaPi-IIb regulation in osteoblasts which could in part explain the factors' control of bone formation.

After entering the osteoblast, phosphate must subsequently be exported to the osteoid to promote mineralization. Although the mechanism of cellular phosphate efflux remains unknown, several different mechanisms have been proposed ranging from diffusion to membrane transporters [6]. In addition to these mechanisms it is, in the case of osteoblasts, also possible that P_i is secreted in matrix vesicles [33]. The mechanism responsible for exporting phosphate from osteoblasts to bone remains to be determined.

Conclusions

We conclude that osteoblasts express two type II Na^+-P_i cotransporters, NaPi-IIa and NaPi-IIb, responsible for the major part of osteoblast P_i uptake, in addition to the previously detected type III cotransporters. As the results were consistent between two different cell lines from two different species they are likely representative of a general osteoblastic phenotype. NaPi-IIa and NaPi-IIb in osteoblasts are regulated by P_i concentration in a manner consistent with the cells' role in maintaining phosphate homeostasis of the organism. Also, our data suggest that an upregulation of P_i uptake and the Na^+-P_i cotransporters NaPi-IIa and NaPi-IIb is a prerequisite for the formation of mineral by osteoblastic cells. This strengthens the hypothesis that the flux of P_i to mineralizing bone is largely dependent on osteoblast type II Na^+-P_i cotransporters.

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