The Pyrophosphate Transporter ANKH is Expressed in Kidney and Bone Cells and Colocalises to the Primary Cilium/Basal Body Complex

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Key Words

ANKH • Epithelial cells • Primary cilia • Cilial localisation motif • Pyrophosphate • Basal body

Abstract

Background/Aims: *ANKH* encodes a putative pyrophosphate transporter named ANKH, which regulates tissue calcification. ANKH is a transmembrane protein with at least 8 predicted transmembrane domains. Sequence analysis reveals a possible cilial localisation motif immediately after the last transmembrane segment. Here we aim to determine the subcellular localisation of ANKH in ciliated epithelial cells and murine tissue and identify colocalisation using ciliary/basal body markers. **Methods:** Using murine kidney, renal epithelial cells and osteoblast cells we investigated the expression and localisation of ANKH using RT-PCR, Western blotting and immunocytochemistry. **Results:** Here we confirm endogenous expression of ANKH mRNA and protein in whole mouse kidney as well as mouse renal epithelial cell lines M1 and mpkCCDcl4 and the osteoblast cell line MC3T3-E1. Using antibodies directed towards ANKH, we confirm cilial and basal body localisation in renal tissues and renal epithelial

cells, in addition to a centrosomal localisation in dividing mpkCCDcl4 cells. We also establish that the osteoblast cell line MC3T3-E1 forms an epithelioid cell layer, with junctional complex formation and primary cilia expression. ANKH is also seen within cilial and basal body structures of MC3T3-E1 cells. An ANKH-3XFLAG construct expressed in mpkCCDcl4 cells also localises to the primary cilium/basal body complex confirming this localisation. **Conclusion:** We conclude that the transmembrane protein ANKH is expressed in cilia and basal body structures, and postulate a sensory role at this location.

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Introduction

Inorganic pyrophosphate (PPi) is a potent inhibitor of apatite precipitation and is present in significant concentrations in both plasma and urine [1]. PPi is a metabolic product from cellular metabolism and also plays roles in calcium sequestration, modification of enzyme function and modulation of mineralisation [2]. Recently, a molecular candidate for a pyrophosphate transporter,

ANKH (alias ANK) has been identified [3, 4]. There is growing evidence that this widely expressed protein may have roles in limiting and regulating calcification within many tissues, including joints, bones and kidney [5].

The discovery of ANKH as a pyrophosphate transporter was first made following the identification of a truncating mutation in *ank* in a mouse model of arthritis [3]. These *ank* mutant mice display a deforming arthropathy and intrarenal calcification. Cultured fibroblasts from these animals displayed increased levels of intracellular pyrophosphate. This defect could be rescued by transfection of the wild-type *ank* into mutant fibroblasts, implicating ANKH protein in PPi export from the cell. Since then, more convincing biophysical data has shown that the ANKH functions at the plasma membrane as a PPi transporter [4].

In previous work we have characterised the expression of ANKH in renal tissues and renal cell cultures, confirming both a plasma membrane and mitochondrial localisation in murine collecting duct cell lines and a collecting duct expression pattern in murine kidney [5]. With regard to human disease, *ANKH* has been recently identified as the gene mutated in craniometaphyseal dysplasia (CMD, OMIM #123000) [6] and calcium-pyrophosphate dihydrate deposition disease (CPPD, OMIM #118600) [7]. Mutations which lead to over activity of the transporter are predicted to give rise to CPPD deposition, whilst mutations leading to under activity may lead to skeletal defects such as CMD. It is postulated that polymorphisms in *ANKH* may be a genetic risk factor for calcium stone formation.

The purpose of the present study was to further elucidate ANKH expression, both in renal and bone cell lines, given the hypothesis that ANKH modulates calcium deposition in both bone and renal tissues. In extending our localisation studies of ANKH to renal cell-lines with morphologically pronounced cilia, we noticed a prominent cilia/basal body localisation for ANKH. The recent identification of a ciliary localisation motif in transmembrane proteins [8] prompted us to examine the amino acid sequence of ANKH for such a motif.

Primary cilia are almost universally expressed within human tissue and are thought to be sensory organelles, with detection of smell [9], vibration [10], gravity [11] and flow [12, 13] being some of their most recent attributes. The cilia location of polycystin-1 (PC-1) and polycystin-2 (PC-2), forming flow-dependent Ca^{2+} -influx pathways highlight the role of cilia in Ca^{2+} -signal transduction [12, 14]. Defects in cilia proteins are implicated in range of human conditions including cystic

kidney disease, blindness, and obesity [10, 15]. Skeletal defects, particularly postaxial polydactyly and skeletal dysplasia have also been linked to basal body and primary cilia defects [16, 17].

ANKH is expressed in multiple tissue types [4] and is likely to have distinct roles in bone and other tissues where ANKH dysfunction may lead to abnormal calcification (including vasculature and the kidney). We speculate that a basal body/cilia localisation may allow ANKH to locally modify inorganic pyrophosphate and calcium activities, so participating in signalling events initiated from the cilium.

The amino acid sequence of ANKH reveals a motif consistent with reported cilia localisation motifs in other transmembrane proteins. We confirm ANKH endogenous expression in renal epithelial and osteoblast cells by RT-PCR and Western blotting. Using ANKH antibody staining, we demonstrate discrete cilia and basal body localisation of ANKH protein in murine kidney and murine cortical collecting duct cells. This ciliary localisation was confirmed using ANKH-3XFLAG overexpression. Furthermore, the osteoblast cell line MC3T3-E1 in monolayer culture, shows both tight junction associated protein expression and prominent primary cilia. ANKH expression is also seen in the basal bodies/base of cilia of these cells, suggesting a common function in renal and bone tissues.

Materials and Methods

Cell Culture

Osteoblastic MC3T3-E1 cells (LGC Promochem) were cultured in Alpha Minimum Essential Medium with ribonucleosides, deoxyribonucleosides, 2 mM L-glutamine and 1 mM sodium pyruvate, but without ascorbic acid (GIBCO) supplemented with 10% FCS. The murine cortical collecting duct principal cell line, mpkCCDcl4 was cultured in HAM's F12/Dulbecco's Modified Eagle's Medium (DMEM) 1:1 v/v with 1g/L Glucose; 60 nM sodium selenate; 5 µg/ml transferrin; 2 mM glutamine; 50 nM dexamethasone; 1 nM triiodothyronine; 10 ng/ml epidermal growth factor; 5 µg/ml insulin; 20 mM D-glucose; 2% FCS; 20 mM HEPES; pH 7.4; 100 U/ml penicillin and 100 µg/ml streptomycin. M1 cells (murine cortical collecting duct cell line) were cultured in HAM's F12/DMEM 1:1 v/v with 4.5g/L glucose, 5% FCS, 5µM dexamethasone, 100 U/ml penicillin and 100 µg/ml streptomycin. All cultures were maintained at 37 °C in a 5% CO_2 -95% humidified air atmosphere.

RT-PCR of ANKH from whole tissue and cell culture lines

Total RNA was obtained from whole mouse kidney, M1, MC3T3-E1 and mpkCCDcl4 cells using the RNeasy midi kit (Qiagen). Extracted RNA was reverse transcribed according to

Fig. 1. Predicted protein domains, amino acid conservation and identification of putative ciliary localisation motif in ANKH. A: Transmembrane topology of the ANKH human protein, demonstrating a predicted 8 transmembrane domains and an intracellular N- and C- terminus. (Domains predicted by SMART <http://smart.embl-heidelberg.de/>). B: Amino acid alignment of ANKH from vertebrate species (homo_s = human, Mus_m = mouse, Xenopus_l = frog, Danio_r = zebrafish, Rattus_n = rat, Gallus_g = chicken, Canis_f = dog). The last transmembrane domain is marked and the conserved “YR” motif of a hydrophobic followed by a basic amino acid residue is boxed. (Alignment performed using Jalview <http://www.jalview.org/>). C: Sequence comparison of known ciliary proteins at the C-terminal juxtamembrane position. Hydrophobic-basic residues are shown in ANKH, Smo (Smoothed), ODR-10 and STR-1 (seven-transmembrane receptors in *C. elegans*) and Rho, rhodopsin.

the protocol using the Omniscript RT kit (Qiagen). Negative controls omitted RNA or reverse transcriptase. PCR used HotStarTaq DNA polymerase (Qiagen). PCR negative controls omitted DNA. Gene-specific oligonucleotide primers (490 bp product; 5'-CAT CAC CAA CAT AGC CAT CG-3' and 5'-CCA GGT GAC TGT GAA GCA AA-3') for murine ANKH were designed from available database sequences (Ensembl). Direct sequencing confirmed the identity of the PCR products.

Western Analysis of endogenous ANKH

MC3T3-E1, M1 and mpkCCDc14 cells were washed twice with ice-cold PBS and then lysed for 30 min at 4°C in lysis buffer (2% Nonidet P40, 0.2% SDS, 1 mM DTT, and complete mini protease inhibitor cocktail (Roche) in PBS). Murine kidney was homogenised in protein lysis buffer (250mM EDTA pH 8, 250mM EGTA pH 8, 320mM sucrose, 500mM Tris pH 7.6, 10% Triton and complete mini protease inhibitor cocktail (Roche) in PBS). The homogenate or lysate was centrifuged at 10,000×g for 10 min at 4°C. The supernatant was retained and protein concentration measured using the Bradford method. 50 µg of each protein sample was heated to 95°C for 10 min and separated on a 10% SDS- PAGE gel. Proteins were transferred to a polyvinylidene difluoride membrane (PVDF) (Hybond-P, Pharmacia Biotech, U.K.).

The membrane was blocked in TBS-T buffer (20 mM Tris, 137 mM NaCl, 0.1% Tween-20, pH 7.6) containing 5% non-fat milk, for 1 h at room temperature (RT). Endogenous ANKH was detected by incubating for 2 h at RT with a rabbit polyclonal anti-ANKH antibody, Ab3 directed towards amino acids 477-492 at the C-terminus (kind gift from D.M. Kingsley, Stanford University) diluted (1:500) in blocking solution. The membrane was washed three times with TBS-T at RT followed by 1 h incubation at RT with anti-rabbit-horseradish peroxidase conjugate (1:10,000 in blocking solution, Amersham Biosciences) for secondary detection. The membrane was washed with TBS-T buffer and protein detected by the enhanced

chemiluminescence method using an ECL Plus Western blotting detection system (Amersham Biosciences).

Renal and subcellular localization of ANKH expression

Monolayers of M1 and mpkCCDc14 cells were prepared by high density seeding (0.5 x 10⁶ cells/filter) onto Transwell culture inserts (0.4-µm pore size, 1 cm² growth area, Corning Costar) and cultured for 7 days with media replacement every 3 days. MC3T3-E1, M1 and mpkCCDc14 cells were also grown to confluency on 13mm coverslips.

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. 10 µm cryosections were cut and mounted onto poly-lysine coated microscope slides. Tissue sections and cells were fixed and permeabilised in methanol at -20°C, prior to immunostaining.

Cells and kidney slices were blocked for 1 h at RT with 3% normal horse serum. ANKH was detected by rabbit anti-ANKH polyclonal antibody (Ab3, 1:250). For colocalisation studies, a ciliary marker, mouse anti-acetylated alpha-tubulin antibody (1:1000, Sigma) or basal body marker, mouse anti-gamma-tubulin antibody (1:2000, Abcam) were incubated overnight at 4 °C. Zona Occludens-1 was detected in cells using mouse anti-ZO-1 (1:100, Zymed).

Secondary block with 3% donkey serum preceded incubation with donkey anti-rabbit FITC (Alexa Fluor 488, Molecular Probes) and donkey anti-mouse TRITC (Alexa Fluor 568, Molecular Probes) or vice versa for M1 cells. Controls omitted the primary antibodies (negative).

Cells or tissue sections were imaged (z or y-series images collected) using confocal laser microscopy (TCS-NT, Leica with Kr-Ar laser) with appropriate excitation and emission filter sets for dual fluorophore detection.

Fig. 2. Expression of ANKH. A: RT-PCR products are visualized on a 0.8% agarose gel using ANKH specific oligonucleotide primers. Products of the expected size (490 bp) are seen using M1, mpkCCDcl4 cells, whole mouse kidney and MC3T3-E1 total RNA as template. Negative controls (not shown) omitted RT enzyme for each template respectively. B: Western blotting to characterise polyclonal antibody anti-ANKH Ab3. In HEK293 cells over expression of ANKH-3XFLAG reveals a single band, detected with anti-FLAG antibodies (left, arrowed) whilst anti-ANKH Ab3 detects endogenous ANKH (arrow head) together with a ~3.5 kDa heavier band (arrowed) consistent with ANKH-3XFLAG expression (right). C: Western blotting of murine cell lines IMCD-3, K2, M1 and murine kidney (left) reveals endogenous expression of ANKH at the expected size (~50 kDa) when detected with anti-ANKH Ab3. Endogenous ANKH expression is also seen in mpkCCDcl4 and MC3T3-E1 cells (right). Additional lower molecular weight bands are also seen at 37 and 42 kDa.

Overexpression of ANKH-3XFLAG

Full length human ANKH cDNA (NM_054027) with an N-terminal 3XFLAG was purchased from Genecopoeia. Subconfluent HEK293 and mpkCCDcl4 cells were transfected with ANKH-3XFLAG using Lipofectamine™ 2000. Cell lysis and protein purification was performed as above. Blots were probed with anti-FLAG conjugated to HRP (Sigma#A8592) 1:10,000 and anti-ANKH (Ab3) and detected as described above.

For immunostaining, ANKH-3XFLAG transfected mpkCCDcl4 cells were fixed in methanol, blocked in horse serum and stained with mouse monoclonal anti-FLAG antibody (1:5000, F3165, Sigma) and anti-ANKH antibody (Ab3). Following donkey serum incubation as a secondary block cells were incubated with donkey anti-mouse TRITC (Alexa Fluor 568, Molecular Probes) and with donkey anti-rabbit FITC (Alexa Fluor 488, Molecular Probes). To avoid cross reaction of secondary antibodies, each antibody was used with no primary control.

Results

ANKH encodes a highly conserved protein with a putative ciliary localisation motif

Amino acid sequence analysis reveals that the ANKH protein is highly conserved. Protein domain analysis (<http://smart.embl-heidelberg.de/>) predicts a single ANKH domain, occupying the entire 492 amino acids of the human ANKH protein. This “ANKH” domain is unique within the whole human genome. The ANKH protein is a putative transmembrane transport protein, and is predicted to have at least 8 transmembrane spanning domains (<http://smart.embl-heidelberg.de/>) (Figure 1A) with intracellular N- and C-termini [18].

The ANKH amino acid sequence is highly conserved in vertebrates, with strong homology between human, mouse, frog and zebrafish (Figure 1B). The predicted transmembrane domains are particularly well conserved. Figure 1B demonstrates that the terminal transmembrane segment maintains an almost identical amino acid composition between vertebrate species including *Danio rerio*. In particular, the last residue of this transmembrane segment and the amino acid immediately following this (comprising the YR motif) are fully conserved (Figure 1B). This combination of a hydrophobic residue followed by a basic residue in the last transmembrane domain has been identified in other transmembrane proteins, whose expression pattern has been shown to be within the primary cilium, such as smoothened (Smo) and rhodopsin (Rho) (Figure 1C).

Fig. 3. ANKH colocalises with acetylated alpha tubulin at the primary cilium/basal body complex. Confocal microscope images (xy sections and xz where indicated) are shown. Columns labelled M1, mpkCCDcl4, MC3T3-E1 represent murine collecting duct cells, murine cortical collecting duct and osteoblast cells, respectively. Antibodies to alpha acetylated tubulin (top row) identify primary cilia structures. ANKH Ab3 antibody identifies ANKH expression (second row), with overlaid images shown (third row). Fourth row demonstrates, in sub apical sections, epithelium cell confluence, using anti-ZO-1 antibodies. Scale bar = 10 μ m.

ANKH is endogenously expressed in kidney and bone epithelium

In order to confirm endogenous expression in renal epithelial cells and osteoblast cells, we performed RT-PCR using gene specific primers and Western blotting using a previously characterised ANKH antibody [3]. RT-PCR confirmed ANKH expression in whole mouse kidney, M1 murine epithelial cells, MC3T3-E1 osteoblast cells and mpkCCDcl4 murine cortical collecting duct cells (Figure 2A). Negative controls (not shown) omitted RT enzyme for each template respectively. Direct sequencing of PCR products confirmed their identity (data not shown).

The full-length ANKH protein is predicted to have a molecular weight of ~54 kDa. In order to confirm

specificity of the anti-ANKH antibody (Ab3), protein from HEK293 cells transfected with full-length ANKH-3XFLAG revealed a single band of ~52 kDa when probed with anti-FLAG antibody (Figure 2B). When endogenous HEK293 protein and HEK293 cells over expressing ANKH-3XFLAG were probed with anti-ANKH (Ab3) a band of identical weight is seen in the transfected cells, whilst endogenous ANKH expression is seen running ~3.5 kDa lower, consistent with the known molecular weight of the 3XFLAG tag (Figure 2B).

Western blotting using anti-ANKH (Ab3) also revealed a band of expected size (~50 kDa) in murine cell lines IMCD-3 cells, K2 (inner medullary collecting duct) cells, M1 (cortical collecting duct) cells, and murine whole kidney as well as mpkCCDcl4 cells and

Fig. 4. ANKH colocalises with gamma tubulin at the basal body complex. Confocal microscope images (*xy* sections and *xz* where indicated) of epithelial monolayers of M1, mpkCCDc14 and MC3T3-E1 cells are shown. Gamma tubulin antibodies (top row) identify centrosomes, including a mpkCCDc14 cell undergoing mitosis (4th column). ANKH Ab3 antibody identifies ANKH expression (middle row), with overlaid images shown (bottom row). Scale bar = 10 μ m.

MC3T3-E1 osteoblast cells. Additional lower molecular weight bands are also seen, of uncertain specificity, since all bands are blocked using peptide preabsorption of Ab3 (data not shown). Databases (www.ncbi.nlm.nih.gov/IEB/Research/Acembly/index.html) predict three isoforms of the ANKH protein in mouse tissues. The commercial anti-ANKH antibody (sc-67242, Santa Cruz Biotechnology) was unable to detect ANKH-3XFLAG when over expressed in HEK293 cells (data not shown).

ANKH expression is seen in the cilia and basal body complex

Our previous data has shown that ANKH is localised to the plasma membrane in both cultured epithelial cells (colocalising with WGA lectin) and in

mouse whole kidney tissue, where it colocalises with aquaporin-2 at the apical pole of cells within cortical collecting duct segments [5]. Given the putative ciliary localisation “YR” motif, identified within the ANKH amino acid sequence (Figure 1), we examined confluent epithelial cell lines for the expression of ANKH in the primary cilia/basal body complex. In order to confirm confluency and tight junction formation in M1 and mpkCCDc14 renal cells and junctional complex formation in MC3T3-E1 osteoblast cells, we stained cell monolayers with Zona Occludens-1 (ZO-1) antibodies. This revealed confluent single cell layers, expressing circumferential ZO-1 staining at the apical pole of M1 and mpkCCDc14 cell lines, and complex interdigitating junctional complexes in MC3T3-E1 cells (Figure 3, bottom row). At or above the plasma membrane/apical surface confocal sections,

Fig. 5. ANKH colocalises with ANKH-3XFLAG at the primary cilium/basal body complex. Confocal microscope images (*xy* sections and *xz* where indicated) of mpkCCDcl4 are shown. Anti-ANKH Ab3 antibody (top row) identifies basal body and cilial-like structures in transfected (arrowed) and untransfected (arrowhead) cells. Expression of ANKH-3XFLAG (second row) colocalises to basal body/cilial-like structures in overlaid images (bottom row). Scale bar = 10 μ m.

distinct punctate cilial staining is seen using the ciliary marker anti- α -acetylated tubulin (Figure 3, top row). Using anti-ANKH Ab3 antibody (Figure 3, second row), there is a striking correspondence of ANKH immunofluorescence with that of acetylated tubulin (Figure 3, third row). This is true for both of the renal epithelial cell lines, M1 and mpkCCDcl4, and osteoclast cells, MC3T3-E1. For mpkCCDcl4 cells the *xz* images show clearly that in addition to what appears to be plasma membrane ANKH immunostaining at both apical and basolateral cell-borders, there is colocalisation of α -acetylated tubulin immunostaining with that of ANKH (Figure 3, 3rd column). In order to substantiate this finding of cilial localisation in native renal cells, murine kidney collecting duct sections were co-stained with antibodies against acetylated tubulin and ANKH (Ab3) (Figure 3, 5th column) showing good correspondence.

To investigate whether the localisation of ANKH also corresponded to basal body structures, gamma tubulin antibody staining was used as a marker for basal bodies/centrosomes. Figure 4 demonstrates colocalisation of gamma tubulin with ANKH in M1 cells, consistent with basal body expression (Figure 4, column 1). This is confirmed in mpkCCDcl4 cells and MC3T3-E1 osteoblast

cells (Figure 4, columns 2 and 3). At these confocal sections, (just below the plasma membrane) lateral membrane/plasma membrane-like expression of ANKH may be also noted, consistent with a role for ANKH in membrane pyrophosphate transport. The intracellular localisation of ANKH is dynamic during the cell cycle (Figure 4, 4th column) corresponding to a centrosomal localisation. Here a dividing cell in metaphase/early anaphase is identified, where colocalisation is evident between centrosomes and ANKH.

In order to verify this cilial pattern of expression we overexpressed ANKH-3XFLAG in mpkCCDcl4 cells. Cells were then stained using anti-FLAG antibodies and antibodies directed towards ANKH (Ab3). Care was taken to avoid any cross reaction of secondary antibodies by using appropriate no primary controls. Figure 5 demonstrates a selection of *xy* and *xz* sections of cells. Anti-ANKH antibodies detect cilial/basal body-like structures, similar to that shown in Figures 3 and 4. In untransfected cells ANKH appears at the basal body (Figure 5, arrowheads). Anti-FLAG antibodies colocalise with ANKH at the base and axoneme of the primary cilia (Figure 5, arrows). Overexpression of ANKH-3XFLAG also increases the ANKH expression

(as detected by both anti-ANKH and anti-FLAG antibodies) at a possible plasma membrane location (Figure 5), consistent with our previously published data [5]. There is no cross-reactivity of anti-FLAG antibody with cilia in neighbouring untransfected cells (Figure 5) and confirmed in experiments with non ANKH-3XFLAG transfected cell layers (not shown).

Discussion

Database searches using the amino acid sequence of ANKH demonstrate its uniqueness within the human genome. The whole amino acid sequence is recognised as a functional domain. The ANKH amino acid sequence is also conserved through evolution in vertebrates including the zebrafish (*Danio rerio*), suggesting a highly conserved functional role for this protein. Although interest in ANKH has primarily been focussed on the role of this protein in control and formation of the skeleton, the wide tissue distribution of expression in non-skeletal tissues, suggests important roles in controlling inappropriate apatite and calcium crystallisation or ionic calcium activity at other cellular locations. Previous work has also demonstrated ANKH expression in brain, with neuronal cell cultures showing ANKH protein within the cell body and cell extensions in dendrites, colocalising with microtubule-associated protein-2 [19]. A functional role for ANKH in neurons, by regulating PPi and ATP release has been postulated [19].

Although the plasma membrane of primary cilium is continuous with the adjacent plasma membrane, traffic through the cilium and concentration of proteins within the ciliary plasma membrane or in the region of the basal body, require specialised sorting mechanisms either encoded by intrinsic sorting signals or by chaperone proteins/partners. Ciliary protein trafficking along the axoneme is facilitated by intraflagellar transport (IFT) rafts such as Polaris/IFT88 and is carried out in the anterograde direction by kinesin-2 molecular motors, whereas retrograde shuttling of proteins is conducted by dynein [20]. Polycystin-2 (PC-2, alias TRPP2) contains an N-terminal R₆VxP motif within its first 15 amino acids which directs localisation to the cilium independently of polycystin-1 (PC-1) [21]. Heterologous proteins containing this conserved motif also traffic to cilia [21]. It should be noted that PC-2 may function as a calcium channel in a multimeric protein complex not only in the ciliary membrane but also at the plasma membrane and endoplasmic reticulum, its function and location

determined by adaptor proteins e.g. PACS proteins [22]. The amino acid sequence of ANKH does not contain the R₆VxP motif.

Dwyer et al. described a ciliary localisation signal in odorant receptor proteins localised to olfactory cilia; deletion of the C-terminal resulted in retention of ODR-10 or STR-1 in the cell body [23]. The two odorant receptors had dissimilar C-terminal sequences except for a hydrophobic/basic amino acid pair immediately following the terminal transmembrane segment. Corbit et al. substantiated such a conserved C-terminal signal sequence for ciliary localisation of smoothened (Smo), a seven transmembrane-segment (7TM) receptor essential for hedgehog signalling [8]. The ciliary localisation of Smo may also be regulated by an extracellular motif, whilst the hydrophobic/basic amino acid pair at the last transmembrane segment remained a requirement for ciliary expression [24]. This motif is retained in other 7TM receptors localised to cilia including the somatostatin 3 receptor (Sstr3), the serotonin 6 receptor (Htr6) and rhodopsin [8]. Subsequent work has also demonstrated another ciliary targeting motif, in the third intracellular loop in Sstr3 and Htr6 [25]. This 5 amino acid consensus domain is not present however, within ANKH amino acid sequence. In contrast, it is apparent from sequence data showing the terminal transmembrane segment that a hydrophobic/basic “YR” motif is present and highly conserved in the ANKH protein. This suggests that the ANKH protein may be targeted to cilia.

Our previous studies in renal cells using an ANK-NT-GFP construct and the ANKH (Ab3) antibody, showed localisation to the plasma membranes and endomembranes (including mitochondria) in mIMCD-3 cells, a model of the inner medullary collecting duct [5]. Most epithelia are ciliated, yet the size of the central cilium can vary enormously from 0.1 µm to 10 µm [26, 27]. We find that in mIMCD-3 cells the central cilia are very short (0.1-0.2 µm) and not readily imaged. In contrast, using cortical collecting duct cells (M1 and mpkCCDcl4 cells) where the cilium is 1-3 µm, there is a striking colocalisation of ANKH immunofluorescence with the cilia/basal body complex detected with either alpha-tubulin or gamma-tubulin. ANKH immunofluorescence is also evident in murine kidney sections where tangential sections across collecting ducts allow apical ciliary profiles to be visualised. The distribution of ANKH immunofluorescence compared to that of alpha and gamma-tubulin in renal collecting cell lines suggests that ANKH is localised to the area of the basal body at steady state.

In addition to renal cells, the osteoblastic cell line MC3T3-E1 cells also expresses a central cilium [28] together with ciliary proteins such as PC-1 and PC-2, Tg737 and Kif3a [28]. ANKH is endogenously expressed in these cells, colocalising to the cilium/basal body complex. The ciliary/basal body localisation of ANKH using anti-ANKH antibody was verified using transfection of an ANKH-3XFLAG construct in mpkCCDcl4 cells. Over expression of ANKH-3XFLAG reveals colocalisation at the base of the cilium as well as the ciliary axoneme (Figure 5). This localisation prompts the question whether mutations in *ANKH* give rise to a ciliopathy. Although there does not seem to be a cystic kidney phenotype (common to many ciliopathies [29]), the bone abnormalities and skeletal phenotypes seen in patients with craniometaphyseal dysplasia secondary to ANKH mutations (OMIM #123000) may be consistent with an additional chemosensory/mechanosensory defect in chondrocytes. Skeletal abnormalities, which may include limb patterning defects, defects in endochondral bone, teeth and craniofacial bones are a feature of several ciliopathy syndromes [30].

The proposed function of the ANKH protein is to mediate the transmembrane flux of pyrophosphate [4]. We propose that the localisation of ANKH to the cilium/basal body may be involved in the vesicular release of pyrophosphate in this region. A pyrophosphate rich-region would allow effective buffering of Ca^{2+} activity and facilitate sequestration without precipitation within Ca^{2+} -stores adjacent to the ciliary structure. In this model the cilium is isolated with respect to Ca^{2+} -signalling from the cell but allows integration and segregation of multiple stimuli within the cilium itself, outputs

consisting of altered protein effectors such as Gli repressors or activators or other diffusible mediators [14].

An interesting feature of the sub-cellular distribution of ANKH is the dynamic positioning of ANKH within the centrosome during the cell cycle. If this distribution reflects functional ANKH, it is possible that, as with the mature cilium, ANKH is involved in the vesicular release of pyrophosphate in this region. A pyrophosphate rich-region may allow local buffering of Ca^{2+} activity or enhanced sequestration within a Ca^{2+} -store adjacent to the centrosomes.

Conclusions

ANKH is a transmembrane protein that is highly conserved throughout evolution. Amino acid sequence analysis predicts a ciliary localisation motif consisting of a hydrophobic and a basic amino acid residue immediately adjacent to the last transmembrane segment. Using ANKH antibodies and expression of a FLAG tagged ANKH protein, we localise ANKH to the cilia/basal body complex. We propose that ANKH facilitates pyrophosphate transport at this location, mediating effective Ca^{2+} buffering.

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