CALRETICULIN 3 GENE POLYMORPHISM IN CELIAC DISEASE AND EFFECTS OF GLUTEN ON CALRETICULIN LOCALIZATION AND EXPRESSION IN EPITHELIAL CELLS *IN VITRO*

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Preface

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Abstract of Master´s Thesis

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Abstract:

Celiac disease (CD) is an inflammatory disease of the small intestine with characteristics of autoimmunity. CD is induced in genetically susceptible individuals by gluten peptides from dietary wheat, barley and rye and leads to mucosal inflammation, villous atrophy, crypt hyperplasia and production of autoantibodies. Majority of these antibodies are against the enzyme transglutaminase 2 (TG2). CD patients are also positive for autoantibodies that recognise other autoantigens, such as calreticulin (CRT), which is a multifunctional, Ca^{2+}-binding chaperone of the endoplasmic reticulum (ER). Its chromosomal location 19p13.1 has previously been linked to CD. CRT is able to modulate the immune system and epithelial cell function and is suspected to have role in CD. *calr3* gene polymorphisms from 25 CD and 25 control patients were determined by Conformation-sensitive Gel Electrophoresis (CSGE), a fast and sensitive method to detect single nucleotide polymorphisms (SNP). CSGE positive samples were confirmed by DNA sequencing. The alterations in CRT localization and expression levels in T84 human epithelial cells after gluten challenge was determined by confocal microscopy and Western blotting, respectively. An intron mutation was found from 9/25 (36%) of control patients against 2/25 (8 %) of CD patients ($p<0,05$). SNPs resulting in amino acid changes were not identified. The found mutation possibly exhibits a protective function against gluten challenge and thus inhibits the pathogenesis of CD and may be an important contribution to the understanding of CD genetics. Gluten challenge resulted in altered localization and downregulation of CRT. The localization change of CRT reflects the gluten-induced stress response and can be used as an additional characteristic in describing the epithelial gluten-response. The down-regulation of CRT by gluten may interfere intracellular signalling and uptake of apoptotic cells by professional phagocytes. The CRT expression and localization data shown here support the theory that CRT has a role in CD.

Keywords: celiac disease, calreticulin, autoimmunity, inflammation, apoptosis

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Tiivistelmä:

Keliakia on ohutsuolen autoimmuunisairaus. Vehnän, ohran ja rukiin gluteenipeptidit aiheuttavat perinnöllisen alttiuden omaavissa henkilöissä suolen limakalvon tulehdusreaktion, villusten atrofian, kryptien hyperplasian ja IgA-luokan autovasta-aineiden tuoton. Pääosa autovasta-aineista kohdistuu transglutaminaasi 2 (TG2) –entsyymiä vastaan, mutta potilailla esiintyy myös vasta-aineita, jotka tunnistavat muita antigeenejä. Osa vasta-aineista tunnistaa kalretikuliinin (CRT), joka on ER:n luumenissa sijaitseva, kalsiumia sitova proteiini. Keliakia potilaan autovasta-aineet ristireagoivat CRT:n kanssa. *calr3*-geeni sijaitsee kromosomaalisessa lokuksessa 19p13.1, jonka on todettu assosioituvan keliakiaan. CRT:n tunnetaan vaikuttavan immuunipuolustukseen ja epiteelisolujen toimintaan ja sillä ajatellaan olevan osa keliakian patogeneesissä. Tässä tutkimuksessa verrattiin 25 keliakiapotilaan ja 25 kontrollihenkilön *calr3*-geenin polymorfismia käyttäen konformaatioherkkää geelielektroforeesia (CSGE). CSGE on nopea ja herkkä menetelmä yksittäisten nukleotipolymorfismien (SNP) tunnistamiseksi. CSGE:llä löydetyt mutaatiot varmennettiin DNA-sekvensoinnilla. Toisena tutkimuskohteena oli gluteenipeptidien vaikutus CRT:n paikantumiseen ja proteiinin ilmentymiseen ihmisen T84-epiteelisoluissa. CRT:n sijainti määritettiin konfokaalimikroskopialla ja proteiinin tuotto Western blotting –menetelmällä. Tutkimuksessa havaittiin intronimutaatio, joka esiintyi yhdeksällä kontrollihenkilöllä (36 %) ja kahdella keliakiapotilaalla (8 %) kahdestakymmenestäviidestä ($p<0,05$). Aminohappomuutoksiin johtavia mutaatioita ei löydetty. Löydetty mutaatio saattaa suojata perinnöllisen altistuksen omaavaa henkilöä keliakian puhkeamiselta tai hidastaa sitä. Tämä mutaatio saattaa olla tärkeä lisä keliakian perinnöllisyyden ymmärtämisessä. Gluteenialtistus epiteelisoluissa johti CRT:n muuttuneeseen paikantumiseen ja vaimennussäätelyyn. CRT:n paikantumisessa havaittu muutos heijastaa gluteenialtistuksen aiheuttamaa stressivastetta ja sitä voidaan mahdollisesti käyttää uutena ominaispiirteenä kuvattaessa epiteelisolujen gluteenivastetta. Havaittu gluteenialtistuksen aiheuttama vaimennussäätely voi johtaa muutoksiin solunsisäisessä viestinnässä ja häiritä apoptoottisten solujen fagosytoosia. Muutokset CRT:n paikantumisessa ja proteiinin ilmentymisessä tukevat teoriaa, jonka mukaan CRT:llä on osa keliakian patogeneesissä.

Avainsanat: keliakia, kalretikuliini, autoimmuniteetti, tulehdus, apoptoosi

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Abbreviations

BSA-PBS	Bovine serum albumin in phosphate buffered saline
calr3	Calreticulin 3 gene
CD	Celiac Disease
CRT	Calreticulin
ER	Endoplasmic reticulum
HLA	Human leukocyte antigen
IL	Interleukin
PBS	Phosphate buffered saline
PT-gliadin	Peptic-tryptic digested gliadin
TG2	Transglutaminase 2
TGF	Tumor necrosis factor

1. Introduction

1.1 Celiac Disease

Celiac disease (CD) is a common disorder of the small intestine. It is induced by dietary gluten from wheat, barley and rye and leads to villous atrophy, crypt hyperplasia and mucosal infiltration of lymphocytes in genetically susceptible individuals. The classical symptoms of CD include chronic diarrhea, failure to thrive, malabsorption, anorexia, abdominal distention and muscle wasting. Today, CD is often characterized by atypical extraintestinal symptoms such as dermatitis herpetiformis, iron deficiency, fatigue, osteoporosis and ataxia (for review see Fasano and Catassi, 2001).

Several screening studies based on highly specific and sensitive serological tests from Europe, South America, Australasia and the USA have revealed that approximately 0.5-1% of the population in these countries may have undetected celiac disease (for review see van Heel, 2006). In the early 90's Catassi and colleagues reported prevalence of 1 in 93 for undetected CD (1994). These results were supported by studies from Finland and UK where patients were tested by serological tests and positive cases were offered small intestinal biopsies. Prevalence of 1 in 100 was found in both studies (Mäki et al, 2003; Bingley et al, 2004). The highest prevalence of 5.6 % was reported in a study of Saharawi children. The reason for this high prevalence is unknown. The authors speculate that it is related to genetic background and that the CD-associated malabsorption may provide protection against infections and parasites (Catassi et al, 1999). Recent study suggests that CD prevalence increases over time in Finnish population (Lohi et al., 2007).

Several pathologies are known to be associated with CD. Association with gastrointestinal malignancies has been reported with patients that do not strictly follow a gluten-free diet, although this view has been questioned. A 1.9 fold increase in mortality rate in CD patients has also been reported. Strictly followed gluten-free diet lowers the mortality rate of patients

to that of general population. Autoimmune diseases such as type I diabetes mellitus, autoimmune thyroiditis and Sjögren syndrome are also known to occur in association with CD (for review see Fasano and Catassi, 2001).

1.2 Genetics

It is well known that CD has a genetic factor that increases susceptibility to the disease with ~ 10 % prevalence in affected families and ~ 70 % concordance rate amongst monozygotic twins (Greco et al, 2002). CD is strongly associated with human leukocyte antigen (HLA) class II genes in DQ locus. HLA association is estimated to be responsible for 40-50 % of the total genetic contribution to the disease (for review see Sollid, 2005). Approximately 95 % of CD patients carry HLA-DQ2 (DQA1*0501/DQB1*0201) and most patients that do not express HLA-DQ2 are HLA-DQ8 (DQA1*0301/DQB1*0302) positive. (Stepniak et al, 2005). The HLA DQ heterodimer can be encoded in *cis* (where the monomer subunits are on the same haplotype) or in *trans* (subunits on different haplotypes). DQA1*05 alfa chain and DQB1*02 beta chain alleles are associated with an increased risk of CD, but the risk is greater according to the number of DQB1*02 molecules (Limongelli et al, 2005). Both DQ2 and DQ8 have characteristic peptide binding motifs that have a preference for negatively charged and hydrophobic amino acids. The association of the HLA molecules to CD can be explained by the binding of DQ molecule to gliadin fragments and presentation to T cells (Vader et al, 2003).

Genetic susceptibility in CD is not explained by HLA alone because approximately 40 % of the general population carries the HLA-DQ2/-DQ8 haplotype (Greco et al, 2002). In addition to HLA in chromosome 6, tumor necrosis factor (TNF) -2 association in the same area has also been reported (Louka et al, 2003). A number of other chromosomal locations from whole genome screening studies are reported to associate with CD. The regions most consistently linked to CD are on chromosome 5 (Greco, 2001) and 19 (Van Belzen et al, 2003). Fine mapping of chromosome 19 has revealed that the gene myosin IXB is the most associated with CD in some populations (Monsuur et al, 2005). In addition to these locations the area 2q33

encoding immune regulatory factors CTLA4/CD28 has been related to CD (Holopainen et al., 2004).

1.3 Gluten

Gluten is the protein fraction of wheat, barley and rye that exhibits the property of stickiness in bread. Gluten can be further subfractioned to ethanol-soluble prolamines, witch have a high content of proline and glutamine residues, and ethanol-insoluble glutenins. Prolamines in wheat, rye and barley are termed gliadin, secalin and hordein, respectively (for review see Ciccocioppo, 2005). Gliadin peptides and the related prolamins are known to be the external triggers for mucosal lesion in CD patients (for review see Sturgess et al, 1991).

Gliadin is the most extensively studied prolamin. It is a heterogenous mixture of proteins consisting of at least 40 components witch can be divided into four major groups: a, ß, ? and ? -gliadins (Jones et al, 1959). A number of gliadin peptides have been identified as toxic (induces mucosal damage) or immunogenic (induces T cell response) to CD patients (for review see Ciccocioppo, 2005). The presence of multiple proline and glutamine residues in gliadin fragments makes them resistant to gastric, pancreatic and intestinal digestive proteases (Hausch et al, 2002).

1.4 Pathogenesis

Sequential progression of CD, based on histopathological findings, was first suggested by Marsh (1992). Stage 1 of the disease is characterized by an increased number of intraepithelial lymphocytes followed by lymphocyte infiltration to *lamina propria.* Crypt hyperplasia is characteristic to stage 2 of CD and is followed by villous atrophy in stage 3.

Under physiological conditions, the epithelium of the small intestine is almost impermeable to macromolecules, such as gliadin. In CD, the permeability of the epithelium is increased and gliadin peptides cross the epithelial barrier and reach *lamina propria* because the integrity of the tight junction system is compromised (Schulzke et al., 1998).

Little is known about the biological activity of gliadin peptides. Gliadin is known to upregulate the expression of zonulin, a protein suggested to be involved in tight junction regulation. The release of zonulin induces a protein kinase C-mediated polymerization of intracellular actin filaments, which are connected to the proteins of the tight junction complex and thus regulate epithelial permeability (Clemente et al., 2003). Interference of the epidermal growth factor receptor endocytosis followed by prolonged activation of the receptor pathway by gliadin has been suggested to contribute to the typical atrophic and proliferative alterations in CD (Barone et al., 2006). Also, presence of the inflammatory mediators, such as tumor necrosis factor-α (TNF-α) and interferon-? increases the permeability of the epithelial barrier, suggesting that the loss of barrier function caused by access of gliadin to submucosa is sustained by the inflammatory response (Madsen et al., 1997).

There is evidence that gliadin peptides are able to reorganize intracellular actin filaments, induce maturation of dendritic cells and induce the secretion of chemokines, such as: interleukine-1 (IL-1) beta, TNF-α, IL-10 and macrophage inflammatory protein-2 (Clemente et al., 2003; Nikulina et al., 2004). Additional evidence of the activation of the innate immune mechanism was provided by the findings that gliadin peptide P31-43 is able to induce the expression of CD83 (mature dendritic cell marker), cyclooxygenase-2 (an enzyme induced in inflammation), CD25 and IL-15 in CD3- cells *ex vivo*. Neutralization of IL-15 by specific antibodies reversed activation of the innate immune mechanism thus suggesting a mediatory role of IL-15 in intestinal mucosal damage (Maiuri et al., 2003). Interestingly, IL-15 upregulates the expression of the natural killer cell ligand NKG2D on intraepithelial lymphocytes and the corresponding receptor MICA on epithelial cells. This has been suggested to be the mechanism of apoptosis of epithelial cells and contribute to tissue remodeling in CD (Meresse et al., 2004). Matrix metalloproteinases are also shown to signifantly contribute to the destruction of intestinal matrix (Pender et al., 1997).

Once the gliadin peptides enter *lamina propria*, they are presented to CD4 positive T cells by antigen presenting cells expressing HLA DQ2 or HLA DQ8. Gluten-reactive CD4+ T cells,

which are the cause of inflammation in the mucosa, can be found from intestinal biopsies of CD patients but not those from healthy individuals. Deamidated gliadin peptides are only recognized by these cells in the presence of DQ2 or DQ8 molecules, although a variety of MHC class II molecules are expressed by CD patients. The strong association of DQ2 and DQ8 molecules in CD can be explained by the preferential HLA presentation of gluten-derived peptides to CD4+ T cells. The expression of DQ2 and DQ8 molecules is thus a necessary but not a sufficient factor for CD (for review see Sollid, 2002). Dendritic cells thus appear to have a dual role in CD specific immune responses: presentation of gliadin peptides to T cells to induce adaptive immune response and secretion of IL-15 to mediate intraepithelial lymphocyte killing of epithelial cells.

1.4.1 Celiac Disease-specific Antibodies

A characteristic feature of CD is the appearance of specific antibodies in serum of CD patients after gluten ingestion. Anti-gliadin serum antibodies of IgA class were identified in the early 80's (Savilahti et al., 1983) but the specificity of these antibodies is low. IgA class anti-gliadin antibodies are also found in CD with high specificity (Unsworth et al., 1981). In addition, serum reticulin IgA antibodies against connective tissue fibers have been used in detection of CD and found to have high specificity (Mäki et al., 1984). At present, reticulin antibody test is often replaced by the more specific and sensitive endomysial antibody test (Chorzelski et al., 1983).

Dieterich and colleagues were the first to report tissue transglutaminase (TG2) as the main autoantigen for the anti-endomysial antibodies (1997). These anti-TG2 antibodies were later shown to fully explain the CD-specific autoantibody binding patterns to endomysium and reticulin tissues (Korponay-Szabo et al., 2003). TG2-spesific autoantibodies are exploited in the diagnosis of CD as they exhibit high specificity and sensitivity (Raivio et al., 2007).

In addition to being present in patient serum, the IgA class autoantibodies have also been found as deposits in the intestinal basement membrane area and on subepithelial fibroblasts

(Karpati et al., 1988). These endomysial antibodies were later shown to be present in intestinal mucosa and secreted to the lumen of the small intestine (Picarelli et al., 1996). These antibodies that recognize TG2, and are also found in the context of capillary vessels in CD patients' mucosa, are early markers of CD and are seen before the appearance of serum IgA-antibodies and mucosal lesion (Korponay-szabo et al., 2004). The IgA deposits are enhanced after gluten intake and gradually disappear after patients switch to gluten-free diet (Kaukinen et al., 2005).

As mentioned in chapter 1.3, gluten peptides are rich in proline and glutamine residues. The high glutamine content makes gluten a suitable substrate for TG2. TG2 is a ubiquitous calcium-dependent enzyme that belongs to the family of transglutaminases. Transglutaminases catalyze a covalent and irreversible cross-link between proteins by connecting a glutamine donor with a glutamine acceptor resulting in an isopeptidyl bond. If an appropriate glutamine acceptor is not present transglutaminases can deamidate residues in donor peptides. TG2 is released from cells during tissue injury or inflammation. It cross-links several matrix proteins and stabilizes connective tissue (for review see Melino, 2000). Because TG2 is able to form covalent complexes with gliadin, it is thought that a possible mechanism of TG2 to become antigenic is via epitope spreading. B cell activation would be the result of cognate help provided by T cells and gliadin would be the carrier molecule for TG2 that provides contact between TG2 and T cells (for review see Sollid, 2000). Another proposed mechanism for autoimmunity in CD is molecular mimicry. This mechanism is probably involved when TG2 autoantibodies recognize transgutaminase 3 in patients with dermatitis herpetiformis (Sardy et al., 2002). Third mechanism proposed for autoimmunity in CD is the release of new epitopes from apoptotic cells during mucosal remodeling to extracellular space. New epitopes may also be presented by proteolytically cleaved proteins or proteins being connected by TG2 activity (for review see Alaedini and Green, 2007).

Deamidation of glutamine residues in gliadin peptides by TG2 is essential for the T cell stimulation in CD because it exposes negatively charged amino acids and result in enhanced

binding to DQ2 and DQ8 molecules. TG2 thus plays a critical dual role in CD pathogenesis by generating the antigenic epitopes that are presented to T cells (Molberg et al., 1998).

Evidence suggests that although TG2 is the primary autoantigen in CD there are secondary autoantibodies involved as well (Uhlig et al., 2000). In general, these antibodies are not highly sensitive or specific to CD, but are possibly associated with extra-intestinal clinical manifestations, such as neurological or dermatological and are present in varying degrees in patients with CD. Furthermore, not all secondary autoantibodies are specific for CD. Tranglutaminase 3 is an autoantigen in dermatitis herpetiformis, which is associated with CD. TG2 autoantibodies probably react with tranglutaminase 3 in this condition (Sardy et al., 2002). Other secondary autoantibodies in CD include: actin (Clemente et al., 2000), collagens (Dieterich et al., 2006), zonulin (Fasano et al., 2000) and calreticulin (CRT) (Tuckova et al., 1997), among others.

Autoantibodies against TG2 or secondary autoantibodies may interfere with small bowel normal mucosal biology and contribute to tissue damage. CD specific IgA antibodies were reported to inhibit epithelial cell differentiation and increase proliferation by mediating TGF-β-induced epithelium-fibroblast crosstalk (Halttunen and Mäki, 1999). Anti-TG2 antibodies have also been shown to increase permeability of the epithelium and induce monocyte activation by binding Toll-like receptor 4 (Zanoni et al., 2006). Evidence also suggests that circulating anti-TG2 antibodies are present in cerebellum of patients suffering from ataxia (Hadjivassiliou et al, 2006). Recently, Myrsky and colleagues showed that TG2 autoantibodies disturb angiogenesis *in vitro* by binding endothelial cells (2007).

1.5 Calreticulin

CRT is a 46-kDa Ca2+-binding chaperone of the endoplasmic reticulum (ER) lumen. It is found across a wide range of species and expressed in all cell types excluding erythrocytes. Classical functions of CRT include regulation of intracellular Ca2+ homeostasis, ER Ca2+ storage capacity and functioning as a molecular chaperone in secretory pathways. The protein

contains several structurally and functionally unique domains with specialized functions. It is known to be essential for cardiac development in mice. Recently, CRT has been attributed with novel functions and has been linked with several disease conditions including autoimmunity and cancer (for review see Gelebart et al., 2005).

CRT gene is entitled *calr* and it is located at 19p13.3-13.2 and another gene homolog *calr3*, which is the subject of this study, is located at 19p13.11. Both genes consist of 9 exons and 8 introns (Entrez Gene, http://www.ncbi.nlm.nih.gov/sites/entrez, 20.05.08). Van Belzen and colleagues reported a significant association to chromosomal location 19p13.1 in Dutch CD study (2003). This makes *calr3* and interesting candidate for CD susceptibility, although myosin IXB from the same region has been nominated for susceptibility locus in CD (Monsuur et al., 2005).

1.5.1 Protein Structure

A model for CRT structure is derived from that of calnexin, which is a homologous integral ER protein (Schrag et al., 2001). The N-terminal domain of CRT, consisting of residues 1-180 is predicted to form a globular domain consisting of eight anti-parallel beta-strands and a single disulphide bridge. This region binds Zn2+ ions and other heavy metals. It is also in interaction with other chaperones of the ER, DNA-binding domain of nuclear receptors and nucleic acids (for review see Michalak et al., 1999). N-domain of CRT is also responsible for the chaperone functions together with the central P-domain of the protein (for review see Michalak et al., 2002). Studies using site-specific mutagenesis show that a single histidine residue (153) is central to the chaperone function (Guo et al., 2003).

The P-domain of CRT (residues 181-290) forms an arm structure that contains a proline-rich region and interacts with other chaperones of the ER. The arm structure is predicted to form an opening accommodating substrate binding. The P-domain consists of three helices, three anti-parallel beta sheets and two sets of amino acid repeats (Schrag et al., 2001).

The highly acidic C-domain of CRT (residues 291-400) binds Ca2+ with high capacity and low affinity (20-30 mol Ca2+/mol protein) and thus functions as a Ca2+ buffer/storage protein in the lumen of the ER (Nakamura et al., 2001). The structure of CRT C-domain is unknown.

1.5.2 Functions of Calreticulin

1.5.2.1 Ca2+ Homeostasis and Signaling

A major function of CRT is the modulatory role in Ca2+ homeostasis. Upregulation of CRT increases the amount of Ca2+ in intracellular stores. Also, CRT deficient cells (CRT-/-) experience a reduced Ca2+ storage capacity (Nakamura et al., 2000). As a consequence, the influx of Ca2+ stored in intracellular compartments to cytosol is affected by the expression level of CRT (Arnaudeau et al., 2002). Furthermore, in CRT-/- cells, agonist-mediated release of Ca2+ from the ER is inhibited, suggesting impairments in Ca2+ handling and misfolding/trafficking of cell surface receptors (Mesaeli et al., 1999). As mentioned above, CRT deficiency is responsible for embryonic lethality in mice. This underlines the importance of CRT in Ca2+ homeostasis and signaling (Guo et al., 2002)

1.5.2.2 Molecular Chaperone Function

A well-known function of CRT is that of molecular chaperone in the ER, the so-called calreticulin/calnexin cycle. CRT binds Glc1Man9GlcNAC2, which is a monoglycosylated high mannose oligosaccharide, by recognizing the terminal glucose and four internal mannose molecules in glycoproteins. An enzyme glucosidase II is able to trim the terminal glucose thus regulating the interaction between CRT and the glycoprotein. A part of the cycle is also the UDP-glucose:glycoprotein transferase which is able to re-glucosylate chains that have been trimmed by glucosidase II. UDP-glucose:glycoprotein transferase can also discriminate between folded and unfolded proteins by only glucosylating unfolded proteins. Together with glucosidase II they control the interaction of the glycoprotein with CRT. This cycle is repeated until the protein is properly folded (for review see Trombetta and Parodi, 2003).

1.5.2.3 Calreticulin and Immunity

CRT plays several roles in the immune system and may contribute to CD through some of these roles. CRT is known to be essential for assembly and antigen-presenting functions of MHC class I molecules. Impaired function of CRT leads to a poor T cell recognition of loaded peptides (Gao et al., 2002). CRT deficiency in T cells leads to lowered threshold of T cell receptor activation, vigorous peripheral T cell response to antigen and increased secretion of inflammatory cytokines. This altered regulation of T cell activation is explained by alterations in cytosolic Ca2+ oscillations following T cell receptor stimulation (Porcellini et al., 2006). CRT is also known to be present in cytotoxic T lymphocyte granules where it is suggested to block pore formation in the granule membranes by binding perforin (Andrin et al., 1998).

CRT has a collagen-like domain, which is able to bind the complement component C1q and other defense collagens (Stuart et al., 1997). CRT forms a complex with CD91 on cell surface, which mediates C1q induced enhancement of phagocytosis *in vitro* (Vandivier et al., 2002). It has also been suggested that cell surface CRT, in complex with lipid raft GPI-anchored protein CD59, functions as a C1q receptor on circulating neutrophils. It was also found that antibody binding to cell surface CRT induced a Ca2+ influx suggesting signaling capability following ligand binding (Ghiran et al., 2003). Incubation of neutrophils with antibodies that block GPI-anchored proteins or disruption of lipid rafts inhibited the C1q induced respiratory burst of neutrophils (Otabor et al., 2004).

1.5.2.4 Novel Functions

In recent years, CRT has been attributed a role in many previously unknown processes. CRT overexpression lowers the threshold of apoptosis by increasing ER Ca2+ buffering capacity and intensifying Ca2+ oscillations from ER to cytosol. This has direct effects on mitochondria that promote apoptosis. Ca2+ oscillations also affect calcineurin function, which activates the intrinsic pathway of apoptosis (for review see Groenendyk et al., 2004). CRT is also an initiator of intake of apoptotic and viable cells (Gardai et al., 2005). CRT is able to mediate cellular adhesion to substratum by binding integrins from cytosol (Zhu et al., 1997) or proteins of the extracellular matrix from the cell surface such as laminin (Yao et al., 2002) and B beta

fibrinogen (Gray et al., 1995). CRT also functions as a receptor for thrombospondin on cell surface and mediates the disassembly of focal adhesions (Goicoechea et al., 2000).

1.5.2.5 Calreticulin in Autoimmunity

Extracellular CRT has been reported to be associated in various conditions of pathology. Autoantibodies against CRT have been found in sera of CD patients (Sanchez et al., 2003) although this data has been suggested to be artifact (Jorgensen et al., 2005). Anti-gliadin antibodies are also known to cross-react with rat enterocytes, and the binding was revealed to be via CRT (Tuckova et al., 1997). In rheumatoid arthritis, CRT binding to shared epitope has been shown to activate an intracellular signaling cascade (Ling et al., 2007).

Anti-CRT antibodies are also found from patients with systemic lupus erythematosus with 40% prevalence and some cases of secondary Sjögren syndrome (Eggleton et al., 2000) and rheumatoid arthritis (Verreck et al., 1995). CRT is able to bind Ro/SS-A antigenic complex and it is suggested that autoimmunity against CRT occurs via epitope spreading (McCluskey et al., 1998). CRT is also known to bind TG2 in the plasma membrane where they function as the two subunits of the G-protein Gh (for review see Mhaouty-Kodja, 2004). This may explain the presence of anti-CRT antibodies in CD by epitope spreading.

2. Purpose of the Study

1. The primary purpose of the study was to identify CD-susceptibility SNPs (single nucleotide polymorphism) in *calr3* gene by comparing promoter, exon, and surrounding intron regions of *calr3* from samples of Hungarian population.

2. The secondary objective was to study the effect of peptic-tryptic digested gliadin (PT-gliadin) on localization and expression level of CRT in T84 intestinal epithelial cells

3. Materials and Methods:

3.1 Patients and DNA

DNA was extracted and purified by DNA Purification Kit (Qiagen, Hilden, Germany) from blood leukocytes of 25 patients seropositive for anti-TG2 antibodies and with small bowel biopsy-confirmed CD. For control patients, negative result for serum anti-TG2 antibodies and normal small bowel mucosal morphology was required. Patients were all of Hungarian origin. The CD diagnosis was performed by an experienced researcher Ilma Korponay-Szabo, who also kindly provided the genomic DNA for the study.

3.2 DNA Amplification

A reaction mixture consisting of 2,5 µl of 10x reaction buffer (GeneAmp 10X PCR buffer, Applied Biosystems, Foster City, CA, USA), 1 µl of 100 ng genomic DNA, 0,2 µl of 20 µM 5’ and 3’ primers (Sigma-Aldrich, St. Louis, MO, USA), 0,2 µl of 5U/µl Taq polymerase (AmpliTaq Gold; Applied Biosystems, Foster City, CA, USA) and 0,6 µl of nucleotides 10 mM each (GeneAmp dNTP, Applied Biosystems, Foster City, CA, USA). Finally, water was added to reach a final volume of 25 µl. Primers, reaction cycles and annealing temperatures are depicted in table 1. Primers were designed using the Primer3 software version 0.4.0 (http://frodo.wi.mit.edu/primer3/input.htm). Primers were designed for amplifying the promoter region and the 9 exons of the *calr3* gene and approximately 50 base pairs surrounding each exon and the primer.

DNA was amplified by thermal cycling at 95 °C for 5 minutes for 1 cycle, followed by 95 °C for 60 seconds, annealing at 57-62 °C for 60 seconds and 72 °C for 90 seconds for 30-35 cycles. Heteroduplex formation was induced by the following steps: 95 °C for 5 minutes and 30 minutes at 68 °C.

Table 1. Promoter region and all 9 exons of the *calr3* gene were studied. Table describes the size of PCR product studied, number of PCR cycles and corresponding annealing temperature and both primers used for each region in PCR.

	Size of PCR product, bp	Number of PCR cycles	Annealing Temperature	5' Primer	3' Primer
Promoter	392	34	62°	AATCGCAGAGTTGTGACGGGAGTTG	AATTTGGGCTAAGGGAAGGGTGAA
Exon 1	340	34	62°	AGACCAATGGCGCCCCGAATCA	GTGGACCCCGTGGCGACTCTGG
Exon 2	380	30	57°	CGTGGTGGCGGAGGTGTAAG	TCATCTGTAAAACGGGAATAAAAG
Exon 3	422	35	51°	ACCCATTATGCATTATTGTAT	AAGTGGGAGGGTTTTCA
Exon 4	306	32	51°	GTGGGTTGGTTATTCTACGGTGAT	CCCCTAAACTGGAAACTGGAAACA
Exon 5	418	34	57°	TTGAGGATTTAATTGTTACGCTTT	TTGGTTCTATAATATTGCCATCGT
Exon 6	249	34	62°	CAGCCTGGGTGAGAGCAAAAC	GCATCCCTGGCATCATACAAAGAG
Exon 7	329	34	62°	GTGTGGCCTCTTTGTATGATGC	CCAGCCTAGGTGACAGAATGAGAC
Exon 8	381	35	62°	CCAAGCCCAGAAGTTCAAATCCAG	TGAGCCGAGACTATGCCACTACA
Exon 9	269	30	57°	TGGGATTACAGGCACGAG	GTCACCAGGGGAGATACAACC

3.3 Conformation-sensitive Gel Electrophoresis

SNPs were identified from PCR products by conformation-sensitive gel electrophoresis (CSGE). Briefly, CSGE is a simple, fast and reliable method for detecting SNPs (Körkkö et al., 1998). The method is based on gel electrophoresis under semi-denaturing conditions, which results in a conformational change and differential electrophoretic properties in heteroduplex PCR-products while homoduplex PCR-products retain original conformation and electrophoretic properties (Figure 1) (Ganguly et al., 1993).

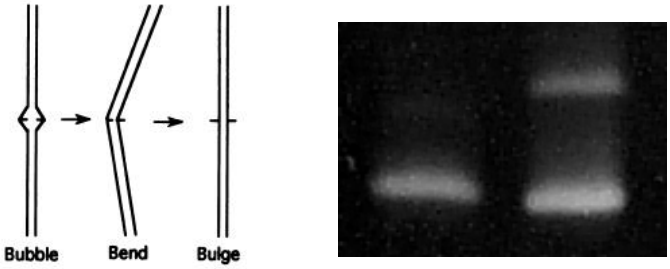

Figure 1. On the left is presented a schematic drawing of double stranded DNA with SNP. SNP induces formation of a bubble structure under non-denaturing conditions, bend structure under semi-denaturing conditions and bulge structure under stronger denaturing conditions. Original picture from Ganguly et al., 1993. On the right is presented a homoduplex, which is formed when there is no polymorphism and a heteroduplex, which is formed by bend structure when polymorphism is present. Bands are visualized under UV-light after CSGE.

A polyacrylamide gel was prepared by mixing 70 ml water, 3,8 ml of 20X TTE-buffer, 37,5 ml 99:1 acrylamide:BAP (Bio-Rad Laboratories, Redmond, WA, USA; Sigma-Aldrich, St. Louis, MO, USA), 22,5 ml Formamide (Fluka Chemie AG, Buchs, Switzerland) and 15 ml ethylene glycole (Riedel-de Haen, Seelze, Germany). A detailed description of the gel components is found in appendix 1. Gel ingredients were mixed with a magnetic stirrer for 10 minutes. After thorough mixing, 750 µl of 20 % ammonium persulfate (Sigma-Aldrich, St. Louis, MO, USA) and 103 µl of N,N,N',N'-Tetramethylethylenediamine (Sigma-Aldrich, St Louis, MO, USA) was added to begin polymerization. The mixture was stirred for another 60 seconds after which the liquid mixture was poured between glass plates sized 30x40 cm separated by a 1-mm spacer. A 25-well comb was applied and the gel was allowed to polymerase for 2 hours. After polymerization, 15 µl of PCR-product was applied to the wells and a 200 V voltage was applied for 20 hours to electrophorize the DNA through the gel matrix. After electrophoresis, the gel was incubated in 0,5 µg/ml ethidium bromide (Sigma-Aldrich, St. Louis, MO, USA) solution for 20 minutes after witch the homo- and heteroduplexes were visualized under UV-light.

3.4 DNA Purification and Precipitation

For DNA sequence analysis, 10 µl of amplified PCR product was loaded to a 12x18 cm 1,5% agarose (Bio-Rad Laboratories, Redmond, WA, USA) gel in TE-buffer (10 mM Trizma,

Sigma-Aldrich, St. Louis, MO, USA, pH 7,5; 1 mM EDTA, Sigma-Aldrich, St. Louis, MO, USA), containing 0,5 µg/ml ethidium bromide. Samples were run for 60 minutes at 100V to separate amplified DNA fragments from other nucleotide material. Following electrophoresis, DNA bands were visualized under UV-light and 0,1 g agarose gel pieces containing the PCR product were collected to sterile 0,5 ml microcentrifuge tubes (Eppendorf, Hamburg, Germany) on top of mineral wool (Isover, Finland). Bottom of the tubes were pierced with a sterile injection needle and tubes were placed inside 1,5 ml collection tubes and centrifuged at 13,000 g (Eppendorf, Hamburg, Germany) for 10 minutes to extract the buffer containing the PCR product from the agarose gel. The supernatant was discarded and the pellet was resuspended in 96 % ethanol and 3 M sodium acetate (pH 5,2) was added to reach a concentration of 0,3 M after witch the extract was incubated for 30 minutes at -20 °C to precipitate high salts. The samples were then sentrifuged for 10 minutes at 13,000 rpm at +4 °C, the supernatant was discarded and the pellet re-suspended in 70 % ethanol. Samples were centrifuged again for 10 minutes at +4 °C 13,000 rpm. After centrifugation, the supernatant was discarded and the pellet was air-dried until the ethanol had evaporated. Finally, the DNA pellet was dissolved in 10 µl water.

3.5 DNA Sequencing

DNA sequencing samples were prepared using Abi Prism BigDye Terminator Ready Detection Kit (Applied Biosystems, Foster City, CA, USA). This method utilizes fluorescent ddNTP nucleotides and PCR. PCR-cycling reaction mix was prepared according to manufacturers instructions. 200 ng of purified DNA, 1,6 pg of primer (described in table 1), 2 µl of 5X buffer, 2 µl of BigDye Terminator were pooled for the reaction mix and water was added to reach a final concentration of 10 µl. Reaction mix was subjected to 25 cycles of PCR by the following protocol: 30 seconds at 96°C, 15 s at 50 °C and 4 minutes at 60 °C.

After cycling, PCR products were precipitated. Contents of each tube were transferred to 1,5 ml centrifuge tubes and 2 µl of 3 M sodium acetate (pH 4,6) and 50 µl of 95% ethanol was added. Tubes were vortexed briefly and left to precipitate for 15 minutes in room temperature.

After incubation the tubes were centrifuged for 20 minutes at 13000 rpm and supernatant was collected and discarded. Pellets were rinsed with 250 µl 70 % ethanol and vortexed briefly. The tubes were again centrifuged for 5 minutes at 13000 rpm and supernatant was discarded. The pellet was air dried and suspended in 12,5 µl of Template Suppression Reagent (Applied Biosystems, Foster City, CA, USA) and denature at 95 °C for 5 minutes after which the samples were cooled on ice.

Data collection and analysis of the samples was done with ABI310 Genetic Analyzer, according to instructions. The used protocol was: 36 cm capillary, POP6 Performance Optimized Polymer and Running Buffer with EDTA (all from Applied Biosystems, Foster City, CA, USA), oven temperature was 50 °C and separation time was 50 minutes. Data was analyzed with ABI sequencing analysis software. Finally, acquired sequences were compared with the *calr3* consensus sequence CCDS12344.1 from NCBI Gene database (http://www.ncbi.nlm.nih.gov/CCDS/CcdsBrowse.cgi?REQUEST=CCDS&GO=MainBrowse&DATA=CCDS12344.1).

3.6 Cell Culture

The Human colon carcinoma cell line T84 was acquired from American Type Culture Collction (ATCC-248). The cells were cultured in medium consisting of Dulbecco's Modified Eagle Medium, containing 1 g/l L-glucose, 25 mM HEPES, pyruvate and L-glutamine, supplemented with 10 % fetal calf serum, 100 U/ml penicillin (Lonza, Walkerville, MD, USA) and 100 U/ml streptomycin (Lonza Walkerville, MD, USA). The cells were passaged twice a week to 10 000 cells/cm2. Passaging was carried out by washing the cells for 2 minutes with phosphate-buffered saline (PBS; 137 mM NaCl, 10 mM Na2HPO4, 2,7 mM KCl, pH 7,4), after which the cells were detached from the culture dish by trypsinization with 0,05% trypsin-EDTA (Gibco, Invitrogen, Carlsbad, CA, USA). The cell suspension was centrifuged at 350 G for 5 minutes and the pellet was resuspended in growth medium and passaged to new culture dishes (Nunc, Naperville, IL, USA).

3.7 Western Blotting

T84 cells were grown on 6 cm culture dishes to approximately 50 % confluency. Cells were treated for 24-48h with 1 mg/ml PT-gliadin. PT-gliadin was a gift of collaboration from professor Herbert Wieser, Research Institute of Food Chemistry, Garching, Germany. After treatment, the culture dishes were kept on ice and washed twice with +4 °C PBS. For cell lysis, cells were suspended in 500 ml RIPA-buffer (150 mM NaCl; 1 % NP-40, Sigma-Aldrich, St. Louis, MO, USA; 0,5 % deoxycholic acid; 0,1 % SDS, Bio-Rad Laboratories Bio-Rad Laboratories, Redmond, WA, USA; 50 mM Trizma, Sigam-Aldrich), collected with a cell screaper (Corning, Corning, NY, USA) and incubated for 10 minutes on ice. Cell lysate was colleced to 1,5 ml microsentrifuge tubes and total protein consentration was determined as later described. 2x Laemmli buffer (Bio-Rad Laboratories, Redmond, WA, USA) was added to complete the sample for Western blotting.

10 µg of protein sample was loaded on 12% polyacrylamide gel (NuPAGE, Invitrogen, Carlsbad, CA, USA) for electrophoresis. Sample movement in gel matrix and molecular weight of detected bands were determined by comparison to molecular standard (All Blue, Bio-Rad Laboratories, Redmond, WA, USA). Samples were run for 10 minutes at 100V, following 70 minutes at 150V until markers were sufficiently separated. After electrophoresis, the gel was incubated for 15 minutes in fresh transfer buffer (20 mM Trizma, Sigma-Aldrich, St. Louis, MO, USA; 150 mM glycine, Sigma-Aldrich, St. Louis, MO, USA; 20 % methanol, Fluka Chemie AG, Buchs, Switzerland and 0,038 % SDS, Bio-Rad Laboratories, Redmond, WA, USA), as well as a nitrocellulose membrane (Whatman, Perkin-Elmer, Boston, MA, USA), filter membranes (Whatman, Perkin-Elmer, Boston, MA, USA) and fiber pads (Bio-Rad Laboratories, Redmond, WA, USA). Filter papers, gel, and the nitrocellulose membrane were assembled to a semi-dry blotter (Bio-Rad Laboratories, Redmond, WA, USA) according to manufacturers instructions. The proteins were blotted to the membrane by a constant current of 400 mA for 60 minutes. Temperature was kept at +4 °C by placing ice on top of the blotter.

The membrane was blocked in 5 % non-fat milk protein (Valio, Finland) in TBS-Tween (150 mM NaCl; 20 mM Trizma, Sigma-Aldrich, St. Louis, MO, USA; Tween 20 0,05%, Sigma-Aldrich, St. Louis, MO, USA) for 60 minutes to block the remaining protein-binding parts of the membrane. After blocking, the membrane was incubated with rabbit anti-human calreticulin primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 60 minutes at a 1 µg/ml concentration in room temperature diluted in milk protein/TBS-Tween. The membrane was washed three times for 5 minutes with milk protein/TBS-Tween and incubated with a horseradish peroxidase-conjugated secondary anti-rabbit antibody (Invitrogen, Carlsbad, CA, USA). After incubation the membrane was washed three times with milk protein/TBS-Tween to remove any traces of antibodies.

Enhanced chemiluminescence detection was carried out using the Amersham ECL Plus Western Blotting Detection Kit (GE Healthcare, Uppsala, Sweden) according to manufacturers instructions. Solutions A and B were combined 1:50 in a dark room. The solution was mixed thoroughly and applied on the western blotting membrane for 1 minute. After incubation the solution was discarded and the membrane was moved to a cassette (Roche Laboratories, Nutley, NJ, NY, USA) with a light-sensitive film (Roche Laboratories, Nutley, NJ, USA). The film was illuminated in the cassette for 0,5-5 minutes depending on the intensity of the signal and the film was developed in an automated developing system (Roche Laboratories, Nutley, NJ, USA). The developed film was photographed with CCD camera (Roche Laboratories, Nutley, NJ, USA) and the intensity of the bands was determined using the ImageJ software (National Institute of Health, http://rsb.info.nih.gov/ij/). The experiment was repeated three times and control level was set to 100 % for statistical analysis.

3.8 Immunofluorescence Staining and Microscopy

T84 Cells were grown on 10 mm coverslips until they reached 70% confluency. After washing the cells with PBS twice for 5 minutes, they were fixed with a fresh solution of 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) for 15 minutes. The cells were washed three times with PBS for 5 minutes before incubation with 0,5 % Triton X-100 (Roche

Laboratories, Nutley, NJ, USA) for 10 minutes to permeabilize cellular membranes. For cell surface CRT staining permeabilization was not done. The cells were washed three times as described before and after a blocking step of 30 minutes with 1% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) dissolved in PBS (BSA-PBS) was done. After the preparative steps described above, the coverslips were incubated with 1 μ/ml anti-calreticulin antibody diluted in 1% BSA-PBS for 60 minutes. The cells were washed three times as described and incubated with 1:1000 dilution of goat anti-rabbit secondary antibody (Alexa Fluor 488, Invitrogen, Carlsbad, CA, USA) diluted in BSA-PBS. Finally, the coverslips were washed three times as described, mounted on microscopy slides with 20 microliters of mounting medium (Vectashield, Vector Laboratories, Burlingame, CA, USA) and nailpolish (Lumene, Finland) was used to fix the coverslips to the microscopy slides.

Samples were examined by confocal scanning laser microscopy, using a Perkin-Elmer-Cetus/Wallac UltraView LCD system (Wellesly, Toronto, Ontario, Canada) with a Plan 100_/1.25 NA oil immersion objective. Acquired images were processed with UltraView 4.0 software and additionally with PhotoShop 7.0 software.

To study the expression of CRT on epithelial cell surface, cells were treated with PT-gliadin as described above, trypsinized and incubated with anti-CRT antibody 10 mg/ml) for 15 minutes on ice in solution. Cells were then washes twice with PBS and incubated with secondary antibody (Alexa Fluor 488, Invitrogen, Carlsbad, CA, USA). After 10 minutes cells were washed again twice with PBS and transferred to microscopy slides and a coverslip was applied on top of cell suspension. Samples were immediately examined under a fluorescence microscope.

4. Results

4.1 *calr3* is Highly Conserved

All nine exons and the promoter region including approximately 50 base pairs of intron region from upstream and downstream of each region from 25 control and 25 CD patients were studied. The *calr3* gene was found to be highly conserved based on identification of only few variants by CSGE. However, a total of 28 exon or intron variants were identified by CSGE. 19 of these 28 variations were observed in the control group and 9 in CD patient group. The nucleotide variants in respect to each studied gene region are depicted in table 2.

Table 2. All 9 exons and the promoter region and surrounding introns of *calr3* gene were studied by CSGE. N-terminus and promoter region were found to be highly conserved where exons 1-5 display some polymorphism. However, none of these SNPs result in amino acid changes on protein level.

	Control Group	CD Patients	Intron/Exon	Nucleotide variant
Promoter	0/25	0/25	-	-
Exon 1	9/25	2/25	Intron	rs60134000
Exon 2	4/25	4/25	Intron/Exon	several
Exon 3	2/25	1/25	Exon	rs12232822
Exon 4	3/25	1/25	Exon	several
Exon 5	1/25	1/25	Intron	rs58659693
Exon 6	0/25	0/25	-	-
Exon 7	0/25	0/25	-	-
Exon 8	0/25	0/25	-	-
Exon 9	0/25	0/25	-	-

DNA samples identified to have nucleotide substitutions were subjected to DNA sequencing and the results were compared to reference sequence from NCBI Entrez Gene database. 12 of 28 variants were located in exon regions and 16 in intron regions. Altered exon DNA sequences were translated to corresponding amino acid sequences to find out whether these variants resulted in amino acid changes on protein level. However, none of the polymorphisms identified by DNA sequencing resulted in amino acid changes highlighting the conserved

nature of *calr3* gene. The distribution of exon polymorphisms between control group and CD group were 8 and 4, respectively. Both exon and intron variants were compared between control and CD group and the polymorphisms were found to be same in both groups.

4.2 Significant Difference in Intron SNP Was Detected

The total number of intron SNPs was 16 of which 10 were in control group and 6 in CD group. A distinct G? C SNP (NCBI Entrez, rs60134000; http://www.ncbi.nlm.nih.gov/sites/entrez) was identified in intron region upstream from exon 1 that was present in 9/25 cases in control group and 2/25 cases in CD patient group. This difference is statistically significant ($p=0.0164$). All polymorphisms identified in this study were previously known and reported in NCBI database.

4.3 Calreticulin Localization is Altered by Gliadin

CRT is localized uniformly in the cytosol in T84 cells (figure 2A). After a 24-hour incubation of the cells with 1 mg/ml PT-gliadin, CRT is partly translocated to the nuclear membrane, while a significant portion of the protein remains in the cytosol (figure 2B). After 48 hours, nuclear envelope localization was abolished and CRT was located in cytosol and probably in the ER. At this time point CRT was observed as cytosolic aggregates in PT-gliadin-treted cells in contrast to the uniform cytosolic localization in control cells (figure 2C). Cellular stress was also induced by UV-irradiation. After 48 hours CRT localization was similar in cells treated with gliadin or irradiated with UV-light (figure 2D). Localization of CRT remained similar in control cells at 24 and 48-hour time points and UV-treated cells were similar to control cells after 24 hours (both data not shown).

4.4 Cell Surface Calreticulin

Cell surface CRT was stained in T84 cells. However, only an extremely weak signal was observed from cells (data not shown) and proper quantification and analysis was not possible.

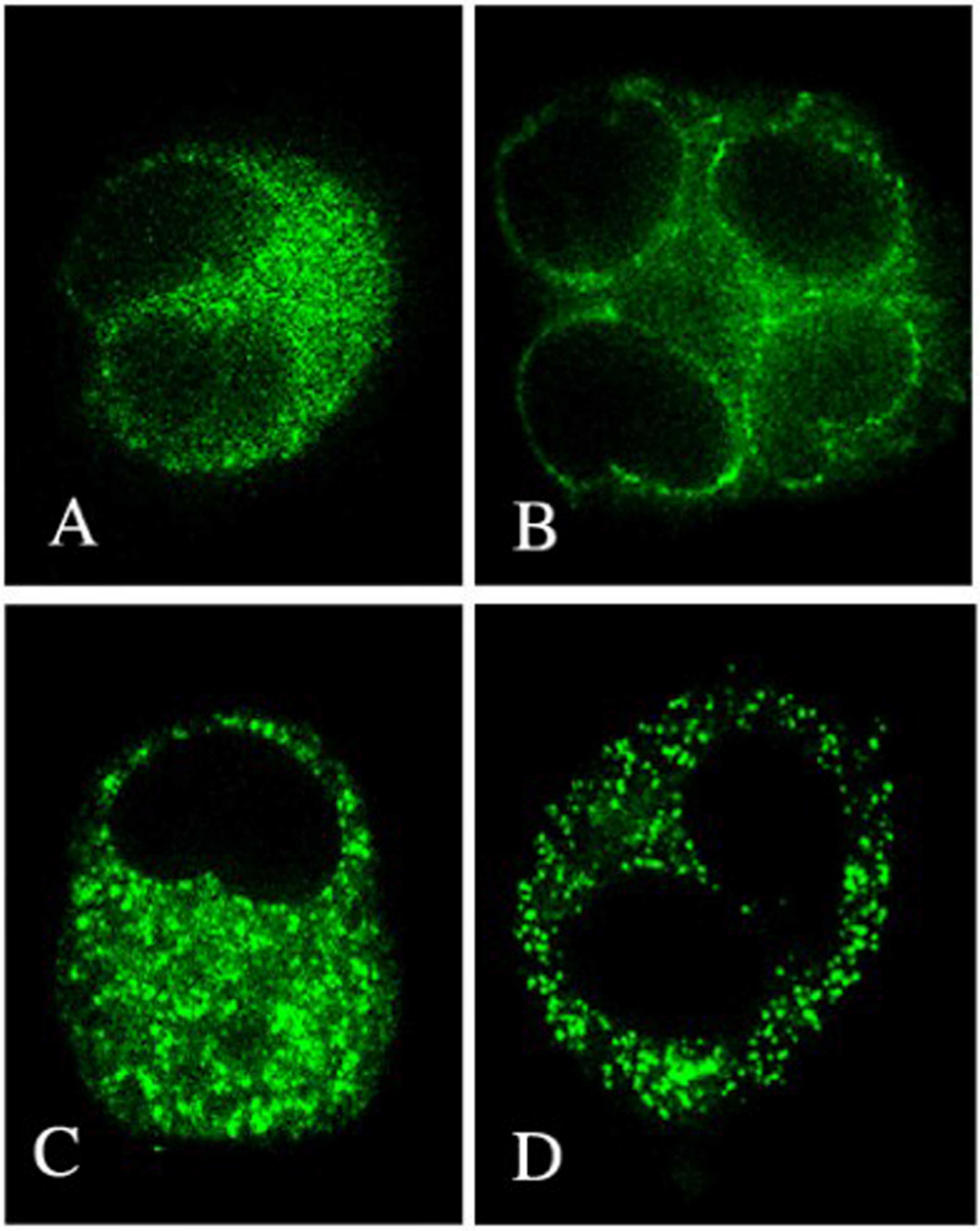

Figure 2. In control cells, CRT is uniformly expressed in the ER and cytosol (A). After 24 hours of gliadin treatment, CRT is partly traslocated to nuclear envelope (B). 48 hours of gliadin challenge results in cytosolic expression of CRT in aggregates or vesicles (C), similar to CRT expression 48 hours after UV irradiation (D).

4.5 Gliadin Downregulates Calreticulin in T84 Cells *In Vitro*

T84 intestinal epithelial cells were grown to confluency and treated subsequently with PT-gliadin for 24 hours. Total protein of western blotting samples was determined and 10 mg of total protein was used for experiments. Gliadin treatment resulted in downregulation of CRT on protein level as indicated by western blotting data in figure 3. The expression level was reduced to 54,2 % of the basal level without gliadin treatment (p=0,0032). Standard deviation was ±12,4 % and ±6,1 % in control cells and gliadin treated cells respectively. Western blotting sample was also prepared from confluent monolayer of T84 cells irradiated with UVB to induce cellular stress. Monolayers that were irradiated expressed 61,5 % of CRT compared to basal level (p=0,0066). The difference between CRT expression levels in gliadin-treated and UV-irradiated groups was not significant.

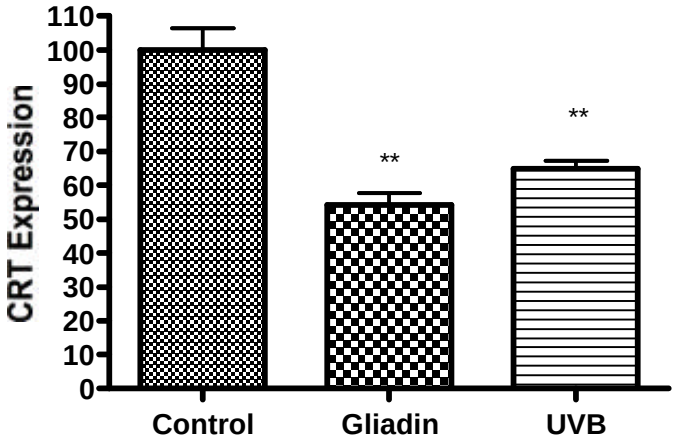

Figure 3. CRT is significantly downregulated in T84 cells after 24-hour gliadin challenge *in vitro*. Treated cell express 54,2 % ±6,1 % of CRT compared to control cells p=0,0032. UV-irradiated cells expressed 61,5 % ±8,0 % compared to basal level p=0,0066. Standard deviation for control group was 12,4 %. Difference between gliadin-treated and UV-irradiated groups is not significant.

5. Discussion

5.1 *calr3* Gene Polymorphisms

Here, we studied the existence of *calr3* gene polymorphisms in CD by comparing the promoter and all 9 exon regions and surrounding intron regions of *calr3* gene of CD patients to those of control patients. We also studied the effects of PT-gliadin peptides on CRT expression and localization in T84 intestinal epithelial cells.

The CSGE analysis identified 28 polymorphisms in *calr3* gene. 19 of these variations were identified in samples from control group and 9 in CD group. When the nature of these polymorphisms was revealed by DNA sequencing, we found that 12 and 16 were located in exonic and intronic regions, respectively. Further analysis revealed that none of the exonic variations in either group changed the corresponding amino acid composition on protein level. 7 of exon polymorphisms were in control group and 5 in CD group. Also, the exonic variants were the same in both groups underlining the similarity of the two groups in exonic region. The promoter and all four N-terminal exons, including surrounding intron regions, did not exhibit any polymorphisms in either group. This data is in line with previous studies of *calr3* cDNA isolated from diverse genera, including mammals (McCauliffe, et al. 1990), insects (Smith, 1992a), nematodes (Smith, 1992b), protozoa (Joshi et al., 1996) and plants (Denecke, et al., 1995) which highlight the conserved nature of CRT in both genomic organization and amino acid sequence through evolution, underlining importance of CRT in cellular function.

We also studied approximately 50 base pairs of intron region upstream and downstream of each exon. 16 of the variations identified by CSGE were located in the intron region and 11 of these resided in intron 1. A distinct G→C SNP was identified in 9 samples of control group and 2 samples of CD group. This result was significant with n=25. This SNP should be studied with a larger sample number to confirm association to CD using CSGE or automated SNP analysis that allows simultaneous analysis of large number of samples. This polymorphism is

possibly an important advance in CD genetics.

The possible biological significance of this polymorphism would be in production of a splice variant and an isoform of CRT with altered biological activity that would affect the onset of CD. Exon 1 codes for the KDEL ER retrieval sequence and a part of the C-terminal globular C-domain. Possible deletion or alteration of the KDEL/C-domain would most probably alter the function of CRT significantly. C-domain of CRT contains the high-capacity and low affinity Calcium-binding domain (Nakamura et al., 2001). If the calcium-binding capacity of CRT is impaired, it would have diverse consequences on cellular function and also on function of CRT itself. The role of CRT in calcium homeostasis and signaling is discussed later. It is also possible that this polymorphism could result in altered expression levels of CRT, possibly downregulation. The effects of CRT downregulation are discussed in chapter 5.3. The other intronic SNPs were equally distributed between the two groups and gene regions and seem to have no possible importance in *calr3* gene or protein expression or function in CD.

5.2 Altered Calreticulin Localization

We also studied the effect of gliadin on CRT localization and expression level in T84 intestinal epithelial cells. Under normal growth conditions CRT was found to be uniformly localized in cytosol but not in nucleus and this same localization pattern was remained throughout the 24- and 48-hour time points. Our results are in line with those of Afshar and colleagues who showed that CRT is proteolytically processed in the ER and subsequently translocated to cytosol (2005). This data supports our evident finding that CRT indeed is localized in cytosol in T84 cells. In cells treated with PT-gliadin for 24 hours, the uniform cytosolic localization was sustained but the CRT was also found on the nuclear envelope where it is normally localized in a variety of cell types (Opas et al., 1991). CRT in known to mediate the nuclear export of glucocortcoid receptor and protein kinase inhibitor (Holaska et al., 2001). Glucocorticoid reseptors are present in the epithelial cells of the small intestine and upon activation, increase proliferation (for review see Tutton and Barkla, 1988). It is likely that PT-gliadin-induced translocation of CRT to the nuclear membrane involves nuclear

import or export of proteins, possibly transcription factors. Possible CRT-mediated alterations in hormone receptor import or export may influence proliferation of intestinal epithelial cells. Further studies to determine whether hormone receptor trafficking is altered upon gliadin challenge are needed.

After 48 hours of PT-gliadin treatment, the nuclear envelope localization of CRT was abolished and CRT was found in cytosol in vesicle-like aggregates. CRT has been found in vesicles in human sperm (Naaby-Hansen et al., 2001) but data of vesicle localization of CRT is scarce. It is possible that under PT-gliadin–induced stress CRT is localized in secretory vesicles targeted to extracellular space or cell surface. CRT, among other KDEL proteins are found on the surface of NG108-15 neuronal cells and interestingly, these proteins are not transported to cell surface under treatment with Brefeldin A, an inhibitor of ER-Golgi trafficking (Xiao et al., 1999a). It is also suggested that CRT is degraded in lysosomes after transport to cell surface (Xiao et al., 1999b). This data suggests that CRT is transported from the ER to cell surface via the secretory pathway. Xiao and colleagues also report that CRT is not found in growth medium but exclusively on cell surface. We studied the possible expression of cell surface CRT but were unable to determine expression reliably. Possible significance of cell surface expression of CRT is discussed in later in this chapter.

We also induced cellular stress to T84 intestinal epithelial cells by treatment of UVB-irradiation. After 48 hours after treatment CRT localization was found to be similar than in cells treated with PT-gliadin. This data suggests that gliadin treatment induces a stress response in T84 cells and that CRT localization is altered in response to stress induced by UV-irradiation or gliadin treatment. Further studies are needed to determine if CRT is localized to vesicles or protein aggregates. Altered localization of CRT described here can possibly be used as an additional marker for gliadin-induced alterations in intestinal epithelial cells. This, however, would require additional *in vivo* or tissue culture data.

5.3 Downregulation of Calreticulin by Gliadin

We found that CRT is downregulated by gliadin in T84 cells after 24-hour gliadin treatment. This result is unexpected given the fact that CRT upregulation is linked to various sources of cellular stresses, including: heat shock, calcium deprivation, heavy metals (Nguyen et al, 1996) and hypoxia–induced cellular stress (Wu et al., 2007). It is also known that gliadin induces cellular stress and apoptosis in epithelial cells (Giovannini et al., 2003). However, we found that gliadin treatment downregulated CRT expression in T84 epithelial cells. This may have various effects on epithelial cell proliferation, differentiation, function and apoptosis.

The most well characterized function of CRT is that of molecular chaperone. Downregulation of CRT can have profound effects on function of cells as CRT knockout in fibroblasts results in acceleration in protein folding, accumulation of unfolded proteins and triggering of the unfolded protein response (Knee et al., 2003). Unfolded protein response-induced stress can lead to apoptosis (Citterio et al., 2008).

Downregulation of CRT is also known to lower the storage capacity of the ER lumen and alter the calcium oscillations from the ER to cytosol. CRT is able modulate inositol 1,4,5-trisphosphate -mediated calcium release from the ER stores (Llewelyn Roderick et al., 1998). It is also shown that CRT is capable of mediating the function of sarcoplasmic/endoplasmic reticulum calcium ATPase 2 (John et al., 1998). Cytosolic calcium, by direct effect on mitochondria or by affecting cytosolic mediators such as calcineurin, promotes the activation of the intrinsic pathway of apoptosis resulting in the release of cytochrome c from the mitochondria and subsequent caspase activation (for review see Groenendyk et al., 2004). Gliadin treatment can thus have a dual role in epithelial cell apoptosis via CRT downregulation: accumulation of unfolded proteins can increase sensitivity to apoptosis as lowered calcium buffering capacity may increase the threshold for apoptosis.

Interestingly, CRT downregulation is associated with human colonic adenocarcinoma and recent evidence suggests that CRT would have a role in cell differentiation. CRT expression

was found to be significantly associated with the differentiation of the tumor (Toquet et al., 2007). Mouse CRT (-/-) stem cells also exhibit an attenuated differentiation capability towards the cardiac phenotype (Rauch et al., 2000). CD is characterized by abnormal crypt-villus axis biology where differentiation of epithelial cells is inhibited. CRT downregulation may have a role in attenuated differentiation of epithelial cells. This phenomenon has been attributed to CD-specific antibody deposits underlining the epithelium (Halttunen and Mäki, 1999). It is possible that these antibody deposits downregulate CRT downstream and affect intracellular signaling and calcium homeostasis in gut epithelial cells *in vivo*.

Downregulation of CRT in T84 cells probably affects both calcium homeostasis and intracellular calcium signaling in a complex manner. Porcellini and colleagues showed that CRT deficiency causes severe immonopathological traits *in vivo*, lowered activation threshold of T cell receptor activation and an exaggerated response to antigen with increased secretion of cytokines (2006). These modulations of the immune system were attributed to altered cytosolic elevations of calcium and subsequent alterations in intracellular signaling.

5.4 Cell Surface Calreticulin

Here we studied the existence of CRT on the surface of cells but only a very weak fluorescent signal was detected witch inhibited detailed characterization of surface expression. This does not exclude the existence of surface CRT. Equally possible explanation is a low sensitivity of the antibody used. Lowered CRT expression in T84 cells probably results in lower CRT expression levels on plasma membrane. The role of cell surface CRT is intriguing because it has been attributed to be a key initiator of clearance of apoptotic or viable cells (Gardai et al., 2005). Furthermore, several isoforms of CRT exist on cell surface (Zhu et al., 1997). The function of these isoforms remains unknown as well as the detailed undestanding of the role of cell surface CRT.

5.5 Calreticulin in Immunity and Autoimmunity

Anti-CRT antibodies have been recognized in sera of CD patients (Sanchez et al., 2003).

Cross-reactivity between anti-gliadin antibodies and rat enterocytes has been shown and the antigen on enterocytes was revealed to be CRT (Tuckova et al., 1997). Anti-gliadin circulating antibody cross-reactivity with cell surface CRT may explain some of symptoms experienced by CD patients. Other autoimmune disorders characterized by anti-CRT antibodies include systemic lupus erythematosus where 40% of patients are positive for CRT autoantibodies, secondary Sjögren syndrome (Eggleton et al., 2000) and rheumatoid arthritis (Verreck et al., 1995). Also, CRT has been shown to be a mediator of signal transduction in rheumatoid arthritis by binding to shared epitope (Ling et al., 2007).

CRT is also able to modulate the immune system by binding the complement component C1q and competing with antibody binding to C1q and inhibiting complement activation (Kovacs et al, 1998). Interestingly, patients who lack C1q frequently develop systemic lupus erythematosus, highlighting the importance of C1q in development of autoimmune diseases (Review Truedsson et al., 2007). Furthermore, CRT is known to bind the Ro/SS-A complex, which is an antigenic ribonucleoprotein complex, and epitope spreading is suggested as the mechanism of autoimmunity of CRT and other molecular chaperones (McCluskey et al., 1998). An intriguing aspect of CRT is its interaction with TG2, the primary autoantigen in CD. The question arises whether epitope spreading is involved between CRT and TG2 in the pathogenesis of CD. Altered localization or exhibition of differential isoforms of CRT or release of CRT from apoptotic or necrotic epithelial cells in CD can result in abnormal interaction of CRT with other proteins. This could modulate the immune system in a variety of ways. Currently there is no answer to the question whether CRT contributes to CD pathogenesis. As discussed here, there are a number of possible mechanisms.

5.6 Conclusions

In this study, we report a SNP that is expressed significantly more frequently in control group than in CD group. This polymorphism may exhibit protection against the onset of CD and be an important advance in CD genetics. We also reported that PT-gliadin downregulates CRT and alters its localization in T84 intestinal epithelial cells. These alterations on protein level

can have profound effects on epithelial cell function, immune responses and autoimmunity in patients with CD.

6. References

Afshar, N., B.E. Black, and B.M. Paschal. 2005. Retrotranslocation of the chaperone calreticulin from the endoplasmic reticulum lumen to the cytosol. *Mol.Cell.Biol.* 25:8844-8853.

Alaedini, A., and P.H. Green. 2008. Autoantibodies in celiac disease. *Autoimmunity.* 41:19-26.

Andrin, C., M.J. Pinkoski, K. Burns, E.A. Atkinson, O. Krahenbuhl, D. Hudig, S.A. Fraser, U. Winkler, J. Tschopp, M. Opas, R.C. Bleackley, and M. Michalak. 1998. Interaction between a Ca2+-binding protein calreticulin and perforin, a component of the cytotoxic T-cell granules. *Biochemistry.* 37:10386-10394.

Arnaudeau, S., M. Frieden, K. Nakamura, C. Castelbou, M. Michalak, and N. Demaurex. 2002. Calreticulin differentially modulates calcium uptake and release in the endoplasmic reticulum and mitochondria. *J.Biol.Chem.* 277:46696-46705.

Auricchio, S., G. De Ritis, M. De Vincenzi, E. Mancini, M. Minetti, O. Sapora, and V. Silano. 1984. Agglutinating activity of gliadin-derived peptides from bread wheat: implications for coeliac disease pathogenesis. *Biochem.Biophys.Res.Commun.* 121:428-433.

Barone, M.V., A. Gimigliano, G. Castoria, G. Paolella, F. Maurano, F. Paparo, M. Maria, M. Nanayakkara, A. Mineo, E. Miele, R. Troncone, and S. Auricchio. 2006. Growth factor-like activity of gliadin, an alimentary protein: implications for celiac disease (CD). *Gut.*

Berridge, M.J. 2002. The endoplasmic reticulum: a multifunctional signaling organelle. *Cell Calcium.* 32:235-249.

Bingley, P.J., and Williams, A.J. Norcross, A.J. 2004. Undiagnosed coeliac disease at age seven: population based prospective birth cohort study. 328:322-323.

Burns, K., B. Duggan, E.A. Atkinson, K.S. Famulski, M. Nemer, R.C. Bleackley, and M. Michalak. 1994. Modulation of gene expression by calreticulin binding to the glucocorticoid receptor. *Nature.* 367:476-480.

Catassi, C., I. Rätsch, and L. Vizzoni. 1999. Why is coeliac disease endemic in the people of the Sahara? *Lancet.* 354:647-648.

Catassi, C., I.M. Ratsch, E. Fabiani, M. Rossini, F. Bordicchia, F. Candela, G.V. Coppa, and P.L. Giorgi. 1994. Coeliac disease in the year 2000: exploring the iceberg. *Lancet.* 343:200-203.

Chorzelski, T.P., J. Sulej, H. Tchorzewska, S. Jablonska, E.H. Beutner, and V. Kumar. 1983. IgA class endomysium antibodies in dermatitis herpetiformis and coeliac disease. *Ann.N.Y.Acad.Sci.* 420:325-334.

Ciccocioppo, R., A. Di Sabatino, and G.R. Corazza. 2005. The immune recognition of gluten in coeliac disease. *Clin.Exp.Immunol.* 140:408-416.

Citterio, C., A. Vichi, G. Pacheco-Rodriguez, A.M. Aponte, J. Moss, and M. Vaughan. 2008. Unfolded protein response and cell death after depletion of brefeldin A-inhibited guanine nucleotide-exchange protein GBF1. *Proc.Natl.Acad.Sci.U.S.A.* 105:2877-2882.

Clemente, M.G., M.P. Musu, F. Frau, G. Brusco, G. Sole, G.R. Corazza, and S. De Virgiliis. 2000. Immune reaction against the cytoskeleton in coeliac disease. *Gut.* 47:520-526.

Clemente, M.G., S. De Virgiliis, J.S. Kang, R. Macatagney, M.P. Musu, M.R. Di Pierro, S. Drago, M. Congia, and A. Fasano. 2003. Early effects of gliadin on enterocyte intracellular signalling involved in intestinal barrier function. *Gut.* 52:218-223.

Dedhar, S., P.S. Rennie, M. Shago, C.Y. Hagesteijn, H. Yang, J. Filmus, R.G. Hawley, N. Bruchovsky, H. Cheng, and R.J. Matusik. 1994. Inhibition of nuclear hormone receptor activity by calreticulin. *Nature.* 367:480-483.

Denecke, J., L.E. Carlsson, S. Vidal, A.S. Hoglund, B. Ek, M.J. van Zeijl, K.M. Sinjorgo, and E.T. Palva. 1995. The tobacco homolog of mammalian calreticulin is present in protein complexes in vivo. *Plant Cell.* 7:391-406.

Dieterich, W., T. Ehnis, M. Bauer, P. Donner, U. Volta, E.O. Riecken, and D. Schuppan. 1997. Identification of tissue transglutaminase as the autoantigen of celiac disease. *Nat.Med.* 3:797-801.

Dieterich, W., B. Esslinger, D. Trapp, E. Hahn, T. Huff, W. Seilmeier, H. Wieser, and D. Schuppan. 2006. Cross linking to tissue transglutaminase and collagen favours gliadin toxicity in coeliac disease. *Gut.* 55:478-484.

Eggleton, P., F.J. Ward, S. Johnson, M.A. Khamashta, G.R. Hughes, V.A. Hajela, M. Michalak, E.F. Corbett, N.A. Staines, and K.B. Reid. 2000. Fine specificity of autoantibodies to calreticulin: epitope mapping and characterization. *Clin.Exp.Immunol.* 120:384-391.

Fasano, A., T. Not, W. Wang, S. Uzzau, I. Berti, A. Tommasini, and S.E. Goldblum. 2000. Zonulin, a newly discovered modulator of intestinal permeability, and its expression in coeliac disease. *Lancet.* 355:1518-1519.

Fasano, A., and C. Catassi. 2001. Current approaches to diagnosis and treatment of celiac disease: an evolving spectrum. *Gastroenterology.* 120:636-651.

Ganguly, A. 2002. An update on conformation sensitive gel electrophoresis. *Hum.Mutat.* 19:334-342.

Gardai, S.J., K.A. McPhillips, S.C. Frasch, W.J. Janssen, A. Starefeldt, J.E. Murphy-Ullrich, D.L. Bratton, P.A. Oldenborg, M. Michalak, and P.M. Henson. 2005. Cell-surface calreticulin initiates clearance of viable or apoptotic cells through trans-activation of LRP on the phagocyte. *Cell.* 123:321-334.

Gelebart, P., M. Opas, and M. Michalak. 2005. Calreticulin, a Ca2+-binding chaperone of the endoplasmic reticulum. *Int.J.Biochem.Cell Biol.* 37:260-266.

Ghiran, I., L.B. Klickstein, and A. Nicholson-Weller. 2003. Calreticulin is at the surface of circulating neutrophils and uses CD59 as an adaptor molecule. *J.Biol.Chem.* 278:21024-21031.

Giovannini, C., P. Matarrese, B. Scazzocchio, R. Vari, M. D'Archivio, E. Straface, R. Masella, W. Malorni, and M. De Vincenzi. 2003. Wheat gliadin induces apoptosis of intestinal cells via an autocrine mechanism involving Fas-Fas ligand pathway. *FEBS Lett.* 540:117-124.

Goicoechea, S., A.W. Orr, M.A. Pallero, P. Eggleton, and J.E. Murphy-Ullrich. 2000. Thrombospondin mediates focal adhesion disassembly through interactions with cell surface calreticulin. *J.Biol.Chem.* 275:36358-36368.

Gray, A.J., P.W. Park, T.J. Broekelmann, G.J. Laurent, J.T. Reeves, K.R. Stenmark, and R.P. Mecham. 1995. The mitogenic effects of the B beta chain of fibrinogen are mediated through cell surface calreticulin. *J.Biol.Chem.* 270:26602-26606.

Greco, L., M.C. Babron, G.R. Corazza, S. Percopo, R. Sica, F. Clot, M.C. Fulchignoni-Lataud, P. Zavattari, P. Momigliano-Richiardi, G. Casari, P. Gasparini, R. Tosi, V. Mantovani, S. De Virgiliis, G. Iacono, A. D'Alfonso, H. Selinger-Leneman, A. Lemainque, J.L. Serre, and F. Clerget-Darpoux. 2001. Existence of a genetic risk factor on chromosome 5q in Italian coeliac disease families. *Ann.Hum.Genet.* 65:35-41.

Greco, L., R. Romino, I. Coto, N. Di Cosmo, S. Percopo, M. Maglio, F. Paparo, V. Gasperi, M.G. Limongelli, R. Cotichini, C. D'Agate, N. Tinto, L. Sacchetti, R. Tosi, and M.A. Stazi. 2002. The first large population based twin study of coeliac disease. *Gut.* 50:624-628.

Groenendyk, J., J. Lynch, and M. Michalak. 2004. Calreticulin, Ca2+, and calcineurin - signaling from the endoplasmic reticulum. *Mol.Cells.* 17:383-389.

Guo, L., J. Groenendyk, S. Papp, M. Dabrowska, B. Knoblach, C. Kay, J.M. Parker, M. Opas, and M. Michalak. 2003. Identification of an N-domain histidine essential for chaperone function in calreticulin. *J.Biol.Chem.* 278:50645-50653.

Hadjivassiliou, M., M. Maki, D.S. Sanders, C.A. Williamson, R.A. Grunewald, N.M. Woodroofe, and I.R. Korponay-Szabo. 2006. Autoantibody targeting of brain and intestinal transglutaminase in gluten ataxia. *Neurology.* 66:373-377.

Halttunen, T., and M. Maki. 1999. Serum immunoglobulin A from patients with celiac disease inhibits human T84 intestinal crypt epithelial cell differentiation. *Gastroenterology.* 116:566-572.

Hausch, F., L. Shan, N.A. Santiago, G.M. Gray, and C. Khosla. 2002. Intestinal digestive resistance of immunodominant gliadin peptides. *Am.J.Physiol.Gastrointest.Liver Physiol.* 283:G996-G1003.

Holaska, J.M., B.E. Black, D.C. Love, J.A. Hanover, J. Leszyk, and B.M. Paschal. 2001. Calreticulin Is a receptor for nuclear export. *J.Cell Biol.* 152:127-140.

Holopainen, P., A.T. Naluai, S. Moodie, S. Percopo, I. Coto, F. Clot, H. Ascher, L. Sollid, P. Ciclitira, L. Greco, F. Clerget-Darpoux, J. Partanen, and Members of the European Genetics Cluster on Coeliac Disease. 2004. Candidate gene region 2q33 in European families with coeliac disease. *Tissue Antigens.* 63:212-222.

John, L.M., J.D. Lechleiter, and P. Camacho. 1998. Differential modulation of SERCA2 isoforms by calreticulin. *J.Cell Biol.* 142:963-973.

Jones, R.W., N.W. Taylor, and F.R. Senti. 1959. Electrophoresis and fractionation of wheat gluten. *Arch.Biochem.Biophys.* 84:363-376.

Jorgensen, C.S., K.B. Hansen, S. Jacobsen, P. Halberg, S. Ullman, D. Hansen, T.L. Mikkelsen, B. Weile, M.H. Madsen, A. Wiik, and G. Houen. 2005. Absence of high-affinity calreticulin autoantibodies in patients with systemic rheumatic diseases and coeliac disease. *Scand.J.Clin.Lab.Invest.* 65:403-412.

Joshi, M., G.P. Pogue, R.C. Duncan, N.S. Lee, N.K. Singh, C.D. Atreya, D.M. Dwyer, and H.L. Nakhasi. 1996. Isolation and characterization of Leishmania donovani calreticulin gene and its conservation of the RNA binding activity. *Mol.Biochem.Parasitol.* 81:53-64.

Karpati, S., I. Kosnai, E. Torok, and J.B. Kovacs. 1988. Immunoglobulin A deposition in jejunal mucosa of children with dermatitis herpetiformis. *J.Invest.Dermatol.* 91:336-339.

Kaukinen, K., M. Peraaho, P. Collin, J. Partanen, N. Woolley, T. Kaartinen, T. Nuutinen, T. Halttunen, M. Maki, and I. Korponay-Szabo. 2005. Small-bowel mucosal transglutaminase 2-specific IgA deposits in coeliac disease without villous atrophy: a prospective and randomized clinical study. *Scand.J.Gastroenterol.* 40:564-572.

Knee, R., I. Ahsan, N. Mesaeli, R.J. Kaufman, and M. Michalak. 2003. Compromised calnexin function in calreticulin-deficient cells. *Biochem.Biophys.Res.Commun.* 304:661-666.

Körkkö, J., S. Annunen, T. Pihlajamaa, D.J. Prockop, and L. Ala-Kokko. 1998. Conformation sensitive gel electrophoresis for simple and accurate detection of mutations: comparison with denaturing gradient gel electrophoresis and nucleotide sequencing. *Proc.Natl.Acad.Sci.U.S.A.* 95:1681-1685.

Korponay-Szabo, I.R., K. Laurila, Z. Szondy, T. Halttunen, Z. Szalai, I. Dahlbom, I. Rantala, J.B. Kovacs, L. Fesus, and M. Maki. 2003. Missing endomysial and reticulin binding of coeliac antibodies in transglutaminase 2 knockout tissues. *Gut.* 52:199-204.

Korponay-Szabo, I.R., T. Halttunen, Z. Szalai, K. Laurila, R. Kiraly, J.B. Kovacs, L. Fesus, and M. Maki. 2004. In vivo targeting of intestinal and extraintestinal transglutaminase 2 by coeliac autoantibodies. *Gut.* 53:641-648.

Kovacs, H., I.D. Campbell, P. Strong, S. Johnson, F.J. Ward, K.B. Reid, and P. Eggleton. 1998. Evidence that C1q binds specifically to CH2-like immunoglobulin gamma motifs present in the autoantigen calreticulin and interferes with complement activation. *Biochemistry.* 37:17865-17874.

Limongelli, M.G., O. Esposito, C. Marano, L. Sacchetti, N. Tinto, D. Gennarelli, R. Troncone, and L. Greco. 2005. Genetic risk of first degree relatives of coeliac patients. 40:667.

Ling, S., X. Pi, and J. Holoshitz. 2007. The rheumatoid arthritis shared epitope triggers innate immune signaling via cell surface calreticulin. *J.Immunol.* 179:6359-6367.

Llewelyn Roderick, H., D.H. Llewellyn, A.K. Campbell, and J.M. Kendall. 1998. Role of calreticulin in regulating intracellular Ca2+ storage and capacitative Ca2+ entry in HeLa cells. *Cell Calcium.* 24:253-262.

Lohi, S., K. Mustalahti, K. Kaukinen, K. Laurila, P. Collin, H. Rissanen, O. Lohi, E. Bravi, M. Gasparin, A. Reunanen, and M. Maki. 2007. Increasing prevalence of coeliac disease over time. *Aliment.Pharmacol.Ther.* 26:1217-1225.

Louka, A.S., B.A. Lie, B. Talseth, H. Ascher, J. Ek, A.H. Gudjonsdottir, and L.M. Sollid. 2003. Coeliac disease patients carry conserved HLA-DR3-DQ2 haplotypes revealed by association of TNF alleles. *Immunogenetics.* 55:339-343.

Madsen, K.L., S.A. Lewis, M.M. Tavernini, J. Hibbard, and R.N. Fedorak. 1997. Interleukin 10 prevents cytokine-induced disruption of T84 monolayer barrier integrity and limits chloride secretion. *Gastroenterology.* 113:151-159.

Maiuri, L., C. Ciacci, I. Ricciardelli, L. Vacca, V. Raia, S. Auricchio, J. Picard, M. Osman, S. Quaratino, and M. Londei. 2003. Association between innate response to gliadin and activation of pathogenic T cells in coeliac disease. *Lancet.* 362:30-37.

Marsh, M.N. 1992. Gluten, major histocompatibility complex, and the small intestine. A molecular and immunobiologic approach to the spectrum of gluten sensitivity ('celiac sprue'). *Gastroenterology.* 102:330-354.

Mazzarella, G., R. Stefanile, A. Camarca, P. Giliberti, E. Cosentini, C. Marano, G. Iaquinto, N. Giardullo, S. Auricchio, A. Sette, R. Troncone, and C. Gianfrani. 2008. Gliadin activates HLA class I-restricted CD8+ T cells in celiac disease intestinal mucosa and induces the enterocyte apoptosis. *Gastroenterology.* 134:1017-1027.

McCauliffe, D.P., F.A. Lux, T.S. Lieu, I. Sanz, J. Hanke, M.M. Newkirk, L.L. Bachinski, Y. Itoh, M.J. Siciliano, and M. Reichlin. 1990. Molecular cloning, expression, and chromosome 19 localization of a human Ro/SS-A autoantigen. *J.Clin.Invest.* 85:1379-1391.

McCluskey, J., A.D. Farris, C.L. Keech, A.W. Purcell, M. Rischmueller, G. Kinoshita, P. Reynolds, and T.P. Gordon. 1998. Determinant spreading: lessons from animal models and human disease. *Immunol.Rev.* 164:209-229.

Melino, G., E. Candi, and P.M. Steinert. 2000. Assays for transglutaminases in cell death. *Methods Enzymol.* 322:433-472.

Meresse, B., Z. Chen, C. Ciszewski, M. Tretiakova, G. Bhagat, T.N. Krausz, D.H. Raulet, L.L. Lanier, V. Groh, T. Spies, E.C. Ebert, P.H. Green, and B. Jabri. 2004. Coordinated induction by IL15 of a TCR-independent NKG2D signaling pathway converts CTL into lymphokine-activated killer cells in celiac disease. *Immunity.* 21:357-366.

Meresse, B., S.A. Curran, C. Ciszewski, G. Orbelyan, M. Setty, G. Bhagat, L. Lee, M. Tretiakova, C. Semrad, E. Kistner, R.J. Winchester, V. Braud, L.L. Lanier, D.E. Geraghty, P.H. Green, S. Guandalini, and B. Jabri. 2006. Reprogramming of CTLs into natural killer-like cells in celiac disease. *J.Exp.Med.* 203:1343-1355.

Mesaeli, N., K. Nakamura, E. Zvaritch, P. Dickie, E. Dziak, K.H. Krause, M. Opas, D.H. MacLennan, and M. Michalak. 1999. Calreticulin is essential for cardiac development. *J.Cell Biol.* 144:857-868.

Mhaouty-Kodja, S. 2004. Ghalpha/tissue transglutaminase 2: an emerging G protein in signal transduction. *Biol.Cell.* 96:363-367.

Michalak, M., E.F. Corbett, N. Mesaeli, K. Nakamura, and M. Opas. 1999. Calreticulin: one protein, one gene, many functions. *Biochem.J.* 344 Pt 2:281-292.

Michalak, M., J.M. Robert Parker, and M. Opas. 2002. Ca2+ signaling and calcium binding chaperones of the endoplasmic reticulum. *Cell Calcium.* 32:269-278.

Molberg, O., S.N. Mcadam, R. Korner, H. Quarsten, C. Kristiansen, L. Madsen, L. Fugger, H. Scott, O. Noren, P. Roepstorff, K.E. Lundin, H. Sjostrom, and L.M. Sollid. 1998. Tissue transglutaminase selectively modifies gliadin peptides that are recognized by gut-derived T cells in celiac disease. *Nat.Med.* 4:713-717.

Monsuur, A.J., P.I. de Bakker, B.Z. Alizadeh, A. Zhernakova, M.R. Bevova, E. Strengman, L. Franke, R. van't Slot, M.J. van Belzen, I.C. Lavrijsen, B. Diosdado, M.J. Daly, C.J. Mulder, M.L. Mearin, J.W. Meijer, G.A. Meijer, E. van Oort, M.C. Wapenaar, B.P. Koeleman, and C. Wijmenga. 2005. Myosin IXB variant increases the risk of celiac disease and points toward a primary intestinal barrier defect. *Nat.Genet.* 37:1341-1344.

Myrsky, E., K. Kaukinen, M. Syrjanen, I.R. Korponay-Szabo, M. Maki, and K. Lindfors. 2008. Coeliac disease-specific autoantibodies targeted against transglutaminase 2 disturb angiogenesis. *Clin.Exp.Immunol.* 152:111-119.

Mäki, M., K. Mustalahti, J. Kokkonen, P. Kulmala, M. Haapalahti, T. Karttunen, J. Ilonen, K. Laurila, I. Dahlbom, T. Hansson, P. Hopfl, and M. Knip. 2003. Prevalence of celiac disease among children in Finland. 348:2517-2524.

Mäki, M., O. Hallstrom, T. Vesikari, and J.K. Visakorpi. 1984. Evaluation of a serum IgA-class reticulin antibody test for the detection of childhood celiac disease. *J.Pediatr.* 105:901-905.

Naaby-Hansen, S., M.J. Wolkowicz, K. Klotz, L.A. Bush, V.A. Westbrook, H. Shibahara, J. Shetty, S.A. Coonrod, P.P. Reddi, J. Shannon, M. Kinter, N.E. Sherman, J. Fox, C.J. Flickinger, and J.C. Herr. 2001. Co-localization of the inositol 1,4,5-trisphosphate receptor and calreticulin in the equatorial segment and in membrane bounded vesicles in the cytoplasmic droplet of human spermatozoa. *Mol.Hum.Reprod.* 7:923-933.

Nakamura, K., E. Bossy-Wetzel, K. Burns, M.P. Fadel, M. Lozyk, I.S. Goping, M. Opas, R.C. Bleackley, D.R. Green, and M. Michalak. 2000. Changes in endoplasmic reticulum luminal environment affect cell sensitivity to apoptosis. *J.Cell Biol.* 150:731-740.

Nakamura, K., A. Zuppini, S. Arnaudeau, J. Lynch, I. Ahsan, R. Krause, S. Papp, H. De Smedt, J.B. Parys, W. Muller-Esterl, D.P. Lew, K.H. Krause, N. Demaurex, M. Opas, and M. Michalak. 2001. Functional specialization of calreticulin domains. *J.Cell Biol.* 154:961-972.

Nikulina, M., C. Habich, S.B. Flohe, F.W. Scott, and H. Kolb. 2004. Wheat gluten causes dendritic cell maturation and chemokine secretion. *J.Immunol.* 173:1925-1933.

Opas, M., E. Dziak, L. Fliegel, and M. Michalak. 1991. Regulation of expression and intracellular distribution of calreticulin, a major calcium binding protein of nonmuscle cells. *J.Cell.Physiol.* 149:160-171.

Otabor, I., S. Tyagi, F.J. Beurskens, I. Ghiran, P. Schwab, A. Nicholson-Weller, and L.B. Klickstein. 2004. A role for lipid rafts in C1q-triggered O2- generation by human neutrophils. *Mol.Immunol.* 41:185-190.

Pender, S.L., S.P. Tickle, A.J. Docherty, D. Howie, N.C. Wathen, and T.T. MacDonald. 1997. A major role for matrix metalloproteinases in T cell injury in the gut. *J.Immunol.* 158:1582-1590.

Picarelli, A., L. Maiuri, A. Frate, M. Greco, S. Auricchio, and M. Londei. 1996. Production of antiendomysial antibodies after in-vitro gliadin challenge of small intestine biopsy samples from patients with coeliac disease. *Lancet.* 348:1065-1067.

Porcellini, S., E. Traggiai, U. Schenk, D. Ferrera, M. Matteoli, A. Lanzavecchia, M. Michalak, and F. Grassi. 2006. Regulation of peripheral T cell activation by calreticulin. *J.Exp.Med.* 203:461-471.

Raivio, T., I. Korponay-Szabo, P. Collin, K. Laurila, H. Huhtala, T. Kaartinen, J. Partanen, M. Maki, and K. Kaukinen. 2007. Performance of a new rapid whole blood coeliac test in adult patients with low prevalence of endomysial antibodies. *Dig.Liver Dis.* 39:1057-1063.

Rauch, F., J. Prud'homme, A. Arabian, S. Dedhar, and R. St-Arnaud. 2000. Heart, brain, and body wall defects in mice lacking calreticulin. *Exp.Cell Res.* 256:105-111.

Sanchez, D., L. Tuckova, T. Mothes, W. Kreisel, Z. Benes, and H. Tlaskalova-Hogenova. 2003. Epitopes of calreticulin recognised by IgA autoantibodies from patients with hepatic and coeliac disease. *J.Autoimmun.* 21:383-392.

Sardy, M., S. Karpati, B. Merkl, M. Paulsson, and N. Smyth. 2002. Epidermal transglutaminase (TGase 3) is the autoantigen of dermatitis herpetiformis. *J.Exp.Med.* 195:747-757.

Savilahti, E., M. Viander, M. Perkkio, E. Vainio, K. Kalimo, and T. Reunala. 1983. IgA antigliadin antibodies: a marker of mucosal damage in childhood coeliac disease. *Lancet.* 1:320-322.

Schrag, J.D., J.J. Bergeron, Y. Li, S. Borisova, M. Hahn, D.Y. Thomas, and M. Cygler. 2001. The Structure of calnexin, an ER chaperone involved in quality control of protein folding. *Mol.Cell.* 8:633-644.

Schulzke, J.D., C.J. Bentzel, I. Schulzke, E.O. Riecken, and M. Fromm. 1998. Epithelial tight junction structure in the jejunum of children with acute and treated celiac sprue. *Pediatr.Res.* 43:435-441.

Sipione, S., C. Ewen, I. Shostak, M. Michalak, and R.C. Bleackley. 2005. Impaired cytolytic activity in calreticulin-deficient CTLs. *J.Immunol.* 174:3212-3219.

Smith, M.J. 1992a. Nucleotide sequence of a Drosophila melanogaster gene encoding a calreticulin homologue. *DNA Seq.* 3:247-250.

Smith, M.J. 1992b. A C. elegans gene encodes a protein homologous to mammalian calreticulin. *DNA Seq.* 2:235-240.

Sollid, L.M. 2000. Molecular basis of celiac disease. *Annu.Rev.Immunol.* 18:53-81.

Sollid, L.M. 2002. Coeliac disease: dissecting a complex inflammatory disorder. *Nat.Rev.Immunol.* 2:647-655.

Sollid, L.M., and B.A. Lie. 2005. Celiac disease genetics: current concepts and practical applications. *Clin.Gastroenterol.Hepatol.* 3:843-851.

Stepniak, D., L.W. Vader, Y. Kooy, P.A. van Veelen, A. Moustakas, N.A. Papandreou, E. Eliopoulos, J.W. Drijfhout, G.K. Papadopoulos, and F. Koning. 2005. T-cell recognition of HLA-DQ2-bound gluten peptides can be influenced by an N-terminal proline at p-1. *Immunogenetics.* 57:8-15.

Stuart, G.R., N.J. Lynch, A.J. Day, W.J. Schwaeble, and R.B. Sim. 1997. The C1q and collectin binding site within C1q receptor (cell surface calreticulin). *Immunopharmacology.* 38:73-80.

Sturgess, R.P., H.J. Ellis, and P.J. Ciclitira. 1991. Cereal chemistry, molecular biology, and toxicity in coeliac disease. *Gut.* 32:1055-1060.

Toquet, C., A. Jarry, C. Bou-Hanna, K. Bach, M.G. Denis, J.F. Mosnier, and C.L. Laboisse. 2007. Altered Calreticulin expression in human colon cancer: maintenance of Calreticulin expression is associated with mucinous differentiation. *Oncol.Rep.* 17:1101-1107.

Trombetta, E.S., and A.J. Parodi. 2003. Quality control and protein folding in the secretory pathway. *Annu.Rev.Cell Dev.Biol.* 19:649-676.

Truedsson, L., A.A. Bengtsson, and G. Sturfelt. 2007. Complement deficiencies and systemic lupus erythematosus. *Autoimmunity*. 40:560-566.

Tuckova, L., K. Karska, J.R. Walters, M. Michalak, P. Rossmann, S. Krupickova, E.F. Verdu, R. Saalman, L.A. Hanson, and H. Tlaskalova-Hogenova. 1997. Anti-gliadin antibodies in patients with celiac disease cross-react with enterocytes and human calreticulin. *Clin.Immunol.Immunopathol.* 85:289-296.

Tutton, P.J., and D.H. Barkla. 1988. Steroid hormones as regulators of the proliferative activity of normal and neoplastic intestinal epithelial cells (review). *Anticancer Res.* 8:451-456.

Uhlig, H.H., J. Lichtenfeld, A.A. Osman, T. Richter, and T. Mothes. 2000. Evidence for existence of coeliac disease autoantigens apart from tissue transglutaminase. *Eur.J.Gastroenterol.Hepatol.* 12:1017-1020.

Unsworth, D.J., M. Kieffer, E.J. Holborow, R.R. Coombs, and J.A. Walker-Smith. 1981. IgA anti-gliadin antibodies in coeliac disease. *Clin.Exp.Immunol.* 46:286-293.

Vader, W., D. Stepniak, Y. Kooy, L. Mearin, A. Thompson, J.J. van Rood, L. Spaenij, and F. Koning. 2003. The HLA-DQ2 gene dose effect in celiac disease is directly related to the magnitude and breadth of gluten-specific T cell responses. *Proc.Natl.Acad.Sci.U.S.A.* 100:12390-12395.

Van Belzen, M.J., J.W. Meijer, L.A. Sandkuijl, A.F. Bardoel, C.J. Mulder, P.L. Pearson, R.H. Houwen, and C. Wijmenga. 2003. A major non-HLA locus in celiac disease maps to chromosome 19. *Gastroenterology*. 125:1032-1041.

van Heel, D.A., and J. West. 2006. Recent advances in coeliac disease. *Gut.* 55:1037-1046.

Vandivier, R.W., C.A. Ogden, V.A. Fadok, P.R. Hoffmann, K.K. Brown, M. Botto, M.J. Walport, J.H. Fisher, P.M. Henson, and K.E. Greene. 2002. Role of surfactant proteins A, D, and C1q in the clearance of apoptotic cells in vivo and in vitro: calreticulin and CD91 as a common collectin receptor complex. *J.Immunol.* 169:3978-3986.

Verreck, F.A., D. Elferink, C.J. Vermeulen, R. Amons, F. Breedveld, R.R. de Vries, and F. Koning. 1995. DR4Dw4/DR53 molecules contain a peptide from the autoantigen calreticulin. *Tissue Antigens.* 45:270-275.

Wu, X., X. Liu, X. Zhu, and C. Tang. 2007. Hypoxic preconditioning induces delayed cardioprotection through p38 MAPK-mediated calreticulin upregulation. *Shock.* 27:572-577.

Xiao, G., T.F. Chung, H.Y. Pyun, R.E. Fine, and R.J. Johnson. 1999a. KDEL proteins are found on the surface of NG108-15 cells. *Brain Res.Mol.Brain Res.* 72:121-128.

Xiao, G., T.F. Chung, R.E. Fine, and R.J. Johnson. 1999b. Calreticulin is transported to the surface of NG108-15 cells where it forms surface patches and is partially degraded in an acidic compartment. *J.Neurosci.Res.* 58:652-662.

Yao, L., S.E. Pike, and G. Tosato. 2002. Laminin binding to the calreticulin fragment vasostatin regulates endothelial cell function. *J.Leukoc.Biol.* 71:47-53.

Zanoni, G., R. Navone, C. Lunardi, G. Tridente, C. Bason, S. Sivori, R. Beri, M. Dolcino, E. Valletta, R. Corrocher, and A. Puccetti. 2006. In celiac disease, a subset of autoantibodies against transglutaminase binds toll-like receptor 4 and induces activation of monocytes. *PLoS Med.* 3:e358.

Zhu, Q., P. Zelinka, T. White, and M.L. Tanzer. 1997. Calreticulin-integrin bidirectional signaling complex. *Biochem.Biophys.Res.Commun.* 232:354-358.

Appendix 1

CSGE Gel Components:

99:1 acrylamide:BAP	37,5 ml
Acrylamide 30 %	
1,4-bis (acryloyl) piperazine (BAP)	
TTE-buffer 20x (for 1 liter)	3,8 ml
216 g Trizma base	
72 g Taurine	
4 g EDTA	
Formamide	22,5 ml
Ethylene glycole	15 ml
H2O	70 ml

After mixing 10 minutes, add 750 µl 20% APS and 103 µl of N,N,N',N'-Tetramethylethylenediamine.