Implication of Gliadin Toxicity on Actin Integrity and Subsequent Protein Trafficking \textit{in vitro}

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Yvonne Reinke
(Wolfsburg)

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Supervisor: Prof. Hassan Y. Naim, PhD

Advisory Committee: Prof. Hassan Y. Naim, PhD
Prof. Dr. Rita Gerardy-Schahn
Prof. Dr. Bernd Schröder

1st Evaluation: Prof. Hassan Y. Naim, PhD
Department of Physiological Chemistry
University of Veterinary Medicine Hannover, Foundation

Prof. Dr. Rita Gerardy-Schahn
Department of Cellular Chemistry
Hannover Medical School

Prof. Dr. Bernd Schröder
Department of Physiology
University of Veterinary Medicine Hannover, Foundation

2nd Evaluation: Prof. Dr. Klaus-Peter Zimmer
Department of Pediatrics
University of Giessen - Marburg

Date of oral exam: 31.7.2009

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The only real voyage of discovery consists not in seeking new landscapes, but in having new eyes.

Marcel Proust (1871 – 1922)

Meiner Ma
in Liebe und Dankbarkeit
und in stillem Gedenken an meinen Vater
(1953 – 1995)
Parts of this project have been already submitted or communicated:

**SCIENTIFIC PRESENTATIONS:**

**Poster:**

**CHANGES IN PROTEIN TRANSPORT AND ACTIN CYTOSKELETON DUE TO TOXIC GLIADIN PEPTIDES**

Yvonne Reinke*, Ina Fischer*, Klaus-Peter Zimmer+, Hassan Y. Naim*
(DVG-FG Biochemie & Physiologie in Gießen (19.2.- 21.2.2006)

**REARRANGEMENT OF THE ACTIN CYTOSKELETON AND ALTERATION OF PROTEIN TRANSPORT IN CELLS TREATED WITH GLIADIN PEPTIDES I**

Yvonne Reinke*, Klaus-Peter Zimmer+ and Hassan Y. Naim*
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**REARRANGEMENT OF THE ACTIN CYTOSKELETON AND ALTERATION OF PROTEIN TRANSPORT IN CELLS TREATED WITH GLIADIN PEPTIDES II**

Yvonne Reinke*, Klaus-Peter Zimmer+ and Hassan Y. Naim*
(Experimental Biology 2007 (ASBMB) in Washington, 28.4.-2.5.2007)

**ALTERATIONS IN ACTIN ORGANIZATION AND MEMBRANE TRANSPORT IN INTESTINAL CACO-2 CELLS DUE TO GLIADIN TOXICITY**

Yvonne Reinke*, Klaus-Peter Zimmer+ and Hassan Y. Naim*
(DVG-FG für Biochemie & Physiologie in Leipzig, 9.3.-11.3.2008)

**Oral:**

**POTENTIAL PATHOMECHANISM IN CELIAC DISEASE: CHANGES IN THE ACTIN CYTOSKELETON AND PROTEIN TRAFFICKING DUE TO GLIADIN TOXICITY**

Y Reinke*, KP Zimmer+, HY Naim*
(Seminars in Biochemistry and Virology, 24.10.2007)

**VERÄNDERUNGEN DES AKTINZYTOSKELETTS UND DES PROTEINTRANSPORTES NACH BEHANDLUNG MIT GLIADINPEPTIDEN**

Y Reinke*, KP Zimmer+, HY Naim*
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**POTENTIELLE PATHOMECHANISMEN IN DER ZÖLIAKIE: VERÄNDERUNGEN DES AKTINZYTOSKELETTS UND DES PROTEINTRANSPORTES DURCH DIE TOXIZITÄT VON GLIADIN PEPTIDEN**

Y Reinke*, KP Zimmer+, HY Naim*
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GLIADIN TOXIC PEPTIDES INTERACT WITH THE ACTIN CYTOSKELETON AND AFFECT THE TARGETING AND FUNCTION OF INTESTINAL PROTEINS
Y. Reinke*, K.-P. Zimmer+ and H.Y. Naim*
*Department of Physiological Chemistry, University of Veterinary Medicine Hannover, Foundation, Germany
+Department of Pediatrics, University of Giessen, Germany
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EFFECTS OF GLIADIN TOXIC PEPTIDES ON ACTIN AND SUBSEQUENT PROTEIN TRAFFICKING
Y. Reinke*, K.P. Zimmer+, H.Y. Naim*
*Department of Physiological Chemistry, University of Veterinary Medicine Hannover, Foundation, Germany
+Department of Pediatrics, University of Giessen, Germany
(Submitted)
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ABBREVIATIONS
ABBREVIATIONS

µg  
microgram
µl  
microliter
AAA  
anti-actin antibody
AEE  
apical early endosomes
ApN  
aminopeptidase N
ARE  
apical recycling endosomes
Arp2/3  
actin-related protein 2 and 3
BB  
brush border
BBM  
brush border membrane
BEE  
basolateral early endosomes
BSA  
bovine serum albumin
cath. D  
cathepsin D
CCP  
clostrin-coated pit
CCV  
clostrin-coated vesicle
CD  
celiac disease
cDNA  
complementary deoxyribonucleic acid
CDxx (e.g. CD28)  
cluster of differentiation xx (e.g. 28)
CE  
common recycling endosomes
CXCR3  
chemokine CXC receptor 3
cytoD  
cytochalasin D
DIG  
detergent insoluble glycolipid-enriched complex
DMEM  
Dulbecco’s modified eagle’s medium
DMSO  
dimethyl sulfoxide
DNA  
deoxyribonucleic acid
DPPIV  
dipeptidylpeptidase IV
DRM  
detergent-resistant membrane
DTT  
dithiothreitol
e.g. for example
ECL enhanced chemiluminescence
EE early endosomes
EEA-1 early endosomal antigen-1
ER endoplasmatic reticulum
FF Frazer’s Fraction
Fig. figure
g gravitation
h hour
HBB human brush border
HLA human leukocyte antigen
HRP horseradish peroxidase
IBD inflammatory bowel disease
IgA Immunoglobulin A
IP immunoprecipitation
kDa kilo Dalton
LPH lactase phlorizin hydrolase
MAPs microtubule-associated proteins
MEM minimum essential medium
mg milligram
MHCII major histocompatibility complex class II molecule
min minute
ml milliliter
MV microvilli
MyD88 myeloid differentiation primary response gene (88)
N-WASP neuronal wiskott-aldrich syndrome protein
OVA ovalbumin
PAGE polyacryl gel electrophoresis
PFA paraformaldehyd
pIgR polymeric immunoglobulin receptor
rpm rounds per minute
<table>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SI</td>
<td>sucrase-isomaltase</td>
</tr>
<tr>
<td>Tf</td>
<td>transferrin</td>
</tr>
<tr>
<td>TfR</td>
<td>transferrin receptor</td>
</tr>
<tr>
<td>TGN</td>
<td>trans-Golgi network</td>
</tr>
<tr>
<td>tTG</td>
<td>tissue transglutaminase</td>
</tr>
<tr>
<td>WGA</td>
<td>wheat germ agglutinin</td>
</tr>
<tr>
<td>YFP</td>
<td>yellow fluorescence protein</td>
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INTRODUCTION
1 INTRODUCTION

1.1 Background

Several pathomechanism of the small intestine originate from errors in processing and transport of proteins in intestinal enterocytes or from villous atrophy caused by damages of the intestinal mucosa. The reason for that can be of endogenous origin when the phenotype of a protein is altered by, for instance, a mutation as shown for sucrase-isomaltase in CSID (congenital sucrase-isomaltase deficiency) or the disease is genetically manifested, as demonstrated for the genetic predisposition HLA-DQ2 and DQ8 in celiac disease. Moreover, environmental factors like food antigens may also play a certain role in the development and maintenance of intestinal disorders, which comes upon celiac disease.

1.2 Celiac Disease (CD)

Celiac disease (CD) is a permanent intolerance to gluten in genetically susceptible individuals. The ingestion of gluten induces inflammation and tissue damage of the small intestine, leading to villous atrophy (see Fig.1-1) and subsequent reduced absorptive capacity. Furthermore, inflammation and tissue damage can result in chronic diarrhea, increased infiltration of lymphocytes, the formation of hyperplastic crypts and a consecutive malabsorption syndrome (CICLITIRA and MOODIE 2003; SOLLID 2002; MARSH 1992). Additionally, several extra-intestinal diseases are also described to be associated with CD, e.g. diabetes mellitus type I or dermatitis herpetiformis (HOLTMEIER and CASPARY 2006; HOURIGAN 2006; CICLITIRA et al. 2005).
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Figure 1-1: The villous architecture of the intestinal mucosa.
The mucosa of healthy subjects shows normal villi and crypts (a; left panel) whereas the celiac mucosa is substantially altered with villous atrophy, hyperplastic crypts and malabsorption (b; right panel).
(SOLLID 2002) doi:10.1038/nri885

1.2.1 Clinical Manifestations and Epidemiology

Today approximately 1% of the Western world population suffer from CD (STEPNIAK and KONING 2006), in which both genetic and environmental factors play a crucial role. (Actually, CATASSI and FASANO (2008) presume that the prevalence for CD is 1% worldwide).

CD can present at any age, but typical cases often manifested in early childhood (FASANO and CATASSI 2005). Furthermore, the etiopathology is not identical for all patients as the clinical spectrum, especially in children, is wide and includes different cases. As demonstrated in Fig. 1-2, it can be distinguished between a) CD with typical or classical symptoms, e.g. fully expressed enteropathy and signs of intestinal malabsorption; b) CD with atypical or non-classical symptoms that are milder and often extra-intestinal but also with fully expressed enteropathy; c) “silent CD” stands for patients with minimal complaints or those that are actually symptom-free although they express a full enteropathy (which is
therefore occasionally discovered by serological screening) and finally d) the potential or latent CD which is manifested in minimal damaged or even normal intestinal mucosa. The patients are sometimes symptomatic and with positive serological screening at first testing. That is why the risk to develop a typical intestinal damage later in life is strongly enhanced (FASANO and CATASSI 2005).

Figure 1-2: Clinical manifestations in children: the iceberg
Celiac disease with classic or non-classic symptoms represents only the tip of the iceberg whereas most of the patients form a latent celiac disease. But in all cases the patients express a genetic susceptibility (DQ2 and/or DQ8) and show an abnormal serology. (FASANO and CATASSI 2005)

Nevertheless, the only available therapy for celiac patients is adherence to a gluten-free diet, although extensive research is being conducted towards finding new improved treatment alternatives. Under normal conditions the intestinal lesion can be reverted to normal, when patients completely abstain from wheat gluten and related proteins of rye and barley (BRANDTZAEG 2006). However, in 5-30% of CD patients a strict gluten-free diet fails to induce clinical and/or histological improvement (HOWDLE and LOSOWSKY 1992). This phenomenon is described as so called refractory CD (DAUM et al. 2005), although a clear definition is still missing.
1.2.2 Genetic Aspects

CD is a polygenic disease with a strong major histocompatibility complex class II molecule (MHCII) association. The genetic disposition is associated with the HLA (human leukocyte antigen) molecules DQ2 and DQ8 (CICLITIRA et al. 2005; KONING et al. 2005; SHIDRAWI et al. 1998; SOLLID and THORSBY 1993). However, there are differences between homozygous and heterozygous individuals, since HLA-DQ2 homozygous individuals have at least a five-fold higher risk to disease development than HLA-DQ2 heterozygous individuals (MEARIN et al. 1983). This correlates with the strength of the gluten-specific T cell response that is stronger in homozygous individuals (VADER et al. 2003). The majority with up to 90-95% of the celiac patients expresses the HLA-DQ2 heterodimer with the remaining 5-10% expressing HLA-DQ8 (HOURIGAN 2006). But since these HLA molecules are also found in healthy individuals, the presence appears to be necessary but not sufficient for the development of CD (HOLTMEIER and CASPARY 2006; HOURIGAN 2006). A number of genome-wide linkage studies have attempted to identify other associated genes or loci (DEWAR et al. 2004). The majority of these loci are expected to point to genes with a small effect (WAPENAAR and WIJME NGA 2005), although there has been recent interest in a locus at chromosome 2q33 containing two such genes: CD28 and CTLA-4 (Cytotoxic T-Lymphocyte Antigen 4; CD152) (AMUNDSEN et al. 2004; POPAT et al. 2002), both expressed on T lymphocytes and involved in facilitating and inhibiting MHC-antigen interactions (DEWAR et al. 2004).

1.2.3 Gluten and related Proteins

Besides the genetic background the environmental factor gluten plays a decisive role in the development of CD. Gluten was identified as a trigger for CD more than fifty years ago (DICKE et al. 1953). Collectively, the disease-activating protein in wheat, rye and barley are widely termed “gluten” (KAGNOFF 2005; DEWAR et al. 2004), but strictly gluten is the scientific name for only the proteins in wheat (KAGNOFF 2005). Gluten can be divided into two main fractions; the prolains and the glutenins. The prolams represent the alcohol soluble fraction that are enriched in proline and glutamine (DIETERICH et al. 2003). Based on the genus type of the grain, the prolams can be distinguished into gliadins from wheat,
secalins from rye and hordeins from barley (MAKI and COLLIN 1997). The high proline content render these proteins relatively resistant to proteolytic digestion by gastric, pancreatic and brush border (BB) enzymes in the human intestine, which results in the presence of relatively large proline- and glutamine-enriched peptides in the small intestine (KAGNOFF 2005). At this, the prolines produce a kink in the polypeptide backbone. Moreover, peptidases are unable to cleave the adjacent peptide bond (CICLITIRA et al. 2005). Therefore, proline residues often dictate the fragments produced by proteolytic digestion. While wheat, rye and barley have a common ancestral origin in the grass family (Triticeae), oat is more distantly related to them (Avenae) (KAGNOFF 2005). This might be a reason why oat proteins are not incompatible for all patients, since only a small number of gluten-sensitive patients display a specific small intestinal T cell response to oat peptides (ELLIS and CICLITIRA 2008). Since wheat is the most common cereal used in the west, several characteristics of wheat gluten have already been ascertained, especially in regard to gliadins. Due to their electrophoretic behavior and primary structure the gliadin proteins are further classified into the fractions of $\alpha$-, $\gamma$- and $\omega$-gliadins (DIETERICH et al. 2003). Digestion of wheat gluten results in a mix of gliadin peptides and glutenin polypeptides and both of them contain so called “toxic” and immunodominant peptides (DEWAR et al. 2006; MOLBERG et al. 2003; VADER et al. 2002; VAN DE WAL et al. 1999; DICKE et al. 1953), which play a critical role in CD. Although more is known about gliadins, several peptides that derive from both gliadins and glutenins are described to have the capacity of T cell stimulation.

The exclusive association of DQ2 and DQ8 with CD suggests a pivotal interaction between gluten peptides and these MHC class II molecules. In fact, the binding affinity of the MHCII is very specific (ELLIS et al. 2003) and critically sensitive to amino acid structures (CICLITIRA et al. 2005). On the one hand, there is the optimal requirement for MHC binding and T cell stimulation that is given by 10-15 residues. On the other hand, the DQ2 molecules show a predilection for negatively charged amino acids in position 4, 6 and 7 of the binding groove (VAN DE WAL et al. 1996). At this, the high content of glutamines plays a decisive role. The glutamines make the gliadins a preferred substrate for the tissue transglutaminase (tTG) (DIETERICH et al. 2003). This enzyme catalyzes the deamidation of glutamine to glutamic acid residues, producing negatively charged gliadin peptides that fit into the groove of the disease-associated HLA-DQ2- and DQ8 molecules. In this context, short peptides of $\alpha$-
and γ- gliadins that stimulate T cells of patients with CD can be deamidated, which transforms them into more potent T cell epitopes (ARENZ-HANSEN et al. 2002; FLECKENSTEIN et al. 2002; ANDERSON et al. 2000; MOLBERG et al. 1998). Some of these peptides are able to induce a T cell response in most of the patients that is why they are called “immunodominant”. An example is the immunodominant sequence of the α-gliadin: α-9 (57-68) QLQPFPQPQLPY (ARENTHANSEN et al. 2000). On the other hand, there are peptides that are described as “toxic”, since they alter the mucosa of celiac patients and induce villous atrophy. A very prominent example for this is the “toxic” epitope within the sequence 31-49 of the α-gliadin (LGQQQPFPQPYPQPQPF), which is also used in the truncated form 31-43 (DE RITIS et al. 1988).

1.3 The Gastrointestinal (GI) Tract

After ingestion, foods and liquids need to be broken down mechanically and chemically into smaller particles and molecules, a process called digestion. These molecules can be absorbed through the wall of the small intestine and transferred around the body via blood vessels to supply nutrients to cells and organs and to provide a source of energy. Moreover, the collection and elimination of waste products is also an important part of digestion. Indigestible food components (e.g. fibers) that line the digestive tract are eliminated from the body as feces.

This pivotal process is accomplished by the gastrointestinal (GI) tract, also known as the digestive tract or gut. The GI tract is the body cavity that extends between the mouth and the anus (Fig. 1-3) and in which food is digested, nutrients absorbed and waste products eliminated.

During digestion the food enters the mouth, goes through the pharynx, the esophagus and reaches the stomach. From here, the chyme (mixture of partly digested food and gastric secretions) enters the small intestine, the main absorptive organ of the GI tract. Thereafter, the waste products reach the large intestine and the rectum and are finally eliminated through the anus (Fig. 1-3). In the mouth, stomach, and small intestine, the mucosa (the inner membrane of an organ) contains tiny glands that produce digestive juices to support the digestion of food. They secrete several enzymes like amylase, pepsin and trypsin that are involved in the
proteolytic cleavage of food proteins. These, so called, accessory organs are represented by the salivary glands, liver, gallbladder, and pancreas (see Fig. 1-3). The final break down of carbohydrates, fats, and proteins occurs through BB enzymes in the small intestine, the organ where almost all nutrients are absorbed. Beside enzymatic assistance, the digestive tract also contains a layer of smooth muscles that supports the food break down mechanically by peristaltic movements which guide the food along the tract.

This part was generated with the aid of the following books: CAMPBELL (1997), BARTELS and BARTELS (1987) and JOHNSON (1987)

1.3.1 The Small Intestine

Since CD is an inflammatory disease of the small intestine, a main topic deals with different processes that take place there during etiopathology.

The small intestine is the largest part of the GI tract with approximately 9m length. Longitudinally, it can be divided into duodenum, jejunum, and ileum (Fig. 1-4), and the
The intestinal wall is composed of 4 tissue layers: the intestinal mucosa, submucosa, muscularis, and serosa.

The small intestine provides the area where the vast majority of digestion takes place and where almost all of the nutrients from food are absorbed into the blood. But at the same time, however, it must also provide an efficient barrier that will exclude undigested dietary molecules, bacterial compounds, environmental toxins, and all other molecules that are present in the food and which would pose a serious threat to health, if allowed to pass through the intestinal mucosa and access the systemic circulation.

**Figure 1-4: The small intestine**
The small intestine can be divided into duodenum, jejunum, and ileum. Moreover, it contains many distinct cellular structures and cell types, which serve specific functions.
(Source: Encyclopedia Britannica Inc.; modified)

Most of the digestive enzymes (from the digestive juices) that act in the small intestine are secreted by the pancreas and enter the small intestine via the pancreatic duct. This occurs in response to the hormone cholecystokinin, which is produced in the small intestine due to the presence of nutrients. Another pancreatic hormone, secretin, causes the release of bicarbonates into the small intestine in order to neutralize the potentially harmful acid coming from the stomach.

The three major classes of nutrients that undergo digestion are: carbohydrates, lipids (fats) and proteins:

Proteins and peptides are degraded into amino acids. Chemical breakdown begins in the stomach and is continued in the small intestine by pancreatic enzymes, including trypsin and
chymotrypsin, which proteolytically cleave proteins into smaller peptides. Pancreatic carboxypeptidase will then process them into amino acids, whereas aminopeptidase and dipeptidase release the terminal amino acid products. 

**Lipids (fats)** are degraded into fatty acids and glycerol. Pancreatic lipase breaks down triglycerides into free fatty acids and monoglycerides with the help of secreted salts from the bile.

**Carbohydrates** are degraded into simple sugars, or monosaccharide’s (e.g., glucose). Pancreatic amylase breaks down carbohydrates into oligosaccharides that are further hydrolyzed by BB enzymes like glucoamylase, which break down oligosaccharides, or by maltase, sucrase and lactase.

Absorption of these nutrient molecules occurs via the intestinal mucosa, which is lined with simple columnar epithelial cells. Structurally, the mucosa is formed by numerous folds, called plicae circulares that are covered by finger-like protrusions, the villi (see Fig. 1-5), and crypts that represent the invaginations of the epithelium around the villi. Furthermore, each individual enterocyte of the villus is also covered with a huge number of finger-like protrusions at the apical membrane, the microvilli. Since the appearance of microvilli is very similar to fringes or bristles, the microvilli-covered membrane of the enterocytes is also termed brush border (BB) or brush border membrane (BBM). Especially the architecture of the BBM, composed of microvilli and small folds, leads to the great enlargement of the intestinal surface and thus to a high efficient absorptive area for nutrients. In general, the BBMs are the largest exposed surfaces in tissues. They constitute the interface between the exterior and the interior milieu of the body in a variety of organs, such as the GI tract (e.g. the small intestine) (NAIM 2005) or the kidney. Furthermore, they mainly maintain the intestinal barrier function of the intestinal epithelium (SNOECK et al. 2005). For this reason, the BBM of enterocytes provide the first area of contact for macromolecules with the intestinal mucosa.

This part was generated with the aid of the following sources: CAMPBELL (1997) and THOMSON et al. (2003a; 2003b)
Figure 1-5: The architecture of the intestinal mucosa

The intestinal mucosa is formed by a large number of folds, so called plicae circulares that are cover by finger-like projections termed villi (single: villus). The epithelium of the villi is composed of numerous intestinal (epithelial) cells that are also covered by a huge number of small finger-like protrusions called microvilli. The apical membrane of the epithelial cells that is covered with densely packed microvilli is also termed brush border membrane (BBM).

[Source: www.med.umich.edu/.../fieldTripIntestine.html; modified]

1.3.2 Uptake of Nutrient Molecules and Oral Tolerance

Internalization of nutritional macromolecules usually occurs by binding to receptors in an unspecific manner, by active or passive uptake via transporter systems, or by fluid phase endocytosis (pinocytosis) as shown for BSA (bovine serum albumin) and HRP (horse radish peroxidase) (BUONO et al. 2007; LAMAZE and SCHMID 1995; ADAMS et al. 1982). As mentioned, the high proline content of gliadin has a protective function against proteolytic digestion during the passage of the GI tract. This results in the appearance of relatively large polypeptides that have to pass the BBM. Until now the underlying mechanism by which these
polypeptides enter the intestinal epithelium is still obscure although some potential mechanisms have been discussed. MATYSIAK-BUDNIK et al. (2008) described the transferrin receptor CD71 to be involved in the retrotranscytosis of toxic peptide 31-49 and LAMMERS et al. (2008) show that gliadin binds to chemokine receptor CXCR3 and leads to MyD88-dependent zonulin release and increased intestinal permeability.

Approximately 90% of oral ingested proteins are absorbed as small peptides whereas only a small, but antigenically significant amount of food proteins enter the enterocytes in the native or intact form (HEYMAN et al. 1989). Processing and presentation of those antigens play a central role in the regulation of immune responses (GERMAIN and MARGULIES 1993) and the induction of oral tolerance. The latter process is described as the down-regulation of the systemic immune response to orally administered antigens via the generation of active cellular suppression or clonal anergy (FRIEDMAN and WEINER 1994). Although enterocytes are not conventional antigen presenting cells, they probably play a role, not only in antigen transport, but also in antigen presentation by MHCII molecules to underlying lymphocytes (BLAND 1996; KAISERLIAN 1996). Here, enterocytes are supposed to have a suppressive activity, as they do not express co-stimulatory factors like CD80 or CD86 under non-inflammatory conditions (BLUMBERG et al. 1999). In contrast, those peptides that escape lysosomal degradation, and therewith its presentation by MHCII, appear to be a small fraction of food antigens, which have functional or immune activity (TERPEND et al. 1998; HEYMAN et al. 1989) as e.g. shown for certain gliadin peptides.

1.3.3 Polarized Epithelial Cells

The intestinal mucosa is unique in its enormous repertoire of functional diversity since a lot of hydrolytic, absorptive and secretory processes take place there. The plasma membrane of epithelial cells like intestinal enterocytes is organized in two different domains; the apical and the basolateral domain (SALAS et al. 1997). The apical domain faces the lumen of the cavity like that of the intestine or the lung and is often characterized by specialized structures like microvilli (see Fig. 1-6). The basolateral domain contacts adjacent cells and the basement membranes. This asymmetric characteristic, also known as polarization, enables epithelia to accomplish their most specialized roles including absorption and secretion or to communicate.
with neighbouring tissues and cells (RODRIGUEZ-BOULAN and NELSON 1989; SIMONS and FULLER 1985). The polarized phenotype of the cell is mainly dependent on actin cytoskeleton, spectrin-based membrane skeleton and microtubules (MAYS et al. 1994). Tight junctions (TJs) also have a fundamental role during development of cell surface polarity, since separation of the two domains occurs via TJs (Fig.1-6), which prevent diffusion of proteins and lipids to the other domain (RODRIGUEZ et al. 1994). Moreover, in collaboration with adherens junctions, TJs regulate epithelial barrier function. While connecting neighboring cells with each other, they mediate vectorial transport of water and electrolytes across the epithelium, but prevent leakage of macromolecules from the lumen (DAUGHERTY and MRSNY 1999). SANDER et al. (2005) were able to demonstrate that treatment with gliadin peptides alter the expression of several tight junction proteins, leading thus to an enhancement of the epithelial permeability. Consequently this will increase the paracellular flux of macromolecules e.g. gliadin peptides, which access the immune system more rapidly and not completely degraded.

The existence of distinct sets of membrane proteins on the apical and basolateral surfaces enables the different functions of these membrane domains. The BBM of enterocytes is, for instance, enriched in several glycoproteins like the disaccharidases sucrase-isomaltase (SI) and lactase phlorizin hydrolase (LPH), or the peptidases dipeptidylpeptidase IV (DPPIV) and aminopeptidase N (ApN) (HAURI et al. 1985) that are responsible for the final digestion of nutrients molecules. The establishment and maintenance of such an epithelial asymmetry, or polarity, in spite of the dynamics of lipids and proteins at either surface, depends on multiple cellular mechanisms (RODRIGUEZ et al. 1994) and requires sophisticated sorting and trafficking mechanisms (SLIMANE and TRUGNAN 2003). The origin depends on the correct sorting of newly synthesized apical and basolateral membrane proteins at the trans-Golgi Network (TGN) (SIMONS and WANDINGER-NESS 1990). From here, the correct transport and specific fusion of the carrier vesicles to the appropriate domain are as important as the retention of the membrane proteins in their correct position (RODRIGUEZ-BOULAN and NELSON 1989). For this purpose, polarized epithelial cells are known to use various cytoskeletal tracks like actin filaments or microtubules (see Fig. 1-6) (ZEGERS et al. 1998; MAYS et al. 1994) that are involved in transport routes of membrane proteins. In this regard,
several glycoproteins that are enriched in the BBM of enterocytes have been well studied and characterized (LOUVARD et al. 1992; HAURI et al. 1985; FUJITA et al. 1972).

Figure 1-6: The polarized epithelial cell
Normally, epithelial cells have two distinct surfaces — apical and basolateral — which are separated by tight junctions. The apical surface of intestinal epithelial cells is rich in microvilli, which are responsible for nutrient absorption. Sorting of newly synthesized proteins occurs in the Golgi complex, from where they were released and transported to either the apical or basolateral membrane. Therefore protein trafficking in polarized cell implicates cytoskeletal tracks like actin filaments or microtubules.

Source: www.nature.com/.../fig_tab/nature01602_F5.html (modified)

Beside primary enterocytes, Caco-2 cells are common as an in vitro model for intestinal epithelial cells. They derived from human colonic adenocarcinomas and have retained the ability to differentiate in culture (ZWEIBAUM et al. 1991). Caco-2 cells have been used by many groups in regard to a broad spectrum of intestinal and epithelial parameters (SAMBUY et al. 2005; LOUVARD et al. 1992; ZWEIBAUM et al. 1991). Upon differentiation, they express several morphological and biochemical characteristics of small intestinal enterocytes.
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(GIULIANO and WOOD 1991; PINTO et al. 1983), although they originate from colon. They provide a BBM similar to that of enterocytes, which consists of densely packed uniformly organized microvilli that are anchored to a subjacent filamentous terminal web (Fig. 1-6), the BB cytoskeleton.

Several glycoproteins of the small intestine are endogenously expressed in the BB of Caco-2 cells, like SI, DPPIV and ApN (HAURI et al. 1985; ZWEIBAUM et al. 1984). In addition, a lot of studies have been performed concerning aspects of BB assembly (COSTA DE BEAUREGARD et al. 1995), interactions between the BBM and the cytoskeleton (PETERSON and MOOSEKER 1993), the “secretory pathway” (HAURI and MATTER 1991; MATTER and HAURI 1991), or endocytosis (JACKMAN et al. 1994) and endocytic pathways (HUGHSON and HOPKINS 1990). Thus, Caco-2 cells provide an excellent tool to study certain aspects of intestinal disorders on the cellular level.

1.4 Intestinal Glycoproteins

In general, a major requirement of proteins to achieve full functionality is to be correctly synthesized, folded, processed and transported to its final destination. Failure with regard to any of these processes can have various consequences.

During the last years, several intestinal glycoproteins that play not only a role in digestion and absorption of nutrients, but also or rather consequently in the development of different intestinal diseases have been identified and characterized. Most of them are disaccharidases and dipeptidases (HAURI et al. 1985), and several groups have examined their expression, their function and regulation, or their transport behavior in intestinal enterocytes (DANIELSEN and COWELL 1985b; FRANSEN et al. 1985) as well as in Caco-2 cells (MATTER et al. 1990a; HAURI et al. 1985). Especially the ability of Caco-2 cells to express a phenotype close to that of enterocytes render them a useful tool in understanding the underlying pathomechanism of certain intestinal disorders related to protein dysfunctions.
1.4.1 Sucrase-Isomaltase (SI)

Sucrase-isomaltase (SI) (EC 3.2.1.48-10) is an integral type II glycoprotein (BLOBEL 1980) with a size of 245kDa and belongs to the family of intestinal disaccharidases or hydrolases. When the precursor-polypeptide reaches the plasma membrane it is proteolytically cleaved by pancreatic Trypsin to sucrase (145 kDa) and isomaltase (130 kDa) (NAIM et al. 1988b; HAURI et al. 1985), but the subunits remain connected (HUNZIKER et al. 1986). SI is located at the apical membrane (BB) of enterocytes and Caco-2 cells and it is responsible for the enzymatic cleavage of disaccharides to monosaccharide’s that can be absorbed by the cells. Therefore, sucrase is cleaved at position $\alpha$-1,2 to glucose and fructose and isomaltose is cleaved at position $\alpha$-1,6, which also results in the production of glucose. This enzymatic activity fails in patients that suffer from CSID. Here, the hydrolysis of sucrose does not occur and the cells are not able to absorb the undigested sugar. Consequently, sucrose causes an osmotic effect in the small intestine and is further fermented in the large intestine, which subsequently leads to abdominal pain and diarrhea (SPODSBERG et al. 2001).

SI contains 1827 amino acids (NAIM et al. 1988b; HUNZIKER et al. 1986) with 5 functional domains and it is synthesized in the endoplasmic reticulum (ER) as a common precursor peptide (pro-SI), which is further integrated in the plasma membrane as an integral glycoprotein. On its way to the plasma membrane, SI undergoes several modifications. The pro-SI fragment provides five $O$-glycosylation sites and each subunit possesses eight $N$-glycosylation sites as well. However, regarding sucrase, only seven of these eight $N$-glycosylation sites get complex glycosylated whereas the eighth remains in the mannose-rich form (NAIM et al. 1988b). $N$-glycosylation starts in the ER lumen where $N$-acetylglucosamine residues are transferred to the amino groups of asparagines (KORNFELD and KORNFELD 1985) and is finished on the way to the trans-Golgi network (TGN). Typical $N$-linked oligosaccharides always contain mannose as well as $N$-acetylglucosamine and usually have several branches, each terminating with a negatively charged sialic acid residue (LODISH et al. 2000). Due to the high content of mannose, the protein is called mannose-rich at this stage. On the other hand, the $O$-glycosidic binding of glycans begins in the Golgi apparatus and ends with a complex glycosylated pro-SI polypeptide. Here, short side chains of about 1 to 4 sugar residues are transferred via $N$-acetylglucosamine to the hydroxyl group of serine or threonine (LODISH et al. 2000). Separation via SDS-PAGE
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(sodium dodecyl sulphate polyacrylamid gel electrophoresis) results in two bands; one at 245kDa, representing the complex glycosylated form of SI (pro-SIc), and one at 210kDa, which indicates the mannose-rich form (pro-SIh) (NAIM et al. 1988b). After all modifications and protein processing steps are completed, the pro-SI is released from the TGN within vesicles and is directly transported to its final destination, the apical plasma membrane (in epithelial cells) (LE BIVIC et al. 1990; MATTER et al. 1990a). The correct sorting of a protein depends on special sorting signals that tell the cell their final location. Therefore, different sorting signals have been described. Basolateral signals are usually located in the cytoplasmic tails of transmembrane proteins, while the signals for apical targeting are diverse in nature, structure and location. Some proteins are linked to a GPI-(glycosylphosphatidyl inositol) anchor (LISANTI et al. 1990), the targeting of other proteins depend on their N- and / or O-glycosylation (ALFALAH et al. 1999; SCHEIFFELE et al. 1995), or the signal is even found in the cytosolic part of the apically sorted protein (RODRIGUEZ-BOULAN and GONZALEZ 1999; FIEDLER and SIMONS 1995). Concomitant with the diversity of sorting signals, there are multiple transport mechanisms that deliver the sorted proteins in distinct carriers to the apical membrane. Some proteins associate with special membrane domains so called detergent-insoluble lipid microdomains that are enriched in glycosphingolipids and cholesterol and that are insoluble with the detergent Triton X-100. Due to their detergent-insolubility they were also termed detergent insoluble glycolipid-enriched complexes (DIGs) (ALFALAH et al. 2002; JACOB and NAIM 2001; JACOB et al. 2000) or DRM’s (detergent resistant membranes). Since these microdomains function as some sort of floating platform for the transport of certain proteins, they were also termed rafts (SIMONS and IKONEN 1997). BROWN and LONDON (2000; 2000; 1998) were able to demonstrate that the association of glycolipids and glycolipid-bound proteins with microdomains already occur in the TGN, from where they are transported along microtubules (GILBERT et al. 1991; ACHLER et al. 1989) and actin filaments (JACOB et al. 2003) to the plasma membrane (MAPLES et al. 1997).

The apical sorting of pro-SI depends on both the O-glycosylation and the association with lipid microdomains or rafts, as inhibition of one of these points or the association with the actin cytoskeleton results in a loss of the apical sorting (JACOB et al. 2003; ALFALAH et al. 1999).
1.4.2 Lactase Phlorizin Hydrolase (LPH)

The human small intestinal lactase-phlorizin hydrolase (LPH) (EC 3.2.123-62) is a homodimeric enzyme complex of about 230kDa size that belongs to the family of disaccharidases or hydrolases. LPH is an integral type I protein as the N-terminus faces the extra cellular site, while the C-terminus lies intracellular. Similar to SI, it represents a glycoprotein with two enzymatic activities (COLOMBO et al. 1973). Lactase is responsible for the hydrolytic digestion of lactose (JACOB et al. 1994), the main disaccharide in milk into the monosaccharide’s glucose and galactose that can be absorbed at the BB. However, the phlorizin hydrolase (‘aryl-β-glucosidase’) catalyzes the hydrolysis of β-glycosyl ceramides (MANTEI et al. 1988), the ‘natural’ substrate found in the food of most vertebrates (LEESE and SEMENZA 1973). If the hydrolysis is incomplete, similar to that of sucrose in CSID, the production of absorptive monosaccharide’s fails and osmotically active di- and oligosaccharides induce sickness, convulsion and diarrhea (PHILLIPS 1981) due to bacterial fermentation in the large intestine.

LPH is synthesized as a 1927 amino acids (MANTEI et al. 1988) long single-chain polypeptide precursor (prepro-LPH) that can be divided into four highly conserved functional domains. This prepro-LPH undergoes a sequential cleavage step in the ER to pro-LPH (215kDa), representing the N-glycosylated high mannose-rich form (pro-LPHh) (NAIM 1992a) with 15 potential N-glycosidic binding sides. Before leaving the ER to reach the Golgi apparatus, two monomeric pro-LPHh molecules come together to form a dimer. Dimerization is essential for the transport competence of pro-LPH and is strongly associated with the presence of an intact transmembrane domain (NAIM and NAIM 1996). In addition, dimerization is also linked to the acquisition of LPH to its biological function and therefore to its enzymatic activity. Failure of dimerization results in retention of the monomeric pro-LPHh molecules in the ER followed by their degradation (NAIM and NAIM 1996). The terminal glycosylation occurs in the Golgi apparatus where the O-glycosylation takes place at several serine and threonine residues and N-glycosylation is completed. The O-glycosylation will further accelerate the hydrolysis of lactose. The complex-glycosylated pro-LPHc (230kDa) leaves the TGN (NAIM et al. 1991; HAURI et al. 1985) and undergoes a cleavage to the mature 160kDa LPH (denoted LPHm) (JACOB et al. 1994; NAIM 1992a). The LPHm
molecule is subsequently targeted to the BBM where it is cleaved by pancreatic trypsin to its final size of 145kDa (JACOB et al. 1996; NAIM et al. 1991).

By contrast to pro-LPH in human enterocytes, the expressed pro-LPHc (230kDa) in COS-1 cells does not undergo intracellular proteolytic cleavage to generate a form similar to the mature enzyme (145kDa) of the BBM. Intracellular cleavage, however, is not essential for the molecule to acquire its enzymatic activity, since the pro-LPH in COS-1 cells shows a similar enzymatic activity as LPH isolated from intestinal BBMs (NAIM et al. 1991).

Unlike SI, LPH does not associate with Triton X-100 insoluble rafts, although both proteins are initially transported in the same vesicle when leaving the TGN (JACOB and NAIM 2001). These post-Golgi vesicles are transported along microtubules before they separate into distinct carriers from which the SI-containing vesicles finish their transport to the apical membrane via actin filaments, whereas LPH is supposed to maintain its route on the microtubules (JACOB et al. 2003).

1.4.3 Dipeptidylpeptidase IV (DPPIV)

Dipeptidylpeptidase IV (DPPIV / CD26) (EC 3.4.14.5) is a multifunctional, intrinsic membrane glycoprotein. It belongs to the family of serine proteases and exerts its different functions depending on cell type and intra- or extracellular conditions in which it is expressed (BOONACKER and VAN NOORDEN 2003). For example, it is widely expressed on the surface of a variety of epithelial, endothelial, and lymphoid cells (DE MEESTER et al. 1999; ABBOTT et al. 1994) whereas a soluble form is also found in plasma (IWAKI-EGAWA et al. 1998). It preferentially cleaves N-terminal dipeptides from polypeptides with proline (proline-specific ectopeptidase) or alanine in the penultimate position (HAVRE et al. 2008) and by doing this, it regulates various physiological processes.

The human DPPIV cDNA encodes for a sequence of 766 amino acids (DARMOUL et al. 1992; MISUMI et al. 1992). The mass (110-150kDa) varies depending on the species, the type of tissue and the level of glycosylation (IKEHARA et al. 1994). DPPIV is a type II membrane glycoprotein that is synthesized with an uncleavable signal sequence that functions as a membrane anchoring domain (FAN et al. 1997; MISUMI et al. 1992). Human DPPIV provides 9 potential N-glycosylation sites that are uniformly distributed
all over the ectodomains of this protein (AERTGEERTS et al. 2004; MISUMI et al. 1992). In Caco-2 cells, processing of the 100-kDa high mannose-rich DPPIVh species to the 124kDa complex glycosylated mature form (DPPIVc) includes an extensive O-glycosylation event, mainly in a Ser / Thr-rich stalk domain adjacent to the membrane anchor (MATTER and HAURI 1991). After maturation in the Golgi apparatus, DPPIV is transported to the apical membrane, either directly from the TGN or along the transcytotic pathway through the basolateral membrane (LOW et al. 1991; LE BIVIC et al. 1990; MATTER et al. 1990a) for which microtubules have been described to be crucial (LEUNG et al. 2000; BREITFELD et al. 1990; HUNZIKER et al. 1990). In Caco-2 cells, the apical sorting of human DPPIV occurs through both complex N-linked and O-linked carbohydrates and implicates its association with Triton X-100 insoluble microdomains, containing cholesterol and sphingolipids (ALFALAH et al. 2002). In contrast to SI, the glycosphingolipids are less crucial for the sorting compared to cholesterol. The membrane-bound DPPIV is present in the microvillar membrane as a homodimer that is formed of two identical monomers (JASCUR et al. 1991). Dimerization occurs immediately after complex glycosylation in the Golgi apparatus (JASCUR et al. 1991) and is essential for the enzyme activity of DPPIV, since the monomeric form alone has no enzyme activity (DE MEESTER et al. 1992).

1.4.4 Aminopeptidase N (ApN)

Aminopeptidase N (ApN / CD13) (EC 3.4.11.2) is a type II transmembrane glycoprotein that is expressed on the surface of a broad variety of cell types, most strongly in intestinal mucosa and kidney tissue (BARNES et al. 1997; LUCIUS et al. 1995). Similar to DPPIV, APN exists in two forms namely the membrane aminopeptidase N and the soluble aminopeptidase N (LUAN and XU 2007). Although not all aspects of its function are fully understood, it is established that the enzyme preferentially cleaves neutral amino acids from the N terminus of oligopeptides, leading thus to degradation of e.g. neuropeptides (AHMAD et al. 1992) or cytokines (KANAYAMA et al. 1995). Depending on its location, APN is also involved in the terminal degradation of small peptides in the intestinal BBM (NOREN et al. 1986). The cDNA of human ApN encodes for a sequence of 967 amino acids (OLSEN et al. 1988), which results in a 115kDa precursor polypeptide (DANIELSEN et al. 1982). This precursor
molecule undergoes further modifications in that it becomes extensively \( N \)- and \( O \)-glycosylated (DANIELSEN et al. 1995; DANIELSEN et al. 1983) in the ER and Golgi-apparatus due to its 10 potential \( N \)-glycosylation sites (OLSEN et al. 1988). Separation via SDS-PAGE results in two bands, one at approximately 160kDa, representing the complex glycosylated form of ApN (ApNc) and one at 130kDa, which indicates the high mannose-rich form of ApN (ApNh) (NAIM et al. 1988a; HAURI et al. 1985). Moreover, ApN dimerizes predominantly before it becomes complex glycosylated in the Golgi and is later expressed as a non-covalent homodimer at the cell surface (DANIELSEN 1994; NAIM 1992b). After completion of all modifications, the protein is released from the TGN and is transported to its final destination, which is the BBM in intestinal cells. Similar to DPPIV, human ApN is either directly sorted to the apical membrane or via transcytosis (LE BIVIC et al. 1990; MATTER et al. 1990a). The apical sorting signal is localized in the catalytic head group of the ectodomain (VOGEL et al. 1992a; VOGEL et al. 1992b). In contrast to SI and DPPIV, the sorting of ApN implicates neither \( O \)-linked nor \( N \)-linked glycans and is driven most likely by carbohydrate-independent mechanisms (NAIM et al. 1999).

1.5 The Cytoskeleton

The cytosol of eukaryotic cells contains an array of fibrous proteins, collectively called cytoskeleton (LODISH et al. 2000). The cytoskeleton is vital to the function of all eukaryotic cells, since it plays a role in mitosis, cytokinesis, cell motility, muscle contraction, maintenance of cell shape, endocytosis, and secretion (BROWN and STOW 1996; MAYS et al. 1994; KELLY 1990). Epithelial cells, which have distinct apical and basolateral plasma membrane domains, exploit cytoskeletal elements to ensure efficient targeting of newly synthesized proteins from the TGN to the appropriate cell surface domain (MAYS et al. 1994). The cytoskeleton also plays a role in endocytosis, exit of cargo from early and late endosomes, and the transport of endocytosed proteins from one plasma membrane domain to the opposite (transcytosis) (MUKHERJEE et al. 1997). In addition to its function in protein and lipid trafficking, the cytoskeleton is also important in protein sorting and can stabilize newly synthesized proteins at one plasma membrane domain or the other (MAYS et al. 1994).
Since, apart from microtubules, the actin cytoskeleton plays a prominent role in most of these processes, especially in the maintenance of cell shape, during endocytosis, and protein transport and sorting, it will be described in more detail.

### 1.5.1 The Actin Cytoskeleton

Actin is one of the most common proteins in eukaryotic cells. In muscle cells, it represents about 20% and in non-muscle cells 5-10% of the total protein (MOLBERG et al. 2003). In non-muscle cells, actin is part of the cytoskeleton and is therefore involved in a wide range of cellular events as described above. The ability to contribute to such different processes within the cells is especially due to the fact that actin co-exists in two forms, a globular form (G-actin) and a filamentous form (F-actin). The G-actin subunit is synthesized as a single polypeptide of about 375 amino acids, which results in a size of approximately 42kDa (PONTE et al. 1984; ELZINGA et al. 1973). However, the F-actin is a helical polymer made of non-covalently bound G-actin subunits. The polymer is considered to consist of two parallel protofilaments that twist around each other in a right-handed helix (ALBERTS et al. 2001).

Due to its rate of acidic amino acids at the N-terminus 3 isoforms can be distinguished: α-, β- und γ-actin (ZECHEL and WEBER 1978). However, the actin cytoskeleton mainly consist of β-actin. The G-actin molecule harbors two binding sites: one of them is a nucleotide binding site that binds ATP (adenosine triphosphate) and the other binds ions like magnesium (Mg^{2+}) or potassium (K^+) (LODISH et al. 2000). The binding of ions induces the assembly of G-actin into F-actin and is termed polymerization. The polymerization starts with aggregation of G-actin into short, unstable oligomers. Once the oligomer reaches a certain length (3 to 4 subunits) it can act as a stable *nucleus* which then rapidly elongates into a filament by further addition of G-actin monomers to both sides of its end. This assembly is accompanied by the hydrolysis of ATP to ADP (adenosine diphosphate) and an inorganic phosphate (P_i), which affects the kinetic of the polymerization (LODISH et al. 2000). In the presence of ATP, actin molecules are no longer identical, which results from ATP hydrolysis after monomer incorporation into filaments. The free actin monomers consist of ATP-actin and the interior of the filaments consists of ADP-actin. The terminal actin molecules at the filament ends may be
at intermediate stages of ATP hydrolysis, containing bound ATP or ADP. P or ADP (WEBER 1999). The actin functions depend on the dynamic interactions of monomers and polymers (WEBER 1999), which means apart from polymerization that the actin filaments have to disassemble once again (FUJIWARA et al. 2002), a process called depolymerization. Actin filaments assemble or disassemble with rising or failing monomer concentration and there are various mechanisms depending on the cell type by which actin binding or capping proteins, like profilin, tropomyosin (WEBER 1999), coflin (CARLIER et al. 1997) or Arp2/3 (POLLARD 2007; SVITKINA and BORISY 1999) alter the free actin monomer concentration, the actin distribution between monomers and polymers, and the rates of actin filament assembly and disassembly (WEBER 1999).

In intestinal enterocytes or cultured epithelial cells that retain features of e.g. Caco-2 cells the actin-based cytoskeleton is implicated in the formation and maintenance of the BBM, which consist of two domains, the microvilli and the terminal web (see Fig. 1-7) The latter is a cytoskeleton-rich region in the apical cytoplasm beneath the microvilli (LOUVARD et al. 1992; PETERSON and MOOSEKER 1992).

Each microvillus contains a core of uniformly polarized actin bundles held together by villin and fimbrin (ALBERTS et al. 2001; PETERSON and MOOSEKER 1992). These cores are linked to the overlying membrane by cross-bridges composed of different proteins like BB myosin I (COLUCCIO 1997; MOOSEKER et al. 1991) or the 110kD-CM (Calmodulin-Myosin) protein complex, a complex of myosin IA and 3 or 4 calmodulins (HOWE and MOOSEKER 1983; GLENNEY et al. 1982; MATSUDAIRA and BURGESS 1982). The actin cores extend as rootlets into the underlying terminal web domain where they are cross linked together by a meshwork of nonerythroid spectrins (COLEMAN et al. 1989) and myosin II oligomers. The actin network that is found in the intracellular underlying body is also termed the cortical actin network (LODISH et al. 2000).

Beside the formation and maintenance of the BB or the cell shape in general, the actin cytoskeleton also takes part in a wide range of other cellular events like protein trafficking (DEPINA and LANGFORD 1999) as mentioned for SI in section 1.4. But not all proteins depend on an intact actin cytoskeleton to be correctly sorted and transported.
For example, the transport of *raft*-associated SI was severely affected through disruption of the actin cytoskeleton due to cell-treatment with fungal toxin cytochalasin D (COOPER 1987) whereas the transport of LPH that is not associated with triton X-100 DRMs was not affected (JACOB et al. 2003). Thus, although SI and LPH initially share *post*-Golgi vesicles (JACOB and NAIM 2001) LPH is transported via an actin-independent pathway that possibly requires microtubules as has been described for other proteins (ACHLER et al. 1989). Therefore, the association with different kinds of DRMs might be a potential sorting signal that routes the transport vesicles either along actin filaments or microtubules.

Figure 1-7: The BB-associated actin cytoskeleton.
The cytoskeleton of the intestinal brush border can be divided into two distinct areas: the microvilli (MV) and the terminal web, which is a cytoskeleton-rich region in the apical cytoplasm beneath the MV. The core of the MV is composed of bundled actin filaments. All filaments show the same polarity, with their nucleation point located near the tip of the MV. The rootlets of these filaments are plunging in the terminal web. (From: The Art of MBoC3 © 1995 Garland Publishing, Inc.)
Moreover, LANGFORD et al. (1994) observed a transition of transport vesicles from microtubules to actin filaments. Both cytoskeletal components are associated via proteins like MAPs (microtubule-associated proteins) (GRIFFITH and POLLARD 1982). This event may enable the distinct transport routes of SI and LPH along either microtubules or actin after division of the common post-Golgi vesicle into smaller vesicles, containing only one of the two proteins, respectively (JACOB and NAIM 2001).

Other proteins, like DPPIV or ApN, do not only implicate the actin cytoskeleton for apical sorting, but also the microtubule cytoskeleton that has been described to be crucial in the basolateral to apical transcytosis (LEUNG et al. 2000; BREITFELD et al. 1990; HUNZIKER et al. 1990). So far, the underlying mechanism by which this combined protein sorting is regulated is still not clear.

Treatment with cytochalasin D (cytoD) gives an example of how the actin cytoskeleton can be affected by environmental factors or reagents, coming from the outside. In CD, the actin cytoskeleton was also found to undergo rearrangements due to the exposure of peptic-tryptic digest of gluten. These alterations were predominantly observed in biopsy specimens of celiac patients (WILSON et al. 2004; HOLMGREN PETERSON et al. 1995), but investigations on permeability (SANDER et al. 2005), activation of the epidermal growth factor receptor (EGFR) (BARONE et al. 2007) and apoptosis (GIOVANNINI et al. 2003) in Caco-2 cells did also reveal reorganizations of the actin cytoskeleton.

Until now the mechanism of the initial steps by which the disruption of the intestinal mucosa is induced, leading thus to villous atrophy and malabsorption, is still obscure. But since the actin cytoskeleton plays a decisive role in the formation and maintenance of the BBM, in the coherence of enterocytes within the intestinal epithelium and during transport events by which intestinal glycoproteins are sorted to the BBM, it might be a great Chance to focus on it.

1.6 Endocytosis

In general, endocytosis is a process in which extracellular substances or particles enter a cell without passing through the cell membrane (LODISH et al. 1995).

All eukaryotic cells exhibit one or more forms of endocytosis. Their reasons for doing so are as diverse as their individual functions. Endocytosis in eukaryotic cells is characterized by the
continuous and regulated formation of a huge number of membrane vesicles at the plasma membrane. These vesicles come in several different varieties, ranging from the actin-dependent formation of phagosomes involved in particle uptake (MUKHERJEE et al. 1997), to smaller clathrin-coated vesicles responsible for the internalization of extracellular fluid and receptor-bound ligands (MELLMAN 1996; LAMAZE and SCHMID 1995), to the point of pinocytosis or fluid phase for the uptake of fluids and solutes via small pinocytic vesicles. In general, each of these vesicle types results in the delivery of their contents to lysosomes for degradation. The membrane components of endocytic vesicles, on the other hand, are subjected to a series of highly complex and interactive molecular sorting events, resulting in their targeting to specific destinations (MELLMAN 1996). In recent years, much has been learned about the function of the endocytic pathway and the mechanisms responsible for the molecular sorting of proteins and lipids. Endocytic mechanisms serve many important cellular functions, including the uptake of extracellular nutrients, regulation of cell-surface receptor expression, recycling of proteins and lipids (SMYTHE and AYSCOUGH 2006), maintenance of cell polarity, and antigen presentation (HARDING and GEUZE 1992). Endocytic pathways are also utilized by viruses, toxins, and symbiotic microorganisms to gain entry into cells (MUKHERJEE et al. 1997; MELLMAN 1996).

As demonstrated in Fig. 1-8 endocytic traffic in polarized cells requires the dynamic capabilities of the cytoskeleton for endocytosis, exit of cargo from early and late endosomes, and the transport of endocytosed proteins from one plasma membrane domain to the opposite (transcytosis) (MUKHERJEE et al. 1997). Here, actin plays a role in internalization at both cell surfaces (step 1A and B) and in collaboration with microtubules, actin is required for efficient transcytosis and delivery of proteins to late endosomes (step 2A and B) and lysosomes (step 5) (APODACA 2001). Microtubules are also important in apical recycling pathways (step 3B) (LEUNG et al. 2000; BREITFELD et al. 1990) whereas, in some polarized cell types, basolateral recycling requires actin.
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Figure 1-8: Model for endocytic traffic in polarized epithelial cells.

Upon internalization, fluid and membrane are delivered to distinct AEE (1A) or BEE (1B). Endocytosis at both surfaces requires actin. Apically internalized fluid can recycle (3A), transcytose (4), or it can be delivered in a microtubule-dependent step to late endosomes (2A) and ultimately lysosomes (5). This latter step is actin-dependent. Basolaterally internalized fluid is primarily delivered to late endosomes (2B) and lysosomes (5). Apical recycling proteins are delivered from the AEE to the ARE (3B) or the CE (3C) before their ultimate release from the apical pole of the cell (8). Delivery between the AEE and ARE requires microtubules. The cytoskeletal requirements, if any, for delivery from the AEE to CE are presently unknown. Basolateral recycling proteins (i.e., receptor-bound Tf) as well as proteins transcytosing in the basolateral to apical direction (i.e., plgR-IgA) enter a shared BEE (1B). Although some receptor-bound Tf may recycle directly from this compartment (6B), a significant fraction is delivered to the CE along with the majority of the plgR-IgA (6A). This translocation step requires actin and microtubules. The majority of the receptor-bound Tf is thought to recycle from the CE (7B); however, a fraction may be delivered to the AEE (7C) and may recycle from this compartment (4). The transcytosing plgR-IgA complexes, as well as apical recycling plgR-IgA complexes, are delivered from the CE to the ARE (7A) and are ultimately released at the apical pole of the cell (8). Actin may be required for efficient recycling of receptor-bound Tf (6B and/or 7B). (APODACA 2001)
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The microtubule motor proteins dynein and kinesin (WOEHLKE and SCHLIWA 2000; VALE 1987) and the class I unconventional myosin motors play a role in many of these trafficking steps (TUXWORTH and TITUS 2000; MERMALL et al. 1998). Actin has been linked to endocytosis in several polarized tissue cell types, including endothelial cells (ALEXANDER et al. 1998), MDCK cells (GOTTLIEB et al. 1993), intestinal epithelial cells and Caco-2 cells (JACKMAN et al. 1994) and one of the best-characterized endocytic mechanisms is receptor-mediated endocytosis via clathrin-coated pits (MUKHERJEE et al. 1997). Structural proteins, including clathrin and adaptor proteins, are recruited from the cell cytosol and assemble on the plasma membrane forming a ~200-nm clathrin-coated pit (CCP). The CCP becomes invaginated and narrows at the neck, forming a constricted coated pit which subsequently pinches off from the plasma membrane, generating a nascent clathrin-coated vesicle (CCV). Finally, the cycle is completed by disassembly of the clathrin coat, which is utilized for subsequent rounds of endocytosis. However, the role of actin during clathrin-mediated endocytosis may vary in dependence of the cell type (APODACA 2001) and the experimental approach (YARAR et al. 2005), but growing evidence has suggested a role for the actin cytoskeleton during this type of endocytosis (MERRIFIELD 2004). For instance, disruption of the actin cytoskeleton in MDCK and Caco-2 cells due to cytoD results in a significant increase of clathrin-coated pits at the apical plasma membrane, indicating an impaired ability of these pits to pinch off (GOTTLIEB et al. 1993). Additionally, MERRIFIELD et al. (2004) suggest that N-WASP and the Arp2/3 complex trigger actin polymerization during a late step in clathrin-mediated endocytosis, and propel clathrin-coated pits or vesicles from the plasma membrane into the cytoplasm. 

Altogether, there is concrete evidence that actin plays a crucial role during several steps of endocytosis although there are differences depending on the tissue and / or the cell type.
AIM OF THE STUDY
2 AIM OF THE STUDY

Since CD is a multifactorial disorder, it is difficult to keep a broad overview of the facts coming together during etiopathology. Besides the genetic background, the environmental factor gluten plays a decisive role in the development of CD. The digestion of wheat gluten results in a mixture of gliadin peptides and glutenin polypeptides and both of them contain so-called “toxic” and immunodominant peptides (DEWAR et al. 2006; MOLBERG et al. 2003; VADER et al. 2002; VAN DE WAL et al. 1999; DICKE et al. 1953) that play a crucial role in the pathogenesis of CD. Although it has been revealed that an altered immune response to these peptides is involved in the pathogenesis of CD (SOLLID 2000; MARSH 1992), the underlying mechanism by which the cereal prolamins damage the small intestine of susceptible individuals is still unclear. That is the main reason why the only available therapy so far is based on a complete elimination of disease-activating proteins from daily food (LONDEI and MAIURI 2004). Respectively, it becomes clear that the delineation of the molecular basis of this disease is a necessity with the ultimate goal of developing innovative treatment strategies that could substantially improve the quality of life of celiac patients.

In recent years, it has been demonstrated that treatment of biopsy samples from celiac patients with gliadin peptides leads to rearrangement of the actin cytoskeleton in enterocytes (WILSON et al. 2004; HOLMGREN PETERSON et al. 1995), which goes along with more severe degrees of villous atrophy (CLEMENTE et al. 2000). Additionally, alterations of the actin cytoskeleton were also observed in Caco-2 cells that have been previously used to study effects of gliadin on metabolism, barrier function and apoptosis (SANDER et al. 2005; GIOVANNINI et al. 2003). Furthermore, with other intestinal inflammatory diseases, such as ulcerative colitis and Crohn’s disease, alterations in actin density and protein expression have been shown (KERSTING et al. 2004). Consequently, several aspects have been investigated in this respect but the biochemical basis of these effects has not been analyzed yet. The questions that arise therefore are: How do gliadin peptides alter the organization of actin cytoskeleton and what are the consequences in terms of subsequent actin dependent processes like protein transport or endocytosis?

Besides its barrier function between the luminal part and the interior side of the intestinal mucosa, the BBM of enterocytes is enriched in a large number of glycoproteins that are
essential for the digestion and absorption of nutritional components like carbohydrates and food peptides (ALPERS 1987). In the past, different transport pathways have been described for several of these intestinal glycoproteins in biopsy specimens (HANSEN et al. 1988; DANIELSEN and COWELL 1985a) and cell culture model (LE BIVIC et al. 1990; MATTER et al. 1990a). Some of these proteins, e.g. SI, require an intact actin cytoskeleton for an efficient sorting and trafficking to the plasma membrane. Other proteins such as LPH are transported via actin-independent pathways (JACOB et al. 2003) that possibly involve microtubules.

The aim of this study was to analyze in which way the actin cytoskeleton is altered by gliadin toxicity and how this effect influences different transport events in the cell. Hence, several intestinal proteins were examined in Caco-2 cells or in non-polar transfected COS-1 cells after cell treatment with a peptic-tryptic gluten digest (Frazer’s Fraction, FF), as a source of gliadin „toxic“ peptides. The transport and the sorting of the intestinal proteins were investigated with particular emphasis on alterations of the actin cytoskeleton. To get an idea of the implication caused by FF, the initial experiments were performed in non-epithelial COS-1 cells. These cells provide an ideal cellular model for the expression of various forms of proteins and protein chimeras as well as for the immediate assessment of the role of the actin cytoskeleton in the absence of other epithelial-specific cytoskeletal proteins (e.g. villin or ezrin). Another advantage is the clearly structured actin cytoskeleton of COS-1 cells rendering them a convenient tool for the assessment of distinct effects in analyses with confocal laser microscopy.

However, the main focus was placed on the implication of FF-treatment in intestinal Caco-2 cells.

 Besides actin-dependent and independent protein transport, special attention was placed on the lipid composition of the BBM of Caco-2 cells, since the transport vesicles that were released from the post-Golgi apparatus contain both proteins and lipids (LOUVARD et al. 1992). Another point we focused on was endocytosis. The actin cytoskeleton plays a crucial role in the endocytic pathway (APODACA 2001) thus an altered actin network will have an impact on endocytic events as well.
EFFECTS OF GLIADIN TOXIC PEPTIDES ON ACTIN AND SUBSEQUENT PROTEIN TRAFFICKING
3 EFFECTS OF GLIADIN TOXIC PEPTIDES ON ACTIN AND SUBSEQUENT PROTEIN TRAFFICKING

Yvonne Reinke*, Klaus-Peter Zimmer+ and Hassan Y. Naim°

*Department of Physiological Chemistry, University of Veterinary Medicine Hannover, Germany
+Department of Pediatrics, University of Giessen, Germany

°Correspondence:
Hassan Y. Naim, PhD
Department of Physiological Chemistry
University of Veterinary Medicine Hannover
Bünteweg 17
D-30559 Hannover, Germany
Tel.: +49 511 953 8780
Fax: +49 511 953 8585
Email: hassan.naim@tiho-hannover.de

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3.1 Abstract

Background information

Celiac disease (CD) is an inflammatory disease of the small intestine triggered by the ingestion of gluten in genetically predisposed individuals. Current concepts have assigned a fraction of the peptic-tryptic digest of gluten, known as Frazer’s Fraction (FF), an essential role in the pathogenesis of the disease. The morphological alterations of intestinal cells in CD suggest that cytoskeletal proteins are directly affected. Here, we investigate in COS-1 cells the immediate effects of FF on the actin cytoskeleton and the subsequent trafficking of actin-dependent and actin-independent proteins.

Results

Confocal laser microscopy of COS-1 cells treated with FF revealed substantial morphological alterations in the actin filaments, which appeared as fragmented and aggregated structures. Interestingly, the drastic reduction in immunoprecipitated actin from biosynthetically-labeled and FF-treated cells suggest a conformational change of the protein and subsequent reduced antibody-binding capacity. On the other hand, the effect induced by cytochalasin D, a depolymerizing agent, differs from that of FF as assessed by disruption of the actin cytoskeleton and its accumulation in dense foci and patches concomitant with an alteration in the cell shape. Moreover, the slightly reduced level of immunoprecipitated actin is compatible with variations in the antibody-binding capacity and possibly different conformation as compared to actin in FF-treated cells. The control employed a peptic-tryptic digest of ovalbumin, which neither affected the actin morphology nor the antibody-binding capacity.

The alterations on the actin cytoskeleton elicit impaired protein trafficking of intestinal sucrase-isomaltase, a glycoprotein that follows an actin-dependent vesicular transport to the cell surface. On the other hand, the actin-independent protein transport, represented by intestinal lactase phlorizin hydrolase, remains unaffected.

Finally, the morphological alteration in the actin cytoskeleton appears to be induced by direct association of FF with actin as demonstrated in co-immunoprecipitation experiments.

Conclusions

Altogether, our data demonstrate that „toxic“ peptic-tryptic digests of gluten represented by FF directly interact with actin and alter the integrity of actin cytoskeleton thus leading to an impaired trafficking of intestinal proteins that depend on an intact actin network.
3.2 Introduction

Celiac disease (CD) is a type of chronic inflammatory diseases. Typically, these diseases have multifactorial etiologies that involve environmental components and several genetic factors. In celiac disease the inflammation of the small intestine comes along with enteropathy, villus atrophy and malabsorption (CICLITIRA and MOODIE 2003; SOLLID 2002; MARSH 1992) in genetically susceptible individuals following dietary ingestion of gluten. Ingestion and digestion of wheat gluten results in a mixture of gliadin peptides and glutenin polypeptides, which contain „toxic“ and immunodominant peptides and play a role in the pathogenesis of celiac disease. Analysis of the gliadin toxicity in vitro has so far employed peptic-tryptic digest of either wheat gluten or isolated gliadin peptides to mimic the in vivo situation (FRAZER et al. 1959). To date, the only available therapy of celiac disease is represented by the adherence to a gluten-free diet, although intensive research is being conducted towards finding new improved treatment alternatives.

It has been demonstrated that treatment of biopsy samples from celiac patients with gliadin peptides leads to rearrangement of the actin cytoskeleton in enterocytes (WILSON et al. 2004; HOLMGREN PETERSON et al. 1995). This phenomenon could be also detected in Caco-2 cells, a human intestinal cell line that has been previously used to study effects of gliadin on metabolism, barrier function and apoptosis (SANDER et al. 2005; GIOVANNINI et al. 2003). In other intestinal inflammatory diseases, such as ulcerative colitis and Crohn’s disease, alterations in actin density and protein expression have been demonstrated (KERSTING et al. 2004). Based on this information we asked whether these effects on the actin cytoskeleton could influence the actin-dependent transport of intestinal glycoproteins.

The actin filaments constitute, in addition to microtubules and intermediate filaments a large part of the cytoskeleton of the cell. Actin forms networks as well as filament bundles (LODISH et al. 1995), whereby the bundles are important in the formation and stabilization of the microvilli of the intestinal epithelial cells (LODISH et al. 1995; RODRIGUEZ et al. 1994). In addition to its role in cell shaping, signal transduction, cell-cell contact, the actin cytoskeleton takes part also in protein and lipid trafficking (MAPLES et al. 1997; FATH and BURGESS 1993; GILBERT et al. 1991; MATTER et al. 1990b), which also implicates interacting microtubules (KUZNETSOV et al. 1992; GRIFFITH and POLLARD 1982).
The epithelial cells of the small intestine or enterocytes harbor a large number of glycoproteins that are implicated in the digestion and absorption of nutritional components. Some of these proteins, e.g. sucrase-isomaltase (SI), depend on an intact actin cytoskeleton for an efficient sorting and trafficking to the plasma membrane. Other proteins such as lactase-phlorizin hydrolase (LPH) are transported via an actin-independent pathway (JACOB et al. 2003) probably based on microtubules.

In view of this discriminating role of the actin cytoskeleton we addressed the effects of gliadin toxicity on the transport of intestinal hydrolases with particular emphasis on alterations of the actin cytoskeleton. For these analyses we utilized COS-1 cells, an ideal cellular model for expression of various forms of proteins and protein chimeras as well as for the immediate assessment of the role of the actin cytoskeleton in the absence of other epithelial-specific cytoskeletal proteins, such as villin or ezrin. Another advantage of these cells is the clear structured actin cytoskeleton rendering them very convenient for assessment of distinct effects in analyses using confocal laser microscopy.

Our data demonstrate that treatment with FF leads to a rearrangement of the actin cytoskeleton concomitant with altered trafficking of proteins that require an intact actin network.

### 3.3 Materials and Methods

#### 3.3.1 Cells and Immunochemical Reagents

COS-1 cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) low glucose (1000mg/l) medium (PAA Laboratories, Pasching, Austria) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (PAA Laboratories, Pasching, Austria). For immunoprecipitation and immunofluorescence the mAbs anti-sucrase-isomaltase HBB 614 (HAURI et al. 1985), HSI2 and HIS3 (BEAULIEU et al. 1989) and anti-lactase-phlorizin hydrolase mLac 1, 3, 4, 5, 8 (MAIURI et al. 1991) and HBB1/909 (HAURI et al. 1985) were utilized. Early endosomes were identified using a rabbit antibody against early endosomes antigen (EEA-1) (Dianova, Hamburg, Germany) and lysosomes with rabbit anti-cathepsin D (cath.D; Dako, Hamburg, Germany). For detection of fluorescent images in confocal laser microscopy.
microscopy AlexaFlour488 (mouse) and AlexaFlour568 (rabbit) (Molecular Probes, Invitrogen detection technologies, Karlsruhe, Germany) were used. Actin was expressed as a chimera fused to pEYFP (the pEYFP-N1 vector was purchased from Takara Bio Europe/Clontech, Saint Germain en Laye, France) and its isolation was performed using a rabbit antibody against GFP (BD Transduction Laboratories, Heidelberg, Germany). Detection of actin on western blots was achieved with a mAb against β-actin (C4) from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). For confocal analysis actin was also labeled with Phalloidin-Rhodamin from Molecular Probes, Invitrogen detection technologies, Karlsruhe. The mAb R5 (MENDEZ et al. 2005) was used to detect the gliadin „toxic“ peptide 31-49. [35S] methionine (1000 Ci/mmol), anti-mouse ECL-peroxidase, and protein A-Sepharose were obtained from Amersham Biosciences GE Healthcare, Freiburg, Germany. The SuperSignal ELISA Femto Maximum Sensitivity Substrate was obtained from Perbio Science, Bonn, Germany. All other chemicals and reagents were of superior analytical grade.

3.3.2 Preparation of Frazer’s Fraction

The procedure used for the preparation of Frazer’s Fraction followed the initial work by FRAZER et al. (1959). Here, 100 g gluten were digested with 0.5 g pepsin (both from Sigma Aldrich Chemie, Taufkirchen, Germany) in 0.1 M HCl (pH 1.8) for 4h at 37°C followed by a further digestion step for 4h with 0.5 g trypsin (Merck, Darmstadt, Germany) at pH 7.8. The digests were then adjusted to pH 4.5 prior to centrifugation for 30 min at 1000 x g. The supernatant obtained is referred to as Frazer’s Fraction III (thereafter indicated as FF). This fraction contains a mixture of gliadin peptides and glutenin polypeptides representing the source for „toxic“ gliadin peptides. In control experiments we used a similarly prepared peptic-tryptic digest of ovalbumin (OVA; Sigma Aldrich Chemie, Taufkirchen) representing thus another nutritional antigen.

3.3.3 Transfection

COS-1 cells were transfected with plasmid DNA encoding the YFP-chimera of actin using DEAE-dextran as described by NAIM et al. (1991) For the intestinal proteins sucrase-isomaltase (SI) and lactase-phlorizin hydrolase, the plasmids pSG8-SI (slightly modified from
pSG5, Promega, Mannheim, Germany) and pCDNA3-LPH (Molecular Probes, Invitrogen detection technologies, Karlsruhe, Germany) were used. The rate of transfection is usually around 10% of the cells.

### 3.3.4 Internalization of FF or OVA

Transfected COS-1 cells were incubated with various concentrations (20 - 150mg) of FF or a peptic-tryptic digest of ovalbumin (OVA) in cell culture medium at 37°C. For confocal laser microscopy the incubation times were from 5 - 60 min and for biochemical analyses extended incubation periods were used to ensure a higher proportion of potentially internalized protein molecules. Essentially, similar effects of FF or OVA were obtained under the different incubation conditions.

### 3.3.5 Immunofluorescence

COS-1 cells were stimulated with FF or OVA 48 h after transfection. Subsequently, the cells were rinsed twice with PBS and then fixed with 4% paraformaldehyde (PFA; Fluka BioChemika, Buchs, Switzerland) for 20 min and further processed according to LEITNER et al. (2006) In brief, the cells were permeabilized for 30 min at room temperature with 0.5% Saponin (Sigma Aldrich Chemie, Taufkirchen) in PBS and 1% BSA (Fluka BioChemika, Buchs, Switzerland) (indicated blocking buffer). The mAb HSI3 (1:200) and HBB1/909 (1:2000) were used in blocking buffer as primary antibodies for detection of SI and LPH respectively. The secondary antibody utilized AlexaFlour488 at 1:1000. Finally, the cells were labeled with Phalloidin-Rhodamin diluted in blocking buffer (1:100), washed with PBS and mounted with mowiol 4-88 (Calbiochem, an Affiliate of Merck, Darmstadt).

Treatment of cells with cytochalasin D (cytoD, Sigma Aldrich Chemie, Taufkirchen) was performed at 10 µM final concentration for 30 min at 37°C followed by actin labeling with Phalloidin-Rhodamin.

Gliadin peptides were immunostained with the antibody R5 (1:75) followed by secondary labeling with goat anti-mouse AlexaFluor488 (1:1000). R5 recognizes a pentapeptide consensus sequence (QQPFP), which is, among other prolamins, present in the toxic gliadin peptide 31-49. Therefore and for reasons of simplification the term toxic gliadin peptide 31-
49 is used as a representative peptide for all peptides recognized by the R5 antibody. EEA-1 (1:200) and cath.D (1:100) were used for the detection of early endosomes and lysosomes followed by secondary labeling with goat anti-rabbit AlexaFlour568 (1:1000).

For surface labeling of SI and LPH the transfected cells were cultured at 20°C overnight to accumulate the protein in the Golgi apparatus. Thereafter, FF treatment was performed for 30 min at 20°C followed by 30 min at 37°C. After removal of FF the cells were incubated at 37°C for further 60 min to allow proteins to be transported to the cell surface. To assess the protein expression levels at the cell surface, anti-LPH (1:2000 in PBS) or anti-SI (1:200 in PBS) were added to intact cells on ice. The cells were washed and immediately treated with the secondary antibody. After further washing procedures the cells were fixed and visualized in the confocal laser microscope the next day.

3.3.6 Confocal Fluorescence Microscopy

Confocal images of fixed cells were acquired using a Leica TCS SP2 microscope with an x63 oil planapochromat lens (Leica Microsystems, Wetzlar). Dual color YFP or AlexaFlour488 and Rhodamin images were obtained by sequential scans with the 468 nm excitation line of an argon laser or the 543 nm excitation line of a He/Ne laser, respectively and the optimal emission wave length for Rhodamin and AlexaFlour568. Further image processing was performed with “Microsoft Picture it! Foto 7.0” for the extraction of enlarged images.

3.3.7 Biochemical Analysis of Actin after Treatment with FF, OVA and CytoD

Transfected COS-1 cells were incubated in methionine-free Minimum Eagle’s Medium (MEM, PAA Laboratories, Pasching, Austria) for 2 h followed by biosynthetic labeling with 50 µCi of [35S]-methionine (Amersham Biosciences GE Healthcare, Freiburg) for 4 h. FF or OVA were added during the last 3 h of labeling. Various concentrations of FF or OVA were used (between 20-150 mg) and comparable results were obtained. The labeling with cytoD was performed at a final concentration of 10 µM for 30 min. Solubilization of the cells and immunoprecipitation were performed essentially according to NAIM et al. (1991) using anti-GPF antibody (BD Transduction Laboratories, Heidelberg). The immunoisolates were further processed by SDS-PAGE on 12% polyacrylamid gels (LAEMMLI 1970) and analyzed by a
phosphor imaging device (Bio-Rad Laboratories, Munich). Whole cell lysates (30µg of total protein) from treated and non-treated cells were also analyzed by SDS-PAGE and immunoblotting against β-actin. Immunostaining of actin was performed with a mAb against β-actin and an anti-mouse ECL-peroxidase as a secondary antibody. The detection was carried out with the SuperSignal ELISA Femto Maximum Sensitivity Substrate via chemiluminescence on x-ray film sheets.

3.3.8 Cell Surface Trypsination of SI and LPH

Transfected COS-1 cells biosynthetically labeled with 50 µCi of [35S]-methionine for either 4h (for SI detection) or 6h (for LPH detection). Stimulation with FF was performed during the final 3h of labeling. Subsequently, the cells were rinsed with ice-cold PBS and further treated with 500µg Trypsin (Sigma Aldrich Chemie, Taufkirchen) in DMEM low glucose medium without fetal calf serum for 45 min at room temperature to monitor the expression of the glycoproteins at the cell surface. The treatment was stopped with a 4-fold concentration of soybean trypsin inhibitor (Sigma Aldrich Chemie, Taufkirchen) for 30 min at 4°C. The non-treated control samples were processed in the same manner. Treated or non-treated cells were centrifuged at 800 x g for 5 min and the pellets were washed twice with ice-cold PBS and processed for immunoprecipitation with anti-SI or anti-LPH antibodies as described for actin (see above).

3.3.9 Co-Immunoprecipitation and Western Blot Analysis

Non-transfected COS-1 cells were incubated for 10, 30, 60 and 180 min with FF in cell culture medium at 37°C. After solubilization with 1% Triton X-100 in PBS the samples were immunoprecipitated with mAb R5 against the „toxic“ peptide 31-49 in the presence of protein A-Sepharose (NAIM et al. 1991). The immunoisolates were further processed by SDS-PAGE on 12% polyacrylamid gels (LAEMMLI 1970) followed by western blot analysis (Hybond-PVDF from Amersham Biosciences GE Healthcare, Freiburg). Immunostaining of actin was performed with a mAb against β-actin and an anti-mouse ECL-peroxidase as a secondary antibody. The detection was carried out with the SuperSignal ELISA Femto Maximum Sensitivity Substrate via chemiluminescence on x-ray film sheets.
Sensitivity Substrate via chemiluminescence on x-ray film sheets. As a control we used cells that were not treated with FF, but immunoprecipitated with the R5 antibody followed by protein A-Sepharose to exclude unspecific binding of the antibody. To exclude potential unspecific binding of actin to protein A-Sepharose or IgG cell lysates of untreated cells or lysates from FF-treated cells were precleared with protein A-Sepharose beads or precipitated with anti-GFP antibody respectively.

Further processing of the scanned blot was performed with “Microsoft Picture it! Foto 7.0” and the quantification was performed with “ImageJ 1.41i” and “Microsoft Office Excel 2003”.

3.4 Results

3.4.1 Rearrangement of the Actin Cytoskeleton in COS-1 Cells

As mentioned before it has already been demonstrated that the actin cytoskeleton of enterocytes form biopsy specimens of CD patients is affected by treatment with peptic-tryptic digests of gluten which results in an rearrangement of the actin cytoskeleton (WILSON et al. 2004; HOLMGREN PETERSON et al. 1995). Here, the immediate effect of gliadin peptides containing FF was investigated via confocal imaging and biochemical analysis. For laser scanning microscopy transfected COS-1 cells were used expressing actin fused to YFP or cells that were treated with Phalloidin-Rhodamin. As shown in Fig. 3-1 stimulation with FF induces marked alterations of the actin cytoskeleton of COS-1 cells. Here, FF changes the density and structure of the actin-YFP network (Fig. 3-1 B). In comparison to the control cells (Fig. 3-1 A) a strong reduction of the perinuclear network (Fig. 3-1 B, arrow) as well as actin-positive vesicular structures (Fig. 3-1 B, dashed arrows) below the plasma membrane is revealed. This pattern of localization could be observed in approximately 50-60% of the transfected COS cells.

Using Phalloidin-Rhodamin the filamentous form of endogenous actin could be revealed (Fig. 3-1 D). There is a strong labeling of the perinuclear network as well as the membrane-associated actin web (arrows). After stimulation with FF these filamentous structures become more dispersed and diffused and as previously shown for actin-YFP the perinuclear web is
also reduced (arrow) and vesicular structures (dashed arrows) can be detected beneath the plasma membrane (Fig. 3-1 E). Here again, almost 50% of the cells revealed this pattern. On the other hand, cytochalasin D (cyto D), an inhibitor of actin polymerization, induces a different kind of rearrangement of the actin filaments, which become reduced and are visible as fragmented structures in the actin-YFP fused form (Fig. 3-1C) or when labeled with Phalloidin-Rhodamin (Fig. 3-1F).

**Figure 3-1:**  Actin rearrangement after treatment with either FF or cyto D (1)
COS-1 cells were transfected with actin-YFP (A-C) and treated with FF or cyto D. In D-F the cells were not transfected, but subjected to treatment with FF and cyto D and labeling with Phalloidin-Rhodamin. Arrows in A and D indicate a dense perinuclear actin network as well as membrane associated actin filaments. Arrows in B and E point to a strong reduction of the perinuclear actin network in FF treated cells. The dashed arrows in C and F indicate accumulations of actin fragments beneath the plasma membrane. Note the alterations induced by cyto D (C and F, star-like structures) differ entirely from those induced by of FF.
The role of FF was also examined in cells that were biosynthetically labeled with $^{35}$S-methionine to assess the effect on de novo synthesized actin. As shown in Fig. 3-2, treatment with FF leads to a dramatic reduction of the actin protein band compared to control cells. These reduced levels suggest a conformational change of the protein and subsequent reduced antibody-binding capacity.

In cyto D treated cells a slight reduction in the levels of immunoprecipitated actin could be also detected, albeit not to a similarly high extent as with FF. This suggests that actin in cyto D treated cells assumes a different conformation compatible with differences in the antibody-binding capacity as compared to actin in FF-treated cells.

The control employed a tryptic-peptic digest of ovalbumin (OVA), an antigen that has been used in comparative analyses of the immune reactions induced by FF in celiac disease (BIAGI et al. 1999; SHIDRAWI et al. 1995). In sharp contrast to FF, and hitherto one major advantage of using this control protein, is the ability of OVA to induce oral tolerance in mice (STROBEL and MOWAT 1998; FURRIE et al. 1994) suggestive of different intracellular site of actions of both proteins.

**Figure 3-2:** Actin rearrangement after treatment with either FF or cyto D (2)

G) Transfected COS-1 cells were biosynthetically labelled with $[^{35}\text{S}]$-methionine and stimulated with either FF or cyto D and the cell detergent extracts were immunoprecipitated with an anti-GFP antibody followed by SDS-PAGE.
Figure 3-3:  Actin-labeling after treatment with OVA
COS-1 cells were treated with OVA fixed with 4%PFA and labeled with Phalloidin-Rhodamin. A) Shows the control cell and B) cells that were stimulated with OVA. Similar perinuclear staining and membrane labeling could be demonstrated in both cell preparations (arrows in A and B).
C) Transfected cells were biosynthetically labeled with [35S]-methionine for 4h and treated with peptic-tryptic digests of OVA. The cell extracts were immunoprecipitated with an anti-GFP antibody and subjected to SDS-PAGE. No alteration in the amount of actin due to OVA stimulation could be detected.

As demonstrated in Fig. 3-3 stimulation with OVA did not affect the organization of the actin cytoskeleton, since there are no alterations in the structure or density of the actin network (see arrows in A and B). Immunoprecipitation of actin from OVA-treated cells supports these data, since no changes in the amount of actin could be detected (Fig. 3-3 C).

To determine whether the reduced levels of actin-YFP revealed after FF exposure and cyto D treatment were the consequence of a causal reduction in the actin and protein expression levels, total lysates of treated or non-treated cells were subjected to western blot analysis and compared. Fig. 3-4 shows that the protein expression levels of control, FF-, OVA-treated samples were virtually the same while only the amount of actin after cyto D treatment
decreases. Since the actin level of FF-treated cells is the same as the control, the substantial reduction in the levels of immunoprecipitated actin (Fig. 3-1) is suggestive of a conformational change of the protein and subsequent reduced antibody-binding capacity rather than causal alteration in the levels of actin. In contrast to this, the slightly reduced level of immunoprecipitated actin in cyto D treated cells is due to a reduction of the total actin expression level.

Figure 3-4: Protein pattern in lysates of stimulated and control cells
Protein pattern in lysates of stimulated and control cells. Transfected COS-1 cells (with actin-YFP) were treated with FF, OVA and cyto D. After cell lysis equal amounts (30µl) of proteins were subjected to SDS-PAGE on 12% slab gels followed by western blotting. Actin was then immunostained with a primary mouse anti-β-actin antibody and a goat anti-mouse-HRP as secondary antibody.
The band pattern for non-treated, FF-and OVA-treated cells show equal amounts of actin while the content is reduced after cyto D-treatment.

3.4.2 Gliadin Peptides Rapidly Interact with the Actin Cytoskeleton in COS-1 Cells
The effects elicited by FF on the integrity of actin in COS-1 cells raise question related to the mode of action of the „toxic“ gliadin peptides on actin. Previously it has been shown in intestinal biopsy specimens that a specific subpopulation of intestinal cells, RACE (rapid antigen uptake into the cytosol of enterocytes), is capable of efficiently internalizing the food antigen OVA (KERSTING et al. 2004; SCHURMANN et al. 1999). The question that arises in this respect is whether the gliadin peptides are internalized in a same rapid manner and if
they directly interact with the actin cytoskeleton thereby disrupting its filamentous morphology as demonstrated above.

This question was addressed by incubating COS-1 cells for either 10 min with FF followed by immunostaining of gliadin peptides and actin labeling with Phalloidin-Rhodamin and further examining by confocal laser microscopy possible co-localizations. In cells that were stimulated with FF for 10 min a clear fluorescent punctuate staining was revealed by the R5 anti-gliadin antibody (Fig. 3-5 F). Several of these structures colocalized with actin as shown in Fig. 3-5 G (arrows in H that shows a higher magnification of G).

By contrast, non-treated control cells did not reveal a similar type of staining and only actin filaments were clearly labeled (Fig. 3-5, A-D). Deterioration of the actin cytoskeleton could be already observed, albeit slightly, within the short 10 min stimulation period with FF. To determine whether the punctuated structures that correspond to the „toxic“ gliadin peptides are located in the cytosol or in endosomal compartments, FF-treated cells were labeled with the R5 antibody and with endosomal protein markers. Staining of lysosomes was performed with cath. D (Fig. 3-5, I-L) and early endosomes with EEA-1 (Fig. 3-5, M-P). The overlay of R5 and cath. D (Fig. 3-5 K) did not reveal a localization of the „toxic“ peptide in lysosomal compartments (see also the enlargement in Fig. 3-5, L). By contrast, several early endosomes that were labeled with the anti-EEA-1 antibody contained also the „toxic“ peptide as shown by yellow staining in Fig. 3-5 O (higher magnification in panel P).
EFFECTS OF GLIADIN TOXIC PEPTIDES ON ACTIN AND SUBSEQUENT PROTEIN TRAFFICKING

control

FF

Cathepsin D

FF

FF

EEA-1
Figure 3-5: Internalization and location of gliadin peptides
COS-1 cells were treated for 10 min with FF fixed with 4%PFA and labeled with mouse-anti Gliadin (R5) followed by AlexaFlour488 as secondary antibody and labeling with Phalloidin-Rhodamin (A-H). Control cells (A-D) do not show a gliadin-labeling and subsequent colocalization with the actin cytoskeleton (C and D). In E-H the cells were stimulated for 10 min with FF. Vesicular or punctuate structures could be detected with the anti-gliadin antibody (F). Slight but definite colocalizations of gliadin peptides with actin are also revealed (G, enlarged and marked by arrows in H). In panels I to P cells were stimulated with FF for 10 min and labeled with mouse-anti gliadin antibody (R5) followed by AlexaFlour488 as a secondary antibody. Thereafter, the cells were labeled with rabbit cath. D or anti-EEA-1 followed by AlexaFlour568 as a secondary antibody. The overlay in panel K (enlarged in L) does not reveal a colocalization of the „toxic“ peptides (R5) with cath. D. In contrast, EEA-1 colocalizes with the gliadin peptide as shown by the yellow staining in O (marked by arrows in P).

These data strongly suggest that FF is rapidly internalized into COS-1 cells following at least two different pathways, the first utilizes early endosomes and the second occurs directly into the cytosol in a manner similar to the rapid uptake of gliadin peptides in intestinal RACE cells (Kersting et al., 2004). It is very likely that the latter pathway facilitates a direct interaction of FF with actin.

Therefore this hypothesis was examined in a co-immunoprecipitation set up. Here, detergent extracts of FF-stimulated cells were immunoprecipitated with mAb R5 that is directed against the „toxic“ peptide 31-49 present in FF (OSMAN et al. 2001; SILANO and DE VINCENZI 1999; SHIDRAWI et al. 1995) and the immunoprecipitates were analyzed by immunoblotting using anti-actin antibodies. Fig. 3-6 A (upper panel) shows definite actin bands in the R5 immunoisolates, but none in the control utilizing protein A-Sepharose (indicated PAS) or the mouse anti-LPH (indicated mαLPH). Remarkably, the intensity of the actin bands increases concomitant with the duration of stimulation of the cells with FF (Fig. 3-6 A, lower panel).

The proportion of immunoprecipitated actin relative to actin in total cell lysates increased steadily from approximately 8% at 10 min of stimulation to 37% after 3 h (Fig. 3-6 B). The increase in the binding capacity during prolonged stimulation supports the specificity of this interaction.
Figure 3-6: Co-Immunoprecipitation of actin and gliadin peptides
COS-1 cells were either incubated with cell culture medium or treated with FF for 10, 30, 60 or 180 min in cell culture medium. Immunoprecipitation was performed with the mAb anti-gliadin antibody (R5) or with rabbit anti-LPH as a control. The immunoprecipitates were subjected to western blot analysis with mAb anti-β-actin. Another control employed protein A-Sepharose used in preclearing of cell lysates (indicated as PAS). The figure compiles data obtained from three individual experiments.

B) Quantification of a representative experiment was performed by defining the band intensity with ImageJ 1.41i and further statistical analysis with Microsoft Office Excel 2003. The immunoisolates show an increase in the precipitated amount of actin concomitant with prolonged stimulation periods.
3.4.3 Alterations in the Organization of the Actin Cytoskeleton Influences Actin-dependent Protein Trafficking

To investigate how rearrangement of the actin cytoskeleton affects the protein trafficking of different glycoproteins that follow different pathways, actin-dependent and actin-independent, to reach the plasma membrane were analyzed. Sucrase-isomaltase (SI), an intestinal hydrolase that depend on an intact actin cytoskeleton, can be detected in the Golgi apparatus as well as at the plasma membrane (Fig. 3-7 B) where it colocalizes (yellow staining) with the actin network (dashed arrows in Fig. 3-7 C). Treatment with FF leads to a reduction of the staining of SI at the cell surface (Fig. 3-7 F) concomitant with an increase in intracellular punctuate staining corresponding to vesicular structures. Concomitantly a reduced appearance of actin in the cell periphery is observed and consequently the colocalization with SI is also reduced.

Further the trafficking of another glycoprotein lactase-phlorizin hydrolase (LPH), which is known to occur in an actin-independent fashion (JACOB et al. 2003) was examined. LPH is transported to the cell surface via microtubules and without an absolute requirement for the actin cytoskeleton. As shown in Fig. 3-7 (G-L) alterations in the transport behavior of LPH could not be identified after stimulation with FF. The cellular distribution of LPH is virtually similar to that in the control cells (see Fig. 3-7, I and L dashed arrows).

To examine only the extent of membrane-integrated protein of both the cell surface expression of SI and LPH was investigated in FF-stimulated but non-permeabilized cells. Control cells revealed strong labeling of both SI (Fig. 3-8, A and B) and LPH (Fig. 3-8, E and F) concomitant with efficient trafficking of these proteins to the cell surface. When cells were stimulated with FF a substantial decrease in the labeling intensity of SI could be observed (Fig. 3-8 C and D) compatible with impaired transport of this protein to the cell surface, supporting the data in Fig. 3-7 F. The transport of LPH, on the other hand, was not affected in the presence of FF (Fig. 3-8, G and H).
Figure 3-7: Co-localization of SI and LPH with the actin cytoskeleton after stimulation with FF
COS-1 cells were either transfected with SI (A-F) or with LPH (G-L), then stimulated with FF, fixed with 4%PFA and labeled with Phalloidin-Rhodamin. SI and LPH were labeled with specific mAbs and visualized using AlexaFlour488. In control cells (A-C) SI is located in the Golgi and at the plasma membrane (B) where it colocalizes (yellow staining) with actin (dashed arrows in C). A, represents actin labeling with Phalloidin alone. After incubation with FF SI is predominantly retained intracellularly and is not localized at the plasma membrane (E). Subsequently, no colocalization of SI with actin could be detected (F). In control cells (G-I) LPH is revealed in the Golgi and at the cell surface in control cells (H), but in contrast to SI does not colocalize with the actin cytoskeleton (I). Treatment of the cells with FF alters the actin cytoskeleton (J), but has no effect on the localization of LPH at the plasma membrane (K, L). G, represents actin labeling with Phalloidin alone.
Figure 3-8: Cell surface expression of SI and LPH with the actin cytoskeleton after stimulation with FF

COS-1 cells were transfected with cDNAs corresponding to SI or LPH. 40 h posttransfection the cells were cultured at 20°C for 18 h to chase the transfected proteins to the Golgi. The cells were then stimulated with FF at 20°C for the initial 30 min followed by a temperature shift to 37°C for another 30 min. After removal of FF the cells were cultured for further 60 min at 37°C, fixed with PFA, but not permeabilized, and anti-SI antibodies or anti-LPH were added followed by AlexaFlour488 as a secondary antibody. All steps were performed on ice to exclude possible internalization of the antibodies. The control cells showed clear staining of SI (A and B) and LPH (E and F) at the cell surface. However, treatment with FF resulted in a substantial reduction of SI staining at cell surface (C and D) while the expression levels of LPH remained the same (G, H) as compared to the control cells (E, F).
In addition to the confocal analyses, the effect of FF stimulation on the transport of glycoproteins to the cell surface was also examined biochemically in biosynthetically labeled cells. Here, the transport of SI and LPH to the cell surface was examined by treatment of intact cells with trypsin, which cleaves SI to the two subunits S and I (NAIM et al. 1988b) and LPH to the LPHβ brush border form (JACOB et al. 1996; NAIM et al. 1991).

**Figure 3-9: Biochemical analysis of glycoproteins**

COS-1 cells were biosynthetically labeled with $[^{35}S]$-methionine for either 4h (for SI detection) or 6h (for LPH detection), taking into consideration the different transport rates of these proteins. The cells were stimulated with FF during the final 3h of labeling. The intact cells were treated with trypsin to monitor the appearance of mature forms of SI and LPH at the cell surface. Thereafter, detergent extracts of the cells were prepared and immunoprecipitated with mAb anti-SI or mAb anti-LPH and subjected to SDS-PAGE. The appearance of the cleaved products I (145 kDa) and S (130 kDa) is indicative of a transport-competence of SI. Likewise, cleavage of LPH to a 160-kDa is concomitant with a cell surface expression of LPH.
In intact cells only the complex glycosylated mature forms of SI (245 kDa, Fig. 3-9, lane 1) and LPH (230 kDa, Fig. 3-9, lane 5), but not the mannose-rich ER forms (210 kDa for SI and 215 kDa for LPH) are expected to be at the cell surface. The appearance of the subunits is therefore indicative of a competent trafficking of the precursor forms to the cell surface. Figure 3-9 shows that surface trypsination leads to a reduction of the complex glycosylated form of SI and produces the I (145 kDa) and S (130 kDa) subunits (lane 2). In cells that have been treated with FF, on the other hand, trypsin treatment did not generate the subunits despite the presence of the complex glycosylated mature form (lanes 3 and 4). This result clearly indicates that mature SI was not further transported to the cell surface after processing in the Golgi apparatus and strongly supports the confocal data that deterioration of the actin cytoskeleton by FF has blocked further transport of SI. By contrast, trypsin treatment revealed in cells treated with FF as well as in non-treated cells a 160-kDa protein band concomitant with a substantial reduction in the intensity of the complex glycosylated mature 230-kDa LPH form (Fig. 3-9, lanes 6 and 8). The results clearly demonstrate therefore that the expression of LPH at the cell surface was not affected by FF and confirming the actin-independent mode of LPH.

Together, the data obtained with these two proteins lend a strong support to the concept of multiple sorting via actin-dependent and –independent pathways from the TGN to the cell surface (SUBRAMANIAN et al. 2008; LAZARO-DIEGUEZ et al. 2007; JACOB et al. 2003).

### 3.5 Discussion

The current paper demonstrates at the cellular and protein levels that stimulation of the non-polarized COS-cells with FF has direct implications on the integrity and morphology of the actin cytoskeleton and subsequently on the trafficking of a population of membrane glycoproteins that follow an actin-dependent sorting pathway.

The expression of an actin-YPF chimera in addition to staining of endogenous actin with Phalloidin provided an adequate readout system to evaluate the effects of FF on the globular form of actin as well as the filamentous forms. In the presence of FF both types of actin undergo a strong reduction and rearrangement of the terminal web and the plasma membrane associated network. Importantly, punctuate structures beneath the plasma membrane
representing fragmented actin appear. By contrast, OVA that represents another food antigen has no effect on the organization of the cytoskeleton pointing thus to a specific effect of FF on the actin cytoskeleton. Importantly, alterations of the actin cytoskeleton and their implication on the cell shape and integrity are not as drastic as those observed with cyto D, a mycotoxin that alters actin polymerization (COOPER 1987). In the latter case, disruption of the actin cytoskeleton and its accumulation in dense foci and patches concomitant with an alteration in the cell shape occur (SCHLIWA 1982).

It is obvious therefore that the mode of action of FF and cyto D on the actin cytoskeleton differ significantly raising questions about putative mechanisms by which gliadin peptides influence the actin cytoskeleton. Our data demonstrate that FF has been rapidly distributed in COS-1 cells and it interacts directly with the actin cytoskeleton. In fact, confocal analyses reveal a clear co-localization of the „toxic“ gliadin peptides 31-49 with actin filament within 10 min concomitant with a direct association with actin or on actin-binding proteins. In addition to their cytosolic location, the „toxic“ peptides are also internalized into compartments of the early endosomal pathway and could not be detected in the lysosomes. Co-immunoprecipitation analyses using a mAb against the „toxic“ gliadin peptide 31-49 support a direct interaction between FF and actin. It is likely that this interaction competes with actin-binding proteins, for example Arp2/3 (MULLINS and POLLARD 1999; SVITKINA and BORISY 1999; MULLINS et al. 1998), interfering thus with the polymerization of actin. This may explain the increase of co-immunoprecipitated actin with the duration of FF-stimulation. It is likely that the „toxic“ gliadin peptides bind more avidly a particular conformation of actin and this binding increases with time until a conformational change of actin has occurred. Along this, it is possible that the fragmented appearance of actin in some areas of the cell observed in confocal images could represent partially polymerized actin with bound FF.

The rapid uptake of FF in COS-1 cells resembles the uptake of other nutritional antigens like OVA or horseradish peroxidase into the cytosol of a specific population of enterocytes (RACE cells) (KERSTING et al. 2004; SCHURMANN et al. 1999).

Importantly, the effect of FF on actin appears to be exclusive for this cytoskeletal protein, since only the trafficking of proteins that depend on an intact actin network is impaired in cells treated with FF. Several proteins use initially microtubules in their intracellular transport.
(GILBERT et al. 1991; ACHLER et al. 1989) and are finally delivered to the cell surface via actin filaments (JACOB et al. 2003). An example of this family of proteins is SI, the transport of which is substantially hampered in FF-treated cells. By contrast, the trafficking of LPH is not affected. LPH follows an actin-independent pathway (JACOB et al. 2003) that exclusively implicates the microtubules, the function of which is apparently not influenced by FF. Although COS-1 cells are not epithelial, sorting pathways at the TGN may well occur as has been shown previously for segregating SI and LPH and also in other non-epithelial cell lines like fibroblasts (CHO, 3T3, BHK) (RUSTOM et al. 2002; PAROLINI et al. 1999; MUSCH et al. 1996; YOSHIMORI et al. 1996).

In conclusion, the direct effect of FF on the actin cytoskeleton and the subsequent protein transport is a possible mechanism that elicits structural and functional alterations in the cell and may constitute one putative mechanism for the pathogenesis of celiac disease. This is in line with previous studies in biopsy specimens, which demonstrated that an increase of cell permeability and impaired and disassembled tight junction (DOLFINI et al. 2005; SANDER et al. 2005) occur upon incubation with gliadin peptides that alter the organization of the actin cytoskeleton (SANDER et al. 2005; CLEMENTE et al. 2003; HOLMGREN PETERSON et al. 1995).

3.6 Acknowledgement

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3.7 Abbreviations

CD, Celiac disease; SI, sucrase-isomaltase; LPH, lactase phlorizin hydrolase, mAb, monoclonal antibody; GFP, green fluorescent protein; YFP, yellow fluorescent protein; FF, Frazer’s Fraction, OVA, ovalbumin; PFA, paraformaldehyde; cyto D, cytochalasin D; EEA-1, early endosomal antigen 1; cath. D, cathepsin D
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GLIADIN TOXIC PEPTIDES INTERACT WITH THE ACTIN CYTOSKELETON AND AFFECT THE TARGETING AND FUNCTION OF INTESTINAL PROTEINS
4 GLIADIN TOXIC PEPTIDES INTERACT WITH THE ACTIN CYTOSKELETON AND AFFECT THE TARGETING AND FUNCTION OF INTESTINAL PROTEINS

Yvonne Reinke*, Klaus-Peter Zimmer+ and Hassan Y. Naim**

*Department of Physiological Chemistry, University of Veterinary Medicine Hannover, Germany
+Department of Pediatrics, University of Giessen, Germany

Short Title: Gliadin affects actin and protein sorting

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*Correspondence:
Hassan Y. Naim, Ph.D.
Department of Physiological Chemistry
University of Veterinary Medicine Hannover
D-30559 Hannover, Germany
Tel.: +49 511 953 8780
Fax: +49 511 953 8585
Email: hassan.naim@tiho-hannover.de
4.1 Abstract

Background & Aims:
Celiac disease (CD) is a multisystemic autoimmune inflammation of the intestinal tract induced by wheat gluten and related cereals in HLA-DQ2/8 positive individuals. An essential role in the pathogenesis of CD is played by a fraction of the peptic-tryptic digest of gluten, Frazer’s Fraction (FF). Here, we investigate the effects of FF on the integrity of intestinal cells with particular emphasis on brush border membrane (BBM) components, their subsequent trafficking and endocytosis.

Methods:
FF was incubated with Caco-2 cells at different concentrations. Thereafter, several protein and lipid components of treated and untreated cells were analyzed at the protein, functional and cellular levels. The control employed tryptic-peptic digests of ovalbumin.

Results:
FF directly interacts with actin in an alternating manner, eliciting substantial alterations in its integrity and extent in the BBM. These alterations lead to an impaired trafficking of SI to the apical membrane and reduction in its enzymatic function. ApN and DPPIV follow a transcytotic pathway and are only partly affected by FF. By contrast, the trafficking of LPH remains unaffected concomitant with its actin-independent trafficking pattern. Finally, the endocytic pathway is substantially blocked in FF-treated cells leading to an accumulation of cholesterol, and sphingolipids in the BBM.

Conclusions:
FF deteriorates the actin cytoskeleton in Caco-2, leading to reduced protein sorting and hampered endocytic events with subsequent alterations in the protein and lipid composition of the BBM. The reduced levels of the disaccharidase SI in the BBM suggest a potential pathomechanism of carbohydrate malabsorption in CD.
4.2 Introduction

Celiac disease (CD) is a chronic inflammatory disease of the small intestine. It is typically characterized by villus atrophy and a consecutive malabsorption syndrome in genetically predisposed individuals (HLA-DQ2/DQ8) due to gliadin toxicity (CICLITIRA and MOODIE 2003; SOLLID 2002; MARSH 1992). Beside the genetic background the environmental factor gluten plays a decisive role in the development of CD. Gluten can be divided into two main fractions the glutelins and prolamins, whereby the latter represents the alcohol soluble fraction that are enriched in prolines and glutamines (DIETERICH et al. 2003). Based on the genus type of the cereal grains the prolamins can be distinguished into gliadins from wheat, secalins from rye and hordeins from barley (MAKI and COLLIN 1997).

Digestion of wheat gluten results in a mixture of gliadin peptides and glutenin polypeptides that contain „toxic“ and immunodominant peptides (VADER et al. 2002; DICKE et al. 1953) and play a crucial role in the pathogenesis of CD. Although it has been revealed that an altered immune response to these peptides is involved in the pathogenesis of CD (SOLLID 2000; MARSH 1992) the underlying mechanism by which the cereal prolamins damage the small intestine of susceptible individuals is still unclear. It is clear that the delineation of the molecular basis of this disease is a necessity with the ultimate goal of development of novel treatment strategies that could substantially improve the quality of life of celiac patients.

The first contact of macromolecular nutrients with the intestinal epithelium occurs through the brush border membrane (BBM) that maintains the intestinal barrier function (SNOECK et al. 2005), whereby their internalization normally occurs by receptor-mediated endocytosis or by fluid phase endocytosis (pinocytosis) as shown for bovine serum albumin (BSA) or horse radish peroxidase (HRP) (BUONO et al. 2007; LAMAZE and SCHMID 1995). The mode of uptake of gliadin through the intestine has not been explored yet and it is not clear whether this uptake is receptor-mediated but MATYSIAK-BUDNIK et al. (2008) were able to show that the transferrin receptor CD71 is involved in the retrotranscytosis of peptide 31-49 and that gliadin binds to chemokine receptor CXCR3 and leads to MyD88-dependent zonulin release and increased intestinal permeability (LAMMERS et al. 2008).

Besides its barrier function between the luminal part and the interior side of the intestinal mucosa, the BBM of enterocytes consists of packed uniformly organized microvilli that provides an expanded surface enriched in a battery of glycoproteins that are essential for the
digestion and absorption of nutritional components like carbohydrates and food peptides (ALPERS 1987). Different sorting pathways have been described for these glycoproteins in biopsy specimens and cell culture model (LE BIVIC et al. 1990; MATTER et al. 1990a). Some of these proteins, e.g. sucrase-isomaltase (SI), require an intact actin cytoskeleton for an efficient sorting and trafficking to the plasma membrane. Other proteins such as lactase-phlorizin hydrolase (LPH) are transported via actin-independent pathways (JACOB et al. 2003) that predominantly implicate microtubules. The actin cytoskeleton of enterocytes in biopsy samples from patients with CD (WILSON et al. 2004; HOLMGREN PETERSON et al. 1995) or of Caco-2 cells (SANDER et al. 2005) has been shown to be affected upon treatment with gliadin peptides. Nevertheless, the sequence of events, leading to the deterioration of the actin cytoskeleton and the mechanism by which gliadin acts on actin are entirely obscure. In the present study we utilized Caco-2 cells to assess the effects of gliadin treatment on the actin-dependent and actin-independent protein transport and to identify potential alterations in the BBM due to impaired membrane trafficking and endocytosis. Our data demonstrate that treatment with gliadin peptides does not only affect the actin cytoskeleton per se leading to morphological alterations, but influences the anterograde and retrograde transport of proteins and lipids concomitantly.

4.3 Materials and Methods

4.3.1 Preparation of Frazer’s fraction

The preparation of Frazer’s Fraction III (thereafter indicated as FF) was performed according to FRAZER et al. (1959). This fraction contains a mixture of gliadin and glutenin peptides representing the source for „toxic“ gliadin peptides. The control experiments employed a similarly prepared peptic-tryptic digest of ovalbumin (OVA; Sigma, Taufkirchen, Germany).

4.3.2 Stimulation of Caco-2 cells

Intestinal Caco-2 cells were cultured in DMEM high glucose (4500mg/l) medium supplemented with 10% fetal calf serum and 1% penicillin/streptomycin (all from PAA
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Laboratories, Pasching, Austria). The cells were incubated with FF or OVA for 48h at 37°C. For this three different concentrations were used, 5mg, 25mg and 100mg.

4.3.3 Cell Fractionation and Western Blotting

Stimulated cells were fractionated using CaCl$_2$ (SCHMITZ et al. 1973) to P1 (basolateral and intracellular membranes) and P2 (BBM). Usually 20-25µg of either P1 or P2 was subjected to western blotting. The following antibodies were used: mAb anti-SI HBB 3/705/60 (1:350), mAb anti-DPPIV (HBB 3/775/42 DPPIV; 1:3000) (both HAURI et al. (1985), polyclonal anti-ApN (1:500) (DANIELSEN et al. 1977) and mAbs mLac 6 and 10 against LPH (1:250) (MAIURI et al. 1991). Due to the low levels of LPH in Caco-2 cells, immunoprecipitation of LPH with a mixture of several mAbs (mLac1,3,4,5,8 (1:40) (MAIURI et al. 1991)) preceded western blotting. Actin was detected using mAb anti-β-actin (C4) (Santa Cruz Biotechnology, Inc., Heidelberg, Germany). The mAb anti-β-tubulin (1:1000) and polyclonal anti α-gliadin antibody (1:1000) were obtained from Sigma (Taufkirchen, Germany). Anti-rabbit IgG-ECL was from Dako (1:10000) (Hamburg, Germany) and anti-mouse IgG-ECL (1:10000) (Amersham Biosciences, Freiburg, Germany) were used as secondary antibodies and the detection employed SuperSignal ELISA Femto (Perbio Science, Bonn, Germany) on x-ray (x-ray Retina XBA) film sheets (Fotochemische Werke GmbH Berlin).

4.3.4 Immunofluorescence

Caco-2 cells were stimulated with FF or OVA for 48 h as described. The cells were rinsed twice with PBS, fixed with 4% paraformaldehyde (PFA; Fluka BioChemika, Buchs, Switzerland) for 20min and further processed according to LEITNER et al. (2006). Permeabilization of the cells was performed for 30min at room temperature with 0.5% Saponin (Sigma Aldrich Chemie, Taufkirchen) in PBS containing 1% BSA (Fluka BioChemika, Buchs, Switzerland) (indicated blocking buffer). The following antibodies were used: mAbs HSI 3 against SI (1:200) (Beaulieu et al. (1989) and HBB 3/775/42 against DPPIV (1:1500) (HAURI et al. 1985). Phalloidin-Rhodamin (1:100) utilized for actin labeling and the anti-mouse IgG AlexaFlour488 (1:1000) as a secondary antibody were from Molecular Probes, (Karlsruhe, Germany). Confocal images were acquired using a Leica TCS
SP2 microscope with an x63 oil planapochromat lens (Leica Microsystems, Wetzlar, Germany) according to JACOB AND NAIM (2001).

4.3.5 Co-Immunoprecipitation of Actin with Anti-Gliadin Antibodies

Caco-2 cells were incubated for different times up to 72h with 100mg FF in cell culture medium at 37°C. FF was replaced every 24h. Cells were lysed for 2h at 4°C with 1% Triton X-100 in PBS and immunoprecipitation with anti-gliadin antibodies was performed. The immunoisolates were further processed by SDS-PAGE followed by western blotting. The control utilized lysates from cells that were not treated with FF, but treated with anti-α-gliadin and protein A-Sepharose (Amersham Biosciences, Freiburg, Germany) as well as lysates of FF-treated cells that were precleared with protein A-Sepharose. Additionally, 20µg of cell lysates of each sample were subjected to western blotting to determine the cellular actin level.

4.3.6 Endocytosis of Wheat Germ Agglutinin (WGA)

Caco-2 cells were stimulated with either FF or OVA, washed twice with ice-cold PBS followed by the addition of 10µg biotinylated WGA (Vector Laboratories, Inc.) to intact cells in a buffer containing 10mM Tris/HCl, pH 7.4, 150mM NaCl2 and 0.1mM CaCl2 for 45min on ice. After washing with ice-cold PBS, internalization of WGA was performed at 37°C in pre-warmed tissue culture medium. The control employed cells that were left at 0°C. Finally, the samples were washed with ice-cold PBS and P1 and P2 were prepared. The samples were subjected to western blotting with streptavidin.

4.3.7 Other Procedures

Sucrase activity in the brush border membranes (P2,) was measured using hexokinase method (Gluco-quant, Roche Diagnostics, Mannheim, Germany). The assessment of sphingomyelin and cholesterol in P1 and P2 utilized the method of BLIGH and DYER (1959). Sphingomyelin was analyzed by HPLC and detected with a Sedex 55 light scatter detector. Cholesterol was applied to HPLC following the method of TAKADATE (1985).
For statistical analyses we used ImageJ 1.41i and GraphPad prism 5 based on the band intensity/area and the ratio between P2/P1. The significance was assessed by one way ANOVA with $p \leq 0.05$ and the Newman-Keuls test.

**4.4 Results**

**4.4.1 FF-Treatment Alters the Integrity of the BBM–associated Cytoskeleton**

It has been described in previous studies that treatment with Frazer’s Fraction (FF) leads to a rearrangement of the actin cytoskeleton in enterocytes (HOLMGREN PETERSON et al. 1995; BAILEY et al. 1989) and in intestinal Caco-2 cells (SANDER et al. 2005). The biochemical basis of these effects is, however, far from being delineated. To analyze whether stimulation of Caco-2 cells with FF has affected the membrane-associated cytoskeleton of Caco-2 cells we separated BBMs from other cellular membranes of Caco-2 cells and determined the distribution of $\beta$-actin and $\alpha$-tubulin in BBM (retained in P2 fraction) as compared to the basolateral membranes and intracellular membranes (retained in P1 fraction). Fig. 4-1A reveals marked reduction in the levels of actin in P1 and P2 upon stimulation of Caco-2 cells with FF. Concomitant with increasing concentrations of FF the levels of actin decrease more substantially in P2 as compared to P1 (Fig. 4-1B, left upper panel). The ratio P2/P1 is indicative of an alteration in the levels of BBM-associated actin. In comparison to the non-stimulated control Caco-2 cells P2/P1 decreases significantly upon treatment with FF ($p \leq 0.01$ and $p \leq 0.001$). Treatment of Caco-2 cells with OVA, as a nutritional control protein, did not generate any changes with respect to the levels of actin in P1 and P2 (Fig. 4-1A) and consequently the ratio P2/P1 (Fig. 4-1B, right upper panel). Substantial effects of FF on the actin cytoskeleton could be also assessed by confocal laser microscopy (Fig. 4-1C, c to f). As shown in Fig. 4-1C (c and d) the membrane-associated actin becomes strongly reduced and its distribution is rather diffuse compared to the control cells (Fig. 4-1C, c and b). By contrast, actin in OVA-treated cells remained unaffected (Fig. 4-1C, e and f).
Figure 4-1: FF-treatment induces alteration in the amount of BBM–associated actin and microtubules cytoskeleton (1)

Caco-2 cells were treated with FF or OVA. The lysates of P1 and P2 were subjected to western blot analysis with mAb anti β-actin and mAb anti α-tubulin. FF-treatment reveals a strong dose-dependent decrease in the amount of β-actin whereas α-tubulin increases the other way around (A). Statistical quantification in B) was performed as described. The significance of the ratio P2/P1 is determined by $p \leq 0.05$ (*), $p \leq 0.01$ (**), and $p \leq 0.001$ (***)
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Figure 4-1: FF-treatment induces alteration in the amount of BBM–associated actin and microtubules cytoskeleton (2)
C) displays cells treated with FF or OVA (10mg), fixed with 4% PFA and labeled Phalloidin-Rhodamin. FF-stimulation substantially reduces actin at the BBM and leads to its diffuse distribution near the basolateral membrane.

The decrease in the levels of actin in P1 and P2 and at the cell surface in the fluorescent images strongly suggests that the integrity of the actin cytoskeleton has been deteriorated and could be a reason for an impaired association of actin with the BBM. The membrane-associated actin in the P2 preparation is likely associated with proteins like class I myosin’s
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(COLUCCIO 1997) or the 110-kDa CM protein complex, a complex of myosin IA and calmodulins. This complex connects actin-derived core bundle of the microvilli with the surrounding plasma membrane via cross-bridges (MOOSEKER and TILNEY 1975). Interestingly, the linkage of actin and the 110-kDa CM complex is determined by the structural features of the actin bundle (LOUVARD et al. 1992) and occurs in an ATP-dependent manner. In view of these mechanisms it is likely that gliadin peptides interact directly with actin filaments or indirectly with actin binding proteins that are involved in processes, such as capping at the barbed end or binding G-actin to the polymerizing filament like capping protein (COOPER and SCHAFER 2000) or profilin (WEBER 1999). These interactions lead ultimately to conformational changes and subsequent loss of interaction with BBM.

In sharp contrast to actin, FF-treatment of Caco-2 cells increased substantially the levels of α-tubulin in P1 and P2. Moreover, concomitant with increasing concentrations of FF we observed a constant increase of α-tubulin in P1. This is strongly supported by quantification of P2/P1 which shows a significant decrease from 1.67 in control cells to approximately 1.0 in cells treated with 100mg FF (Fig. 4-1B, left lower panel). As shown above with actin, OVA did not affect the arrangement of α-tubulin regardless of the concentration. Taking into consideration that the intracellular membranes are also included in P1 it is possible that the increase in the levels of α-tubulin leads to an enrichment of microtubular structures and may thus compensate the loss of an intact actin network.

4.4.2 Co-Immunoprecipitation Experiments Reveal an Alternating Binding Pattern of Gliadin to Actin

Gliadin peptides can be rapidly internalized into the cytosol of a specialized population of enterocytes known as RACE cells (KERSTING et al. 2004). We therefore asked whether gliadin peptides directly interact with the actin cytoskeleton and examined the temporal requirements in a time span between 1 to 72h. The detergent extracts of FF-stimulated cells were immunoprecipitated with an anti-gliadin and further analyzed by immunoblotting using a β-actin antibody. Fig. 4-2 (upper panel) shows an alternating increase and decrease in the concentration of actin in the immunoisolates. Thus, the initial high levels of actin that bound
to gliadin within 1h of stimulation with FF decreased substantially with increasing time points of 3h and 6h, increased again after 12h, reached a minimum at 48h followed by a marked elevation within 60h of stimulation. Nevertheless, the levels of actin that interacted with gliadin did not reach those of the initial 1h. The levels of actin in the total cell lysates of treated cells did not differ from the control non-treated cells (Fig. 4-2, lower panel).

Figure 4-2: Alternating amounts of actin in immunoisolates due to prolonged FF-treatment

Caco-2 cells were stimulated for 1h up to 72hrs with 100mg FF. Immunoprecipitation was performed with the antibody against gliadin and western blot analysis with mAb anti β-actin. The control provides total lysates. Prolonged stimulation results in an alternating increase and decrease in the concentration of actin in the immunoisolates while the general concentration of actin disclosed in total lysates remains unaffected.

Control (C); immunoprecipitation (IP)

Therefore, treatment with FF alters the binding capacity of actin concomitant with the duration of stimulation. Nevertheless, the overall expression levels of actin per se remain unaffected. The actin cytoskeleton undergoes permanent polymerization and depolymerization dynamics making it very suitable for a set of functions within the cell (FUJIWARA et al. 2002). These functions can be divided into functions that depend on stable actin filaments and functions that require the monomer-polymer transition (WEBER 1999). Since the highest amount of co-immunoprecipitated actin was detected at 1h of FF-treatment, our results strongly suggest that gliadin peptides prefer an interaction with the filamentous form of actin that prevails at 1h of FF-treatment. Stimulation with FF for 3h or 6h decreased the amount of actin in the immunoisolates, while the total amount of actin in the cell lysates
remained unchanged. This result indicates that other forms of actin, possibly monomeric globular forms, with lower affinity to FF have been formed. DRAAIJER et al. (1989) have observed that the ratio of globular to filamentous changes while the total amount of actin remains constant.

4.4.3 The Actin-dependent Transport is Impaired after Treatment with Gliadin Peptides

The actin-dependent post-Golgi vesicular protein transport is directly linked to the association of proteins with detergent-resistant membranes (DRMs) or lipid rafts (JACOB et al. 2003). SI associates with DRMs and is targeted to the cell surface in an actin-dependent manner whereas another disaccharidase, LPH, does not require an intact actin network in its transport to the cell surface (JACOB et al. 2003). While SI and LPH are directly transported to the apical membrane in intestinal Caco-2 cells, other brush border proteins such as ApN and DPPIV utilize the direct as well as transcytotic pathway from the basolateral to the apical membrane (LE BIVIC et al. 1990; MATTER et al. 1990a). Therefore, FF-treated Caco-2 cells provide an exquisite model system to analyze how an impaired actin cytoskeleton can influence the sorting of BBM proteins. Caco-2 cells were stimulated for 48 h with FF, during this time the strongest effect on the actin cytoskeleton could be observed (see Fig. 4-2, upper panel). This incubation time is also suitable given the turnover of the proteins under investigation (DUDLEY et al. 1993). The results obtained revealed differential effects of FF on the levels of the glycoproteins, SI, ApN, DPPIV and LPH in the BBM (Fig. 4-3A). Thus the ratio of P2 versus P1, utilized here as a measure for transport competence to BBM, was significantly reduced for SI and partly also for ApN, but not for LPH (Fig. 4-3B). The effects of FF on the overall expression of DPPIV, particularly in BBM did not follow a uniform pattern. The P2 versus P1 ratio slightly decreased when the cells were stimulated with 5 mg or 25 mg FF and restored its normal levels at 100 mg FF (Fig. 4-3A and B).
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Figure 4-3: FF-Stimulation alters the expression levels of some glycoproteins, especially in the BBM-fraction (1)

A) elicits Caco-2 cells treated with FF. The lysates of P1 and P2 were subjected to western blot analysis with the respective antibodies against SI, ApN, DPPIV and LPH. SI is significantly decreased in FF-treated cells especially in P2. An alleviated effect can be observed for ApN. DPPIV is just slightly affected and LPH not at all.

B) provides the appending statistical data. The significance of the ratio P2/P1 is determined by $p \leq 0.05$ (*) and $p \leq 0.001$ (**).
Figure 4-3: FF-Stimulation alters the expression levels of some glycoproteins, especially in the BBM-fraction (2).

Immunostaining of SI (HIS 3) and DPPIV (HBB 3/775/42) in FF-treated cells reveals a strong reduction in the BBM-expression level of SI whereas the remaining protein is rather intracellular distributed (b). DPPIV is obviously not affected by FF.

The analysis of SI and DPPIV via confocal imaging (Fig. 4-3 C-F) supports the data obtained from the BBM-preparation. SI is substantially reduced at the cell surface of FF-treated cells (Fig. 3D), whereas the cell surface expression of DPPIV is only slightly altered (Fig. 4-3, E and F). OVA did neither induce changes in actin cytoskeleton (see Fig. 4-1A) nor in the transport competence of SI to the cell surface in OVA-stimulated cells (Fig. 4-4, A and B).

Together, the results indicate an altered actin cytoskeleton due to FF stimulation impairs the apical transport of SI and, to a lesser extent, ApN and leads to their intracellular accumulation.
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**Figure 4-4:** OVA-treatment has no effect on the expression level of SI

Caco-2 cells were treated with OVA. The lysates of P1 and P2 were subjected to western blot analysis with the mAb anti-SI. Stimulation with OVA has no influence on the expression levels of SI (A) which is also shown in the diagram (B). The significance of the ratio P2/P1 is assessed as $p \leq 0.05$ and revealed no significance.

**4.4.4 Decrease in the Enzymatic Activity of SI in the BBM upon FF-Stimulation**

The effects assessed at the protein level were further substantiated by enzymatic activity measurements of SI in FF and OVA-treated cells. We have chosen to measure the activity of SI, since it is directly affected in malabsorption due to CD and moreover, it reveals the most dramatic effect of all intestinal glycoproteins (see Fig. 4-3). As shown in Fig. 4-5 the activity of SI in P2 was significantly reduced in FF-stimulated Caco-2 cells whereas by contrast, OVA-stimulated cells revealed normal levels of SI activities.
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Figure 4-5: Concomitant decrease of SI at the protein level and enzyme activity in cells treated with FF
Caco-2 cells were treated with FF or OVA. The enzyme activity of SI was measured in the BBM-fraction (P2). The concentration of metabolized glucose was assessed per mg of total protein in P2 and is provided in the diagram as µg glucose/mg protein. The appraisal of results was performed as described and shows a clear reduction in the enzyme activity of SI for all concentrations in FF-treated cells. The significance is determined by $p \leq 0.01$ (**).

4.4.5 Accumulation of Cholesterol and Sphingomyelin in the BBM upon FF-Stimulation
Since the actin-dependent trafficking to the cell surface is affected in FF-stimulated cells we asked whether these effects are translated to alterations in the membrane composition of intestinal cells. Since actin-dependent sorting to the apical membrane implicates DRMs that are enriched in cholesterol and sphingolipids (JACOB and NAIM 2001) we assessed the levels of these lipids in the BBM-fraction of FF-stimulated or non-stimulated cells. Interestingly, significant elevation of the levels of both cholesterol and sphingomyelin were observed (Fig. 4-6). On the other hand, the control OVA-stimulated cells did not reveal any difference in the levels of these lipids. This result provides therefore a further strong support to the notion that the described effects in Caco-2 cells are specific for FF.
The content of brush border lipids alters in FF-stimulated cells
Caco-2 cells were treated with 100mg FF or OVA. The lipids were extracted from the P2 fractions followed by measurement of cholesterol and sphingomyelin. The lipid concentrations were assessed per mg total protein and are represented as µg lipid per mg protein. FF-treatment induces significant elevations of both cholesterol and sphingomyelin. The significance is determined by $p \leq 0.05$ (*) and $p \leq 0.01$ (**).

4.4.6 Impairment of Endocytosis due to Gliadin Treatment
In view of the observations mentioned above we examined the hypothesis that the increase in the lipid levels is the result of an impaired endocytosis. We therefore analyzed the endocytosis of biotinylated wheat germ agglutinin (WGA) in Caco-2 cells in the presence or absence of FF. This assay is based on the capability of WGA to bind glycoproteins including those that undergo endocytosis. Internalized biotinylated WGA will then increasingly appear in the intracellular fraction (P1) with concomitant reduction in BBM (P2). In case endocytic events have been hampered in FF-stimulated cells then the ratio P2/P1 should increase as compared to non-stimulated cells. First, we examined the validity and specificity of our assay by comparing the levels of WGA bound to the BBM at 0°C and after raising the temperature to 37°C. As demonstrated in Fig. 4-7 (A and B) the WGA band detected in the intracellular membranes (P1) of cells left on ice was several folds lower than its counterpart in cells subjected to 37°C. We then examined the internalization capacity of Caco-2 cells after FF-
stimulation. Fig. 4-7 (C, upper panel and D, left panel) shows that the capacity of Caco-2 cells to internalize WGA decreased with increasing concentrations of FF. In fact, the levels of WGA retained at the cell surface in P2 increased substantially resulting in a significant 1.5 to 2-fold increase in P2/P1 ratio at FF concentrations of 25 mg and 100 mg respectively. OVA stimulation of the cells did neither change the levels of WGA in the intracellular membranes (P1) nor in BBM (P2) (Fig. 4-7C, lower panel and D, right panel) clearly indicating that the observed effects are restricted to FF stimulation. Altogether, the internalization of WGA was strongly hampered in FF-stimulated cells lending strong support to the notion that the composition of BBM at the protein and lipid levels is indeed altered due to impaired endocytic processes elicited by disrupted structural organization of the actin cytoskeleton.

Figure 4-7: An internalization assay with biotinylated WGA reveals disturbances in endocytotic processes (1)
After stimulation of Caco-2 cells with FF and OVA the internalization assay was performed with biotinylated WGA as described. The lysates of P1 and P2 were subjected to western blot analysis with a streptavidin-antibody.
A) shows the internalization of WGA at 0°C versus 37°C and the ratio P2/P1 is more than 3-fold higher compared to that of control cells at 37°C (B).
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Figure 4-7: An internalization assay with biotinylated WGA reveals disturbances in
endocytotic processes (2)
In C) the amount of internalized WGA in P1 decreases markedly with rising concentrations of FF,
especially at 100mg FF; concomitant with diminished amounts of WGA in P2.
D) displays the ratio of P2/P1 and reveals a significant reduction of WGA in P1 for cells treated with
25mg FF with \( p \leq 0.05 \) (*) and for 100mg FF with \( p \leq 0.05 \) (**).
4.5 Discussion

The current study demonstrates that gliadin represented by FF alters the integrity of the actin cytoskeleton with subsequent effects on the actin-dependent protein transport to the BBMs as well as endocytic processes. In this respect our data support and substantiate previous studies that treatment with gliadin peptides leads to a rearrangement of the actin cytoskeleton in enterocytes (HOLMGREN PETERSON et al. 1995) and intestinal cells (SANDER et al. 2005). Nevertheless, the biochemical basis of these effects has not been analyzed yet. Our data provide direct evidence that FF interacts with actin, preferentially with the filamentous form and this binding constitutes one putative mechanism for the actin rearrangement. These alterations presumably induce causal disturbances in the homeostasis of the vesicular transport within the cell suggesting a potential mechanism in CD.

In fact, an altered actin cytoskeleton impairs the apical transport of a population of brush border proteins, such as SI and, to a lesser extent, ApN and leads to their intracellular accumulation. That ApN is not affected in its trafficking pattern as SI is presumably due to the transcytotic pathway that this protein partially follows to the apical membrane (MATTER et al. 1990a) that presumably does not require actin, but rather microtubules (BREITFELD et al. 1990; HUNZIKER et al. 1990). This explains why the cell surface levels of DPPIV, which follows the direct and transcytotic pathways (LE BIVIC et al. 1990; MATTER et al. 1990a), are partially reduced at low FF concentrations, but are increased at higher FF concentrations concomitant with the elevation of α-tubulin in the intracellular membrane fraction P1.

The specific effect of FF on the targeting of a specific population of proteins and the discriminatory role of actin is further supported by the observation that LPH, which is known to be transported via an actin-independent pathway to the BBM (JACOB et al. 2003), remains unaffected by stimulation with gliadin peptides. Finally, our conclusions are strengthened by the observation that OVA, another food protein that has been frequently utilized as a negative control in the analysis of pathomechanism of celiac disease, does neither induce changes in the actin cytoskeleton nor in the transport competence of SI and other actin-dependent proteins to the cell surface.

The dramatic reductions in the activity level of brush border SI that occur concomitant with FF-stimulation are directly associated with an altered trafficking and could partially contribute to the onset of carbohydrate malabsorption in patients with CD. An interesting
observed in this study are the elevated levels of lipids in the BBM that are presumably due to a general block of membrane flow and recycling elicited by disturbances of the actin network. The actin cytoskeleton plays a crucial role in endocytosis of particular regions of the plasma membrane destined for recycling and degradation (MUKHERJEE et al. 1997) and the deterioration of actin is therefore not only associated with an inhibition of the anterograde transport, but also membrane internalization. In Caco-2 cells polymerized actin that is associated with the plasma membrane is involved in endocytosis, especially in the formation of coated pits at the apical plasma membrane (GOTTLieB et al. 1993). The disruption of the actin network due to gliadin toxicity may therefore lead to an impairment of membrane internalization and recycling resulting in the accumulation of lipids in the apical membrane. Indeed, the internalization of WGA is strongly hampered in FF-stimulated cells lending strong support to our hypothesis that the composition of the BBM at the protein and lipid levels is indeed changed due to impaired endocytic processes elicited by disrupted structural organization of the actin cytoskeleton.

Interestingly, the overall reduction of the WGA levels in the BBM correlates well with the observed reduction of some glycoproteins, such as SI and ApN, in FF-stimulated cells. Along this, it is likely that many apical glycoproteins in Caco-2 cells follow a similar targeting pathway as SI and partly also ApN and display thus reduced levels in the BBM upon FF-stimulation. Consequently the targets to which the lectin WGA would bind become also reduced leading thus to the observed reduction in the WGA intensity in BBM.

The consequences of the protein and lipid targeting impairment could lead to morphological alterations that are associated with an intracellular accumulation of SI, and also other proteins following similar targeting pathways, and subsequent malfunction. Morphological alterations upon down-regulation of structural brush border proteins, such as villin (COSTA DE BEAUREGARD et al. 1995), are often associated with missorting and defective trafficking of proteins as well as their intracellular accumulation and loss of function (MULLER et al. 2008).

Our study demonstrate that a potential pathogenetic mechanism of celiac disease could be elicited by the direct interaction of gliadin with the actin cytoskeleton in a subset of intestinal cells leading to the dramatic effects on the protein targeting and function. This direct interaction assumes that FF at least partially diffuses into the cytosol. Caco-2 cells possess
this characteristics in partial uptake of FF into the cytosol (unpublished data) although not to the same extent as a subset of intestinal cells referred to as RACE cells (KERSTING et al. 2004). While it is certain that FF binds actin, the binding site and the competition with actin binding proteins are the next questions that should resolved towards better understanding of the pathogenesis of celiac disease.

4.6 Acknowledgment

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4.7 Abbreviations

BBM, brush border membrane; SI, sucrase-isomaltase; LPH, lactase phlorizin hydrolase, mAb, monoclonal antibody; DPPIV, dipeptidylpeptidase IV; ApN, aminopeptidase N; FF, Frazer’s Fraction, OVA, ovalbumin; PFA, paraformaldehyde; cytoD, cytochalasin D

4.8 References


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DISCUSSION
5 DISCUSSION

During the past decades several studies have focused on different aspects of the pathophysiology in CD, especially in the field of diagnostic tools and reliable markers (ANDERSON 2008; SETTY et al. 2008), genetic aspects and predisposition (CICLITIRA et al. 2005; KONING et al. 2005; SHIDRAWI et al. 1998; SOLLID and THORSBY 1993), the toxicity of common cereals (DEWAR et al. 2006; MOLBERG et al. 2003; VADER et al. 2002; VAN DE WAL et al. 1999; AURICCHIO et al. 1985; DICKE et al. 1953) and immune responses due to interactions between specific gliadin peptides and discriminative or restricted T-cells (ARENȚZ-HANSEN et al. 2002; FLECKENSTEIN et al. 2002; MOLBERG et al. 1997; GJERTSEN et al. 1994).

It has been reported that gliadin treatment leads to a disorganization of the microvilli architecture and the associated actin cytoskeleton suggesting a role in mucosal lesion and villous atrophy (WILSON et al. 2004; BAILEY et al. 1989). Nevertheless, the underlying mechanism of how this rearrangement is induced is still obscure. BRANDTZAEG (2006) indicates that the celiac lesion reflects an immune-driven remodeling of mucosal architecture with only a minor inflammatory component, which most likely results from innate signals. He therefore suggests the innate and the adaptive immunity as the main possible models in the pathogenesis of celiac disease. In fact, apart from the immune reaction against the extracellular matrix, CLEMENTE et al. (2000) described an immune reaction against the cytoskeleton of enterocytes in patients with CD as there is a close link between the presence of anti-actin antibodies (AAA) and the severity and persistence of tissue damage. Nevertheless, they suggest that AAA could be secondary to intestinal mucosa damage in that it unmask cryptic antigens. Since the cytoskeletal actin network of intestinal microvilli has been found to be rapidly disorganized after gluten challenge in CD (HOLMGREN PETERSON et al. 1995), there must be another mechanism. The first contact of macromolecular nutrients with the intestinal epithelial cells or enterocytes occurs through the BBM that maintains the intestinal barrier function (SNOECK et al. 2005). Because of this and the fact that treatment of cultured intestinal cells like Caco-2 cells or HT29 cells reveals several effects concerning apoptosis (GIOVANNINI et al. 2003), cell permeability (SANDER
et al. 2005) or internalization and antigen presentation (FISCHER 2007), the most obvious location for initial steps of tissue damage seem the enterocytes themselves. Very little is known about the biochemical basis of the effects of gliadin peptides on the actin cytoskeleton and subsequent actin dependent transport events. The aim of this study was to investigate these effects on the trafficking of several intestinal proteins in intestinal Caco-2 cells or in non-polar transfected COS-1 cells that have been treated with Frazer’s Fraction (FF).

5.1 FF- treatment alters the integrity of the actin cytoskeleton

The immediate effect of FF on the actin cytoskeleton was initially addressed to non-polar COS-1 cells that provide an ideal cellular model for the assessment of the role of the actin cytoskeleton in the absence of other epithelial-specific cytoskeletal proteins, e.g. villin or ezrin. Therefore the expression of an actin-YFP chimera in addition to staining of endogenous actin with Phalloidin provided an adequate readout system in COS-1 cells to evaluate the effects of FF on the globular form of actin as well as the filamentous forms. It is quite obvious that in both displayed formats of actin the presence of FF induced a strong reduction and rearrangement of the terminal web and the plasma membrane associated network. Concomitantly, punctated structures beneath the plasma membrane representing fragmented actin appeared. WILSON et al. (2004) observed similar structures in biopsy specimens of CD patients and described them as actin-coated structures. The morphological alterations of the actin cytoskeleton can be observed within 30 min, which is in line with a rapid impairment of the actin cytoskeleton observed in CD patients due to gluten challenge by several working groups like HOLMGREN PETERSON et al. (1995). In contrast, OVA that represents another food antigen, had no effect on the organization of the cytoskeleton pointing thus to a specific effect of FF on the actin cytoskeleton. To classify the effect of gliadin peptides it was compared with the alterations of the actin cytoskeleton caused by cytoD, a mycotoxin that inhibits actin polymerization and depolymerization (COOPER 1987). Here, the disruption of the actin cytoskeleton led to its accumulation in dense foci and patches, a process that goes along with alteration in the cell shape (SCHLIWA 1982). Since these alterations are more
distinct and destructing to the cells compared to those induced by FF it is obvious that the mode of action of FF and cytoD on the actin cytoskeleton differ significantly. This raises the questions about the putative mechanisms by which gliadin peptides influence the actin cytoskeleton. In this respect, BARONE et al. (2007) observed that treatment with peptic-tryptic gliadin and the truncated „toxic“ peptide 31-43 leads to rearrangements of the actin cytoskeleton that are very similar to that induced by epidermal growth factor (EGF). This is manifested in a rapid and large membrane ruffling at the edge of clustered Caco-2 cells. Membrane ruffling in EGF-stimulated cells originates from newly formed actin filament barbed ends that accumulate at the leading edge (ZEBDA et al. 2000). Here, coflin, an actin depolymerizing protein, is supposed to play a role (CHAN et al. 2000). The process constitutes a prerequisite for massive actin polymerization at the leading edge required for EGF induced lamellipodia extension and cell motility (CONDEELIS 1993). Initial steps of actin nucleation are supposed to occur in dependence of the Arp2/3 complex (BAILLY et al. 1999). Since gliadin-treatment did not reveal lamellipodia extension or cell motility after gliadin-induced membrane ruffling, the Arp2/3 complex may play a role in this context.

In contrast to COS-1 cells, epithelial Caco-2 cells are able to generate an asymmetric plasma membrane, which is divided into two different domains, the apical and the basolateral domain (SALAS et al. 1997; SIMONS and FULLER 1985). A similar characteristic is found in the actin-based cytoskeleton of intestinal cells that also consists of two domains, the microvilli and the subjacent terminal web (LOUVARD et al. 1992; PETERSON and MOOSEKER 1992). Since the cores of microvilli contain uniformly polarized actin bundles that are connected to the overlying membrane via cross-bridges (MOOSEKER et al. 1991), isolation of the BBM with a modified procedure according to SCHMITZ et al. (1973) provides a suitable method to separate the BBM-associated actin cytoskeleton from the intracellular and basolateral connected actin network. Moreover, the BBM-fraction does not only contain actin but also microvillar proteins like BBM-hydrolases (PETERSON and MOOSEKER 1992). The data demonstrate at the protein and the cellular level that stimulation with FF results in a dramatic reduction of membrane-associated actin. Biochemical analyses reveal that the actin level decreases in both fractions P1 and P2, indicating that the integrity of the actin cytoskeleton is extremely disturbed. Additionally, confocal images display a diffuse distribution of actin in FF-treated cells concomitant with a decrease in the BBM-expression
level. The decrease of actin in both fractions suggests a conformational alteration of actin as a reason for its impaired association with membranes per se and with the BBM in particular. The membrane-associated actin is coupled to the BBM via different proteins like class I myosins (COLUMIO 1997; MOOSEKER et al. 1991) or the 110kD-CM protein complex, a complex of myosin IA and calmodulin, which connects the actin derived core bundle of the microvilli to the surrounding plasma membrane via cross-bridges (HOWE and MOOSEKER 1983; GLENNEY et al. 1982; MATSUDAIRA and BURGESS 1982). Interestingly, the linkage of actin and the 110kD-CM complex is determined by the structural feature of the actin bundle (LOUVARD et al. 1992) and occurs in an ATP-dependent manner so that ATP treatment leads to a dissociation of the lateral bridges from the cytoskeleton and Solubilization of the 110kD-CM complex (CONZELMAN and MOOSEKER 1987). ATP plays a crucial role in the dynamic actin monomer-polymer interaction (WEBER 1999) as free actin monomers consist of ATP-actin that is hydrolyzed to ADP-actin after its incorporation into filaments (WEBER 1999). Therefore, conformational changes of actin may prevent ATP binding or the incorporation of ATP-actin, thus leading to an increase in the normal physiological ATP concentration and subsequently to a loss of the actin-BBM connection. The conformational changes could possibly be induced by direct or indirect interactions of gliadin peptides with actin filaments or actin binding proteins that are involved in processes like capping filaments at the barbed end, binding G-actin to the polymerizing filament, or inducing the spreading via filament branches. Those proteins can be capping protein (COOPER and SCHAFER 2000; SCHAFER and COOPER 1995), profilin (WEBER 1999; SCHLUTER et al. 1997) or the Arp2/3 complex (POLLARD 2007).

In contrast to actin, α-tubulin which represents the microtubule cytoskeleton reveals a completely different expression pattern after FF-treatment. Concomitant with raising concentrations of FF α-tubulin increases in the fraction of intracellular and basolateral membranes (P1). This suggests a balancing for decreasing amounts of membrane-associated actin since the microtubule system is also a major part of the cytoskeleton that plays a role in e.g. protein and lipid trafficking (MAPLES et al. 1997; FATH and BURGESS 1993; GILBERT et al. 1991) as well as in endocytic traffic (APODACA 2001).

Interestingly, even though the cell lines and the experimental approaches used are different, the bottom line for the main result is that FF alters the integrity of the actin cytoskeleton in
both cell types. This phenomenon indicates that treatment with gliadin peptides in general affects the integrity of the actin cytoskeleton regardless of the cell type or tissue-specific proteins like microvilli marker proteins or the tissue transglutaminase (tTG) that is absent in COS-1 cells. The latter plays a crucial role in the pathogenesis in CD (SAKLY et al. 2006). It catalyzes the deamidation of glutamine, which commonly appears in gliadin peptides, to glutamic acid residues producing negatively charged gliadin peptides, thus rendering them into more potent T cell epitopes (ARENTZ-HANSEN et al. 2002; FLECKENSTEIN et al. 2002; ANDERSON et al. 2000; MOLBERG et al. 1998). It was shown in Caco-2 cells that deamidation of certain glutamines in specific peptides alters their mode of action in respect to the inhibition of cell growth or activation of tTG (SAKLY et al. 2006), which seems not to be the case in the actin cytoskeleton.

5.2 Gliadin peptides interact with the actin cytoskeleton

The alteration in the integrity of the actin cytoskeleton suggested a direct or indirect interaction of gliadin peptides with the actin cytoskeleton or, more likely, actin binding proteins.

In fact, confocal imaging reveals a rapid uptake of FF in COS-1 cells within 10min which resembles the uptake of other nutritional antigens like OVA or horseradish peroxidase (HRP) into the cytosol of a specific population of enterocytes termed as RACE cells (rapid uptake of antigens into the cytosol of enterocytes) (KERSTING et al. 2004; SCHURMANN et al. 1999). The mechanism by which those peptides enter the cytosol of enterocytes is still unclear. Referring to this, OKADA and RECHSTEINER (1982) and MOORE et al. (1988) described a cytosolic distribution of HRP and OVA after osmotic lysis of pinocytic vesicles. For this reason, pinocytosis was induced by cell treatment with a hypotonic cell medium whereas the osmotic lysis was induced by changing the conditions using a hypertonic medium. With regard to gliadin peptides, it has not been analyzed yet, whether gliadin peptides change the osmolarity in enterocytes. Hence, it is still obscure whether their cytosolic distribution results from a similar release of gliadin peptides from endosomal vesicles.
Nevertheless, the cytosolic distribution of gliadin peptides, represented by FF, enables a direct interaction of those peptides with cytoskeletal components which could be demonstrated through a clear co-localization of the „toxic“ gliadin peptide 31-49 (DE RITIS et al. 1988) with actin filaments. In addition to its cytosolic location, the „toxic“ gliadin peptide 31-49 was also internalized into compartments of the early endosomal pathway but could not be detected in the lysosomes. These observations correspond with internalization studies in HT29 cells (FISCHER 2007). Although COS-1 cells are non-polarized cells, the data may also support the view that, under certain conditions, food antigens follow different types of internalization and subsequent targeting through enterocytes and the intestinal barrier. Consequently, this will either lead to the induction of oral tolerance or not (ZIMMER et al. 2000).

The co-immunoprecipitation analyses in COS-1 cells using an antibody against the „toxic“ gliadin peptide 31-49 support a direct interaction between FF and actin. It is likely that this interaction competes with actin-binding proteins, for example Arp2/3 (MULLINS and POLLARD 1999; SVITKINA and BORISY 1999; MULLINS et al. 1998), interfering thus with the polymerization of actin. This might explain the increase of co-immunoprecipitated actin with the duration of FF-stimulation. Furthermore, it is likely that the „toxic“ gliadin peptides bind more avidly to a particular conformation of actin and that this binding increases with time until a conformational change of actin has taken place. Along with this change, it is possible that the fragmented appearance of actin in some areas of the cell, which was observed in confocal images, could represent partially polymerized actin with bound FF.

Based on these results a similar approach was performed in Caco-2 cells, but FF-stimulation was prolonged to 72h. The data did also reveal a direct interaction between gliadin peptides and actin or actin binding proteins, but in contrast to COS-1 cells the total amount of immunoisolated actin decreases with prolonged FF-stimulation even though there are alternating peaks in cells stimulated for 12 h and 60 h. The different effects that could be observed in both cell lines might result from the fact that generally the incubation in Caco-2 starts with 1 h, as opposed to 10, 30, 60 and 180 min in COS-1 cells, and was prolonged upon 72 h, which does not permit a direct comparison. Another explanation could stem from the use of two different antibodies; in one case actin was isolated with an antibody against the „toxic“ gliadin peptide 31-49 from COS-1 cells, in the other case with a polyclonal anti-
gliadin antibody from Caco-2 cells. As the „toxic“ gliadin peptide 31-49 derives from a peptic-tryptic digest of gliadin, the ratio of the precipitated peptide 31-49 in COS-1 cells is unproportional compared to the whole amount of gliadin in Caco-2 cells, which presumably results in a variable effect. The necessity to use two different antibodies emerged from the situation that the polyclonal rabbit anti-gliadin antibody did not work in COS-1 cells, something that sometimes originates from interferences between different species. Moreover, there is a difference between the relative simple organization of the non-polar COS-1 actin cytoskeleton and the complex ordered actin cytoskeleton in Caco-2 cells which to some extent requires a different and wide set of actin interacting proteins like villin (FRIEDERICH et al. 1999), ezrin (BERRYMAN et al. 1993) or several tight junction proteins (IVANOV 2008). This circumstance can lead to an altered initial situation for this experiment, although there are obviously no differences concerning the question of actin integrity.

Interestingly, the stimulation of Caco-2 cells revealed an alteration in the amount of immunoisolated actin that is related to the duration of FF-treatment, while the total expression level of actin remains the same in all samples. This observation supports the hypothesis that treatment with FF presumably induces conformational changes of actin by either direct binding of actin or indirect binding of actin binding proteins without altering the expression of actin per se. Similar aspects were described by DRAAIJER et al. (1989) when observing an increase in the ratio of globular to filamentous actin without changes in the total amount of actin.

It is therefore likely that gliadin peptides bind more avidly to a particular conformation of actin in Caco-2 cells. The actin cytoskeleton undergoes permanent polymerization and depolymerization events making it very suitable for a set of functions within the cell (FUJIWARA et al. 2002). These functions can be divided into those that depend on stable actin filaments like the maintenance of the microvilli structure and other that require the monomer-polymer transition like cell motility or endocytosis (WEBER 1999). Presumably, gliadin peptides prefer binding to actin filaments or filament-associated proteins since there is a very strong actin band after 1h FF-treatment. Under normal cell culture conditions, the actin cytoskeleton consists to a high extent of filamentous actin, for example to maintain cell shape, cell-cell contact and transport events. Moreover, confocal images of control cells also revealed a distinct filamentous actin network, especially in COS-1 cells. This could explain
the increase of actin over time in COS-1 cells and also the high amount of co-immunoprecipitated actin in Caco-2 cells within the first hour of FF-treatment. The following decrease in the immunoisolates after 3 h and 6 h may therefore indicate the point where the conformational changes of actin have been occurred, leading thus to reduced amounts of actin. There is a possibility that the alternating amounts of immunoprecipitated actin emerge from a fragmentation of actin filaments due to their interaction with gliadin peptides and that the structure and the size of these fragments change over time (see Fig. 5-1). If the gliadin peptides avidly bind the filamentous, potentially fragmented form of actin, it is possible that not all eligible peptides will bind at the same time. It is therefore likely that the remaining gliadin peptides bind to partially newly synthesized actin filaments or actin binding proteins after 12h, which consequently leads to the small increase in the amount of actin. Although the ““toxic“” gliadin peptides could not be detected in lysosomes of COS-1 cells after short times of stimulation with the R5 antibody the reduction of actin in the immunoisolates after 3 h and 6 h could be due to degradation. Since FF contains a large number of different gliadin peptides that are presumably involved in this process certain fragments consisting of gliadin peptides and actin or actin-binding proteins may be destined for degradation. Moreover, synthetic peptides like the immunodominant peptide G3 (α56-68) are described to co-localize with Lamp-2 (lysosomal associated membrane protein-2), a marker for lysosomes (FISCHER 2007). In this case, the synthesis of new actin filaments may elucidate the unvarying levels of actin in the total lysates, whereas the alternating levels of co-immunoprecipitated actin could result from the refreshment of FF in the medium every 24h.

The ability of the actin cytoskeleton to recover from the FF-induced reorganization by newly synthesized actin was demonstrated when stimulating Caco-2 cells for 48h with FF as described, followed by treatment with normal high glucose media (DMEM high glucose) for another 24 h or 48 h. Here, treatment with DMEM results in a recovery of actin in cells treated with 5mg and 25mg FF to approximately 80-90% compared to control cells (data not shown). This result indicates that to a certain extent the alteration of the actin cytoskeleton is presumably reversible.
Figure 5-1: A model: The potential interaction between actin and gliadin peptides
The Model shown here may explain how the gliadin peptides interact with the actin cytoskeleton. When adding FF to the cells a pool of gliadin peptides immediately binds to the actin cytoskeleton either directly or indirectly via actin-binding proteins, while other peptides presumably remain in the cytosol. The interaction will lead to a coarse disruption of the actin network, ending in larger filamentous structures within the first hour. During a 3h stimulation this process may therefore proceed as further free gliadin peptides bind to the originate structures, producing single protofilaments. These protofilaments are not suitable substrates for remaining gliadin peptides in the cytosol to bind and produce even smaller fragments during 3h and 6h of stimulation. This process might therefore explain the decrease in the amount of actin as shown in the immunoisolates on the right hand of the panel. However, the increase of actin after 12h could in turn be due to the fact that gliadin peptides bind more avidly to a certain filamentous form of actin and the small fragments present after 6h are no longer suitable. Since the dynamic of the actin cytoskeleton is given by assembly and disassembly of filaments, the few remaining free gliadin peptides may bind to partly newly synthesized actin filaments the gliadin peptides are more attracted to.

However, the question about the type of interaction requires further experiments focusing on specific binding sides of all potential components, including ions (e.g. Ca\(^{2+}\), Mg\(^{2+}\)) (PIAZZA and WALLACE 1985) or nucleotides (e.g. ATP) (WEBER 1999) that may play a role during the polymerization and depolymerization of actin.
5.3 Impairment of the actin-dependent transport due to FF-stimulation

As previously indicated the function of a correctly folded and mature protein depends on its correct transport and sorting. Therefore, several proteins initially use microtubules in their intracellular transport (GILBERT et al. 1991; ACHLER et al. 1989) and are delivered to the cell surface via actin filaments (JACOB et al. 2003). An example of this family of proteins is SI, whose transport is substantially hampered in FF-treated COS-1 cells as has been demonstrated by a set of different experimental approaches. SI is known to associate with sphingolipid and cholesterol enriched DRMs (JACOB and NAIM 2001), which serve as a sorting platform to the apical membrane (ALFALAH et al. 1999). Moreover, a putative link between DRMs or membrane rafts and the actin cytoskeleton has been described to be mediated through adapter proteins (BRDICKOVA et al. 2001), which would support the actin dependent transport of SI and therefore explain its impaired trafficking. In contrast, the trafficking of LPH is not affected. This conforms with the results of JACOB and co-workers (2003) who demonstrate that LPH follows an actin-independent pathway that presumably implicates microtubules alone, whose function is apparently not influenced by FF.

COS-1 cells provide a suitable model to assess the transport behavior of intestinal epithelial glycoproteins, although these cells are not epithelial. In fact, sorting pathways at the TGN occur as has been previously shown for segregating SI and LPH (JACOB and NAIM 2001) and also in other non-epithelial cell lines like fibroblasts (CHO, 3T3, BHK) (RUSTOM et al. 2002; PAROLINI et al. 1999; MUSCH et al. 1996; YOSHIMORI et al. 1996).

Unlike COS-1 cells, Caco-2 cells endogenously express a set of intestinal brush border glycoproteins that follow different transport routes to the BBM. Here, an altered actin cytoskeleton also impairs the apical transport of SI and, to a lesser extent, ApN, but since the amounts of both proteins in P1 decrease as well, it does neither induce a missorting to the basolateral membranes nor a stack in the intracellular compartments. KERSTING et al. (2004) were able to demonstrate that SI is reduced at the BBM in Crohn’s disease, an inflammatory bowel disease (IBD), but it increases in the cytosol. This is in line with a down-regulation of SI in human intestinal epithelial cells by interleukin-6 (ZIAMBARAS et al. 1996) which is enhanced in IBD (ATREYA et al. 2000). Additionally, down-regulation of villin using the anti-sense RNA affects the brush border assembly in Caco-2 cells leading to an increase of SI within the cytosol as well (COSTA DE BEAUREGARD et al. 1995).
cytosolic distribution may therefore explain the disposition of the proteins. In addition to its impaired trafficking to the apical membrane resulting from an altered cytoskeletal actin, the strong decrease in the BBM expression of SI could be due to a loss of a particular amount of SI that has already reached the BBM. DANIELSON (1995) found that SI also resides in DRMs in the BBM and TYSKA and MOOSEKER (2004) suggest that an interaction of the actin-based motor protein myosin IA with those DRMs is responsible for the retention of SI in the BBM, since a loss of SI is concomitant with low levels of myosin IA. This interaction would also provide a link from SI to the underlying cytoskeleton. TYSKA and MOOSEKER (2004) presume that in the absence of a cytoskeletal linkage, SI may be more susceptible to removal from the cell via the continuous apical membrane shedding that occurs at microvillar tips in Caco-2 cells. In contrast to the latter model, JACOB et al. (2003) reported that SI-containing apical vesicles contain myosin IA that triggers the apical transport along actin filaments. This is in line with observations of FATH et al. (1994), who proposed an active role of myosin IA in the apical targeting of Golgi-derived vesicles. As mentioned before, myosin IA is also a component of the 110kD-CM protein complex that connects the actin derived core bundle of the microvilli with the BBM. The disruption of this complex by, for instance, ATP leads to a dissociation of actin from the BBM. Altogether, these observations suggest that myosin IA could be one of the actin-associated proteins that is also affected by FF-stimulation. The affected trafficking pattern of ApN compared to SI presumably results from a transcytotic pathway (see Fig. 5-2) that this protein partially follows to the apical membrane (MATTER et al. 1990a) and that does probably not require the actin cytoskeleton. In fact, microtubules have been described to be crucial in the basolateral to apical transcytosis (Fig. 5-2) (LEUNG et al. 2000; BREITFELD et al. 1990; HUNZIKER et al. 1990). This may another fact that explains why the cell surface levels of DPPIV that is targeted to the apical membrane both directly and via transcytosis (see Fig. 5-1) (LE BIVIC et al. 1990; MATTER et al. 1990a), are partially reduced at low FF concentrations, while they are increased at higher FF concentrations. At high FF concentrations, an elevation of α-tubulin in the intracellular membrane fraction P1 was observed. The fact that Caco-2 cells represent a polarized cell line with an apical and a basolateral domain imposes an entirely different challenge compared to non-polarized COS-1 cells. One essential process in preserving this
cellular polarity is the delivery of newly synthesized proteins and lipids to specific sites at the cell surface (JACOB and NAIM 2001),

![Figure 5-2: Cytoskeletal tracks and transport routes in polarized epithelial cells](https://www.nature.com/figure01602_F5.html)

In polarized cells, proteins follow different pathways to reach either the apical or the basolateral membrane domain. Proteins like SI follow a direct pathway to the apical surface that implicates the actin cytoskeleton. In contrast, the transport of LPH, which is also directly transported to the apical membrane, occurs in an actin independent manner that rather implicates microtubules. ApN and DPPIV represent proteins that follow both a direct and an indirect route to the apical membrane. Here, the indirect transport occurs via transcytosis from the basolateral to the apical membrane that possibly requires microtubules.

Source: www.nature.com/figure01602_F5.html (modified)

which includes transcytosis of certain proteins. These events are maintained by a sophisticated sorting process within the TGN that implicates specific signals for the apical and basolateral domains (GRIFFITHS and SIMONS 1986), and an organized post-TGN protein traffic that requires coordinated interactions between different cytoskeletal systems (MAYS et al. 1994; FATH et al. 1993). Therefore, the discriminative sorting signals of ApN and DPPIV may lead to differences in their appearance at the BBM, although both of them follow a direct as well as an indirect pathway to reach the latter one. While the apical sorting of DPPIV occurs through both complex N-linked and O-linked carbohydrates and implicates
its association with microdomains containing cholesterol and sphingolipids (ALFALAH et al. 2002), the sorting of ApN implicates neither O-linked nor N-linked glycans and is driven most likely by carbohydrate-independent mechanisms (NAIM et al. 1999). Although the apical sorting of DPPIV has more in common with the sorting of SI than with the one of ApN, the impaired transport to the BBM after FF-treatment of the latter one shows more similarities to that of SI. However, transport studies in Caco-2 with microtubule reducing agents revealed a transport competence of ApN to the BBM of approximately 80% of the newly synthesized protein, whereas DPPIV is rather resident at the basolateral membrane (GILBERT et al. 1991). The high rate of apically transported ApN to the BBM in the absence of microtubules suggests a direct route that implicates actin, whereas only a small amount of ApN (approximately 10%) follows a transcytotic pathway along microtubules. In contrast, the transport of DPPIV is apparently more hampered by disruption of the microtubule system (GILBERT et al. 1991). Together with the present results on FF, it can be presumed that DPPIV follows a direct route along actin to a certain extent, which would explain the decrease after treatment with 5mg and 25mg FF. On the contrary, the main transport of DPPIV presumably occurs through the basolateral membrane via microtubules, which in turn leads to the increase of DPPIV in the BBM fraction concomitant with elevated amounts of $\alpha$-tubulin in cells treated with 100mg FF.

In comparison to SI, ApN and DPPIV, the targeting of LPH remains unaffected by stimulation with gliadin peptides, as previously demonstrated in COS-1 cells. Although it has been shown here that the BBM-expression of LPH is not affected by FF-treatment which presumably results from its actin independent transport to the apical membrane several studies reveal a certain correlation between CD and lactose intolerance (RADLOVIC et al. 2009). For example, OJETTI et al. (2005) found a high prevalence of CD in patients with lactose intolerance. Lactose intolerance is the most common intestinal disorder associated with an absence or drastically reduced levels of intestinal LPH (NAIM 2001), which opposed to the results of this study. The generally low expression level of LPH in Caco-2 cells consequently leads to the creation of an over-expressing hybrid clone. Although no evidence could be found that the over-expression has altered the behavior of LPH or the Caco-2 cells per se, it cannot be excluded that the nature of LPH in enterocytes differs from that of Caco-2 cells. Contrarily, the decrease in the levels of LPH in enterocytes of patients with CD that possibly
trigger lactose intolerance may also be derived from mucosal lesions or villous atrophy (PRASAD et al. 2008) which often results in a consecutive malabsorption syndrome (CICLITIRA and MOODIE 2003; SOLLID 2002; MARSH 1992).

However, the specific effect of FF on the targeting of a specific population of proteins and the discriminatory role of actin is supported by the observation that OVA, which has been frequently utilized as a negative control in the analysis of pathomechanisms of CD (SHIDRAWI et al. 1995), does neither induce changes in the actin cytoskeleton nor in the transport competence of SI. Since OVA-stimulation has no impact on the enzymatic activity levels of brush border SI, the dramatic reduction in its activity levels after FF-stimulation is directly associated with an altered trafficking and could partially contribute to the onset of carbohydrate malabsorption in patients with CD.

5.4 **FF treatment hampers endocytic events and subsequent membrane flow and recycling**

An interesting observation in this study is the elevated levels of cholesterol and sphingomyelin in the BBM. Since the transport vesicles emerging from the post-Golgi consist of both proteins and lipids (LOUVARD et al. 1992) one would rather assume that concomitant with reduced levels of certain proteins in the BBM, the amount of lipids will also decrease; this applies especially to cholesterol and sphingolipids enriched in DRMs with which SI is associated (JACOB and NAIM 2001). Apart from its function as a biosynthetic transport system, the actin cytoskeleton plays a crucial role in endocytosis of particular regions of the plasma membrane destined for the recycling of proteins and lipids and degradation (MUKHERJEE et al. 1997). The deterioration of actin is therefore not only associated with an inhibition of the anterograde transport, but also with membrane internalization. Thus, the elevated levels of cholesterol and sphingomyelin could result from a general block of membrane flow and recycling elicited by disturbances of the actin network. While the functions of the microtubule cytoskeleton in endocytic processes are well established and focused rather on the post-endocytic traffic than on internalization (APODACA 2001; DURRBACH et al. 1996; BOMSEL et al. 1990), the actin regulated endocytosis is described to be important in specific cell types or tissues and is therefore
depending on the type of endocytosis, like clathrin-dependent and -independent mechanisms (DA COSTA et al. 2003; APODACA 2001). In Caco-2 cells polymerized actin that is associated with the plasma membrane, is supposed to be involved in endocytosis, especially in the formation of clathrin coated pits (CCPs) at the apical plasma membrane (HYMAN et al. 2006; APODACA 2001). The disruption of the actin network due to gliadin toxicity may therefore lead to an impairment of membrane internalization and recycling, resulting in the accumulation of lipids in the apical membrane. A similar effect was described for cytoD where proteins were found to accumulate at the plasma membrane because the pits were prevented to pinch off (GOTTLIEB et al. 1993).

The utilization of biotinylated WGA provides a suitable reagent to assess the capability of endocytosis in FF-treated cells. WGA is lectin that selectively recognizes sialic acid and N-acetylglucosamine sugar residues (GALLAGHER et al. 1985) which are predominantly found in plasma membrane glycoproteins (LODISH et al. 2000).

The data show that the internalization of WGA is strongly hampered in FF-stimulated cells lending strong support to the hypothesis that the composition of the BBM at the protein and lipid level is indeed changed due to impaired endocytic processes, which again is elicited by disrupted structural organization of the actin cytoskeleton. Interestingly, the overall reduction of the WGA levels in the brush border membrane correlates well with the observed reduction of some glycoproteins, such as SI and ApN, in FF-stimulated cells. Along with this observation, it is likely that many apical glycoproteins in Caco-2 cells follow a similar targeting pathway as do SI and to some extent also ApN, and that they thus display reduced levels in the brush border membrane upon FF-stimulation. Consequently, the targets to which the lectin WGA would bind become reduced too, leading to the observed reduction in the WGA intensity in BBM.

The consequences of an impaired protein and lipid targeting could lead to morphological alterations that are associated with an intracellular accumulation of SI and other proteins following similar targeting pathways, and to a subsequent malfunction. Morphological alterations upon down-regulation of structural brush border proteins, such as villin (COSTA DE BEAUREGARD et al. 1995), or in various forms of IBD (ATREYA et al. 2000) are often associated with missorting and defective trafficking of proteins as well as their intracellular accumulation and loss of function (MULLER et al. 2008).
Moreover, an altered ratio of membrane proteins to membrane lipids may influence the membrane homoeostasis, thus leading to additional impairments of cellular functions that depend on the asymmetric characteristic of epithelial cells, such as the vectorial transport of molecules across the cell layer (DRUBIN and NELSON 1996).

5.5 Concluding remarks

The current study demonstrates at cellular and protein level that gliadin represented by FF affects the actin cytoskeleton and subsequent protein trafficking of both non-polarized COS-1 cells and intestinal epithelial Caco-2 cells. Moreover, the results of this study provide direct evidence that FF interacts with actin, preferably with the filamentous form; and that this binding constitutes one putative mechanism for the actin rearrangement. These alterations presumably induce causal disturbances in the homeostasis of the vesicular transport within the cell, suggesting a potential mechanism in CD.

While it is certain that FF binds actin or actin-binding proteins, the identification of the binding site and the competition with actin-binding proteins are the next questions that should be addressed towards a better understanding of the pathogenesis of CD.
Celiac disease (CD) is a chronic inflammation of the small intestine due to ingestion of gluten in genetically predisposed individuals (HLA-DQ2/DQ8). While there has been great progress in some areas of CD, e.g. diagnostic, genetic aspects, or immunology, the molecular basis of the mucosal damage, especially at the outset of etiopathology, is poorly understood.

It has been described that treatment with gliadin peptides leads to morphological alterations of the actin cytoskeleton in both biopsy specimens and Caco-2 cells. This points to a possible role of actin during early steps in the pathogenesis of CD, since actin is a key player in a variety of cellular events e.g. cell shape, protein transport and sorting, and endocytosis.

For these reasons, the present study was aimed to elucidate the gliadin-induced rearrangements of the actin cytoskeleton on the molecular level and to investigate this effect on subsequent protein trafficking.

Employment of discriminative experimental procedures revealed new insights into immediate effects on two different cell types (COS-1 and Caco-2) due to exposure with Frazer’s Fraction (FF), the source of gliadin peptides. Initial investigations with COS-1 cells that were treated with FF revealed morphological alterations in the actin filaments, which appeared as fragmented and aggregated structures in confocal laser microscopy. This observation indicates deterioration in the integrity of the actin cytoskeleton. Interestingly, there was a decrease of immunoprecipitated radio-active labeled actin, which suggests conformational changes. Leading thus to an altered antibody-binding capacity of actin. In contrast to FF, cytochalasin D (cytoD) induced a very drastic disruption of actin, which led to its accumulation in dense foci and patches concomitant with alteration in the cell shape. Moreover, actin was only partially reduced in immunosolates from cytoD-treated cells. Therefore, the effect of FF appears to be different from that of cytoD. On the other hand, a peptic-tryptic digest of ovalbumin (OVA), that represents another food antigen, has no effect on actin neither in COS-1 cells nor in Caco-2 cells indicating that the effect on actin is gliadin-specific.
Stimulation of Caco-2 cells with FF revealed also changes in the integrity of the actin network. In intestinal epithelial cells, the brush border is formed by a huge number of microvilli that are maintained by numerous actin filaments connected to the overlying brush border membrane (BBM) and the adjacent actin network. Isolation of BBMs from FF-stimulated Caco-2 cells showed a strong reduction of actin in the BBM-fraction which suggests a decrease in the association of actin with the BBM. Interestingly, α-tubulin displayed in turn an increase in the protein level indicating a compensational effect.

The alteration of the actin cytoskeleton is presumably induced by direct interaction of FF-derived gliadin peptides with actin or, more likely, actin binding proteins. Here, confocal imaging of FF-treated COS-1 cells showed a clear co-localization of the “toxic” gliadin peptide 31-49 with the actin cytoskeleton and co-immunoprecipitation revealed an increase of actin in the immunoisolates with time. On the other hand, Caco-2 cells displayed an alternating binding pattern of co-immunoprecipitated actin after prolonged stimulation up to 72h although the amount of actin in total lysates remains the same. A varying binding pattern together with the appearance of actin fragments in confocal images after FF-stimulation suggests a favored binding of gliadin peptides to the filamentous form of actin.

The alterations of the actin cytoskeleton elicited impaired protein trafficking of intestinal sucrase-isomaltase (SI), a glycoprotein that follows an actin-dependent vesicular transport to the cell surface. This came along with reduced enzymatic function of SI in the BBM-fraction of Caco-2 cells. The transport of aminopeptidase N (ApN) and dipeptidylpeptidase IV (DPPIV) was only partially affected by FF. This is presumably due to their property to follow both a direct and a transcytotic pathway to the BBM. The latter pathway involves predominantly microtubules. By contrast, the actin-independent protein transport, as monitored by transport of the representative protein intestinal lactase phlorizin hydrolase (LPH), remains unaffected.

In addition to anterograde transport processes, actin-dependent retrograde events are also found to be hampered by FF stimulation. Vesicles that are released from the trans-Golgi network do not only contain proteins but also lipids as SI associates with membrane microdomains enriched in cholesterol and sphingomyelin. Normally, these lipids are often internalized and recycle back to the plasma membrane. The establishment of an assay using biotinylated wheat germ agglutinin (WGA) provides an opportunity to assess the endocytic
capability of treated and non-treated Caco-2 cells. The data revealed a substantially block of
the endocytic pathway in FF-treated cells that led to an accumulation of cholesterol, and
sphingolipids in the BBM.
Altogether, the data demonstrate that FF deteriorates the actin cytoskeleton in COS-1 cells
and Caco-2 cells by direct interaction with actin or, more likely, actin binding proteins leading
thus to altered integrity. Consequently, this results in reduced protein trafficking and
hampered endocytic events with subsequent alterations in the protein and lipid composition of
the BBM. The reduced levels of the disaccharidase SI in the BBM suggest therefore a
potential pathomechanism of carbohydrate malabsorption in CD.
ZUSAMMENFASSUNG
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Yvonne Reinke – Die Auswirkung der *in vitro* Gliadin Toxizität auf die Integrität des Aktinzytoskeletts und den nachfolgenden Proteintransport


Abnahme von Aktin in den Immunisolaten festgestellt werden, was ebenfalls auf eine andere Art von Interaktion hinweist. Im Gegensatz dazu hat pepsin-trypsin verdautes Ovalbumin (OVA), das als Kontrollantigen eingesetzt wurde, keinerlei Einfluss auf das Aktinzytoskelett, weder in COS-1 Zellen noch in Caco-2 Zellen, was auf einen Gliadin-spezifischen Effekt hindeutet.

In Caco-2 Zellen ruft die Stimulation mit FF ebenfalls eine Änderung in der Integrität des Aktinnetzwerk hervor. In intestinalen Epithelzellen wird der Bürstensaum von einer großen Anzahl von Mikrovilli gebildet, die durch zahlreiche Aktinfilamente, die mit der Bürstensaummembran (BBM) und dem darunterliegenden Aktinnetzwerk verbunden sind, aufrechterhalten wird. Die Isolation der BBM von FF-behandelten Caco-2 Zellen zeigte eine starke Reduktion von Aktin in der BBM-Fraktion, die auf eine geringere Assoziation von Aktin mit der BBM schließen lässt. Interessanterweise konnte für α-Tubulin eine Zunahme in der BBM-Fraktion beobachtet werden, die auf einen ausgleichenden Effekt hindeutet.


werden, wobei der zuletzt genannte Weg vor allem über Mikrotubuli erfolgt. Im Gegensatz dazu ist der Aktin-unabhängige Transport, der durch die intestinale Laktase Phlorizin Hydrolase repräsentiert wird, in keiner Weise eingeschränkt.


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Erklärung

Hiermit erkläre ich, dass ich die Dissertation „Implication of Gliadin Toxicity on Actin Integrity and Subsequent Protein Trafficking in vitro“ selbständig angefertigt habe.


Ich habe meine Dissertation an der folgenden Institution angefertigt:
Institut für Physiologische Chemie, Stiftung Tierärztliche Hochschule Hannover

Die Dissertation wurde bisher nicht für eine Prüfung der Promotion oder für einen ähnlichen Zweck zur Beurteilung eingereicht.

Ich versichere, dass ich die vorstehenden Angaben nach bestem Wissen vollständig und der Wahrheit entsprechend gemacht habe.

Hannover, den

____________________________
Yvonne Reinke
DANKSAGUNG
DANKSAGUNG

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