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ATP protects against FITC labeling of *Solanum lycopersicon* and *Arabidopsis thaliana* Ca^{2+} -ATPase ATP binding domains

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Abstract

Ca^{2+}-ATPases are integral membrane proteins that actively transport Ca^{2+} against substantial concentration gradients in eukaryotic cells. This active transport is energized by coupling ion translocation with ATP hydrolysis. In order to better understand this coupling mechanism, we studied the nucleotide specificities of isolated ATP binding domains (ABDs) of *Solanum lycopersicon* Ca^{2+}-ATPase (LCA), a type IIA non-calmodulin regulated P-type pump found in tomato plants that is very similar to mammalian sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), and *Arabidopsis* Ca^{2+}-ATPase, isoform 2 (ACA2), a type IIB calmodulin regulated P-type ATPase found in the endoplasmic reticulum of *Arabidopsis* cells. We used nucleotide protection against FITC labeling as a measure of binding since both LCA and ACA contained the KGAP(S,V,F)E motif, which has been shown to be modified by fluorescein isothiocyanate (FITC) in P-type pumps from animal cells. We demonstrated that the heterologously expressed GST-tagged ABDs from both LCA and ACA2 were modified by FITC and that ATP protects against this modification. Moreover, GTP was able to reduce, but not eliminate, the level of FITC labeling in both ABD constructs, suggesting that these plant pumps may also bind GTP with low affinity, which is in contrast to mammalian SERCA and PMCA type pumps which do not bind GTP.

1. INTRODUCTION

Ca^{2+} -ATPases are integral membrane proteins that actively transport Ca^{2+} ions “uphill” in eukaryotic cells and help establish and maintain steep Ca^{2+} gradients across membranes. In addition, they play a crucial role in maintaining calcium homeostasis, essential for Ca^{2+} mediated signaling networks in the cell. Ca^{2+} -ATPases belong to the P-type family of ATPases, characterized by the formation of an acylphosphate intermediate during the transport cycle. Plant Ca^{2+}-ATPases are broken down into two subgroups, IIA and IIB, based on their sequence similarity to animal sarco/endoplasmic reticulum and plasma membrane Ca^{2+} -ATPases, (SERCA and PMCA, respectively) [1, 2, and 3].

LCA, *Solanum lycopersicon* Ca^{2+} -ATPase, is a type IIA Ca^{2+}-ATPase (ER type) and has been reported to localize to plasma membrane (PM), and tonoplast (TN) in tomato plants, but not the endoplasmic reticulum (ER) despite containing a characteristic KXKXX ER retention signal (i.e. KLKAA) at its extreme C-terminus [4, 5, and 6]. On the other hand, ACA2, an Arabidopsis Ca^{2+} -ATPase isoform 2, is a type IIB Ca^{2+} -ATPase which is

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exclusively located in ER [6, 7]. Both LCA and ACA2 contain conserved motifs that are characteristic of all P-type ATPases including the PGD, PAD, TGES motifs of the small cytoplasmic loop, the PEGL motif, and the catalytic phosphorylation site DKTGT that contains the phosphate accepting aspartate (Fig 1). The large cytosolic domain also contains the KGAP(S,V,F)E motif that has been shown to participate in nucleotide binding via high resolution structures in some P-type pumps [8, 9, and 10]. Modification of the initial lysine residue in this motif has been shown to inhibit P-type pumps [11, 12]. Also conserved in the large cytoplasmic domain is the DPPR motif, which contains aspartate and proline residues that are thought to be crucial in binding inorganic phosphate. The MV(I)TGD motif, which is part of the ATP binding domain (ABD) in the large cytoplasmic loop, and the MTGDGVN motif, in a putative "hinge" region, are also conserved in LCA (Fig 1). The "hinge" region is thought to be reasonably flexible so as to allow the ABD and the phosphorylation domain to come together. The six amino acid residues that coordinate the transport of the two Ca^{2+} ions in animal SERCAs are fully conserved in LCA but only three of these six are conserved in ACA2, similar to its mammalian ortholog, PMCA [10, 13]. The stoichiometries of plant LCA and ACAs have not been determined. Since the residues that coordinate the Ca^{2+} binding sites in SERCA and PMCA are conserved in LCA and ACA2 respectively, it is likely that LCA transports two Ca^{2+} ions and ACA2 transports one Ca^{2+} ion per catalytic cycle [13]. However, the cation binding site within LCA is not a strict mimic of mammalian SERCA, as LCA appears to also transport Mn^{2+}, whereas SERCA does not [14].

For the present study, we focus on the ABDs from LCA and ACA2, which represent the two subgroups of plant Ca^{2+} -ATPases. These ABDs were heterologously expressed and purified from *Escherichia coli* as GST-tagged fusion proteins. We characterized the isolated ABDs of these plant Ca^{2+} -ATPases and determined their nucleotide specificity. Given that both LCA and ACA have the conserved KGAP(S,V,F)E motif modified by fluorescein isothiocyanate (FITC) in other P-type pumps, we used nucleotide protection against FITC labeling as a measure of nucleotide binding [15]. We observed that the isolated ABDs from both LCA and ACA2 were modified by FITC and that ATP protects against this modification, consistent with ATP binding to the isolated domains. A full-length type IIB Ca^{2+} -ATPase (i.e. ACA2-like) from radish has been shown to be modified by FITC in a nucleotide dependent manner [35], but this is the first demonstration that LCA (a plant type IIA Ca^{2+} -ATPase) can be modified by fluorescein derivatives. Moreover, GTP was able to reduce, but not eliminate, the level of FITC labeling in both ABD constructs, suggesting that these plant pumps may also bind GTP. In contrast, mammalian SERCA and PMCA type pumps do not bind GTP [15].

2. RESULTS

The main focus for the present study was to compare the nucleotide specificity of two plant Ca^{2+} -ATPases, LCA and ACA2. In order to specifically look at the nucleotide binding, we expressed and purified the respective nucleotide binding domains from these transporters and performed all the experiments utilizing the affinity purified GST-tagged ABDs of LCA and ACA2.

2.1. Overexpression and Purification of Plant Ca^{2+} -ATPase ATP Binding Domains

LCA-ABD and ACA2-ABD were PCR amplified and cloned into pGEX-4T3 to make GST fusion constructs (at the N-terminus). The plasmids were subsequently transformed into BL21-DE3 pLysS *E. coli* cells for protein expression. GST-tagged ABDs of LCA and ACA2 were purified by glutathione affinity chromatography, which yielded about 3-4 mg of purified protein from a culture volume of one liter (Fig 2). Different fractions of the purification process including the eluent were subjected to sodium dodecyl sulfate poly-

acrylamide gel electrophoresis (SDS PAGE) by running a 12% gel. Coomassie staining of the SDS-PAGE gel revealed GST-ABD proteins of the predicted size of approximately 72 kDa. In many instances the GST-constructs appeared as a doublet, likely indicating a slight degradation (Fig 2A). Western blotting was performed on the same fractions with anti-GST antibody to ensure that the eluted products were indeed the GST-tagged proteins of interest (Fig 2B).

2.2. FITC labeling of LCA and ACA2 ABDs

Fluorescein isothiocyanate (FITC) is a fluorescent amine-reactive molecule that readily reacts with the epsilon amino group of Lysine residues. Isothiocyanates are known to label Lys^{501} in Na,K-ATPase [15, 16], and the equivalent conserved lysine in other animal P-type ATPases [17]. Here we predicted that the similarly conserved lysine residues in the plant pumps, LCA and ACA2, (Lys^{530} and Lys^{578}, respectively) would also be modified by FITC in an ATP protectable manner. Simultaneous presence of ATP has been shown to prevent FITC labeling in Na^+,K^+-ATPase [11, 18], as well as other animal P-type ATPases [11, 12, 18, 19]. There is also a single report of covalent FITC modification in a plant P-type ATPase [35].

FITC labeling experiments were performed using 6 μg of both GST-ABDs in the presence or absence of ATP (5 mM) in 50mM Tris buffer (pH 7.4). Purified Na,K-ATPase from sheep kidney was used as the positive control. After a 15 min incubation of the proteins with 50 μM FITC at room temperature (pH 7.4; RT 23-25°C), the reactions were terminated by the addition of 4X Laemmli sample buffer and rapid freezing; aliquots were run on a 12% SDS-PAGE gel. Successful FITC labeling was imaged using a UV light box.

We determined the relative affinities of GST-ACA2-ABD and GST-LCA-ABD for FITC binding. We found that the labeling of both ABDs increased with increasing concentrations of FITC, with maximal binding observed at concentrations above approximately 50 μM FITC (Fig 3). Although there appeared to be no significant differences in the affinity between the two plant ABDs for FITC binding, we did observe that the affinity of both ABDs for FITC was lower than Na^+,K^+-ATPase, which exhibits maximal binding at approximately 10 μM FITC (data not shown).

Figures 4 and 5 show that FITC clearly modified both GST-ABDs as well as the purified Na^+,K^+-ATPase. Moreover, simultaneous incubation with ATP and FITC completely abolished FITC labeling, demonstrating that ATP does bind to both isolated nucleotide binding domains (Fig 4A). After imaging, the gel was stained with coomassie brilliant blue to show that equal amounts of protein were loaded in each lane in the gel (Fig 4B).

It has been reported that although ATP is the preferred substrate for plant Ca^{2+}-ATPases, in the absence of ATP, some plant Ca^{2+} -pumps can utilize other high energy nucleotide triphosphates such as GTP [20, 21, 22]. Consequently, we wanted to determine whether either tomato plant LCA or Arabidopsis ACA2 might be able to use GTP. Thus, we performed the same FITC labeling assay as above in the presence or absence of GTP (5 mM) to determine if GTP would protect against FITC labeling. Here, the purified Na^+,K^+-ATPase was used as the negative control, as GTP has been shown to not protect against FITC labeling [15, 23]. Figure 5 reveals that GTP is not as effective as ATP in protecting the isolated ABDs from FITC labeling. Nonetheless, there is a noticeable difference in the fluorescence intensities between the FITC-only samples and the FITC/GTP samples (Fig 5A). The gel was subsequently stained with coomassie brilliant blue to show that equal amounts of protein were loaded in each lane (Fig 5B).

3. DISCUSSION

Results from this study show that FITC covalently labels isolated plant Ca^{2+} - pump ATP-binding domains and ATP prevents this modification; we used representatives of both type IIA (LCA) and IIB (ACA2) plant Ca^{2+} -ATPases. The site of FITC modification in Na^+,K^+-ATPase has been identified as Lys^{501} which has been shown to reside within the nucleotide binding domain via recent crystal structures of this enzyme and indeed Lys^{501} is an ATP coordinating residue [24, 25, 26]. In SERCA, FITC selectively binds to Lys 515, which also resides in the nucleotide binding of the N-domain [12, 17, 27]. An equivalent lysine residue exists in a conserved sequence among most P_2-ATPases including LCA and ACA2. However, the sequences differ slightly; in Na^+,K^+-ATPase Lys^{501} is in the sequence, KGAPE, whereas the equivalent lysine residues reside in sequences KGAFE and KGASE for LCA and ACA2 respectively (Fig 1). Since it is known that proline residues can significantly alter the secondary structure (e.g. breaking alpha helixes), it is possible that the absence of proline in LCA and ACA2 can alter the ability of the conserved lysine to interact with ATP or FITC (or both). Here, we demonstrate that although the conserved sequences in these plant pumps differ from mammalian P-type pumps (e.g. Na^+,K^+-ATPase and SERCA), the lysine residue is still important for ATP binding. Clearly, the definitive identification of all ATP coordinating residues in these plant pumps awaits high-resolution crystal structures of these ATPases.

An interesting characteristic observed in P-type ATPases where investigated is the appearance of two kinetically distinct ATP effects on ATPase activity. There is a high affinity ATP effect (K_m 0.1-1.0 μM) which catalytically phosphorylates the enzyme and a low affinity ATP effect (K_m 100-500 μM) that accelerates the rate-limiting conformational change resulting in cation access moving from extracytoplasmic to cytoplasmic [36]. Whether these two ATP effects are the result of a single physical binding site that has different ATP affinities in different conformations, versus two physically distinct sites remains a matter of debate. The dose-response to FITC labeling suggests only a single site is labeled (Fig. 3). A similar single site with low ATP affinity was observed in the isolated ATP-binding domain of the Na,K-ATPase [15].

3.1. Plant Pumps vs. Mammalian Pumps

Comparing the sequence alignments of LCA and ACA2 to Na^+,K^+-ATPase revealed that the three pumps share roughly 30% sequence identity and approximately 45% similarity. A chemically accessible lysine residue, which is labeled by FITC, is present in a highly conserved region in all the three pumps (highlighted in Fig 1). Interestingly, only this single lysine is able to nucleophilically attack amine-reactive reagents, like isothiocyanates, at physiological pH. This suggests that the local pK of this lysine residue must be lower than that of a typical lysine residue (~ pK = 10.5). That is, with a pK of 10.5 essentially all of the lysine residues exist in the protonated – NH_3^+ form, which is unable to act as a nucleophile as its lone pair of electrons is no longer available. The observation that FITC can modify this lysine residue in all of the P_2-type pumps tested thus far, suggests that the local conformation of the enzyme active site sufficiently lowers the pK. The lower pK concomitantly increases the fraction of this lysine residue that exists in the non-protonated – NH_2 form, which is highly nucleophilic and readily conjugated.

While ACA2 is found exclusively in the ER of *Arabidopsis* plants, LCA has been reportedly found in the PM and TN of tomato plants [28]. The ABDs of both pumps appear similar in size, being approximately 47 kDa. The results of this study show that both pumps equally bind ATP and are able to use it to protect against FITC labeling (see Fig 4). The results also indicate that the ABDs might bind GTP as well, but with a much lower affinity than ATP. Moreover, ACA2 appears to bind GTP better than LCA (see Fig 5). Studies have shown that

there exist substrate preferences between ECAs (ER type Ca^{2+} -ATPase) and ACAs. ACAs are able to use GTP or ITP as substrates, as an alternative to ATP, unlike ECAs that show a strong preference for ATP as the substrate [13, 20, 29, 30]. This can be attributed to subtle differences in the conformation of the two ABDs. GTP is slightly larger than ATP, due to an extra amino group on the purine ring, and it may be that the ABD of ACA2 is slightly larger than the ABD of LCA, thus ACA2 may be able to accommodate GTP more readily than LCA. On the other hand, ITP and ATP are similar in size, yet ACAs are known to utilize ITP as substrate as well as ATP [6]. Similarly, the ABD of the mammalian Na^+,K^+-ATPase is also around 46 kDa. Like LCA, the Na^+,K^+-ATPase ABD was shown to protect against FITC labeling in the presence of ATP but not in the presence of GTP [15]. It is possible that mammalian pumps have been selected against utilizing nucleotides with substituents in the 2-position of the purine ring and a similar selection exists in LCA (and possibly all plant type IIA ATPases).

4. MATERIALS AND METHODS

4.1. Construction and overexpression of LCA and ACA2 ABD plasmids

Both GST-LCA-ABD and GST-ACA2-ABD constructs were made by PCR amplification of the corresponding ~1200 bp fragments encoding M4M5, which were subsequently ligated into SacI and NotI sites of pGEX-4T3 vector (ACA2 gene was a generous gift from Dr. Jeffrey Harper, University of Nevada-Reno).

GST-LCA-ABD and GST-ACA2-ABD plasmids were transformed into BL21 (DE3) pLysS cells for protein expression and purification. Cultures with an OD_{600} of 0.6 - 0.8 were induced with 0.1 mM Isopropyl-β-D-1-thiogalactopyranoside (IPTG) and grown overnight at room temperature (RT). Overnight cultures were pelleted and resuspended in 30 ml of lysis buffer, which consists of 50 mM Tris,100 mM NaCl, pH 7.9, 5 mM dithiothreitol, 5 μM antipain, 5 μM leupeptin, 5 μM pepstatin, 2 μM chymostatin, 5 μM protease inhibitor cocktail (Sigma), and 1 mM phenylmethanesulphonylfluoride. The cells were lysed using a French Press. The lysate was then spun down at 13,500 × *g* for 45 minutes at 4°C. The insoluble pellet was discard ed while the soluble supernatant was subjected to glutathione-affinity column purification.

4.2. Affinity Column Chromatography

A 1 ml volume of reduced glutathione-conjugated sepharose resin was washed with 14 ml of 1X TBS (50 mM Tris-Cl, pH 7.5 and 150 mM NaCl). The resin and the buffer were centrifuged at 500 × *g* for 5 minutes at 4°C. The supernatant was discarde d and the soluble protein was added to the resin. The sample was then mixed for 1 hour at 4°C on a rotisserie mixer. After thorough mixing, it was spun at 500 × *g* for 10 minutes at 4°C. The supernatant was collected and labeled as “Binding Flow Through”. About 3 ml of 1X TBS was added to the protein bound resin, which was then added to a glass column filter (5 ml volume). The resin was allowed to settle and the column was washed overnight with 1X TBS at 4°C. Any remaining proteins bound to the column were then eluted using 3 ml of 10 mM reduced glutathione in 1X glutathione reconstitution buffer (Novagen). Protein concentrations were determined by the method of Lowry using bovine serum albumin as a standard [31].

4.3. SDS-PAGE and Western Blotting

A 6 g quantity of cell pellet, soluble fraction, Binding Flow Through, Column Flow Through, and Eluent fractions for both GST-ACA2-ABD and GST-LCA-ABD were subjected to SDS-PAGE [32] on 12% acrylamide gels. Protein samples were mixed with Laemmli sample buffer (1:1:1 v/v 8 M urea, 10% SDS, 125 mM Tris-HCl, pH 6.8 with 5% v/v β-Mercaptoethanol) to give a final volume of 50 μL. Gels were stained with coomassie

brilliant blue (5% v/v glacial acetic acid, 50% v/v methanol, and 0.1% w/v Coomassie Brilliant Blue R-250) and de-stained with 10% v/v acetic acid, 10% v/v isopropanol overnight to detect proteins of interest and determine their purity.

After electrophoresis, proteins were transferred onto PVDF membranes by electroblotting in 10 mM CAPS, 10% v/v methanol, pH 11.0, for 2 hr at 180 mA constant current [33]. The PVDF membrane was blocked with soymilk [34] and subsequently incubated with 1:2500 goat anti-GST (Amersham) for an hour at RT. After three washes with PBS/0.1% v/v Tween-20, it was incubated with HRP-conjugated rabbit anti-goat at 1:5000 (Pierce) for 1h at RT. The membrane was then washed five times with PBS/0.1% Tween-20 and the proteins were visualized by chemiluminescent detection of peroxidase activity using the SuperSignal substrate kit (Pierce).

4.4. FITC-Labeling Assay and Nucleotide Binding Determination

FITC-labeling assays were performed utilizing purified preparations of GST-ACA2-ABD and GST-LCA-ABD. Purified endogenous Na^+,K^+ -ATPase from sheep kidney was used as the positive control. Briefly, after incubating the proteins in the presence or absence of 5 mM of nucleotide (ATP or GTP), 6 μg protein and 50 mM Tris, pH 7.4 at RT for 10 minutes, freshly prepared FITC (50 μM final concentration and final reaction volume of 37.5 μL) was added and the reactions were further incubated for 15 minutes at room temperature (23-25°C). The chemical modification reactions were stopped by the addition of 12.5 μL of 4X Laemmli sample buffer and snap freezing and the samples were stored in the freezer. The 50μL samples were subsequently resolved on 12% gels. The gel was imaged using UV light to visualize the FITC labeled bands and stained thereafter with coomassie brilliant blue to show that equal amounts of protein had been loaded in each lane.

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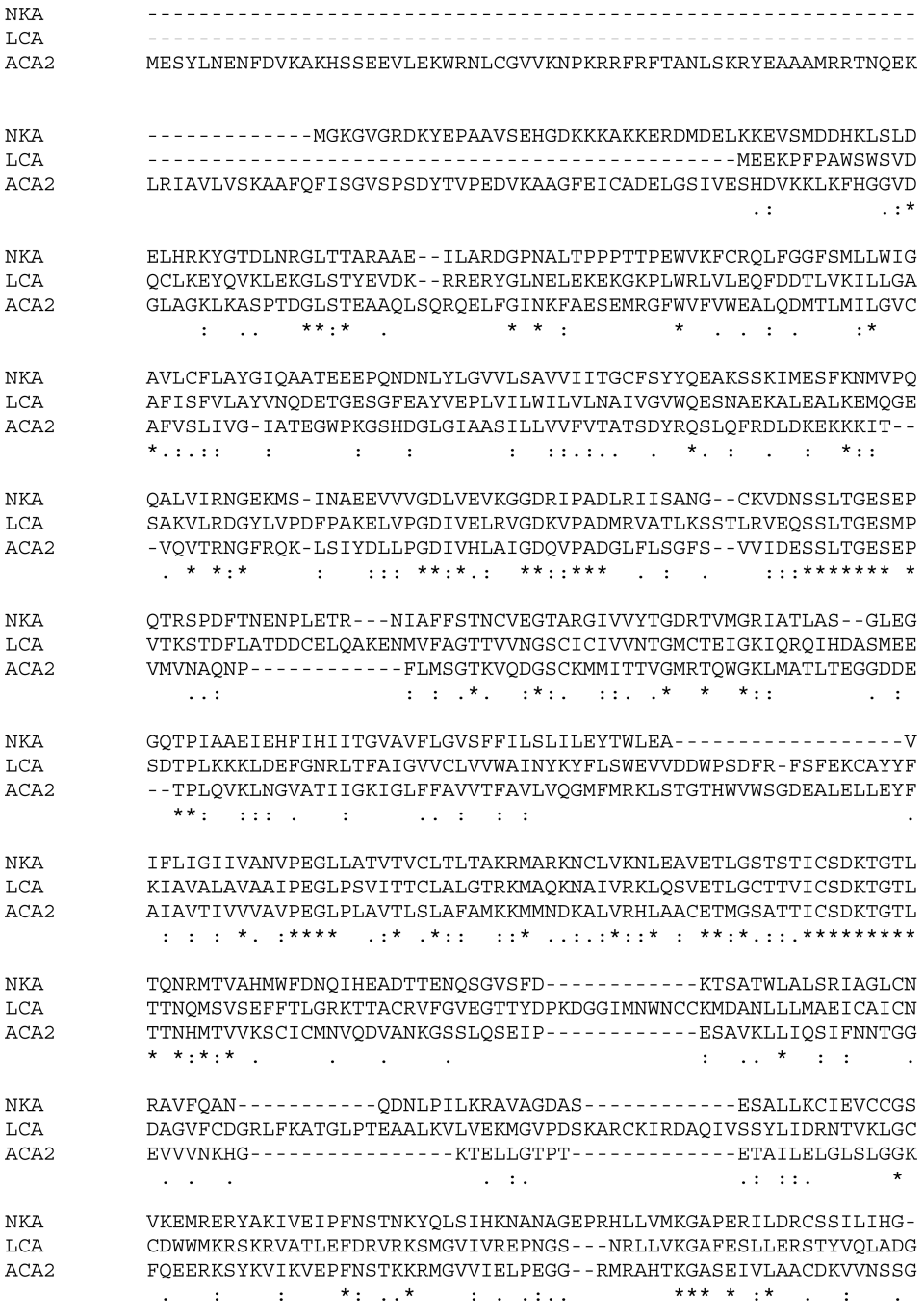
HIGHLIGHTS

1. FITC labels the ATP site in tomato and Arabidopsis Ca-ATPases (LCA and ACA2).
2. ATP binding protects against FITC modification of LCA and ACA2 ATP binding domain.
3. GTP binding reduces but does not abrogate FITC modification of LCA and ACA2 ABDs.



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NKA         KEQPLDEELKDAFQNAYLELGGLGERVLGFCHLMLPDEQ--------FPEGFQFDTDDVN
LCA         STVPLDESCRQLLLLKQLEMSSKGLRCLGLAYKDDLGELSGYYAATHPAHKKLLDPSCYS
ACA2        EVVPLDEESIKYLNVTINEFANEALRTLCLAYMDIEGGFS----------------PDDA
            .  ****.  . :     *:.. . * * :.:    .

NKA         FPVDNLCFVGLISMIDPPRAAVPDAVGKCRSAGIKVIMVTGDHPITAKAIAKGVGIISEG
LCA         SIESDLVFVGVVGLRDPPREEVHRAVNDCRRAGIKIMVITGDNKSTAEAVCREIQLFSNG
ACA2        IPASGFTCVGIVGIKDPVRPGVKESVELCRRAGITVRMVTGDNINTAKAIARECGILTDD
               ..:  **:.:: ** *  *  :*  ** ***.: ::***:  **:*:.:   :::::.

NKA         NETVEDIAARLNIPVSQVNPRDARACVVHGSDLKDMTPEQLDDILKYHTEIVFARTSPQQ
LCA         E-------------------NLRGSSFTGKEFMAFSSQQQIEILSQDGGKVFSRAEPRH
ACA2        G-----------------------IAIEGPVFREKNQEELLELIP--KIQVMARSSPMD
                                    . *  :   . ::  :::      *::*:.* .

NKA         KLIIVEGCQR-QGAIVAVTGDGVNDSPALKKADIGVAMGIAGSDVSKQAADMILLDDNFA
LCA         KQEIVRMLKE-MGEIVAMTGDGVNDAPALKLADIGIAMGITGTEVAKEASDMVLADDNFS
ACA2        KHTLVKQLRTTFDEVVAVTGDGTNDAPALHEADIGLAMGIAGTEVAKESADVIILDDNFS
            *  :*.  :   . :**:****.**:***: ****:****:*::*:*:::*::: ****:

NKA         SIVTGVEEGRLIFDNLKKSIAYTLTSNIPEITPFLIFIIANIPLPLGTVTILCIDLGTDM
LCA         TIVSAVAEGRSIYNNMKAFIRYMISSNVGEVISIFLTAVLGIPECLIPVQLLWVNLVTDG
ACA2        TIVTVAKWGRSVYINIQKFVQFQLTVNVVALVVNFSSACLTGSAPLTAVQLLWVNMIMDT
            :**: .  ** :: *::  : : :: *:  :   :       .  * .* :* :::  *

NKA         VPAISLAYEQAESDIMKRQPRNPQTDKLVN-----ERLISMAYGQIGMIQALGGFFTYFV
LCA         PPATALGFNPADVDIMQKPPRK-NTDALINSWVFFRYMVIGSYVGIATVGIFIVWYTQAS
ACA2        LGALALATEPPNDELMKRLPVGRRGNFITN-------AMWRNILGQAVYQFIVIWILQAK
              * :*. : .: ::*:: *   . : : *        :       .    :  :

NKA         IMAENGFLPNHLLGIRVTWDDRWINDVEDSYGQQWTYEQRKIVEFTCHTAFFVS------
LCA         FLGINIVSDGHTLVELSQLRNWGECSTWTNFTVSPFKAGNRLITFSDPCEYFTVGKVKAM
ACA2        GKAMFGLDGPDSTLMLNTLIFN-------------------------------------
              .   .   .

NKA         ------IVVVQWADLVICKTRRNSVFQQG-MKNKILIFGLFEETALAAFLSYCPGMGVAL
LCA         TLSLSVLVAIEMFNSLNALSEDNSLIKMPPWRNPWLLVAMSLSFALHSVILYVPFLADIF
ACA2        -----CFVFCQVFNEISSREMEEIDVFKGILDNYVFVVVIGATVFFQ--IIIIEFLGTFA
                  :*  :  : : .    :  .      *  ::. :     :   :     :.

NKA         RMYPLKPTWWFCAFPYSLLIFVYDEVRKLIIRRRPGGWVEKETYY
LCA         GIVPLSLYEWLLVILLSAPVILIDEVLKFVGRRRRRTKLKAA---
ACA2        STTPLTITQWIFSIFIGFLGMPIAAGLKTIPV-------------
               **.   *:  :  .   :      * :
```

Figure 1.

Alignment of three P-type ATPases. Na^+,K^+-ATPase alpha-1 subunit from *Ovis aris*, LCA, and ACA2 amino acid sequences were aligned using ClustalW. NKA is the Na^+,K^+-ATPase. Conserved motifs in NKA, LCA, and ACA2 are highlighted.

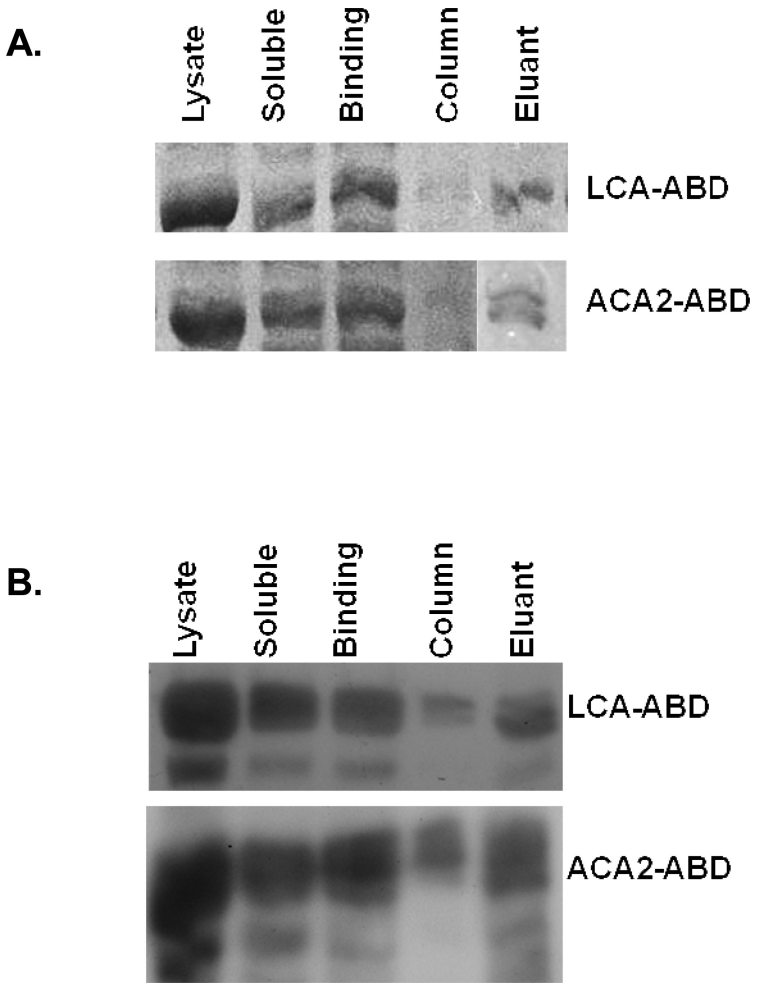

Figure 2.

Purification of GST-LCA-ABD and GST-ACA2-ABD proteins. **(A)** Coomassie stained gel images showing GST-LCA-ABD and GST-ACA2-ABD fractions. The Fermentas PageRuler prestained protein ladder was used as a molecular weight standard for this 12% SDS-PAGE gel. The lanes labeled “Lysate” contain a sample from the lysate fractions of the protein prep, “Soluble” is the sample from the soluble fraction of the protein, “Binding” is the flow through of the binding step of the prep, “Column” is the column flow through and “Eluent” is the protein eluted from the GST column. Each lane contains 6 μg of protein, as determined by Lowry assay. In each lane, except in the column flow through lanes (“Column”) a band at approximately 72 kDa is apparent, which is the approximate molecular weight of both GST-LCA-ABD and GST-ACA2-ABD. **(B)** Western blot analysis of GST-LCA-ABD and GST-ACA2-ABD. Each lane of this 12% SDS-PAGE gel was loaded with 6 μg of protein, as determined by Lowry assay. After protein transfer, the PVDF membrane was blocked with soymilk following by probing with primary (goat anti-GST; 1:2500) and secondary (rabbit anti-goat; 1:5000) antibody. Detection was done using Pierce ECL Western Blotting Substrate. The top bands in all of the lanes are the ABDs, while the bottom bands in these lanes appear to be slightly degraded products of the ABDs.

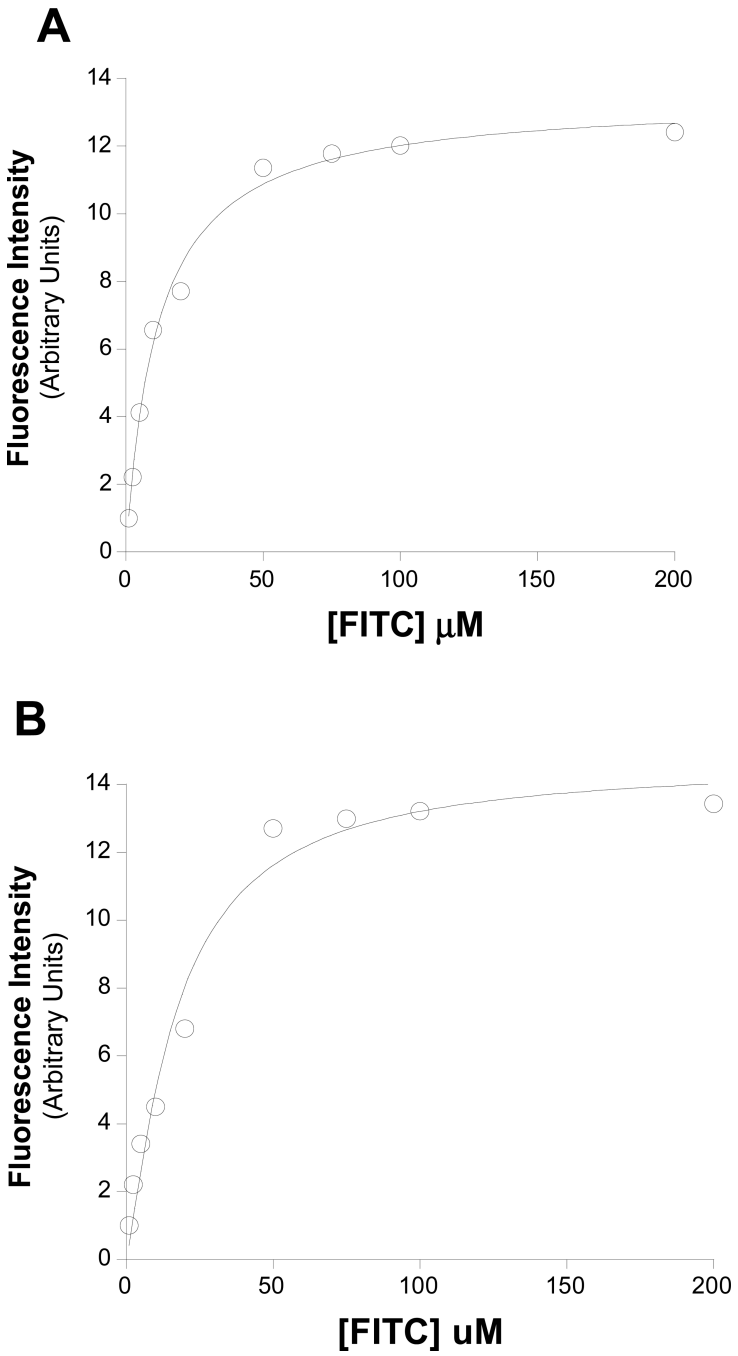

Figure 3.

Quantitative analysis of the affinities of GST-ACA2-ABD and GST-LCA-ABD for FITC. Equal amounts (6 μg) of GST-ACA2-ABD (A) or GST-LCA-ABD (B) were incubated with increasing concentrations of FITC. Fluorescein labeling of both ACA2-ABD and LCA-ABD increased with higher concentrations of FITC up to about 50 μM, above which no further FITC incorporation was observed. Fluorescence intensity of FITC-labeled ABDs was determined via pixel density using Photoshop (version 7.0). The figure is a representative of three replications.

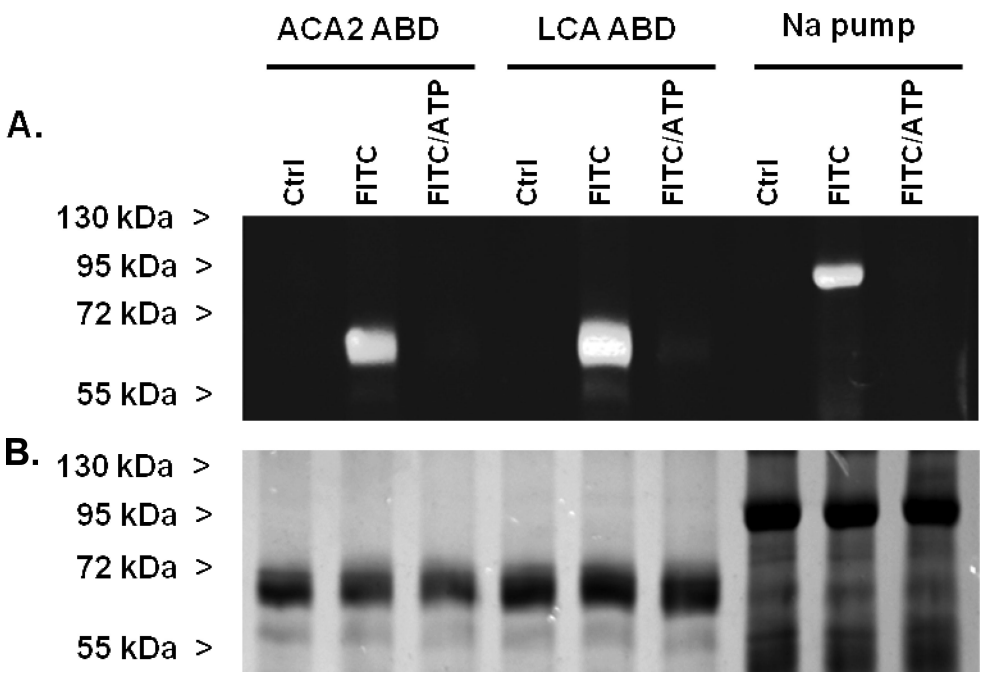

Figure 4.

FITC labeling of LCA-ABD and ACA2-ABD in the presence of ATP. (A) UV image of FITC/ATP gel. This is a UV image of 12% SDS-PAGE gel loaded with samples used in the FITC labeling assay in the presence or absence of 5 mM ATP. Fermentas PageRuler prestained protein ladder was loaded as the molecular weight standard. The lanes labeled "Ctrl" are the control lanes that contain only Tris buffer and 0.5 mg/ml enzyme. The lanes labeled "FITC" are lanes that contain 6 μg protein, Tris buffer, and 50 μM FITC. The lanes labeled "FITC/ATP" contain 6 μg protein, Tris buffer, 50 μM FITC, and 5 mM ATP. In both LCA-ABD and ACA2-ABD samples, a clear fluorescent band can be seen at approximately 72 kDa in the "FITC" lanes and a fluorescent band in the Na Pump "FITC" lane at approximately 110 kDa, while there is no fluorescent product in any of the "ATP" lanes. The figure is a representative experiment of three replications. (B) The FITC/ATP gel stained with Coomassie brilliant blue R-250. The gel shows that equal amounts of proteins were loaded in all lanes thus confirming that the fluorescence seen in Fig 4A is not due to differing amounts of protein in the gel. In ACA2-ABD and LCA-ABD lanes, ABDs can be seen at approximately 72 kDa with degradation products apparent below 72 kDa. The α-subunit of the Na pump, which is responsible for ATP and FITC binding, can be seen at approximately 110 kDa, while the β-subunit can be seen at approximately 55 kDa.

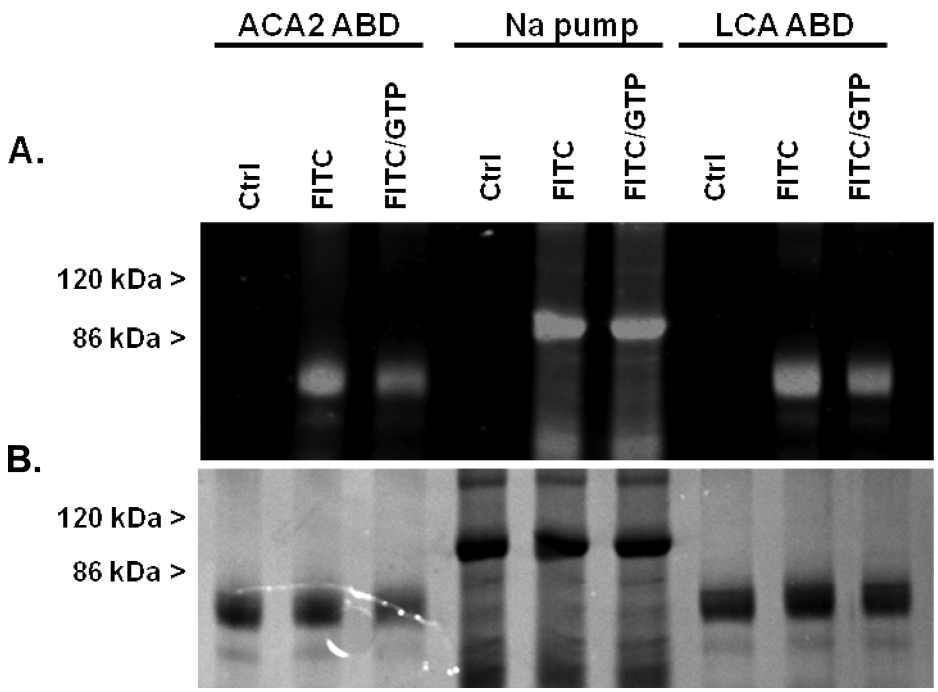

Figure 5.

FITC labeling of LCA-ABD and ACA2-ABD in the presence of GTP. (A) UV image of FITC/GTP gel. This is a UV image of a gel loaded with samples used in the FITC labeling assay performed in the presence of GTP. In both the “FITC” lane and the “FITC/GTP” lane for all samples (ACA2, Na pump, LCA) a fluorescent band can be seen. This band is present at approximately 72 kDa in the ACA2 and LCA “FITC” and “FITC/GTP” lanes and at about 110 kDa in the Na Pump “FITC” and “FITC/GTP” lanes. The figure is a representative experiment of three replications. (B) FITC/GTP gel stained with Coomassie brilliant blue R-250 to indicate that equal amounts of protein were loaded.