Secretory IgA mediates retrotranscytosis of intact gliadin peptides via the transferrin receptor in celiac disease

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Celiac disease (CD) is an enteropathy resulting from an abnormal immune response to gluten-derived peptides in genetically susceptible individuals. This immune response is initiated by intestinal transport of intact peptide 31–49 (p31-49) and 33-mer gliadin peptides through an unknown mechanism. We show that the transferrin receptor CD71 is responsible for apical to basal retrotranscytosis of gliadin peptides, a process during which p31-49 and 33-mer peptides are protected from degradation. In patients with active CD, CD71 is overexpressed in the intestinal epithelium and colocalizes with immunoglobulin (Ig) A. Intestinal transport of intact p31–49 and 33-mer peptides was blocked by polymeric and secretory IgA (SIgA) and by soluble CD71 receptors, pointing to a role of SIgA–gliadin complexes in this abnormal intestinal transport. This retrotranscytosis of SIgA–gliadin complexes may promote the entry of harmful gliadin peptides into the intestinal mucosa, thereby triggering an immune response and perpetuating intestinal inflammation. Our findings strongly implicate CD71 in the pathogenesis of CD.
CD4+ adaptive immune response (11) and activate cytotoxic activity and IFN-γ production in intraepithelial lymphocytes (12–14).

This activation of the local immune system implies that undigested gliadin fragments present in the intestinal lumen somehow cross the intestinal epithelium. Indeed, apical to basal transport and processing of gliadin peptides, particularly of p31-49 and 33-mer, are severely altered in active CD, leading to the release of intact peptides on the basolateral side, whereas the same peptides are almost entirely degraded during their intestinal transport in control individuals and treated CD patients (15, 16). Several lines of evidence argue against simple nonspecific paracellular leakage of gliadin peptides across the celiac mucosa. In particular, a 12-mer gliadin peptide, p57-68, is completely degraded during intestinal transport in patients with active CD, suggesting no paracellular leakage of molecules of this size or larger. In addition, only 0.3% of the apical peptide crosses the epithelium during a 3-h incubation period, arguing against free diffusion across a damaged mucosa. The “protected” transport of p31-49 thus involves a transcellular pathway (15, 16), which enables the peptide to escape lysosomal degradation. As active CD is associated with high concentrations of antigliadin IgA antibodies in the intestinal lumen (17, 18), we postulated that the transport of intact gliadin peptides might result from abnormal retrotranscytosis of IgA–gliadin complexes. Indeed, although antigliadin IgA antibodies are a hallmark of CD, they have no known pathogenic role. We obtained evidence that polymeric/secretory IgA (pIgA/SIgA) can mediate protected transport of p31-49 and 33-mer gliadin peptides through their binding to CD71, the transferrin (Tf) receptor. Importantly, we found that this receptor was abnormally expressed at the apical pole of enterocytes in patients with active CD. Although initially implicated in endocytosis of iron-loaded Tf, CD71 was recently recognized as an IgA receptor mediating mesangial deposition of IgA1 complexes in IgA nephropathy (19).

RESULTS
High molecular weight immune complexes containing gliadin-specific IgA and transglutaminase (Tgase) are present in patients with active CD

Active CD is associated with enhanced intestinal secretory immunity (17, 20) and with higher antigliadin antibody titers in serum and jejunal secretions than in controls and treated CD patients (17, 20, 21). In addition, gliadin colocalizes with Tgase (the autoantigen in CD) in the epithelium and subepithelium of patients with active CD (22). We postulated that, as in IgA nephropathy, high molecular weight immune complexes (23) might be present in CD patients. As shown in Fig. 1A, antigliadin IgA antibodies and Tgase were detected within high molecular weight immune complexes, which were found in larger amounts in the serum and duodenal secretions of patients with active CD than in those of treated CD patients and controls. The presence of Tgase in these immune complexes may be related to the cross-linking capacity of this enzyme, contributing to the formation of IgA–gliadin–Tgase complexes.

Consistent with a possible role of gliadin-specific IgA in the luminal uptake of gliadin peptides in active CD, immunofluorescence studies of duodenal biopsies revealed large amounts of IgA at the apical pole of the surface epithelium in patients with active CD, whereas in control subjects and treated CD patients, IgA was restricted to the crypts and was not observed in villous epithelium (Fig. 1B and Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20071204/DC1). In patients with active CD, colocalization of IgA with cytokeratin or alkaline phosphatase (a brush-border enzyme) confirmed the presence of IgA at the apex of surface epithelial cells (Fig. 1C).

CD71 is overexpressed in the apical pole of enterocytes in active CD

IgA involvement in the intestinal transport of gliadin peptides would imply the presence of IgA receptors on the apical pole of enterocytes. In addition to the plg receptor (plgR; also called membrane secretory component [SC]), which permits transcytosis of dimeric IgA (dIgA) from the lamina propria and its delivery into the intestinal lumen, complexed to the cleaved extracellular part of plgR (bound SC) in the form of SlgA, enterocytes express a second receptor able to bind IgA, namely CD71 (19, 24). In normal intestinal epithelium, CD71 mediates the rapid endocytosis/recycling of Tf necessary to deliver iron to rapidly proliferating epithelial cells (25). The level of CD71 expression is also linked to body iron stores and correlates negatively with the serum iron concentration. CD71 can be up-regulated in CD patients with iron deficiency anemia (26). The recent demonstration that CD71 can bind IgA1 at the surface of mesangial cells in IgA nephropathy (19), together with a previous report indicating up-regulation of intestinal CD71 by IL–15 in CD (27), led us to investigate whether CD71 mediates abnormal IgA retrotransport in CD.

The distribution of CD71 in active CD was assessed in duodenal biopsies by both immunoperoxidase and immunofluorescence detection (Fig. 2, A and B). In control biopsies, CD71 expression was confined to the basolateral pole of villous epithelial cells and was observed at both the basal and apical poles of crypt epithelial cells. In contrast, in active CD, CD71 was strongly expressed all over the epithelial layer, including the flat surface epithelium. Staining was not restricted to the basolateral compartment but was also detected at the apical pole of epithelial cells in a subapical rather than strictly apical location, possibly corresponding to the apical recycling compartment from which this receptor is rapidly recycled to the cell surface (28). A similar abnormal distribution of CD71 expression was observed in patients with refractory celiac sprue who have a flattened overproliferating mucosa. In contrast, CD71 expression was normal in treated CD patients who had recovered a normal or subnormal epithelial architecture. The strong expression of CD71 contrasted with the lack of significant expression of CD89, the myeloid IgA Fc receptor (29), on either enterocytes or immune cells in the lamina propria of controls and CD patients (unpublished data) (30).
Collectively, these results were compatible with a role of CD71 in IgA binding at the apical surface of enterocytes in active CD.

IgA colocalizes with CD71 and p31–49 at the apical pole of enterocytes in active CD

The possible role of apically expressed CD71 in the binding of IgA–gliadin peptide complexes in patients with active CD was first addressed by performing colocalization immunofluorescence studies on frozen sections. Colocalization (white) of IgA (green) and CD71 (blue) was observed at the apical pole (and the basal pole) of epithelial cells (Fig. 2 C) in fresh biopsies from patients with active CD but not from treated CD patients or controls. The presence of CD71 at the brush-border membrane of enterocytes in patients with active CD and its colocalization with IgA was confirmed by immunogold electron microscopy (Fig. 2 D and Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20071204/DC1).

As SIgA is the main IgA subtype present in the intestinal lumen, we examined whether it can bind CD71. The capacity of pIgA and SIgA to bind to Daudi cells, which express CD71 as the only IgA receptor (19), was studied by flow cytometry. Both pIgA and SIgA were able to bind Daudi cells, and this

Figure 1. IgA and IgA–gliadin complexes are overexpressed in CD. (A) Analysis of IgA immune complexes (IC) isolated from the serum and duodenal secretions of patients with active or treated CD and controls. PEG precipitates containing high molecular weight IC were analyzed by ELISA. Plates were coated with Frazer’s fraction (pepsin/trypsin gliadin hydrolysate) or anti-Tgase antibody, followed by anti-human IgA–horseradish peroxidase. Results are presented as OD values obtained with all sera or duodenal secretions tested. A significant increase in IC recognizing gliadin peptides and containing Tgase was observed in the active CD group compared with the treated CD and control groups in both serum and duodenal secretion. Horizontal lines represent medians. *, P < 0.0005 and 0.01 compared with the control and treated CD groups, respectively; #, P < 0.01 and 0.02 compared with the control and treated CD groups, respectively. (B) IgA is concentrated at the apical pole of surface enterocytes in active CD. Cryosections of duodenal mucosa from control subjects, patients with active or treated CD, and patients with refractory celiac sprue were labeled with anti-IgA–FITC antibody and TOPRO-3 (blue nuclei). In controls, treated CD patients, and patients with refractory celiac sprue, IgA staining of epithelial cells was located at the basal pole of villous cells and at the apical and basal poles of crypt cells. In contrast, in patients with active CD, IgA staining was concentrated at the apical pole of the surface epithelium (and of crypt cells; Fig. S1). Results are representative of three controls, seven patients with treated CD, and six patients with active CD. (C) IgA overexpression observed at the apical pole of epithelial cells of patients with active CD was located inside the cell, including the brush border membrane, as shown by its colocalization with cytokeratin and alkaline phosphatase, a marker of apical membranes. No such colocalization was seen in controls. Bars, 50 μm.
CD71 expression and colocalization with IgA and p31-49 in duodenal biopsies. (A) Expression of CD71 (immunoperoxidase labeling) on duodenal biopsies from controls, patients with active or treated CD, and patients with refractory celiac sprue. Compared with controls, CD71 was overexpressed in patients with active CD and in patients with refractory celiac sprue. At higher magnification (bottom), strong CD71 expression was observed all over the surface epithelium in patients with active CD, whereas in controls and treated CD patients CD71 expression was only observed at the basal pole of villous epithelial cells and in crypt cells. (B) CD71 overexpression by surface epithelium of patients with active CD was confirmed by immunofluorescent labeling. The fluorophore was a cy5-conjugated secondary antibody (blue staining). Results in A and B are representative of three control subjects, four treated CD patients, eight patients with active CD, and two patients with refractory celiac sprue. (C) Double immunofluorescence labeling of IgA–CD71 in duodenal biopsies. Colocalization (white) of IgA (green) and CD71 (blue) was observed at the apical surface of the epithelium in active CD (n = 5), but not in controls or in treated CD patients (n = 3). (D) Immunogold electron microscopy with double labeling of IgA (10-nm particles; arrowhead) and CD71 (15-nm particles; arrow). In active CD, IgA and CD71 were expressed in the brush border membrane and subepithelial compartments, and IgA–CD71 colocalization was frequent (boxes). No such colocalization was observed in controls (see additional images of one control subject and two patients with active CD in Fig. S2). (E) IgA can bind CD71 at the cell surface of a B cell line (Daudi cells) known to express CD71 as the only IgA receptor. Cells were incubated for 30 min at 4 °C with 500 μg/ml SlgA or pigpA1 in the presence or absence of 500 μg/ml of soluble CD71. IgA was revealed with biotinylated anti-IgA and allophycocyanin-labeled streptavidin (green line). Both pigpA1 and SlgA specifically bound CD71, as the binding was inhibited by soluble CD71 receptors (pink line). The black line indicates the isotype control. (F) Colocalization (yellow) of IgA (green) and p31-49 (red) in duodenal biopsies from two patients with active CD, mounted in Ussing chambers and exposed to p31-49–TAMRA on the apical side for 15 min at 37°C before being fixed, cryosectioned, and stained with anti-IgA–FITC antibodies. No colocalization was found in two treated CD patients or in a control (not depicted). Bars, 50 μm.
IgA mediates intestinal transport of intact p31-49 through CD71

To directly address the role of IgA in the protected apical to basal transport of gliadin peptides, duodenal biopsies were mounted in Ussing chambers and ^3^H-labeled p31-49 was applied to the apical side. Intestinal transport was evaluated by analyzing ^3^H-labeled fragments in the basal compartment 3 h later. Confirming our published results (15), a large fraction of ^3^H-labeled p31-49 was transported intact across the duodenal mucosa of patients with active CD who have both a flat mucosa and IgA lining the surface epithelium (Fig. 3 A). In contrast, this peptide was almost totally degraded during transport through samples from control subjects and treated CD patients who had a normal intestinal epithelium with much less CD71 and IgA than patients with active CD. Furthermore, the peptide was similarly degraded during transport across duodenal biopsies.
IgG and human Tf were unable to inhibit the protected transport pathway (Fig. 3 E), indicating that gliadin-specific IgG does not play a major role in the intestinal absorption of gliadin peptides in patients with active CD. The failure of Tf to inhibit p31–49 transport is in line with its inability to inhibit IgA binding to CD71 on Daudi cells (31) and suggests that IgA and Tf do not share the same binding site on CD71. In addition, the anti-CD71 mAb A24 did not inhibit the transport of intact p31–49 in active CD, suggesting that A24, like Tf, does not bind CD71 at the same site as IgA (Fig. S3 A, available at http://www.jem.org/cgi/content/full/jem.20071204/DC1) (32). Also, an mAb directed to SC was not able to block the transport of IgA–p31–49 complexes (Fig. S3 B). Finally, we used a mAb to Tgase II (anti–Tgase II, 6B9) or dansyl cadaverin, an inhibitor of Tgase activity, to test the involvement of Tgase II, and no inhibitory effect was observed.

To confirm the role of CD71 in the transport of intact p31–49, we tested the capacity of a soluble CD71 receptor to competitively inhibit this transport in duodenal biopsies from six patients with refractory celiac sprue, a complicated form of CD associated with severe villous atrophy, CD71 apical overexpression but no detectable IgA on the epithelial cell surface (Fig. 1 B and Fig. 3 A). The latter result indicates that nonspecific leakage caused by epithelial flattening is not sufficient to explain the increased transport of the intact peptide observed in active CD. The percentage of intact p31–49 and active fragments found in the basal compartment in active CD is significantly higher than that observed in controls and treated CD patients (Fig. 3 B). Adding an excess of dIgA, pIgA, or SIgA to the apical compartment of duodenal biopsies from patients with active CD, as competitive inhibitors of endogenous gliadin-specific IgA, strongly inhibited the transport of intact p31–49 (Fig. 3, C and D), indicating that large IgA molecules are involved in this transport. In contrast, monomeric IgA (mIgA) had no inhibitory effect, as expected in view of its low affinity for CD71 (31).

To better understand the mechanism underlying intestinal transport of p31–49, we tested potentially inhibitory molecules.
patients with active CD using Ussing chambers. When soluble CD71 receptor was added to the apical compartment, reversed-phase HPLC (RP-HPLC) analysis of the radioactive material recovered in the basal compartment after intestinal transport of \(^3\)H-labeled p31-49 showed that transport of the intact peptide was significantly inhibited (Fig. 4, A and B). In contrast, soluble CD89 receptor had no inhibitory effect, in accordance with the absence of CD89 expression in epithelial cells.

**Discussion**

This study reveals that protected transport of gliadin peptides in CD is driven by retrotranscytosis of SIgA through the Tf receptor (CD71), which is abnormally expressed on the apical side of the intestinal epithelium. This process may sustain anti-gliadin immune responses and aggravate intestinal inflammation in CD patients.

IgA mediates the intestinal transport of intact 33-mer via CD71

Intestinal transport of 33-mer in the form of the intact peptide and large immunogenic 12-mer (16) fragments (35 and 28%, respectively) was significantly higher in active CD than in controls (4 and 9%) and in treated CD (12 and 8%) (Fig. 5, A and B, left), confirming our previous results (15, 16). In active CD, competitive inhibition of 33-mer transport was attempted with dIgA, pIgA, and soluble CD71 (Fig. 5 B, right). The percentage of 33-mer crossing the intestinal mucosa in intact form was significantly reduced by dIgA and pIgA (7 and 14%, respectively) and by soluble CD71 (19%) compared with the peptide alone (46%; P < 0.01), further supporting CD71 mediation of protected IgA–33-mer complex retrotransport.

![Figure 5. Duodenal transport of 33-mer in patients with active CD, and competitive inhibition by IgA and soluble CD71.](image)

(A) Typical RP-HPLC elution profile of \(^3\)H-labeled 33-mer after intestinal transport across duodenal biopsies from a control individual and a patient with active CD, mounted in Ussing chambers. \(^3\)H-labeled radioactive material present in the basal compartment 3 h after adding \(^3\)H-labeled 33-mer to the apical compartment is shown. The percentages of the different eluted fractions (proline, small and large fragments, and intact 33-mer) were quantified with Radiostar software. The control tissue almost totally degraded the 33-mer peptide, whereas digestion was incomplete in the sample from the patient with active CD. (B, left) Mean percentages of 33-mer and its fragments after intestinal transport. The duodenal mucosa of patients with active CD does not completely degrade 33-mer, as 38 and 22% of intact peptide and large fragments, respectively, were recovered in the basal compartment, compared with 4 and 9% in control subjects. Treated CD patients had an intermediate profile (12 and 8%). (right) dIgA, pIgA, and soluble CD71 (sCD71) significantly inhibited the transport of intact 33-mer but not of large fragments. *, P < 0.04 compared with control; #, P < 0.04 compared with peptide alone.
through CD23, the low-affinity IgE receptor, has been shown in allergic patients and might elicit rapid activation of intestinal mast cells (33–35). Comparable protected transport of IgG–antigen complexes via the neonatal Fc receptor has also been demonstrated. This receptor, initially described at the apical surface of enterocytes in newborn rodents (36) and human fetal intestine (37, 38), could transport IgG into the intestinal lumen and recycle IgG–antigen complexes back into the lamina propria, thereby promoting a specific immune response (38).

In this study, we tested the hypothesis that, in active CD, SlgA–gliadin immune complex retrotransport could be mediated by CD71 and, thus, explain the protected transport of gliadin peptides in celiac patients. In healthy individuals, vectorial intestinal transport of IgA consists mainly of basal to apical transcytosis of dIgA via the plgR, leading to the release of SlgA in the intestinal lumen (39), where it retains microbial and food antigens and confers protective mucosal immunity. Although basal to apical transfer of IgA is the norm in epithelia, apical to basal retrotransport of SlgA through M cells overlying Peyer’s patches has been documented in mice (40, 41) even though specific IgA receptors are not identified. Several lines of evidence support retrotransport of SlgA–gliadin complexes in active CD. First, the intestinal lumen of patients with active CD contains elevated levels of antigliadin IgA antibodies (17), which can bind a set of gliadin peptides (42, 43) including the 33-mer (44) and p31–55 (45). We show the presence of high molecular weight gliadin–specific IgA immune complexes containing Tgase. This is an important finding, as IgA in form of high molecular weight complexes binds CD71 with high affinity (31). Second, we show that IgA is more strongly concentrated at the apical pole of surface epithelial cells in active CD than in treated CD patients, refractory celiac patients, and control subjects, and that this pattern correlates with substantial transport of intact p31–49 and 33-mer in active CD, whereas this transport is negligible in controls, treated CD patients, and patients with refractory celiac sprue. Finally, IgA involvement in the protected transport of these gliadin peptides is indicated by the competitive inhibition of their transport by plgA observed in this study. Although inhibition of 33-mer transport by IgA concerns the intact peptide only and not its large 12-mer fragments, these latter fragments have been shown to stimulate T cells much less potently than the intact peptide (6). One possible explanation is partial resistance of 33-mer to lysosomal degradation during fluid-phase transcytosis occurring in parallel with receptor-mediated transcytosis.

A role of IgA in protected transcytosis of gliadin peptides would imply the presence of a receptor able to bind plgA/SlgA at the apical surface of enterocytes. Our findings highlight the role of the Tf receptor CD71, a newly identified IgA receptor. Previous studies have shown that CD71 binds plgA but not mlgA (31). In this study, we show that CD71 can also bind SlgA, the main form of IgA present in the intestinal lumen. In the normal intestine, CD71 is mainly expressed in crypts (restricted to the basolateral membrane of epithelial cells) (46) with little expression in the villous epithelium. In active CD, villous flattening, increased epithelial renewal, and iron-deficiency anemia are all associated with substantial CD71 up-regulation all over the surface epithelium, and this may be responsible for its missorting toward the apical pole of enterocytes. This is compatible with a role of CD71 in the endocytosis of IgA complexes from the apical cell surface. Previous studies have shown in nonpolarized cells that plgA binding to CD71 induces the internalization and rapid addressing of IgA-loaded CD71 into recycling vesicles (31). The colocalization that we observed between IgA and CD71 and between IgA and p31–49 in active CD but not in controls suggests that IgA–gliadin complexes might bind CD71. These findings, combined with the demonstration that soluble CD71 receptors (as well as dIgA, plgA, and SlgA) can block the protected transcellular transport of intact p31–49 and 33-mer, provide strong evidence that CD71 is the receptor that allows gliadin peptides bound to SlgA to translocate from the intestinal lumen into the lamina propria in active CD. Interestingly, the capacity of CD71 to mediate plgA binding to mesangial cells of patients with IgA nephropathy also underlines the pathogenic role of CD71–IgA interactions in IgA nephropathy (19, 31). The presence of glomerular IgA deposits in a significant proportion of newly diagnosed CD patients (47) and the abnormally elevated incidence of CD in patients with IgA nephropathy (48) provides a link between these diseases.

The specific retrotranscytosis of SlgA–gliadin complexes in CD may appear puzzling given the presence of SlgA with diverse specificities in the intestinal lumen. First, large IgA complexes bind CD71 with higher affinity than smaller IgA species such as SlgA (31). Second, SlgA–gliadin complexes could be selectively retrotranscytosed with the help of tissue Tgase, which we detected in the high molecular weight IgA immune complexes found in duodenal secretions. Indeed, Tgase can cross-link gliadin peptides and promote receptor–mediated endocytosis (49), particularly the internalization step of CD71 (50), and was recently detected at the surface of enterocytes in active CD (51). However, neither Tgase antibodies nor an inhibitor of Tgase activity could inhibit the protected transport pathway in biopsy specimens from patients with active CD, suggesting that Tgase is not directly involved in the transport process or that Tgase effect is irreversible in biopsies from patients with active CD mounted in Ussing chambers. Finally, the plgA and SlgA concentrations used in our Ussing chamber experiments were high enough, in view of the small exposed surface area (0.025 cm²), for competitive inhibition of IgA–gliadin transport to occur.

Gliadin-specific SlgA is present at high titers in the intestinal lumen of patients with active CD but is also found in healthy individuals (52, 53). In contrast, apical to basal delivery of intact gliadin peptides is only observed in active CD. Our data indicate that CD71 overexpression and misaddressing to the apical pole of enterocytes is a key event in the intestinal retrotransport of SlgA–gliadin complexes (Fig. 6). They also indicate that the normal function of SlgA, namely the containment of harmful antigens in the intestinal lumen, is deficient in CD. This could account for the abnormal immune response to gluten in genetically susceptible (HLA–DQ2/8) individuals.

It is unclear whether this abnormal transport is the triggering event in CD or whether it becomes operational secondarily,
perpetuating inflammation once the mucosa has flattened. Several environmental factors may serve as initial triggers for SLgA–gliadin complex entry into intestinal tissue. Among these factors, iron deficiency anemia, by inducing CD71 up-regulation, could promote the delivery of the SLgA–gliadin complex, triggering abnormal intestinal responses in susceptible individuals. In addition, strong intestinal epithelial cell proliferation secondary to epithelial damage by intestinal infection might also stimulate CD71 overexpression and thereby favor the onset of CD in susceptible individuals. It is noteworthy that frequent rotavirus infection is associated with a higher risk of autoimmunity, defined as positivity for tissue Tgase, in early childhood (54).

Finally, if CD71 is necessary for CD to develop, why do patients with treated CD react so rapidly to the ingestion of gluten in the absence of CD71 expression? Some CD patients on a gluten-free diet still have minor small bowel abnormalities, and there is evidence that the celiac epithelium may be persistently activated even after successful treatment and mucosal healing (55). It is possible that residual expression of CD71 at the apical membrane of enterocytes may drive the entry of small amounts of gliadin peptides that are nonetheless sufficient to reactivate gluten-sensitive memory T cells and participate in rapid relapse.

In conclusion, we describe a novel mechanism of CD71-mediated IgA transcytosis that enables gliadin peptides to enter the lamina propria of patients with active CD. Inhibition of this protected transport pathway might provide a new therapeutic option, blocking the cascade that perpetuates innate and adaptive immune responses to gluten in patients with active CD.

**MATERIALS AND METHODS**

**Patients**

We studied 26 patients with active CD, 13 treated CD patients who had been on a gluten-free diet for at least 1 yr, 4 patients with refractory celiac sprue, and 10 nonceliac control subjects. Diagnosis of active CD was based on subtotal or total villous atrophy and positivity for antigliadin, anti-Tgase, and antidentomysium IgA antibodies. Refractory celiac sprue was defined as CD resistant to a gluten-free diet, characterized by the persistence of villous atrophy in the absence of antigliadin IgA antibodies. The control subjects underwent upper endoscopy for routine diagnostic purposes (e.g., dyspepsia and chronic diarrhea) and had a normal intestinal mucosa. All the patients underwent duodenal endoscopy, during which four to six additional biopsy samples were taken from the distal duodenum for research purposes. In some cases, duodenal secretions were obtained by infusion–aspiration of 50 ml of phosphate-buffered saline. Local ethics committee (Comité de Protection des Personnes Ile de France II) approval was obtained, and all of the patients signed an informed consent to participate in the study.

**Purification of IgA and synthesis of soluble IgA receptors**

Competing IgA used in the Ussing chamber studies was human myeloma IgA1 protein prepared according to Chevailler et al. (56). mlgA, dlgA, and plgA were separated by gel filtration on columns (>98% pure; Sephacryl S300 [GE Healthcare]) (57). SlgA consisting of pooled human colostrum was obtained from Fitzgerald or Biotrend. The soluble IgA receptors sCD71 (TRc) and sCD89 were used. Both soluble receptors were expressed in a lytic baculovirus/insect cell expression system, as described previously (19).
Measurement of IgA and IgA-containing complexes in serum and duodenal secretions

IgA immune complexes in serum samples were analyzed by precipitation with polyethylene glycol 6000 (PEG 6000), as previously described (58). PEG precipitates were dissolved in 500 μl of 0.1 M phosphate buffer, pH 7.4, containing 0.5 M NaCl and 0.05% Tween 20. IgA immune complex in duodenal secretions was measured after precipitation with saturated ammonium sulfate. Total immune complex content was estimated by measuring OD at 280 nm. The specificity of the immune complex present in serum and duodenal secretions and the presence of Tgase in these complexes was checked by ELISA. 96-well plates were coated with either 5 μg/ml of pepsin/trypsin gliadin hydrolysate or 5 μg/ml of rabbit polyclonal anti-Tgase antibody (US-Biological), respectively, blocked with 5% gelatin in borate buffer, and incubated overnight at 4°C with serum or PEG immune complex diluted 1:10 and 1:100 in PBS containing 0.05% Tween 20 or with undiluted immune complex from duodenal secretions. After washing, 1,200 alkaline phosphatase (AP)–anti-IgA (SouthernBiotech) was added for 2 h at 37°C. The reaction was developed by adding AP substrate, and absorbance was read at 405 nm. Results are expressed as OD.

Flow cytometry analysis of SIgA binding to CD71

SIgA binding was examined with an indirect immunofluorescence assay in which 0.5 × 10^6 Daudi cells, which express CD71 as the only IgA receptor (19), were incubated with 10 μl SIgA (0.5 mg/ml; Fitzgerald) for 1 h on ice before washing and incubation for 20 min at 4°C with a biotinylated anti-IgA mAb clone (CH-EB6-8) that recognizes both IgA1 and IgA2 (59). After washes, allophycocyanin-streptavidin (SouthernBiotech) was used as a developing reagent. For inhibition studies, SIgA was preincubated with soluble CD71 receptor at 0.5 mg/ml for 1 h before adding the cells. Immunofluorescence was analyzed by flow cytometry (FACScalibur; Becton Dickinson).

Immunohistochemical analysis of duodenal biopsies

Immunoperoxidase labeling, CD71, CD89, and SC were detected on 6-μm cryosections of duodenal biopsies fixed in cold acetone for 15 min at −20°C and rehydrated with 0.1% PBS/BSA. Endogenous peroxidase and biotin were blocked with 3% H2O2 and the Blocking Biotin system (Dako), respectively, and other nonspecific binding sites were blocked with horse serum. Sections were incubated for 60 min with 22 μg/ml of anti-CD71 mAb A24 (57), 10 μg/ml of biotinylated anti-CD89 mAb A77 (57), or 10 μg/ml of anti-hSC mAb (Monosan), respectively. After rinsing in 0.1% PBS/BSA, primary antibodies were detected with an indirect biotin/streptavidin-peroxidase labeling kit (ChemMate; Dako) with DAB as substrate. Sections were counterstained with Mayer’s hematoxylin.

Immunofluorescence labeling. Immunofluorescence studies comprised two parts: (a) a study of fresh-frozen duodenal biopsy sections and (b) a study of duodenal biopsy specimens incubated for 15 min at 37°C in Using chambers in the presence of fluorescent TAMRA-p31-49 in the apical compartment before freezing and cryosectioning.

In the first part, fresh duodenal biopsies were frozen in Tissue-Tek (−80°C; Euromedex), cut into 6-μm-thick sections, and kept at −20°C until staining. Biopsy sections were thawed, fixed in cold acetone, and rehydrated in 0.1% PBS/BSA, and nonspecific sites were blocked for 30 min with antibody diluent (ChemMate; Dako). Primary antibodies—24 μg/ml of polyclonal goat anti-IgA–FITC (Abcam), 22 μg/ml of anti-CD71 (A24) mAb (57), 50 μg/ml of anti-human cytokeratin (epithelial) mAb (US-Biological), or 25 μg/ml of rabbit polyclonal antialkaline phosphatase (Abcam)—were incubated for 60–90 min. After rinsing, secondary antibodies to A24 (15 μg/ml of Cy5-conjugated goat anti-mouse IgG [H+L] antibody), antityrosoketan mAb (30 μg/ml of Texas red–conjugated goat anti–mouse IgG [H+L]), or antialkaline phosphatase antibody (20 μg/ml of Texas red–conjugated sheep polyclonal anti–rabbit IgG [H+L]; Abcam) were added for 30 min. Tissue sections were mounted with antifade mounting medium (Vectashield) and stored at 4°C until analysis.

All immunolabeling experiments included negative controls in which the primary antibody was replaced by concentration-matched isotype controls (Becton Dickinson). The isotype control used for the anti-IgA–FITC primary conjugate was a goat IgG–FITC (Abcam). In some experiments, nuclei were labeled with TOPRO-3 (blue fluorescence; Invitrogen). Slides were read with a laser scanning confocal microscope (LSM 510; Carl Zeiss, Inc.).

In the second part, 200 μg/ml of p31-49–TAMRA was added to the apical compartment of duodenal biopsies from patients with active CD, patients with treated CD, and control patients, placed in Using chambers. After 15 min at 37°C, the biopsies were removed, embedded in Tissue-Tek, cryosectioned, and submitted to immunofluorescent staining with 24 μg/ml of a goat anti-IgA–FITC (colocalization peptide–IgA).

Immunogold electron microscopy

Biopsies were fixed with 2% paraformaldehyde/0.2% glutaraldehyde (Electron Microscopy Sciences) in 0.2 M of phosphate buffer, pH 7.4. Free aldehyde groups were quenched with 50 mM glycine in PBS, and cells were embedded in 10% gelatin, infused in 2.3 M sucrose, and frozen in liquid nitrogen. Ultrathin cryosections were prepared with an ultracrystomicrotome (FCS-Leica) and double immunogold-labeled with anti-CD71 mAb (H68.4; Invitrogen), followed by rabbit anti–mouse Ig (Dako) and protein A conjugated to 15-nm gold particles and a goat polyclonal anti–human IgA antibody (Abcam), followed by rabbit anti–goat Ig (Dako) protein A conjugated to 10-nm gold particles. Protein A conjugated to gold particles was purchased from Cell Microscopy Centre. To avoid crossover of protein A binding, double labeling was performed sequentially, and sections were treated with glutaraldehyde between the two incubations to prevent blending of the different antibody–gold complexes (60).

Synthesis and radiolabeling of gliadin peptides

p31-49 (19-mer, LGQQQQPPQQPQPQPQPQPQPQP; mol wt, 2221) and p56-88 (33-mer, LQLQPQPQPQLPQPQLPQPQLPQ; mol wt, 3903) were synthesized (Covalab) and radiolabeled (tritiated) on selected proline residues as previously described (15). The specific activity of the radiolabeled peptide batches used in this study was between 2 and 3 Ci/mmol. Radiolabeling allowed us to follow peptide degradation during intestinal transport, using RP-HPLC chromatography with online detection of radioactivity. Immunofluorescence studies were performed with p31-49 coupled to 5-TAMRA (5-carboxytryramethylrhodamine) via a spacer (Alx) at the N-terminal portion of the peptide (Covalab).

Transport of p31-49 and 33-mer across duodenal biopsies in Using chambers

Four to six duodenal biopsies from each patient were mounted in adapted Using chambers, exposing a surface area of 0.025 cm², as previously described (15). Each biopsy was used to quantify apical to basolateral flux of radiolabeled p31-49 or 33-mer and to analyze tritiated peptide fragments in the basolateral compartment. p31-49 or 33-mer was placed on the apical side at 0.2 mg/ml, together with 1850 kBq (50 μCi) of H-labeled peptide.

All inhibitors tested, namely nonspecific IgA1 (mIgA, dIgA, and pIgA at a final concentration of 50 μg/ml, respectively, prepared as described in Purification of IgA...), 50 μg/ml SIgA (Biotrend), 30 μg/ml of soluble IgA receptors (sCD71 or sCD89), 50 μg/ml IgG (Biotrend), 10 μg/ml of human Tf (T330; Sigma–Aldrich), 20 μg/ml of anti-CD71 mAb A24 (57), 30 μg/ml of anti-human SC mAb (SC-05; Monosan), 10μg/ml of anti-Tgase II mAb 6B9 (a gift from T. Issekutz, Dalhousie University, Halifax, Canada), and 500 μM of dansyl cadaverin, a Tgase II inhibitor (Sigma–Aldrich), were added to the apical compartment 3 min before adding the peptide. Finally, preliminary experiments indicated no nonspecific binding of H-labeled p31-49 to myeloma plgA1, SIgA, or sCD71 (unpublished data).

RP-HPLC analysis

p31-49 or p56-88 (33-mer) and their fragments present in the apical and basolateral compartments bathing the duodenal biopsies in the Using chamber were analyzed after 3 h of incubation by radio–RP-HPLC, as previously described (15). Acquisition, integration, and calculation of the percentage of
radioactivity eluted in each peak were performed with Radiostar software (Berthold). In this setting, free $^{14}$C-labeled prolinc (the radiolabeled amino acid in the peptide sequence) is eluted with a retention time (Rt) of 4 min, whereas intact p31-49 and 33-mer are eluted at 24.5 and 30 min, respectively. As already reported (15), there was no degradation of p31-49 or 33-mer in intestinal digestion. The results are reported as means ± SD or as scatter plots and medians. Multiple comparisons (analysis of variance) followed by group-to-group comparisons were performed with the general linear model procedure or the nonparametric Wilcoxon test of the SAS software package (SAS Institute). Differences were considered to be statistically significant at P < 0.05.

Online supplemental material
Fig. S1 examines IgA expression in crypt and surface epithelium of control subjects and active celiac patients. Fig. S2 presents an immunogold electron microscopy study of IgA and CD71 expression or colocalization in duodenal biopsies from one control subject and two patients with active CD. Fig. S3 describes the attempted inhibition of intact $^{14}$C-labeled p31-49 transport in active celiac patients in the presence of anti-CD71 mAb, anti-SC mAb, anti-Tgase II mAb, or the Tgase inhibitor dansyl cadaverin. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20071204/D1C1.

The authors wish to thank Dr. Axel Baijan for his help in recruiting celiac patients, Hélène Cohen and Marie-Anne Lelaï for excellent technical assistance, and Graça Raposo for the expertise in electron microscopy. This work was supported by the Institut de Recherche des Maladies de l’Appareil Digestif, the French Association for Gluten Intolerance, the Princess Grace of Monaco Foundation, and INSERM. The authors have no conflicting financial interests.

Submitted: 13 June 2007
Accepted: 20 November 2007

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