Expression and Localisation of the Pyrophosphate Transporter, ANK, in Murine Kidney Cells

Georgina Carr^a, John A. Sayer^{a,b} and Nicholas L. Simmons^a

^aEpithelial Transport Research Group, Institute for Cell and Molecular Biosciences and ^bSchool of Clinical Medical Sciences, Medical School, University of Newcastle upon Tyne

Key Words

Renal stone formation • Pyrophosphate • ANK • mIMCD-3 cells • Inner medulla • Mitochondria

Abstract

Background/Aims: Mutation of the pyrophosphate transporter, ANK, results in progressive arthritis in mice. ANK is expressed in non-skeletal tissues including kidney. The aim was therefore to investigate ANK location at the cellular and subcellular level in renal cells. **Methods:** RT-PCR identified a murine cell-line, mIMCD3, expressing ANK. The intra-renal distribution of ANK was determined by immunohistochemistry and the subcellular distribution in mIMCD3 cells by transfection of an ANK-NT-GFP fusion protein. Furthermore, an inactivating mutation of murine *ank*, Glu440X, and a gain of function mutation, Met48Thr, were tested to determine whether membrane traffic contributed to a transport defect. **Results:** ANK is expressed in cells of the cortical collecting duct, as assessed by colocalisation with aquaporin 2 and at the lateral and apical plasma

membranes of mIMCD-3 epithelial cells, as assessed by colocalisation with wheat germ agglutinin lectin (WGA). ANK-NT-GFP was also present in endoplasmic reticulum, Golgi, acidic endosomes and mitochondria. mIMCD3 expression of Glu440X ANK-NT-GFP shows evidence of Golgi retention whereas Met48Thr ANK-NT-GFP is unaltered at the plasma membrane compared to wild type. **Conclusion:** The intra-renal and subcellular localisation of ANK is consistent with pyrophosphate export from collecting duct cells and supports a role for ANK in limiting intra-renal calcium-crystal formation.

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Introduction

Renal stone disease (nephrolithiasis) affects over 10% of males and 5% of females over a lifetime [1] and causes substantial morbidity. Urinary concentration results in supersaturation, with respect to calcium salts, of fluid within the papillary interstitium and the collecting duct so

predisposing these areas to formation of abnormal crystal deposits [2-4]. Notwithstanding this supersaturation of calcium with either phosphate or oxalate ions, stone formation does not occur in the majority of normal individuals. This indicates the presence of intrinsic mechanisms to prevent stone precipitation at points of maximal urinary concentration (the loop of Henle, the renal papilla and the inner medullary collecting duct (IMCD)). A number of inhibitory anions and proteins exist in urine which are likely to act synergistically to limit crystallisation [5, 6]. Inorganic pyrophosphate (PP) is present in normal urine at μM levels to potentially inhibit crystallisation [7, 8].

A key question concerning PP presence in urine is its origin. Recently, ANKH, the protein encoded by the human *ANKH* gene and its murine homologue ANK have been identified as multi-pass plasma membrane PP transporters involved in the export of PP from the cell interior to the extracellular fluid [9-11]. In a mouse model of arthritis, where the *ank* gene is mutated (Glu440X, leading to a C-terminal deletion), there is decreased export of PP to the extracellular medium and enhanced levels of intracellular PP [9]. This decrease in extracellular PP is also associated with abnormal deposition of hydroxyapatite crystals in articular surfaces (progressive arthritis) [9]. The phenotype of *ank* mice, though reminiscent of human arthritis, does not precisely mimic any human clinical condition [9]. In fact, certain natural mutations associated with the human *ANKH* (e.g. mutation of the highly conserved Met 48 to Thr) give rise to a different phenotype, associated with gain of function [11]. Increased PP transport, in this instance, gives rise to local elaboration of CPPD (Ca-pyrophosphate dihydrate) crystals and chondrocalcinosis [11]. The *ank* gene is not only expressed in joints but is also expressed in non-skeletal tissue including the kidney [9]. In mutant *ank* mice, adults show renal calcification, indicating that lack of ANK function disrupts normal protective mechanisms operating to limit nephrocalcinosis / nephrolithiasis [9]. Although the clinical picture of ANKH deletion has not included renal disease, a recent retrospective cohort study has identified spondyloarthropathy as an independent risk factor for kidney stones [12].

The primary purpose of the present study was to substantiate renal expression of ANK by determining the sites of intra-renal expression. Since we identify the cortical collecting duct as a primary site of ANK expression we have identified a murine collecting duct cell-line (mIMCD-3) [13] with endogenous ANK expression in order to determine the sub-cellular

distribution of the ANK protein, using a combination of immunohistochemistry and expression of an ANK-NT-GFP fusion construct. Since altered membrane traffic may explain both diminished and elevated transport function, the effects of both the Glu440X and the Met48Thr mutations on the subcellular distribution of ANK-NT-GFP in renal cells have been determined.

Material and Methods

Cell culture and molecular reagents

mIMCD-3 cells were cultured as previously described [13-15]. Monolayers of mIMCD-3 cells were prepared by high density seeding (10^5 cells/filter) onto Transwell culture inserts (Costar) and cultured for 14 days with media replacement every 3 days. Alternatively, mIMCD-3 cells were grown to near confluency on 13mm coverslips [14-16]. mIMCD-3 or mouse kidney total RNA was isolated (Qiagen) and used as the template for RT-PCR reactions using mouse gene specific primer pairs for the *ank* transcript (490 bp product; 5'-CAT CAC CAA CAT AGC CAT CG-3' and 5'-CCA GGT GAC TGT GAA GCA AA-3'). Image clone 13877021 (full-length murine *ank* corresponding to BC054379 *mus musculus* progressive ankylosis) was obtained from MRC Geneservice (Babraham, Cambridge UK). High fidelity PCR using Expand PCR enzyme mixture (Roche) and 13877021 as template with full-length primers (5'-GCG GGG ACT ATG GTG AA-3' and 5'-CAG CAG CCT GTG CTT ACT-3') amplified full-length *ank* product that was directly cloned into expression vector pcDNA3.1/NT-GFP (Invitrogen Life Technologies). Sequencing confirmed orientation and fidelity of the ANK-GFP fusion protein construct. Mutagenic reverse sequence primers 5'-CTA GCA GGG TTT GTG GGA TAA TCT ACC CAT GGT TGC-3' and 5'-GGA GGA TGC AGT AGAAAC GCT GGC CAG CTACGG-3' were used to generate mutant ANK-NT-GFP constructs corresponding to the mouse loss of function mutant Glu440X (Glu440X ANK-NT-GFP) and the human gain of function mutant, Met48Thr (Met48Thr ANK-NT-GFP) respectively (QuikChange, Stratagene).

Renal and subcellular localization of ANK expression

Mice were sacrificed by cervical dislocation and their kidneys rapidly excised into ice-cold phosphate-buffered saline. Kidneys were decapsulated and bisected using coronal or sagittal cuts with a sharp razor. Tissue blocks were mounted in OCT on cork squares and rapidly frozen using liquid nitrogen cooled isopentane. Frozen kidney blocks were then stored at -80°C . $10\mu\text{m}$ cryosections were cut and mounted onto poly-lysine coated microscope slides. Tissue sections were then fixed in methanol at -20°C , prior to immunostaining. mIMCD-3 cells grown on coverslips were fixed and permeabilised with methanol. ANK was detected, after incubation in 3% horse serum by a rabbit anti-ANK polyclonal antibody (Ab3 raised against residues 477-492 of the mouse ANK sequence, generously provided by D.M.Kingsley, Stanford University

CA [9]) at 1:250. For cultured cells a secondary block with goat serum preceded incubation with goat anti-rabbit TRITC (1:100 & 1:25, Sigma). Control experiments omitted the primary antibody or used pre-immune rabbit serum instead of primary antibody (both negative). For kidney sections, identification of collecting ducts was achieved by co-incubation of Ab3 with goat anti-aquaporin2 antibody (sc9882, 1:250, Santa Cruz Biotechnology) followed by a secondary block with 3% donkey serum and donkey anti-rabbit FITC (Alexa Fluor 488, Molecular Probes) and donkey anti-goat TRITC (Alexa Fluor 568, Molecular Probes). Controls omitted the primary antibodies.

Localisation of ANK in mIMCD-3 cells was also determined in cells transiently transfected with ANK-NT-GFP using Lipofectamine 2000 reagent (Life Technologies) as per manufacturer's protocol. 24 post transfection, live cells were incubated with TRITC-conjugated wheat germ agglutinin (TRITC-WGA) lectin (1:50, Sigma) for 2min at 37°C to label the plasma membrane [13], or 75 nM LysoTracker Red (Molecular Probes) for 30min at 37°C to label acidic endosomes [14]. Co-transfection with pDsRed2-ER (BD Biosciences, targeted to endoplasmic reticulum with a calreticulin sequence) allowed identification of endoplasmic reticulum (ER). Alternatively, co-transfection using pDsRed2-mito was performed (BD Biosciences, incorporating the mitochondrial targeting sequence from subunit V111 of human cytochrome c oxidase). For co-transfection a ratio of 25:1 (μ gDNA, ANK-NT-GFP (or mutant): pDsRed was used). In addition to co-transfection, mitochondrial localisation was confirmed in methanol-fixed cells using the mouse monoclonal 7H10 against the α subunit of the F_1F_0 -ATPase (Molecular Probes/Invitrogen) at a dilution of 1:250 followed by goat anti-mouse Alexa-fluor (Invitrogen) for secondary detection. For staining of the Golgi apparatus (intermediate-to-*trans* stacks), cells were fixed and permeabilised 24h post transfection with methanol then incubated (room temperature) with biotinylated *Griffonia* lectin (20 μ g/ml, Vector Laboratories) for 30min, followed by streptavidin-Cy5 (20 μ g/ml Southern Biotech) for 1h at 37°C [16]. Nuclei were stained after fixation in methanol (above) and incubation with 25 μ g/ml propidium iodide (diluted in PBS) for 10min at room temperature.

In order to further demonstrate the specificity of antibody Ab3, immunofluorescence (using goat anti-rabbit TRITC) was determined after transient expression of ANK-NT-GFP or Glu440X ANK-NT-GFP lacking the epitope recognised by Ab3 under identical conditions. Quantification of cellular fluorescence was made using the Leica Qwin software. Individual cells were selected and component pixels highlighted, summated pixel intensity then being determined within the highlighted areas.

Microscopy

Cells or tissue sections were imaged using confocal laser microscopy (TCS-NT, Leica with Kr-Ar laser) with appropriate excitation and emission filter sets for dual fluorophore detection and z-series images were collected. Mid-cell (*x-y*) sections of cells transfected with ANK-NT-GFP (co-stained with TRITC-WGA), or mutants Glu440X ANK-NT-GFP, Met48Thr ANK-NT-GFP together with appropriate controls (empty vector GFP) were used to quantify ANK protein expression by analysis of

pixel intensity. For 'membrane' localization, circumferential transects were drawn across the cell. Pixel intensity for TRITC-WGA was then determined from background (near membrane but extracellular) values to the adjacent (often peak) pixel at the cell periphery [14]. This was then compared to equivalent pixel intensities for GFP fusion proteins [14]. A similar approach was used to analyse the expression of ANK-NT-GFP with the intracellular distribution of pDsRed2-mito. In order to quantify membrane ANK densities for control versus the Met48Thr ANK-NT-GFP construct, transfections were made under identical conditions and ANK at the plasma membrane determined as above but with 4 circumferential transects per cell [15] being drawn progressing clockwise around the cell under identical conditions of imaging, illumination intensity and photo-multiplier settings. This averaged intensity (8 readings per cell) then defined membrane (peripheral) ANK staining.

Analysis of cell volumes were made using the Leica TCS-NT quantification tools; transfectants were identified and imaged mid-cell. Cell boundaries were directly delimited on screen using the drawing tool and cell area calculated. Cell height was determined from the number of optical sections between cell apex and substrate.

All images are representative of multiple individual transfections (e.g. 15 wild-type ANK-NT-GFP, 7 transfections of Glu440X ANK-NT-GFP and 5 transfections of Met48Thr ANK-NT-GFP in which >3 transfectants were captured per transfection).

Pyrophosphate measurement

Inorganic PP was measured with a spectrophotometric method using the PP dependent fructose-6-phosphate kinase activity coupled to the disappearance of NADH [17] (Sigma Chemical Co. Product P7275). In order to increase the sensitivity, 0.4ml of sample or standard (0-40nmol NaPP) was added to 0.8ml of reconstituted reagent. mIMCD-3 cell layers were cultured in phenol-red free media for 48h and the apical and basal bathing solutions sampled directly. Cell layers were extracted with 0.5ml 1M perchloric acid for >2h on ice, neutralised with 0.5ml 1M KOH, samples then being centrifuged (1000*g, 2min) to remove the precipitate.

Statistics

Results are expressed as mean $\pm$ s.e.mean, n represents the number of monolayers or transfectants in image analysis. Student's *t* test (2-tailed, unpaired means) was used to test the significance of difference between mean values.

Results

Using RT-PCR we confirm the expression of ANK mRNA in murine kidney together with endogenous expression of ANK mRNA in the collecting duct cell line, mIMCD-3 (Figure 1A). Using a rabbit polyclonal anti-ANK antibody, Ab3, raised against a C-terminal ANK

Fig. 1. A) RT-PCR of an internal (490bp) *ank* product. PCR products are seen in the mouse terminal collecting duct epithelial cell line (mIMCD-3, lane 1) as well as whole murine kidney (lane 2); lane 3 water control. PCR products were confirmed as *ank* by direct sequencing. B) Cellular localisation of ANK in mIMCD-3 cells; *x-y* mid-cell confocal section showing ANK staining using the primary antibody Ab3 and AF 488 goat anti-rabbit Ig. C) *x-y* mid-cell confocal section showing GFP transfected cell, counterstained with TRITC-WGA lectin. D) *x-y* mid cell confocal section of ANK-NT-GFP transfected cell, E) same cell counterstained with primary antibody Ab3/AF 568. F) Overlay of D+E. G) *x-y* mid cell confocal section of ANK-NT-GFP transfected cell, H) same cell counterstained with TRITC-WGA lectin. I) Overlay of G+H showing co-localisation at the plasma membrane. Scale bars=5µm.

Fig. 2. Effect of the Met48Thr and Glu440X mutations on the membrane localisation of ANK. Mid-cell *x-y* sections of ANK-NT-GFP A), Met48Thr ANK-NT-GFP D) and Glu480X ANK-NT-GFP G) showing the comparison between wild type and mutant cells stained with the plasma membrane marker TRITC-WGA lectin after 2min incubation. Blue bars indicate mid-cell transect used to compare the pixel intensity of GFP fluorescence intensity with that of TRITC-WGA lectin (A, D, G for B, E, H respectively). Note the co-incidence of peak fluorescence for GFP and TRITC-WGA for wild-type ANK-NT-GFP and Met48Thr ANK-NT-GFP, but not for Glu440X ANK-NT-GFP. *X-Z* section showing prominent lateral localisation of ANK-NT-GFP C) and Met48Thr ANK-NT-GFP F) but note absence of membrane staining for Glu440X ANK-NT-GFP I). Scale bars = 10µm.

Fig. 3. Sub-cellular localisation of ANK. (A, C, E, G, I) Wild type ANK-NT-GFP. (B, D, F, H, J) Glu440X NT-GFP. A, B) Transfected cells counterstained with propidium iodide. Note the signet ring appearance of the Glu440X ANK-NT-GFP. C, D) Co-transfection with pDsRed2-ER, show limited colocalisation. E, F) Transfected cells counterstained with the *Griffonia* lectin Golgi marker; note the extensive colocalisation with the Glu440X mutant. G, H) transfected cells counterstained with the acidic endosome marker LysoTracker Red show limited colocalisation. I, J) Cells cotransfected with pDsRed2-mito, note the extensive localisation with wild type ANK-NT-GFP. Scale bars=10µm.

peptide [9] we localise endogenous expression of ANK protein within mIMCD-3 cells (Figure 1B). ANK is expressed in a vesicular manner in the cytoplasm and at a near plasma membrane location but excluding the nucleus (Figure 1B). Expression of control GFP vector results in a homogeneous distribution of GFP throughout the cytoplasm and nucleus with no co-localisation with TRITC-WGA at the plasma membrane (Figure 1C).

Expression of the fusion protein ANK-NT-GFP in mIMCD-3 cells confirms the cytoplasmic, vesicular nature of expression (Figure 1D). Co-staining with Ab3 confirms a good correlation between the two methods of ANK detection (Figure 1E,F). Plasma membrane localisation of ANK-NT-GFP (Figure 1G) is confirmed by co-localisation with TRITC-WGA (Figure 1H,I) [14].

Figure 2 illustrates a quantitative comparison of ANK-NT-GFP with TRITC-WGA. A transect taken across an *x-y* confocal section of an ANK-NT-GFP transfected cell co-stained with TRITC-WGA (Figure 2A) shows that maximum pixel densities of the fusion protein and TRITC-WGA are coincident (Figure 2B). Inspection of the *x-z* section shows that there is concentration of ANK-NT-GFP expression at the lateral membranes together with apical membrane expression (Figure 2C).

Using confluent monolayers of mIMCD-3 cells grown upon permeable supports we have determined the amount of endogenous PP production in the bathing media by enzymatic determination. The mean values of apical and basal bathing solution were $11.9 \pm 4.1 \mu\text{M}$ ($n=6$) and $3.8 \pm 0.5 \mu\text{M}$ ($n=6$) respectively, corresponding to a production rate of $9.6 \pm 2.7 \text{ nmol/filter/48h}$ ($n=6$) and $6.1 \pm 0.8 \text{ nmol/filter/48h}$ ($n=6$) (note that apical incubation

volume 0.8ml, basal incubation volume 1.6ml). Intracellular PP was $9.9 \pm 2.8 \text{ nmol/filter}$ ($n=6$). The membrane localisation of ANK together with PP presence in both bathing solutions is consistent with ANK function as a PP transporter to both the lumen (urine) and across the

Fig. 4. Mitochondrial localisation of ANK. A) Colocalisation of ANK-NT-GFP with pDsRed2-mito. Coloured bars indicate transect used to compare the pixel intensity of GFP fluorescence intensity with that of pDsRed2-mito B). C) Colocalisation of ANK using Ab3 with monoclonal 7H10 antibody. Scale bars=10 μ m.

Fig. 5. Immunolocalisation of ANK in mouse renal cortex. A-D) Ab3, secondary antibody, donkey anti-rabbit FITC (green) with anti-aquaporin 2, secondary antibody, donkey anti-goat TRITC (red). A, B) Low power images from 2 separate animals. C, D) High power images. Note that both principal (staining positively (red) with aquaporin 2 antibody) and intercalated (aquaporin 2 negative) cells are evident and positive for ANK. Note colocalisation of ANK with aquaporin 2. G=glomerulus. Scale bars=20 μ m.

basal membrane (to the medullary interstitium).

Additional assessment of the subcellular distribution of ANK in renal collecting duct cells was obtained using subcellular markers (Figure 3). Counter-staining of fixed and permeabilised cells with propidium iodide confirms the absence of nuclear expression of ANK-NT-GFP (Figure 3A). There is minor co-localisation of ANK-NT-GFP with pDsRed2-ER (endoplasmic reticulum, Figure 3C), *Griffonia* lectin (Golgi, Figure 3E) and Lysotracker Red (acidic endosomes, Figure 3G). Interestingly there is substantial co-localisation of ANK-NT-GFP with mitochondria (Figure 3I).

In order to substantiate the localisation of ANK to renal mitochondria, the distribution of ANK-NT-GFP was analysed with respect to pDsRed-mito or endogenous ANK to an antibody against the α subunit of the F_1 - F_0 -ATPase, 7H10 (Figures 4A, C). Transects taken over a region of cytoplasm containing a number of mitochondria clearly show a coincidence of pixel intensity from ANK-NT-GFP with the mitochondrial marker pDsRed2-construct (Figures 4B). Furthermore, localisation of endogenous ANK using the rabbit anti-ank antibody (Ab3) was also coincident with the mitochondrial marker (pDsRed2-mito) (Figure 4C).

Murine ANK mutations, associated with loss of function (Glu440X) and human ANK gain of function mutations (Met48Thr) have been described [9-11]. Since the mouse protein differs only slightly from the human protein (9 amino acid changes) we used site-directed mutagenesis of the ANK-NT-GFP fusion protein (verified by direct sequencing (data not shown)) to investigate the effect of these diverse mutations on the subcellular distribution of ANK.

The subcellular localisation of Met48Thr ANK-NT-GFP is not substantially altered compared to wild type (not shown). Quantification of wild-type ANK-NT-GFP versus Met48Thr ANK-NT-GFP at the plasma membrane (as illustrated in Figure 2D, E, F) showed a modest increase (control pixel intensity at plasma membrane 69.5 ± 10.6 arbitrary units $n=6$ versus Met48Thr ANK 92.6 ± 13.6 arbitrary units $n=6$) that did not reach statistical significance ($p>0.2$). Cell volume (hence membrane surface area) in Met48Thr transfectants was also slightly raised (from $1938 \pm 375 \mu\text{m}^3$ in wild type ($n=10$) to $2519 \pm 210 \mu\text{m}^3$ ($n=10$)), but this was not statistically significant ($p=0.19$). It should be noted that transfection efficiency was not substantial enough to allow quantification of PP export in comparison to wild type. In contrast to Met48Thr ANK, Figure 2G, H, I and 3 shows that truncation of the C-terminal of ANK markedly alters the

subcellular distribution; Glu440X ANK-NT-GFP transfectants display a "signet ring" appearance. Cell volume is slightly reduced (to $1579 \pm 273 \mu\text{m}^3$ ($n=10$), n.s. versus wild type). Whereas there is only minor co-localisation of Glu440X ANK-NT-GFP with both pDsRed2-ER (Figure 3D) and Lysotracker Red (Figure 3H), there is substantial co-localisation of Glu440X ANK-NT-GFP with *Griffonia* lectin (Figure 3F) in contrast to wild type suggesting a reduction of membrane traffic of this construct from Golgi. Whilst an apparent reduction in mitochondrial density is observed, Glu480X ANK-NT-GFP still co-localises with pDsRed2-mito (Figure 3J).

Since the deletion mutant Glu440X ANK-NT-GFP lacks the epitope to Ab3, antibody specificity (Figures 1B, F and 4C) has been confirmed by comparing the immunofluorescence detected by Ab3 using goat anti-rabbit TRITC in Glu440X ANK-NT-GFP transfected cells, to ANK-NT-GFP transfected cells. Ab3 immunofluorescence (summed pixel intensity 332.4 ± 25.2 $n=8$ a.u.) is markedly elevated compared to Glu440X ANK-NT-GFP transfected cells (10.9 ± 6.3 arbitrary units, $n=5$, $p<0.001$) whereas GFP fluorescence in ANK-NT-GFP cells (62.4 ± 13.4 a.u. $n=8$) does not differ from Glu440X ANK-NT-GFP cells (53.2 ± 13.8 a.u. $n=5$, n.s.).

Since RT-PCR confirms expression of ANK in native murine kidney (Figure 1A), we have determined the intra-renal distribution of ANK revealed by Ab3 (Figure 5). Cortical collecting tubules (confirmed by co-expression of aquaporin 2) are the primary site of ANK expression with only weak expression in adjacent glomeruli. Within the collecting duct both aquaporin positive (principal cells) and aquaporin negative (intercalated cells) show strong immunoreactivity.

Discussion

There is now convincing evidence that the ANK protein mediates pyrophosphate (PP) export [9, 11]. The *ank* gene is expressed in skeletal tissue, but *ank* cDNA probes also detect 3.5 and 4kb transcripts in heart, brain, liver, spleen, lung, skeletal muscle and kidney [9]. The present data confirm renal expression of the ANK protein and show that expression is concentrated in cortical collecting duct epithelial cells in both principal and intercalated cells. PP is present in urine with daily outputs of 12 to $45 \mu\text{mol}/24\text{h}$ [17]. Given that the PP plasma half life will be limited at cell surfaces by the actions of alkaline phosphatase and ectonucleotide pyrophosphatases [18] it is likely that the source of urinary PP may be, in part,

from glomerular filtration but also operation of the ANK protein at the level of the renal tubular epithelial cell. In addition we show that ANK is present in a collecting duct cell-line, mIMCD-3, where it traffics to both the apical and lateral plasma membranes. Since PP can be detected in both apical and basal solutions, it is apparent that collecting duct cells may export PP across both the apical and basal plasma membranes. The present data showing ANK colocalisation with aquaporin 2 within principal cells requires further work to determine whether surface membrane expression is controlled similarly for aquaporin 2 and ANK.

PP is a potent inhibitor of crystal formation from both natural and artificial urines [5, 6]. PP concentrations attained in the bathing media of mIMCD-3 cells (12 and 4 μ M for apical and basal solutions respectively) are within the range where such inhibition is observed. Thus ANK protein expression by kidney cells may serve a physiological role in preventing nephrocalcinosis/nephrolithiasis [2]. In terms of mineralisation within the skeletal system, extracellular PP acts as an inhibitor of hydroxyapatite deposition [9]. In both Glu440X and null *ank*^{-/-} mice, the loss of PP transport to the extracellular space results in abnormal deposition of hydroxyapatite in joints [9, 19]. In idiopathic stone formers Evan *et al* [4] have demonstrated that abnormal apatite deposits (Randall's plaques) originate in the medullary interstitium adjacent to loops of Henle. Furthermore, in the same study, hypercalciuric calcium-oxalate stone formers possess initial (seeding) calcium phosphate crystals [4]. Intratubular stone formation in intestinal bypass patients showing hyperoxaluria is also initiated by calcium phosphate (apatite) crystals [4]. Taken together with the demonstration of renal epithelial cell expression of ANK and PP export to apical/basal bathing media *in vitro*, we postulate that pyrophosphate transport to either interstitium or tubular lumen comprises an important mechanism that protects against nephrocalcinosis/nephrolithiasis. Indeed hypopyrophosphaturia has been documented in a group of recurrent stone-forming patients [20]. Ho *et al* mention that *ank*^{-/-} animals show renal calcification [9].

Given the wide tissue distribution of *ank* transcripts, additional functions for pyrophosphate transport are indicated. The present data show that as well as expression at the plasma membrane in IMCD-3 cells, ANK is expressed at endoplasmic reticulum, acidic endosomes and most strikingly, mitochondria. Co-expression of ANK with a calreticulin-targeting sequence suggests that accumulation of PP within the lumen of the

endoplasmic reticulum may enhance sequestration of stored Ca²⁺ so allowing sustained Ca²⁺-signalling after activation of IP₃/Ryanodine sensitive pathways [21].

The marked colocalisation of the ANK protein to renal mitochondria suggests an unidentified physiological role for PP transport in mitochondrial function. Mitochondria are able to generate PP, mitochondrial metabolism being connected with both regulation of intracellular and extracellular PP (reviewed in [22]). Mitochondria may also contain significant quantities of soluble adenylate cyclase in the outer mitochondrial space [23], generation of cAMP from ATP being accompanied by PP generation. Further work is required to define both the intra-mitochondrial localisation of ANK and a possible role for ANK/PP in cellular energy charge and mitochondrial function in renal cells.

The progressive ankylosis phenotype in *ank*^{-/-} mice arises from a nonsense mutation Glu440X which truncates the protein within the terminal transmembrane segment [9]. This mutation causes an increased cellular PP in *ank*^{-/-} fibroblasts together with a decrease in PP export to the extracellular medium [9] and abolishes ³²PP uptake when expressed in *Xenopus* oocytes [24]. Since Ab3 is immunoreactive only after cell permeabilisation [9], the C-terminal must be intracellular. In the present study we show that Glu440X ANK-NT-GFP does not reach the plasma membrane of mIMCD-3 cells but is retained at an intracellular location mostly within Golgi suggesting that protein misfolding/targeting prevents normal cell traffic. Though the Glu440X mutation abolishes ³²PP uptake [24] consistent with the absence of plasma membrane expression, it is unclear whether protein function is abolished. Mitochondrial targeting of the mutant protein is not prevented.

The cellular basis of the Met48Thr ANK mutation has been investigated by Pendleton *et al.* [11]. Reconstitution of the Met48Thr ANK mutation within a CMV-based expression vector and expression of mutant ANK in COS-7 cells resulted in a reduction in intracellular PP levels that was equivalent to that seen with wild type controls (101%) [11]. Similarly, expression of the Met48Thr mutant protein in *Xenopus* oocytes resulted in surface expression and wild-type levels of ³²PP uptake [24]. The results reported here are in agreement with this data since no major change in the sub-cellular distribution nor significant increase in surface expression of Met48Thr ANK-NT-GFP was observed. Neither the data of Pendleton *et al.* [11] nor that reported here explain the increase in deposition of CaPPD crystals seen in

affected individuals [11].

In summary, the renal expression of ANK is likely to have important physiological roles within the renal tubule. Given the importance of ANK in controlling deposition of apatite within the extracellular matrix of the skeletal system, we propose that renal ANK function has a similar local role, limiting abnormal apatite formation

both in the medullary interstitium and in the collecting tubule lumen, in conditions of urinary concentration.

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