Proteomics Identification of Acyl-acceptor and Acyl-donor Substrates for Transglutaminase in a Human Intestinal Epithelial Cell Line

IMPLICATIONS FOR CELIAC DISEASE*

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Transglutaminase (TG)-catalyzed cross-linking of both intracellular and extracellular proteins is an important biochemical event. However, increased concentrations of cross-linked proteins have been observed in many disorders. Moreover, TG-catalyzed modification of proteins might generate new self-antigens responsible for the autoimmune response, as in celiac disease. The identification of available substrates may offer an understanding of how the TG-catalyzed post-translational modification has an impact on physiology and disease. We used a proteomic approach to identify TG-modified protein targets in human intestinal epithelial cells to determine the extent to which transglutaminase specifically contributes to celiac disease. Two probes were used for endogenous TG activity: 5-(biotinamido)pentylamine, which represents the acyl-acceptor, and a biotinylated glutamine-containing peptide, which represents the acyl-donor. This approach identified >25 proteins, which range from 30,000 to 300,000 Daltons and can serve as acyl-acceptor and/or acyl-donor for transglutaminase. Some of them were known transglutaminase substrates, whereas others had not been previously identified. These targets include proteins involved in cytoskeletal network organization, folding of proteins, transport processes, and miscellaneous metabolic functions.

Transglutaminases (TGs)† (EC 2.3.2.13) constitute a family of enzymes that catalyze the post-translational modification of proteins. Their calcium-dependent catalytic activity is exhibited toward γ-carboxamide groups of peptide-bound glutamine residues and ε-amino groups of peptide-bound lysines, leading to an intrachain or interchain isopeptide bond. Low molecular weight amines may substitute lysines in transamidating reactions. In the absence of suitable amines, water may act as an acyl-acceptor substrate with the consequent deamidation of protein-bound glutamine residues (1, 2). Nine different TG genes have been characterized in mammalian species to date (3, 4). However, the biological significance of some gene products is completely unknown. Currently, distinct widely expressed TG molecular forms have been described, including plasma Factor XIIIα, keratinocyte TG (type I), tissue TG (type II), epidermal TG (type III), prostate TG (type IV), and TG V (4). TG enzymes are involved in many fundamental biological processes including the fibrin-clotting cascade (1), seminal vesicle coagulation (5), and cornification of the epidermis, hair, and nails (6, 7).

Despite the fact that tissue TG (tTG) has been studied extensively, its function remains an open question. tTG is expressed in selected mammalian tissues and seems involved in the regulation of several biological events including cellular proliferation, differentiation, and apoptosis (3, 8). Moreover, tTG has been found associated with the extracellular surface where it has been implicated in extracellular matrix organization (9, 10). tTG functions might therefore depend on its peculiar location in tissues. In particular, the presence of different accessible proteins able to act as substrates for the enzyme in specific cell type may determine the activity of tTG and its control. Up to now, few cases of tTG activity have been reported to be linked to the modulation of the function of specific substrate proteins. The cross-linking of retinoblastoma protein has been shown to be a key signal for the initiation of apoptosis (11, 12). Latent transforming growth factor-β that is considered the major autocrine/paracrine inhibitory regulator in the intestinal epithelium is activated by tTG (13). Finally, tTG-mediated post-translational modifications of proteins may represent pathogenic mechanisms in diseases, including celiac disease (CD) (14). CD is an autoimmune pathology triggered in genetically predisposed patients by exposure to gliadin, a flour protein, thus evoking local immune reactions and mucosal atrophy (15). A model was postulated on the basis of the finding that tTG is the main target of immune response in CD (16) and that gliadin is able to act as a tTG substrate (17, 18). This model suggests that tTG catalyzes in situ the deamidation of specific glutamine residues present in immunodominant gluten peptides, which are recognized by CD4+ DQ2-restricted mucosal T cells (19, 20). However, the mucosal environment should be
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without amine donors to allow the occurrence of such reactions in vivo. Apart from the deamidation of gliadin peptide, tTG acts as hapten in the generation of antibodies against gliadin and itself, catalyzing the synthesis of heteromeric gliadin-tTG complexes that may provoke an immune response to tTG by stimulating normally silent B cells specific for tTG (21, 22). However, where tTG comes in contact with gliadin in the intestinal mucosa and how tTG-catalyzed post-translational modifications may occur remain to be investigated. It is conceivable that a primary pathogenic event of CD could happen at the level of the enterocytes, which are crucial to maintain barrier function between the luminal milieu and the internal environment. There is increasing evidence that enterocytes modulate immunological functions of the intestinal mucosa by expressing HLA class II antigens and presenting antigens to T-lymphocytes (23). Recently, an increased expression of tTG in celiac patients was reported in enterocytes, both at brush border and cytoplasm levels, as well as in the extracellular matrix. Moreover, by performing tTG assays in situ, it was discovered that accessible proteins act as acyl-acceptor and acyl-donor substrates in intestinal mucosa (24). Therefore, we focused on the identification of putative substrates of tTG in an enterocyte-like system. Achieving this goal represents a crucial step to clarify the role of the enzyme in CD. CaCo-2 cells, a human colon adenocarcinoma cell line, that represent a suitable model for the study of the enterocytes function in vitro (25) were used. In situ and in vitro tTG assays were performed using two probes for endogenous tTG activity: 5-(biotinamido)pentylamine, which represents the acyl-acceptor tTG substrate, and a synthetic biotinylated glutamine-containing peptide, which represents the acyl-donor tTG substrate. A proteomic approach was used to identify >25 endogenous proteins, both acyl-acceptor (Lys-donor) and acyl-donor (Gln-donor), that may represent the target of tTG catalytic action. Biological relevance of such potential in vitro tTG substrates is discussed relatively to the role of intestinal epithelial cells in CD pathogenesis.

EXPERIMENTAL PROCEDURES

Cell Culture—The human colon adenocarcinoma cell line CaCo-2 and Madin-Darby canine kidney (MDCK) cells were obtained from the Integrated Cell Line Collection (Centro di Biotecnologie Avanzate, Genova, Italy). CaCo-2 cells were cultured in 100 × 10-mm Petri dishes containing Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) non-essential amino acids, 0.2 mM l-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin (Invitrogen). MDCK cells were grown in minimum essential medium (Invitrogen) supplemented with 10% (v/v) fetal bovine serum, 10% (v/v) Earle’s balanced salt solution, 50 units/ml penicillin, 50 μg/ml streptomycin, and 1% (v/v) non-essential amino acids. Cells were maintained at 37 °C in a 5% CO2, 95% air-humidified atmosphere and passaged twice a week. In this study, CaCo-2 cells were used between the 16th and 25th passages. For the experiments, the cells were seeded at a density of 30 × 104/cm2. On the 3rd and 12th day post-confluence, cells were used for in situ treatments and alternatively were harvested to perform in vitro assays. A stable MDCK cell clone, MDCK-tTG, expressing recombinant tTG was previously obtained (26) and used for in situ assays. In situ tTG Activity Assay—Two probes were used for labeling proteins in intact cells: 5-(biotinamido)pentylamine (BNPH2, Pierce), which represents the acyl-acceptor probe, and a biotinylated glutamine-containing hexapeptide (biotinyl-TQVQEL, A25 peptide) (27), which represents the acyl-donor probe. Cells were preincubated for 1 h in the presence of 1 mM BNPH2 or 1 mM A25 peptide in standard medium. To activate intracellular tTG, cells were treated for 30 min with the Ca2+ ionophore A-23187 (ICN) at the concentration of 10 μM. As A-23187 was dissolved in dimethyl sulfoxide, the maximal dimethyl sulfoxide concentration to which the cells were exposed was 0.1%. A trypan blue exclusion test was performed to determine the cell viability after A-23187 challenge. After the treatments, cells were washed twice with calcium-free and magnesium-free phosphate-buffered saline (PBS) solution and scraped in ice-cold buffer (HEPES, pH 7.5) containing 1 mM EGTA and protease inhibitors (20 μg/ml leupeptin, 0.5 mM benzamidine, 20 μM aprotinin, and 0.2 mM phenylmethylsulfonyl fluoride (PMSF)). The cell suspension was sonicated on ice for four times for 10 s each and centrifuged at 15,000 × g for 10 min at 4 °C. The supernatant was used for further experiments.

In vitro tTG-mediated Labeling Assay—To perform in vitro tTG-mediated protein labeling, CaCo-2 cells were washed twice with PBS solution and scraped in ice-cold 10 mM HEPES, pH 7.5, containing 5 mM dithiothreitol and protease inhibitors. Cells were sonicated four times for 10 s each, and the homogenate was centrifuged at 15,000 × g for 10 min at 4 °C. The obtained supernatants (500 μg) were incubated at 37 °C for 2 h in a 50 mM HEPES buffer, pH 9.0, containing 10 mM dithiothreitol and 5 mM CaCl2 in the presence of 1 mM BNPH2 or, alternatively, 1 mM A25 peptide (final volume, 200 μl). In control samples, CaCl2 was replaced by 0.05% Tween 20. The samples were incubated for 2 h with the HRP-conjugated goat anti-mouse secondary antibody (Bio-Rad) diluted 1:3000 in the blocking buffer. The blots were washed as above, and immunocomplexes were revealed using the chemiluminescence detection kit (Pierce) according to manufacturer’s instructions.

Cytochemistry—For cytochemical staining, cells were plated onto coverslips and preincubated for 1 h at 37 °C in the presence of 1 mM BNPH2 or 1 mM A25 peptide. Cells were then fixed in paraformaldehyde (3% in PBS) for 20 min and permeabilized with Triton X-100 (0.2% in PBS) for 4 min. After the incubation with 1% bovine serum albumin in PBS to reduce the background, cell were probed with fluorescein isothiocyanate-conjugated streptavidin (Santa Cruz Biotechnology) diluted 1:30 in 1% bovine serum albumin for 30 min at room temperature in the presence of no biotinylated substrate.Cells were finally observed with an Axioscope 2 microscope (Zeiss), and images were acquired and analyzed by using the KS400 software.

Purification of Labeled tTG Protein Substrates—The protein mixture containing tTG protein substrates labeled by BNPH2 was removed from the excess of the acyl-donor probe using a Sephadex PD10 pre-packed column (Amersham Biosciences) with PBS as eluent. 1-mL fractions were collected and monitored at 280 nm. The eluted proteins were pooled and analyzed by affinity chromatography with a pre-packed monomeric avidin column (Pierce) according to the procedure previously (29, 30). Biotinylated proteins were specifically eluted with PBS buffer containing 2 mM biotin. The labeled tTG protein substrates were pooled, desalted, and concentrated by Centricron-10 (Amicon) up to a final volume of 0.5–1 ml. Finally, the biotin-labeled protein mixture was analyzed by the SDS-PAGE on 10% linear slab gels. Molecular masses of protein bands were estimated by using Bio-Rad precision protein standards.

The protein mixture containing tTG protein substrates labeled by the A25 peptide was dialyzed against PBS at 4 °C for 24 h to remove the excess of acyl-donor probe. The protein mixture was then purified following the procedure described above.

In Gel Digestion of Proteins—Coomassie Blue-stained protein-containing bands were excised from the gel, cut in pieces, and washed first with H2O and then with 1 mM ammonium bicarbonate for 30 min at room temperature in the dark as described previously (31). The gel pieces were then washed with 0.1 mM ammonium bicarbonate and acetoniTrile. Enzymatic digestions were carried out in modified trypsin (Roche Applied Science) digestion buffer containing 10 mM ammonium bicarbonate, pH 8.5, at 4 °C for 45 min. The enzymatic solution was then removed, and a new aliquot of the buffer solution was added to the gel pieces. The digestions were incubated at 37 °C for 18 h. A minimum reaction volume sufficient for complete rehydration of the gel was used. Peptides were then extracted by washing the gel pieces with three steps: (1) 10 mM Tris-Cl, pH 7.4, for 10 min at 4 °C; (2) 0.2% trifluoroacetic acid; and (3) acetoniTrile at 37 °C for 15 min/step and then lyophilized.

Peptide Mapping—MALDI-ToF spectra were recorded using a reflectron Voyager DE PRO MALDI-time-of-flight mass spectrometer (Applied Biosystems). An aliquot of the peptide mixture (1 μl) was added to a
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MALDI matrix solution, and then 1 µl was spotted onto a stainless steel sample plate and allowed to dry. The MALDI matrixes used in this study were α-cyano-4-hydroxycinnamic acid (Sigma, 10 mg/ml in acetonitrile, 0.2% trifluoroacetic acid, 80% acetonitrile, 0.2% trifluoroacetic acid, 6:4 (v/v)) in a sample:matrix ratio of 1:1 and sinapinic acid (Sigma, 10 mg/ml in acetonitrile, 0.2% trifluoroacetic acid, 7:3 (v/v)) in a sample:matrix ratio of 1:5. Spectra were acquired both in a linear and in a reflectron mode. The mass range was calibrated using the [M + H]⁺ ions from the standard peptide mixture provided by the manufacturers. Raw data were analyzed using computer software provided by the manufacturers.

In cases where the identity of the proteins present could not be established by MALDI analysis alone, the peptide mixtures were further analyzed by LC/MS/MS using a Q-ToF hybrid mass spectrometer (Micromass) equipped with a Z-spray source and coupled on-line with a capillary chromatography system. After loading, the peptide mixture (10 µl) was first concentrated and washed at 30 µl/min onto a reverse-phase pre-column (Waters) using 0.1% formic acid as eluent. The sample was then fractionated using a C₁₈ reverse-phase capillary column (Micromass) equipped with a Z-spray source and coupled on-line with a data-dependent MS/MS mode where a full scan spectrum (m/z range from 500 to 2000 Da) was followed by a tandem mass spectrum. The precursor ion was selected as the most intense peak of the previous scan. A ramp of collision energy between 30 and 60 eV was applied depending on the mass and charge of the precursor ion. ProteinLynx Global Server software, provided by the manufacturers, was used to analyze raw MS and MS/MS data and identify proteins.

RESULTS

In situ tTG-mediated Labeling of Cellular Proteins—CaCo-2 cells that constitutively express tTG (32) were used for in vitro transamidation experiments. In particular, CaCo-2 cells were studied both after 3 days post-confluence when they were still colonocytes to a large extent (undifferentiated cells) and after 12 days post-confluence when they were most similar to ileal enterocytes. To detect the acyl-donor (Gln-donor) and the acyl-acceptor (Lys-donor) tTG substrates, the acyl-acceptor BPNH₂ and the acyl-donor A25 peptide were used as probes, respectively, as described under “Experimental Procedures.” To investigate the probes uptake, a cytochemistry approach was used. Fig. 1 shows the intracellular localization of both biotinylated probes were detected using fluorescein isothiocyanate-conjugated streptavidin. NT, non-treated cells.

In situ tTG-mediated Labeling of Cellular Proteins—CaCo-2 cells were fractionated using a C₁₈ reverse-phase capillary column (Waters) using 0.1% formic acid as eluent. The sample was subjected to SDS-PAGE, transferred to a nitrocellulose membrane, and probed with an HRP-conjugated streptavidin (Fig. 2A, lanes 1 and 3). Because tTG is a calcium-activated enzyme, intracellular levels of calcium were elevated by treating the cells with the Ca²⁺-ionophore A-23187. Increasing intracellular calcium levels resulted in a significant increase in tTG-catalyzed incorporation of BPNH₂ into proteins in both different stages of CaCo-2 cells (Fig. 2A, lanes 2 and 4). The profile of proteins modified by tTG in the cells did not differ between the two extremes of CaCo-2 cell expression. However, the cross-linking was somewhat more evident in differentiated cells than in undifferentiated one, judging from the total amount labeled (compare lane 4 with lane 2). To determine whether the increase in the level of cross-links in situ observed in differentiated cells coincided with an increase of tTG expression, blots were stripped and reprobed with the monoclonal tTG antibody (Fig. 2B). tTG protein levels were higher in 12-day-post-confluent CaCo-2 cells than in 3-day-post-confluent CaCo-2 cells (Fig. 2B, lanes 3 and 4 compared with lanes 1 and 2). Moreover, in the presence of increased calcium ions level, the decrease of the tTG monomer migrating at 78 kDa contemporary with the presence of a band at high molecular mass revealed that tTG takes part in the formation of heteropolymers together with protein substrates.

To establish the specificity of the tTG-modified intracellular proteins, MDCK cells stably transfected with wild-type tTG were used as a control. The drastically decreased number of proteins that might act as acyl-donors in the transamidating reaction (Fig. 2A, lanes 5 and 6) suggests that proteins in CaCo-2 cells are selectively modified by tTG, even though tTG protein levels are higher in MDCK-tTG cells than in CaCo-2 cells as shown in Fig. 2B, lanes 5 and 6.

Parallel experiments were performed to detect the acyl-acceptor (Lys-donor) tTG substrates in CaCo-2 cells using the acyl-donor A25 peptide (Fig. 2C). Also, in this case, it was possible to detect the presence of several protein bands spread over a wide molecular weight range only after intracellular tTG activation (lanes 2 and 4). Moreover, the labeled protein patterns seemed not to be different between 3 and 12 days post-confluence. It is worth noting that the Lys-donor tTG substrates pattern seemed to be different from the Gln-donor pattern (see panel C and compare with panel A), thus indicating that different proteins can act as tTG substrates when using the two selected probes. Very high molecular weight polymers, including tTG not able to enter the gel mesh, were also detected in the gel wells when the blot was stripped and reprobed with the monoclonal antibody CUB 7402 (Fig. 1D) (33).

To isolate the Gln-donor and Lys-donor tTG protein substrates in CaCo-2 cells, the biotin-labeled proteins were separately purified from the whole CaCo-2 protein homogenate (8 mg) by an avidin-affinity chromatographic step. Such a strategy led to the isolation of the labeled proteins in a single step, thus simplifying the protein mixture. The eluted proteins were finally separated by SDS-PAGE analysis. The obtained Coomassie Blue-stained patterns displayed very few faint protein bands (data not shown). Considering the low efficiency of the in situ assay increasing the amount of tTG labeled proteins so that they could be successfully purified and visualized on a Coomassie Blue-stained gel, the substrate reactivity of CaCo-2 cell proteins was investigated by an in vitro assay.

In vitro tTG-mediated Labeling of Cellular Proteins—Homogenates from 3- and 12-day-post-confluence cultures were incubated separately with BPNH₂ and A25 peptide as described in “Experimental Procedures.” An aliquot (20 µg) of each reaction mixture was assayed by performing a Western...
CaCo-2 cells were incubated with both the acyl-acceptor homogenates (8 mg) obtained from 3-day-post-confluent label the Gln-donor and Lys-donor tTG substrates. Cellular served. Similar results were obtained when the A25 peptide distribution of the bands with few minor differences was observed. Hence, an in vitro assay was performed to labeling to in situ the correspondent patterns obtained in situ (Fig. 3A, lanes 3 and 4) and in the presence (+) of the Ca²⁺-ionophore A-23187 as described under “Experimental Procedures.” Moreover, MDCK-tTG cells were also treated with BPNH₂ (C, lanes 5 and 6). For Western blot analyses, 80 µg of proteins for each sample of CaCo-2 cells and 80 µg of proteins for each sample of MDCK-tTG cells were loaded and probed with HRP-conjugated streptavidin. Blots were then stripped and reprobed with the monoclonal anti-tTG CUB 7402 to visualize the tTG content in cells treated with BPNH₂ (B) or with A25 peptide (D). Positions at which molecule mass standards (kDa) migrated are indicated at the right with arrows.

Identification of tTG Protein Substrates in CaCo-2 Cells—Fig. 4 displays the electrophoretic pattern of the BPNH₂-labeled proteins, whereas the A25 peptide-labeled species are shown in Fig. 5. The better electrophoretic resolved bands were excised from the gel slabs. In the case of the Gln-donor proteins selected by BPNH₂ (Fig. 4), two protein bands, A_Q and B_Q, were removed in the mass range between 150 and 250 kDa. Three species, C_Q, D_Q, and E_Q, were selected from the 75–100-kDa mass range. The bands F_Q, G_Q, I_Q, and L_Q were cut out from the gel in the mass range between 50 and 75 kDa. Finally, the species M_Q was selected in the mass range below 37 kDa. In the case of the Lys-donor species specifically labeled by the A25 peptide (Fig. 5), one band, A_K, was excised in the mass range between 150 and 250 kDa; two species, B_K and C_K, were excised from the 75–100-kDa mass range; four protein bands, D_K, E_K, F_K, and G_K, were selected between 50 and 75 kDa; and finally, bands I_K and L_K were selected from the 37–50-kDa range. Each protein band was reduced, carboxymethylated, and digested with trypsin as described under “Experimental Procedures.” The peptide mixtures were then directly analyzed using MALDI/MS. Such treatments meant that diagnostic peptide mixtures could be produced for each unknown protein and translated in a list of peptide molecular masses. For each sample, MALDI/MS measurements were repeated at least three times, changing MALDI matrix and/or acquisition parameters. m/z signals registered in the different mass spectra obtained were then collected as a single set. The different sets of values were separately investigated by searching the non-redundant NCBI protein data base through several web-available programs to identify tTG protein substrates.

As an example, MALDI/MS analyses related to band A_Q (Fig. 4) at approximately 250 kDa gave rise to >100 m/z signals. These signals were analyzed by independent search programs exploring the non-redundant NCBI protein data base. This search ranked fatty-acid synthase (molecular mass of 275 kDa) in the first position of the output list of probable candidates with a sequence coverage of 30%. Some of the original m/z signals were not assigned to the fatty-acid synthase sequence, and the search performed on the unassigned signals led to identification of another two Gln-donor proteins, filamin 1 (molecular mass of 283 kDa) and spectrin α (molecular mass of 285 kDa), characterized by a sequence coverage of 21 and 19%, respectively.

In some cases it was not possible to identify protein species by their MALDI spectra. This inconvenience was because of several factors such as low protein amounts and complex co-migrating protein mixtures. These samples were then analyzed by μLCMS/MS to confirm previous identification and to extend the characterization to non-assigned protein gel bands. The eluate of the reverse-phase chromatography was directly analyzed using the Q-ToF mass spectrometer, which was able to fragment “on-line” up to 8 co-eluting peptides. The peptide mass data and their relative internal sequence information were then used to explore NCBI nr using the ProteinLynx Global Server software. This procedure led to the unambiguous identification of the proteins as the peptide mass along with internal sequence information represent a unique fingerprint for a protein. The accuracy and sensitivity of the μLCMS/MS analysis meant that multiple proteins could be identified within a single protein band.

Figs. 4 and 5 report the Gln-donor and Lys-donor protein substrates of tTG identified in this study both by MALDI/MS.
and by μLCMS/MS analysis. Identifications obtained by means of MALDI/MS are followed by the sequence coverage of the peptide mapping reported in brackets.

The whole strategy employed in this study led to the definition of 14 proteins in the Gln-donor group and 18 in the Lys-donor set. It is interesting to note that among the protein substrates, five of them acted as complete tTG substrates: tumor rejection antigen-1; heat shock (Hsp) 90-kDa protein; Hsp 70-kDa protein 8; Hsp 70/90-organizing protein; and T-complex protein-1α subunit.

**DISCUSSION**

The interaction of tTG with multiple substrates, both intracellular and extracellular, is essential for those proteins to carry out their biological functions. The tTG-catalyzed post-translational modification of the substrate proteins, either by polymerization or by covalent linkage to soluble low molecular weight molecules such as amines or peptides, can alter the physical-chemical characteristics of the substrates, thus playing a key role in modulating their biological activity. However, in some circumstances, tTG-catalyzed post-translational mod-
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The proteomic strategy meant that it was possible to identify acyl-acceptor transglutaminase protein substrates. SDS-PAGE analysis of A25 peptide-labeled proteins. Excised Lys-donor protein bands are labeled. A list of the identified proteins is reported. *, the percentage in brackets of the sequence coverage obtained by MALDI/MS analysis is shown.

In the absence of viable cell lines from human small intestine, we used the CaCo-2 cell line. Although this cell line is derived from a colonic neoplasm, it shows features of small intestine and therefore may offer an understanding of the role of the enzyme in CD.

The first group is represented by proteins involved in the organization of the cytoskeleton. The intestinal epithelium forms a barrier made of polarized cells joined by complex cell-cell adhesion systems that are connected to the cytoskeleton. Proteins of the cytoskeleton play an important role in both generation and maintenance of epithelial polarity. The basolateral membrane domain of the mature enterocytes is scaffolded by a spectrin-based membrane cytoskeleton consisting primarily of actin, myosin, spectrin, and ankyrin. In this case, it was shown that spectrin and myosin are able to act as acyl-donor and acyl-acceptor, respectively. Furthermore, we identified the protein filamin known to link actin filaments to membrane glycoproteins as the acyl-donor. Cytoskeletal proteins have been shown to be cross-linked by tTG, especially following the loss of cellular integrity, inducing apoptosis to prevent an inflammatory response. Moreover, it has been reported that tTG is associated with elements of the cytoskeleton, thus indicating its involvement in the organization of the cytoskeleton.

To note is that some proteins were shown to be complete substrates of tTG, such as tumor rejection antigen-1, Hsp 90-kDa protein, Hsp 70-kDa protein 8, Hsp 70/90-organizing protein, and T-complex protein-1 subunit.

In relation to their biological significance, the identified proteins fall into four groups (Table I). These targets include proteins involved in cytoskeletal network organization, folding of proteins, and transport processes. A fourth group finally consists of proteins involved in miscellaneous metabolic functions.

The first group is represented by proteins involved in the organization of the cytoskeleton. The intestinal epithelium forms a barrier made of polarized cells joined by complex cell-cell adhesion systems that are connected to the cytoskeleton. Proteins of the cytoskeleton play an important role in both generation and maintenance of epithelial polarity. The basolateral membrane domain of the mature enterocytes is scaffolded by a spectrin-based membrane cytoskeleton consisting primarily of actin, myosin, spectrin, and ankyrin. In this case, it was shown that spectrin and myosin are able to act as acyl-donor and acyl-acceptor, respectively. Furthermore, we identified the protein filamin known to link actin filaments to membrane glycoproteins as the acyl-donor. Cytoskeletal proteins have been shown to be cross-linked by tTG, especially following the loss of cellular integrity, inducing apoptosis to prevent an inflammatory response. Moreover, it has been reported that tTG is associated with elements of the cytoskeleton, thus indicating its involvement in the organization of the cytoskeleton.

tTG was in fact shown to be co-localized with the stress fibers via cross-linking to myosin by virtue of autocatalytic activity of the enzyme. In this study, we report that ezrin/radixin/moesin-binding phosphoprotein-50, a member of ezrin/radixin/moesin family of membrane-cytoskeletal adapters involved in the formation of microvilli, cell-cell adhesion, maintenance of cell shape, cell motility, and membrane trafficking, is able to act as a tTG Lys-donor substrate. Two elongation factors, types α and γ, were shown to be able to act as tTG Lys-donor substrates. Elongation factor 1α is known as an actin-binding protein besides a cofactor of polypeptide elongation. Finally, Rho-associated coiled-coil-containing protein kinase 2 (ROCK-2) was shown to be a tTG acyl-donor substrate in CaCo-2 cells. ROCK-2 is critical for the assembly of proteins may generate auto-antibodies as it happens in autoimmune disorders such as CD. Such a phenomenon was explained by a mechanism where tTG, acting as a hapten in the generation of antibodies against gliadin and itself, catalyzes the synthesis of heteromeric gliadin-tTG complexes. Such complexes may provoke an immune response to tTG by stimulating normally silent tTG-specific B cells. Biagi et al. (23) have suggested that enterocytes might have a role in the induction of the immune response in CD, acting as antigen-presenting cells. Recently, an accumulation of active tTG as well as the presence of Glu-donor and Lys-donor tTG substrates have been demonstrated in the enterocytes of celiac patients compared with controls (24, 34). The identification of the potentially dangerous substrates of tTG in intestinal mucosa may therefore offer an understanding of the role of the enzyme in CD.
of the apical junctional proteins and actin-cytoskeleton organization during junctional formation in intestinal epithelia (44). Recent evidence suggests that ezrin/radixin/moesin proteins, ROCK-2 and elongation factor 1, participate in signaling pathways involving the activation of members of the Rho-GTPase, a member of the Rho family (45). In summary, the present findings support the hypothesis that tTG-catalyzed post-translational modifications of proteins involved in cytoskeletal rearrangement are required to modulate their biological properties and influence their interactions with other proteins important for enterocyte survival.

The second group of proteins represents proteins with chaperone activities such as the Gln-donors Bip and T-complex protein-1 subunit and the acyl-acceptor chaperonin 3 (that belongs to the chaperonin family Hsp60), Hsp70/90 organizing protein, nuclease-sensitive element-binding protein 1, and T-complex protein 1 subunit. The T-complex protein-1 subunit, which is able to act as a complete TGG-substrate, belongs to the T-complex protein chaperonin family, and it assists in vitro in the folding of actin and tubulin (46). In cellular model produced by us, several members of high molecular weight Hsp family and Hsp-like were found that are able to act as complete substrates for tTG. tTG is very specific, and the number of proteins able to act as glutaminyl and lysyl substrates is highly restricted. On this basis, it is interesting to underline that, in CaCo-2 cells, the number of proteins acting as complete substrates is exceptionally high. The expression of constitutively Hsps such as Hsp60, Hsp70, Hsp72, and Hsp90 and Bip (47) by enterocytes may be part of a protective mechanism developed by the intestinal epithelium to deal with noxious components in the intestinal lumen.

A third group represents proteins involved in transport processes. It was found that importin is able to act as a Lys-donor, whereas valosin and clathrin act as Gln-donors. It has been reported that tTG is involved in the receptor-mediated endocytosis process in different cellular systems (48). Monodansylcadaverine, a synthetic amine substrate of tTG widely used to inhibit tTG activity in vivo, was shown to inhibit the internalization of ligands via the clathrin-mediated endocytotic pathway (49). More recently, it has been reported that there is a functional and/or physical interaction between the cytoskeleton and the clathrin-mediated endocytotic machinery (50). The present results suggest that the interaction between the Gln-donor clathrin and a Lys-donor cytoskeletal protein could be mediated by tTG activity. Based on this finding, it is interesting to note that gliadin peptides are transported inside enterocytes and processed by the intestinal mucosa (23). However, the mechanism of internalization of gliadin peptides is unknown. Therefore, it might be suggested that the transport process of gliadin is probably mediated by the clathrin-mediated endocytotic pathway and that an alteration of this process may be involved in the pathogenesis of CD enterocytes.

A fourth group is represented by miscellaneous proteins that are involved in different metabolic processes such as phosphoglycerate dehydrogenase that catalyzes the initiating step in the phosphorylated pathway of serine biosynthesis or fatty-acid synthase that is a key enzyme in lipid metabolism.

The data reported here demonstrate that in CaCo-2 cells there are tTG substrates. Obviously, substrate identification alone cannot explain CD specificity, but these results provide a platform that could support a broader understanding of how tTG participates in endocytotic metabolic processes. It is intriguing to suggest that tTG could come in contact with gliadin inside enterocytes and modify gliadin peptides by cross-linking to itself or to other acyl-acceptor substrates, thus originating neo-antigens recognized by the immune system. This mechanism could explain the existence of auto-antibodies in CD with several distinct specificities (51). An immune reaction was observed against the cytoskeleton in both children and adults with CD. In particular, anti-actin antibodies are shown to be more strongly associated with more severe degrees of villous atrophy (52). Furthermore, it is possible to hypothesize that gliadin peptides can trigger a cascade of events leading to the inappropriate presentation of tTG and cross-linked substrates to the immune system contributing to the immune aspect of CD. This mechanism could in part explain the increased prevalence of concomitant auto-immune diseases, such as collagen diseases, type 1 diabetes, auto-immune alopecia, hypophysitis, and others in CD patients with prolonged gluten exposure (53).

Studies are in progress to identify tTG substrates in biotopic specimens from CD patients and from controls. An alteration of the substrate profile might mean a different distribution of gliadin in the enterocytes of healthy controls and CD patients that is responsible for the deleterious effects evoked by gliadin in the celiac intestine.

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Proteomics Identification of Acyl-acceptor and Acyl-donor Substrates for Transglutaminase in a Human Intestinal Epithelial Cell Line: IMPLICATIONS FOR CELIAC DISEASE

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