Biochimica et Biophysica Acta 1848 (2015) 702–711

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Molecular mechanism of regulation of villus cell Na-K-ATPase in the chronically inflamed mammalian small intestine

Prosenjit Saha [1], Palanikumar Manoharan [2], Subha Arthur, Shanmuga Sundaram, Ramesh Kekuda [3], Uma Sundaram *

Department of Clinical and Translational Sciences, Joan C. Edwards School of Medicine, Marshall University, Huntington, WV 25701, USA

ARTICLE INFO

Article history:
Received 11 August 2014
Received in revised form 3 November 2014
Accepted 6 November 2014
Available online 22 November 2014

Keywords:
Inflammatory bowel disease
Na-nutrient co-transport
Villus cell
Chronic intestinal inflammation
Na-K-ATPase
Ankyrin

ABSTRACT

Na-K-ATPase located on the basolateral membrane (BLM) of intestinal epithelial cells provides a favorable intracellular Na^+ gradient to promote all Na dependent co-transport processes across the brush border membrane (BBM). Down-regulation of Na-K-ATPase activity has been postulated to alter the absorption *via* Na-solute co-transporters in human inflammatory bowel disease (IBD). Further, the altered activity of a variety of Na-solute co-transporters in intact villus cells has been reported in animal models of chronic enteritis. But the molecular mechanism of down-regulation of Na-K-ATPase is not known. In the present study, using a rabbit model of chronic intestinal inflammation, which resembles human IBD, Na-K-ATPase in villus cells was shown to decrease. The relative mRNA abundance of α-1 and β-1 subunits was not altered in villus cells during chronic intestinal inflammation. Similarly, the protein levels of these subunits were also not altered in villus cells during chronic enteritis. However, the BLM concentration of α-1 and β-1 subunits was diminished in the chronically inflamed intestinal villus cells. An ankyrin–spectrin skeleton is necessary for the proper trafficking of Na-K-ATPase to the BLM of the cell. In the present study, ankyrin expression was markedly diminished in villus cells from the chronically inflamed intestine resulting in depolarization of ankyrin-G protein. The decrease of Na-K-ATPase activity was comparable to that seen in ankyrin knockdown IEC-18 cells. Therefore, altered localization of Na-K-ATPase as a result of transcriptional down-regulation of ankyrin-G mediates the down-regulation of Na-K-ATPase activity during chronic intestinal inflammation.

© 2014 Elsevier B.V. All rights reserved.

1. Introduction

Na-K-ATPase is an integral protein present in the BLM of mammalian epithelial cells. It is composed of α and β subunits, where the α-1 subunit is essential for the functioning of Na-K-ATPase while the β-1 subunit is important for stabilizing the correct folding of the α-1 polypeptide. The ankyrin–spectrin skeleton is necessary for the proper trafficking of Na-K-ATPase to the BLM of the cell. Thus, alteration at any one of these critical steps can affect the functioning of Na-K-ATPase.

Na-K-ATPase is responsible for establishing and maintaining high intracellular K^+ and low intracellular Na^+ concentrations that result in a favorable intracellular Na^+ gradient [1]. In theory, the nutrient-coupled uptake of Na^+ entry across the BBM increases $[Na^+]_i$, which, in turn, increases Na^+ extrusion across the BLM through Na-K-ATPase. This favorable transcellular Na^+ gradient is efficiently maintained by BLM Na-K-ATPase and is in turn essential for the proper functioning of BBM Na-coupled nutrient co-transporters, which are the primary means of absorption of many (macro) nutrients (*e.g.* glucose, amino acid), some vitamins and certain nucleic acids (*e.g.* adenosine). Thus, the proper functioning of Na-K-ATPase is essential for efficient nutrient absorption and subsequent maintenance of good health. For this reason, Na-K-ATPase has been found to play a significant part in the management and treatment of disease conditions such as diarrheal diseases. For example in diarrhea, though a significant malabsorption of nutrients is seen, Na-K-ATPase function is preserved thus forming the basis for oral rehydration therapy where the functional Na-K-ATPase efficiently moves the Na^+ salt and glucose in the oral rehydration fluid through the intestinal epithelial cells along with water molecules resulting in the rehydration of the patients [2,3]. Also, in the chronically inflamed small intestine (*e.g.* IBD and Crohn's disease), the cellular alteration of a variety of BBM Na-solute co-transport processes is at least partially, a result

* Corresponding author at: 1600 Medical Center Drive, Joan C. Edwards School of Medicine, Marshall University, Huntington, WV 25701, USA. Tel.: +1 304 691 1841; fax: +1 304 691 1840.
E-mail address: sundaramu@marshall.edu (U. Sundaram).
[1] Present address: Chittaranjan National Cancer Institute, Kolkata 700 026, India.
[2] Present address: Department of Molecular Genetics, Biochemistry & Microbiology, University of Cincinnati, Cincinnati, OH 45267, USA.
[3] Present address: Department of Pediatrics, West Virginia University, Morgantown, WV 26506, USA.

http://dx.doi.org/10.1016/j.bbamem.2014.11.005
0005-2736/© 2014 Elsevier B.V. All rights reserved.

of the diminished Na-K-ATPase activity on the BLM of absorptive villus cells [4–7]. For example, villus BBM Na-glutamine co-transporter B0AT1 is down-regulated during inflammation partially due to the down-regulation of BLM Na-K-ATPase leading to the loss of the intracellular Na^+ gradient [7]. However, when Na-K-ATPase activity was restored by treatment with a corticosteroid, the BBM B0AT1 was also restored in villus cells, thus establishing that Na-K-ATPase is, at least in part, responsible for the loss of a BBM Na-nutrient co-transporter [8]. Finally, Na-K-ATPase is also important for the osmotic balance and volume regulation of cells [9].

Na-K-ATPase is a transmembrane heterodimer consisting of two dissimilar subunits, α and β, present in equimolar amounts. The α subunit regulates catalytic activity and contains intracellular binding sites for ATP and Na^+, a phosphorylation site and extracellular binding sites for K^+ [10]. The glycosylated β subunit is essential for Na-K-ATPase function as it facilitates α/β heterodimer formation and subsequent transport of the holoenzyme to the plasma membrane [11]. Multiple isoforms of Na-K-ATPase α (α-1, α-2, α-3 and α-4) and β (β-1, β-2 and β-3) subunits are expressed in a developmental and tissue-specific pattern [12–17]. Subunits α-1 and β-1, are constitutively expressed in the majority of tissues, whereas the α-2, α-3 and α-4 genes, as well as the β-2 and β-3 genes, have limited expression [18]. A third small polypeptide, the γ subunit, is found in association with α and β dimer in a tissue-specific manner and does not seem to be required for functional Na-K-ATPase and may play a regulatory role [19]. The cytoplasmic domain of the α subunit interacts with the N-terminal domain of ankyrin [20], a protein that connects the Na-K-ATPase to the spectrin (fodrin) based membrane skeleton and stabilizes Na-K-ATPase at the plasma membrane [21].

Multiple mechanisms can possibly regulate the activity of Na-K-ATPase subject to either physiological or pathophysiological conditions. A dysfunction or deficiency of Na-K-ATPase has been identified in chronic neurodegenerative disorder, and cardiovascular and renal diseases [22–25]. Several studies have described a decreased activity of Na-K-ATPase in acute and transient infectious enteritis [26–30]. In chronic diarrheal diseases characterized by chronic intestinal inflammation resulting in malabsorption and thus malnutrition, alterations in Na-K-ATPase function maybe even more important. For example, in an animal model resembling IBD, it has been shown that Na-glucose (SGLT1), Na-alanine (ATB0), Na-glutamine (B0AT1), Na-taurocholate (ASBT), and Na-adenosine (DMT1) co-transport is diminished in intact villus cells. In these instances described there is also a decrease in BLM Na-K-ATPase [4–7]. Thus, it has been suggested that the decrease of Na and nutrient absorption in diarrheal diseases characterized by chronic intestinal inflammation is, at least in part, due to a down-regulation of Na-K-ATPase in the BLM of villus cells. However, the mechanism of the alteration of Na-K-ATPase during chronic intestinal inflammation is not known. Therefore, in this study, the mechanism of Na-K-ATPase down-regulation in villus cells from the chronically inflamed intestine was determined.

2. Methods

2.1. Induction of chronic inflammation

New Zealand white rabbits (10–12 week old males; 2.0–2.2 kg) were intragastrically inoculated with approximately 10,000 *Eimeria magna* oocytes. The animals gradually developed chronic intestinal inflammation with the peak being on days 13 and 14 post inoculation when they showed symptoms of watery diarrhea. The animals were euthanized on day 14 with an overdose of pentobarbital sodium and the intestinal cells were isolated from the ileum immediately after euthanization. Intestinal inflammation was confirmed in the rabbits through histological characteristics like villus blunting, crypt hyperplasia and fusion, as well as immune cell (lymphocytes, plasma cells, neutrophils) infiltration of the mucosa by H&E staining. Usually, 80–90% of the inoculated rabbits develop intestinal inflammation and in the present study all the animals that were inoculated with the oocytes successfully developed intestinal inflammation. Control rabbits were sham inoculated with saline and did not show any signs of intestinal inflammation as confirmed through histological examination. All the animal handling and maintenance were done according to Institutional Animal Care and Use Committee regulations.

2.2. Villus cell isolation

Villus cells were isolated from normal and chronically inflamed rabbit small intestine by a Ca^{++} chelation technique as previously described [29]. Briefly, a 3-ft section of ileum was filled with buffer containing 0.15 mM EDTA, 112 mM NaCl, 25 mM $NaHCO_3$, 2.4 mM K_2HPO_4, 0.4 mM KH_2PO_4, 2.5 mM L-glutamine, 0.5 mM β-hydroxybutyrate, and 0.5 mM dithiothreitol, and gassed with 95% O_2 and 5% CO_2, pH 7.4, at 37 °C. The intestine was incubated in this solution for 3 min and gently palpitated for another 3 min to facilitate cell dispersion. The fluid was then drained from the ileal loop, phenylmethylsulfonyl fluoride was added, and the suspension was centrifuged at 1000 *g* for 3 min. Villus cells from fraction 2 were used for total RNA isolation, BLM, BBM and cell extract preparations and immunocytochemistry.

2.3. RTQ-PCR studies

Total RNA was isolated from normal and chronically inflamed rabbit intestinal villus cells by using an RNeasy Plus total RNA purification mini kit obtained from Qiagen. Real-time quantitative PCR (RTQ-PCR) was performed using total RNA isolated by a two-step method. First-strand cDNA synthesis from total RNA was performed by using SuperScript III from Invitrogen Life Technologies using an equal mixture of oligo (dT) primer and random hexamers. The cDNA generated was used as a template for real-time PCR using a TaqMan Universal PCR master mix from Applied Biosystems (Foster City, CA) according to the manufacturer's protocol. Table 1 describes rabbit Na-K-ATPase α-1, Na-K-ATPase β-1, ankyrin and spectrin specific primer and probe sequences that were used for the RTQ-PCR studies. Rabbit specific β-actin primers and probes were run along with all the RTQ-PCR as internal control. The expression of β-actin was used to normalize the expression levels between the individual samples. The sequences of the rabbit β-actin primers and probes are also listed in Table 1. All experiments were performed in triplicates.

2.4. Western blot studies

Western blotting for Na-K-ATPase α-1, Na-K-ATPase β-1, ankyrin-G and spectrin was performed according to the standard protocols. BLM protein and cell extracts were prepared from rabbit normal and chronically inflamed ileal villus cells. BLM were prepared by the sucrose-density gradient and differential centrifugation method, as described previously [31]. BBM from rabbit intestinal villus cells were prepared by $CaCl_2$ precipitation and differential centrifugation as previously described [29]. The final pellet of the membrane was resuspended in RIPA buffer (50 mM Tris–HCl pH 7.4, 1% Igepal, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM Na_3VO_4, and 1 mM NaF) containing a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Cell extracts were prepared by sonication after incubating the cells in RIPA for 30 min at 4 °C. An equal volume of 2× SDS/sample buffer (100 mM Tris, 25% glycerol, 2% SDS, 0.01% bromophenol blue, 10% 2-ME, pH 6.8) was added and the proteins were separated on a 9% polyacrylamide gel. The separated proteins were transferred onto a polyvinylidene difluoride membrane and individually probed with the monoclonal primary antibodies, mouse anti-Na-K-ATPase α-1 and mouse anti-Na-K-ATPase β-1 (Upstate Cell Signaling Solutions, Lake Placid, NY). For the expression

Table 1
List of primers and probes for Na-K-ATPase-α-1, β-1, ankyrin-G, spectrin and β-actin RTQ-PCR.

Na-K-α1	Forward primer	5′-TCTCCTACTGCCCTGGAA-3′
	Reverse primer	5′-GAGTAGGGGAAGGCACAGAA-3′
	TaqMan probe	5′-FAM-CCCTTAGGATGTATCCCCTCAAGCCT-TAMRA-3′
Na-K-β1	Forward primer	5′-ATGTGCTGCCGGTTCAGT-3′
	Reverse primer	5′-ACCAGCGTAATCACCCATTC-3′
	TaqMan probe	5′-FAM-AGATGAAGATAAGGAAGTTGGAAGCATGG-TAMRA-3′
Ankyrin	Forward primer	5′-TGTGCCAGAAAAACACGATG-3′
	Reverse primer	5′-GACTCCTCGGCAGTAGAGTTC-3′
	TaqMan probe	5′-FAM-CCAGAGGAACAGGGTCAGCAAGAAGA-TAMRA-3′
Spectrin	Forward primer	5′-CATGCAGCACAACCTGGA-3′
	Reverse primer	5′-GAATTCTTTGAGGGCCTCCT-3′
	TaqMan probe	5′-FAM-CAGATCCAGACCAGGAACACAACGG-TAMRA-3′
β-Actin	Forward primer	5′-GCTATTTGGCGCTGGACTT-3′
	Reverse primer	5′-GCGGCTCGTAGCTCTTCTC-3′
	TaqMan probe	5′-FAM-AAGAGATGGCCACGGCGCGAAC-TAMRA-3′

of ankyrin-G and spectrin the membranes were probed with rabbit anti-ankyrin-G and rabbit anti-spectrin antibodies (Santa Cruz Biotechnology, CL). Secondary antibodies coupled to horseradish peroxidase were used to monitor the binding of the primary antibody. ECL Western blotting detection reagent (GE Healthcare Bio-Sciences, Piscataway, NJ) was used to detect the specific protein signal. Ezrin (81 kDa) was used as a loading control and all the experiments were performed in triplicate.

2.5. Immunocytochemistry

Immunolocalization of Na-K-ATPase α-1, Na-K-ATPase β-1 and ankyrin-G protein was determined by confocal immunofluorescence microscopy. Villus cells from normal and chronically inflamed rabbit intestine were fixed on a poly-L-lysine coated coverslip in 4% paraformaldehyde (Electron Microscopy Science, PA) and permeabilized by dipping cells for 10 s in chilled (−20 °C) 100% methanol. Nonspecific binding sites were blocked by 20 minute incubation with 10% goat serum at room temperature. The cells were then incubated overnight with mouse anti-Na-K-ATPase α-1 (Upsate, Lake Placid, NY) in 1:500 dilution and mouse anti-Na-K-ATPase β-1 (Upsate, Lake Placid, NY) in 1:200 dilution and rabbit anti-ankyrin-G primary antibody (Santa Cruz Biotechnology, CL) in 1:500 dilution at 4 °C. Excess antibody was removed by washing with PBS and incubated with Alexa Fluor® 647 goat anti-mouse IgG (H + L, Molecular Probes, Inc.) for α-1 and β-1 and Alexa Fluor® 488 goat anti-rabbit IgG (H + L, Molecular Probes, Inc.) for ankyrin-G at room temperature for 1 h in 1:500 dilution. Excess secondary antibody was removed by washing with PBS and the coverslips were mounted by ProLong Gold Antifade Reagent (Invitrogen Life Technologies). Specific filters were applied to visualize the signals generated by the probes under a Zeiss LSM510 confocal microscope (647 nm for Alexa Fluor® 488 and 519 nm for Alexa Fluor® 488), and the appropriate visuals generated by the two probes were captured individually. Visuals generated from each individual probe were also superimposed for further data analysis.

2.6. Co-immunoprecipitation (co-IP)

Villus cells were lysed for 20 min at 4 °C in 2 ml of immunoprecipitation (IP) buffer [10 mM Tris–HCl, 150 mM NaCl, 5 mM EDTA, 1 mM EGTA, 0.5% deoxycholate, 1% Nonidet P-40, and 1× Complete protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO)]. The lysates were centrifuged for 1 min at 10,000 g and the protein concentrations of the supernatant were estimated with a Bio-Rad protein assay kit (Hercules, CA) using BSA as standard. An equal amount of protein (500 μg) from normal and chronically inflamed intestinal villus cell lysates were pre-cleared by incubation with 25 μl of pre-immune serum and 100 μl of 50% Immobilized protein G slurry (Pierce, IL) at 4 °C for 1 h with gentle shaking. The cleared supernatant was incubated with 20 μl of the monoclonal Na-K-ATPase α-1 antibody (Upsate, Lake Placid, NY), overnight at 4 °C and an additional hour with 100 μl of a 50% Immobilized protein G slurry at room temperature with gentle shaking. The immunoadsorbants were centrifuged for 1 min at 10,000 g, washed three times with IP buffer prior to the addition of 50 μl of Laemmli sample buffer (62.5 mM Tris–HCl, pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.01% bromophenol blue) and incubation in room temperature for an hour. The immunoprecipitated proteins were then resolved by SDS-PAGE followed by Western blot analysis to detect the levels of Na-K-ATPase α-1 and its interacting ankyrin protein.

2.7. Cell culture

The normal, diploid, rat small intestinal IEC-18 cell line from American Type Culture Collection (ATCC) was maintained in culture as described earlier [32]. In brief, the cells between passage numbers 12 and 24, were maintained in DMEM (with 4.5 g/l glucose and 3.7 g/l Na bicarbonate) containing 2 mM L-glutamine, 10% (vol/vol) bovine fetal serum, 0.2 U/ml insulin, 0.5 mM β-hydroxybutyric acid, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 10% CO_2 at 37 °C. The cells were fed with fresh medium every 2 to 3 days. At around 90% confluence, the cells were passaged by trypsinization with 0.1% trypsin/0.04% EDTA in phosphate buffered saline.

2.8. RNA interference

Silencer® pre-designed rat ankyrin specific siRNA (siRNA ID s168097) and negative control (Cat. no. 4611) were obtained from Ambion. 1.5 μg ankyrin or negative control siRNAs resuspended in nucleofector solution, pH 7.4, containing 7.1 mM ATP, 11.6 mM $MgCl_2 \cdot 6H_2O$, 13.6 mM $NaHCO_3$, 84 mM KH_2PO_4 and 2.1 mM glucose were individually nucleofected into IEC-18 cells using a Nucleofector II device (Amaxa Inc.) according to the manufacturer recommended protocol. The cells grown for 3 days after electroporation were used for Na-K-ATPase activity and Western blot analyses.

2.9. Na-K-ATPase assay

Na-K-ATPase activity was measured in cellular homogenates as the amount of inorganic phosphate (P_i) liberated using a calorimetric method by Forbush [33] with few modifications. Briefly, 20 μg of cellular homogenate was added to an assay medium containing 50 mM Tris–HCl (pH 7.2), 5 mM $MgCl_2$ with 20 mM KCl and 100 mM NaCl, with or without 1 mM Ouabain. The enzyme activity was initiated by the addition of 20 mM ATP–Tris to the assay mixture with the cellular homogenate followed by incubated at 37 °C for 15 min. The assay reaction was stopped at 15 min by the addition of a 1 ml ice cold mixture of 2.8% ascorbic acid, 0.48% ammonium molybdate, and 2.8% sodium dodecyl sulfate (SDS)–0.48 M HCl solution followed by incubation in ice for

10 min. The reaction mixture was then incubated at 37 °C for 10 min after the addition of 1.5 ml of a solution containing 2% each of sodium citrate, sodium arsenate and acetic acid. Readings were obtained at 705 nm in a UV spectrophotometer (Shimadzu UV-1601). Na-K-ATPase activity was determined as the difference between the P_i released from assays of identical samples with and without Ouabain. Enzyme specific activity was finally expressed as nanomoles of P_i released per milligram protein per minute.

2.10. Data presentation

Where data were averaged, means ± SE are shown, except when error bars are inclusive within the symbol. The number (n) for any set of experiments refers to the number of rabbits. Student's *t*-test was used for statistical analysis.

3. Results

3.1. Na-K-ATPase activity

Na-K-ATPase activity was measured in homogenates of villus cells isolated from rabbit normal and chronically inflamed small intestine. Na-K-ATPase activity was reduced approximately 3 fold in villus cells from the chronically inflamed intestine (6.6 ± 0.3 nmol/mg protein·min) compared with the normal intestine (17.3 ± 1.2 nmol/mg protein·min) ($n = 3$, $p < 0.001$). These data indicated that Na-K-ATPase activity was diminished in villus cells during chronic intestinal inflammation.

3.2. Na-K-ATPase α-1 and β-1 subunit protein expression

To further delineate the molecular mechanism of this decrease, the levels of the immuno-detectable protein of Na-K-ATPase α-1 and β-1 subunits expressed in the villus cells and BLM were quantitated by Western blot analysis. Immunoblotting of cell extract and BLM fractions with monoclonal Na-K-ATPase α-1-antisera revealed a protein of 97 kDa (Fig. 1A), whereas the β-1-antisera recognized a 45-kDa band (Fig. 1B). The levels of α-1 and β-1 immunoreactive proteins were unaffected in villus cell homogenates in control compared to that from the chronically inflamed ileum. However, BLM levels of α-1 and β-1 immunoreactive proteins were markedly diminished in villus cells during chronic enteritis (Fig. 1A and 1B). Densitometric quantitation showed an approximately 50% decrease ($n = 3$, $p < 0.01$) of α-1 and an approximately 40% decrease in β-1 protein levels in the BLM of villus cells from

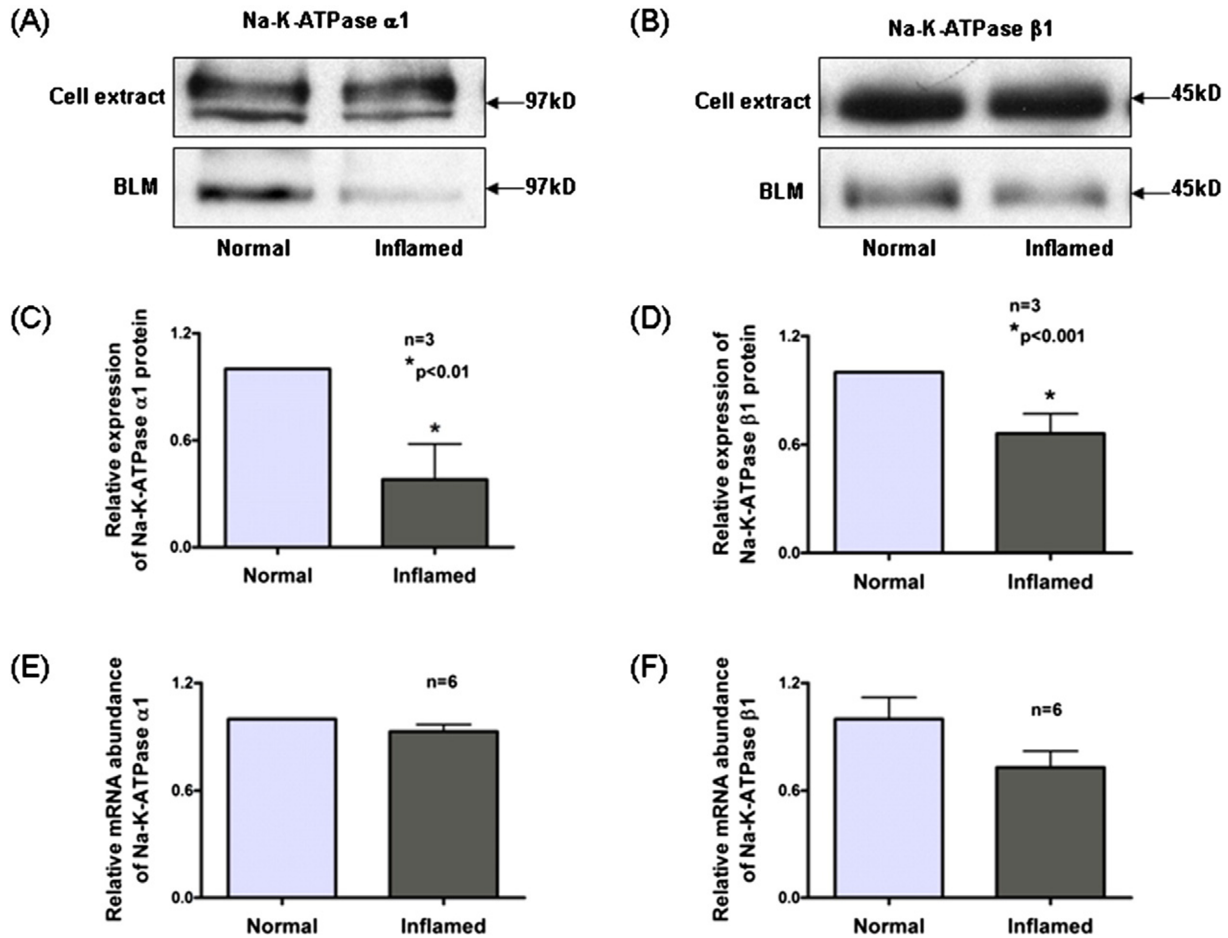

Fig. 1. Na-K-ATPase α-1 and β-1 subunit proteins and mRNA expression in villus cells during chronic enteritis. (A) Representative Na-K-ATPase α-1 Western blot from 3 independent experiments. Na-K-ATPase α-1 protein was markedly diminished in villus cell basolateral membrane (BLM) during chronic enteritis while unaffected in the total cell extract. (B) Representative Na-K-ATPase β-1 Western blot from 3 independent experiments. Na-K-ATPase β-1 subunit protein was also markedly diminished in villus cell BLM during chronic enteritis while unaffected in the total cell extract. (C) Densitometric quantification is represented as relative Na-K-ATPase α-1 expression in inflamed cells compared to total expression in normal cells equalized to 1. Densitometric quantitation of Na-K-ATPase α-1 expression in the BLM showed a significant decrease during chronic enteritis ($n = 3$, $^*p < 0.01$). (D) Densitometric quantification is represented as relative Na-K-ATPase β-1 expression in inflamed cells compared to total expression in normal cells equalized to 1. Densitometric quantitation of Na-K-ATPase β-1 expression in BLM showed a significant decrease during chronic enteritis ($n = 3$, $^*p < 0.001$). (E) In villus cells, Na-K-ATPase α-1 mRNA abundance was not affected during chronic intestinal inflammation ($n = 6$). (F) Na-K-ATPase β-1 mRNA abundance was also not altered during chronic enteritis ($n = 6$). The relative abundance of mRNA was normalized using β-actin in all conditions.

the chronically inflamed rabbit intestine compared to control (Fig. 1C and 1D). These results indicate that, down-regulation of Na-K-ATPase activity during chronic enteritis is a result of reduced levels of α-1 and β-1 subunit proteins on the BLM.

3.3. Na-K-ATPase α-1 and β-1 subunit mRNA abundance

To determine whether alterations in Na-K-ATPase activity and reduced expression of α-1 and β-1 subunit proteins in the BLM observed during chronic enteritis may be secondary to alterations in the corresponding mRNAs encoding these subunits, RTQ-PCR was carried out. There was no alteration in α-1 subunit mRNA abundance in villus cells from the chronically inflamed intestine (Fig. 1E). Similarly the β-1 subunit mRNA abundance was also not statistically different from control (Fig. 1F). These results confirmed that reduction of α-1 and β-1 subunit proteins in the villus cell BLM during chronic enteritis was likely the result of post-transcriptional regulation.

3.4. Localization of Na-K-ATPase α-1 and β-1 subunits in villus cells

Intestinal villus cells are polarized epithelial cells with a well differentiated BBM and BLM. To confirm the expression pattern of Na-K-ATPase α-1 and β-1 subunit proteins in villus cells during chronic enteritis, confocal immunofluorescence microscopy, using antibodies specific for Na-K-ATPase α-1 and β-1 subunits was performed. Na-K-ATPase α-1 and β-1 subunit proteins were shown to be distinctly present in the BLM of villus cells from normal rabbit (Fig. 2A and 2C). In contrast, both the proteins were localized to the cytoplasm instead of the BLM in the villus cells from chronically inflamed intestine (Fig. 2B and 2D). Thus, these confocal microscopic studies corroborate the results obtained by the Western blot studies of the BLM and cell extract. These data indicate that down-regulation of Na-K-ATPase in villus cells during chronic intestinal inflammation parallels a corresponding decrease of α-1 and β-1 protein expression in the BLM. Thus, the mechanism responsible for the alteration of Na-K-ATPase in villus cells during chronic enteritis is defective trafficking of Na-K-ATPase to the BLM.

3.5. Expression of ankyrin-G and spectrin during chronic enteritis

Trafficking and stabilization of Na-K-ATPase to the BLM depend on the ankyrin–spectrin based cytoskeleton. To determine whether this may be altered during chronic enteritis, the expression pattern of both of the proteins in total cell extract and in the BLM of villus cells from normal and chronically inflamed intestine was performed

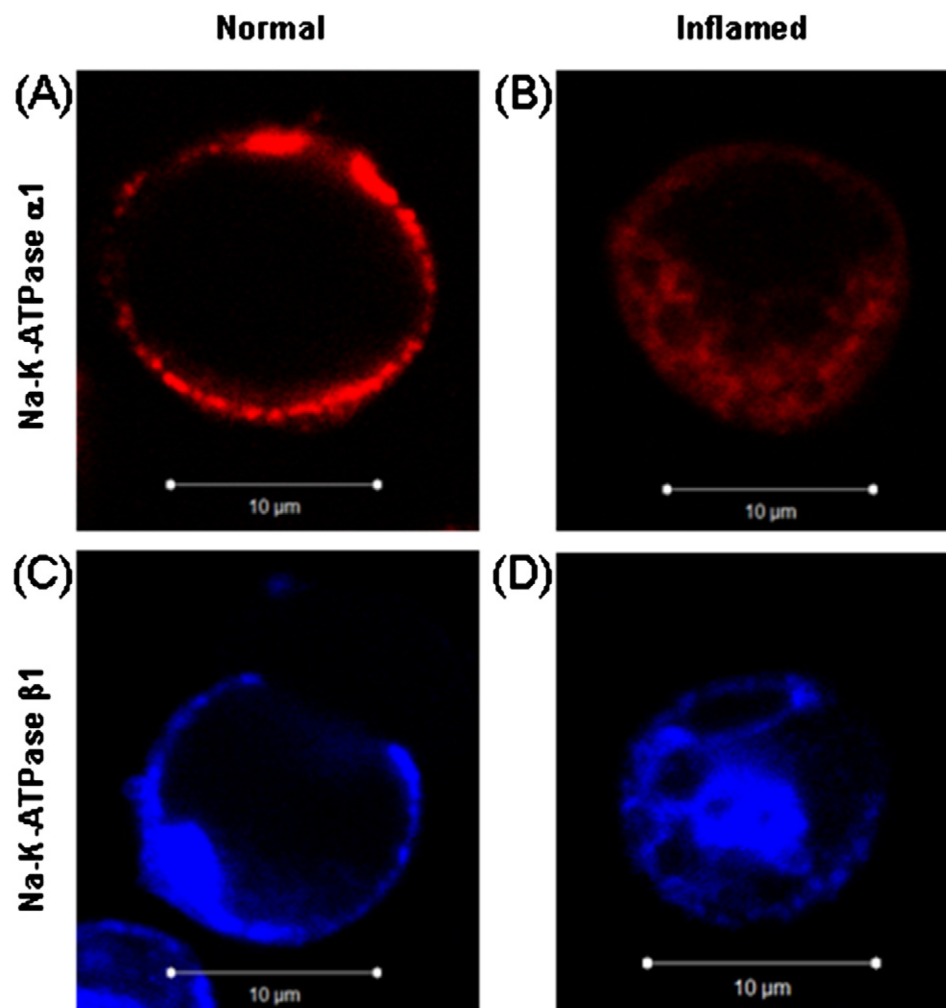

Fig. 2. Localization of Na-K-ATPase α-1 and β-1 immunoreactive proteins in villus cells during chronic enteritis. (A) The Na-K-ATPase α-1 subunit protein was present in the BLM of villus cells from normal rabbit intestine (B) whereas, the Na-K-ATPase α-1 protein was mostly localized in the cytoplasm instead of the BLM in the villus cells from chronically inflamed intestine. (C) Na-K-ATPase β-1 was expressed along the entire length of the lateral and basal plasma membrane and no staining was found on the apical membrane of villus cells from normal intestine. (D) Most of the immunoreactive Na-K-ATPase β-1 was present in the cytoplasm of chronically inflamed intestinal villus cell.

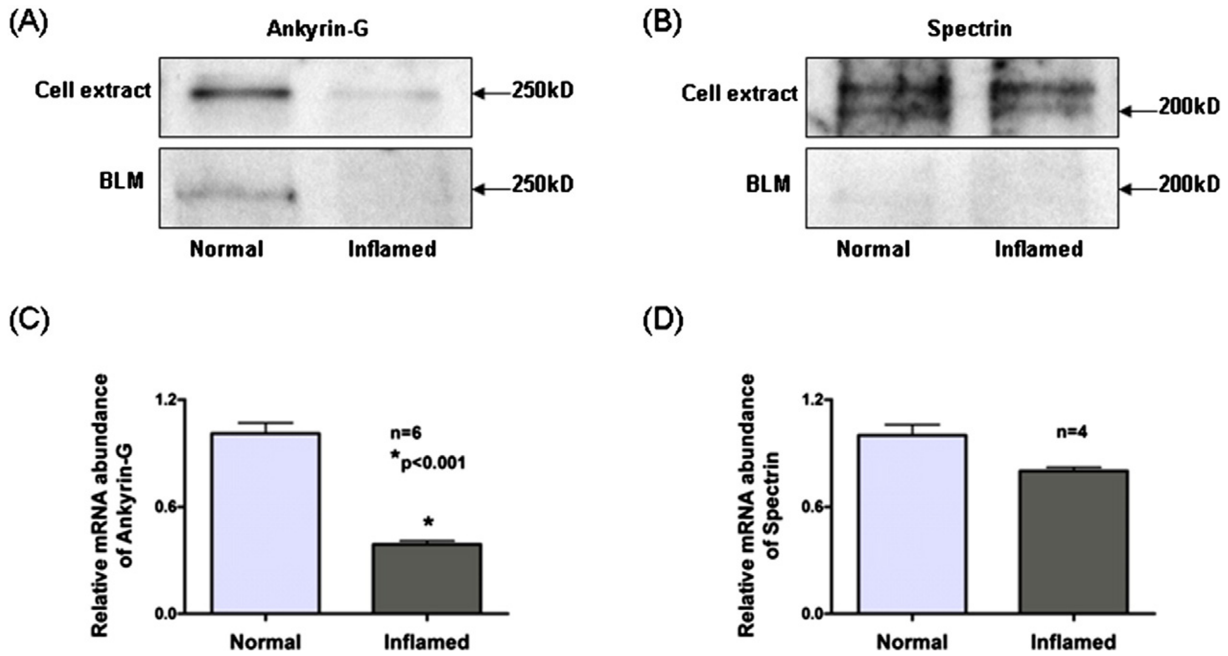

Fig. 3. Ankyrin-G protein and spectrin protein and their mRNA expression in villus cells during chronic enteritis. (A) Representative ankyrin-G Western blot from 3 independent experiments. Ankyrin-G protein was markedly diminished in villus cell BLM and cell extract during chronic enteritis. (B) Representative Western blot of spectrin from 3 independent experiments. Spectrin protein was unaffected in villus cells during chronic enteritis and there was no expression in the BLM. (C) Relative mRNA abundance is represented as abundance in inflamed cells compared to that in normal cells equalized to 1. In villus cells, ankyrin-G mRNA abundance quantitated by RTQ-PCR was significantly reduced during chronic intestinal inflammation ($n = 6$, $p < 0.001$). (D) Spectrin mRNA abundance was not statistically affected during chronic enteritis ($n = 4$) and was statistically insignificant. The relative abundance of mRNA was normalized to β-actin in all experiments.

by Western blot. Ankyrin expression showed a significant 2–3 fold decrease ($n = 3$, $p < 0.01$) in the total cell as well as in the BLM of villus cells during chronic enteritis (Fig. 3A). In contrast, while spectrin expression was not altered in the total cell extract during chronic enteritis, spectrin was nearly absent during chronic enteritis in the villus cell BLM (Fig. 3B).

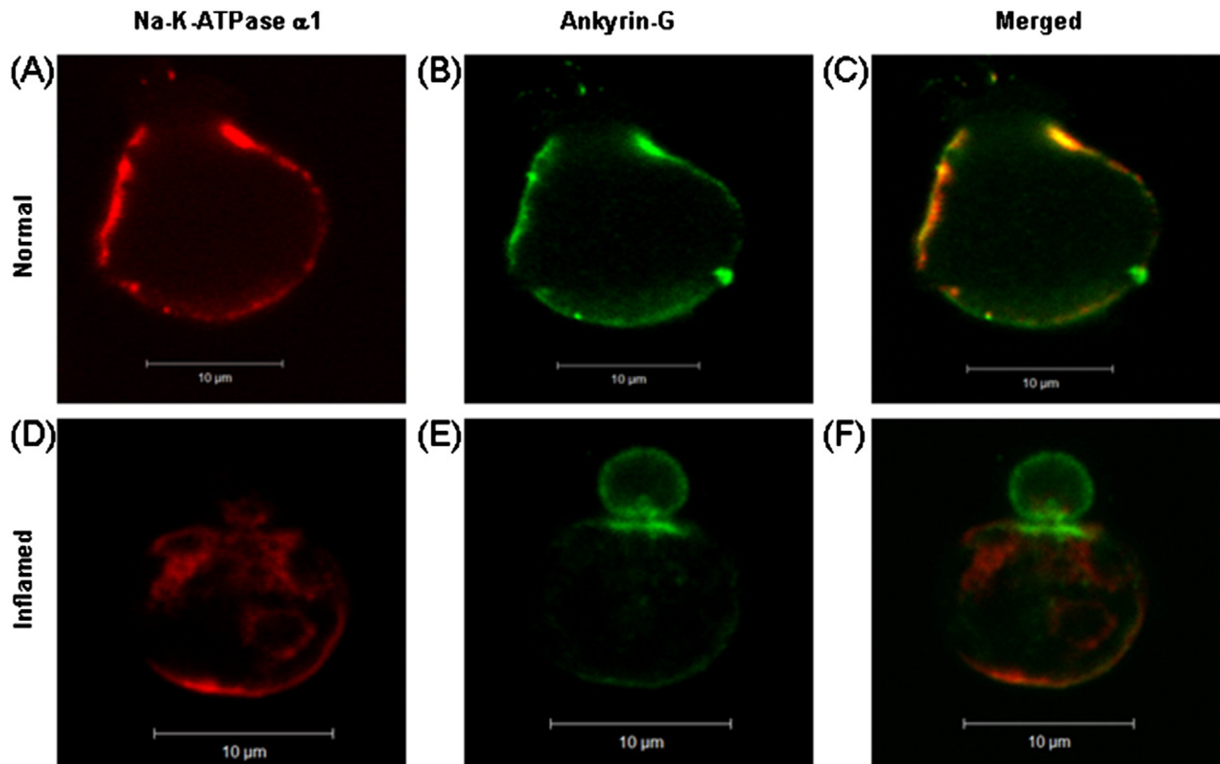

Fig. 4. Co-localization of Na-K-ATPase α-1 and ankyrin-G in villus cells during chronic enteritis. (A) Na-K-ATPase α-1 was expressed along the entire length of the lateral and basal plasma membrane while little or no staining was found on the BBM of villus cells from normal intestine. (B) Immunoreactive ankyrin-G was also distinctly present in the basolateral side of villus cells from normal rabbit. (C) Merged picture of Na-K-ATPase α-1 and ankyrin-G showed co-localization of both of the proteins. (D) Na-K-ATPase α-1 was present in the cytoplasm of chronically inflamed intestinal villus cells (E). In contrast, ankyrin was localized along the apical side instead of the BLM in the villus cells from chronically inflamed intestine (F). Merged picture of Na-K-ATPase α-1 and ankyrin-G showed that both the proteins were localized to different regions of villus cells during chronic enteritis.

3.6. Relative mRNA abundance of ankyrin-G and spectrin

To determine the mechanism of alteration of ankyrin in villus cells during chronic enteritis RTQ-PCR analysis was done. The relative abundance of ankyrin was significantly reduced ($n = 6$, $p < 0.001$) in the villus cells from chronically inflamed intestine (Fig. 3C). In contrast, there was no significant alteration in the relative abundance of spectrin, the other cytoskeleton partner of Na-K-ATPase (Fig. 3D). These results indicate that the transcriptional inhibition of linker protein ankyrin might be responsible for the altered trafficking of Na-K-ATPase α-1 to the BLM.

3.7. Co-immunolocalization of Na-K-ATPase α-1 and ankyrin-G

Alterations in Na-K-ATPase α-1 and ankyrin co-localizations during chronic enteritis was determined using immunofluorescence microscopy of villus cells. Na-K-ATPase α-1 stained with Alexa Fluor® 647 antibody showed a strong signal along the entire length of the BLM and little or no staining was found on the BBM of villus cells from normal intestine (Fig. 4A). Staining of same cell with Alexa Fluor® 488 antibody for ankyrin showed it's presence at the BLM (Fig. 4B). The merged picture of Alexa Fluor® 647 and Alexa Fluor® 488 showed a distinct overlap in the distribution of Na-K-ATPase α-1 and ankyrin (Fig. 4C). In contrast, Na-K-ATPase α-1 was mostly present in the cytoplasm instead of BLM in the villus cells from chronically inflamed rabbit intestine (Fig. 4D). More surprisingly, staining with Alexa Fluor® 488 antibody for ankyrin showed strong staining at the BBM and little or no staining on the BLM (Fig. 4E). The merged picture of Alexa Fluor® 647 and Alexa Fluor® 488 shows that α-1 and ankyrin are distributed differently in villus cells during chronic enteritis (Fig. 4F). These results indicate that depolarization of α-1 and ankyrin, and transcriptional down-regulation of ankyrin are responsible for the altered trafficking of Na-K-ATPase α-1 during chronic enteritis. Further, this is the first report of apical localization of ankyrin during an intestinal pathophysiological condition.

3.8. Expression of ankyrin-G in the villus BBM during chronic enteritis

To confirm the immunolocalization of ankyrin to the apical side of villus cells during chronic enteritis Western blot analysis of brush border membranes (BBMs) from normal and chronically inflamed intestinal villus cells was performed. Immunoreactive ankyrin-G was present only in the BBM of chronically inflamed intestinal villus cells while there was no expression in the BBM of normal rabbit villus cells (Fig. 5).

3.9. Interaction of Na-K-ATPase α-1 and ankyrin-G during chronic enteritis

To determine the interaction between Na-K-ATPase α-1 and ankyrin co-IP using monoclonal Na-K-ATPase α-1 antibody was performed in normal and chronically inflamed intestinal villus cells followed by Western blot analysis. The results showed reduced expression of ankyrin in chronically inflamed intestinal villus cells whereas no change was seen in Na-K-ATPase α-1 expression corroborating the previous findings (Fig. 6), which indicates that interaction between Na-K-ATPase α-1 and ankyrin was diminished in villus cells during chronic enteritis.

3.10. Na-K-ATPase activity in ankyrin knockdown IEC-18 cells

All the data from the *in-vivo* model of chronic intestinal inflammation indicate that the activity of Na-K-ATPase is decreased due to the transcriptional down-regulation and depolarization of ankyrin which affect trafficking of Na-K-ATPase α-1 to the BLM. To determine *in vitro* whether the activity of Na-K-ATPase was indeed altered secondary to ankyrin, the enzyme activity in ankyrin knockdown IEC-18 cells was measured. There was about a 60% decrease in the BLM localized Na-K-ATPase ($n = 3$, $p < 0.005$) after ankyrin knockdown (Fig. 7A). Also, ankyrin expression level was lower in knockdown cells in comparison to non-specific siRNA transfected IEC-18 (Fig. 7B) cells. These *in vitro* studies directly demonstrated that ankyrin is critical in the regulation of Na-K-ATPase activity during chronic enteritis.

4. Discussion

This study demonstrated that in the chronically inflamed rabbit small intestinal villus cells Na-K-ATPase is decreased. The relative mRNA abundance and protein expression of α-1 and β-1 subunits were not altered in villus cells during chronic intestinal inflammation. The mechanism of reduction in BLM Na-K-ATPase activity was secondary to diminished BLM localization of α-1 and β-1 subunits. The abhorrent trafficking of the Na-K-ATPase α-1 subunit was a result of transcriptional down-regulation and depolarization of ankyrin-G protein. In fact, a similar decrease in BLM localized Na-K-ATPase was observed in ankyrin siRNA transfected IEC-18 cells. Therefore, yet to be determined immune-inflammatory mediator(s) inhibits the synthesis of ankyrin-G, which in turn as a key mediator of Na-K-ATPase trafficking to the BLM reduces the activity of this enzyme during chronic intestinal inflammation.

Na-K-ATPase is a ubiquitous enzyme and is responsible for the ATP-driven 3 Na^+ for 2 K^+ transmembrane exchange to maintain the transcellular Na^+ gradient which is essential to facilitate a variety of Na-solute co-transport processes on the BBM of intestinal epithelial cells [4,5]. While there is a growing body of information pertaining

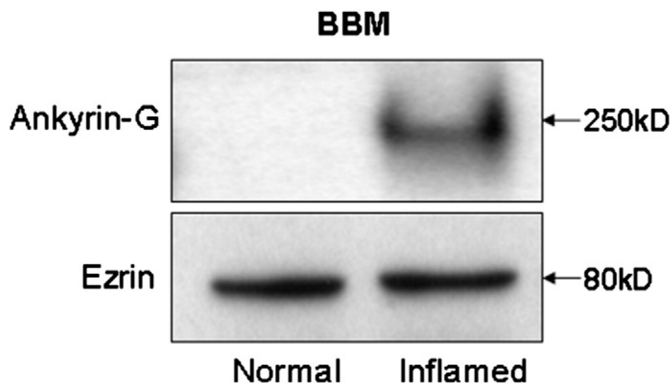

Fig. 5. Expression of ankyrin-G in the BBM during chronic enteritis. Representative ankyrin-G Western blot showed the presence of ankyrin in the BBM of chronically inflamed villus cells but not in the normal villus cell BBM ($n = 3$, $p < 0.001$).

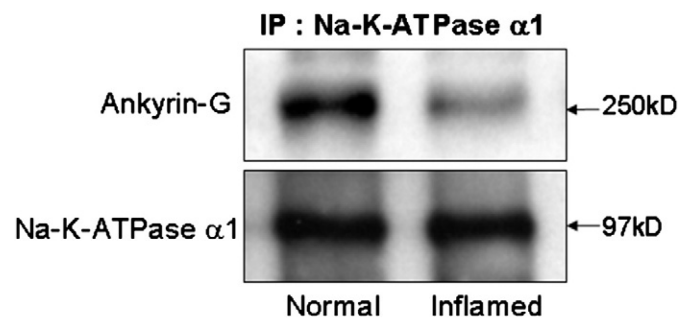

Fig. 6. Interaction of Na-K-ATPase α-1 and ankyrin-G during chronic enteritis. Ankyrin-G expression was significantly decreased in the co-immunoprecipitated Na-K-ATPase α-1 protein in villus cells from chronically inflamed intestine ($n = 3$, $p < 0.05$). Expression of Na-K-ATPase-α-1 was unaffected.

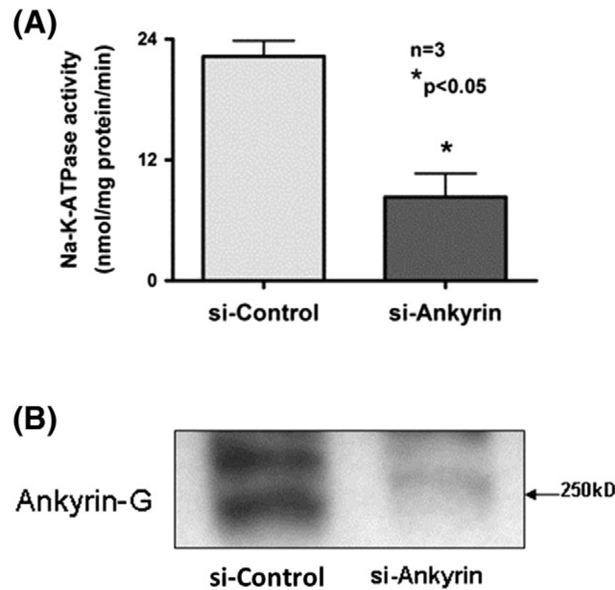

Fig. 7. Na-K-ATPase activity in ankyrin knockdown IEC-18 cells: (A) A significant 3 fold decrease of Na-K-ATPase activity was seen in the ankyrin siRNA transfected IEC-18 cells in comparison to scrambled siRNA transfected cells ($n = 3$, $p < 0.005$). (B) Representative Western blot showed that ankyrin expression was significantly reduced in the ankyrin siRNA transfected IEC-18 cells but not in non-specific siRNA transfected cells.

to the molecular structure, function and regulation of Na-K-ATPase in a variety of tissues and cell types [34–36], few studies have examined the regulation of Na-K-ATPase in the small intestine. In acute diarrheal diseases where there is serious fluid and electrolyte loss and decrease of coupled NaCl absorption, Na-K-ATPase dependent Na-nutrient pathways appear to be preserved [15,37,38]. Cholera is such a condition in which Na:H exchange, responsible for coupled NaCl absorption mediated by Na:H and Cl:HCO_3 exchange on the BBM, is decreased [38,39]. But Na-glucose and possibly Na-amino acid co-transport that are secondarily active and thus dependent on the transcellular Na gradient generated by Na-K-ATPase, are preserved [39]. Indeed, this is the physiologic basis for oral rehydration therapy for severe diarrheal conditions such as cholera. Oral rehydration therapy is the most efficacious and cost effective therapy for diarrheal diseases in infants in the developing parts of the world where this preventable condition nevertheless results in more than a million deaths a year [2,3].

In the Western hemisphere, inflammatory bowel diseases (Crohn's disease and ulcerative colitis) afflict more than a million people and the most common clinical concern in patients with these chronic diarrheal diseases is malabsorption of electrolytes and nutrients resulting in malnutrition [40,41]. Na-K-ATPase activity has shown to be decreased during human IBD and ulcerative colitis [26–30]. These observations have implications for Na and solute absorption at the cellular level in the intestine. Indeed, in a rabbit model of chronic intestinal inflammation resembling IBD, it has been demonstrated that Na-glucose (SGLT1), Na-amino acid (-alanine (ATB0), -glutamine (B0AT1)), Na-bile acid (ASBT) and Na-adenosine co-transport are reduced in the villus cells [4–7]. At least part of this decrease is secondary to the diminished Na-extruding capacity of these cells as evidenced by the down-regulation of Na-K-ATPase. However, the molecular mechanisms of down-regulation of Na-K-ATPase in villus cells during chronic intestinal inflammation were previously not well understood. The present study provides new insights into the regulation of villus cell Na-K-ATPase during chronic intestinal inflammation.

In a rat model of acute intestinal inflammation induced by 2,4,6-trinitrobenzenesulfonic acid it was reported that there was decrease of Na-K-ATPase activity in total enterocytes [42]. But, in normal intestine approximately 80% of the enterocytes are absorptive villus cells while the remainder is the secretory crypt cells. Further, villus cells almost exclusively possess Na-K-ATPase driven Na-solute co-transport processes as compared to primarily secretory crypt cells in the small intestine [39]. During acute inflammation there is villus atrophy and crypt hypertrophy and thus, measuring Na-K-ATPase activity in a mixture of enterocytes, as was done in this study, will not provide mechanistic information. Nevertheless, in TNBS-induced ileitis it was reported that there was a decrease in the expression of α-1 and β-1 subunits in enterocytes *in toto* [42]. However, in the current study, during chronic intestinal inflammation, in absorptive villus cells there was no alteration in the relative abundance of α-1 and β-1 mRNA levels (Fig. 1). Further, α-1 and β-1 protein expression pattern in whole cell extract corroborates the results obtained by RTQ PCR analysis (Fig. 1). Species differences, chronic *versus* acute inflammation, and villus *versus* a mixture of enterocytes may account for these differences.

Despite the absence of change in the steady state levels of α-1 and β-1 mRNA and protein in villus cells in the chronically inflamed intestine, current study showed that α-1 and β-1 protein concentrations were significantly reduced in the BLM of villus cells from chronic inflamed intestine (Fig. 1). Indeed, when immunocytochemical studies were performed in order to localize the subunit proteins in the villus cells from inflamed intestine, α-1 and β-1 subunit proteins were distinctly absent at BLM, but were seen disseminated in the cytoplasm (Fig. 2). These results indicate that down-regulation of Na-K-ATPase activity during chronic enteritis was paralleled by corresponding decreases of α-1 and β-1 subunit protein levels in the BLM, likely due to altered intracellular trafficking.

A study in renal tubule cells (MDCK) has suggested that ankyrin has an important role in facilitating the intracellular trafficking of Na-K-ATPase in addition to stabilizing the Na-K-ATPase membrane scaffold [36]. In the present study, for the first time, the correlation of the functional activity of Na-K-ATPase with ankyrin-G in the mammalian intestinal villus cells has been reported. There was a significant down-regulation of ankyrin-G protein expression and mRNA abundance in villus cells from chronically inflamed intestine (Fig. 3). Immunostaining of the α-1 subunit and ankyrin showed that both the proteins were co-

localized along the BLM in normal villus cells. In the villus cells from chronically inflamed intestine, α-1 was present in the cytoplasm in a scattered fashion and ankyrin mostly localized towards the BBM (Fig. 4). To confirm that the depolarization of ankyrin during chronic enteritis is a unique phenomenon, Western blot analysis using villus BBM from normal and chronically inflamed rabbits was done. This also showed the presence of ankyrin in villus cell BBM from the chronically inflamed intestine but not in villus cell BBM from the normal intestine (Fig. 5). These data suggested that the separation of the two co-localized proteins during chronic enteritis might be the result of the loss of interaction between ankyrin and the ankyrin binding sequence of the α1 subunit of Na-K-ATPase. To assess this interaction IP using Na-K-ATPase α-1 monoclonal antibody was performed followed by Western blot analysis. The results showed the presence of a lesser amount of ankyrin interaction during chronic enteritis (Fig. 6). The loss of interaction was probably the result of the unavailability of ankyrin due to transcriptional down-regulation and/or depolarization. These findings were confirmed by ankyrin knockdown in IEC-18 cells where Na-K-ATPase was decreased (Fig. 7). Further studies are required to understand the intracellular mechanism that may be responsible for the down-regulation of ankyrin during intestinal inflammation. A recent publication has shown that an increase in miR-342-5p down-regulates the expression of ankyrin-G [43]. Also, dinucleotide deletion in the ankyrin promoter and mutation in its intronic sequence have been shown to decrease its expression in disease conditions [44,45].

In conclusion, the results of the current study demonstrated that the mechanism of down-regulation of Na-K-ATPase in the villus cells during chronic intestinal inflammation is secondary to diminished enzyme levels at the BLM. This defective localization of Na-K-ATPase to the BLM is a result of decreased production of and depolarization of ankyrin protein in villus cells from the chronically inflamed intestine. Understanding the molecular mechanisms of alterations of Na-K-ATPase in the intestine, especially diarrheal diseases as done in this study, is important since at the intact cell level alterations in the BLM Na-K-ATPase contribute, at least partially, to the changes in the absorption of Na and nutrients. Thus, regulation of Na-K-ATPase provides another option in designing treatments. Restoring the polarization/localization of Na-K-ATPase in these conditions may, again, at least partially, restore all altered Na-solute co-transport processes in the inflamed intestine.

Acknowledgements

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Research Grants DK-45062 and DK-58034 to U. Sundaram.

References

[1] J.D. Robinson, M.S. Flashner, The ($Na^+ + K^+$)-activated ATPase enzymatic and transport properties, Biochim. Biophys. Acta 549 (1979) 145–176.
[2] J.N. Ruxin, Magic bullet: the history of oral rehydration therapy, Med. Hist. 38 (1994) 363–397.
[3] K.L. Hamilton, Robert K. Crane—Na(+)-glucose cotransporter to cure? Front. Physiol. 4 (2013) 53.
[4] U. Sundaram, S. Wisel, V.M. Rajendren, A.B. West, Mechanism of inhibition of Na^+-glucose cotransport in the chronically inflamed rabbit ileum, Am. J. Physiol. 273 (1997) G913–G919.
[5] U. Sundaram, S. Wisel, J.J. Fromkes, Unique mechanism of inhibition of Na^+-amino acid cotransport during chronic ileal inflammation, Am. J. Physiol. 275 (1998) G483–G489.
[6] S. Coon, R. Kekuda, P. Saha, U. Sundaram, Glucocorticoids differentially regulate Na-bile acid cotransport in normal and chronically inflamed rabbit ileal villus cells, Am. J. Physiol. Gastrointest. Liver Physiol. 298 (2010) G675–G682.
[7] P. Saha, S. Arthur, R. Kekuda, U. Sundaram, Na-glutamine co-transporters B(0)AT1 in villus and SN2 in crypts are differentially altered in chronically inflamed rabbit intestine, Biochim. Biophys. Acta 1818 (2012) 434–442.
[8] S. Arthur, P. Saha, S. Sundaram, R. Kekuda, U. Sundaram, Regulation of sodium-glutamine cotransport in villus and crypt cells by glucocorticoids during chronic enteritis, Inflamm. Bowel Dis. 18 (2012) 2149–2157.
[9] K.V. Pavlov, V.S. Sokolov, Electrogenic ion transport by Na^+,K^+-ATPase, Membr. Cell Biol. 13 (2000) 745–788.
[10] T.A. Pressley, J.C. Allen, C.H. Clarke, T. Odebunmi, S.C. Higham, Amino-terminal processing of the catalytic subunit from Na(+)-K(+)-ATPase, Am. J. Physiol. 271 (1996) C825–C832.
[11] K. Geering, The functional role of the beta-subunit in the maturation and intracellular transport of Na K-ATPase, FEBS lett. 285 (1991) 189–193.
[12] R.M. Young, J.B. Lingrel, Tissue distribution of mRNAs encoding the alpha isoforms and beta subunit of rat Na^+,K^+-ATPase, Biochem. Biophys. Res. Commun. 145 (1987) 52–58.
[13] J. Orlowski, J.B. Lingrel, Tissue-specific and developmental regulation of rat Na K-ATPase catalytic alpha isoform and beta subunit mRNAs, J. Biol. Chem. 263 (1988) 10436–10442.
[14] P. Martin-Vasallo, W. Dackowski, J.R. Emanuel, R. Levenson, Identification of a putative isoform of the Na, K-ATPase beta subunit. Primary structure and tissue-specific expression, J. Biol. Chem. 264 (1989) 4613–4618.
[15] N. Malik, V.A. Canfield, M.C. Beckers, P. Gros, R. Levenson, Identification of the mammalian Na, K-ATPase 3 subunit, J. Biol. Chem. 271 (1996) 22754–22758.
[16] E. Arystarkhova, K.J. Sweadner, Tissue-specific expression of the Na, K-ATPase beta3 subunit. The presence of beta3 in lung and liver addresses the problem of the missing subunit, J. Biol. Chem. 272 (1997) 22405–22408.
[17] O.I. Shamraj, J.B. Lingrel, A putative fourth Na^+,K^+-ATPase alpha-subunit gene is expressed in testis, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 12952–12956.
[18] J.B. Lingrel, J. Orlowski, M.M. Shull, E.M. Price, Molecular genetics of Na K-ATPase, Prog. Nucleic Acid Res. Mol. Biol. 38 (1990) 37–89.
[19] A.G. Therien, R. Blostein, Mechanisms of sodium pump regulation, Am. J. Physiol. Cell Physiol. 279 (2000) C541–C566.
[20] C. Jordan, B. Puschel, R. Koob, D. Drenckhahn, Identification of a binding motif for ankyrin on the alpha-subunit of Na^+,K^+-ATPase, J. Biol. Chem. 270 (1995) 29971–29975.
[21] A.M. Rubtsov, O.D. Lopina, Ankyrins, FEBS Lett. 482 (2000) 1–5.
[22] N.B. Chauhan, J.M. Lee, G.J. Siegel, Na,K-ATPase mRNA levels and plaque load in Alzheimer's disease, J. Mol. Neurosci. 9 (1997) 151–166.
[23] A. Ziegelhoffer, K. Kjeldsen, H. Bundgaard, A. Breier, N. Vrbjar, A. Dzurba, Na,K-ATPase in the myocardium: molecular principles, functional and clinical aspects, Gen. Physiol. Biophys. 19 (2000) 9–47.
[24] J. Patrick, P.J. Hilton, Characterization of sodium-transport disorders in disease: different effects upon sodium and potassium of changes in the sodium pump and in membrane permeability, Clin. Sci. (Lond.) 57 (1979) (1979) 289–293.
[25] A.M. Rose, R. Valdes Jr., Understanding the sodium pump and its relevance to disease, Clin. Chem. 40 (1994) 1674–1685.
[26] D. Rachmilewitz, F. Karmeli, P. Sharon, Decreased colonic Na-K-ATPase activity in active ulcerative colitis, Isr. J. Med. Sci. 20 (1984) 681–684.
[27] H. Allgayer, W. Kruis, G. Paumgartner, B. Wiebecke, L. Brown, E. Erdmann, Inverse relationship between colonic ($Na^+ + K^+$)-ATPase activity and degree of mucosal inflammation in inflammatory bowel disease, Dig. Dis. Sci. 33 (1988) 417–422.
[28] J. Ejderhamn, Y. Finkel, B. Strandvik, Na,K-ATPase activity in rectal mucosa of children with ulcerative colitis and Crohn's disease, Scand. J. Gastroenterol. 24 (1989) 1121–1125.
[29] U. Sundaram, A.B. West, Effect of chronic inflammation on electrolyte transport in rabbit ileal villus and crypt cells, Am. J. Physiol. 272 (1997) G732–G741.
[30] J.H. Tripp, D.P. Muller, J.T. Harries, Mucosal (Na^+-K^+)-ATPase and adenylate cyclase activities in children with toddler diarrhea and the postenteritis syndrome, Pediatr. Res. 14 (1980) 1382–1386.
[31] V.M. Rajendran, M. Oesterlin, H.J. Binder, Sodium uptake across basolateral membrane of rat distal colon. Evidence for Na–H exchange and Na-anion cotransport, J. Clin. Invest. 88 (1991) 1379–1385.
[32] J.R. Talukder, R. Kekuda, P. Saha, U. Sundaram, Mechanism of leukotriene D4 inhibition of Na-alanine cotransport in intestinal epithelial cells, Am. J. Physiol. Gastrointest. Liver Physiol. 295 (2008) G1–G6.
[33] B. Forbush III, Assay of Na K-ATPase in plasma membrane preparations: increasing the permeability of membrane vesicles using sodium dodecyl sulfate buffered with bovine serum albumin, Anal. Biochem. 128 (1983) 159–163.
[34] R. Woroniecki, J.R. Ferdinand, J.S. Morrow, P. Devarajan, Dissociation of spectrin–ankyrin complex as a basis for loss of Na-K-ATPase polarity after ischemia, Am. J. Physiol. Ren. Physiol. 284 (2003) F358–F364.
[35] J. Inserte, D. Garcia-Dorado, V. Hernando, J. Soler-Soler, Calpain-mediated impairment of Na^+/K^+-ATPase activity during early reperfusion contributes to cell death after myocardial ischemia, Circ. Res. 97 (2005) 465–473.
[36] P.R. Stabach, P. Devarajan, M.C. Stankewich, S. Bannykh, J.S. Morrow, Ankyrin facilitates intracellular trafficking of alpha1-Na^+-K^+-ATPase in polarized cells, Am. J. Physiol. Cell Physiol. 295 (2008) C1202–C1214.
[37] A.N. Charney, M. Donowitz, Prevention and reversal of cholera enterotoxin-induced intestinal secretion by methylprednisolone induction of Na^+-K^+-ATPase, J. Clin. Invest. 57 (1976) 1590–1599.
[38] E.M. Wright, B.A. Hirayama, D.F. Loo, Active sugar transport in health and disease, J. Intern. Med. 261 (2007) 32–43.
[39] M. Field, Intestinal ion transport and the pathophysiology of diarrhea, J. Clin. Invest. 111 (2003) 931–943.
[40] P.L. Lakatos, Recent trends in the epidemiology of inflammatory bowel diseases: up or down? World J. Gastroenterol. 12 (2006) 6102–6108.
[41] S. Massironi, R.E. Rossi, F.A. Cavalcoli, S. Della Valle, M. Fraquelli, D. Conte, Nutritional deficiencies in inflammatory bowel disease: therapeutic approaches, Clinical nutrition (Edinburgh, Scotland) 32 (2013) 904–910.

[42] G.E. Wild, A.B. Thomson, Na(+)-K(+)-ATPase alpha 1- and beta 1-mRNA and protein levels in rat small intestine in experimental ileitis, Am. J. Physiol. 269 (1995) G666–G675.
[43] X. Sun, Y. Wu, M. Gu, Y. Zhang, miR-342-5p decreases ankyrin G levels in Alzheimer's disease transgenic mouse models, Cell reports 6 (2014) 264–270.
[44] P.G. Gallagher, D.G. Nilson, C. Wong, J.L. Weisbein, L.J. Garrett-Beal, S.W. Eber, D.M. Bodine, A dinucleotide deletion in the ankyrin promoter alters gene expression, transcription initiation and TFIID complex formation in hereditary spherocytosis, Hum. Mol. Genet. 14 (2005) 2501–2509.
[45] H. Huang, P. Zhao, K. Arimatsu, K. Tabeta, K. Yamazaki, L. Krieg, E. Fu, T. Zhang, X. Du, A deep intronic mutation in the ankyrin-1 gene causes diminished protein expression resulting in hemolytic anemia in mice, G3 (Bethesda) 3 (2013) 1687–1695.