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The Role of ANK in Calcium Pyrophosphate Deposition Disease

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Abstract

The protein product of the progressive ankylosis gene, known as ANK, is a 492-amino acid multi-pass transmembrane protein. This protein is critical for the regulation of pyrophosphate, and gain of function ANK mutations is associated with calcium pyrophosphate deposition disease. Much about the structure, function, and regulation of ANK remain unstudied. This review of the current literature examines recent contributions to our understanding of ANK. We focus on new work on the function, binding partners, and regulators of ANK. A more complete understanding of this important protein may help to identify future therapeutic targets for the treatment of calcium pyrophosphate deposition disease.

Keywords

ANK; ANKH; Progressive ankylosis gene; Calcium pyrophosphate; Pseudogout; Chondrocalcinosis

Introduction

Calcium pyrophosphate deposition (CPPD) disease is arthritis characterized by articular calcifications composed of calcium pyrophosphate (CPP). While CPPD was initially described in patients with gout-like arthritis, there are several different phenotypes observed among patients who suffer from CPPD. The most commonly recognized form is acute CPP crystal arthritis (aka pseudogout) which presents with sudden onset of debilitating pain, swelling, warmth, and erythema of the affected joint. Many patients with CPPD disease suffer from a more indolent, chronic arthritis with episodic acute flares and severe joint damage. CPPD can mimic unusually distributed osteoarthritis or resemble rheumatoid arthritis. Risk factors for CPPD include advanced age, prior joint injury, and a handful of interesting metabolic syndromes, such as hyperparathyroidism, hemochromatosis, and hypophosphatasia.

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Conflict of Interest EMF, CMG, BB, and AKR declare that they have no conflicts of interest.

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The etiology of CPPD remains unclear. As a consequence, we lack specific therapies for this common and painful arthritis. Elegant work by Ryan et al. demonstrated a key role for local excess of pyrophosphate (PPi) in cartilage from patients with this disease [1]. Based on these studies, the prevailing theory of pathogenesis is that in affected patients, high levels of extracellular PPi complex with ambient calcium in the chondrocyte pericellular matrix to form CPP crystals. Thus, PPi plays an analogous role to that of urate in gout, and understanding the factors that regulate local PPi levels should result in the identification of therapeutic targets for CPPD.

Studies dating back as early as 1963 report familial cases of CPPD disease. With this observation came a potential to improve our understanding of disease mechanisms by identifying genetic abnormalities associated with this phenotype [2, 3]. Two loci, termed CCAL1 and CCAL2, were noted to be associated with CPPD. The protein product of CCAL1 remains unidentified. In contrast, the identification of CCAL2 on chromosome 5p as the progressive ankylosis gene product known as ANK has significantly contributed to our understanding of CPPD and is the focus of this review.

Structure and Distribution

ANK is a relatively recently discovered protein. It was described in 2000, by Ho et al. as the mutated gene product responsible for the abnormal phenotype in the *ank/ank* mouse [4]. The mouse protein is known as ANK, and the human homologue is correctly referred to as ANKH. For our current purposes, we will refer to both the mouse and human proteins using the term “ANK.” Human ANK is 492 amino acids in length and has a molecular weight of 53 kD. Kyte-Doolittle hydropathy plots showed 9–12 hydrophobic stretches, suggesting multiple loops through the membrane. The exact orientation of these domains remains to be elucidated. There are three *N*-glycosylation sites and multiple loci of potential phosphorylation [4].

ANK is a surprisingly understudied protein considering its high level of conservation across species and wide distribution. The human and mouse homologues of ANK share 483 of its 492 amino acids. A search of NCBI shows that this protein is seen in over 150 different tissues and cells including the eye, prostate, brain, lung, spermatozoa, cementum, and cartilage. Careful localization studies in various cell types and tissues have not been completed to date.

While there are multiple CPPD kindreds with ANK mutations [5], Uzuki et al. recently showed that ANK levels are higher in cartilage from patients with sporadic CPPD than those seen in the articular tissues of both osteoarthritic (OA) and normal patients [6••], thus confirming the earlier work of Hirose et al. [7]. ANK was found not only in the hyaline articular cartilage but also in the meniscus and the synovium. In situ hybridization revealed an increase of ANK messenger RNA (mRNA) as well as protein levels and high concentrations of ANK mRNA and protein in the vicinity of CPP crystal deposits. This work provides additional evidence that this protein is critically important in both sporadic and familial CPPD. Interestingly, in OA chondrocytes, ANK was found in the cytoplasm and not in the membrane of the chondrocytes. A role for ANK in OA warrants further study,

particularly as we learn more about the potential role of ATP as a potent stimulus of both nociceptive and innate immune responses.

Function

Major work on ANK's function coincided with the first gene description of *ank* in 2000. Ho et al. suggested that ANK was a PPi transporter, based on evidence showing that overexpression of wild-type ANK in Cos-7 cells increased levels of extracellular PPi and decreased levels of intracellular PPi [3]. The mutation of ANK seen in *ank/ank* mice appeared to be a loss of function mutation, and lower levels of extracellular PPi were noted when mutated ANK protein was overexpressed compared to those seen with wild-type ANK. Thus, *ank/ank* mice should have lower PPi levels in their articular tissues. Subsequent attempts to prove that ANK directly transports PPi using techniques such as expression in *Xenopus* oocytes have been inconclusive [8].

PPi is a critical regulator of pathologic calcification, based on its ability to inhibit calcium phosphate mineralization. This correlates nicely with the phenotypic changes seen in *ank/ank* mice, which develop periarticular calcifications and joint ankylosis composed of calcium phosphate. Calcification disorders associated with ANK might seem paradoxical but are clearly linked through the effects of PPi. ANK overexpression or overactivity leads to CPPD deposition in cartilage due to high levels of PPi. For complex reasons, CPPD deposition rarely occurs in non-cartilage tissue. ANK underexpression or loss of activity leads to calcium phosphate deposition in multiple tissues, due to loss of PPi's inhibitory effects on calcium phosphate formation and the ubiquitous nature of calcium phosphate mineralization.

Researchers hypothesized a role for ANK in ATP transport in chondrocytes [9] and in brain tissue [10, 11] as early as 2003. Extracellular ATP can be converted to PPi through the action of ecto-enzymes, such as ectonucleotide pyrophosphatase 1 (ENPP1), common to many cell types. Thus, an ATP transport function for ANK is also compatible with the data obtained by Ho et al. Recent studies contribute additional support for a role for ANK in ATP transport in chondrocytes [12••]. Rosenthal et al. transfected primary porcine articular chondrocytes with a short interfering RNA targeting ANK and measured extracellular ATP levels. ATP efflux was significantly reduced in ANK-silenced cells compared to a scramble control. To investigate a possible role for reduced PPi levels in this effect, exogenous PPi was added to ANK-silenced cells and ATP levels were measured. This did not restore ATP levels in the silenced cells compared to the scrambled control, which demonstrates that ATP efflux is not mediated by ePPi concentrations. Probenecid, which blocks ANK activity, also suppressed ATP efflux in chondrocytes. Whether ANK itself is a PPi transporter, an ATP transporter, or merely a regulator of these transport mechanisms will require further elucidation.

ANK has been demonstrated to affect cell differentiation, which may be dependent on its effects on PPi and ATP. Las Heras et al. used *ank/ank* mice to examine the progression of chondrocyte hypertrophy in postnatal articular cartilage [13]. Hypertrophy is an essential process of differentiation of growth plate chondrocytes during mineralization. They found

increased numbers of hypertrophic chondrocytes in the *ank/ank* mutant mice compared to wild-type controls and concluded that this loss-of-function ANK mutation may result in premature bone formation and joint ankylosis by accelerating chondrocyte hypertrophy. Using immunohistochemistry, they also found nuclear β-catenin in the young *ank/ank* mice, which has previously been found only in mature chondrocytes which stimulates hypertrophy and mineralization. ANK levels also modulate differentiation of erythrocytes, osteoblasts, and osteoclasts [14–16], although the mechanisms of these effects are not fully delineated.

Mutations and Single Nucleotide Polymorphisms

ANK mutations resulting in clinical phenotypes have important implications for our understanding of ANK functions. In addition to the phenotype observed in *ank/ank* mice, mutated ANK proteins are seen in some kindreds with familial CPPD and in craniometaphyseal dysplasia (CMD). CMD is characterized by overgrowth and sclerosis of the facial bones along with disordered metaphyseal modeling in long bones [17]. While there are differences in the mutated regions of *ank* that produce CMD compared to those associated with CPPD [18], ANK's role in CMD has not been approached mechanistically, but it seems to be a reduced function of the protein.

Spurred by the recognition of familial mutations in ANK associated with CPPD, numerous laboratories have conducted studies of these mutations in vitro. ΔE490 and M48T are two mutations that have been studied in the laboratory and are believed to be gain-of-function mutations. The ΔE490 mutation is a 3-amino acid deletion on the carboxyl terminus of ANK which results in late onset CPPD. Wang et al. transfected chondrogenic ATDC5 cells with wild-type or ΔE490 mutant proteins [19]. Overexpression of wild-type ANK led to decreased hypertrophic markers compared to untransfected cells after chondrogenic induction at 17 and 23 days. Overexpressing the ΔE490 ANK protein resulted in similar levels of chondrogenic markers compared to untransfected cells. Ultimately, the authors concluded that normal differentiation is disrupted in cells overexpressing ANK and delayed in ANK ΔE490 mutants.

A recent case report from Gruber et al. characterized a previously undescribed ANK exon 1 mutation in a young woman with (widespread) early-onset CPPD in which proline replaces serine at codon 5. The patient's father also displayed radiographic chondrocalcinosis and shared the same mutation. Two other known polymorphisms were also noted. Metabolic abnormalities in this family, including hyperparathyroidism and secondary phosphaturia have not been observed in other kindreds with ANK mutations [20•].

Abhishek and colleagues [21] utilized two hospital-based datasets to examine the correlation of chondrocalcinosis and a specific polymorphism in the 5′UTR of ANK, namely −4bpG>A transition. Chondrocalcinosis is the radiographic correlate of CPPD, but does not necessarily capture patients with clinically important arthritis. There was a strong association between the presence of this ANK polymorphism and chondrocalcinosis, which was independent of age and OA in this population. Previous studies have demonstrated that this polymorphism reduces intracellular PPi and increases ANK expression. However, these studies did not directly demonstrate an increase in PPi transport across the membrane [22,

23]. This important work provides further evidence for the importance of increased ANK levels and activity in CPPD.

Homeostatic Regulators of PPi

The role of ANK in CPPD must be considered in the context of other factors that regulate and participate in PPi metabolism. The ratio of PPi/Pi is a key regulator of mineralization in many tissues. There is ample evidence supporting coordinate regulation of the many participants in this critical process. The enzyme ENPP1 deserves mention as it may be regulated in concert with ANK. The *ENPP1* gene encodes a protein expressed in a variety of cells and tissues including matrix vesicles of bone and cartilage. This membrane-bound enzyme cleaves ATP to AMP and PPi. ENPP1-deficient *ttw/ttw* mice show articular calcification postnatally, a similar phenotype to the *ank/ank* mouse [4, 24]. A recent study of disc degeneration confirmed coordinate regulation of ANK and ENPP1 [25•]. Cadaveric cervical intervertebral discs (IVD) were used to examine the expression of proteins commonly associated with IVD degeneration and calcification including osteopontin (OPN), alkaline phosphatase (TNAP), transforming growth factor β1 (TGF-β1), ANK, and ENPP-1. In specimens with more severe IVD degeneration, ANK and ENPP-1 expression was suppressed. Tomaszewski and colleagues hypothesized that low levels of these proteins promoted calcium phosphate deposition and disc calcification. The other major factors in the PPi pathway that may be coordinately regulated with ANK include TNAP which hydrolyzes PPi and Pit-1 which acts as a sodium/phosphate transporter [26].

Binding Partners

The three-dimensional structure of ANK remains unknown, and it is not clear whether it acts as a transporter or simply regulates another transport mechanism. Wang et al. hypothesized an interaction between ANK and Pit-1, based on an observation that an altered response to Pi in cells expressing the M48T mutation [27], but a physical association between ANK and Pit-1 has not been proven. An important study of ANK-binding partners was recently undertaken in hopes of elucidating additional structural and functional aspects of ANK and its clinically important mutations. Using co-immunoprecipitation and yeast two-hybrid screenings, Minashima et al. found that ANK strongly interacts with two proteins. These include Myb-binding protein 1a (MYBBP1a) and sphingosine kinase 1 (SPHK1). SPHK1 stimulates NF-κB signaling, while MYBPP1a is involved in cell stress and senescence. MYBBP1a binds at the C-terminal loop of ANK, and ANK mutations at this location are associated with CMD [28••]. The authors showed that ANK levels as well as mutated ANK affected the cellular location of MYBBP1. SPHK1, on the other hand, binds to the N-terminal of ANK; mutations at this location are associated with CPPD. Decreased ANK function was associated with decreased SPHK1 activity which led to a reduction in the nuclear p65 subunit of NF-κB in IL-1β-treated articular chondrocytes. This interesting work suggests that ANK may regulate the location and functions of these proteins and thus modify the inflammatory and stress responses in cartilage.

ANK Regulation

Little is known about the regulation of ANK in cells and tissues (Table 1). Several cytokines, implicated in cartilage health and disease, have been demonstrated to regulate levels of ANK. For example, TGF-β1 increases levels of extracellular PPi in cartilage, and investigators have shown that TGF-β1 increases *ank* gene expression [7, 29]. ANK plays an important role in vascular calcification since the presence of PPi acts as an inhibitor of calcium phosphate mineralization. Zhao et al. showed that the cytokine, TNFα, induced vascular calcification [30•]. Overexpression of p65, a component of activated NF-κB, also induced calcification and suppressed ANK in the presence of TNFα. Therefore, the authors concluded that TNFα via NF-κB promotes vascular calcification by suppressing the expression of ANK and reducing PPi levels. These results were further supported by immunohistochemistry of human atherosclerotic lesions from patients with chronic kidney disease, which showed decreased ANK expression and activated NF-κB compared to vessels from healthy donors.

Additional support for a role for inflammatory mediators in regulating ANK levels was recently supplied by Nasi et al. They showed that IL-6, another important cytokine in crystal-induced arthritis, upregulated ANK, Annexin 5, and Pit-1 gene expression in murine cartilage explants [31].

ANK levels are regulated by mechanical stress, hypoxia, and injury [23, 32–34]. Recently, ANK regulation has been explored in models of cervical endplate degeneration using both rat and human in vitro models. The recent findings of Xu et al. suggest a role for JNK phosphorylation in ANK regulation [35•, 36•]. Effective degeneration was established by sequentially subculturing rat chondrocytes and noting the downregulation of typical chondrocyte markers, aggrecan, and type II collagen. Subsequently, they found JNK phosphorylation increased with cell passaging and ANK was significantly downregulated. Degenerative cells were then treated with a broad spectrum JNK inhibitor (SP600125) which reduced phosphorylation of JNK and increased ANK levels. This group next compared human cervical endplates from controls (cervical fracture) and degenerated samples and found similar results.

Pi levels also regulate ANK expression. Rendenbach et al. stimulated primary murine osteoblasts with extracellular Pi and performed a genome-wide expression analysis to identify potential gene targets [37]. Among the genes relevant to Pi homeostasis, *ank*, *ENPP1*, and *Slc20a1* (Pit-1) were upregulated after 6 h of Pi treatment. When MC3T3-E1 cells were transfected with a Pit-1-specific shRNA, levels of *ank* were unresponsive to Pi. This work demonstrates that ambient Pi levels regulate *ank* expression and reinforces the importance of an interaction between ANK and Pit-1.

The interactions between PPi concentrations and *ank* expression levels form a complicated feedback loop. Foster et al. studied ANK and ENPP1 levels during acellular cementum formation [38••]. Cementum covers the tooth root and is the “glue” that attaches the tooth root to the underlying bone. These researchers found that the morphology and extracellular matrix of the acellular cementum were dramatically altered by the disruption of local PPi

levels. During cementum apposition when PPi homeostasis is lost, cementoblasts in the area coordinately adapt the expression of ANK, ENPP1, and osteopontin to compensate. Another study showed elevated PPi levels depressed ANK expression in osteoblasts [24]. These findings support a “pathway” of PPi production which involves coordinate regulation of both ANK and ENPP1.

Conclusions

Ample evidence supports ANK’s association with CPPD and CMD, and increasing evidence supports its function as a transporter of either PPi or ATP. The contribution of ANK to the critical ratio of PPi to Pi is currently well supported, and we are beginning to delineate key regulators and binding partners of this protein. Thus, ANK may be important in the many settings in which pathologic or physiologic mineralization occurs. It is certainly an attractive potential therapeutic target in CPPD. Recent work demonstrates that probenecid, an FDA-approved gout drug, reduces ANK’s ability to transport ATP and PPi in vitro. Additional proof-of-concept studies are needed before we recommend using probenecid clinically. Certainly, the widespread distribution and highly conserved structure of ANK support potential roles in other cellular processes, and further work on this interesting protein is warranted.

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Table 1

Modulators of ANK levels

Modulator	Direction/endpoint	Cell/tissue	Pathway	Reference
TGFβ	Increase mRNA and protein	Cartilage; human and rat	Ras/Raf-1/ERK and calcium-dependent PKC	[39]
IL-1β	Decrease		? NF-κB	[40]
Mechanical stress	Increase and decrease mRNA	Rat endplate chondrocytes	?	[32, 41]
Methotrexate	Increase mRNA	Liver	?	[42]
Renal failure	Decrease mRNA	Vascular wall	? NF-κB	[30•]
TNFα via NF-κB	Decrease mRNA	Human aortic smooth muscle	Tristetraprolin (RNA destabilizer) mimics effects	[30•]
Hypoxia	Decrease mRNA	Cartilage and disc	HIF1 in articular cartilage; HIF1 and HIF2 in nucleus pulposus	[33]
IL-6	Increase	Murine chondrocytes	Crystal induced	[31]
Pi	Increase	Murine osteoblasts	? ERK	[37]