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Osteocyte-Specific Monoclonal Antibody MAb OB7.3 Is Directed Against Phex Protein

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ABSTRACT

Osteocytes are the most abundant cells in bone; however, relatively little is known about their properties and functions. The development of monoclonal antibody MAb OB7.3 directed against chicken osteocytes enabled us to purify osteocytes from enzymatically isolated bone cells. Cultures of purified osteocytes were used to gain better insight into the role of osteocytes in bone metabolism. Until now, the antigen of MAb OB7.3 has not been elucidated. In this study, we examined the antigen to which this osteocyte-specific antibody is directed. Immunoprecipitation and purification of the protein, followed by amino acid sequence analysis of two isolated peptides, revealed that the antigen has high homology to human and murine PHEX/Phex protein sequences (*PH*osphate-regulating gene with homology to *E*ndopeptidases on the *X* chromosome). The OB7.3 antigen was therefore identified as chicken Phex protein. In addition, using suppression subtractive hybridization, we obtained a complementary DNA (cDNA) sequence of 502 base pairs (bp) with high homology to the human and murine *PHEX/Phex* genes. This method was applied to identify genes, which are differentially expressed in osteocytes compared with osteoblasts. The results also suggest that Phex is expressed at higher levels in chicken osteocytes compared with osteoblasts. Reverse-transcription polymerase chain reaction (RT-PCR) and Northern blot analyses supported these findings. The function of Phex is not completely understood. However, it is known that the gene is preferentially expressed in bone and that mutations in *PHEX/Phex* lead to X-linked hypophosphatemia and bone mineralization abnormalities. Our findings suggest that osteocytes play an important role in the Phex-regulated phosphate handling in the kidney and in bone. (J Bone Miner Res 2002;17:845–853)

Key words: osteocytes, OB7.3, Phex, suppression subtractive hybridization

INTRODUCTION

THE MOST common cells of bone are the highly differentiated osteocytes. During bone formation a number of osteoblasts are embedded in newly-formed matrix, where they develop from cuboidal into more stellate-shaped cells, which ultimately differentiate into osteocytes. During this process, the cells remain in contact with each other and with the osteoblasts on the bone surface via slender cell processes, thus forming a three-dimensional cellular network throughout the bone. Osteocyte morphology and position have led several investigators to suggest a pivotal role for osteocytes in the perception of local changes in strain and in the adaptation of bone mass and structure to mechanical loading.[1,2] However, this same position of osteocytes, embedded in the mineralized matrix, makes them quite inaccessible to study.

In 1986 we developed a monoclonal antibody (MAb OB7.3), which was directed against avian osteocytes.[3] This antibody proved to be highly specific for osteocytes

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and recognizes an antigenic site on the osteocyte surface.[3–5] It has enabled us to purify osteocytes from mixed–bone-cell preparations, enzymatically isolated from fetal chicken calvariae.[4] In culture, osteocytes readily extend cell processes to form cellular networks, but they do not proliferate.[6] Several studies using isolated osteocytes have pointed out the high sensitivity of these cells to mechanical loading,[7–10] corroborating the above suggested role of osteocytes in the mechanoregulation of bone.[1,2,11,12]

Until now, the nature of the OB7.3 antigen has not been elucidated. In this study, we have identified the antigen to which MAb OB7.3 is directed. We have used MAb OB7.3 to immunoprecipitate the antigen from whole body lysates of 10-day-old fetal chickens. Amino acid sequence analysis of the purified protein resulted in two fragments; both showed homology to human and murine PHEX/Phex (formerly PEX). PHEX (*PH*osphate-regulating gene with homology to *E*ndopeptidases on the *X* chromosome), is a protein constructed of 749 amino acids, with a short *N*-terminal domain, one transmembrane domain, and a large extracellular *C*-terminal domain with a zinc-binding motif and 10 cysteine residues.[13] It belongs to the M13 family of metalloendopeptidases. Mutations in *PHEX* have been shown to result in the dominantly inherited disorder, X-linked hypophosphatemia (XLH). In XLH, renal phosphate retention and vitamin D metabolism is impaired, and patients with XLH show hypophosphatemia, rachitic or osteomalacic bones, and growth retardation.[14–16] Phex protein and messenger RNA (mRNA) has previously been localized in murine bone.[17] In this study the osteocyte-specificity of Phex protein expression was confirmed by our results obtained with suppression subtractive hybridization.[18] We used this method to identify genes differentially expressed in osteocytes compared with osteoblasts. One of the genes found to be preferentially expressed in osteocytes is *Phex*. The obtained complementary DNA (cDNA) stretch was 502 base pairs (bp) long and showed high homology to human and murine *PHEX/Phex* sequences. Reverse-transcription polymerase chain reaction (RT-PCR) and Northern blot analyses supported this finding by showing that Phex mRNA expression was higher in the isolated chicken osteocyte population than in the osteoblast and osteoblast precursor populations.

MATERIALS AND METHODS

Isolation and culture of chicken bone cells

Fetal chicken calvarial cells were isolated as described before.[6] In short, calvariae were aseptically dissected from 18-day-old chicken fetuses. The periostea were removed and sequentially treated with collagenase (1 mg/ml in phosphate-buffered saline [PBS]; Sigma, Zwijndrecht, The Netherlands) in PBS, to isolate periosteal fibroblasts (PF), a population representing osteoblastic precursor cells. After isolation, PF were cultured in "complete medium" consisting of minimum essential medium (αMEM; Gibco BRL, Breda, The Netherlands) supplemented with 2% chicken serum (Gibco BRL), 200 μg/ml glutamine (Sigma), 50 μg/ml gentamicin sulfate (Sigma), 50 μg/ml L-ascorbic acid (BDH, Amsterdam, The Netherlands), and 1 mg/ml D-glucose (BDH).

After removal of the periostea, calvariae were treated sequentially with collagenase (Sigma), and 4 mM EDTA in PBS (to obtain a mixed population of osteoblasts and osteocytes [OBmix]). The OBmix cell population was cultured in "complete medium." The next day, osteoblasts (OB) were separated from osteocytes (OCY) by immunomagnetic separation, using the OCY-specific monoclonal antibody MAb OB7.3.[3] Briefly, OBmix cells were harvested by 3 minutes of 0.05% trypsin/0.01% EDTA treatment at 37°C. The obtained cell suspension was filtered through a nylon filter (pore size, 30 μm). The resulting single cell suspension was incubated with MAb OB7.3 bound to immunomagnetic beads via a DNA linker (Dynal, Etten-Leur, The Netherlands). The cell suspension was placed in a magnetic field, which attracted the bead-bound OCY to one side of the tube. The nonbound cells (OB) were removed, leaving only OCY behind. The OB population was treated a second time with MAb OB7.3 bound to magnetic beads to ensure the removal of all OCY from the OB population.

Surface-labeling of cells with iodine-125

OBmix cells were isolated and cultured for 24 h as described above. Approximately 4×10^6 cells were harvested by 3 minutes trypsin/EDTA treatment at 37°C, washed 3 times in PBS, and subjected to lactoperoxidase-catalyzed cell surface iodination.[19] After labeling, cells were incubated in lysis buffer[20] for 2 h at 4°C. The resulting lysates were centrifuged at 13,000*g* for 15 minutes at 4°C to remove insoluble material. Supernatant was used for immunoprecipitation.

Immunoprecipitation of iodinated protein with MAb OB7.3

Radioactively labeled lysate, obtained as described above, was precleared according to Jordens et al.[20] with some adjustments. In short, 100 μl of protein A Sepharose beads (100%) (Amersham Pharmacia Biotech, Roosendaal, The Netherlands) and 5 μg of normal mouse immunoglobulin G (IgG) (Sigma) were added for the first preclear step. For the second preclear step, 100 μl of protein A Sepharose beads (100%) were added to the supernatant. Next, immunoprecipitation was carried out by incubating the supernatant with 7.5 μg of MAb OB7.3 for 1 h at room temperature, while continuously mixing. Subsequently, 30 μl of protein G Sepharose beads (Amersham Pharmacia Biotech) (50%) were added, and the suspension was incubated for another hour. The sample was centrifuged for 30 s at 13,000*g*, the supernatant discarded, and the pellet washed 4 times with lysis buffer. After this, the pellet was resuspended in gel-loading buffer (containing 2.5% β-mercaptoethanol, 60 mM Tris, 1.25% sodium dodecyl sulfate [SDS], 12.5% glycerol, and 0.0125% bromophenol blue) and incubated for 20 minutes at room temperature and for 3 minutes at 100°C, followed by analysis on 7% SDS-polyacrylamide gel elec-

trophoresis (PAGE). The gel was dried, and radioactive protein was visualized on an autoradiogram.

Isolation of large quantities of MAb OB7.3 precipitate

To isolate large quantities of the antigen of MAb OB7.3 for sequencing purposes, 10-day-old chicken embryos (from which the head, legs, and wings had been removed) were minced. Minced fragments were washed with Hanks' balanced salt solution and centrifuged, and the pellet was resuspended in the lysis buffer (10 embryos in 50 ml) mentioned earlier. The suspension was mixed well and incubated overnight at 4°C, with continuous mixing, to ascertain complete lysis. The next day, samples were sieved and centrifuged for 15 minutes at 4°C at 400*g* to remove all insoluble material. Supernatant was used for immunoprecipitation as described above, except 1 ml of protein A Sepharose beads (50%) and 25 μg of normal mouse IgG for the first preclear, 400 μl of protein A Sepharose beads (50%) for the second preclear followed by 125 μg of OB7.3 (incubated for 6 h at 4°C), and 80 μl of protein G Sepharose beads (50%) (incubated overnight at 4°C) for immunoprecipitation. Immunoprecipitates were run on a 7% SDS-PAGE gel (under reducing conditions), which was stained with Sypro Ruby (Molecular Probes, Leiden, The Netherlands) overnight (without fixing the gel). Appropriate bands were cut out and kept frozen until further use. Purified protein samples were commercially analyzed using Edman degradation (Eurosequence, Groningen, The Netherlands).

Suppression subtractive hybridization

We used the PCR-Select cDNA Subtraction Kit (Clontech, Heidelberg, Germany), according to the manufacturer's instructions, to compare gene expression in isolated chicken osteocytes with that in osteoblasts. In short, cDNA was amplified from total RNA of both cell populations, using the SMART cDNA Amplification Kit (Clontech). The resulting cDNA samples were digested with *Rsa*I. Subsequently, the OCY cDNA was subdivided into two portions and each was ligated to a different double-stranded cDNA adaptor. In the first hybridization step, an excess of OB cDNA was added to each sample of OCY cDNA, and the samples were heat-denatured and allowed to hybridize for 6 h at 68°C. Hereafter, the two primary hybridization samples were mixed, a fresh aliquot of denatured OB cDNA was added, and the samples were allowed to hybridize for 16 h at 68°C. During hybridization, several double-stranded cDNA hybrids were formed,[15] but only hybrids containing two different adaptors could be exponentially amplified by suppression PCR. These fragments represent genes, which are differentially expressed in OCY. The resulting subtracted cDNA pool was cloned using the TOPO TA Cloning Kit (Invitrogen Life Technologies, Groningen, The Netherlands).

RNA isolation, cDNA synthesis, and PCR

For RT-PCR experiments, equal amounts of OCY, OB, and PF were isolated as described above. Cells were lysed in appropriate volumes of RNAzol B (Campro Scientific, Veenendaal, The Netherlands). Total RNA was extracted according to the manufacturer's instructions. Obtained RNA was used to reverse transcribe cDNA. To check for genomic DNA contamination, controls were incorporated in which RT enzyme was omitted.

PCR reactions were done using specific primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) chicken mRNA[21] and for chicken Phex mRNA. GAPDH primers: 5′-CACGCCATCACTATCTTC-3′ and 5′-CACAATGCCAAAGTTGTC-3′. Phex primers: 5′-GTGAAACCACTGTTAAGCATCC-3′ and 5′-TCGTGAAGCATTTGCACC-3′. All primer combinations were designed using the Prime program of the Wisconsin Sequence Analysis Package (Genetics Computer Group, Madison, WI, USA). PCR reactions with GAPDH primers were used to correct for the starting amount of cDNA in the different samples. To compare Phex mRNA expression relative to GAPDH mRNA expression in the various populations, semiquantitative RT-PCR was performed, using the titration method.[22] After an initial denaturing of 5 minutes at 94°C, serial dilutions of the cDNA samples were amplified in a thermal cycler (2400; Perkin Elmer, Wellesley, MA, USA) for 32 cycles of 30 s of denaturation at 94°C, 30 s of annealing at 56.3°C (GAPDH)/57.5°C (Phex), and 30 s of extension at 72°C, followed by a final extension of 72°C. PCR reaction mix consisted of 1× PCR buffer (Perkin Elmer), 2 mM $MgCl_2$ (GAPDH) or 1.5 mM $MgCl_2$ (Phex), 0.2 mM dNTPs, 0.4 μM of each primer, and 0.5 U AmpliTaq (Perkin Elmer). PCR fragments were separated by gel electrophoresis and blotted to Hybond-N^+ nylon membranes (Amersham Pharmacia Biotech) in 0.4 M NaOH. The resulting blots were hybridized with probes specific for GAPDH and Phex chicken mRNAs, which were made by PCR with the primers described above, isolated from gel (Qiagen Gel Extraction Kit; Qiagen, West Sussex, UK), and sequenced to ascertain their correct sequence. Isolated fragments were labeled with [α-^{32}P]deoxycytidine triphosphate (dCTP) using the Ready-To-Go DNA Labeling Beads (Amersham Pharmacia Biotech). Unincorporated nucleotides were removed by running the probes over a ProbeQuant G-50 Micro Column (Amersham Pharmacia Biotech). Prehybridizations and hybridizations were performed at 65°C in NaPi/SDS hybridization buffer (0.4 M Na_2HPO_4, 0.1 M NaH_2PO_4, 7% SDS, and 1 mM EDTA). Blots were hybridized overnight and were washed the next day as follows: 2 times at 65°C with 2× saline-sodium citrate (SSC)/0.1% SDS for 20 minutes, 2 times for 20 minutes with 1× SSC/0.1% SDS, and once with 0.3× SSC/0.1% SDS. Blots were exposed to a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA, USA), scanned the next day, and net-intensities were determined using ImageQuant software (Molecular Dynamics). The experiment was repeated six times.

Northern blot analysis

For Northern blot analysis, cells were isolated as described above. Cells were lysed in appropriate volumes RNAzol B (Campro Scientific). Total RNA was then ex-

tracted according to manufacturer's instructions. RNA pellets were resuspended in water; OCY in 5 μl and OB and PF in 20 μl. Quality and quantity of the RNAs were analyzed on a 1% Tris-borate-EDTA (TBE) agarose gel stained with ethidium bromide.

RNA samples, approximately 5 μg, were fractionated by electrophoresis on a 1% TBE agarose gel with 5 mM guanidine thiocyanate salt.[23] Gels were stained with ethidium bromide afterwards, denatured in 7.5 mM NaOH, and transferred onto Hybond-N^+ membranes. Northern blots were prehybridized and then hybridized overnight at 55°C in NaPi/SDS hybridization buffer with radioactively labeled GAPDH and Phex probes. Both probes were obtained by PCR with the primers discussed earlier. The blots were washed the next day 2–3 times in 2× SSC/0.1% SDS at 60°C and analyzed as described above. The experiment was repeated six times.

RESULTS

Immunoprecipitation with MAb OB7.3

Immunoprecipitation experiments with MAb OB7.3 on protein lysates obtained from OBmix cultures labeled with ^{125}I revealed a distinct band at approximately 89 kDa on SDS-polyacrylamide gels under reducing conditions. This band was absent in the negative control, which was obtained by using an antibody specifically directed against human monocytes for immunoprecipitation (Fig. 1A). Because only small numbers of OBmix (and thus OCY in particular) can be isolated, we used protein lysates of minced 10-day-old chicken embryos to obtain enough material for the quantitative isolation of the 89-kDa protein. After staining with Sypro Ruby, one of the bands obtained from this large immunoprecipitate on SDS-PAGE (Fig. 1B; SR) was found to run at exactly the same position as the radioactively labeled 89-kDa band, when running both samples in the same lane (Fig. 1B; RA). Using 10 minced 10-day-old chicken embryos for immunoprecipitation, 800 ng to 1.8 μg could be isolated. Bands of several experiments were collected, pooled, and commercially analyzed for amino acid sequence using Edman degradation. Two peptide sequences were obtained, consisting of 10 and 14 amino acids respectively. These sequences clearly demonstrated that MAb OB7.3 precipitated a protein, which is most probably the chicken homologue of the Phex protein.

The amino acid sequence of the peptide, consisting of 14 amino acids, differed only one amino acid from the murine Phex protein sequence, having a threonine instead of a serine at position 498 (NP_035207). It differed by two amino acids from the human PHEX protein sequence, again having a threonine instead of a serine, but also a serine instead of an alanine at position 500 (NP_000435; Fig. 2A). The sequence of the other peptide, 10 amino acids long, had 100% identity with both the human and murine PHEX/Phex proteins. Both fragments represent part of the extracellular domain of the chicken Phex protein (Fig. 2A).

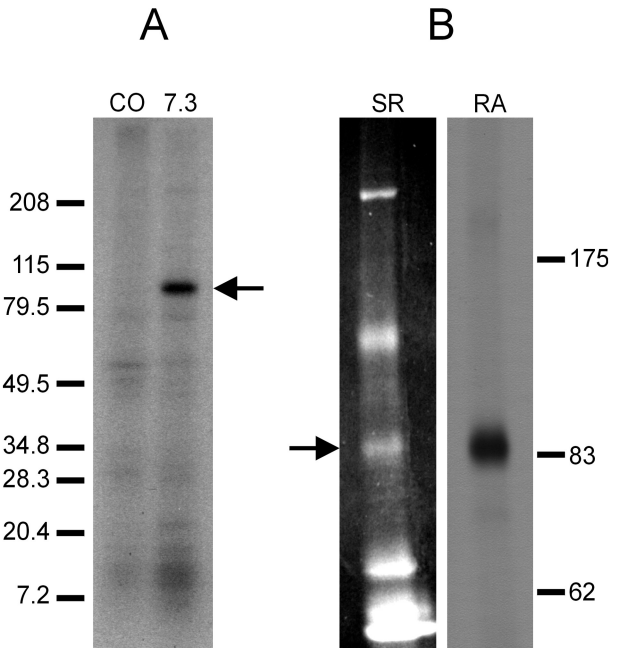

FIG. 1. Immunoprecipitation with MAb OB7.3. (A) Radioactively labeled OBmix lysate, immunoprecipitated with FK24 (an antibody specifically directed against human monocytes [CO]), and lysate immunoprecipitated with MAb OB7.3 (7.3) were run on a 5–20% gradient SDS-PAGE. MAb OB7.3 precipitated one band of ~89 kDa (arrow). (B) Embryo lysate immunoprecipitated with MAb OB 7.3 was run on a 7% SDS-PAGE, while radioactively labeled immunoprecipitate was loaded in the same lane. The gel was stained with Sypro Ruby (SR), photographed under ultraviolet (UV) illumination, dried, and visualized by autoradiography (RA).

Suppression subtractive hybridization

We have used suppression subtractive hybridization to compare gene expression in isolated chicken osteocytes and osteoblasts. A large number of clones was obtained, and sequence analysis revealed that approximately one-third of the clones showed homology to known genes (A. van der Plas, K. E. de Rooij, and P. J. Nijweide, unpublished data, 1998). Two of these clones contained sequences (GenBank Accession No. AF093205) that showed 77% and 76% identity with the human and murine *PHEX*/*Phex* genes, respectively (Fig. 2B). The predicted amino acid sequence showed 82% identity and 91% homology to the human and murine PHEX/Phex proteins, respectively, and is part of the large extracellular domain of the protein (Fig. 2B).

Expression of Phex mRNA in isolated bone cells

Primers were constructed from the 502-bp sequence representing chicken Phex cDNA and were used in semi-quantitative RT-PCR experiments, using RNA derived from OCY, OB, and PF (Fig. 3). OCY seemed to have a significantly ($p < 0.05$, Students t-test with Bonferroni correction, $n = 6$) higher expression of Phex mRNA compared with the other two cell types (22.9 $\pm$ 5.6 times higher compared with OB and 8.4 $\pm$ 2.7 compared with PF

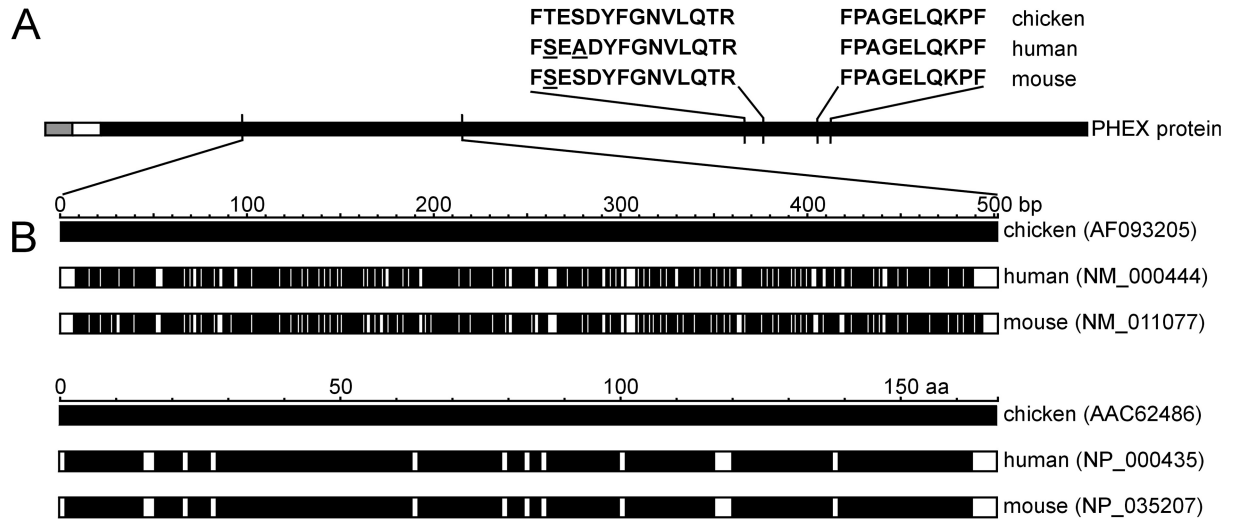

FIG. 2. Alignment of protein fragments and cDNA against human and murine PHEX and their position on the PHEX protein. (A) Amino acid sequences of the peptide fragments, which were obtained by immunoprecipitation with OB7.3, are aligned against PHEX/Phex protein amino acid sequences of human and mouse. The most *N*-terminal peptide shows one difference with the murine protein while it differs in two amino acids from the human sequence. The most *C*-terminal peptide shows complete homology to both human and mouse proteins. (Plain letters indicate identity, underlined letters indicate homology.) (B) Alignment of the cDNA fragment and its predicted amino acid sequence, obtained by suppression subtractive hybridization, aligned against human and murine PHEX/Phex cDNA. The black areas in the nucleotide alignment depict identities (overall, 77% and 76% for human and mouse, respectively), while the black bars in the amino acid alignment depict identities and homologies (overall, 82% identity and 91% homology for both human and mouse). In the PHEX protein, the black area indicates extracellular domain, the white area depicts transmembrane domain, and the gray area depicts the cytoplasmic domain.

[means ± SEM]). RT-PCR of GAPDH was used to correct for differences in RNA content of the cDNA samples.

These results were supported by Northern blot analysis. Northern blots of OCY, OB, and PF RNA hybridized with a chicken Phex cDNA probe, resulted in an ~8-kb band for all three cell types (Fig. 4A). Relative expression intensities (Phex/GAPDH) were determined and statistically analyzed after square-root transformation (because the original data were not normally distributed). Again, OCY showed a higher expression of Phex mRNA compared with OB or PF (Fig. 4B; $p < 0.05$, Students *t*-test with Bonferroni correction, $n = 6$). In particular, the signal in OB was very low compared with OCY and PF. Only when three times as much OB RNA was loaded on the gels could a signal be detected. Even then, the signal was much lower than the PF signal (Fig. 4B). Remarkably, the transcript found in OCY seemed to be somewhat larger than the transcript observed in PF (OCY ~8.5 kb vs. PF ~8.2 kb; means of six observations).

DISCUSSION

In this study we investigated the identity of the antigen of monoclonal antibody MAb OB7.3, which earlier was found to be highly specific for osteocytes in chicken bone tissue sections and chicken bone cell isolates.[3] Using MAb OB7.3 in immunoprecipitation experiments, we isolated an 89-kDa protein. Amino acid sequence analysis of two peptides of this protein showed that it is highly homologous to human and murine PHEX/Phex protein. The protein was therefore designated chicken Phex. The fact that we isolated an 89-kDa protein, while others have isolated a 97–100-kDa protein from mice,[24,25] probably indicates differences between species; for instance, in glycosylation patterns or in amino acid sequence. This idea is supported by the fact that the expected molecular weight of human PHEX is calculated to be 86 kDa (ExPaSy Accession No. P78562; www.expasy.org). Furthermore, Lipman et al.[26] found that in vitro translation of human PHEX cRNA leads to an ~86-kDa protein in the absence of microsomal membranes, whereas addition of canine microsomal membranes lead to a protein of ~100 kDa. This is consistent with *N*-glycosylation of PHEX at the eight predicted glycosylation sites. More recently, Ruchon et al.[25] also showed in mice that *Phex* encodes a 100-kDa glycoprotein, which migrated with an apparent mass of 82 kDa after peptide-N-glycosidase F (PN-Gase F) treatment, also suggesting that the unglycosylated protein is approximately 82–86 kDa, whereas the glycosylated protein is approximately 100 kDa.

Previous studies have already shown that PHEX/Phex mRNA, in both human and mouse, is preferentially expressed in bone and (developing) teeth.[27,28] Furthermore, Ruchon et al.[25] reported that monoclonal MAb 13B12, raised against human PHEX, specifically stained osteoblasts and osteocytes in sections of mouse bone tissues. MAb OB7.3 was previously shown to be highly specific for avian bone tissues. Liver, spleen, small intestine, skin, endothelium, and kidney, of avian origin, were all found to be negative for the antibody.[3] In bone, MAb OB7.3 specifically stained osteocytes,[3,4] but not osteoblasts, in contrast to the antibodies used by Ruchon et al.[28] and Miao et

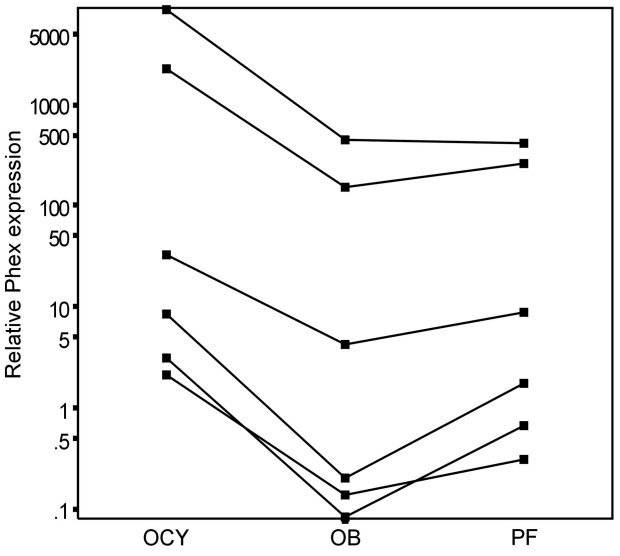

FIG. 3. Expression of Phex mRNA in isolated bone cells determined by RT-PCR. RNA was isolated from osteocytes (OCY), osteoblasts (OB), and periosteal fibroblasts (PF); reverse transcribed; and used in RT-PCR experiments to determine the relative expression of Phex mRNA corrected for GAPDH mRNA expression in the three cell populations. The expression compared with OB (OCY[Phex/GAPDH expression]/OB[Phex/GAPDH expression]) in the individual experiments are shown. OCY seemed to have a significantly ($p < 0.05$, Students *t*-test with Bonferroni correction, $n = 6$) higher expression of Phex mRNA compared with the other two cell types.

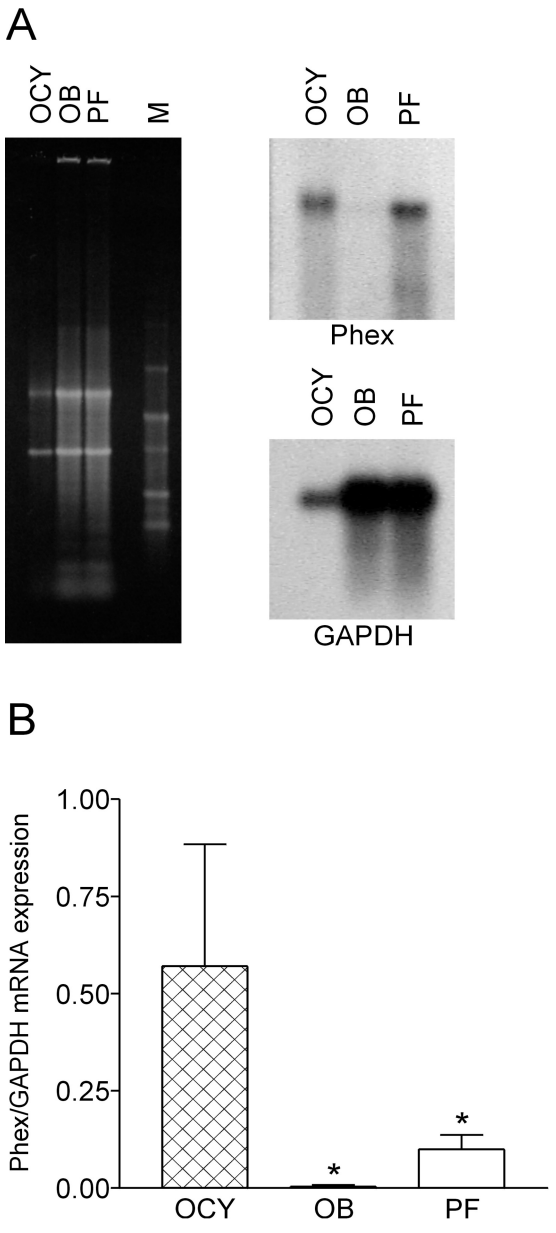

FIG. 4. Expression of Phex mRNA in isolated bone cells determined by Northern blot. Total RNA of osteocytes (OCY), osteoblasts (OB), and periosteal fibroblasts (PF) was fractionated on a 1% agarose gel, stained with ethidium bromide, and blotted onto a nylon membrane. The blot was hybridized with radioactively labeled GAPDH and Phex probes. (A) Example of a representative ethidium bromide–stained gel and its Northern blot. (B) Graphical representation of the relative expression of Phex mRNA corrected for GAPDH mRNA expression in the three cell populations. OB and PF show a significantly lower expression of Phex mRNA compared with OCY (*$p < 0.05$, Students *t*-test with Bonferroni correction, $n = 6$). M, RNA marker (NEB #362; New England Biolabs, Leusden, The Netherlands).

al.,[29] which stained both osteocytes and osteoblasts in murine bone tissue, although according to Miao et al.,[29] the immunohistochemical staining was stronger in osteocytes than in osteoblasts.

This difference in immunocytochemical staining, mouse versus chicken, may derive from species differences. The osteocyte specificity of MAb OB7.3 was further demonstrated when the antibody was used to purify MAb OB7.3 positive cells from enzymatically isolated bone cell populations,[4] and in culture, these cells behaved very much like osteocytes in situ. Their morphology in culture was very similar to that of osteocytes in situ, stellate cells with long, slender, often branched cell processes. They formed networks of separate, but cell processes-connected cells. Such networks were formed either by moving away from each other when the cells were seeded in clumps[3] or by reaching for each other with their processes when seeded sparsely.[4] This specificity of MAb OB7.3 for osteocytes was recently confirmed by the elegant study of Kamioka et al.[5] These authors clearly showed in a three-dimensional model that only the osteocyte syncytium was positive for the antibody, whereas the osteoblasts on the bone surface were not.

Chicken *Phex* is one of the genes that, according to the suppression subtractive hybridization procedure, was found to be highly expressed in osteocytes. Considering the immunocytochemical staining results reported in our previous papers and the amino acid sequence of the immunoprecipitated OB7.3 antigen presented in this paper, we do not find this surprising. Using suppression subtractive hybridization, we have obtained a 502-bp long cDNA stretch, which showed high homology with human and murine *PHEX/Phex*. Subsequently, RT-PCR experiments using primers designed from the obtained cDNA sequence, as well as

Northern blot experiments using the RT-PCR amplicon as a probe, confirmed that chicken Phex mRNA is expressed to a much higher extent in the OCY population than in OB and PF populations. As far as the OB population is concerned, this finding is not unexpected, because the OB population was obtained from the OBmix by removal of MAb OB7.3 positive cells (OCY). Nevertheless, in culture, OB cells are clearly osteoblast-like, considering their relatively high alkaline phosphatase activity and a square, nonstellate morphology.[6] Because they still proliferate, the OB population probably represents young, nonterminally differentiated osteoblasts.[6] In addition, others[14,30] have found that Phex mRNA expression is up-regulated during osteoblast differentiation in vitro. Furthermore, Miao et al.[29] also described that in murine bone tissue, immunohistochemical staining of Phex protein was stronger in osteocytes than in osteoblasts, which also supports our findings that osteocytes have a higher Phex expression than either osteoblasts or periosteal fibroblasts. Remarkable however, is the fact that PF cells were negative in immunocytochemistry studies with MAb OB7.3, while RT-PCR and Northern blot experiments indicated the expression of Phex mRNA in these cells. Differences in sensitivity of the techniques may be an explanation. However, the fact that OCY may possibly have a somewhat larger transcript in Northern blot experiments than PF may indicate alternative splicing in the two cell types, leading to a slightly different Phex protein in OCY compared with PF. Further experiments should be done to confirm this result. Furthermore, it should be mentioned that others have found smaller transcripts in humans, mice, and rats.[17,26–28,31] This discrepancy may result from differences in the untranslated regions of the genes between the different species. Furthermore, differences in the RNA transcripts may also explain (part of) the differences between the molecular weight of the proteins in the different species.

PHEX/Phex protein is a metalloendopeptidase for which the substrate is not yet known. Mutations in the human/murine *PHEX/Phex* gene have shown to be responsible for XLH in humans[32] and hypophosphatemia in the *Hyp* mice.[27] Parabiosis of normal mice to *Hyp* mice resulted in rapid development of *Hyp* phenotypic features in the normal mouse.[33] Separation of the normal and *Hyp* mouse resulted in normalization of the normal mouse. Several studies[34,35] have led to the hypothesis that PHEX/Phex enzyme either inactivates a circulating inhibitor of phosphate reabsorption or activates an inactive pro-hormone, which once activated, stimulates phosphate reabsorption.[13,14] The nature of this putative circulating factor is still unknown; however, recent indications have suggested that fibroblast growth factor (FGF)-23 may be the circulating substrate for Phex.[36–38] However, Guo et al.[39] did not find any evidence for cleavage of FGF-23 by recombinant wild-type Phex proteins.

Other studies demonstrated that abnormalities in a phosphate-regulating factor in the blood circulation are not the only explanations for the aberrant bone phenotype of XLH. Osteoblast dysfunctions also seem to be part of the disease.[40] Immortalized *Hyp* osteoblasts, which did not express Phex mRNA,[24] showed decreased mineralization of in vitro produced bone matrix compared with immortalized osteoblasts from normal, Phex mRNA–expressing mice.[41] Surprisingly, it was also found that *Hyp* osteoblasts produced a diffusible factor, which inhibited mineralization by normal osteoblasts in co-culture experiments. This suggests that bone cells not only express Phex but also produce a factor (by Phex activity) that regulates (local) phosphate metabolism.

The data shown in this paper that particularly osteocytes express Phex, combined with the fact that the osteocyte is by far the most abundant cell type in bone suggests exciting new ideas about osteocyte function. Osteocytes may be responsible for the regulation of mineralization by regulation of local bone phosphate metabolism. In addition, osteocytes may regulate the phosphate handling by the kidney, through Phex activity. Therefore, the osteocyte syncytium may have to be considered as a kind of secondary gland.

The most prominent role that has been attributed to the osteocyte, is that of the mechanosensory cell.[2] Today, modulation of the canalicular fluid flow resulting from compression/relaxation of the bone matrix is considered to be the most probable mechanism of mechanosignaling in bone. However, this strain-derived flow, for which experimental evidence is reported by Knothe Tate et al.[42] and Knothe Tate and Knothe,[43] may not only act as the mechano-signal–multiplying system of bone but also may improve osteocyte viability by facilitating exchange of nutrients and waste products.[44,45] Similarly, this mechanism may be crucial in the transport of the hypothetical phosphate-regulating factor to the osteocytes, where it is to be activated/deactivated by Phex. Therefore, it would be interesting to study whether immobility leading to decreased canalicular fluid flow also leads to disturbed phosphate metabolism and hypophosphatemia.

Although the complete sequence of chicken *Phex* is not yet elucidated, we conclude from the results obtained that the *Phex* gene is highly expressed in chicken osteocytes, both as protein and as mRNA. The PHEX/Phex enzyme is reported to be of crucial importance for phosphate metabolism in the body and locally in bone. Osteocytes are dependent on strain-induced movement of interstitial fluid through the lacuno-canalicular system for their survival, and they function as mechanosensory cells. The same movement of interstitial fluid and related facilitated diffusion of solutes may also be crucial for the metabolization of the yet unknown substrate of PHEX/Phex enzyme.

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