Trafficking of SLC26A3 in Intestinal Epithelial Cells:

Effect of Enteropathogenic E. coli Infection

By

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THESIS

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To My Husband

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LIST OF ABBREVIATIONS

5-HT	5-Hydroxy Tryptamine
AE	Anion exchanger
ANOVA	Analysis of Variance
BN-PAGE	Blue Native- Polyacrylamide gel Electrophoresis
BSA	Bovine Serum Albumin
cAMP	cyclic Adenosine Monophosphate
CCP/CCV	Clathrin-coated Pit/Vesicle
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
cGMP	cyclic Guanosine Monophosphate
CLD	Congenital Chloride Diarrhea
CME	Clathrin Mediated Endocytosis
CPZ	Chlorpromazine
CTX	Cholera Toxin
DAG	Diacyl glycerol
DIDS	4, 4'-diisothiocyanostilbene-2, 2' disulfonic acid
DNA	Deoxyribonucleic Acid
DPC	Diphenylamine-2 carboxylate
DRA	Down Regulated in Adenoma
DSDI	Detergent Soluble and Detergent Insoluble
DYN	Dynasore hydrate
ECL	Enhanced Chemiluminescent
EE	Early Endosomes
EGFR	Epidermal Growth Factor Receptor
EPEC	Enteropathogenic *E. coli*
FBS	Fetal Bovine Serum
GLUT	Glucose Transporter
GPCR	G-protein coupled receptor
IBD	Inflammatory Bowel Disease
IFNγ	Interferon-γ
IL-8	Interleukin-8
IRF-1	Interferon Regulatory Factor-1
JAK	Janus Kinase
KCl	Potassium Chloride

LIST OF ABBREVIATIONS (continued)

LA	*Lactobacilus acidophilus*
LE	Late Endosomes
LPA	Lysophosphatidic Acid
LPP	Lipid Phosphate Phosphatase
LPS	Lyso Phosphatidyl Serine
MT	Microtubule
NFA	Niflumic Acid
NGS	Normal Goat Serum
NHE	Sodium Hydrogen Exchanger
NHERF	Sodium Hydrogen Exchanger Regulatory Factor
NO	Nitric Oxide
NPY	Neuropeptide Y
OCT	Optimal Cutting Temperature
PA	Phosphatidic Acid
PAF-AH	Platelet Activating Factor Acetylhydrolase
PAT-1	Putative Anion Transporter-1
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PDGF	Platelet derived growth factor
PDZ	Post synaptic density protein, Drosophila disc large tumor suppressor and Zonula occludens-1 protein
PI3K	Phosphatidyl Inositol 3-Kinase
PKC	Protein Kinase C
RE	Recycling Endosomes
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SDS-PAGE	Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis
SEM	Standard Error Mean
SLC	Solute Carrier
STAS	Sulfate Transporter and Anti-Sigma factor antagonist
STAT1	Signal Transducers and Activators of Transcription 1
TBS	Tris Buffer Saline
TNF-α	Tumor Necrosis Factor-α

LIST OF ABBREVIATIONS (continued)

TTSS	Type III Secretion System
UC	Ulcerative Colitis

SUMMARY

Diarrhea resulting from infections caused by food-borne pathogens such as *Enteropathogenic E.coli* (EPEC) remains a major cause of morbidity and mortality globally. A dysregulation of Sodium Hydrogen Exchanger (NHE3) and Down Regulated in Adenoma (DRA), the main Na^+/H^+ and $Cl^-/OH^-(HCO_3^-)$ exchangers, respectively, has been implicated in diarrhea associated with intestinal disorders such as enteric infections and inflammatory bowel disease. Previous studies have shown that EPEC inhibits both DRA function and surface expression in the intestinal epithelial cells, via virulence factors EspGs (EspG1 and EspG2), known to disrupt host microtubule network. However, cellular mechanisms underlying EPEC induced modulation of DRA surface expression are currently unknown. Also, very little is currently known about the role played by microtubule disruption during EPEC infection in decreasing DRA function and membrane levels. Similarly, recycling mechanisms involved in regulating DRA membrane expression under basal conditions remain elusive. Understanding the cellular and molecular mechanisms responsible for maintaining DRA surface levels under physiological conditions as well as the inhibition of DRA during EPEC infection is therefore, critical for the development of novel therapeutic modalities against infectious diseases. The focus of this dissertation is to elucidate the basal DRA trafficking mechanisms and investigate EPEC mediated modulation of these mechanisms resulting in decreased DRA surface expression in intestinal epithelial cells.

Two specific aims were proposed for this research: Specific Aim 1: to elucidate the role of cellular trafficking mechanisms and microtubules in basal DRA surface expression and Specific Aim 2: to investigate implication of EPEC mediated modulation of trafficking mechanisms and microtubule disruption in decreased DRA surface expression. Our results are summarized in the following paragraphs.

Specific Aim 1:

I. Apical $Cl^-/OH^-(HCO_3^-)$ exchange activity, measured by DIDS sensitive $^{125}I^-$ uptake, was stimulated by endocytosis inhibitor with dynasore (80 μM, 1 hour) in both Caco-2 and T-84 cells.

II. Dynasore did not alter the basal NHE3 activity as measured by S3226 sensitive $^{22}Na^+$ uptake in Caco-2 cells.

III. Similar to dynasore, chlorpromazine, an inhibitor of clathrin-mediated endocytosis (60 μM, 1 hour) stimulated apical $Cl^-/OH^-(HCO_3^-)$ exchange activity in Caco-2 cells. The role of caveolin-mediated pathway was not examined in these studies because of lack of endogenous expression of caveolin-1 in Caco-2 cells grown in 2D or 3D monolayers.

IV. Utilization of biochemical techniques showed that treatment with dynasore (80 μM, 1 hour) increased surface expression (as measured by cell-surface biotinylation and immunoflourescence studies) via decreasing the endocytosis of DRA.

V. Separation of lipid raft fractions from total cellular membrane, at 4°C, by a detergent-based membrane solubilization technique showed that increased association of DRA with membrane lipid-raft domains accompanied the increase in function and surface expression of DRA in the presence of dynasore.

VI. Disruption of microtubules in the cold, followed by recovery at 37°C in the presence of microtubule disrupting agent nocodazole (66 μM) decreased basal DRA exocytosis as compared to control. This was supported by confocal microscopy data, which showed that disruption of microtubules reduced DRA expression on the apical membrane in confluent Caco-2 monolayers.

VII. Results from reverse cell-surface biotinylation after microtubule disruption and recovery in the presence or absence of nocodazole (66 μM), suggested that basal DRA endocytosis was not dependent upon intact microtubules.

VIII. *Ex vivo* surface biotinylation and immunostaining studies in mouse colon showed reduced surface DRA, and, increased sub-apical expression of DRA in colchicine (3mg/kg b.wt) treated mice as compared to control mice.

To summarize, results from Specific Aim 1 showed that the level of DRA proteins on plasma membrane is dependent upon clathrin-mediated endocytosis and microtubule-dependent exocytosis in Caco-2 cells.

Specific Aim 2:

I. EPEC infection of Caco-2 cells for 30 minutes decreased DRA surface levels via modulation of trafficking mechanisms, which was partly due to increased DRA endocytosis in Caco-2 cells.

II. The decrease in apical $Cl^-/OH^-(HCO_3^-)$ exchange activity induced by EPEC was, however, unchanged in the presence of endocytosis-inhibitor dynasore, suggesting that inhibition of dynamin was unable to block the EPEC mediated decrease in chloride uptake.

III. Parallel to apical $Cl^-/OH^-(HCO_3^-)$ exchange activity, dynasore did not significantly alter EPEC mediated inhibition of NHE3 activity as measured by S3226-sensitive ^{22}Na uptake in Caco-2 cells.

IV. Biochemical assays to measure exocytic insertion into plasma membrane, demonstrated that EPEC infection decreased exocytosis of DRA in Caco-2 cells.

V. The EPEC mediated decrease in exocytosis was prevented by infection of Caco-2 cells with deletion mutants for effector molecules ESPG1/G2, indicating that intact microtubular network is essential for DRA exocytosis.

VI. The *ΔespG1/G2* mutants prevented the increase in DRA endocytosis induced by EPEC infection, further indicating that EPEC induced modulation of DRA trafficking is EspG1/G2 dependent.

VII. EPEC infection significantly decreased the association of DRA with detergent insoluble fraction, suggesting a redistribution of DRA within the membrane microdomains.

VIII. Similar to functional studies, the decrease in DRA association with lipid-rafts upon EPEC infection was unaltered in the presence of dynasore.

IX. Culture medium from the probiotic *Lactobacillus acidophilus* showed abrogation of EPEC induced decrease in apical $Cl^-/OH^-(HCO_3^-)$ exchange activity in Caco-2 cells.

Collectively, these data indicate that inhibition of DRA surface expression in response to EPEC infection occurs via modulation of cellular trafficking events involving both increased endocytosis and decreased exocytosis of DRA in Caco-2 cells.

Overall, the studies presented in this thesis, establish the role played by clathrin-mediated endocytosis and intact microtubules in the basal surface expression of DRA and define the cellular trafficking mechanisms underlying EPEC induced decrease in DRA function and apical expression in Caco-2 cells. Further elucidation of these pathways may help identify targets for development of novel therapeutics against diarrheal disorders.

I. INTRODUCTION

Enteropathogenic *E.coli* (EPEC) infection causes profuse diarrhea especially in infants and children under the age of five. Previous studies showed that perturbation in electrolyte transport processes contributes to rapid diarrhea associated with EPEC infection (1). In this regard, function of DRA (Down Regulated in Adenoma), the apical Cl^-/HCO_3^- exchanger was decreased concomitant with a reduction in surface expression (2). Physiologically, maintenance of surface levels involves constitutive recycling of DRA at the plasma membrane (3). The focus of this dissertation is to elucidate the cellular mechanisms controlling basal and EPEC-modulated DRA trafficking in intestinal epithelial cells. The following sections provide a brief summary of the current knowledge of the pathophysiology of diarrhea and intestinal ion transport with a focus on anion exchangers responsible for chloride absorption, diarrheal pathogen EPEC and its mode of action as well as the cellular trafficking mechanisms involved in internalization of surface proteins.

A. Diarrheal Disorders and Intestinal Ion Transport

In 2013, the world health organization (WHO) reported approximately 1.7 billion cases of diarrheal diseases annually. A significantly large percentage of these cases are associated with death especially in infants and children under the age of five. For example, in 2013, approximately 760,000 cases of diarrhea-related death were reported in young children, making it the second leading cause of child-fatality (WHO). It is essential, therefore, to gain a better understanding of the pathophysiology of diarrhea, to reduce the associated malaise and mortality.

Diarrheal diseases result from a disturbance in the normal physiological fluid and electrolyte balance. The mammalian intestine has a tremendous absorptive capacity and plays an essential role in the maintenance of fluid homeostasis in the body. The daily fluid load presented to the intestine is approximately 9 liters of which approximately 98.8% is efficiently

reabsorbed. These 9 liters of fluid is made up of dietary intake (~2.0 L), secretions from the salivary glands (~1.5 L), the gastric mucosa (~2.5 L), pancreas (~1.5 L), bile and the intestine (~1.5 L) **(Figure 1)**. The bulk of fluid reabsorption occurs in the small and large intestine. A loss of this absorptive function, an increase in secretion or both may lead to the development of diarrheal conditions. Since solute movement is coupled to the movement of water, a dysregulation in net uptake of sodium and chloride or an increase in the secretion of chloride ions underlies the pathophysiology of diarrhea (5). For example, diarrhea associated with toxigenic strains of bacteria like *Vibrio cholerae* and *Enterotoxigenic E coli* (ETEC) results predominantly from an increase in chloride ion secretion (1). On the other hand, infection caused by *Enteropathogenic E coli* (EPEC) is suggested to involve a severe downregulation of Na^+/Cl^- absorption resulting in diarrhea. Also, an increase in the levels of inflammatory cytokines leads to a significant reduction in Na^+/Cl^- absorption contributing to diarrhea associated with inflammatory bowel disease (IBD) (5). Thus, understanding mechanisms involved in modulating Na^+ and Cl^- transport in health and disease is critical for the development of novel therapeutic agents for the treatment of diarrhea. The following paragraphs give a short overview of normal intestinal NaCl absorption and secretion with a special focus on Cl^- absorption.

B. Mechanisms of Sodium and Chloride Transport

The intestinal lumen is lined by a monolayer of polarized epithelial cells (IECs) with distinct apical (luminal) and basolateral (serosal) membranes. Movement of fluid and ions from the luminal to serosal side or vice versa may occur via paracellular or transcellular pathways. Tight junctions connecting adjacent epithelial cells, restrict movement of substances across the passive paracellular pathway.

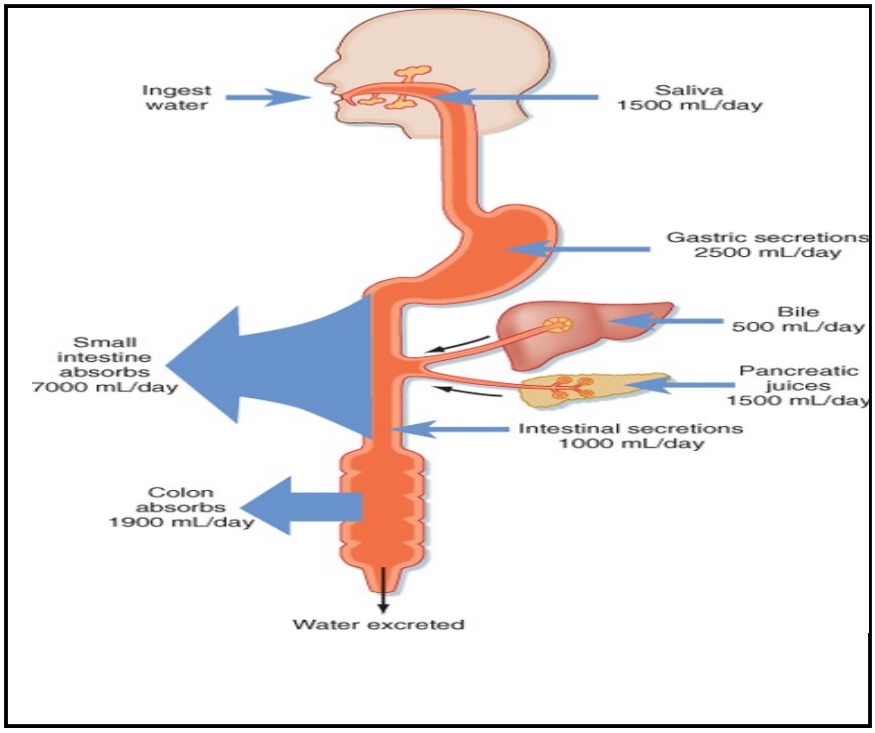


Figure 1. Intestinal Fluid Homeostasis. Berne and Levy (115)
Permission (Appendix B)

The transcellular pathway, which may be either active or passive, is mainly responsible for the regulated movement of fluid and electrolytes. The apical and basolateral plasma membranes of intestinal epithelial cells express distinct channels, pumps and carrier proteins mediating absorption or secretion of water and solutes via the transcellular pathway (6). Although designed to absorb a wide range of electrolytes and nutrients, the overall fluid balance in the gut is maintained by three important transport processes: **chloride secretion, electroneutral NaCl absorption and electrogenic Na^+ absorption.**

B.1. Chloride Secretion

Cystic fibrosis transmembrane conductance regulator (CFTR) is the main transporter responsible for Cl^- secretion across the apical membrane. Genetic mutations in CFTR lead to cystic fibrosis (CF), an autosomal recessive disorder characterized by dysfunctional Cl^- secretion leading to a thick and viscous mucus production. Chloride secretion through CFTR involves the activity of transporters such as $Na^+/K^+/2Cl^-$ cotransporter (NKCC1) and potassium channels. The ubiquitously expressed basolateral membrane Na^+/K^+ ATPase pump creates a low intracellular Na^+ level by expelling $3Na^+$ out of the cell in exchange for $2K^+$ ions utilizing one ATP molecule. This creates a low intracellular sodium level utilized by the basolateral NKCC1 co-transporter to drive coupled electro-neutral entry of $2Cl^-$ ions into the cell, while the K^+ ions are recycled out of the cell via potassium channels **(Figure 2)**. This recycling of potassium ions establishes a net negative driving force for the secretion of chloride ions through the apical CFTR transporter (7).

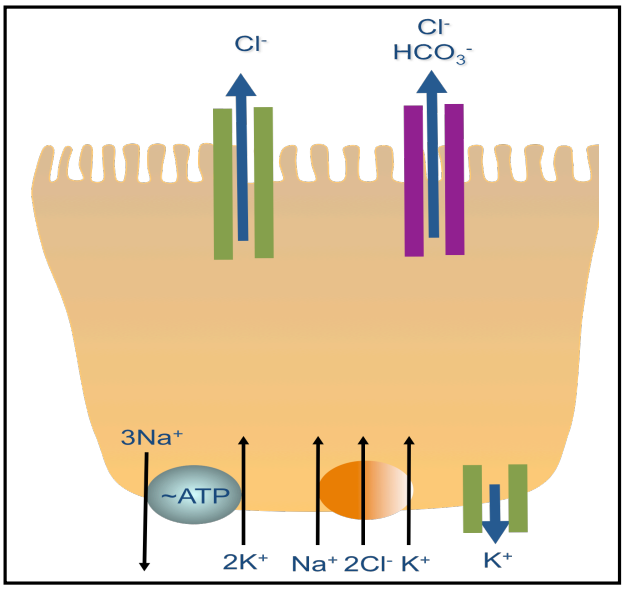


Figure 2: Chloride secretion.

B.2. Electrogenic Sodium Absorption

The exit of sodium ions basolaterally through the Na^+/K^+ ATPase pump is also responsible for the paracellular absorption of water. In a variety of epithelia including the colon, kidney, lung and sweat glands, the apical epithelial sodium channel (ENaC), drives the transcellular entry of sodium followed by a paracellular entry of chloride ions and water via tight junctions **(Figure 3)**. The ENaC channel is regulated by hormones like, aldosterone secreted in response to low blood pressure, thereby increasing the active sodium and water absorption (8). Studies have shown that in the intestine, the ENaC-mediated amiloride sensitive electrogenic transport mechanism is responsible for sodium absorption only in the distal colon.

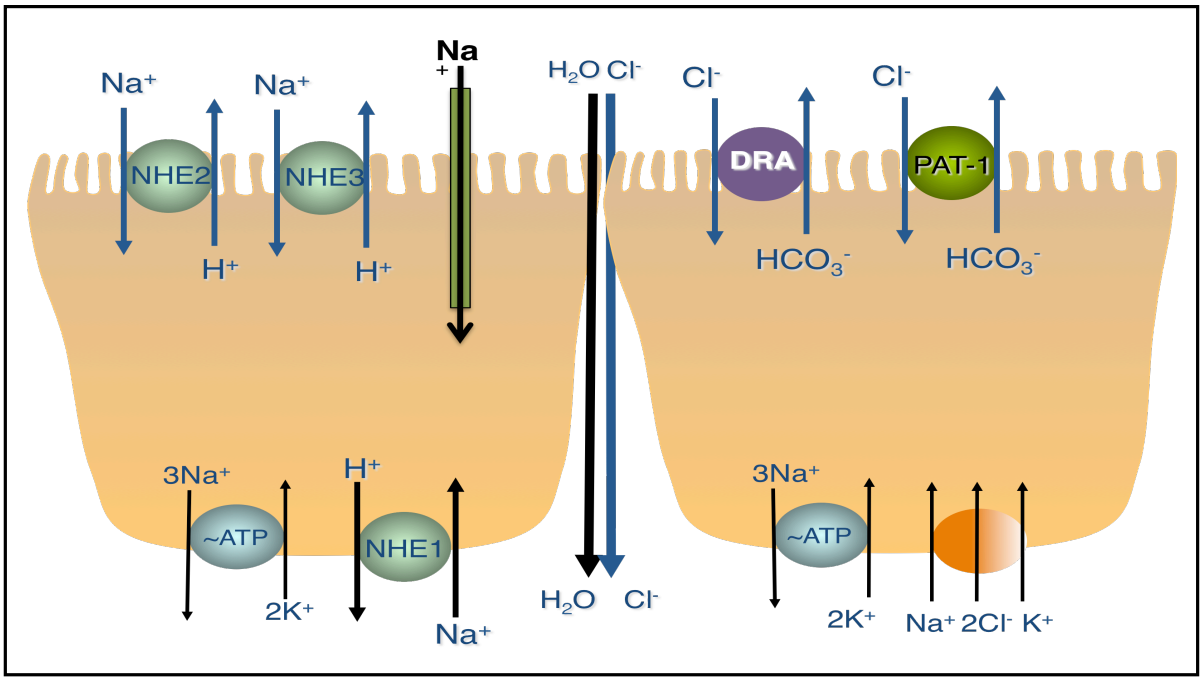


Figure 3. Ion transport processes involved in electroneutral and electrogenic sodium absorption.

B.3. Electroneutral Sodium Chloride Absorption

In vivo perfusion studies and studies utilizing plasma membrane vesicles from organ donors showed that coupling of the Na^+/H^+ exchange process and the $Cl^-/OH^-(HCO_3^-)$ exchange processes allows for electro-neutral sodium chloride absorption in the intestine. The Na^+ gradient created by the Na^+/K^+ ATPase pump provides the driving force for coupled electro-neutral NaCl absorption. The Na^+/H^+ exchange drives H^+ ions out of the cell making the intracellular environment alkaline with subsequent activation of the apical $Cl^-/OH^-(HCO_3^-)$ exchangers. The H^+ and HCO_3^- required for exchange with Na^+ and Cl^- are provided by the intracellular enzyme carbonic anhydrase (CA) **(Figure 4)**. CA utilizes CO_2 and H_2O to generate H^+ and HCO_3^- ions and studies showed that electro-neutral NaCl uptake is sensitive to changes in CO_2 and HCO_3^- concentrations (9-11). The following sub-sections discuss the identity of ion transporters involved in mediating electroneutral sodium chloride absorption.

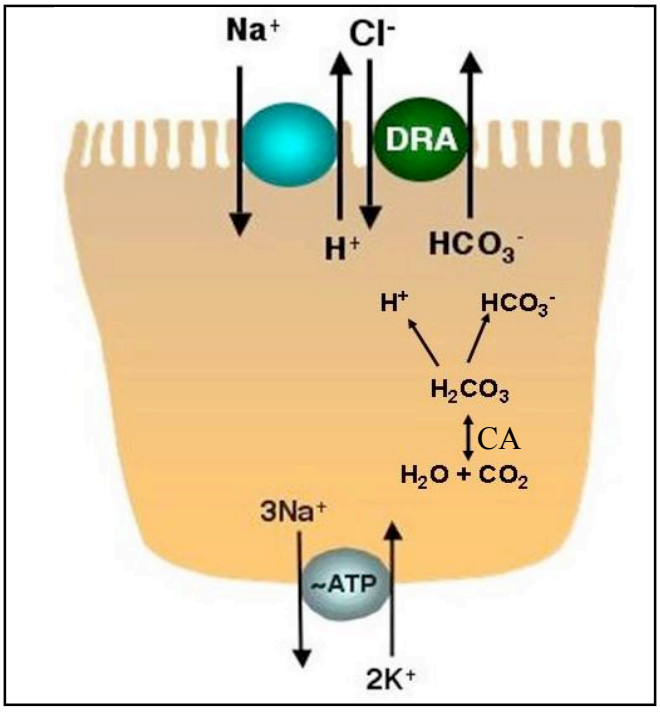


Figure 4: Role of carbonic anhydrase in intestinal NaCl absorption

B.3.1. Na^+/H^+ Exchangers

Ten NHE isoforms, designated NHE1-10 have been identified so far. NHE1, NHE2 and NHE3 are mainly expressed in the intestine, while the intestinal expression of NHE8 is limited to the early stages of life. Functionally, NHE1 is responsible for maintaining intracellular volume and pH and NHE2 and NHE3 are the main transporters that facilitate Na^+ uptake across the apical membrane in the small intestine and proximal colon. Studies from NHE2 and NHE3 knockout mice show NHE3 to be the major sodium absorbing isoform as only NHE3 knockouts exhibit a diarrheal phenotype (12). With regard to their intracellular localization, studies showed that NHE2, NHE3 and NHE8 are expressed on the apical membrane, whereas NHE1 is expressed on the basolateral membrane (13).

B.3.2. Cl^-/OH^- (HCO_3^-) Exchangers

Perfusion studies in normal humans and patients with a genetic disorder called congenital chloride diarrhea (CLD) have established the Cl^-/OH^- (HCO_3^-) exchange process as the major chloride absorptive mechanism in human ileum and colon (14). CLD, a rare genetic disorder is characterized with severe fluid depletion and metabolic alkalosis, and a stool chloride content exceeding 90mmol/L compared to control values of about 10-15mmol/L. However, the identity of the apical Cl^-/OH^- (HCO_3^-) exchangers remained elusive for many years. In recent years, studies utilizing purified apical membrane vesicles from rat distal colon showed the presence of two distinct exchangers, Cl^-/OH^- and Cl^-/HCO_3^-. Similar studies in the rabbit and human ileum and colon, however, showed a single transporter responsible for transporting OH^- or HCO_3^- in exchange for Cl^-. Several lines of evidence implicate the sulfate permease family also known as the Solute Carrier 26 (SLC26) gene family in the apical $Cl^-/OH^-(HCO_3^-)$ exchange process in the intestine (15). The 11 members of the SLC26 gene family namely SLC26A1-SLC26A11, are anion exchangers known to transport a wide range of monovalent and divalent anions like sulfate, iodide, chloride, bicarbonate, oxalate, formate and hydroxyl ion (16). The

genes of the SLC26 family are well conserved in fungi, plant, bacteria, animals and humans. They each encode for membrane proteins that show homology in their hydrophobic transmembrane domains. Mutations in the carboxy terminus in three members of the SLC26 family, namely SLC26A3, A4 and A2 have been associated with diseases such as congenital chloride diarrhea, Pendred syndrome and diastrophic dysplasia, respectively (17) (18). Of the 11 members of the SLC26 family, SLC26A3 (DRA) and SLC26A6 (PAT-1) are the two major apical membrane $Cl^-/OH^-(HCO_3^-)$ exchangers in the intestinal epithelial cells. PAT-1 is expressed in many tissues such as heart, brain, lung, intestine, pancreas, kidney liver and placenta (19). PAT-1 deficiency has not yet been associated with a human disease, but SLC26A6 deficient mice exhibit enhanced oxalate absorption and subsequent increase in urine oxalate excretion and kidney oxalate stone formation (121). In the renal proximal tubule cells it acts as a chloride formate exchanger and is expressed both on the apical and basolateral membranes. With regard to the intestine, PAT-1 is a chloride bicarbonate exchanger functional predominantly in the small intestine. Studies in PAT-1 knockout mice show that although the apical $Cl^-/(HCO_3^-)$ exchange activity is reduced, these mice do not exhibit a diarrheal phenotype, suggesting, that chloride uptake via PAT-1 is not directly coupled to water movement (20). Studies also show that DRA knockout mice but not PAT-1 knockout mice present with a phenotype similar to CLD patients (21). Additionally, mutations in DRA are now known to be responsible for CLD, establishing DRA as the main chloride transporter in the intestine. Since the research presented in this dissertation is focused on elucidating basal and pathogen induced trafficking mechanisms of SLC26A3, the following section will address the current knowledge of its structure, expression, function, and regulation.

C. SLC26A3 or DRA (Down Regulated in Adenoma)

DRA or Down Regulated in Adenoma was originally isolated as a candidate tumor suppressor gene. The gene was mapped to chromosome 7 and a decrease in its expression was found to be associated with adenocarcinomas and colorectal tumorigenesis (22). However, subsequent studies found mutations in DRA to be associated with the autosomal recessive disease congenital chloride diarrhea (CLD). More than 55 mutations in the DRA gene have been identified in CLD patients so far, thus making it an important candidate gene responsible for chloride absorption in the intestine (23) .

C.1. Structure

Recently, studies performed in HeLa cells show DRA to be a large transmembrane protein spanning the membrane 12 times (24). Native-PAGE gels indicate that DRA exhibits a dimeric subunit stoichiometry. Made up of 764 amino acids, both its C and N terminal ends are intracellular. Highly conserved residues at the NH2 terminus are responsible for pore formation and selective anion transport (25). The C-terminus of DRA is also highly conserved and shown to be essential for ion transport function. This end of the protein contains a domain homologous to SpoIIAA family of anti-sigma factor antagonists, which control sporulation in bacteria like *Bacillus subtilis*, called the Sulfate Transporter and Anti-Sigma factor antagonist (STAS) domain (26) **(Figure 5)**. The STAS domain is considered important for protein-protein interaction. For example, studies show that phosphorylation of the STAS domain in SLC26A3 is required for its interaction with CFTR in humans and mice, and is essential for Cl^- secretion, implicating its relevance in the pathophysiology of cystic fibrosis (27). Further, mutations in the STAS domain have been linked to congenital chloride diarrhea, where these mutations are known to cause misfolding and mistargeting of the protein (28). SLC26A3 mutants with a deletion of the STAS domain showed a complete loss of Cl^- transport activity. The C-terminus of DRA also interacts with other cytosolic scaffolding proteins such as NHE Regulatory Factor (NHERF) family of

proteins and ezrin (29). These interactions are believed to be important for plasma membrane expression and association of DRA with specialized regions of the membrane called lipid rafts (LR). Lipid rafts are cholesterol rich membrane microdomains that are resistant to detergent solubilization at 4°C. Studies from our group and others have shown that functional DRA is known to be associated with lipid rafts and this compartmentalization is sensitive to regulatory factors like hormones such as Neuropeptide Y and pathological agents (30).

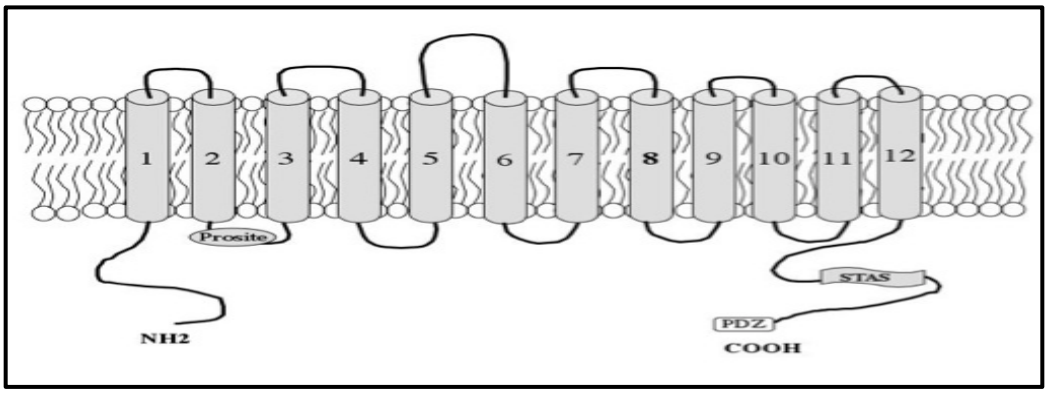


Figure 5: Predicted topology of SLC26 gene family.

C.2. Expression

SLC26A3 is an epithelial-specific protein, expressed in tissues such as the intestine, pancreas, sweat glands and seminal vesicles. In the mammalian intestine its expression is limited to the apical membrane of the colonic epithelium as well as the small intestine although its expression is significantly higher in the human colon (31). In the mouse intestine DRA

expression is highest in cecum followed by distal and proximal colon (32). Along with variation in expression along the length of the intestine, in situ hybridization studies show the expression of SLC26A3 differs along the vertical axis with highest expression in the upper crypt region and surface epithelium of the human colon.

C.3. Function

Studies performed over the last decade have established the role of DRA in (i) NHE3 coupled electro-neutral NaCl absorption in the ileum and colon and (ii) HCO_3^- secretion which is also shown to require functionally active CFTR in the duodenum (5). Expression of human wild type DRA and mutant construct V317del (a mutation found in CLD patients) in *Xenopus* oocyte system showed that wild type DRA mediates both chloride and sulfate transport and that this transport was found to be defective in mutated DRA (24, 32). Other *in vitro* studies using stably transfected HEK293 cells showed a $Cl^-/(HCO_3^-)$ exchange process dependent upon functional DRA (33). Further, studies utilizing brush border vesicles isolated from SLC26A3 KO mice confirmed DRA as the major intestinal $Cl^-/(HCO_3^-)$ exchanger (116). In addition, some studies in *Xenopus* oocyte showed DRA mediated sulfate and chloride transport to be inhibited by DIDS (4,4'-diisothiocyanate-2,2'-stilbene disulfonate), a stilbene derivative known to be an anion exchange inhibitor. However, conflicting reports on the sensitivity of SLC26A3 function to DIDS come from studies in HEK 293 cells that show a very low sensitivity to DIDS. This variability could be due to SLC26A3 being expressed in heterologous expression systems. SLC26A3 has also been shown to be sensitive to anti-inflammatory drugs like niflumic acid and tenidap (28). Taken together, the functional data suggest that DRA is a major intestinal $Cl^-/(HCO_3^-)$ exchanger that is impaired in patients with CLD (24).

C.4. Regulation

Chloride bicarbonate exchange mediated by SLC26A3 is regulated by various stimuli. These stimuli may be extracellular signals such as hormones, neurotransmitters, immune system modulators and gut microorganisms or their intracellular mediators such as cAMP, cGMP, kinases and intracellular Ca^{2+}. Studies have shown DRA to be regulated via both post-translational and transcriptional mechanisms. Additionally, studies in intestinal epithelial cells have shown that increased levels of intracellular cAMP and Ca^{2+} increase DRA endocytosis and downregulate DRA via modulation of trafficking pathways.

C.4.1 Mechanisms Underlying Stimulation of DRA

Studies from our group have shown that several pro-absorptive agents such as neuropeptide Y (NPY), probiotics and lysophospatidic acid (LPA) stimulate DRA via distinct post-translational mechanisms. For example, pro-aborptive hormone NPY mediated increase in intestinal $Cl^-/OH^-(HCO_3^-)$ exchange activity was attributed to increased association of DRA with lipid-raft fractions. Acute treatment with LPA increased DRA surface expression via cellular signaling intermediates PI3K/AKT dependent pathway, whereas, the acute effects of probiotic *Lactobacillus acidophilus* increased DRA membrane levels via PI3K dependent but AKT independent pathways (30, 34, 35). In addition to short-term effects, both LPA and *L. acidophilus* have been shown to induce DRA expression via transcriptional mechanisms in intestinal epithelial cells (36, 37). DRA expression is also transcriptionally upregulated by the short chain fatty acid butyrate via transcription factors YY1 and GATA1. These studies project DRA upregulation as an important therapeutic target to harness for the treatment of diarrheal disorders.

C.4.2 DRA Modulation Under Pathophysiological Conditions

Several lines of evidence implicate down-regulation of DRA in cases of inflammation (38, 39). Recent studies in patients with ulcerative colitis (UC) show decreased $Cl^-/OH^-(HCO_3^-)$ activity as compared to healthy controls. Pro-inflammatory cytokines including IL-1β, TNFα and IFNγ have been shown to decrease DRA expression (40, 41). IFNγ induced inhibition in DRA function and mRNA levels occurred via involvement of the JAK/STAT pathway and inhibition of DRA promoter activity (42). Recent work from our group has shown that NF-κB activation also represses DRA expression via transcriptional mechanisms. Inflammatory mediator H_2O_2 implicated in inflammatory bowel disease has also been shown to have acute inhibitory effects on chloride absorption via PI3K/PKC dependent pathways and long-term treatment with H_2O_2 results in suppression of DRA expression. Pathogens like *Enteropathogenic E coli* (EPEC) acutely inhibit $Cl^-/OH^-(HCO_3^-)$ exchange activity. EPEC attaches to the plasma membrane and delivers its virulence factors via a Type III Secretion System (TTSS). Studies utilizing mutants for EPEC virulence factors EspG1 and EspG2 suggest that these proteins may be involved in modulating cellular trafficking and decreased surface levels and function of DRA via disruption of host cytoskeletal components (2).

Since this dissertation is focused on elucidation of the basal and EPEC modulated trafficking mechanisms influencing DRA function and expression, relevant details regarding the pathophysiology of EPEC infection and its effect on sodium chloride absorption will be discussed in the subsequent sections.

D. Enterpathogenic *E. coli* (EPEC)

Escherichia coli is a gram-negative facultative anaerobe naturally occurring and colonizing in the intestinal microflora (2). Usually co-existing with the host in a beneficial way, some strains of *E. coli* have acquired, through horizontal gene transfer, virulence attributes that make them pathogenic (43). Enteropathogenic *E. coli* is a minimally invasive pathogen with the

ability to form characteristic attaching and effacing (A/E) lesions that allow it to attach to and infect human intestinal epithelial cells. Worldwide, especially in developing countries, EPEC is a major cause of persistent watery infantile diarrhea accompanied by low-grade fever. The diarrhea is highly infectious, quickly transmitted by the oral-fecal route and is associated with a high rate of mortality and morbidity (44). It therefore, necessitates a better understanding of the mechanistic details of the pathophysiology of EPEC induced diarrhea. The following section provides details on the mode of action of Enteropathogenic *E. coli* and its effect on ion transporters involved in electroneutral NaCl absorption.

D.1 Mode of Action – Effects on Host Cell

Unlike typical enteric pathogens, EPEC is modestly invasive and does not produce enterotoxins such as heat labile toxin (LT) from Enterotoxigenic *E. coli* (ETEC) to attack host cells. The attachment of EPEC to host cells is believed to occur in three stages; initial localized adherence or non-intimate attachement, translocation of virulence factors into host cell via type 3 secretion system leading to cytoskeletal disruption and finally intimate attachment with host cells, formation of actin pedestals and A/E lesions (45). Initial attachment is rapid and requires the EPEC adherence factor (EAF) plasmid, which encodes for the bundle forming pilus (BFP) adhesin. Stages two and three are encoded for by the locus of enterocyte effacement (LEE) pathogenicity islands (PAI), which are inserted into many chromosomal loci in EPEC strains. The second stage of EPEC infection involves signal transduction via translocated EPEC virulence factors into the host cells (46). This is achieved by the formation of the type III secretion system, which is like a molecular syringe or pore designed to translocate virulence factors directly into target cell (47). There are at least 8 EPEC secreted proteins relevant to the current study, that are known to translocate into the host cell some of which hijack host cell processes resulting in disruption of tight junctions and dysregulation of electrolyte movement. Virulence factor EspA, a 25kDa protein forms short surface-associated filaments that form a

bridge between the pathogen and host cell during attachment and is essential for the second stage of EPEC attachment. EspB and EspD are required for secretion of effector proteins and A/E lesion formation. Virulence factor EspF secreted in a T3SS dependent manner is not required for A/E lesion formation, instead it has been shown to disrupt the host actin cytoskeleton (48). Finally, 44kDa proteins secreted by *espG* gene, EspG1 and EspG2 have been shown to translocate into the host cell and hijack the host microtubule network (49). Previous studies have shown virulence factors EspG1/G2 and EspF to be implicated in perturbations in sodium chloride transport (2).

Finally in the third stage of its attachment to the host cells EPEC expresses a 94kDa outer wall ahdesin, intimin, and its receptor Tir (Hp90) that is translocated into the host cell via the T3SS and inserts itself into the host plasma membrane and acts as a receptor for the pathogen. Intimin then binds Tir resulting in an intimate adherence of the pathogen to the host cell. LEE pathogenicity islands capacitate the pathogen to produce the typical attaching and effacing (A/E) lesions characterized by a rearrangement of cytoskeletal components like actin to form a pedestal or cup like structure underneath the bacterial attachment sites and cause localized effacement of microvilli (50).

The profound loss of absorptive surface due to the formation of A/E lesions contributes greatly to the diarrheal phenotype during EPEC infection. However, rapid diarrhea that occurs within < 3 hours after EPEC ingestion suggests that other mechanisms of dysregulation in water and solute absorption are at play (51). Recent studies from our group and others have shown that the onset of early diarrhea during EPEC infection results from a direct inhibition of ion transport processes involved in solute and electrolyte movement (2, 52).

D.2 Effect on Sodium and Chloride Transport

Studies suggest that although EPEC induced early diarrhea is multifactorial, it is predominantly a result of impaired sodium chloride absorption. While the total Na^+/H^+

exchangers (NHE) mediated Na^+ uptake is increased by EPEC infection in Caco-2 cells, the effect on the different NHE isoforms is distinct. Function of the major apical sodium absorbing isoform NHE3 is inhibited while the basolateral NHE1 and apical NHE2 are upregulated during EPEC infection. The upregulation of NHE1 and NHE2 is thought to be a host compensatory mechanism in response to a loss of NHE3 activity. It should be noted, however, that increased NHE2 activity may compensate for the decrease in NHE3 in maintaining intracellular homeostasis only but not Na^+ absorption or water movement, since the upregulation of NHE2 does not prevent diarrheal conditions associated with NHE3 knockout mice and NHE2 knockout mice do not exhibit diarrhea. The inhibition of NHE3 activity by EPEC has also been shown to require interaction with scaffolding proteins, namely NHE regulatory factors (NHERF1-NHERF4). For example, studies show that EPEC induced inhibition of NHE3 is increased when cells are co-transfected with NHERF1 and NHERF2 (2,53,54).

Studies in Caco-2 and T-84 cells show inhibition of the apical $Cl^-/OH^-(HCO_3^-)$ exchange activity upon EPEC infection. Of the two main apical $Cl^-/OH^-(HCO_3^-)$ exchangers, biochemical and immunoflorescence studies show that EPEC infection inhibits apical surface levels of DRA but not PAT-1. Mice infected with EPEC show a severe downregulation in $Cl^-/OH^-(HCO_3^-)$ exchange activity along with a redistribution of DRA from the apical surface to the sub-apical compartment in the colon. Similar decrease in DRA expression is also seen in mice infected with *C. rodentium* the murine model of EPEC infection (55). Recently studies have shown that the EPEC translocation machinery T3SS, and its translocated virulence factors EspF, EspG1 and EspG2 play a major role in the downregulation of both NHE3 and DRA **(Figure 6)**. Studies with EPEC mutants revealed the importance of intact T3SS and the virulence factor EspF in NHE3 inhibition. Similarly studies from Gill et al have shown that virulence factors EspG1 and EspG2, known to disrupt host microtubule network, are essential for EPEC induced reduction in apical DRA expression and $Cl^-/OH^-(HCO_3^-)$ exchange activity (2, 53).

Additionally EPEC infection also inhibits the activity of SGLT-1, the sodium-glucose co-transporter present on the apical membrane of intestinal epithelial cells further resulting in loss of water absorption (54).

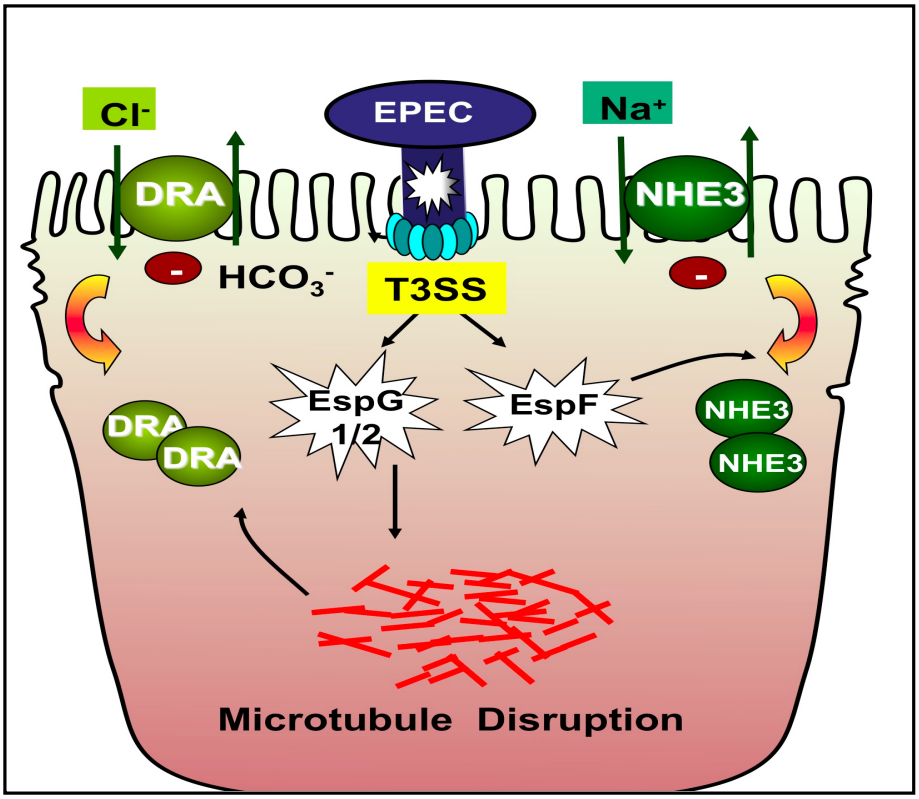


Figure 6. EPEC – Effects on NaCl transport (2).

Overall, the decrease in function and apical surface expression of DRA is a result of modulation of cellular trafficking events by EPEC. However, the molecular basis or the underlying cellular events responsible for altered DRA trafficking are not yet defined. The critical need to delineate these trafficking mechanisms forms the basis of the research presented in this

thesis. The following section presents a background of cellular trafficking mechanisms of endocytosis and exocytosis processes and the role played by cytoskeletal components with special emphasis on the current knowledge of DRA trafficking and its modulation in pathophysiological conditions in intestinal epithelial cells.

E. Cellular Trafficking Mechanisms in Polarized Epithelial Cells

Polarity is a fundamental property of the epithelial cells lining the gastrointestinal tract. Generation of this polarity requires tightly regulated distribution of proteins and lipids to the two distinct membrane surfaces (56, 57). Proteins once synthesized in the endoplasmic reticulum, are trafficked along the microtubules for delivery to specific cellular locations. This distribution is accomplished via the cellular trafficking and sorting machinery, the components of which can broadly be classified as cargo, adaptors and effectors. Cargo such as proteins have intrinsic or post-translationally added motifs or sequences that enable them to be sorted to their final destination (58, 59). For example, the cytoplasmic terminus of many basolateral proteins contains leucine (LL, DXXLL) or tyrosine (NPXY) based motifs that allow basolateral sorting. Not much is known about apical sorting motifs, the presence of *N* and/or *O* glycosylation, however, in a majority of apical proteins is considered important for their apical sorting and providing protection against luminal proteases (60). Adaptor proteins from the Adaptor protein (AP) family and the Golgi-localized, γ adaptin ear-containing (GGA) family, have multiple subunits that allow packaging of cargo into vesicles and facilitate their interaction with motor proteins and microtubules. The role of these adaptors in basolateral sorting is more clearly defined than in apical delivery of proteins. Although less well studied the association of a large number of apically destined proteins with lipid-rafts domains may confer directionality to apical sorting of cargo. The cargo-adaptor complex interacts with accessory proteins that are specific to different stages of trafficking and are essential for vesicle formation and fusion (56). These

effector proteins include GTPases from the Rab family such as Rab4, Rab5 and Rab22 found in early endosomal (EE) compartments, Rab7 associated with late endosomes (LE) usually destined for lysosomal degradation and Rab11 a marker for recycling endosomes (RE) normally destined to the apical membrane (61). Other effector molecules such as soluble N-ethylmaleimide-sensitive factor attachment receptors (SNARES) facilitate fusion of cargo containing vesicle to the membrane. For example, target membranes have target SNARES or t-SNARES and the approaching vesicles express v-SNARES for docking and fusion. Different sets of SNARE proteins are involved in vesicle fusion at either membrane in polarized cells. For example in MDCK cells the t-SNARE, syntaxin 3 is predominantly expressed at the apical membrane, whereas the basolateral membrane expresses syntaxin 4 (60).

With regard to intestinal transport proteins, several ion transporters such as CFTR, NHE3 and DRA contain a class I PDZ domain in their cytosolic termini. Like sorting motifs, PDZ domains enable the proteins to interact with effector molecules like Rabs and accessory scaffolding proteins such as NHE Regulatory Factor proteins (NHERFs). Further, studies show that NHERF mediated interactions of transport proteins with cytoskeletal elements and membrane raft domains are important for post-delivery stabilization and retention of proteins on the apical membrane (62-64). For example, the PDZ domain, ETKF present in carboxy terminus of DRA is known to be required for its interaction with Rab11a positive recycling endosomes that mediate its apical delivery. Recent studies in HEK cells stably transfected with EGFP-DRA or EGFP-DRA-ETKF minus show that the movement of DRA from early endosomal compartments to Rab11a positive recycling endosomes is dependent on the ETKF domain (3). Studies have also shown regulated NHE3 trafficking to be dependent on its interaction with PDZ domain containing proteins PDZK1, IKEPP and E3KARP (62, 63). For example, studies show that Ca2+ dependent decrease in surface levels of both DRA and NHE3 in stably transfected HEK and PS120 cells respectively, is dependent on their C-terminus PDZ domain interactions with scaffolding proteins like PDZK1 and E3KARP (65, 66).

The role of DRA trafficking and its interaction with lipid rafts, cytoskeletal components and accessory trafficking proteins to maintain surface levels is just emerging. Modulation of DRA trafficking events may underlie the decrease in surface levels associated with the pathophysiology of EPEC infection, making it critical to understand these pathways in greater detail.

E.1 Endocytic Pathways

Endocytosis plays an important role in the internalization and directional sorting of membrane proteins in polarized epithelial cells. Endocytosis is an invagination of the plasma membrane and formation of a vesicle containing endocytic cargo. Endocytic mechanisms may be classified as clathrin-dependent or clathrin-independent mechanisms (67) **(Figure 7)**. Clathrin-dependent endocytosis is well studied and involves formation of plasma membrane invaginations coated with clathrin, a soluble cytosolic protein to form clathrin coated pits (CCPs), which are pinched off and internalized with the help of GTPase dynamin. In contrast clathrin-independent mechaninsms are relatively less widely studied and are only now beginning to be fully understood (68). These include caveolin-dependent endocytosis, which is the most well understood dynamin dependent but clathrin-independent endocytic pathway. Literally translated ‘caveolae’ means little caves. Caveolae, consisting mostly of caveolin-1 protein, are often associated with cholesterol rich regions of the membrane and a form of endocytosis, which is sensitive to cholesterol depletion. Caveolin-1 binds to cholesterol in the plasma membrane and forms small, coated vesicles, which pinch off and fuse to form larger caveosomes. The pinching off of these vesicles requires the activity of the GTPase dynamin (68, 69). Caveosomes by-pass the traditional endosomal pathways and fuse directly with target intracellular membranes such as early endosomes and hence this pathway is often used opportunistically by invasive pathogens. Internalization of several sphingolipids, toxins, such as cholera-toxin and pathogenic

viruses and bacteria has been shown to be caveolin-dependent. Although the caveolin-dependent pathway has been studied in polarized epithelial cell lines such as MDCK and T-84, previous studies have shown that Caco-2 cells, our main cell culture model for these studies, do not express caveolin-1 or caveolin-2. Consistent with previous studies we did not find any endogenous expression of caveolin-1 in Caco-2 cells (70, 71). An endocytic pathway recently discovered while studying the internalization of IL-2R-β, the β chain of the interluekin -2 receptor was found to be a clathrin and caveolin independent but dynamin and RhoGTPase dependent pathway (72). However, it is unclear from these studies whether dynamin and RhoGTPase are required for directing IL-2R-β to an enodcytic pathway or for the endocytosis itself and demand further investigation. Other endocytic routes independent of both clathrin and dynamin include pathways that use either small cellular GTPases from the Arf family, importantly Arf6 or CDC-42 from the Rho family (68). Although, this clathrin-independent pathway is sensitive to Arf6 inhibition, it is believed that Arf6-GTPase is not required for the endocytic process itself but more so for the recycling of cargo from this pathway. Further, many vesicles originating from clathrin-independent pathways are internalized into common endosomal vesicles with clathrin-coated cargo and follow similar routes for either lysosomal degradation or recycling (68). Finally, there are other specialized clathrin-independent endocytic mechanisms such as phagocytosis and macropinocytosis. These are actin-dependent, dynamin-independent pathways, which require the activity of Rho-family of GTPases. For the purpose of this thesis, the following paragraphs explain in greater details the clathrin-dependent endocytic pathway.

The clathrin molecule has a triskelion structure formed of three heavy and three light chain molecules (69). The clathrin molecule polymerizes into a lattice that then assembles into clathrin coated pits (CCPs) and vesicles (CCVs) with the help of special proteins called adaptor proteins. AP-2 is the major adaptor responsible for recruiting clathrin triskelions and other accessory proteins to the plasma membrane (73). Once formed the CCVs undergo scission facilitated by a GTPase, dynamin.

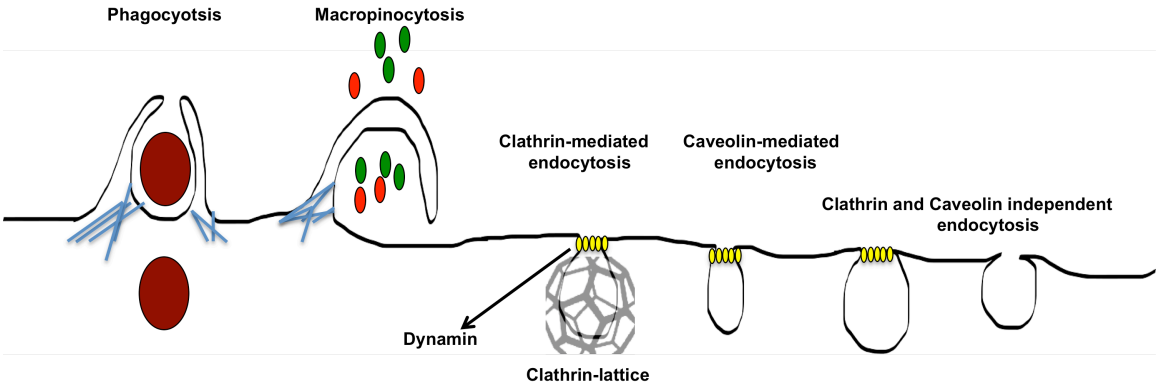


Figure 7. Endocytic Pathways.

There are three known isoforms of dynamin; dynamin1, 2 and 3, of which 1 is restricted to the brain, 2 is ubiquitously expressed and 3 is expressed mostly in the testis and brain. Stimulation of the GTPase activity of the assembled dynamin molecules at the CCV allows for a conformational change in dynamin arrangement, which is responsible for pinching off of the vesicle. Inhibitors such as chlorpromazine, which does not allow the recruitment and assembly of clathrin triskelions along with adaptor proteins at the plasma membrane and inhibitors of dynamin 1 and 2 such as dynasore, have been extensively used to demonstrate the clathrin dependent endocytosis of various receptors, ion channels and even pathogens (74). For example, cAMP regulated internalization of NHE3 has been shown in MDCK cells to be dependent upon clathrin (75). Studies utilizing dominant negative dynamin mutants show constitutive internalization of water channel Aquaporin-2 (AQ2) and chloride channel CFTR to be mediated via a clathrin-dependent pathway (76, 77). These studies and several others establish the role of clathrin-dependent internalization in the endocytic retrieval of several

membrane proteins from the surface of epithelial cells. However nothing is known about the clathrin dependency of DRA internalization in intestinal epithelial cells under physiological or pathophysiological conditions and demands further investigation.

E.2 Cytoskeletal Elements in Ion Transport

Cellular scaffolding or cytoskeleton plays an important role in functions such as maintenance of cell shape, motility, cell division, endocytosis and secretion in eukaryotic cells. In polarized epithelial cells, which require directional sorting of proteins in order to maintain polarity the cytoskeletal elements such as actin and microtubules, are essential for the polarized distribution of proteins and lipids. Various studies suggest that the cytoskeleton is not only important for the stabilization and delivery of ion transporters and channels to the membrane but also their interaction with actin associated proteins influences the ion transport activity. The following paragraph attempts to shed light on the current knowledge of the relationship between the cytoskeleton and membrane proteins in epithelial cells (78).

Actin filaments have been associated with vesicular transport in multiple endocytic pathways. Actin filaments are generally shorter than microtubules and mediate slow transport of endosomal vesicles brought about by actin-associated myosin motors. Almost 13 different classes of myosin motors are known however only Class I, V, VI and VII have been shown to be associated with organelle and vesicle movement. With regard to the intestinal epithelial cells, myosin I has been shown to be present in the brush border, where it helps attach actin bundles beneath the microvilli to the plasma membrane. Other actin-binding proteins present in the IECs include myosin II, V and VI, tropomysoin, filamin, β-spectrin etc (79).

Aside from regulating the movement of vesicles containing ion transport proteins, the actin cytoskeleton and actin-binding proteins regulate ion transport function by influencing membrane fluidity, cell morphology and motility and by regulating signaling cascades. Actin-disrupting class of fungal metabolites, cytochalasins have been widely used in epithelial cells to

identify the link between cytoskeleton and protein function. Cytochalasin D treatment in MDCK and Caco-2 cells has varied effects on apical and basolateral endocytosis of membrane proteins (80, 81). Further, these studies suggested that actin microfilaments are more important for proteins internalized from the apical surface. For example studies show that apical endocytosis of ferritin (clathrin-dependent) and fluid-phase marker Lucifer yellow is inhibited in MDCK cells treated with cytochalasin D, while basolateral internalization of transferrin and lucifer yellow remained unaffected (82). Similarly internalization of horseradish peroxidase (HRP) from apical surface of small intestinal tissue and apical water channels from bladder epithelial cells is inhibited in the presence of cytochalasin D (119). Recently however studies in MDCK and T-84 cells showed the involvement of actin in basolateral endocytosis (117). Another essential role of actin filaments is as cellular scaffolding that allows assembly of trafficking machinery and associated cargo. For example, the Na^+/K^+ ATPase pump is shown to directly bind to actin-binding proteins spectrin and ankyrin, which restrict its expression to lateral membranes. With regard to DRA, studies from Chernova et al showed that neither actin-disrupting cytochalasin D nor actin-stabilizing drug NBD-phallicidin altered DRA activity (28). In contrast, NHE3 activity and expression on the apical membrane is dependent upon its PDZ domain mediated interactions with the actin cytoskeleton (63). PDZ domain containing proteins NHERF1 and NHERF2 act as intermediates in facilitating the linking of NHE3 to actin-binding protein ezrin. Further studies show that cytochalasins B and D and latrunculin B significantly inhibit NHE3 activity (83). Although there is no direct evidence implicating actin cytoskeleton in regulation of DRA, functional coupling to NHE3 might bring about secondary regulation and needs further investigation.

Microtubules govern several important aspects of the apico-basolateral polarity in epithelial cells. They are polar structures with a fast growing plus end and a slow growing minus end. In non-polarized cells such as fibroblasts, microtubules have a centrosomal alignment with the minus end anchored towards the centrosome and the plus end facing the cell cortex. In

contrast, in polarized epithelial cells microtubules arrange themselves in a non-centrosomal fashion following the apico-basolateral polarity of the cells with minus ends facing the apical membrane and plus ends directed towards the basal membrane (86). Microtubule filaments are longer than actin flaments and mediate rapid transport generally directed towards the apical or basolateral membranes. They are made up of 13 protofilaments arranged around a hollow central core. Protofilaments in turn are made up of alpha and beta tubulin molecules arranged in a head to tail fashion. Directional arrangement of tubulin provides microtubules with their intrinsic polarity. Typically in the intestinal and kidney epithelial cells microtubules are arranged with their minus ends directed towards the apical surface and their plus ends directed towards the cell interior (84, 85). These characteristics make microtubules perfect for rapid, directional movement of ion channels and transporters to and from the polarized surface membrane. Vesicle movement along the microtubules is mediated by two microtubule motor proteins, kinesin and cytoplasmic dyenin, that require ATP to move cargo. Kinesin is a multimeric protein made up of two heavy and two light chains and directs plus ended transport or centrally directed transport along the microtubules. On the other hand dynein is made up two heavy chains, two or three intermediate chains and several light chains and directs minus ended transport mediated towards the membrane surface in epithelial cells (86).

With regard to cellular trafficking, most receptor-based endocytosis is unaffected by microtubule disruption, however some forms of fluid-phase endocytosis have previously been shown to be sensitive to microtubule-disrupting drug nocodazole. On the other hand, movement between common endosomes, apical early endosomes and recycling endosomes is rapid and well suited to microtubule based transport (84). Studies show that movement of cargo from early to late endosomal compartments is dependent upon microtubules. For example, studies in MDCK cells show that while the kinetics of internalization of transferrin receptor are largely unaffected by nocodazole, movement from basolateral early endosomes to common endosomes is impaired. Further, live-imaging studies from epithelial cells show that post-Golgi

exocytic vesicles containing GFP-tagged apical (NTRp75) and basolateral (VSVG) markers bind to microtubules and are associated with microtubular motors and that disruption of microtubules retards both apically and basolaterlly targeted exocytosis. However many studies now show that apical exocytosis is affected to a much greater extent. With regard to transcytosis, there are conflicting reports. While some studies show the transcytosis of proteins such as IgG to be unaltered by microtubule disruption, other reports show that apical to basolateral movement of viruses such as HIV is dependent upon intact microtubules (120).

Membrane delivery of ion channels, pumps and transporters has been shown to be dependent upon microtubular transport. For example studies in proximal tubule cell line LL-CK, showed that segregation of Na^+ - hexose carrier protein to the apical membrane was inhibited upon treatment with colchicne and nocodazle (84). Further, basolateral expression of Na^+/K^+ ATPase was also found to be sensitive to drugs targeting microtubules. Consistent with these studies other studies in colchicine treated rats showed a redistribution of gp330 membrane protein to the cytoplasmic and basolateral membranes contrasting with its apical localization in control rats. Further, a study showed that increased in water uptake in toad urinary bladder cells upon vasopressin treatment was dependent upon intact microtubules (87,88). A study in T-84 cells showed that intact microtubules are also essential for chloride secretion and exocytosis of CFTR induced by forskolin (118). These studies emphasize the importance of microtubules in the exocytic trafficking of membrane proteins. Not much is known about the role of microtubules in DRA trafficking. Previous studies from our group have shown that EPEC mediated decrease in DRA function and surface expression was dependent upon EPEC virulence factors EspG1 and EspG2 known to disrupt host microtubular network (2). Further these studies showed a similar decrease in DRA function in Caco-2 cells treated with colchicine and nocodazole. However the mechanistic details underlying decreased DRA function and surface levels associated with disrupted microtubules are currently unknown and need further investigation.

F. Hypothesis and Specific Aims

Maintenance of function and surface expression of DRA under basal conditions, involves recycling at the plasma membrane. However, little is known about the detailed cellular trafficking of DRA under physiological or pathophysiological states in intestinal epithelial cells. Recent studies (as mentioned above) suggested that EPEC via a type 3-secretion system decreased the activity and surface expression of the apical Cl^-/HCO_3^- exchanger (SLC26A3/DRA – Down Regulated in Adenoma), via modulation of cellular trafficking mechanisms and disruption of host cytoskeletal elements.

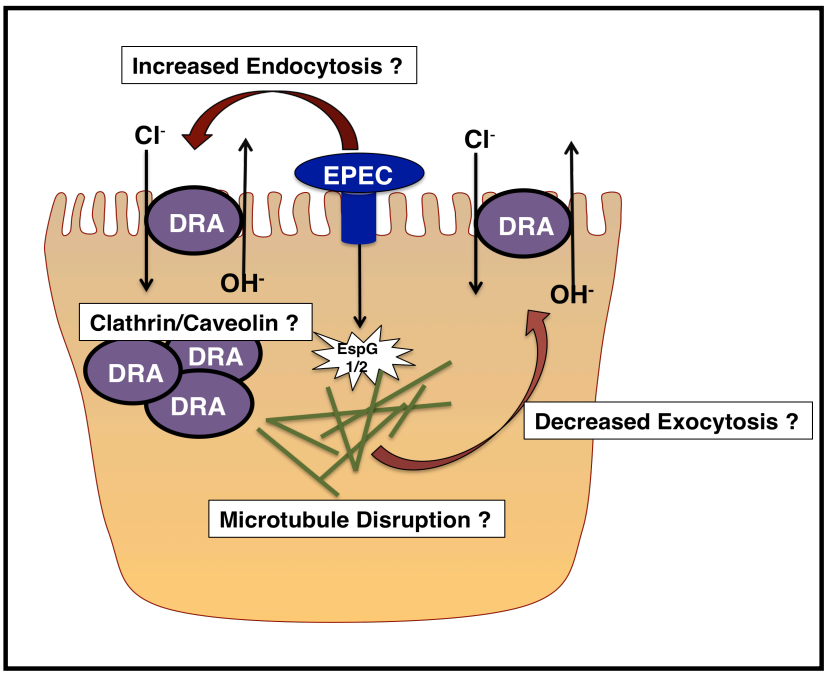


Figure 8. Proposed model

We hypothesized that endocytic and/or exocytic pathways are involved in maintenance of surface levels of DRA under basal conditions and that EPEC induced modulation of cellular trafficking pathways via disruption of host microtubule network decreases DRA function and surface expression in intestinal epithelial cells.

Two specific aims were designed for these studies:

1. Elucidate the role of cellular trafficking mechanisms and microtubules in basal DRA surface expression.
2. Investigate implication of EPEC mediated modulation of trafficking mechanisms and microtubule disruption in decreased DRA surface expression.

II. MATERIALS AND METHODS

A. Materials

Eagle's Minimum Essential Medium (EMEM), Dulbecco's modification of Eagle's Minimum Essential Medium (DMEM F-12), Caco-2 and T-84 cells and *Lactobacillus acidophilus* strain (LA4357) were obtained from American Type Culture Collection (ATCC, Manassas, VA). For 3D culture of Caco-2 cells, Growth Factor Reduced Matrigel was obtained from BD Biosciences (BD No.354230). For uptake studies, radionuclide ^{125}I (NaI) was procured from Perkin Elmer (Boston, MA) and 4, 4'-diisothiocyanostilbene-2, 2' disulfonic acid (DIDS), was obtained from Sigma-Aldrich. Pharmacological inhibitors dynasore and chloropromazine and anti-actin antibody were purchased from Sigma-Aldrich (St. Louis, MO). Sulfo-NH-SS-biotin, and Sulfo-NH-SS-acetate and reduced glutathione (GSH) were obtained from Thermoscientific (Rockford, IL). Microtubule disrupting agent nocodazole and colchicine were obtained from Biomol (Plymoth Meeting, PA) and Sigma-Aldrich respectively. Affinity purified DRA antibody was custom synthesized by the Research Resources Center (RRC), University of Illinois at Chicago. All other chemicals were of at least reagent grade and were purchased from Sigma or Fisher Scientific (Pittsburgh, PA).

B. Cell Culture

Human colonic adenocarcinoma cell line, Caco-2, were grown routinely in EMEM supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 2 μg/ml gentamycin and 20% fetal bovine serum in 5% CO_2-95% air environment at 37°C in T-150 cm^2 plastic flasks. The colonic crypt like cell line, T-84, was grown in a mixture of Ham's F12 and DMEM medium supplemented with 2.5 mM L-glutamine, 5% FBS and the antibiotics as described for Caco-2 cells. Both Caco-2 and T-84 cells have been shown to express DRA and it has previously been shown that 20% serum helps in enhanced expression of ion transporters in Caco-2 cells (40, 89).

Most of the studies were performed in Caco2 cells. Only functional results were confirmed in T84 cells to rule out the possibility that the effects could be cell line specific. For the uptake studies, biotinylation experiments and lipid-raft studies, cells between passages 25 and 45 were plated on 24 well plates at a density of 2 X 10^4 cells/well. Fully differentiated confluent monolayers were used for the experiments (10-12 days post plating). To study the effect of endocytosis inhibitors, dynasore (80 μM) or chlorpromazine (60 μM), on apical Cl^-/HCO_3^- exchange activity and DRA protein expression, cells were incubated with inhibitor in serum-free culture medium for 60 minutes at 37°C (90). Cell monolayers grown on transwell inserts at a density of 4×10^3 cells/well were used for immunofluorescence staining at 10 day post plating.

For 3D cell culture of Caco-2 cells, Matrigel was allowed to thaw on ice overnight at 4°C. Immediately before plating, Matrigel, culture plates, pipette tips were pre-cooled in ice. Forty μl of Matrigel was added to each well of an eight well glass chamber slide and spread evenly using the tip of a P-200 pipet. Slides were placed in a cell culture incubator to allow the Matrigel to solidify. Trypsinized Caco-2 cells were seeded at a density of 5000 cells/well and maintained in 5% CO_2-95% air environment at 37°C.

C. Assessment of Cl^-/OH^- Exchange Activity

Cl^-/HCO_3^- exchange activity was determined by measuring DIDS-sensitive $^{125}I^-$ uptake in base loaded cells as previously described by us with minor modification from the $^{36}Cl^-$ uptake method. $^{125}I^-$ was used in place of $^{36}Cl^-$ to measure the exchange activity as the vendor discontinued $^{36}Cl^-$. Use of $^{125}I^-$ to measure Cl^-/HCO_3^- exchange activity has been validated in the native intestine in our previous study (30). For uptake studies with EPEC, cells were pre-treated with inhibitor for 30 minutes followed by a 30 minute EPEC infection in the presence or absence of inhibitor. Following treatment or infection, the cell monolayers were incubated with loading buffer, containing 20 mM HEPES pH 8.5, for 30 min at room temperature. The medium was

removed after treatment, and the cells were rapidly washed with 1 ml tracer-free mannitol uptake buffer containing 260 mM mannitol, 20 mM Tris/2-N-Morpholino) ethanesulfonic acid (Tris-MES), pH 7.0. The cells were then incubated with the uptake buffer with or without 600 µM DIDS containing 1.0 µCi of ^{125}I (3 mM) sodium iodide (specific activity: 17.4 Ci/mg) for 5 min. This time period was chosen as it was within the linear range of I^- uptake. Uptake was stopped by removing the radioactivity containing buffer and washing the cells rapidly twice with ice-cold phosphate-buffered saline (PBS), pH 7.2. Finally, the cells were solubilized by incubation with 0.5 N NaOH for 4 h and protein concentration was measured by Bradford method. Radioactivity was measured with a Packard Tri-Carb 1600 TR liquid scintillation analyzer (Packard Instruments; Perkin Elmer, Boston, MA). The Cl^-/HCO_3^- exchange activity was assessed as DIDS-sensitive $^{125}I^-$ uptake, and the specific activity is expressed as nanomoles per milligram protein per 5 min.

D. EPEC Culture and Cell Infection

The following EPEC strains were used: (i) wild-type EPEC strain (ii) **Δ**espG/**Δ**espG2 kindly provided by Dr Gail Hecht (Loyola University, Chicago, USA). Bacterial strains were grown overnight in 5 ml Luria Broth in the presence of appropriate antibiotics, at 37°C in a shaker at 250rpm. On the day of infection, 300 µl of overnight bacterial culture was transferred to 10 ml of serum- and antibiotic-free T84 cell culture medium supplemented with 0.5% mannose, to promote formation of type III secretion system as previously described (48,94). Bacteria were grown approximately for 3 hours till they reached to an OD600nm of 0.4. Caco-2 monolayers were infected at a multiplicity of infection (MOI) of 100. Non-adherent bacteria were removed by washing in PBS after 30–60 minutes.

E. *Lactobacillus acidophilus* Culture and Cell Treatment

L. acidophilus (LA) was grown in Mann-Rogosa-Sharpe (MRS) broth (Difco) for 24 h at 37°C with no shaking. The cultures was then centrifuged at 3000 × g; 10 min at 4°C. The supernatant obtained was filtered through a 0.22-μm filter (Millex, Millipore) to sterilize and remove all bacterial cells and was designated as conditioned/culture medium (CM). For treating the Caco-2 monolayers, LA culture supernatant was diluted 50 times in culture media.

F. Cell Lysates

After treatment with dynasore (80 μM) or following EPEC infection, control or treated cells were washed with ice-cold 1X-PBS to remove residual media. Total protein was extracted by suspending the cell pellet in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA) supplemented with protease inhibitor cocktail from Roche (Indianapolis, IN) and phosphatase inhibitor (Sigma). For biotniylation studies, the cells were lysed by sonication (three pulses for 20 s each) and the lysate was centrifuged at 13000 rpm for 10 min at 4°C to remove cell debris. The supernatant containing the total cell proteins was collected and protein concentration was determined by the Bradford method (91). For lipid-raft studies, cell lysates were homogenized in a detergent-free buffer and subsequently passed through a 25-gauge needle about 8-10 times. The homogenized lysate was centrifuged at 13000 rpm for 10 min at 4°C to remove cell debris and saved.

G. Sodium Dodecyl Sulfate-PAGE and Western Blotting

To examine the expression levels of DRA, equal amounts (75 μg/sample) of whole cell lysates were solubilized in SDS-gel loading buffer ((250 mM Tris-HCl pH 6.8, 10% sodium dodecyl sulfate, 250 mM dithiothreitol, 50% glycerol and 0.2% bromophenol blue) and boiled for 5 min. Proteins were loaded on a 7.5% SDS-polyacrylamide gel and transblotted to

nitrocellulose membrane after electrophoretic separation. After one hour of incubation in blocking bufer (1X-PBS and 5% non-fat dry milk) the membrane was probed with anti-DRA (1:100 dilution) or anti-Actin antibody (Sigma; 1:3,000 dilution) in 1X-PBS and 2.5% nonfat dry milk overnight at 4°C. The membrane was washed five times with the wash buffer (1X-PBS and 0.1% Tween-20) for 5 min and probed with HRP-conjugated goat anti-rabbit antibody (1:2000 dilution) for 1 h followed by ECL (enhanced chemiluminescence, from Bio-Rad, Hercules, CA) detection.

H. Cell Surface Biotinylation

Cell surface biotinylation studies were performed in Caco-2 monolayers utilizing Sulfo-NH-SS-Biotin (1.5 mg/ml; Pierce, Rockford, IL) in borate buffer (in mM: 154 NaCl, 7.2 KCl, 1.8 $CaCl_2$, 10 H_3BO_3, pH 9.0) as described previously (34). Labeling of surface proteins with biotin was allowed to proceed for 60 min at 4°C to prevent endocytosis and internalization of antigens. Neutravidin agarose beads were used to pull down the biotinylated antigens. The biotinylated proteins were released by boiling in gel loading buffer containing 100 μM dithiothreitol. Proteins were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Membranes were then probed with anti-DRA antibody. The surface DRA levels were compared with total cellular DRA as determined by immunoblotting in solubilized cell extract.

I. Endocytic Internalization Assay

To measure the extent of endocytosis, Caco-2 cells were plated at a density of 1 X 10^5 cells on 6 well plates. Cell surface was labeled with Sulfo-NH-SS-Biotin (1.5 mg/ml; Pierce, Rockford, IL) in borate buffer (in mM: 154 NaCl, 7.2 KCl, 1.8 $CaCl_2$, 10 H_3BO_3, pH 9.0) at 4°C for 60 minutes (92). Following surface biotinylation, cells were treated with dynasore or vehicle (DMSO) at 37°C for 60 minutes. For studies with EPEC, cells were infected with EPEC for 60 minutes at 37°C. Immediately after treatment cells were rinsed with ice cold 1X PBS twice at

4°C. Un-internalized, surface biotin was cleaved by 150 mM reduced glutathione (GSH) in 1X PBS and the biotinylated freshly endocytosed proteins were protected from cleavage by GSH. Cells were solubilized in lysis buffer and biotinylated proteins were retrieved and assayed for endocytosed DRA as described above.

J. Exocytic Insertion into Plasma Membrane

To measure the exocytic insertion of DRA into plasma membrane following microtubule disruption, NHS reactive sites on Caco-2 cell surface were masked by pretreatment with sulfo-NHS-acetate at 4°C for 60 minutes, followed by quenching in 1 M glycine as previously described (92). Cells were then placed at 4°C for 1 hour to allow for cold induced microtubule disruption, followed by recovery of microtubules at 37°C for 3 hours in the presence or absence of microtubule disrupting agent nocodazole (66 μM). For EPEC studies, cells were infected with wild-type EPEC, able to disrupt microtubules and *ΔespG1/G2* mutants unable to disrupt microtubules at 37°C for 60 min. After microtubule disruption and recovery, cells were subjected to cell-surface biotinylation as described above. The biotinylated fractions, which represent the newly inserted membrane proteins, were precipitated with neutravidin agarose beads and the precipitate was subjected to SDS-PAGE and western blotting with anti-DRA antibody.

K. Isolation of Detergent Insoluble and Detergent Soluble Fractions from Caco-2 Cell Membranes

Caco-2 cells lysates prepared in detergent free buffer (described above) were subjected to ultacentrifugation at 100,000 *g* for 30 min at 4°C. The resulting total membrane pellet obtained was resuspended in a detergent based MES buffer (50 mM MES (pH 6.5), 60 mM NaCl, 3 mM EGTA, 5 mM $MgCl_2$, 1% Triton X-100, and 1X Complete protease inhibitor cocktail) and incubated at 4°C on a rotatory shaker for 30 min. The mixture was then ultracentrifuged at

100,000 *g* for 30 min at 4°C to separate detergent soluble and detergent insoluble membranes. The supernatant obtained was referred to as the detergent soluble (DS) fraction. The pellet was resuspended in a RIPA lysis buffer (5 mM HEPES (pH 7.4), 150 mM NaCl, 10 mM EDTA, 1 mM DTT, 1% Triton X-100, 0.1% SDS and 1× Complete protease inhibitor cocktail) and designated the detergent insoluble (DI) fraction (93). Both DS and DI fractions obtained after treatment of Caco-2 cells with dynasore (or infection with EPEC) were analyzed by Western blotting as described above.

L. Immunofluorescence Staining in Caco-2 Cells

Caco-2 cells grown on 12 well Transwell inserts at 4 X 10^3 cell/well were treated from the luminal side with dynasore for 60 min. Cells were then washed twice in 1X PBS containing 1 mM $CaCl_2$, pH 7.4 and fixed with 2% paraformaldehyde for 1.5 h. Following this, cells were permeabilized with 0.08% saponin, washed with 1X PBS-$CaCl_2$, and blocked in 5% normal goat serum (NGS) for 2 hours at room temperature. Monolayers were then incubated with rabbit anti-human DRA antibody (1:100) for 2 h followed by 3 washes 5 min each with 1X-PBS containing $CaCl_2$ and saponin. Cells were incubated in Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody at 1:100 dilution (Invitrogen) and rhodamine-phalloidin (1:60 dilution; Invitrogen) for 60 min at room temperature. The immunostained inserts were removed from transwells and mounted on glass slides with Slowfade gold antifade reagent from Invitrogen. Images were taken with x63 water immersion objective on a Zeiss LSM 510 META confocal microscope. Beams at 488nm and 534nm from an Ag/Kr laser and 361 nm from a UV laser were used for excitation of fluorophores. Green and red fluorescence emissions were detected using LP505 and 585 filters respectively. A series of images was taken in the *z* axis plane and *z* stack obtained was used to make orthogonal sections in the *xz* plane. Images were processed using ZenLite imaging software from Carl Zeiss (2)

M. Animal Protocol

Eight-week old C57BL6 mice weighing between 20-25 g were used in all studies. Animals were acclimatized for one week prior to the experiment on standard mouse pellet diet with free access to water. The animal experiments were conducted according to the guidelines of Animal Care Committee of University of Illinois at Chicago and Jesse Brown VA Medical Centre **(Appendix A).** Mice were sacrificed and a segment of distal colon was dissected, flushed with PBS and used for ex-vivo biotinylation studies and immunoflorescence.

N. Immunofluorescence Staining in Optimal Cutting Temperature (OCT) Tissue Sections

Distal colon sections from control and colchicine treated mice were snap-frozen in optimal cutting temperature embedding medium (Tissue-Tek O.C.T compound; Sakura) and stored at –80°C till used for tissue sectioning. Five µm sections were cut uitilizing a Microme cryostat. Multiple sections were taken from at least 3 animals from each treatment and analyzed by immunofluorescence staining. Sections were fixed with 1% paraformaldehyde in 1X PBS for 15 minutes at room temperature and washed with 1X PBS 3 times. Tissues were then subjected to permeabilization using 0.5% NP-40 in 1X PBS for 5 minutes, followed by 2 washes with 1X PBS. Sections were then blocked in 2.5% NGS (Normal Goat Serum) for 1.5 h at room temperature followed by incubation with primary antibodies (rabbit anti-DRA (1:100) and mouse anti-villin (1:100)) in 1% NGS for 2 h at room temperature. After 4 washings with 1% NGS, sections were incubated with the secondary antibodies, (Alexa Fluor 568-conjugated goat anti-rabbit IgG and Alexa Fluor 488-conjugated goat anti-mouse IgG) for 60 min and were washed 5 times with 1% NGS in 1X PBS. Mounting on glass slides was done utilizing slow fade DAPI. Microscopy was performed using a Zeiss LSM 710 laser scanning confocal microscope equipped with a 63x water immersion objective (2).

O. Statistical Analysis

Results are expressed as Mean ± SEM and represent the data from 3-5 independent experiments. One way ANOVA with Tukey multiple comparison test was used for statistical analysis. For some experiments, unpaired t-test was used as a statistical parameter. Differences between control and treated groups were considered significant at $p < 0.05$.

III. RESULTS (AIM 1)

As described in the following paragraphs, specific aim 1 identifies clathrin-mediated endocytosis as the pathway involved in DRA internalization and delineates the role played by microtubules in DRA exocytosis.

A. Endocytosis Inhibitor Dynasore Stimulates Apical $Cl^-/OH^-(HCO_3^-)$ Exchange Activity

The first part of this aim examined the cellular endocytic pathway responsible for basal DRA internalization. Our first set of studies utilized the endocytosis inhibitor dynasore that is known to target dynamin, a 100kDa GTPase required to pinch off coated vesicles from the plasma membrane (90). Post-confluent differentiated Caco-2 cells were serum starved overnight and treated with different doses of dynasore (40 μM – 120 μM) in serum free media for 1 hour. Apical Cl^-/OH^- (HCO_3^-) exchange activity was assessed by the measurement of DIDS (4,4'-diisothiocyanate-2,2'-stilbene disulfonate) sensitive $^{125}I^-$ uptake in base loaded Caco-2 cells. Inhibition of endocytosis with dynasore showed no stimulation of apical $Cl^-/OH^-(HCO_3^-)$ exchange activity at 40 μM, with maximal stimulation at 80 μM (~ 2 fold) followed by a similar increase with 120 μM **(Figure 9A).** These results support the notion that DRA undergoes endocytic recycling in intestinal epithelial cells.

Clathrin and caveolin dependent endocytosis are well-studied cellular endocytic routes previously shown to be involved in the basal and regulated internalization of ion transporters. Since inhibition of dynamin prevents both clathrin and caveolin dependent endocytosis, in our second set of studies, we utilized an inhibitor, chlorpromazine, known to specifically inhibit clathrin-mediated endocytosis (CME) without affecting other endocytic pathways (74).

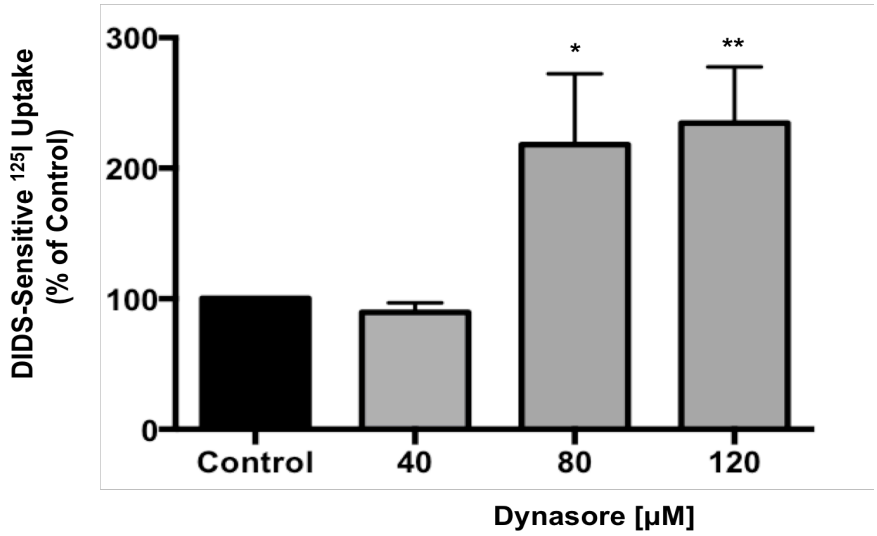
Chlorpromazine prevents the assembly of clathrin-coated vesicles at the plasma membrane. Caco-2 cells were serum starved overnight and then treated with chlorpromazine (60 μM) in serum free media for 1 hour. Apical $Cl^-/OH^-(HCO_3^-)$ exchange activity was assessed by the measurement of DIDS sensitive $^{125}I^-$ uptake in base loaded Caco-2 cells. Similar to dynasore, chlorpromazine treatment resulted in an increase (~ 2 fold) in apical $Cl^-/OH^-(HCO_3^-)$ exchange activity **(Figure 9B)**. These data indicate that $Cl^-/OH^-(HCO_3^-)$ exchange activity in Caco-2 cells is regulated by clathrin-dependent endocytosis.

Previous studies have shown that Caco-2 cells do not express caveolin-1 or caveolin-2, the two major caveolin isoforms (70, 71). However, these studies were carried out in Caco-2 cells cultured in the standard 2D culture system. To examine if culturing Caco-2 cells in 3D may change gene expression profile, we investigated the expression of endogenous caveolin-1 in both 2D and 3D culture systems. Total protein lysates from Caco-2 cells and two other intestinal cell lines, T-84 and HT-29 were run on a 10% SDS-PAGE gel followed by immunoblotting with anti-caveolin-1 antibody. Our results showed no expression of caveolin-1 in Caco-2 cells grown either in 2D monolayers or as 3D system **(Figure 10)**. On the other hand, caveolin expression was detected in both T-84 and HT-29 cells cultured in 2D system. Hence, the role of caveolin-dependent DRA trafficking in Caco-2 cells was not examined further.

B. <u>Stimulation of Apical $Cl^-/OH^-(HCO_3^-)$ Exchange Activity by Dynasore is Specific.</u>

The $Cl^-/OH^-(HCO_3^-)$ exchange process is functionally coupled to the Na^+/H^+ exchange in the intestine. To rule out the possibility that increase in $Cl^-/OH^-(HCO_3^-)$ exchange activity is not secondary to increased Na^+/H^+ exchange, it was of interest to examine whether dynasore affects NHE3 activity. NHE3 activity was assessed in serum-starved Caco-2 cells treated with different doses of dynasore (40 μM – 120 μM) for 1 hour. NHE3 activity was measured as S3226-sensitive $^{22}Na^+$ uptake in acid loaded cells.

(A)



(B)

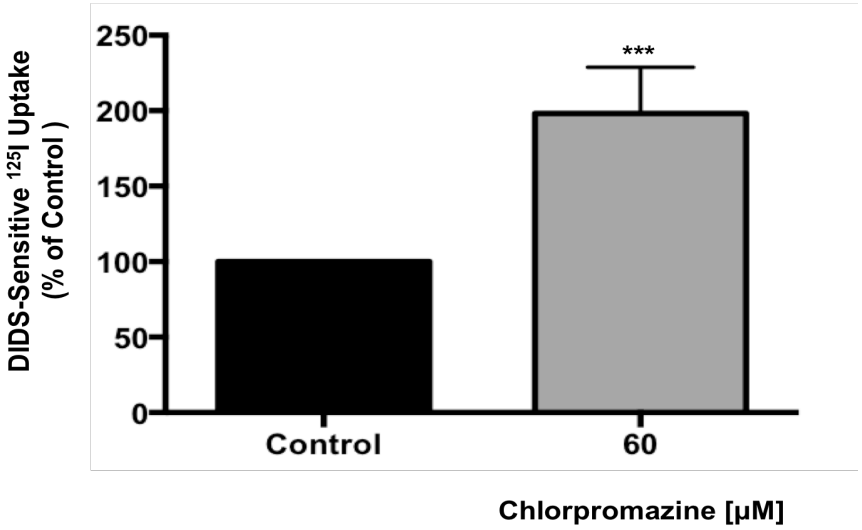


Figure 9. Dynasore and Chlorpromazine stimulate apical Cl^-/HCO_3^- exchange activity in Caco-2 cells. Serum starved Caco-2 cells were treated with **(A)** different doses of dynasore (40-120 μM) for 1h or **(B)** 60 μM Chlorpromazine for 1h. Cl^-/HCO_3^- exchange activity was measured as DIDS-sensitive (600 μM) $^{125}I^-$ uptake in base loaded Caco-2 cells. Results are expressed as % of control and represent the mean ± SEM of 4 different experiments performed in triplicate. *p<0.01, **p<0.006 and ***p<0.0007 as compared to control.

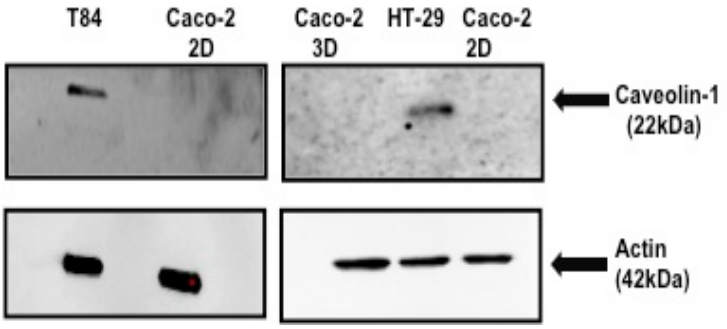


Figure 10. Caco-2 cells do not express caveolin-1 in 2D and 3D cultures. Post-confluent Caco-2, HT-29 and T-84 cells were used to prepare protein lysates. Protein lysate was also prepared from Caco-2 cells cultured in 3D. Total protein lysates were run on a 7.5% SDS-PAGE gel and then analysed by immunoblotting for the expression of caveolin-1 and actin.

The results in **Figure 11** showed that treatment with dynasore did not affect basal NHE3 activity in Caco-2 cells, suggesting that basal NHE3 endocytosis is a dynamin-independent phenomenon and that the observed effects of dynasore are specific to $Cl^-/OH^-(HCO_3^-)$ exchange.

Further, to exclude the possibility that regulation of apical $Cl^-/OH^-(HCO_3^-)$ exchange activity by CME inhibitor dynasore is cell line specific, we investigated the effect of 80 μM dynasore on $Cl^-/OH^-(HCO_3^-)$ exchange activity in a human colonic epithelial cell line, T-84. Serum starved T-84 cells were treated with 80 μM dynasore for 1 hour and $Cl^-/OH^-(HCO_3^-)$ exchange activity was measured as DIDS-sensitive $^{125}I^-$ uptake in base loaded cells. As shown in **Figure 12**, similar to the effects in Caco-2 cells, 80 μM dynasore treatment increased apical $Cl^-/OH^-(HCO_3^-)$ exchange activity (~ 2 fold) in T-84 cells. These results suggest that the effect of dynamin inhibition on $Cl^-/OH^-(HCO_3^-)$ exchange is not cell line specific and that basal $Cl^-/OH^-(HCO_3^-)$ exchange activity is sensitive to the inhibition of dynamin dependent endocytosis in intestinal epithelial cells.

C. Dynasore Increases Apical Surface Expression of DRA via Decreased Endocytosis in Caco-2 Cells

To examine if the increase in $Cl^-/OH^-(HCO_3^-)$ exchange activity in the presence of CME inhibitors correlates with an increase in surface levels of DRA, Caco-2 monoloayers were treated with dynasore (80 μM, 1 hr) and surface levels of DRA were assessed by a cell-surface biotinylation assay. Our results showed that treatment with dynasore significantly increased DRA surface levels as compared to control. As shown in **Figure 13A and 13B** densitometric analysis of protein bands showed a 40-50% increase in DRA surface levels in cells treated with dynasore as compared to untreated controls, whereas, no change was observed in the total cellular DRA levels. Immunoflourescent studies (**Figure 14B**) showed that DRA (shown in green) was increased on the apical membrane (shown in xz plane) in dynasore (80 μM, 1 hr) treated Caco-2 cells as compared to untreated controls **(Figure 14A)**. These data are consistent with an increase in apical $Cl^-/OH^-(HCO_3^-)$ exchange activity observed in the presence of endocytosis inhibitor dynasore and further establish the role of clathrin-mediated endocytosis in basal DRA trafficking in intestinal epithelial cells.

To establish, if the increase in apical $Cl^-/OH^-(HCO_3^-)$ exchange activity and surface expression of DRA in Caco-2 cells treated with dynasore was indeed a result of inhibition of DRA endocytosis, we performed a biotin-based endocytosis assay, termed reverse cell-surface biotinylation in treated and untreated Caco-2 cells. Caco-2 cells grown on plastic support were surface biotinylated at 4°C followed by incubation with dynasore (80 μM, 1 hr) or vehicle (DMSO) in culture medium at 37°C. Since the excess bitoin outside the cell is cleaved with glutathione the biotinylated fractions represent the internalized or endocytosed pool of DRA.

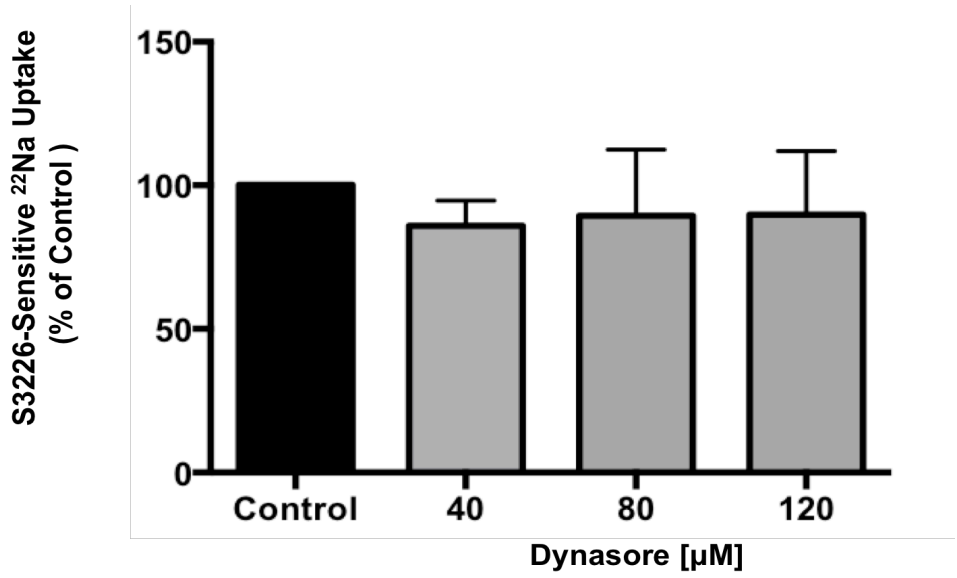


Figure 11. Effect of dynasore on NHE3 activity in Caco-2 cells. Serum starved Caco-2 monolayers were treated with different doses of dynasore (40-120 μM) in culture medium for 1hour. S3226-sensitive $^{22}Na^{+}$ uptake was measured in acid loaded cells. Results are expressed as % of control and represent the mean ± SEM of three experiments performed in triplicate.

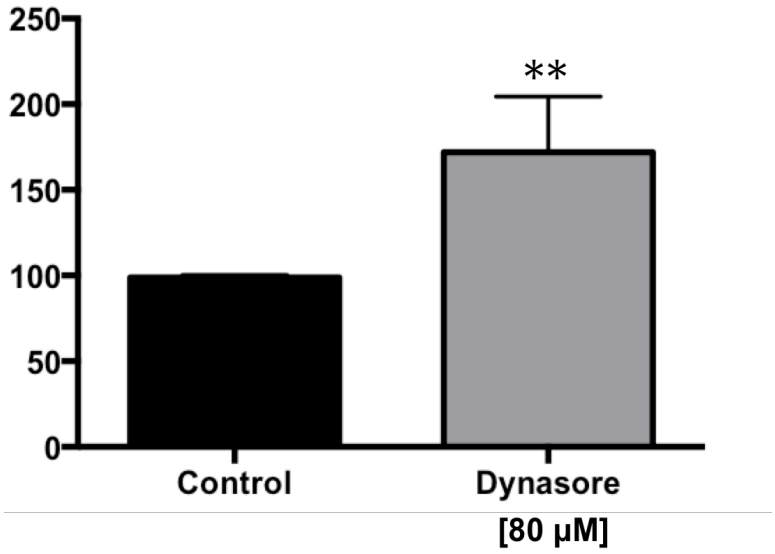


Figure 12. Stimulation of apical Cl^{-}/HCO_3^{-} exchange activity by endocytosis inhibitor dynasore is not cell line specific. T-84 cells were serum starved overnight and treated with dynasore (80 μM) for 1 hour. Cl^{-}/HCO_3^{-} exchange activity was measured as DIDS-sensitive (600 μM) $^{125}I^{-}$ uptake in alkali loaded cells. Results represent mean ± SEM of 3 experiments performed in triplicate.

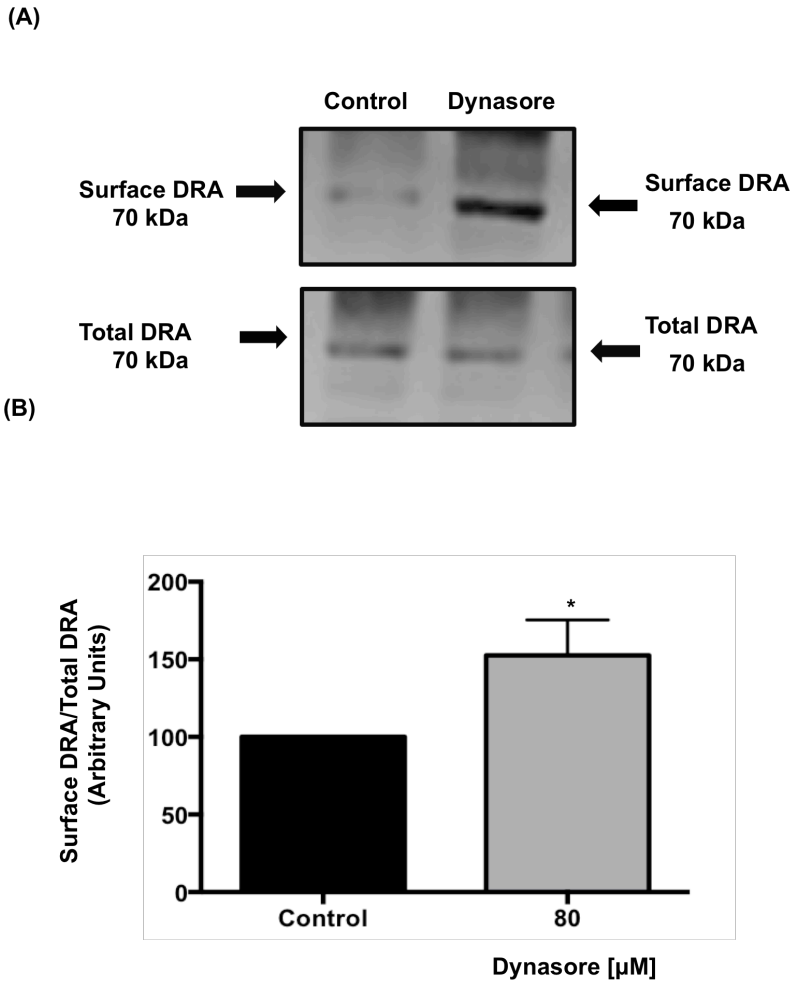
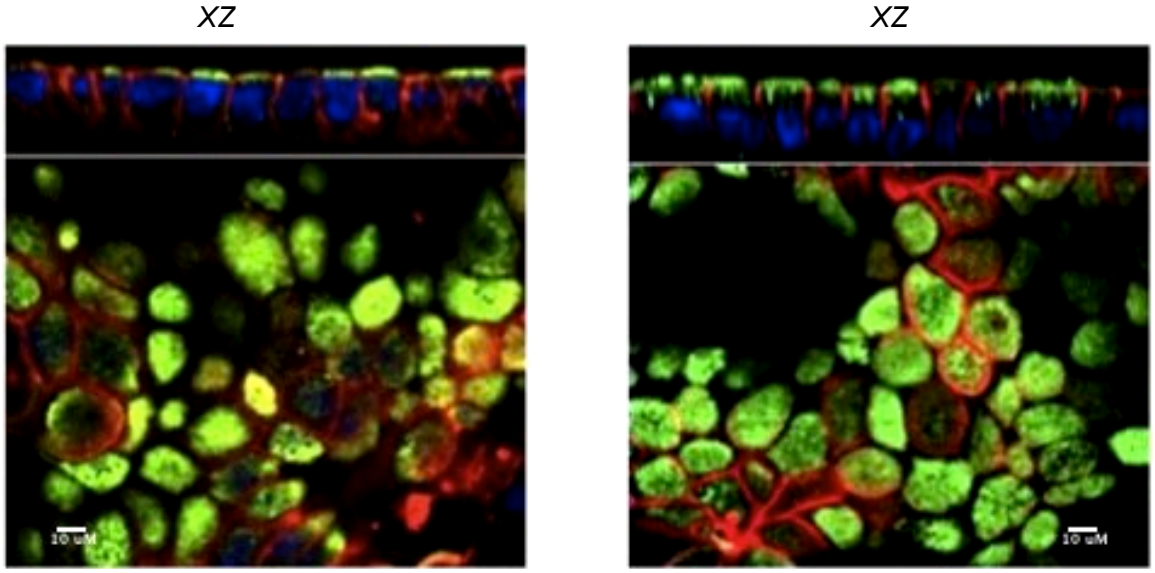


Figure 13. Dynasore increases the surface expression of DRA in Caco-2 cells. Cells treated with dynasore (80 μM) were subjected to cell-surface biotinylation. Biotinylated proteins were pulled down with neutravidin-agarose and both surface and total fractions were run on a 7.5% SDS-polyacrylamide gel. The representative blot shown was probed with rabbit anti-DRA antibody **(A)**. The biotinylated fraction represents surface DRA expression. Densitometric analysis shown below is representative of 3 separate experiments **(B)**. Results are expressed as a ratio of surface DRA/total DRA. Values represent mean ± SEM of 3 different experiments. *$p<0.05$ as compared to control.



Control (*XY*) Dynasore (*XY*)

Figure 14. Dynasore increases the apical surface expression of DRA in Caco-2 monolayers. Caco-2 cell monolayers grown on Transwell filter inserts were apically treated with dynasore 80 μM, for 1 h. Cells were then fixed with paraformaldehyde, followed by incubation with anti-DRA antibody (1:100) and secondary incubation with Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (1:100) and rhodamine-phalloidin (1:60) for 60 min. DRA is shown in green and actin in red. Orthognal *xz* images were obtained with a Zeiss LSM 510 confocal microscope.

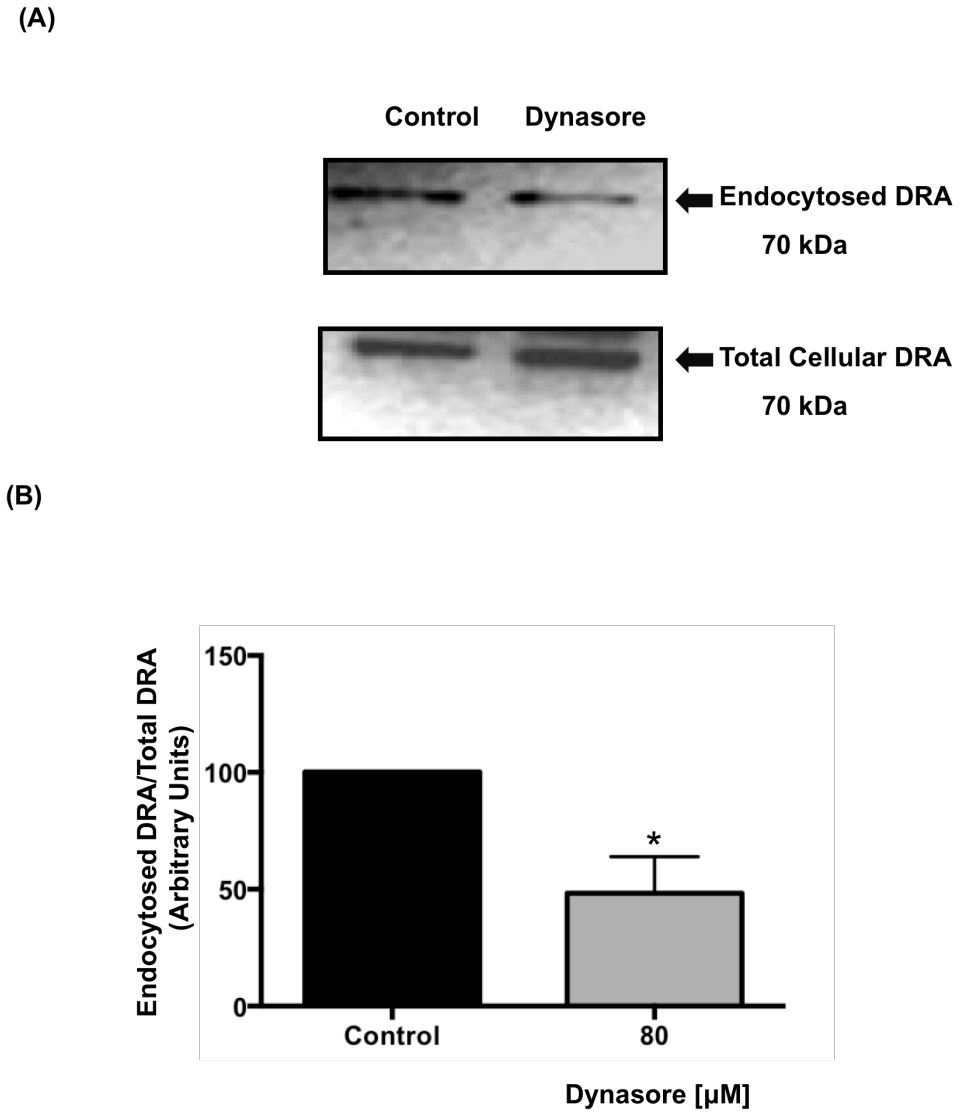


Figure 15. Dynasore increases surface expression of DRA via a decrease in DRA endocytosis in Caco-2 cells. Caco-2 monolayers were used for performing reverse cell-surface biotinylation at 4°C followed by incubation with dynasore (80 μM) or vehicle (DMSO) at 37°C. Excess biotin was cleaved by glutathione. Biotinylated proteins were pulled down with neutravidin-agarose and both biotinylated and total fractions were run on a 7.5% SDS-polyacrylamide gel. The western blot was probed with rabbit anti-DRA antibody and a representative blot is shown here **(A)**. The biotinylated fraction represents the endocytosed pool of DRA. Amount of endocytosed DRA was quantified by densitometric analysis **(B)**. Results are expressed as endocytosed DRA/total DRA. Values represent mean ± SEM of 3 different experiments. *$p<0.05$ as compared to control.

As shown in **Figure 15A and 15B** densitometric analysis of protein bands showed that dynasore significantly decreased basal DRA endocytosis by 50-60% as compared to control, thus confirming the role played by the clathrin-dependent pathway in basal DRA trafficking. Taken together, these data establish that the internalization of DRA from the surface under basal conditions occurs via clathrin-mediated endocytosis in intestinal epithelial cells.

D. DRA Association with Lipid-Rafts is Increased by Dynasore

Previous studies from our laboratory and others have shown that increase in DRA function is also associated with increased DRA in lipid raft or detergent resistant fractions with no change in total surface expression (30). We next examined if DRA is preferentially partitioned into lipid raft domains by treatment with dynasore. Caco-2 cells grown on plastic support were treated with dynasore or vehicle (DMSO) lysed, and ultracentrifuged, to obtain the total membrane pellet. Detergent-soluble (non-raft) and detergent-insoluble (raft) fractions were separated by solubilization in a TritonX-100 based buffer at 4°C. As shown in **Figure 16A and 16B** densitometric analysis of detergent insoluble (lipid-raft) fractions revealed that dynasore treatment increased DRA associated with lipid-rafts (1.5) fold as compared to control. Data for detergent soluble fractions in this and subsequent experiments are not shown, as these fractions are highly dilute making it challenging to detect and quantify the DRA protein bands. These data suggest that inhibiting DRA endocytosis increases its levels on plasma membrane and association with lipid raft domains enhancing its function.

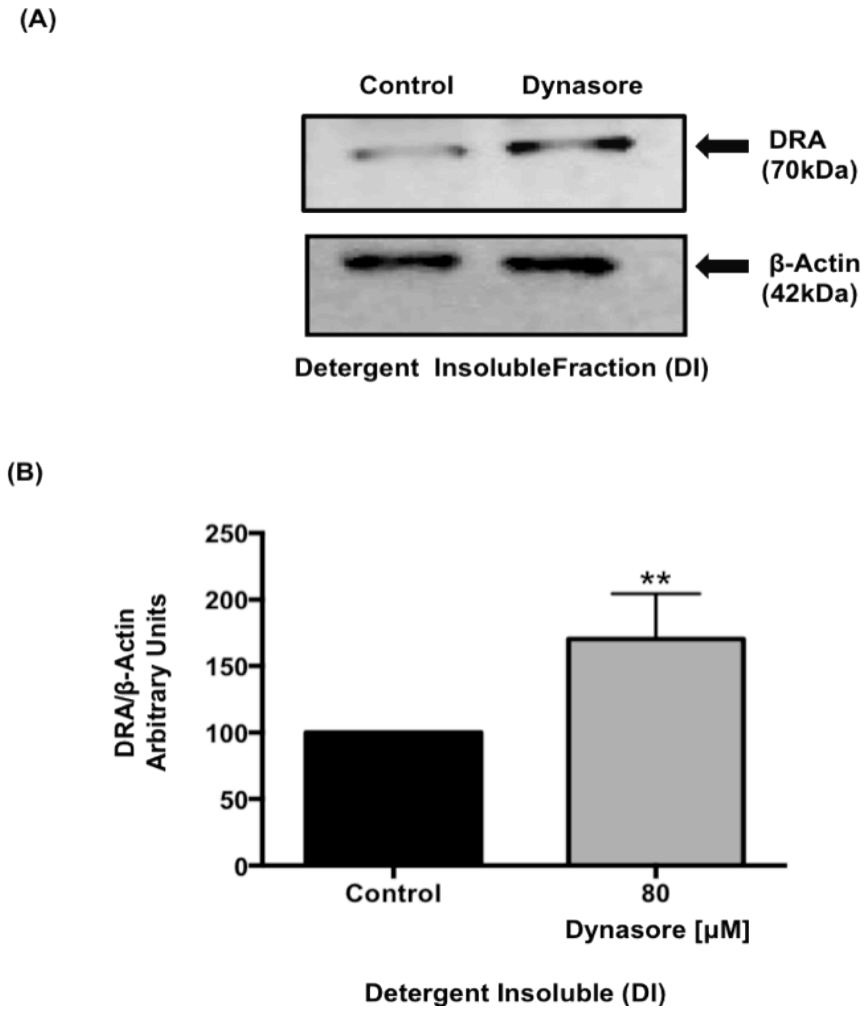


Figure 16. Dynasore increased DRA association with detergent insoluble (DI) fraction. Post-confluent Caco-2 monolayers were treated with dynasore (80 μM) for 1 h. Membrane pellets obtained from total cell lysate by ultracentrifugation were solubilized at 4°C in a Triton X-100 detergent buffer to separate lipid-raft (DI) and non-raft fractions (DS). DI fractions were run on a 7.5% SDS-PAGE gel and analyzed by western blotting for DRA and β-actin expression as shown in the representative blot **(A).** Data were quantified by densitometric analysis and are representative of 4 separate immunoblots **(B)**. Results are expressed as DRA/β-Actin and values represent mean ± SEM of 4 experiments. **p<0.006 as compared to control.

E. Role of Microtubules in DRA Endocytosis and Exocytosis

Cellular trafficking of membrane proteins involves movement of endocytic and exocytic vesicles along cytoskeletal components. Microtubules for example, have been shown to be involved in the trafficking of various membrane proteins. With regard to DRA, previous studies utilizing colchicine, a microtubule-disrupting drug, showed a decrease in DRA function in colchicine treated Caco-2 cells (2). It was thus, logical, to examine the role of microtubules in endocytic and exocytic routes of DRA trafficking.

To elucidate the importance of intact microtubules in DRA endocytosis, Caco-2 cells grown on plastic support were subjected to surface biotinylation followed by disruption of microtubules in the cold and recovery at 37°C in the presence or absence of microtubule disrupting agent nocodazole (66 μM). The biotinylated fractions pulled down with neutra-avidin beads represent the endocytosed proteins. As shown in **Figure 17A and 17B** there was no significant change in DRA endocytosis in Caco-2 cells with microtubule disruption as compared to the enodcytosed pool of DRA in cells with recovered (intact) microtubules. Thus, our studies suggest that microtubules are not involved in the endocytosis of DRA in Caco-2 cells.

Having established that intact microtubules are not essential for DRA endocytosis, we examined if the decrease in DRA surface expression by microtubule-disrupting agents such as colchicine resulted from decreased DRA exocytosis. A previously described biotinylation-based exocytosis assay was performed in post-confluent Caco-2 cells. Cells were incubated with sulfo NH-SS-acetate at 4°C to mask cell surface proteins. Microtubules were disrupted in the cold followed by recovery at 37°C in the presence or absence of nocodazole (66 μM). The newly exocytosed proteins were then assessed by cell-surface biotinylation. The results in **Figure 18A and 18B show** that disruption of microtubules in the presence of nocodazole reduced DRA exocytosis by ~ 4 fold as compared to cells where microtubules had been recovered. This suggests that microtubules play an important role in exocytic insertion of DRA. The role of microtubules in surface expression of DRA was further supported by our immunoflourescent

studies carried out in Caco-2 monolayers. Caco-2 cells were subjected to cold induced microtubule disruption followed by recovery at 37°C in the presence or absence of nocodazole for 3 hours (94). Images shown in **Figure 19A** clearly suggest that disruption of microtubules significantly decreases DRA expression on the apical membrane and that recovery of microtubules at 37°C **(Figure 19B)** restores surface DRA in Caco-2 cells. Hence, our results indicate that intact microtubules are important for basal exocytic trafficking and apical expression of DRA in Caco-2 cells.

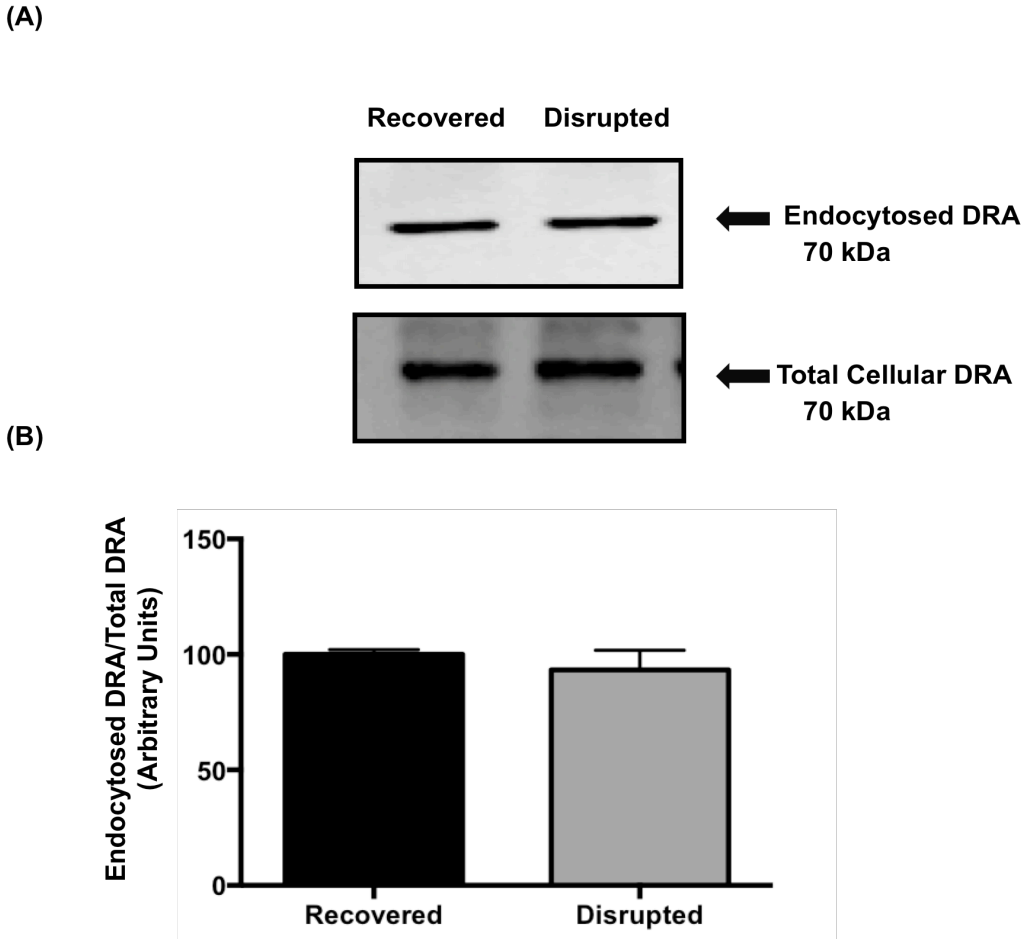


Figure 17. Disruption of microtubules does not affect DRA endocytosis in Caco-2 cells. Caco-2 monolayers were incubated with sulfo-NHS-SS-S biotin (1.5mg/ml) at 4°C for 1 hour followed by disruption of microtubules in the cold. The cells were then kept at 37°C to allow for recovery of microtubules in the presence or absence of nocodazole (66 μM) for 3 hours. Surface biotin was cleaved by glutathione. Total protein lysate was incubated at 4°C overnight with neutravidin beads to pull down the biotinylated proteins. Total and biotinylated fractions were run on a 7.5% SDS-PAGE gel followed by transfer to a nitrocellulose membrane for immunoblotting with anti-DRA antibody. The DRA protein bands shown in a representative blot in **(A)** were quantified by densitometry shown in **(B)**. Results are expressed as endocytosed DRA/total DRA and values are mean ± SEM of 3 different blots.

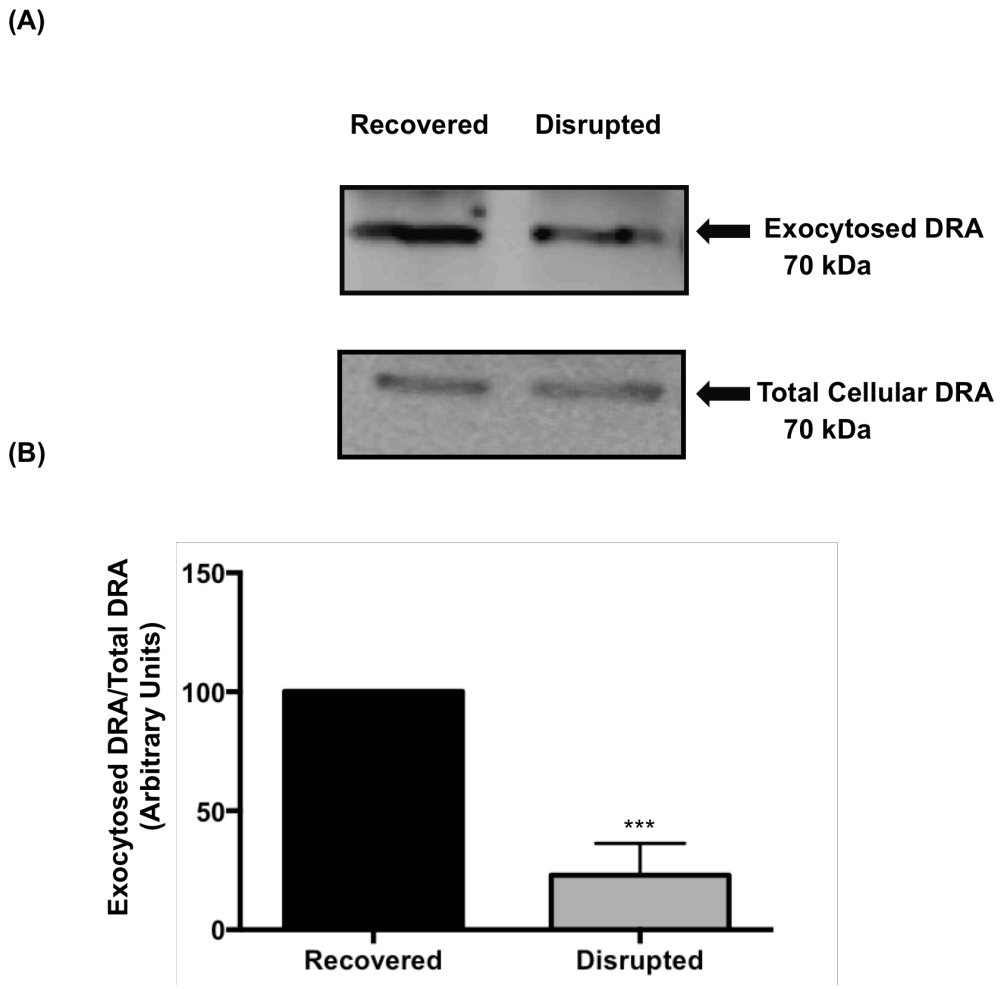
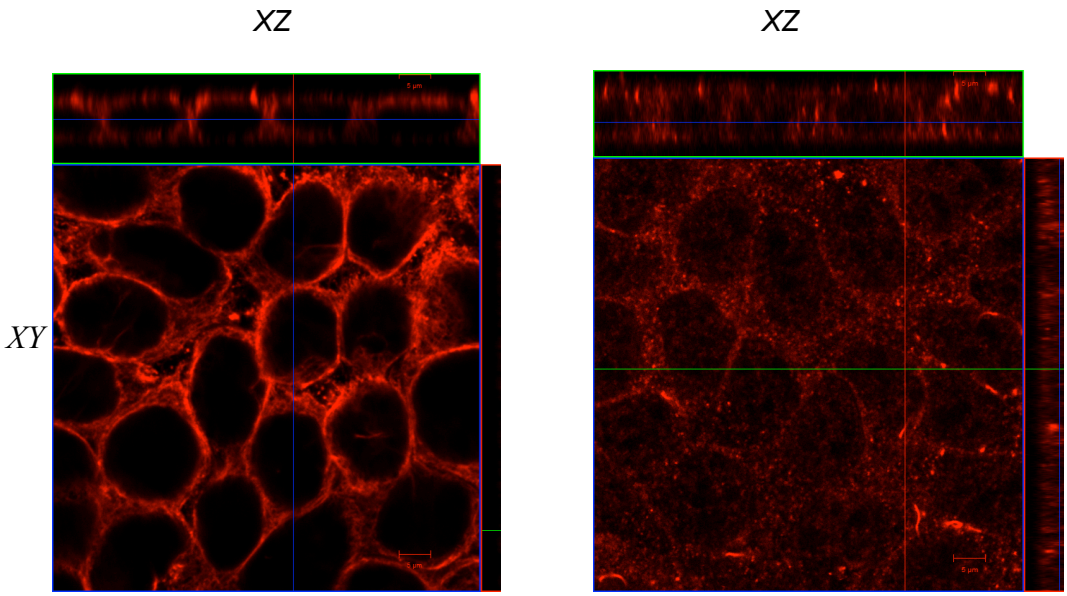


Figure 18. DRA exocytosis is dependent upon intact microtubules. Cell-surface of well-differentiated Caco-2 was masked with NH-SS-Acetate at 4°C for 1 hour. Cells were then kept in the cold for 1 hour to allow disruption of microtubules, followed by recovery in the presence or absence of nocodazole (66 μM) at 37°C for 3 hours. The amount of newly exocytosed protein was assessed by cell-surface biotinylation. Biotinylated proteins were pulled down with neutravidin-agarose beads and both biotinylated and total lysates were run on a 7.5% SDS-polyacrylamide gel. The blot was probed with anti-DRA antibody **(A)** and quantitated by densitometric analysis **(B).** Results are expressed as exocytosed DRA/total DRA and are mean ± SEM of 3 different blots. ***$p<0.001$ as compared to recovered cells.

(A)



(B)

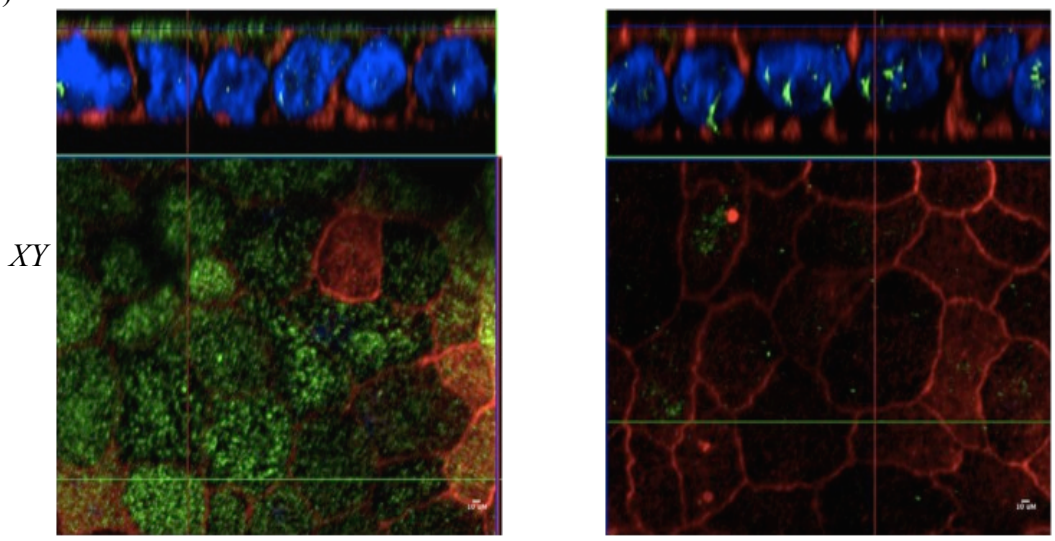


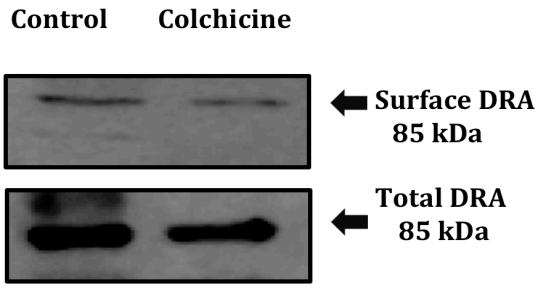
Figure 19. Disruption of microtubules in vitro decreases DRA surface membrane expression in Caco-2 cells. Caco-2 cells were subjected to cold + nocodazole treatment for microtubule disruption. Cells were stained for α-tubulin shown in red to confirm microtubule disruption and recovery **(A).** Caco-2 cells were immunostained for localization of DRA (green) and Alexa Fluor 568–conjugated phalloidin (actin; red) and DAPI was used to label the nuclei (blue) in cells with recovered and disrupted microtubules **(B).**

F. Disruption of Microtubules *In Vivo* Reduces DRA Surface Levels

To examine if disruption of microtubules *in vivo* resulted in a similar decrease in DRA surface levels, 8 weeks old C57BL/6 mice were treated with microtubule disrupting agent colchicine (3mg/kg b.wt. 24 h) administered intraperitoneally. *Ex vivo* surface biotinylation was performed in intact treated and un-treated mouse distal colon segments. As shown in **Figure 20,** disruption of microtubules with colchicine resulted in a significant decrease in apical surface levels of DRA (~40%) with no change in total level of DRA. This observation was further supported by immunoflourescent studies performed in tissue sections from colchicine treated and untreated mouse colon. As shown in **Figure 21,** colchicine treated mice showed increased DRA internalization from the apical membrane and accumulation in sub-apical pools as compared to control mice. Combined with our *in vitro* results, these results further support the hypothesis that disruption of microtubules decreases DRA surface expression perhaps via decreased exocytic insertion of DRA.

In summary, our results from Specific Aim 1 indicate that DRA undergoes clathrin-dependent endocytic internalization that is independent of microtubules. Further, microtubules play an essential role in the exocytic insertion and surface expression of DRA on the apical membrane in intestinal epithelial cells.

(A)



(B)

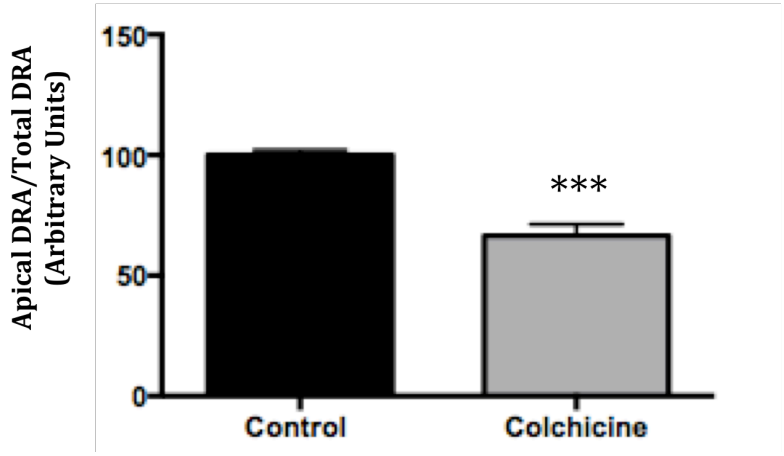
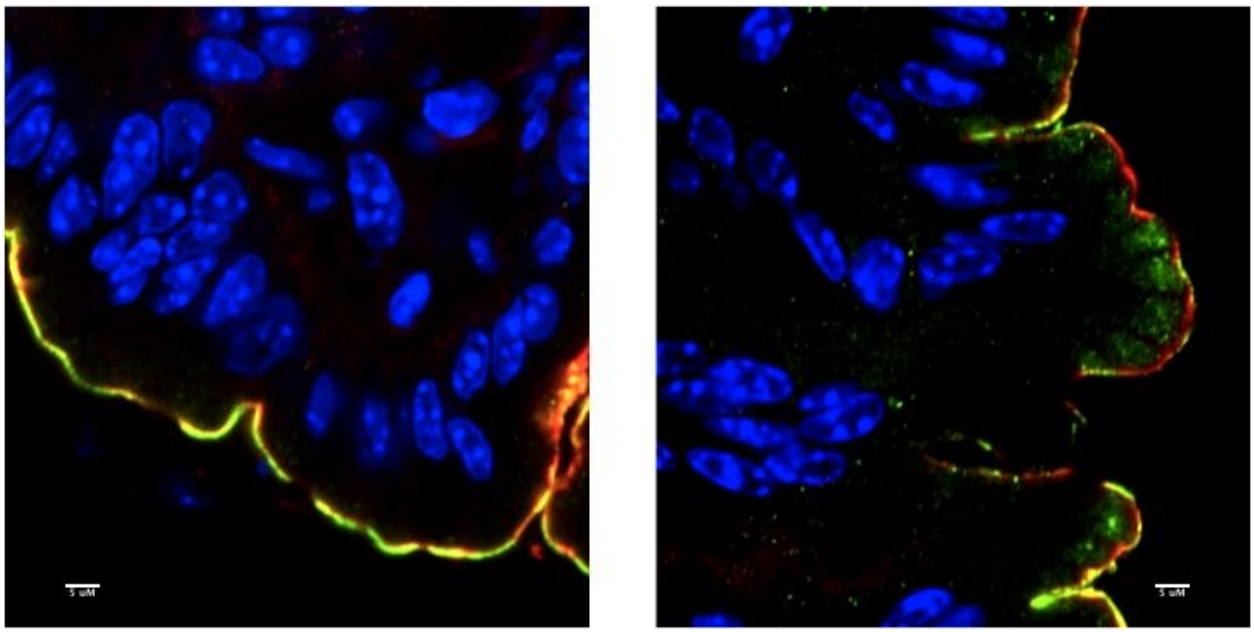


Figure 20. *In vivo* disruption of microtubules results in DRA internalization from the apical membrane of mouse colon. *Ex vivo* biotinylation was performed in colon from control and colchicine treated (3mg/kg b.wt. 24 h) mice. Total lysates from control and treated mice were incubated with agarose beads overnight at 4°C to pull down the biotinylated proteins. Biotinylated and total fractions were run on a 7.5% gel followed by transfer to nitrocellulose membrane. Membrane was probed with anti-DRA antibodies and protein bands **(A)** were quantified by densitometric analysis **(B)**. Results are expressed as a ratio of surface DRA/total DRA. Values represent mean ± SEM of 3 different experiments. ***p< 0.006 and **(A)** is representative blot of 3 experiments.



Control Colchicine

Figure 21. Colchicine internalizes DRA from the apical surface of mouse distal colon. Immunofluorescent staining was performed in colonic tissue sections from control and colchicine (3 mg/kg b.wt.) treated mice. DRA staining is shown in green and staining for villin is shown in red and nuclei are stained blue with slowfade DAPI. A representative image from 3 different animals for each treatment is shown. Images are taken in a Zeiss LSM 710 confocal microscope with a 63x objective. Control and colchicine images shown here are taken at same plane.

IV. RESULTS (AIM 2)

Results from Specific Aim 2 detailed below indicate increased endocytosis and a microtubule dependent decrease in exocytosis to be involved in EPEC mediated decrease in DRA surface levels.

A. EPEC Infection Decreases DRA Surface Levels via Increased Endocytosis

We utilized cell surface and reverse biotinylation **(Figure 22A and 22B**) studies to investigate if EPEC induced reduction of DRA surface levels is a result of increased DRA endocytosis in Caco-2 cells infected with EPEC. Consistent with previous studies, 60 min EPEC infection of Caco-2 cells decreased surface expression of DRA in Caco-2 cells (~ 50%) as compared to control. This decrease in surface expression of DRA was also evident by confocal microscopy studies performed in EPEC infected Caco-2 cells grown on filter support **(Figure 23).** This decrease in DRA surface levels was found to be partly associated with increased endocytosis, as reverse biotinylation studies showed that infection with EPEC significantly increased DRA endocytosis (35-45%) as compared to uninfected control cells. Also, increase in endocytosis upon EPEC infection was not observed **(Figure 22C)** when the experiment was performed on ice to block cellular trafficking, providing further proof that EPEC mediated decrease in cell surface level of DRA is a result of increased endocytic trafficking.

B. Dynasore did not Alter EPEC Induced Decrease in Apical $Cl^-/OH^-(HCO_3^-)$ Exchange Activity or NHE3 Activity

Since our previous results in this study suggested that basal DRA endocytosis is clathrin-dependent in Caco-2 cells and that EPEC modulates DRA surface levels via increased endocytosis, we next examined the role played by clathrin-mediated endocytosis in EPEC induced inhibition of $Cl^-/OH^-(HCO_3^-)$ exchange activity in Caco-2 cells. Serum starved Caco-2 monolayers were pretreated for 30 minutes with dynasore (80 μM) followed by a 30 minunte

EPEC infection in the presence or absence of dynasore (80 μM). DIDS sensitive $^{125}I^-$ uptake to measure $Cl^-/OH^-(HCO_3^-)$ exchange activity was performed in base loaded Caco-2 cells. An inhibition of apical $Cl^-/OH^-(HCO_3^-)$ exchange activity on infection with EPEC was observed as shown in previous studies. However, dynasore (80 μM) failed to block EPEC mediated inhibition of $Cl^-/OH^-(HCO_3^-)$ exchange activity (**Figure 24)**. Thus, our results suggest that inhibition of apical $Cl^-/OH^-(HCO_3^-)$ exchange in Caco-2 cells by EPEC infection remains unaltered by blocking dynamin activity.

We further examined if EPEC induced inhibition of NHE3 activity occurs as a result of increased enodytosis. Serum-starved Caco-2 cells were preincubated with 80 μM dynasore for 30 minutes followed by a 30 minute EPEC infection in the presence or absence of dynasore (80 μM). NHE3 activity was measured as S3226-sensitive $^{22}Na^+$ uptake in acid loaded cells. The results in **Figure 25** indicate that dynasore did not alter EPEC mediated inhibition of NHE3 activity, as there was no significant difference between NHE3 activity in cells infected with EPEC alone and those infected with EPEC in the presence of 80 μM dynasore.

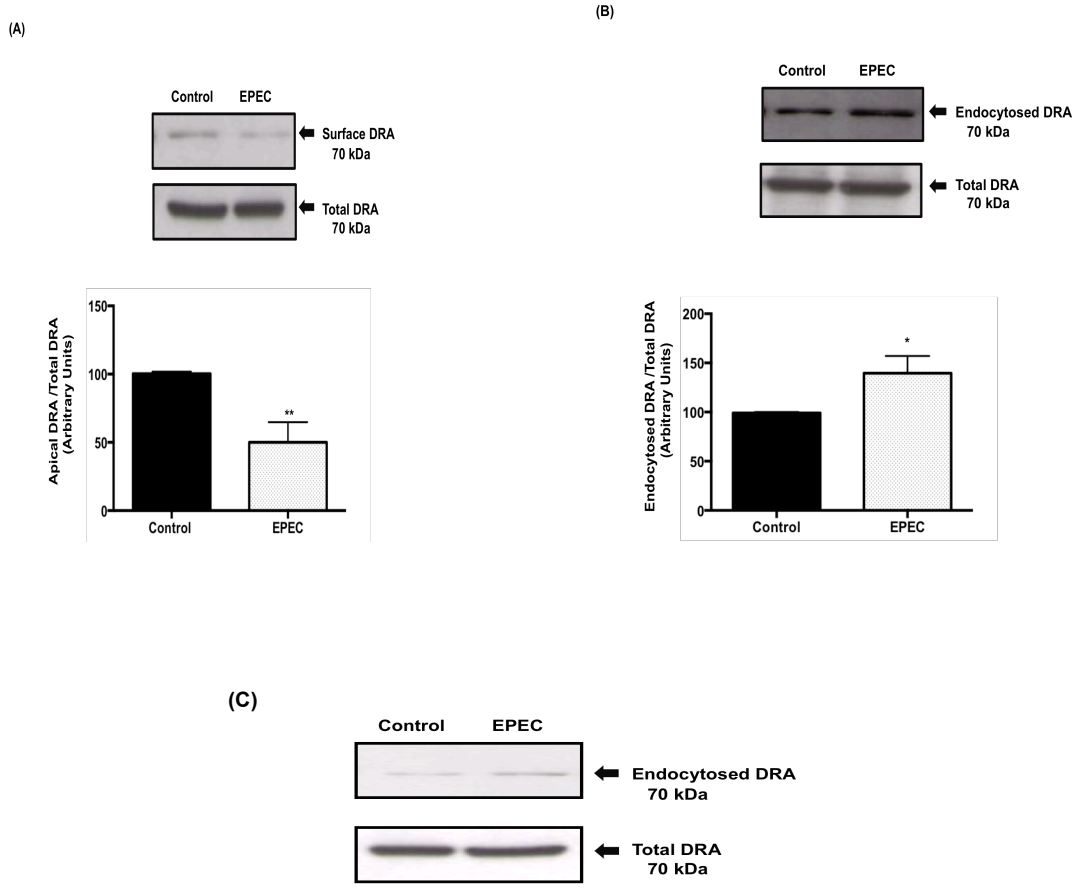


Figure 22. EPEC decreases DRA surface expression partly via increased DRA endocytosis in Caco-2 cells. Cells infected with EPEC for 60 minute were subjected to **(A)** cell-surface biotinylation or **(B)** reverse cell-surface biotinylation and **(C)** reverse-biotinylation carried out on ice (negative control, n=1). Neutravidin-agarose beads were used to pull down the biotinylated proteins. Biotinylated and total protein fractions were run on a 7.5% SDS-polyacrylamide gel. The blot was immunostained with rabbit anti-DRA antibody. The biotinylated fractions in **(A)** and **(B)** represent the apical and endocytosed pool of DRA respectively. Densitometric analysis presented below each blot is representative of three separate experiments. Results are expressed as apical DRA/total DRA for **(A)** and endocytosed DRA/total DRA for **(B**). Values represent mean ± SEM of 3 different experiments. *p<0.05 and **p<0.004ascomparedto control.

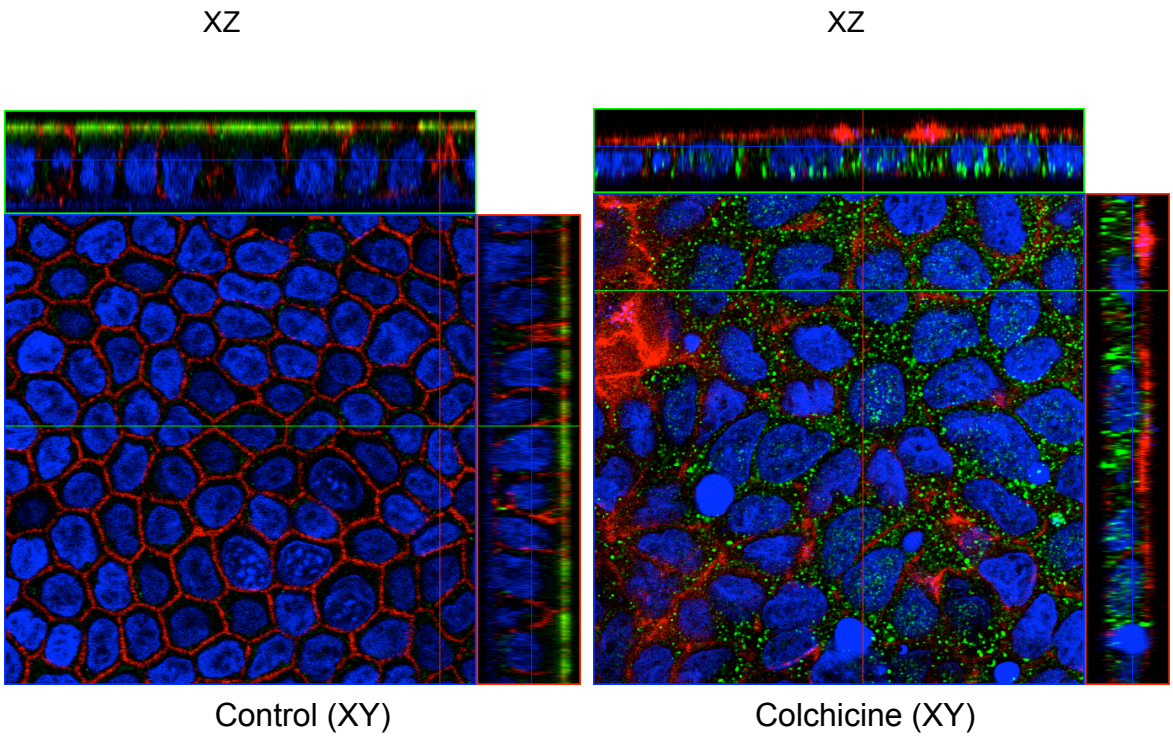


Figure 23. EPEC decreases DRA surface expression in Caco-2 cells. Caco-2 monolayers grown on transwell inserts were infected with EPEC for 60 min. Cells were then fixed with paraformaldehyde, followed by incubation with anti-DRA antibody (1:100) and secondary incubation with Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (1:100) and rhodamine-phalloidin (1:60) for 60 min. DRA is shown in green and actin in red. Orthognal *xz* images were obtained with a Zeiss LSM 510 META confocal microscope. Similar planes (XY) for control and EPEC are shown here.

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(A)

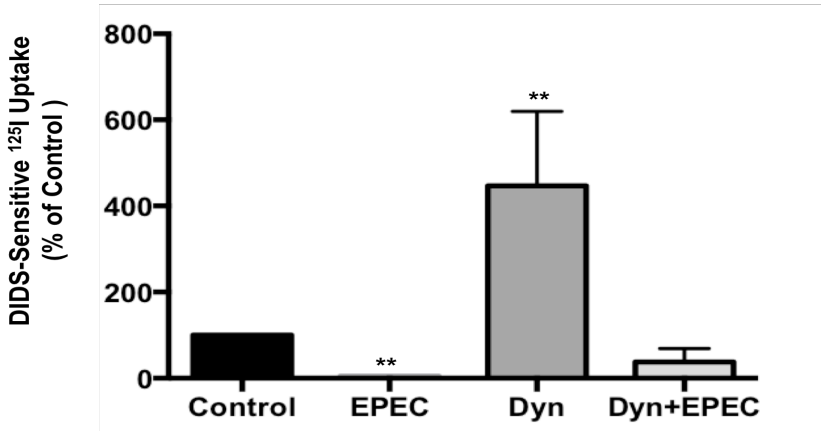

Figure 24. Dynasore does not alter EPEC induced reduction in apical Cl^-/HCO_3^- exchange in Caco-2 cells. Overnight serum starved Caco-2 cells were preincubated with 80 μM dynasore for 30 minutes followed by a 30 minutes EPEC infection in the presence or absence of the inhibitor. Apical Cl^-/HCO_3^- exchange activity was measured as DIDS-sensitive (600 μM) $^{125}I^-$ uptake in base loaded cells. Results represent mean ± SEM of 4 experiments performed in triplicate. **p<0.001 as compared to control.

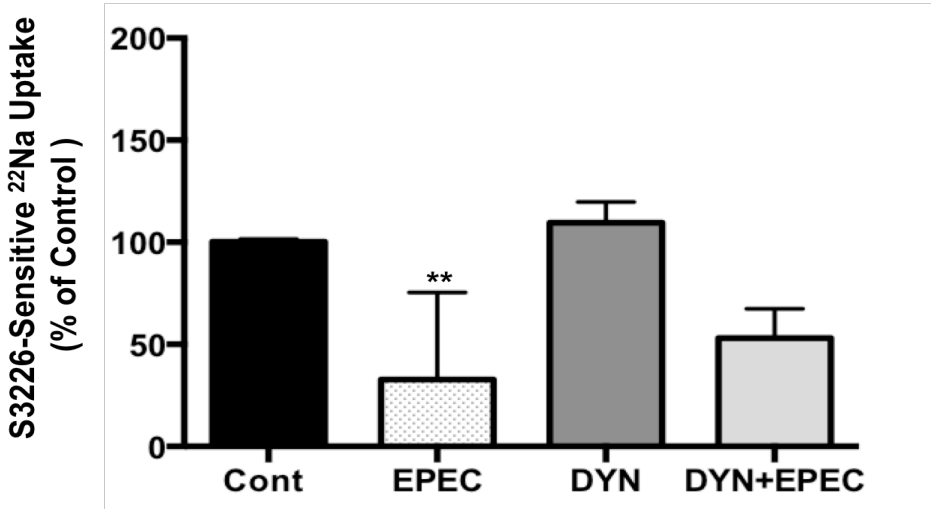

Figure 25. Dynasore does not alter EPEC induced reduction in NHE3 activity. Serum-starved Caco-2 cells were pre-incubated with dynasore (80 μM) for 30 minutes followed by infection with EPEC in culture medium for 30 minutes. Cells were infected with EPEC both in the presence and absence of dynasore. S3226-sensitive $^{22}Na^+$ uptake was measured in acid loaded cells. Results are representative of mean ± SEM of 4 separate experiments performed in triplicate. **p<0.001compared to control.

C. EPEC Infection Reduces DRA Association with Lipid-Rafts

Further, to investigate the effect of EPEC infection on the association of DRA with membrane raft domains, Caco-2 cells were infected with EPEC for 60 minutes and the total cell lysate from infected and uninfected cells was ultra-centrifuged to prepare membrane pellets. Lipid-raft and non-raft fractions were separated from the total membrane by a detergent based solubilization at 4°C. **Figure 26A and 26B** clearly show that infection with EPEC results in ~40% decrease in DRA association with lipid-rafts as compared to control. Our results, thus, indicate that EPEC infection induced decrease in DRA function and increased endocytosis is parallel to decreased presence of DRA in lipid-rafts. Further, when cells were infected with EPEC in the presence or absence of dynasore **(Figure 27A and 27B)**, changes in DRA association with lipid-rafts paralleled those observed in apical $Cl^-/OH^-(HCO_3^-)$ exchange activity as mentioned above **(Figure 24)**.

D. EPEC infection decreased DRA exocytosis via microtubule disruption by EspG1 and EspG2.

Recent studies from our group and others have shown that EPEC induced decrease in DRA function and surface level was dependent on virulence proteins EspG1 and EspG2. EspG1 and EspG2 are known to disrupt the host microtubule network (2). Since our studies on control Caco-2 cells indicated that microtubules are involved in DRA exocytosis, it was logical, to examine their role in EPEC induced decrease in DRA surface expression. To elucidate if EPEC mediated disruption of microtubules is implicated in the decreased surface levels of DRA in Caco-2 cells, we performed a biotin-based exocytosis assay in cells infected with wild-type EPEC and *ΔespG1/G2* mutants for 60 minutes. Our results indicated that compared to mutant EPEC strains, infection with wild-type EPEC, resulted in ~70% lesser DRA in the exocytosed pool **(Figure 28A and 28B).** We conclude that EPEC induced decrease in DRA surface levels in

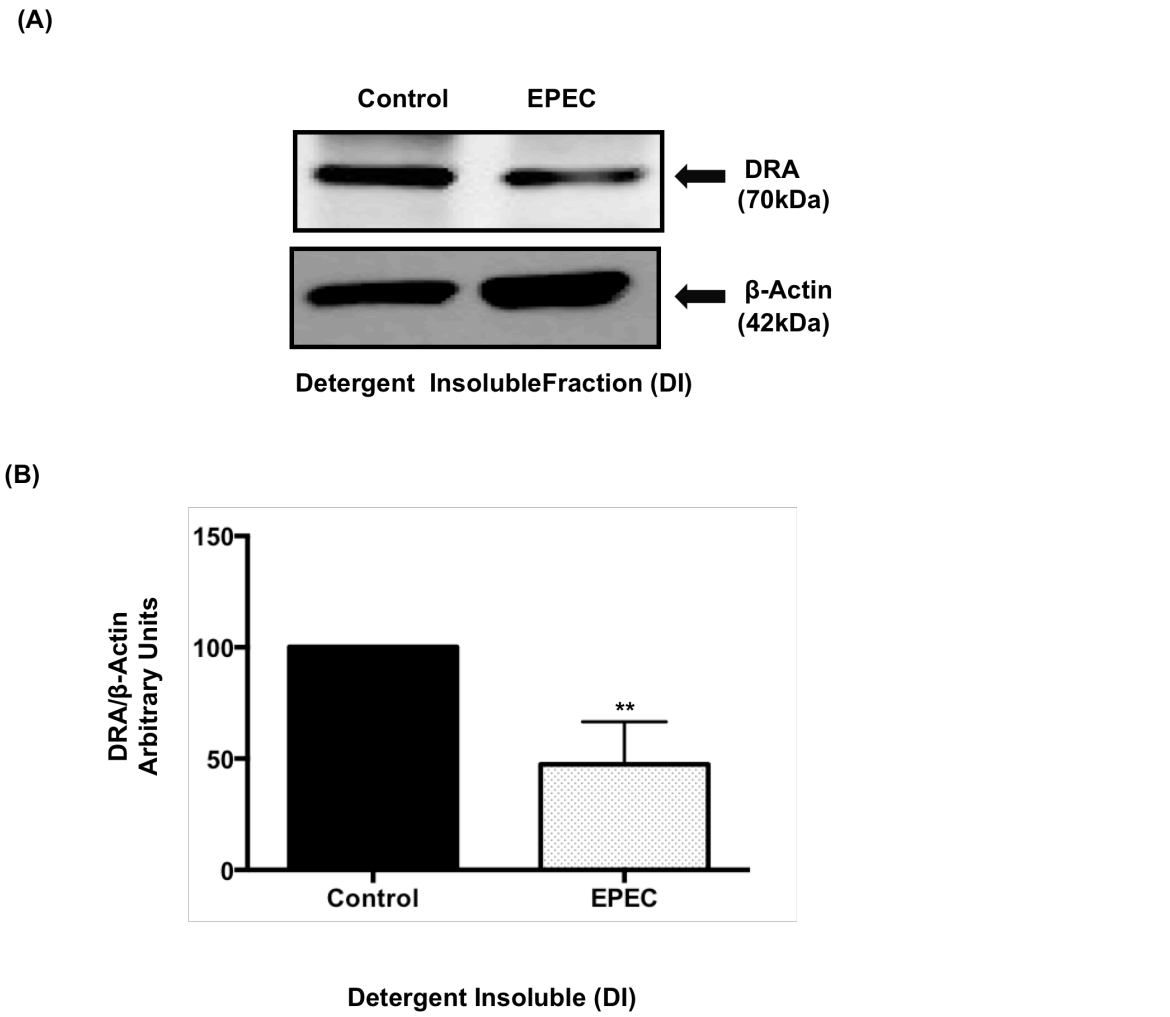

Figure 26. EPEC infection decreases DRA association with the detergent insoluble or lipid-raft membrane fraction in Caco-2 cells. Caco-2 monolayers were infected with EPEC for 60 minutes. Total membrane pellet was obtained from control and EPEC infected cell lysates by ultracentrifugation. Lipd-rafts were separated by solubilization in a Triton X-100 detergent buffer at 4°C. Lipid-raft (DI) fractions were run on a 7.5% SDS-PAGE gel and immunoblotted with anti-DRA and anti-actin antibodies **(A).** The blot shown above is representative of four separate experiments/blots that were quantitated by densitometric analysis **(B).** Results are expressed as DRA/β-Actin and values represent mean ± SEM of 4 different experiments. **p<0.001 as compared to control.

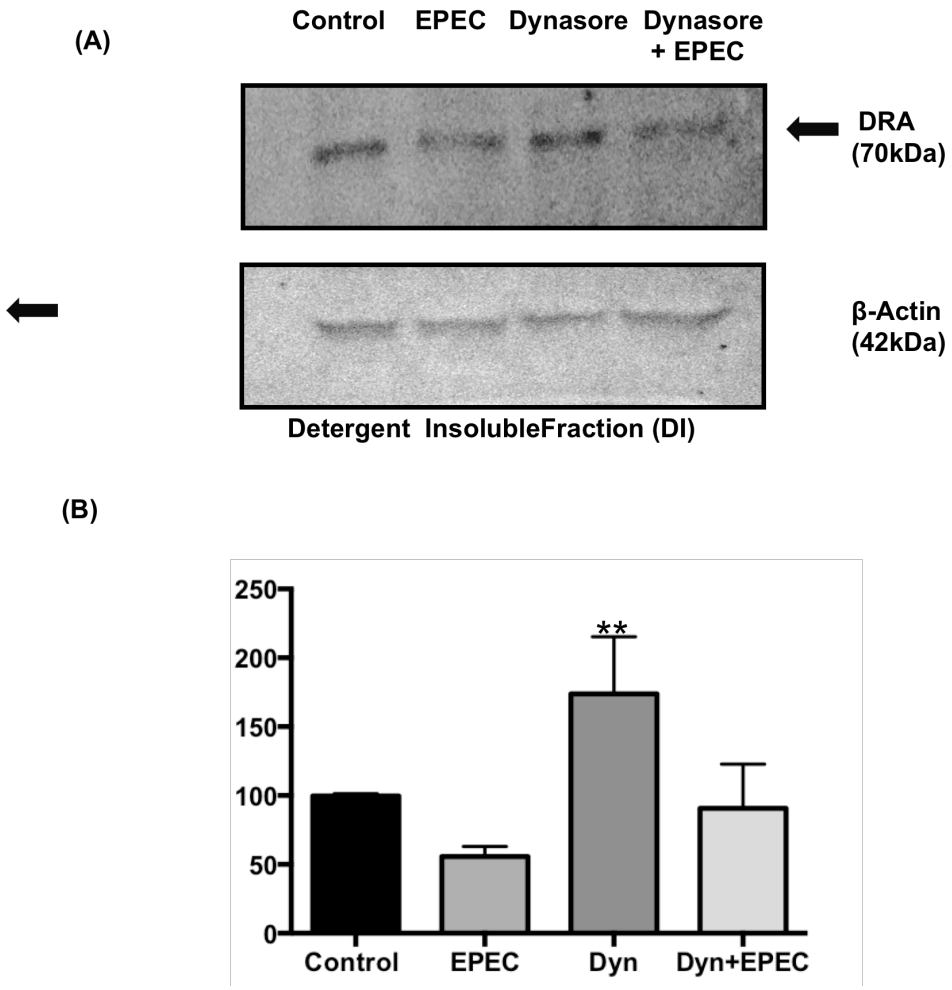

Figure 27. Dynasore does not alter EPEC induced decrease in DRA association with lipid-raft fraction in Caco-2 cells. Caco-2 monolayers were infected with EPEC in the presence or absence of dynasore for 60 minutes. Total membrane pellet was obtained from control and EPEC infected cell lysates by ultracentrifugation. Lipd-rafts were separated by solubilization in a Triton X-100 detergent buffer at 4°C. Lipid-raft (DI) fractions were run on a 7.5% SDS-PAGE gel and immunoblotted with anti-DRA and anti-actin antibodies **(A).** The blot shown above is representative of four separate experiments/blots that were quantitated by densitometric analysis **(B).** Results are expressed as DRA/β-Actin and values represent mean ± SEM of 4 different experiments. **p<0.001 as compared to control

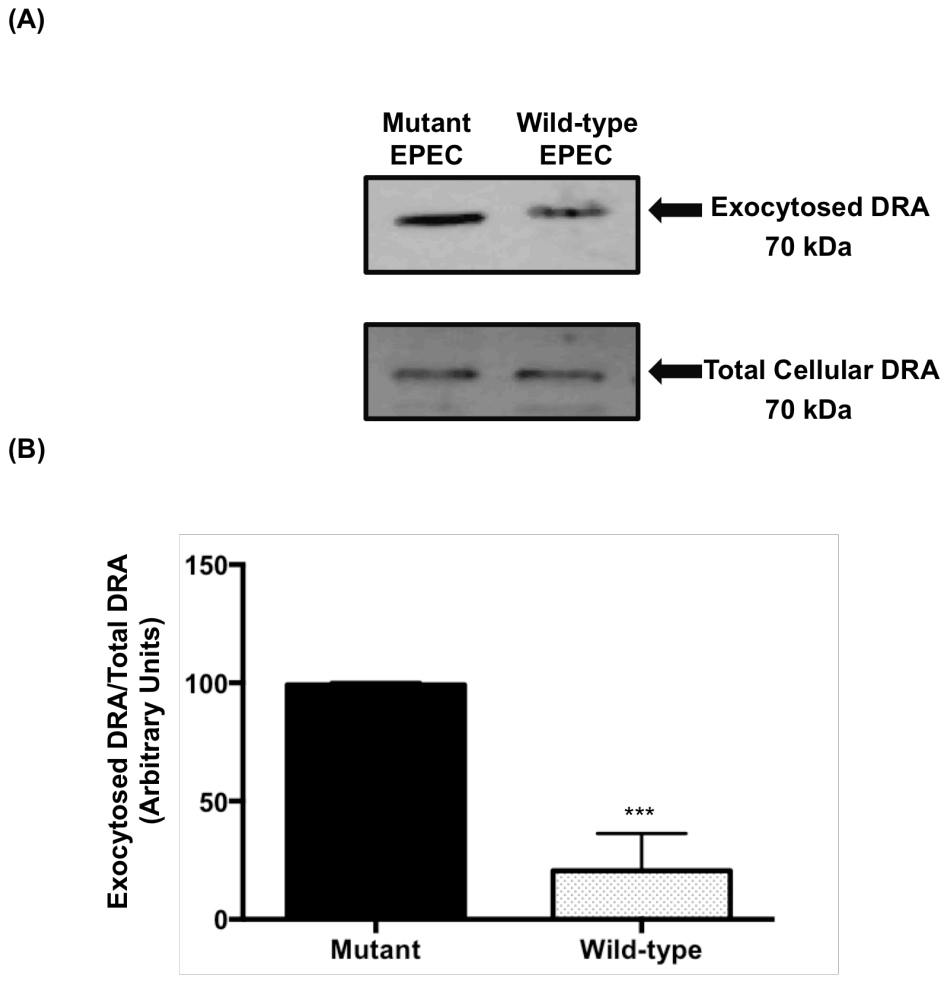

Figure 28. EPEC induced decrease in DRA exocytosis is dependent on EspG1 and EspG2. Surface NHS reactive sites on Caco-2 cells grown on plastic support were masked with NH-SS-Acetate at 4°C for 1 hour. Cells were then infected with either wild-type EPEC or Δ*espG1/G2* mutant for 60 minutes. The amount of protein exocytosed to the surface was measured by cell-surface biotinylation using sulfo-NH-SS-S-biotin at 4°C for 1 hour. The biotinylated proteins were pulled down with neutra-avidin agarose from equal amounts of total cellular lysates. Biotinylated and total fractions were run on a 7.5% SDS-PAGE gel and transferred to nitrocellulose membrane. The blot was probed with anti-DRA antibody and analyzed by densitometry. Results are expressed as exocytosed DRA/total DRA and values are mean ± SEM of 3 different blots. ***$p<0.005$ as compared to mutant EPEC.

Caco-2 cells results partly from disruption of microtubules, which are otherwise essential for DRA exocytosis and surface expression.

E. Culture Supernatant from *Lactobacillus acidophilus* Attenuates EPEC Induced Decrease in Apical $Cl^-/OH^-(HCO_3^-)$ Exchange Activity.

Probiotics are emerging as novel anti-diarrheal therapeutics. Previous studies from our laboratory have shown the stimulatory effect of probiotic *Lactobacillus acidophilus* (LA) on DRA via both transcriptional and post-translational mechanisms (34). As a therapeutic approach we examined if EPEC infection mediated decrease in DRA function was reversed by treatment with culture supernatant from *L. acidophilus.* Post confluent Caco-2 cells were preincubated for 3 hours with culture supernatant from *L. acidophilus* diluted in a 1:10 ratio in cell culture medium based on a previous study from our laboratory (34). This was followed by a 30 min EPEC infection in the presence or absence of LA culture supernatant. DIDS sensitive $^{125}I^-$ uptake to measure apical $Cl^-/OH^-(HCO_3^-)$ exchange activity was performed in base loaded Caco-2 cells. As shown in previous studies, EPEC alone decreased, while LA supernatant alone increased the apical $Cl^-/OH^-(HCO_3^-)$ exchange activity. Interestingly, our results show that pre-incubation with LA supernatant was almost entirely able to prevent EPEC mediated decrease in $Cl^-/OH^-(HCO_3^-)$ exchange activity **(Figure 29)** (LA-CM+EPEC is statistically different from EPEC but not from control). However the mechanistic details underlying this reversal of EPEC mediated decrease in apical $Cl^-/OH^-(HCO_3^-)$ exchange activity and DRA surface expression by LA supernatant remain to be determined.

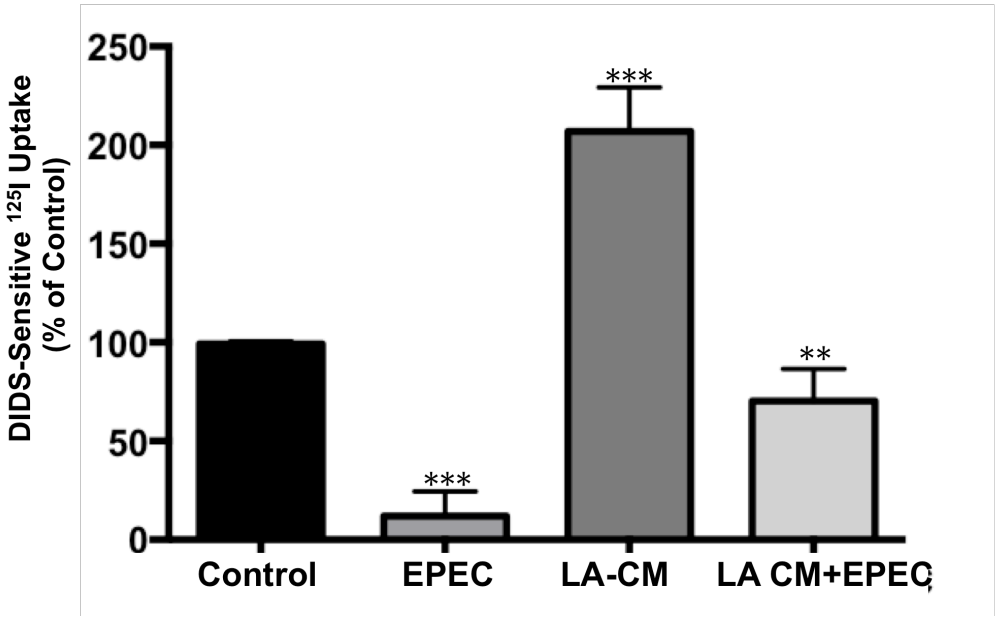

Figure 29. EPEC induced decrease in apical Cl^-/HCO_3^- exchange activity is partially attenuated by *L. acidophilus*. Serum starved Caco-2 cells were preincubated with culture supernatant from L acidophilus (LA-CM) followed by EPEC infection in presence or absence of LA-CM. Apical Cl^-/HCO_3^- exchange activity was measured as DIDS-sensitive (600 μM) $^{125}I^-$ uptake in base loaded cells. Results represent mean ± SEM of 3 experiments performed in triplicate. **p<0.05 as compared to EPEC and ***p<0.001 as compared to control. (LA-CM+EPEC vs Control is not significant.)

Overall, our results showed a clathrin-dependent basal endocytosis of DRA in Caco-2 cells. The endocytosis inhibitor dynasore was however, unable to prevent EPEC mediated decrease in apical $Cl^-/OH^-(HCO_3^-)$ exchange activity or NHE3 activity in Caco-2 cells. While retention of DRA in the detergent resistant fractions was increased in presence of dynasore, EPEC significantly decreased DRA association with membrane lipid-raft domains. Further, our results showed a microtubule dependent exocytic reinsertion of DRA at the plasma membrane. Microtubule disruption was also implicated in EPEC mediated decrease in DRA exocytosis.

V. DISCUSSION

SLC26A3 or DRA (Down Regulated in Adenoma) is an integral membrane transporter expressed on the apical membrane of epithelial cells of a variety of exocrine tissues including the small intestine and colon. Mutations in the SLC26A3 gene are associated with congenital chloride diarrhea (CLD), characterized by voluminous diarrhea with high fecal chloride content (95). DRA knockout mice exhibit severe diarrhea and a phenotype similar to that of congenital chloride diarrhea (CLD) patients (21). Owing to its important role in Cl^- uptake, DRA is subject to extensive regulation both at the cellular and molecular level (5). Emerging studies from our laboratory and others indicate that post-translational modifications of DRA protein may underlie the pathophysiology of diarrheal diseases. For example, CLD-causing mutations induce diarrhea by causing loss of functional DRA protein at the plasma membrane, possibly via its misfolding and mistrafficking. Studies from our laboratory have shown that *Enteropathogenic E coli* (EPEC) infection decreases DRA function and surface levels to induce early diarrhea, however, the underlying membrane trafficking mechanisms are not fully understood. Thus, investigation of the cellular pathways responsible for maintaining appropriate DRA levels on the apical membrane under physiological and pathophysiological conditions is significant for understanding disease pathogenesis and development of novel anti-diarrheal therapies. Results presented in this study, for the first time, demonstrate the involvement clathrin dependent pathway in basal DRA endocytosis in intestinal epithelial cells and further implicate EPEC induced host microtubule disruption in altering exocytosis of DRA as discussed in the following sections:

A. Mechanisms of basal DRA recycling:

Membrane proteins, channels and ion transporters undergo both constitutive and regulated endocytic retrieval followed by delivery to the plasma membrane to maintain steady surface levels. This mode of rapid regulation of membrane proteins is of critical importance

considering the rapidly changing milieu of the luminal environment in the gut. Rapid regulation of membrane proteins involves post-translational modifications such as alterations in i) phosphorylation levels of the protein; ii) membrane trafficking events; iii) protein turnover (activity) or iv) association with specialized membrane domains such as lipid-rafts. Previous studies in HEK293 cells stably transfected with DRA have shown that DRA undergoes basal e ndocytic recycling. This study and others show that once internalized, DRA enters the sub-apical pool of early endosomes from where it is sorted to recycling endosomes to be delivered back to the apical membrane (3, 96, 97). However, the role of clathrin and caveolin mediated pathways in basal DRA recycling in IECs has not been fully investigated.

A.1. Role of clathrin and caveolin mediated endocytosis in regulating apical Cl^-/HCO_3^- exchange activity

Dynamin, a 100kDa protein, is a GTPase required to pinch off coated vesicles involved in endocytosis. Dynamin binding proteins such as adaptor proteins, bind to its C-terminal proline rich domain and either stimulate its GTPase activity or direct it towards the plasma membrane where it participates in the endocytic cleavage process. There are three known isoforms of dynamin – dynamin 1, 2 and 3 and smaller mitochondrial dynamin GTPases. Dynamin 2 is the main isoform present in intestinal tissue. Dynamin molecules readily assemble into a ring like structure at the neck of coated endocytic vesicles and intrinsic GTPase activity allows for vesicle scission (98). The role of dynamin in clathrin and caveloin- mediated endoctyosis is well established and inhibition of dynamin is believed to affect both endocytic pathways. Further, some studies also show a RhoGTPase and actin dependent endocytic pathway to be dependent on dynamin activity, however this function of dynamin is relatively less well characterized and appears to specific to cell type and cargo. Dynasore is a pharmacological/chemical inhibitor known to specifically inhibit dynamin-1, dynamin-2 and mitochondrial dynamins, and prevent both clathrin and caveolin-mediated endocytosis (90).

Previously, studies have shown that a 30-minute treatment with an 80 μM dose of dynasore efficiently blocks clathrin dependent transferrin receptor uptake, activity and intracellular accumulation in HeLa cells and caveolin dependent uptake of cholera-toxin (CTx) with no observed cell toxicity. The extent of inhibition of transferrin and CTx uptake by dynasore was found to be similar to that observed upon overexpression of a dominant negative dynamin mutant (90). Similar to the above studies our functional studies in Caco-2 cells utilizing dynasore (80 μM, 60 minutes) showed an increase in apical Cl^-/HCO_3^- exchange activity indicating that maintenance of basal apical Cl^-/HCO_3^- exchange is dynamin dependent. Further, as dynasore treatment showed a similar increase in Cl^-/HCO_3^- exchange activity in another crypt like cell line T-84, our results suggest that the effects of dynasore on chloride uptake are not cell line specific. Our results are consistent with previous studies investigating the effect of the stress response element SERP1 on the ENaC channel activity showing a similar stimulation of amiloride sensitive transport in the presence of dynasore in human airway epithelial cell lines, thus suggesting that ion transport function in epithelial cell lines may be sensitive to inhibition of endocytic pathways (99).

Since dynamin regulates both clathrin and caveolin-dependent endocytic routes, we next performed functional studies utilizing an inhibitor specific to clathrin-mediated endocytosis. Chlorpromazine is a cationic drug that, when used in the range of 50–100 μM inhibits clathrin-mediated endocytosis (74). At these concentrations chlorpromazine is known to inhibit calcium-calmodulin and decrease intracellular calcium levels. However, previous studies from our group in Caco-2 cells showed no change in Cl^-/HCO_3^- exchange activity in the presence of BAPTA-AM a chelator of intracellular calcium (123). Thus, it is unlikely that inhibition of calcium via chlorpromazine may contribute to increased Cl^-/HCO_3^- exchange activity. However, further studies are needed to investigate this aspect. Chlorpromazine prevents translocation and assembly of clathrin and adaptor proteins (required for recruitment of cargo and clathrin) to the site of endocytosis at the plasma membrane. Previous studies in COS-7 and Vero, both kidney

epithelial cell lines, showed that chlorpromazine at 100 μM specifically inhibited transferrin (clathrin-dependent) uptake, while the uptake of LacCer (clathrin-independent) was largely unaffected. Our results showed a significant increase in apical Cl^-/HCO_3^- exchange activity in Caco-2 cells treated with 60 μ*M* of chlorpromazine. These results show dependency of the basal Cl^-/HCO_3^- exchange activity on clathrin mediated endocytic pathways. Since the above conclusion is based on pharmacological inhibition of CME, further studies utilizing imaging techniques like electron microscopy where the formation and pinching off of the clathrin-coated pits can be clearly observed are required to support this data.

Interestingly, our functional studies in Caco-2 cells treated with dynasore showed no significant modulation of NHE3 activity. These results suggest that even though NHE3 and Cl^-/HCO_3^- exchange processes are coupled to mediate electroneutral NaCl absorption, the mechanisms regulating their basal apical membrane expression appear to be distinct. An earlier study, however, in a non-polarized CHO derived cell line, used elevation of medium osmolarity (hypertonicity) and potassium depletion to inhibit clathrin-dependent endocytosis and showed that internalization of an exogenously expressed HA-tagged NHE3 occurs via a clathrin-dependent pathway (100). However, this study did not present any data regarding NHE3 function in presence of CME inhibitors. The observed differences between our studies and the study of Orlowski *et al* may also be due to different cell lines used in the study i.e non-polarized cell line Chinese hamster ovary (CHO) vs Caco-2 cells used for studying NHE3 (100).

We also proposed to examine the role of caveolin dependent pathways in regulating basal Cl^-/HCO_3^- exchange activity in Caco-2 cells. However our results showed that Caco-2 cells do not express caveolin-1. This is consistent with previous reports suggesting that Caco-2 cells lack endogenous caveolin-1 and caveolin-2 (70). Additionally, since 3D culture systems often show a gene expression profile closer to native intestine than the standard 2D culture, we also examined the expression of endogenous caveolin-1 in Caco-2 cells grown in a 3D Matrigel system. Similar to our data in 2D system, no expression of caveolin1 was detected in Caco-2

cells grown in 3D culture. Hence the role of caveolin-dependent DRA trafficking was not examined further in our studies. Nevertheless, T-84 cells and native mouse intestinal cells express caveolin-1 and 2 and further studies are needed to investigate the role of caveolin, if any, in the cellular trafficking of DRA.

A.2. Cellular DRA expression in response to inhibition of endocytosis

It is important to mention here that apical Cl^-/HCO_3^- exchange activity in intestinal epithelial cells is contributed by two major apical anion exchangers: DRA and putative anion transporter (PAT-1). Several lines of evidence have shown that DRA is subject to extensive regulation, whereas, PAT-1 remains unaltered in response to many physiological or pathophysiological stimuli including EPEC infection (2). In addition, DRA (but not PAT-1 KO) mice exhibit diarrheal phenotype, further emphasizing the important role of DRA in NaCl absorption and implication in diarrheal disorders. Additionally, increased apical Cl^-/HCO_3^- exchange activity observed in our studies correlates well with increased apical membrane DRA protein levels as assessed by biochemical and immunoflorescence studies as discussed in the following paragraph.

Many studies have shown that function and surface levels of membrane transport proteins complement each other (2, 36). In this regard, studies from our group and others have previously shown a parallel regulation of apical Cl^-/HCO_3^- exchange activity and surface levels of DRA in response to various stimuli (5). Our current studies in Caco-2 cells treated with dynasore showed a similar increase (~ 50%) in apical surface levels of DRA as measured by cell-surface biotinylation and visualization by confocal microscopy. Further, as expected, our results also showed a significant decrease in DRA endocytosis as measured by reverse cell-surface biotinylation, confirming that basal DRA internalization is mediated via a dynamin-dependent pathway. These results are consistent with previous biochemical studies carried out in renal epithelial cells of the thick ascending limb that show inhibition of endocytosis with a

dominant negative dynamin mutant or by inhibitors dynasore and chlorpromazine, which resulted in decreased endocytosis of the sodium-potassium-chloride cotransporter (NKCC2) (101). Another study in HeLa cells stably transfected with CFTR showed that inhibition of endocytosis with dynasore resulted in decreased endocytosis and consequently increased surface expression of CFTR (77). Collectively our results indicate that apical Cl^-/HCO_3^- exchange activity and DRA endocytosis are mediated via a clathrin-dependent pathway. This notion is further strengthened by a lack of caveolin expression in our Caco-2 cell culture model. However, further studies utilizing siRNA-mediated knockdown of clathrin heavy/light chain may provide a more robust picture of the clathrin-dependency of DRA and NHE3 recycling under basal conditions. These studies are currently ongoing in our laboratory.

A.3. Lipid-raft association of DRA by endocytosis inhibitors

Further elucidation of the mechanisms of basal DRA endocytosis in Caco-2 cells revealed that association of DRA with lipid rafts is altered in response to dynasore. This was evident by an increase in the association of DRA with detergent insoluble fractions correlating with dynasore stimulated apical Cl^-/HCO_3^- exchange activity. Lipid rafts are cholesterol and sphingolipid rich membrane microdomains, identified and characterized based on their insolubility in cold Triton X-100. Although, originally linked to caveolae and endocytosis, lipid rafts have now emerged as key docking complexes consisting of various sets of signaling molecules, adaptor and membrane proteins. Thus, these complexes influence several cellular processes including signaling, membrane sorting, recycling and protein trafficking. Previous studies from our group have shown DRA to be associated with lipid rafts in both human colonic apical membrane vesicles (AMVs) and Caco-2 cells. Also while treatment with methyl-beta-cyclodextrin (MβCD) decreased DRA in detergent insoluble fractions; the hormone neuropeptide Y (NPY) stimulated both DRA function and surface expression in Caco-2 cells by increasing its association with DI fraction (30). Another similar study from Lamprecht et al indicated that DRA

is associated with lipid rafts in an intracellular fraction such as recycling vesicles and its exocytic insertion to the apical plasma membrane depends upon raft integrity and PI3K (97). Thus, it is possible that in our study, dynasore increases DRA function and surface expression partly as a consequence of increased exocytosis. However, a 2006 study from Newton et al clearly demonstrated that dynasore did not affect exocytosis of vesicles but rather inhibited only dynamin dependent endocytosis (102). We speculate that dynasore mediated decrease in endocytosis causes retention of DRA on the plasma membrane leading to increase in its association with lipid rafts on the plasma membrane resulting in an increase in its activity. Since our data indicate that dynasore inhibits DRA endocytosis while increasing its association with lipid rafts, another possible explanation might be that the newly exocytosed DRA protein is preferentially inserted into the lipid raft fractions. This maybe best addressed by disrupting exocytosis (e.g. by nocodazole, see below) and then examining the lipid raft distribution of DRA.

A.4. Microtubules and basal DRA recycling

Rapid directed movement of ion transporters in the polarized cells is a consequence of cytoskeletal elements (such as actin and microtubules) based vesicle transport (78). Microtubules have so far been considered to be more important for the delivery of membrane proteins to the apical or basolateral surfaces than the endocytosis of these proteins from the respective membrane surfaces. For example, Hauri et al showed that while disruption of microtubules in Caco-2 cells with nocodazole retarded both apical and basolateral delivery of membrane glycoproteins, the endocytosis of these proteins was largely unaffected by treatment with nocodazole (103). Earlier studies from our laboratory showed decreased apical Cl^-/HCO_3^- exchange activity in Caco-2 cells treated with colchicine (2). These studies were complemented by our confocal microscopy data showing reduced apical surface DRA in Caco-2 cells with disrupted microtubules. Our results examining the effect of microtubule disruption on DRA endocytosis in Caco2 cells are consistent with these previous studies. Disruption of

microtubules with cold and nocodazole treatment did not significantly alter the endocytosed pool of DRA as compared to control cells with recovered microtubules. Thus we speculate that microtubule disruption decreases DRA surface expression via a decrease in DRA exocytosis rather than increased DRA endocytosis. Our biotinylation-based studies on basal DRA exocytosis in Caco-2 cells in the presence of nocodazole indeed showed decreased DRA exocytosis as compared to cells with intact microtubules. Our results are similar to previous studies where disruption of microtubules by colchicine and nocodazole has been implicated in decreased delivery of apical recycling proteins to the membrane. For example, an earlier study by Eilers et al showed that nocodazole retarded the apical delivery of plasma membrane proteins like aminopeptidase N in Caco-2 cells (104). In vivo studies also show the decreased exocytosis of V-ATPase in kidney epithelial cells of mice administered with colchicine. Another study by Brown et al in A-intercalated cells of the kidney showed perturbed exocytic insertion of H^+-ATPase pump (normally apical) upon colchicine treatment, resulting in increased punctate cytoplasmic localization of proton pumps (105).

Our data regarding the role of intact microtubules in DRA exocytosis and surface expression was further supported by results from in vivo biotinylation studies performed on mice treated with colchicine (3mg/kg b.wt.), a microtubule-disrupting agent. Although both nocodazole and colchicine have been used for in vivo disruption of microtubules, our studies utilizing colchicine were based on previous trafficking studies on the NHE3 transporter carried out in colchicine-treated rats (106). *In vivo* biotinylation in mouse colon showed a significant decrease in apical DRA levels in colchicine treated mice as compared to untreated control mice. These results were further validated by immunostaining data, where colchicine treated mouse colon showed significantly more sub-apical DRA as compared to untreated controls. These results are similar to a previous in vivo study where NHE3 and NHERF were shown to be mistargeted to the basolateral membrane in proximal tubules of colchicine treated rats (106).

B Mechanisms of DRA recycling in response to EPEC infection

Enteropathogenic E. coli is a food borne pathogen causing persistent watery diarrhea associated with a high incidence of morbidity and mortality especially in infants. Although, diarrhea caused by EPEC infection is multifactorial, rapid diarrhea that develops in <3hrs of pathogen ingestion is suggested to be associated with a dysregulation in function of ion transporters (1). Initial localized adherence or non-intimate attachment of EPEC to host cells and translocation of virulence factors via the type III secretion system results in disruption of host cytoskeletal network and inhibition of ion transport processes leading to early diarrhea (50). Previous studies have shown an inhibition in the activity of several intestinal ion transporters such as SGLT-1, serotonin transporter (SERT) (107), monocarboxylate transporter (MCT1), NHE3 and apical Cl^-/HCO_3^- exchange in response to EPEC infection. Further, recent studies from our laboratory showed that inhibition in apical Cl^-/HCO_3^- exchange activity was accompanied by a concomitant reduction in surface levels of DRA. Consistent, with this, the results from our current study showed that reduction in surface levels of DRA in Caco-2 cells infected with EPEC occur via modulation of DRA trafficking events in the intestinal epithelial cells.

B.1. Endocytosis of DRA in response to EPEC infection

Previous studies from our laboratory have shown that EPEC deletion mutants for virulence factors: EspA, necessary for formation of a filamentous bridge between the pathogen and host, and, EspB and EspD, which are required for formation of the translocation pore, lack the type III secretion ability and are unable to inhibit apical Cl^-/HCO_3^- exchange activity (47). Further, virulence factor EspF, known to disrupt tight junctions via host actin cytoskeleton and to mediate a decrease in NHE3 surface levels, was shown not to be involved in EPEC mediated decrease in DRA function (2). Rather, decrease in DRA function and surface expression was dependent upon virulence factors EspG1/G2, known to disrupt microtubular network. The

decrease in surface levels of DRA by EPEC infection could occur as a consequence of increase in DRA endocytosis and/or a decrease in DRA exocytosis. Our findings from biochemical and confocal microscopy studies performed in Caco-2 cells showed that EPEC significantly increased DRA endocytosis as compared to uninfected cells. Since our data showed increased apical Cl^-/HCO_3^- exchange activity in response to treatment with dynasore, we investigated if EPEC mediated decrease in apical Cl^-/HCO_3^- exchange activity in Caco-2 cells was altered in the presence of endocytosis inhibitor dynasore. However, infection of Caco-2 cells with EPEC resulted in decrease in apical Cl^-/HCO_3^- exchange activity both in the presence or absence of dynasore. These results are in agreement with recent studies carried out in T-84 cells, which showed that although CFTR internalization is mediated via a clathrin-dependent pathway, the inhibition of this pathway did not significantly alter EPEC mediated mislocalization of CFTR (108). Additionally similar to apical Cl^-/HCO_3^- exchange activity, the EPEC mediated decrease in NHE3 activity, as seen by S3226 sensitive $^{22}Na^+$ uptake, was unaltered in the presence of dynasore in Caco-2 cells. These results are suggestive of the notion that EPEC mediated decrease in apical Cl^-/HCO_3^- exchange activity and Na^+/H^+ exchange activity is independent of dynamin mediated cellular trafficking events. Due to marginal cytotoxicity of chlorpromazine, experiments with EPEC infection in the presence or absence of chlorpromazine were not performed. However, biochemical endocytosis assays in intestinal epithelial cells infected with EPEC in the presence or absence of dynasore are needed to validate these results. Further it would be of interest to assess the effect of EPEC infection in the presence of varying doses of dynasore.

It is important to mention here that both DRA and PAT-1 contribute to luminal chloride uptake, however EPEC has differential effects on these two apical anion exchangers. Previous studies from our group show that while EPEC induced a decrease in DRA surface expression PAT-1 surface expression was unchanged in cells infected with EPEC. For these reasons changes in expression of PAT-1 were not examined in the current study. Further, it could be

argued that anion exchangers AE2 and AE3 also contribute apical Cl-/HCO3- exchange activity measured in our experiments. However since these transporters are expressed on basolateral membrane and DIDS sensitive iodine uptake was measured from apical side in fully differentiated Caco-2 monolayers, we do not believe our data reflects a contribution from potential changes in AE2 or AE3 activity. Whether EPEC affects AE2 or AE3 within 30 minutes of infection is unknown and needs further study. It has also been shown that EPEC induced change in transepithelial electrical resistance (TER) does not occur until 3 hours post infection. Thus changes in apical Cl^-/HCO_3^- exchange activity at 30 minutes- 60 minutes post infection most likely reflect the activity of apical DRA (present study, 48, 54).

B.2. Effect on exocytic trafficking of DRA in response to EPEC infection

In addition to an increase in DRA endocytosis, EPEC infection of Caco-2 cells also significantly decreased the DRA levels in the exocytosis pool. Further, this decrease was found to be dependent upon virulence factors EspG1/G2 as Caco-2 cells infected with wild type EPEC, able to cause microtubule disruption, resulted in significantly lesser DRA exocytosis as compared to cells infected with *ΔespG1/G2* mutant, which is unable to disrupt host microtubules. Although, our data rule out the involvement of microtubules in basal DRA endocytosis, the integrity of microtubular network in EPEC mediated modulation of DRA exocytosis appears to be critical. Similar to our data *in vivo* studies utilizing *C. rodentium* infected mice, the equivalent murine model for EPEC, showed the mislocalization of the water channels, aquaporins (AQP2 and 3) from the plasma membrane to the cytoplasm partially via an EspF and EspG dependent mechanism (109).

B.3. Association of lipid-rafts with DRA in response to EPEC infection

Taking cues from previous studies and our current data showing a functionally parallel redistribution of DRA in the membrane lipid-raft domains upon dynasore treatment, we

investigated DRA membrane distribution between rafts and non-raft compartments in response to EPEC infection. Our findings demonstrated that EPEC infection significantly re-distributed DRA from the lipid-rafts to the detergent soluble non-raft membrane fractions, which may, thus, influence its function on the apical membrane. An in vivo study similarly showed that EPEC infection causes redistribution of tight junction proteins occluding and claudin-1 from detergent insoluble raft fractions to detergent soluble fractions causing a loss of the tight junction barrier function (110). In addition, previous studies from our laboratory have shown that increased DRA association with lipid rafts caused an increase in apical Cl^-/HCO_3^- exchange activity without a change in total membrane levels of DRA (30). Studies of Lamprecht et al showed that DRA association with lipid rafts was important for PI3K dependent exocytic insertion of DRA into plasma membrane (97). Further, the inhibition of apical Cl^-/HCO_3^- exchange activity observed during EPEC infection in the presence or absence of dynasore, paralled the decreased association of DRA with lipid-rafts, further strengthening the notion that DRA function is dependent upon its association with lipid-rafts. It is, however, unknown if lipid-raft dissociation of DRA in response to EPEC infection is a result of increased endocytosis, or a consequence of decreased re-insertion of DRA into the plasma membrane and warrants further examination. Further, the total membrane pellet obtained in our experiments includes plasma membrane as well as vesiclular membranes. Exclusive surface biotinylation of plasma membrane followed by detergent based raft separation is required to study the effect of EPEC infection on apical DRA protein. Collectively, these data indicate that EPEC infection modulates DRA trafficking via i) increased endocytic mechanism; and ii) decreased DRA exocytosis dependent upon disruption of host microtubular network. These data highlight the importance of membrane trafficking events in diarrhea induced by EPEC infection. Thus, rapid activation of DRA in case of enteric infections may prove to be a possible therapeutic alternative.

Our results from functional studies suggested a partial attenuation in EPEC induced decrease in apical Cl^-/HCO_3^- exchange activity in cells pre-treated with culture supernatant from

L. acidophilus (LA-CM). In this regard, studies from our group showed acute stimulatory effects of probiotic *L. acidophilus* on apical Cl^-/HCO_3^- exchange and surface expression of DRA, via activation of PI3K dependent pathway (34). It has been previously shown that EPEC inhibits PI3K activity and that PI3K plays an important role in trafficking of several membrane proteins. We speculate that changes in activation and/or inhibition of PI3K by LA-CM and EPEC may play a role in PI3K mediated trafficking events. Whether these changes in PI3K activity induced by LA-CM may be responsible for counteracting the effects of EPEC is uninvestigated. It will be interesting to examine the cellular mechanisms and signaling pathways involved in LA-CM mediated attenuation of DRA inhibition in response to enteric pathogenic infections.

Overall, our results for the first time demonstrate novel mechanisms of DRA cellular trafficking under physiological conditions and in response to EPEC infection. Further elucidation of these pathways will enhance our understanding of the mechanisms underlying the pathophysiology of EPEC induced early diarrhea and help identify novel targets for the treatment of diarrheal disorders.

VI. CONCLUSIONS AND FUTURE DIRECTIONS

Research presented in this dissertation focuses on delineating the role of cellular trafficking pathways in physiological maintenance of DRA surface levels and in EPEC induced decrease in DRA surface expression in IECs and *in vivo* mouse model. Our findings support the conclusion that DRA recycling at the apical membrane is governed by a clathrin-dependent endocytic mechanism and a microtubule dependent exocytic reinsertion into the plasma membrane. Further, our results indicate that EPEC modulates both endocytic and exocytic DRA trafficking resulting in decreased surface expression of DRA.

Specific Aim 1

1. Inhibition of dynamin-dependent endocytosis by dynasore (80 μM) and clathrin-mediated endocytosis by chlorpromazine (60 μM) increased apical $Cl^-/OH^-(HCO_3^-)$ exchange activity in Caco-2 cells. A similar increase was seen in T-84 cells treated with dynasore, indicating that the effects of dynasore are not cell line specific. Dynasore treatment however, failed to stimulate NHE3 activity in Caco-2 cells suggesting that increased apical $Cl^-/OH^-(HCO_3^-)$ exchange activity was not secondary to increased apical Na^+/H^+ activity.

2. Further, the lack of endogenous caveolin-1 expression in Caco-2 cells cultured in 2D and 3D culture systems rule out any involvement of caveolin pathways and strengthened the notion that DRA endocytosis in Caco2 cells is clathrin-dependent.

3. Parallel to an increase in function, dynasore treatment increased surface levels of DRA, as measured by biotinylation studies, with no change in total cellular levels of DRA. Immunoflourescence studies in Caco-2 cells complemented our biochemical results showing increased apical DRA in dynasore treated Caco-2 monolayers. The increase in surface expression upon dynasore treatment was attributed to decreased DRA endocytosis.

4. Our results from lipid-raft studies indicated that increased DRA function and surface expression was accompanied by an increase in DRA associated with lipid-raft (detergent-insoluble) fractions isolated from total cellular membranes.

5. Disruption of microtubules in Caco-2 cells followed by recovery in the presence or absence of nocodazole (66 μM) suggested that intact microtubules are essential for basal DRA exocytosis as measured by biochemical studies. Confocal microscopy results also revealed that disruption of microtubules decreased DRA surface levels in Caco-2 cells as compared to cells with recovered microtubules. Disruption of microtubules did not however affect DRA endocytosis.

6. Consistent with in vitro data, mice treated with microtubule disrupting agent colchicine (3mg/kg b.wt. 24h) showed a decrease in apical surface levels of DRA and an increase the sub-apical pool of DRA in the colon as seen by immunostaining. This decrease in surface levels of DRA with colchicine treatment was also supported by in vivo biotinylation studies performed in the mouse colon.

Specific Aim 2

1. Our biochemical studies showed that a 60 minute EPEC infection in Caco-2 cells resulted in increased DRA endocytosis which was not observed when similar studies were carried out on ice to block cellular trafficking. This suggested that EPEC-mediated decrease in DRA surface expression is partially mediated via increased endocytic trafficking of DRA.

2. Functional studies in Caco-2 cells infected with EPEC showed that the presence of dynasore (80-120 μM) did not significantly alter the EPEC mediated decrease in apical $Cl^-/OH^-(HCO_3^-)$ or apical Na^+/H^+ exchange activity.

3. Investigation of the exocytic arm of DRA trafficking in Caco-2 cells infected with EPEC revealed a significant decrease in DRA exocytosis in EPEC infected cells as compared to uninfected controls. Further, infection of Caco-2 cells with EPEC double deletion mutants for effector molecules EspG1/G2 prevented the decrease in the DRA exocytosis induced by

infection with wild type EPEC. Since EspG1/G2 are implicated in host microtubule disruption, these results support our in vitro data emphasizing the importance of intact microtubules in DRA exocytosis.

4. Modulation of endocytic and exocytic pathways by EPEC infection was accompanied by a redistribution of DRA in the lipid-raft domains in Caco-2 cells infected with EPEC. Our results from a detergent based raft isolation technique showed that infection with EPEC significantly decreased the association of DRA with detergent-resistant membrane fractions.

5. Functional studies in Caco-2 cells pre-incubated with culture supernatant from the probiotic *Lactobacillus acidophilus* showed an attenuation of EPEC mediated decrease in apical Cl^-/OH^- (HCO_3^-) exchange activity in cells pre-treated with LA culture-supernatant (LA-CS) followed by EPEC infection in the presence of LA-CS as compared to cells infected with EPEC alone.

To conclude, as shown in figure 29, we propose a model that indicates the involvement of the clathrin-dependent endocytic pathway in basal DRA endocytosis. Further, the EPEC induced disruption of microtubules, previously implicated in decreased DRA function, is shown to be involved in decreased DRA exocytosis. Our data also indicate that EPEC infection increases DRA endocytosis, however further studies need to be carried out exhaustively to identify the cellular pathway underlying the pathophysiology of EPEC induced diarrhea.

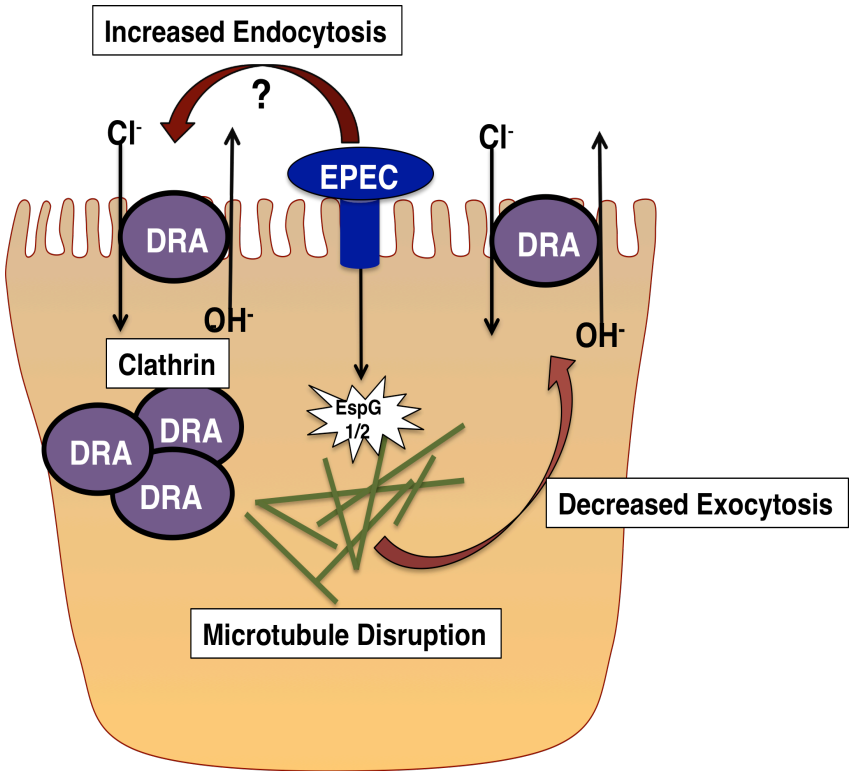

Figure 30. Conclusion

VII. FUTURE DIRECTIONS

Specific Aim 1

1. Utilize immunofluorescence techniques, to examine the association of DRA with markers of early endosomes (EEA1, Rab5) and recycling endosomes (Rab4, Rab11) in intestinal epithelial cells treated with dynasore and chlorpromazine. We expect that inhibition of CME will lead to accumulation of DRA in sub-apical vesicle pools.
2. Delineate the role of PDZ domain interaction of DRA with NHERF proteins in endocytic and exocytic trafficking utilizing EKTF mutant (lacking PDZ domain).
3. Examine regulation of DRA association with lipid-rafts only at the apical membrane surface via combined biotinylation and detergent based raft separation techniques in the presence of dynasore or EPEC infection.
4. Establish the role of dynamin dependent endocytosis in DRA trafficking utilizing dynamin specific siRNA (as an alternative approach to use of chemical inhibitors) in Caco-2 cells.
5. Examine the possible contribution of other endocytic recycling pathways such as lipid-raft and caveloin-dependent endocytosis in DRA trafficking in cell lines that express caveolin 1.
6. Investigate the contribution of PAT-1 in dynasore-mediated effects in intestinal epithelial cells by biochemical studies in Caco-2 cells treated with dynasore and chlorpromazine.
7. Isolation of clathrin coated vesicles first from Caco-2 cells followed by native mouse intestine to examine both *in vitro* and *in vivo* association of DRA with clathrin.
8. Investigate the role of kinesin and dynein motor proteins in DRA trafficking utilizing motor-specific inhibitors and siRNA techniques in intestinal epithelial cells.

Specific Aim 2

1. Investigate the role of clathrin-dependent endocytosis in EPEC-induced decrease in DRA surface expression utilizing biotinylation assays in the presence of dyansore and chlorpromazine. We expect these experiments to support our current functional data.
2. Determine whether EPEC double deletion mutants for effector molecules EspG1/G2 alter DRA endocytosis.
3. Examine alternate endocytic pathways such Arf-6 dependent endocytosis in EPEC-mediated decrease in DRA surface levels in intestinal epithelial cells utilizing siRNA methodology.
4. Examine the association of DRA in the native intestine with endosomal markers in control and EPEC gavaged mouse models. We expect EPEC infection to result in increased accumulation of DRA in endosomal vesicles.
5. Investigate the role of kinesin and dynein motor proteins in DRA trafficking in response to EPEC utilizing motor-specific inhibitors and siRNA techniques in intestinal epithelial cells.
6. Investigate the role of actin in EPEC mediated DRA inhibition utilizing actin disrupting agent cytochalasin D or actin stabilizing agent jasplakinolide in vitro. Actin does not appear to affect basal DRA trafficking.
7. Delineate the cellular endocytic and exocytic mechanisms responsible for *L. acidophilus* mediated abrogation of decreased apical $Cl^-/OH^-(HCO_3^-)$ exchange activity and DRA surface expression in intestinal epithelial cells infected with EPEC.

VIII. SIGNIFICANCE AND SPECULATION

Despite advances in treatments, diarrheal disorders continue to be a major cause of morbidity and mortality in children below 5 years of age (113). Diarrhea occurs due to the decreased absorption or increased secretion or both of electrolytes and nutrients. Impaired absorption of electrolytes and nutrients in children has been associated with impairment in growth, fitness and cognition and results in long-term developmental disabilities (114). Various factors can lead to diarrhea such as food borne pathogens, radiation injury, genetic mutations, hormonal alterations, malabsorption and inflammatory bowel disease (IBD). For example, EPEC, a food-borne pathogen, is a major cause of infantile diarrhea worldwide. Previously, our studies have shown that EPEC induced early diarrhea results from a perturbation in intestinal ion transport. Further, EPEC infection was found to decrease the activity of NHE3 and DRA, the apical Na^+/H^+ and Cl^-/OH^- exchangers via decreased surface expression of the transporters in intestinal epithelial cells. Therefore, a better understanding of cellular trafficking pathways involved in physiological and pathophysiological surface expression of these transporters is critical for designing novel strategies for therapeutic interventions. In this regard, my studies provide an insight into basal trafficking pathways that regulate the surface expression of DRA in intestinal epithelial cells identifying the role played by clathrin-dependent endocytic and microtubule dependent exocytic mechanisms in the constitutive recycling of DRA. Further, the results from these studies implicate altered cellular trafficking and microtubule disruption in EPEC mediated internalization of DRA. Therefore, these cellular pathways could be potential targets for the development of improved anti-diarrheal therapeutics. We speculate that other cellular pathways may also be involved in EPEC mediated decrease in apical Cl^-/HCO_3^- exchange activity. For example, the T3SS proteins such as EspT and EspH are known to alter the activity of RhoGTPases like CDC42, Rac1 and Rho. The disrupted activity of RhoGTPases leads to subversion of actin dynamics in the host cell. Previous studies showed that the

inhibition of NHE3 activity during EPEC infection is dependent upon actin disruption by the pathogens. Whether the modulation of RhoGTPases may be involved in EPEC induced decrease in apical NHE3 or Cl^-/HCO_3^- exchange activity is currently unknown. Further, EspG which was originally believed to be involved in microtubule disruption, has very recently also been shown to bind to p21 activated kinases (PAKs), and ADP-ribosylation factor family of GTPases (ARFs) and regulate host signaling cascades (112). Regulation of ARFs might be another a potential pathway for EPEC mediated effects on DRA function and surface expression. Future studies will focus on examining the role of these pathways in basal and EPEC induced modulation of DRA trafficking.

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APPENDICES

APPENDIX A

Department of Veterans Affairs

Memorandum

Date: February 22, 2013

From: Rhonda kineman, Ph.D, Chairman
R&D Institutional Animal Care and Use Committee (IACUC) (537/151)

To: Dr. Pradeep K. Dudeja
Protocol Entitled: "Transporter Trafficking Mechanisms in Infectious Diarrhea",
IACUC #13-04 (Mice)

RE: Animal Care and Use Approval

At the **February 13, 2013** meeting of the IACUC, your protocol entitled: **"Transporter Trafficking Mechanisms in Infectious Diarrhea"** (Mice) **IACUC #13-04** was brought before the IACUC and received **Full Approval** on **February 20, 2013** The Project Personnel listed are: **Dudeja, Gill, Borthakur, Saksena, Tyagi, Kumar, Priyamavada and Anbazhagan** the funding source is: **NIH**.

Please note that this approval is valid only for animals that are housed at **JBVAMC VMU.**

Please verify the information below. If corrections are required please notify Robin McWherter, IACUC Coordinator at Robin.McWherter@va.gov immediately.

a. USDA Category: B (303) , C (3247), and D (1200)
b. Species: Mice
c. Strain: (C57BL/6J/NHERF2 -/-), (C57BL/6J/NHERF2 +/-), (C57BL/6J/NHERF2 +/+), (C57BL/6J/NHERF1 -/-),(FVBN/NHERF3 -/-), (C57BL/6J mice/FVBN)
d. # of Animals Approved: 4750**Total**
e. Approval Date: February 14 2013
f. Approval Termination: February 13, 2016
g. IACUC **#13-04**

Should it become necessary to make any additional changes in this protocol, you must submit a modification request for approval prior to initiating changes. In the case in which animal work is being done at an affiliate institute, you must obtain approval from both the affiliate and the JBVAMC prior to initiation. Failure to comply with these provisions can result in a suspension of the research.

If you have any questions, or need further assistance, please contact Robin McWherter, IACUC Coordinator at Robin.McWherter@va.gov.

Sincerely,

Rhonda Kineman, Ph.D.
Chair, IACUC

APPENDIX A (CONTINUED)

UIC UNIVERSITY OF ILLINOIS AT CHICAGO

Office of Animal Care and Institutional
Biosafety Committee (OACIB) (M/C 672)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612

10/16/2013

Pradeep K. Dudeja
Medicine/Digestive and Liver Diseases
JAVAMC, R&D, MP-151
820 S. Damen, Suite 6215
Chicago, IL 60612

Dear Dr. Dudeja:

The protocol indicated below was reviewed in accordance with the Animal Care Policies and Procedures of the University of Illinois at Chicago and **renewed on 10/16/2013.**

Title of Application:	**Ion Transporter Mechanisms in Infectious Diarrhea**
ACC NO:	**12-190**
Original Protocol Approval:	**12/4/2012 (3 year approval with annual continuation required).**
Current Approval Period:	**10/16/2013 to 10/16/2014**

Funding: ***Portions of this protocol are supported by the funding sources indicated in the table below.***
Number of funding sources: 1

Funding Agency	**Funding Title**			**Portion of Funding Matched**
NIH	*Transporter Trafficking Mechanisms In Infectious Diarrhea*			*All matched*
Funding Number	**Current Status**	**UIC PAF NO.**	**Performance Site**	**Funding PI**
RO1 DK09211 (original yrs 1-4)	*Funded*	*2011-01559*	*JBVAMC*	*Pradeep Dudeja*

This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare, NIH. **This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the grant are matched to this ACC protocol.**

Thank you for complying with the Animal Care Policies and Procedures of the UIC.

Sincerely,

Bradley Merrill, PhD
Chair, Animal Care Committee

BM/kg
cc: BRL, ACC File, Seema Saksena

Phone (312) 996-1972 • Fax (312) 996-9088

APPENDIX B

Gmail by Google

taru gujral <tarun1323@gmail.com>

RE: Obtain Permission - Book request

1 message

Jones, Jennifer (ELS-OXF) <J.Jones@elsevier.com> Wed, Jun 25, 2014 at 4:15 AM
To: "tgujra2@uic.edu" <tgujra2@uic.edu>

ELSEVIER

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VITA

TARUNMEET GUJRAL

EDUCATION

B.S. Biotechnology, Panjab University, Chandigarh, India, 2007
M.S. Biotechnology (Honors School), Dept of Biotechnology, Panjab University, India, 2009
M.S. Physiology and Biophysics, College of Medicine, University of Illinois at Chicago, 2014

WORK EXPERIENCE

Project Assistant, Institute of Microbial Technology (IMTECH), India, 2009-10
Graduate Research Assistant, Jesse Brown VA Medical Centre, and University of Illinois at Chicago, 2011-14

ABSTRACTS AND PRESENTATIONS

1. Priyamvada S, Anbazhagan AN, Kumar A, **Gujral T**, Borthakur A, Saksena S, Gill R, Alrefai WA and Dudeja PK. All-Trans-Retinoic Acid Increases SLC26A3 (DRA) Expression via HNF-1β. **Gastroenterology**, Vol. 144, Issue 5, S-130–S-131, **2013.**
2. Kumar A, Alakkam A, Gill RK, **Gujral T**, Soni V, Anbazhagan AN, Malakooti J, Alrefai WA and Dudeja PK. Inhibition of DRA Expression by NF-κβ Pathway in Human Intestinal Epithelial Cells. **Gastroenterology**, Vol. 144, Issue 5, S-664–S-665, **2013**
3. **Gujral T**, Kumar A, Priyamvada S, Gill RK, Alrefai WA, Hecht, GA and Dudeja PK. Trafficking of SLC26A3 (DRA) in Intestinal Epithelial Cells: Effect of EPEC Infection. **Invited Speaker,** Gut Epithelial and Mucosal Club Meeting, Chicago**, 2013**
4. **Gujral T**, Kumar A, Priyamvada S,. Gill RK, Alrefai WA, Hecht, GA and Dudeja PK. Trafficking of SLC26A3 (DRA) in Intestinal Epithelial Cells: Effect of EPEC Infection. **Gastroenterology,** 146 (5), S-653, **2014. Poster presentation** – Digestive Diseases Week (DDW), **2014, Chicago (Manuscript under preparation)**
5. Kumar A, **Gujral T**, Gill RK, Coffing H, Anbazhagan AN, Borthakur A, Alrefai WA and Dudeja PK. TNF Inhibits SLC26A3 Expression via Activation of NF-κB Pathway. **Gastroenterology,** 146 (5), S-650, **2014. Poster of Distinction** - Digestive Diseases Week (DDW), **2014, Chicago**
6. Anbazhagan AN, Priyamvada S, **Gujral T**, Alrefai WA, Dudeja PK and Borthakur A. Distinct Roles of the Long-Chain Fatty Acid Receptor GPR120 in Different Cell Types of the Intestinal Epithelium. **Gastroenterology**, Vol. 146, Issue 5, S-110–S-111, **2014**
7. Priyamvada S, Anbazhagan AN, Kumar A, **Gujral T**, Borthakur A, Saksena S, Gill R, Alrefai WA and Dudeja PK. All-Trans-Retinoic Acid (ATRA) Counteracts Inhibition of DRA Function and Expression in Intestinal Inflammation. **Gastroenterology**, Vol. 146, Issue 5, S-650, **2014. Poster of Distinction** - Digestive Diseases Week (DDW), **2014, Chicago**
8. Anbazhagan AN, Priyamvada S, Kumar A, **Gujral T,** Dulari J, Mugarza E, Gill RK, Alrefai WA, Onyuksel H and Dudeja PK. A Novel Role of GLP-1 Nanomedicine in Amelioration of Gut Inflammation. **Gastroenterology**, Vol. 146, Issue 5, S-654, **2014**