The CPPDD-Associated ANKH M48T Mutation Interrupts the Interaction of ANKH with the Sodium/Phosphate Cotransporter PiT-1

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ABSTRACT. Objective. Numerous dominant human homolog of progressive ankylosis (ANKH) mutations have been identified in familial calcium pyrophosphate dihydrate crystal deposition disease (CPPDD). Due to the dominant nature of these mutations, we investigated whether ANKH interacts with other proteins; and if so, whether any CPPDD-associated ANKH mutation might disrupt such protein interactions.

Methods. Stable ATDC5 ANKH wt- and ANKH M48T-transfectants were generated. Lysates from these transfectants were used to identify candidate protein interaction with ANKH by coimmunoprecipitation followed by Western blot analysis. The effect of high phosphate on the expression of genes involved in modulating Pi (inorganic phosphate)/PPi (inorganic pyrophosphate) homeostasis in these transfectants was assessed.

Results. We showed that ANKH protein associates with the sodium/phosphate cotransporter PiT-1, and that ANKH M48T mutant protein failed to interact with PiT-1. We also showed that upon high phosphate treatment, the normally coordinated upregulation of endogenous Ank and Pit1 transcript expression was disrupted in ANKH M48T transfectants.

Conclusion. Our results suggested that there is a coordinated interrelationship between 2 key participants of Pi and PPi metabolism, ANKH and PiT-1. (First Release April 15 2009; J Rheumatol 2009;36:1265–72; doi:10.3899/jrheum.081118)

Key Indexing Terms:
CALCIUM PYROPHOSPHATE DIHYDRATE CRYSTAL DEPOSITION DISEASE ANKH PIT-1 INORGANIC PHOSPHATE INORGANIC PYROPHOSPHATE

Elegant molecular studies on the recessive ank (progressive ankylosis) mouse led to cloning of the Ank gene that codes for a regulator of inorganic pyrophosphate (PPi) transport, and identification of the molecular basis of the ank mutation in this mutant mouse1. Since then, in humans, various dominant human homolog of Ank (ANKH) mutations were detected in patients with 2 rare diseases [craniometaphyseal dysplasia (CMD)2,3 and familial calcium pyrophosphate dihydrate crystal deposition disease (CPPDD)4-8]. The precise mechanisms whereby these ANKH mutations lead to disease development are not entirely clear. Studies using transgenic mice bearing a couple of these mutations showed that it is likely that patients with CMD have dominant negative ANKH mutations, while patients with familial CPPDD have “gain-of-function” ANKH mutations9. The dominant nature of ANKH mutations in these 2 diseases suggests that the ANKH protein might interact with a membrane or signaling component(s). To date, there is no published report on proteins that interact with ANKH. Examinations of the functional consequences of CPPDD-associated ANKH mutations have thus far been focused on the effect of these mutations on extracellular PPi levels, and the results remain controversial4,7,10. In situations where gain-of-function mutations are subjected to feedback transcriptional downregulation, the outcome measured from cell culture systems will depend on the delicate balance between the gain-of-function conferred by the mutation and the intrinsic compensatory dampening of the response, leading to inconsistent and contradictory results from different laboratories. Similar to an Ank wild-type transgene, a BAC transgene with the M48T mutation rescued the joint phenotype in Ank null mice. However, unlike patients with the ANKH M48T mutation, no pathological CPPDD was found in Ank null mice with the Ank M48T transgene9. It remains unclear whether this was due to the lack of an appropriate environment for CPPD crystal formation in mice.

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The control of systemic inorganic phosphate (Pi) homeostasis is known to be important for mineralization and bone formation. *ank/ank* mice and *Enpp1/–/–* mice on high Pi diet showed increased mineralization of bones, and ectopic mineralization in the extracellular matrix (ECM) of arteries and skin, implying that these mutant mice cannot maintain a balance in Pi/Pi homeostasis. Numerous studies in chondrocytes, cementoblasts, and osteoblasts showed that the expression profiles of genes that regulate [Pi] and [Pi] (such as *Ank*, *Enpp1*, *TNAP*, and *Pit-1*) are in turn changed by the Pi and Pi levels, indicating that the balance of Pi/Pi is strictly controlled by a complex interplay of these genes. Based on work with embryonic chick hypertrophic chondrocytes, Wang and colleagues proposed the following model for how Ank expression might affect Pi and Pi homeostasis. Enhanced Ank PiP transport activity results in an increased e[Pi] and hydrolysis of e[Pi] by TNAP. Pi resulting from PiP hydrolysis then enters the cell through Na+/Pi cotransporters (such as Pit-1). Transport of Pi into the cell results in a further stimulation of TNAP expression. The regulation of TNAP expression and activity by Ank ensure that sufficient TNAP activity is available at the outer membrane surface of growth plate chondrocytes and matrix vesicles to remove PiP, which is a potent inhibitor of mineralization. It is not entirely clear whether this model of regulation of TNAP expression by Ank in chicken hypertrophic growth plate chondrocytes is applicable to mammalian chondrocytes.

We recently showed that the *ANKH ΔE490* mutation in CPPD affects TNAP activities in stable ATDC5 transfectants, suggesting an interplay of *ANKH* and TNAP activities. In our study, we investigated whether another CPPD-associated mutation, *ANKH M48T*, might affect Pi homeostasis.

**MATERIALS AND METHODS**

*DNA transfection.* *ANKH* cDNA (*wt* and *M48T*) was subcloned into plasmid cytomegalovirus (pCMV) Tag 5A (Stratagene, La Jolla, CA, USA) in frame with the c-myc tag at the 3′ end. All constructs were sequenced to ensure that no mutations occurred during the cloning process. The *ANKH* constructs or the empty vector (*neo*) were transfected into ATDC5 cells, using Fugene 6 (Roche). The transfectants were selected and maintained in G418. To avoid clonal bias, populations of stable transfectants (from 2 separate transfections) were used in all experiments. Differentiation of ATDC5 transfectants was induced in culture medium with 1% ITS (insulin, transferrin and selenium; Sigma). The cultures were fed with fresh ITS-containing medium every 2 days.

*Immunoprecipitation and immunoblotting.* ATDC5 transfectants were lysed with 50 mM Tris·HCl, pH 7.5, with 150 mM NaCl, 1% NP40, and a mixture of protease inhibitors (leupeptins, pepstatin A, and antipain). An aliquot of the lysates was first immunoprecipitated with an anti-myc antibody (kindly provided by Dr. J.A. Winkles, University of Maryland School of Medicine, Baltimore, MD, USA) or an anti-Pit-1 antibody (Alpha Diagnostic) for 45 min and then with horseradish peroxidase-conjugated anti-goat antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 30 min. Specific signals were detected by chemiluminescence using Supersignal® West Femto maximum sensitivity substrates (Pierce Biotech) and imaging (BioRad).

*Real-time reverse transcription-polymerase chain reaction (RT-PCR).* RNA from the ATDC5 transfectants was prepared using the Trizol method. Oligo-dT primers were used for RT. Multiple dilutions of the RT mix were used for amplification to ensure linearity. The same primer pairs were used as reported. β-actin expression was used for normalization. PCR reactions in triplicates were carried out for each sample using IQ SYBR mix (BioRad). Melt curves were done at the end of each PCR. Relative quantification of gene expression was carried out using the 2–ΔΔCt method. Each sample was compared to the expression of *neo* controls at Day 0. At least 2 different RNA preparations and 4 separate experiments were carried out for relative quantification of expression of each gene transcript. For each gene, statistical significance (p value < 0.05) among the transfectants was calculated using 2-way analysis of variance (ANOVA) and pairwise multiple comparison procedures (Holm-Sidak method).

**RESULTS**

Effect of *ANKH M48T* mutation on the interaction of *ANKH* with *Pit-1*. We recently reported that ATDC5 transfectants stably expressing the *ANKH ΔE490* mutation had low alkaline phosphatase activities throughout its treatment due to lower TNAP protein expression and the presence of intracellular low molecular weight inhibitors. In view of recent reports that PiP homeostasis is strictly controlled by a complex interplay of genes that regulate Pi and PiP concentrations, we investigated whether *ANKH* physically associates with Pit-1 due to the dominant nature of the CPPD-associated *ANKH* mutations. Using various transfectant lysates, we immunoprecipitated the tagged ANKH proteins using an anti-myc antibody. The Western blot of the immunoprecipitates was probed with an anti-Pit-1 antibody. The same Western blot was also reprobed with an anti-ANK antibody. As the *neo* control transfectants expressed no myc-tagged ANKH proteins, we detected neither the ANKH-myc nor the Pit-1 bands (Figure 1A, lane *neo*). From the *ANKH wt*-transfectants, Pit-1 was detected in the anti-myc immunoprecipitates, suggesting that Pit-1 associated with ANKH wt proteins (Figure 1A, lane wt). Pit-1 was also coimmunoprecipitated with the myc-tagged ANKH ΔE490 mutant proteins. Interestingly, Pit-1 failed to coimmunoprecipitate with the myc-tagged ANKH M48T proteins. Similar amounts of ANKH-myc proteins were detected in the immunoprecipitates from all the transfectants except the *neo* control (Figure 1A, bottom panel). Further, similar amounts of Pit-1 protein were present in the lysates of *ANKH wt-* versus *ANKH M48T*-transfectants, indicating that the failure to coimmunoprecipitate Pit-1 with ANKH M48T proteins was not due to the lack of Pit-1 proteins in the lysates (Figure 1B).

*Coordinated upregulation of Ank and Pit1 gene expression was disrupted in ANKH M48T-transfectants upon treatment with high Pi.* We next assessed the functional outcome of the disrupted ANKH-Pit1 interaction consequent to the *ANKH*
M48T mutation. Our hypothesis is that ANKH-PiT-1 interaction ensures a balance in PPI export and Pi import. We anticipated that expression of genes involving Pi/PPI homeostasis would be dysregulated by treating ANKH M48T transfectants with high Pi. Thus, we treated ITS D17 ANKH transfectants (wt and M48T) and the neo controls with 0, 2, and 5 mM of Pi for 48 h and determined whether PiT-1, Ank, and TNAP transcript expressions were differentially affected. Real-time RT-PCR was carried out to assess the relative expression of PiT-1, Ank, TNAP, and PiT-1, and normalizations were carried out with \( \beta \)-actin expression. The primer-pair for Ank amplified both endogenous Ank and transfected ANKH transcripts. We do not expect that the transfected ANKH levels would be modulated by Pi treatment, as the transfected cDNA is driven by a CMV promoter. Treatment of neo controls with Pi in the culture medium resulted in a 1.6 to 2.7-fold increase in PiT-1 transcript expression (Figure 2A). Compared to neo controls, ANKH wt-transfectants had 1.6-fold higher, while ANKH M48T-transfectants had ~1.7-fold less PiT-1 expression in the absence of exogenous Pi. There were no significant changes in the levels of PiT-1 expression when either wt- or M48T-transfectants were treated with 2 or 5 mM Pi in the culture medium (Figure 2A). Neo controls cultured in 5 mM Pi showed a 1.8-fold increase in Ank transcripts (Figure 2B). No significant changes of total Ank/ANKH expression were detected in untreated or Pi treated ANKH wt-transfectants. In contrast, ANKH M48T-transfectants treated with 2 or 5 mM Pi showed a 2.4 and 1.9-fold increase in Ank transcript, respectively (Figure 2B). There was no change in TNAP expression in untreated or Pi treated neo controls (Figure 2C). Untreated ANKH wt-transfectants had 6-fold more TNAP transcripts than the neo controls. The TNAP transcript expression was downregulated (~4-fold) when the ANKH wt-transfectants were treated with 5 mM Pi (Figure 2C). Interestingly, untreated ANKH M48T-transfectants had 60-fold higher TNAP levels than the neo controls and Pi treatment led to a downregulation of TNAP expression in a dose-response manner. However, even at 5 mM Pi, ANKH M48T transfectants had ~9 to 14-fold more TNAP transcript than neo controls and ANKH wt-transfectants, respectively.

In summary, in neo controls, high [Pi] induced upregulation of Ank and PiT-1 expression coordinately. However, in ANKH M48T-transfectants, high [Pi] induced upregulation of Ank, but not PiT-1 expression. In addition, TNAP expression was significantly higher in untreated and Pi treated ANKH M48T-transfectants. We also investigated whether the expression of ENPP-1 (which codes for a PPI-generating ectonucleotidase) is affected by the overexpression of wt or mutant ANKH in the presence or absence of exogenous Pi. The expression of ENPP-1 was similar in ANKH wt-transfectants and neo controls (Figure 2D) in the absence of exogenous Pi. High [Pi] induced a 2-fold increase in ENPP-1 expression in ANKH wt-transfectants, but resulted in a ~2-fold decrease in ENPP-1 expression in neo controls. In contrast, in the absence of exogenous Pi, ANKH M48T-transfectants had a 2-fold increase in ENPP-1 expression compared to that of neo controls, and this level of ENPP-1 expression was maintained upon high [Pi] treatment (Figure 2D). As mentioned, these experiments were carried out in ATDC5 transfectants treated with ITS for 17 days (Day 17 ITS). The same set of experiments was also carried out in transfectants without ITS treatments, and similar results were obtained (data not shown).

Does overexpression of ANKH M48T change the kinetics of chondrocyte differentiation in ATDC5 transfectants? Neo controls and ANKH-transfectants (wt or M48T) were induced with ITS for 10, 17, and 23 days (ITS Day 10, Day 17, Day 23). At each timepoint, real-time RT-PCR was carried out to assess the relative expression of chondrocyte markers [3 Sox transcription factors: Sox 9, Sox 5, and Sox 6; 1 early chondrocyte marker: collagen IIa1 (Col 2a1); and 3 hypertrophic chondrocyte markers: bone sialoprotein (BSP), collagen X (ColIX), and matrix metalloproteinase (MMP-13). After normalization with \( \beta \)-actin expression, the results were expressed as fold-changes relative to the expression in Day 0 neo controls. For each timepoint, we also determined the ratio of expression (listed below each plot at each timepoint in Figure 3). The stable ANKH-transfectants (wt or M48T) had similar ANKH transcript and ANKH protein expression (data not shown). Overexpression of ANKH wt-proteins in ATDC5 cells at the prechondrocyte stage (Day 0 cells with no ITS treatment) appears to disturb the normal induction of chondrogenesis by ITS. Similar to ANKH wt-transfectants, the ANKH M48T transfectants also appeared to be more differentiated than the neo controls at
Figure 2. Expression of Pit-1 (A), Ank/ANKH (B), TNAP (C), and ENPP-1 (D) transcripts in different transfectants untreated (0) or treated with Pi (2 and 5 mM). Relative expression of different transcript was expressed as fold relative to that of neo controls (0 mM Pi). *Statistically significant difference compared to that of the corresponding transfectant without Pi treatment (0 mM Pi), using Holm-Sidak method of pairwise multiple comparison. Arrow bars denote standard deviation (n = 4).
Figure 3. Comparison of the kinetics of expression of Sox9, Sox5, Sox6, Col2a1, ColX, BSP, and MMP13 in ANKH wt-transfectants (solid lines) versus neo controls (dotted lines) versus ANKH M48T-transfectants (dashed lines) at Day 0, Day 10, Day 17, and Day 23 of ITS treatment. Expression of β-actin was used for normalization. “ratio” denotes expression in ANKH transfectants (either wt- or M48T-) over that of neo-controls. Arrow bars denote standard deviation (n = 4). *p < 0.001 (Holm-Sidak method of pairwise multiple comparison).
the prechondrocyte stage (Day 0), while lower levels of hypertrophic chondrocyte markers (such as ColX and BSP) were expressed at ITS Day 23 treatment (Figure 3 and Table 1). Thus, overexpression of ANKH M48T proteins affected the kinetics of normal hypertrophic chondrocyte differentiation in ATDC5 cells.

DISCUSSION
To mimic the dominant ANKH mutations found in familial CPPDD cells, we transfected ATDC5 cells that express Ank proteins with wt or mutant ANKH-myc constructs. Clonal ATDC5 ANKH transfectants have been used to study the effects of overexpressing either wild-type or various CPPDD-associated ANKH mutant proteins, and the results differed from our study. For example, Zaka, et al showed that overexpression of wt Ank protein had a 4.5-fold increase in Col 10α expression at ITS Day 2810, while we showed that overexpression of wt ANKH protein in a mixed population of ATDC5 stable transfectants had a 10-fold decrease in Col X expression at ITS Day 23. The reason for this discrepancy is not clear, but could be due to clonal bias in the study by Zaka, et al10. One of the differences between clonal versus mixed populations relates to the ratio of transfected ANKH (wt or mutant) and the endogenous Ank present in the ANKH transfectants. Further, we found that ITS stimulated the upregulation of endogenous Ank, but not the transfected ANKH (wt or mutant) and the endogenous Ank present in the ANKH transfectants. Thus during the course of induced hypertrophic differentiation of the ATDC5 transfectants, the ratio of the endogenous versus transfected forms also changed. In addition, Pi/PPi homeostasis is a dynamic process, likely subjected to feedback mechanisms. Taken together, in vitro culture systems investigated in different laboratories might have different [Pi]/[PPi] conditions and different ratio of transfected ANKH and endogenous Ank, leading to the generation of inconsistent and even contradictory results.

The physiological ionic extracellular [Pi] level is around 1 mM (varies from 0.8 to 1.2 mM). Feeding wild-type as well as anklank mice with a high phosphorus diet resulted in a 2-fold increase in serum [Pi], but enhanced joint mineralization was detected only in the anklank mice11. For this reason, we chose to treat the ATDC5 transfectants with 2 mM and 5 mM Pi, which would allow us to detect dose-response effects under conditions mimicking pathological [Pi] levels. We chose to transflect ATDC5 cells for the ease of generating mixed populations of stable transfectants and for the ability to follow the kinetics of endochondral chondrocyte differentiation. Although articular chondrocytes normally do not undergo hypertrophy in the uncalcified cartilage, chondrogenic events prior to chondrocyte hypertrophy are generally similar to those in endochondral chondrocytes. For example, similar levels of the major ECM proteins such as aggregan and collagen II were found in both surface articular chondrocytes and the growth-plate (resting zone) chondrocytes20. Thus, we chose to examine the effects of high Pi treatment on various ATDC5 transfectants at ITS Day 17, just prior to chondrocyte hypertrophy.

Treatment of the neo transfectants (ITS Day 17) with 5 mM Pi resulted in a coordinated upregulation of Pit-1 and endogenous Ank transcript, while there was no change in TNAP transcript expression. Treatment of the ANKH wt-transfectants (ITS Day 17) with 5 mM Pi did not lead to any significant changes in Pit-1 and Ank-ANKH expression, but there was a significant drop in TNAP expression (about 4-fold less than that of the ANKH wt transfectants with no Pi treatment). It is possible that the combination of both Pit-1 and Ank/ANKH expression was adequate to maintain Pi/PPi homeostasis in the presence of extracellular high Pi. However, an uncoordinated upregulation of Ank transcript, but not that of Pit-1, was detected when ANKH M48T-transfectants were treated with 5 mM Pi, possibly due to the inability of the mutant ANKH M48T protein to interact with Pit-1. Similar to the Pi-treated ANKH wt-transfectants, there was also a significant drop in TNAP expression when the ANKH M48T-transfectants were treated with 5 mM Pi (about 4-fold less than that of the ANKH M48T transfectants

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ANKH: human homolog of progressive ankylosis; Col: collagen; BSP: bone sialoprotein; MMP: matrix metalloproteinase; ND: not done.
with no Pi treatment). Possibly, these changes reflect an attempt to compensate for the Pi excess and restore an optimal Pi/PPi ratio. This notion is supported by the fact that a 2-fold increase in ENPP-1 transcript expression was detected in both untreated and Pi-treated ANKH \(M48^{T}\) transfectants (Figure 2D). As shown in Table 1, ANKH \(M48^{T}\)-transfectants had 2-fold higher alkaline phosphatase activities at Day 0, ITS Day 10, and Day 17. Taken together, our results suggest that the ANKH \(M48^{T}\) mutation leads to dysregulation of participants (such as PiT-1, ENPP-1, and TNAP) involved in the Pi and Ppi homeostasis.

Very little is known regarding the structure-function of the ANK/ANKH proteins. It is not clear how many transmembrane domains are present. Structural prediction of the ANKH protein using 11 different programs showed 7 to 12 predicted transmembrane domains\(^5\). We showed that the ANKH M48T proteins failed to interact with PiT-1. However, it is not clear whether ANKH binds to PiT-1 directly, and M48 might be a crucial contact point of the interaction. If the latter were true, M48 would be located intracellularly and thus would favor the 7-transmembrane model as predicted by the PRED-TMR program\(^5\). It is also unclear whether the ANKH M48T protein has the same conformation as the ANKH wt protein. Whether this disruption of ANKH-PiT-1 interaction is sufficient for the development of CPPDD is not known. The French kindred with the \(\Delta ANK^{H}\) mutation in ANKH-deficient mice bearing an ANKH \(M48^{T}\) transgene\(^9\), results from our studies using the ATDC5 transfectants also did not show any evidence that the ANKH \(M48^{T}\) mutation is an activating mutation. However, based on our results, avoiding high-phosphorus diet might be beneficial to patients with the ANKH \(M48^{T}\) mutation.

Previously, we showed that the CPPDD-associated ANKH \(\Delta E490\) mutation affects TNAP activities\(^15\). In this study, we showed that the CPPDD-associated ANKH \(M48^{T}\) mutation affects the interactions between ANKH and PiT-1, leading to a dysregulated expression of genes relating to Pi and Ppi homeostasis. Taking these results together, we hypothesize that CPPDD-associated ANKH proteins lead to a dysregulation of modulators of Pi/Ppi homeostasis such as PiT-1 and TNAP. It is likely that in familial CPPDD patients, the dynamic interplay of ANKH, PiT-1, and TNAP, consequent to the various ANKH mutations (ANKH \(\Delta E490\) and ANKH \(M48^{T}\)), led to the fluctuating local [Pi]/[Ppi] ratio, resulting in the formation of CPPD/HA crystals in the joints.

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