Physiol Rev 91: 151–175, 2011;
doi:10.1152/physrev.00003.2008.

Zonulin and Its Regulation of Intestinal Barrier Function: The Biological Door to Inflammation, Autoimmunity, and Cancer

ALESSIO FASANO

Mucosal Biology Research Center and Center for Celiac Research, University of Maryland School of Medicine, Baltimore, Maryland

Fasano A. Zonulin and Its Regulation of Intestinal Barrier Function: The Biological Door to Inflammation, Autoimmunity, and Cancer. *Physiol Rev* 91: 151–175, 2011; doi:10.1152/physrev.00003.2008.—The primary functions of the gastrointestinal tract have traditionally been perceived to be limited to the digestion and absorption of nutrients and to electrolytes and water homeostasis. A more attentive analysis of the anatomic and functional arrangement of the gastrointestinal tract, however, suggests that another extremely important function of this organ is its ability to regulate the trafficking of macromolecules between the environment and the host through a barrier mechanism. Together with the gut-associated lymphoid tissue and the neuroendocrine network, the intestinal epithelial barrier, with its intercellular tight junctions, controls the equilibrium between tolerance and immunity to non-self antigens. Zonulin is the only physiological modulator of intercellular tight junctions described so far that is involved in trafficking of macromolecules and, therefore, in tolerance/immune response balance. When the finely tuned zonulin pathway is deregulated in genetically susceptible individuals, both intestinal and extraintestinal autoimmune, inflammatory, and neoplastic disorders can occur. This new paradigm subverts traditional theories underlying the development of these diseases and suggests that these processes can be arrested if the interplay between genes and environmental triggers is prevented by reestablishing the zonulin-dependent intestinal barrier function. This review is timely given the increased interest in the role of a "leaky gut" in the pathogenesis of several pathological conditions targeting both the intestine and extraintestinal organs.

I. INTRODUCTION

In recent years much has been discovered about the structure, function, and regulation of intercellular tight junctions (TJ). However, the precise mechanism(s) by which they operate is/are still incompletely understood. The discovery of zonula occludens toxin (Zot), an enterotoxin elaborated by *Vibrio cholerae* that affects the TJ competency, has shed light on the intricate mechanisms involved in the modulation of the intestinal paracellular pathway. Our Zot structure-functional analysis demonstrated that the COOH-terminal portion (that we called ΔG) of the toxin is involved in specific proteinase activating receptor $(PAR)_2$ binding and activation of intracellular signaling leading to reversible opening of intercellular TJ (47, 58). Taken collectively, our data suggested that Zot regulates TJ in a rapid, reversible, and reproducible fashion,

likely activating intracellular signals that are operative during the physiological modulation of the paracellular pathway. Based on this observation, we postulated that Zot may mimic the effect of a functionally and immunologically related endogenous modulator of epithelial TJ. The combination of affinity-purified anti-Zot antibodies and the Ussing chamber assay allowed us to identify an intestinal Zot homolog that we named zonulin (62, 172). Isolation from human intestinal cadavers revealed that zonulin is a 47-kDa protein that increases the intestinal permeability in nonhuman primate intestinal epithelia (172). Zonulin has been observed to be involved in intestinal innate immunity (53) and to be upregulated in several autoimmune diseases, including celiac disease (CD) and type 1 diabetes (T1D), in which TJ dysfunction seems to be the primary defect (35, 50, 62, 138).

II. INTESTINAL BARRIER AND ITS REGULATION

The paracellular route is the dominant pathway for passive solute flow across the intestinal epithelial barrier, and its functional state depends on the regulation of the intercellular TJ (185). The TJ is one of the hallmarks of absorptive and secretory epithelia. As a barrier between apical and basolateral compartments, it selectively regulates the passive diffusion of ions and small water-soluble solutes through the paracellular pathway, thereby compensating for any gradients generated by transcellular pathways (93). Due to the high resistance of the enterocyte plasma membrane, variations in transepithelial conductance have been ascribed to changes in the paracellular pathway (98). The TJ represents the major barrier within this paracellular pathway with electrical resistance of epithelial tissues dependent on the number and complexity of transmembrane protein strands within the TJ, as observed by freeze-fracture electron microscopy (99). Evidence now exists that TJ, once regarded as static structures, are in fact dynamic and readily adapt to a variety of developmental (66, 105, 136), physiological (68, 100, 114, 139), and pathological (34, 61, 63, 101) circumstances.

To meet the diverse physiological challenges to which the intestinal epithelial barrier is subjected, TJ must be capable of rapid and coordinated responses. This requires the presence of a complex regulatory system that orchestrates the state of assembly of the TJ multiprotein network. While knowledge about TJ ultrastructure and intracellular signaling events has progressed significantly during the past decade, only recently we have seen effort focused on elucidating the link between TJs and many pathophysiological states (57, 58, 160, 161).

III. THE ZONULIN SYSTEM

The discovery of Zot, an enterotoxin elaborated by *Vibrio cholerae* that reversibly opens TJ (59), increased our understanding of the intricate mechanisms that regulate the intestinal epithelial paracellular pathway. Zot action is mediated through a cascade of intracellular events that lead to protein kinase C (PKC)-α-dependent polymerization of actin microfilaments and subsequent TJ disassembly (60). Using immunofluorescence binding studies, we have shown that Zot binding varies within the intestine, being detectable in the jejunum and distal ileum, but not in the colon, and decreasing along the villous-crypt axis (64). This binding distribution coincides with the differential intestinal epithelial barrier responsiveness and actin reorganization that occurs along the villous axis (109) as well as with the regional effect of Zot on intestinal permeability (60, 64). These combined data demonstrate that Zot regulates TJ in a rapid, reversible, and reproducible fashion. Based on these observations, we postulated that Zot may mimic an immunologically related, endogenous modulator of epithelial TJ that we identified and named zonulin (172). Affinity-purified zonulin reduced transepithelial electrical resistance (TEER) compared with the media control in both monkey jejunum (35.3% decrement) and ileum (25.6% decrement), but not in the colon (172). *V. cholerae*-derived Zot and human zonulin both act on intestinal TJ (12, 47, 59) and display the same regional barrier responsiveness (64) coincident with Zot receptor distribution within the intestine (60, 162). The physiological role(s) of the zonulin system remains to be established. This pathway appears to be involved in several functions, including TJ regulation responsible for the movement of fluid, macromolecules, and leukocytes between the bloodstream and the intestinal lumen, and vice versa (57). Another potential physiological role of intestinal zonulin is the protection against microorganism colonization of the proximal intestine (innate immunity) (53).

A. Identification of Zonulin as Pre-Haptoglobin 2

Since zonulin is overexpressed in tissues and sera of subjects affected by autoimmune diseases, we elected to use sera from zonulin-positive and zonulin-negative T1D and CD subjects to characterize further the molecular nature of zonulin. Through proteomic analysis of human sera, we have recently identified zonulin as pre-haptoglobin (HP) 2 (159), a molecule that, to date, has only been regarded as the inactive precursor for HP2, one of the two genetic variants (together with HP1) of human HPs. Mature human HPs are heterodimeric plasma glycoproteins composed of α- and β-polypeptide chains that are covalently associated by disulfide bonds and in which only

the β chain is glycosylated (73) (Fig. 1). While the β chain (36 kDa) is constant, the α chain exists in two forms, i.e., α1 (~9 kDa) and α2 (~18 kDa). The presence of one or both of the α-chains results in the three human HP phenotypes, i.e., HP1–1 homozygote, HP2–1 heterozygote, and HP2–2 homozygote.

Despite this multidomain structure, the only function assigned to HPs, to date, is to bind Hb to form stable HP-Hb complexes, thereby preventing Hb-induced oxidative tissue damage (6). In contrast, no function has ever been described for their precursor forms. Our data demonstrated that the same anti-Zot antibodies used to detect serum zonulin by ELISA (62, 138, 173) showed a unique immunoreactivity against HP in human serum. These antibodies showed strong reaction against HP α1- and α2-chains allowing easy typing of the sera into the three major HP phenotypes (159) (Fig. 1). A consistently weak recognition of a ~45 kDa was also detected only in HP2–2 sera (Fig. 1) (159). The anti-Zot antibodies showed restrictive recognition for this ~45-kDa band specific to the HP2–2 phenotype. Interestingly, despite the fact that the anti-Zot antibodies recognize both α1- and α2-chains, under nondenaturing conditions they immunoreacted only with HP2–2 phenotype (159), suggesting that the ELISA used to measure zonulin serum concentration (62, 138, 172) is most likely detecting the ~45-kDa protein band present only in HP2. MS/MS analysis identified the 45-kDa band as pre-HP2. These data and the fact that human zonulin molecular mass (~47 kDa) (62, 172) and pre-HP2 molecular mass (45 kDa) are very similar support the hypothesis that they are the same molecule.

Additional studies using purified human HP1–1 and HP2–2 further defined that the anti-Zot antibodies specifically recognize the human pre-HP2 (159). The primary translation product of mammalian Hp mRNA is a polypeptide that dimerizes contranslationally and is proteolytically cleaved while still in the endoplasmic reticulum (179). Conversely, zonulin is detectable in an uncleaved form in human serum, adding another extremely intriguing aspect of the multifunctional characteristics of HPs. HPs are unusual secretory proteins in that they are proteolytically processed in the endoplasmic reticulum, the subcellular fraction in which we detected the highest zonulin concentration (50). Wicher and Fries (179) found that the complement C1r-like protein mediates this cleavage in a specific manner, since the enzyme did not cleave the proform of complement C1s, a protein similar to Pre-HP2. Therefore, it is conceivable to hypothesize that the activity of Cr-like protein modulates the zonulin pool.

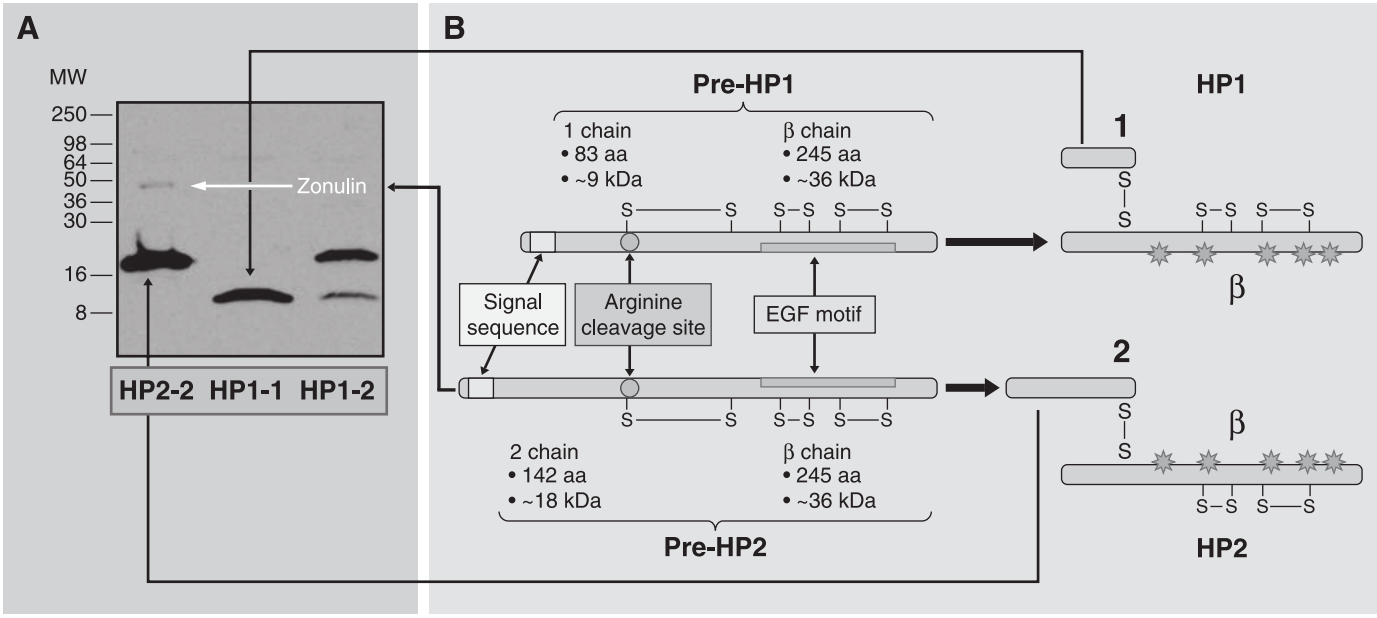

FIG. 1. *A*: Western blotting using zonulin cross-reacting anti-Zot polyclonal antibodies on CD patient sera. Three main patterns were detected: sera showing a 18-kDa immunoreactive band and a fainter ~45-kDa band (*lane 1*), sera showing only a 9-kDa band (*lane 2*), and sera showing both the 18- and 9-kDa bands (*lane 3*). *B*: cartoon showing the structure of both pre-haptoglobin (HP) 1 and pre-HP2 and their mature proteins. HPs evolved from a complement-associated protein (mannose-binding lectin-associated serine protease, MASP), with their α-chain containing a complement control protein (CCP), while the β-chain is related to chymotrypsin-like serine proteases (SP domain) containing an epidermal growth factor-like motif. The gene encoding the α2-chain of pre-HP2 originated in India almost 2 million years ago through a chromosomal aberration (unequal crossing over) of HP1. Pre-HPs are translated as single-chain precursor proteins. Pre-HPs may be proteolytically cleaved intracellularly into α- and β-chains that remain disulfide linked, referred to as cleaved, two-chain mature HPs. The two-chain mature HPs are abundant plasma glycoproteins and are composed of their α- and β-subunits covalently associated by three disulfide bonds in which the carbohydrate groups are exclusively linked to the β-chain. [Modified from Camarca et al. (29).]

B. Evolutionary and Structural Biology of HPs

HPs are mammalian α2-globulins whose name comes from their ability to bind proteins. The primary function of HP is to bind free Hb to inhibit its oxidative activity. Clearance of the HP-Hb complex can be mediated by the monocyte/macrophage scavenger receptor CD163 (6). Several HP variants have been identified in addition to the main types, and evidence of genic evolution through duplication (by unequal crossover) and subsequent independent mutations has been reported (179), providing a rationale for the high HP intra- and interspecies variability.

By analyzing available genomic sequences, Wicher and Fries (178) found that bony fish, but not more primitive animals, have a gene coding for a protein homologous to mammalian HP (Fig. 2). Interestingly, in the chimpanzee there are three genes in the HP family [HP1; HP-related (HPR), and HP-chimpanzee, (HPCh)], whereas only two genes mapped on chromosome 16 exist in humans (HP and HPR) (Fig. 3) as we confirmed with our sequence analysis of intestinal tissues (159). The two-gene cluster of the human species was formed after the separation of the human and chimpanzee lineages (that occurred 2.5 millions years ago) by an unequal homologous crossover that deleted most of the third gene. The 3-gene HP cluster in chimpanzees shows evidence of many recombinations, insertions, and deletions during its evolution, suggesting a high rate of mutation in this region of chromosome 16. One interesting observation is that the α2-chain gene (and, therefore, zonulin) is found only in humans and originated 2 millions years ago (500,000 years after that humans and chimpanzee lineages split) through a chromosomal aberration (unequal crossover) in a humanoid in India who was heterozygous α-1F/α-1S (103). The α2-chain is nearly twice as long as the α1-chain and consists of portions of α-1F and α-1S (16). The HP*2 allele has an internal duplication of 1.7 kb that includes two of the α-chain exons. Since we have detected zonulin in other mammals (53, 157, 173), it is likely that high zonulin polymorphism secondary to high mutation rate during

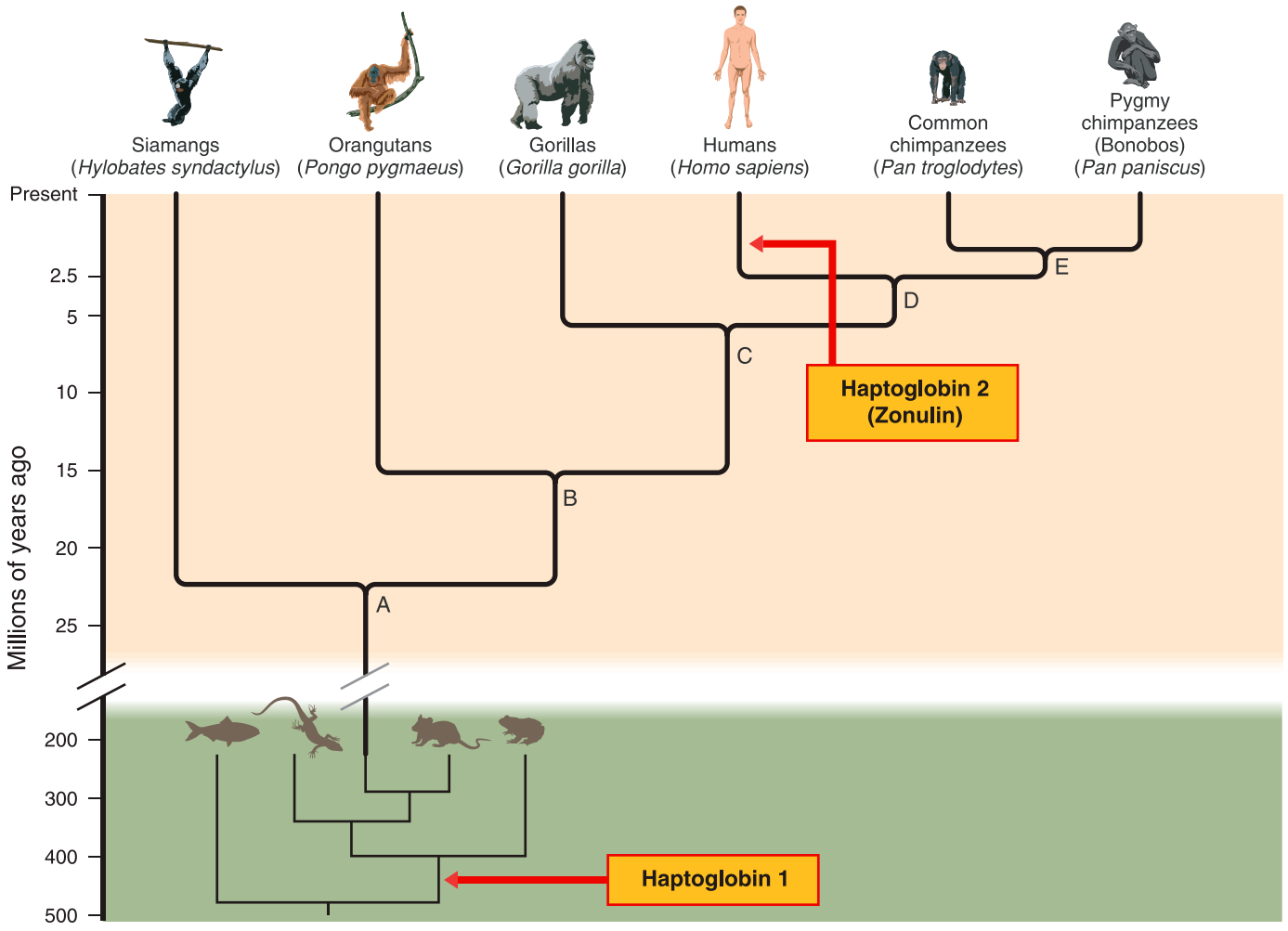

FIG. 2. Evolutionary tree of HP gene. The appearance of the gene encoding HP1 has been mapped ~450 millions years ago, soon after the split between bony fish, reptiles, and mammals. HP2 appeared much later, 500, 000 years after, then chimpanzee and human split 2.5 millions years ago.

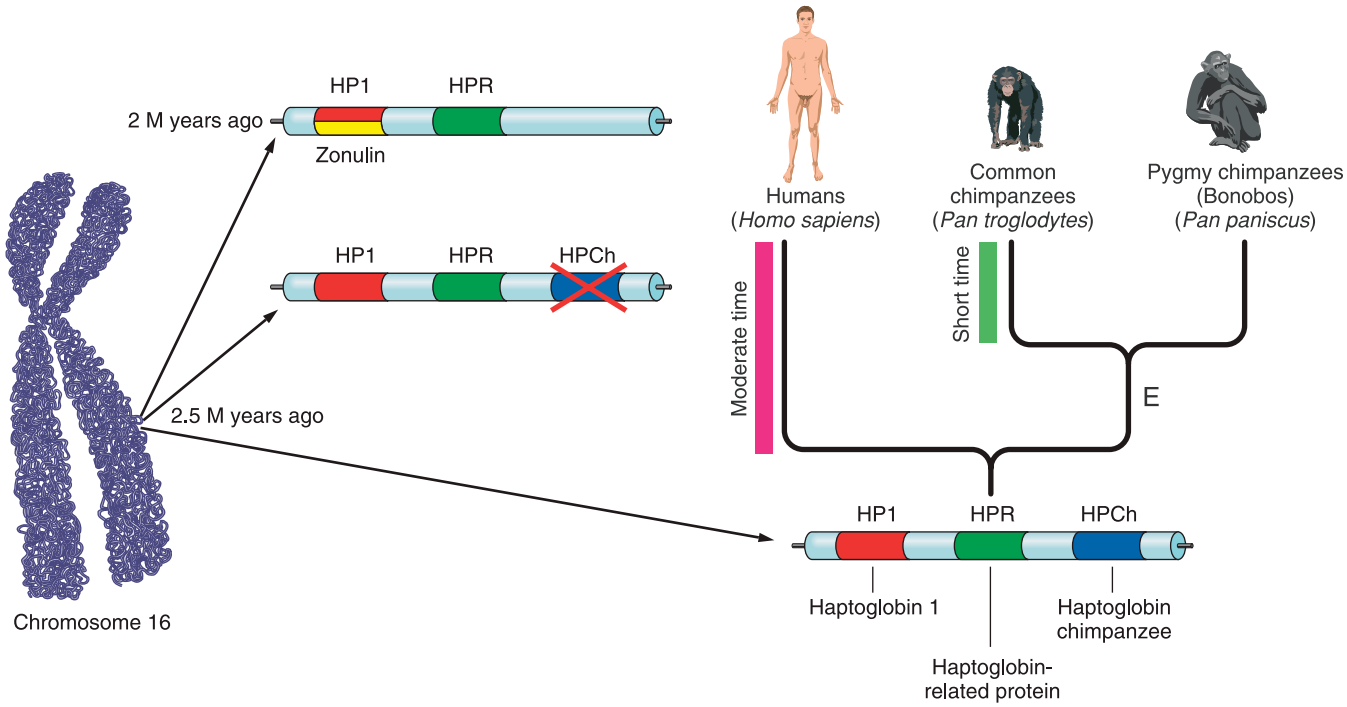

FIG. 3. Differences in HP gene clustering on chromosome 16 between human and chimpanzee. The HP gene complex is located on chromosome 16 and is composed by different variants in chimpanzee versus humanoid. In chimpanzee, HP1 is followed by HP-related gene (HPR) and HP chimpanzee (HPCh). The 2-gene cluster of the human was formed after the separation of the human and chimpanzee lineages by an unequal homologous crossover that deleted most of the HPCh. HP2 is found only in humans and originated 2 millions years ago through a chromosomal aberration (unequal crossover) in a humanoid in India who was heterozygous α-1F/α-1S (103).

evolution led to interspecies zonulin variability. Alternatively, the zonulins identified in other species may belong to other members of the mannose-binding lectin-associated serine protease (MASP) family (see below).

C. Structural Characterization of Zonulin and Its Subunits

Additional phylogenetic analyses suggest that HPs evolved from a complement-associated protein (MASP), with their α-chain containing a complement control protein (CCP) (this domain activates complement), while the β-chain is related to chymotrypsin-like serine proteases (SP domain) (89, 178). However, the SP domain of HP lacks the essential catalytic amino acid residues required for protease function; recent structure-function analyses have implicated this domain in receptor recognition and binding (123, 133).

Although not a serine protease, zonulin shares ~19% amino acid sequence homology with chymotrypsin, and their genes both map on chromosome 16. Alignment of the β-chain sequence of zonulin to that of several serine proteases is remarkably consistent except for an insertion of 16 residues in the region corresponding to the methionyl loop of the serine proteases. Comparison of the zonulin α-β junction region with the heavy-light-chain junction of tissue-type plasminogen activator strengthens the evolutionary homology of zonulin and serine proteases. The active-site residues typical of the serine proteases, histidine-57 and serine-195, are replaced in zonulin by lysine and alanine, respectively. Because of these mutations, during evolution zonulin most likely lost its protease activity despite that zonulin and serine proteases evolved from a common ancestor (89). Therefore, zonulin and the serine proteases represent a striking example of homologous proteins with different biological functions. Other members of the MASP family include a series of plasminogen-related growth factors [epidermal growth factor (EGF), hepatocyte growth factor (HGF), etc.] involved in cell growth, proliferation, differentiation and migration, and disruption of intercellular junctions.

D. Zonulin Functional Characterization

Since no biological function has ever been described for pre-HP2, several experiments were performed to confirm its identity as zonulin. Pre-Hp-2 gene was cloned in an inset vector system and expressed using a Baculovirus expression system. The protein was recognized by the zonulin cross-reacting anti-Zot antibodies and was cleaved both by trypsin (159) and Caco2-derived trypsin

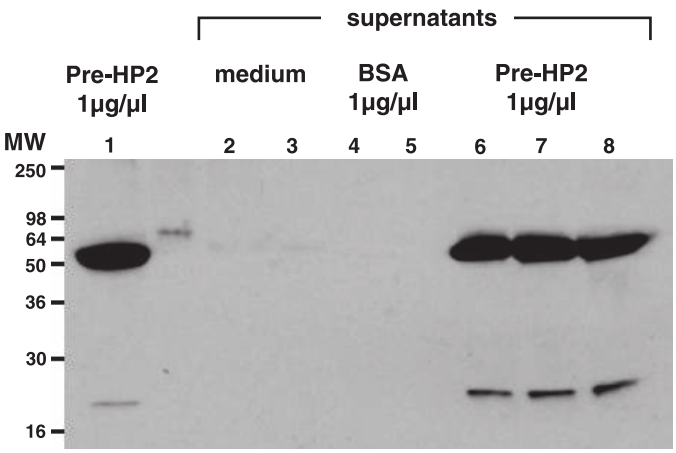

FIG. 4. Caco2-derived trypsin IV cleaves zonulin in its two subunits. Western immunoblotting of recombinant zonulin before (*lane 1*) and after (*lanes 6–8*) incubation on Caco-2 cells. The intensity of the ~18-kDa band (identified by NH_2-terminal sequencing as the a2 chain) increased after 120-min incubation in Caco2 cells. Media (*lanes 2* and *3*) and bovine serum albumin (BSA; *lanes 4* and *5*) were also tested to confirm antibody specificity.

into its α- and β-chains at the predicted Arg^{161} cleavage site (Fig. 4).

Since we have reported previously that the key biological effect of zonulin is to affect the integrity of intercellular TJ, we specifically focused our efforts on demonstrating that the recombinant pre-HP2 alters intestinal permeability. Indeed, ex vivo experiments showed that recombinant pre-HP2 induced a time- and dose-dependent reduction in TEER when added to murine small intestinal mucosa (159). These results were validated independently in an in vivo intestinal permeability assay in which zonulin, but not its cleaved form, induced a significant and reversible increase in both gastroduodenal and small intestinal permeability (Fig. 5). The evidence that zonulin cleaved in its α- and β-subunits lost the permeating activity further supports the notion that pre-HP2 (alias, zonulin) and mature HP2 exert two different biological functions most likely related to the different folding of the protein in its cleaved or not cleaved form. The importance of HP folding in dictating its structure and function was further supported by the finding that anti-Zot antibodies recognize α1-chain under denaturing conditions but fail to recognize nondenatured HP1 (159).

E. Zonulin Signaling

Structural analysis of zonulin revealed similarities with several growth factors. Like zonulin, growth factors affect intercellular TJ integrity (83, 78). Our data showing

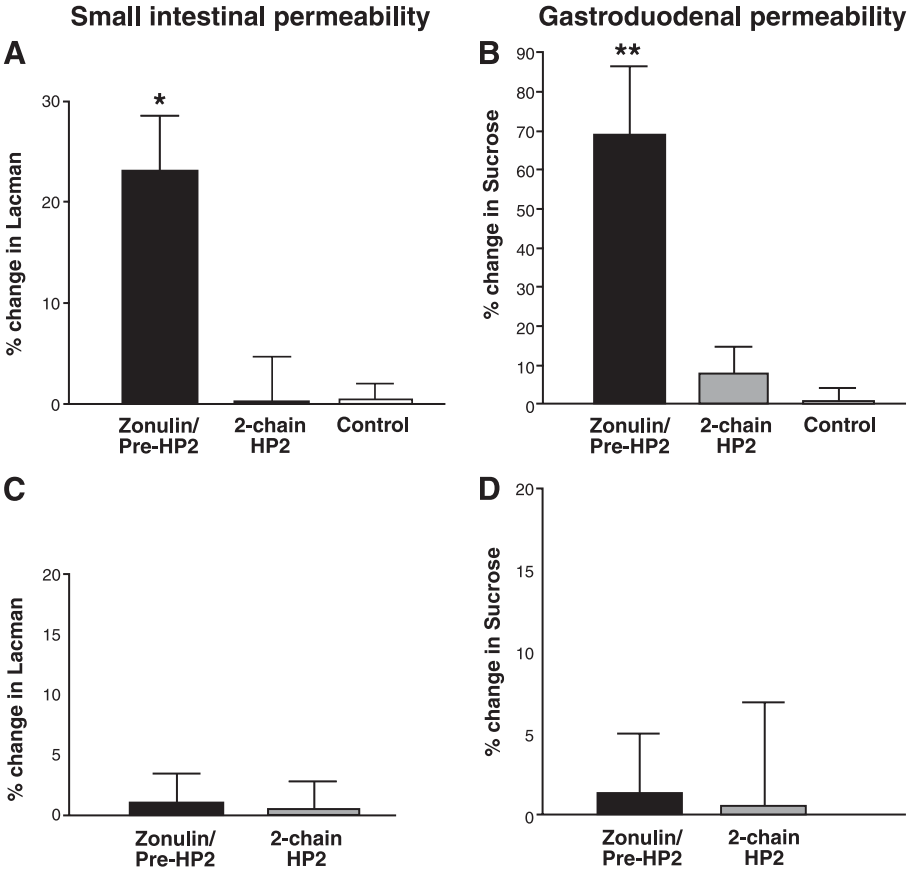

FIG. 5. Effect of zonulin on gastrointestinal permeability in vivo. Zonulin (closed bars) increases both small intestinal (*A*) and gastroduodenal (*B*) permeability compared with BSA-treated controls (open bars). The differences in lacman ratio (small intestinal permeability) and sucrose fractional excretion (gastroduodenal permeability) are shown as percentage of change in permeability between the measurements on the challenge day and 3 days before challenge. Mature two-chain HP2 (dotted bars) caused no changes in either small intestinal or gastroduodenal permeability. The effect of zonulin was completely reversible, since both small intestinal (*C*) and gastroduodenal (*D*) permeability returned to prechallenge values within 48 h. The differences in lacman or sucrose fractional excretion are shown as percentage of permeability change between the value of 2 days after the challenge and the challenge day. *Lacman $P < 0.0024$ compared with both BSA control and 2-chain HP2; **Sucrose $P < 0.0049$ compared with both BSA control and 2-chain HP2 ($n = 10$ for each group of treatment). [Modified from Tripathi et al. (159).]

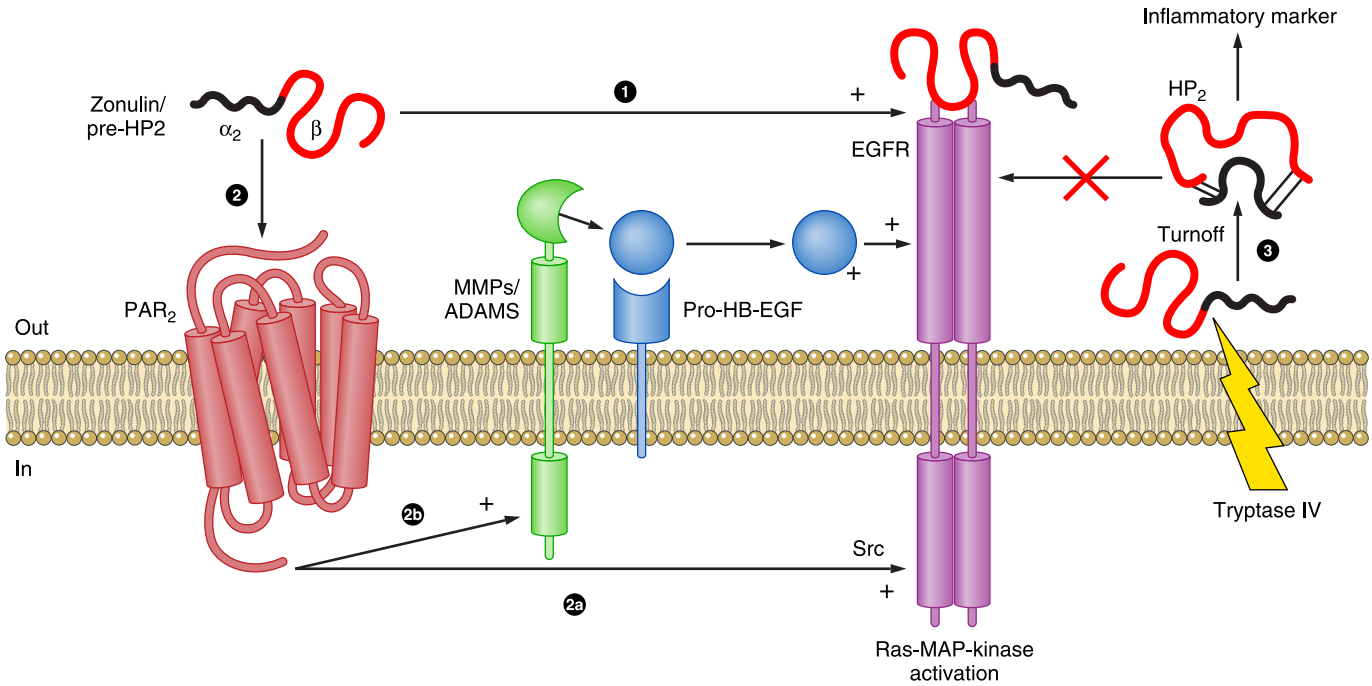

FIG. 6. Proposed mechanisms through which zonulin activates EGFR. Zonulin can activate EGFR through direct binding (*1*) and/or through PAR_2 transactivation (*2*). This second mechanism can be mediated by either Src signaling (*2a*) or by the release of MMPs and/or ADAMS that in turn will activate Pro-HB-EGF. Processing of zonulin into its two-chain mature form, for example, via proteolytic cleavage by intestinal tryptase IV, induces conformational changes in the molecule that abolish its ability to bind to EGFR (*3*), but instead enables a different function (e.g., Hb binding), and it becomes an inflammatory marker.

that zonulin but not its cleaved subunits activate EGF receptor (EGFR) (159) and that its effect on TEER was prevented by the EGFR tyrosine kinase inhibitor AG-1478 (159) suggest that zonulin is properly folded to activate EGFR and, therefore, to cause TJ disassembly only in its uncleaved form. Several G protein-coupled receptors (GPCR), including PAR_2, transactivate EGFR (164). Zonulin prokaryotic counterpart Zot active peptide FCIGRL (AT1002) has structural similarities with PAR_2-activating peptide (AP), SLIGRL, and causes PAR_2-dependent changes in TEER (31), a finding that we have demonstrated in wild-type (WT) but not $PAR2^{-/-}$ mice. Therefore, it was not totally unexpected that experiments in Caco2 cells in which PAR_2 was silenced showed decreased EGFR Y1068 phosphorylation in response to recombinant zonulin compatible with PAR_2-dependent transactivation of EGFR (159). To further establish a role for PAR_2 in EGFR activation in response to zonulin, we conducted small intestinal barrier function studies using segments isolated from either C57BL/6 WT or $PAR_2^{-/-}$ mice. As expected, zonulin decreased TEER in intestinal segments from C57BL/6 WT mice, while it failed to reduce TEER in small intestinal segments from $PAR_2^{-/-}$ mice (159), so linking zonulin-induced PAR_2-dependent transactivation of EGFR with barrier function modulation.

To summarize, we have reported for the first time the novel characterization of zonulin as pre-HP2, a multifunctional protein that, in its intact single-chain form, regulates intestinal permeability caused by EGFR transactivation through PAR_2, while in its cleaved two-chain form acts as a Hb scavenger (Fig. 6).

Interestingly, it has been recently reported that gliadin, the environmental trigger of CD, fully reproduces the effects of EGF on actin cytoskeleton (11), effects that are very similar to those we reported for zonulin (35, 53, 172). Since gliadin induces zonulin release from both intestinal cells (35, 53) and whole intestinal tissues (50, 53, 157, 172) through CXCR3 binding (92), it is likely that the gliadin-related EGF effects are indeed secondary to its capability to induce zonulin release.

F. Stimuli That Cause Zonulin Release in the Gut

Among the several potential intestinal luminal stimuli that can trigger zonulin release, we identified small intestinal exposure to bacteria and gluten as the two more powerful triggers (Fig. 7). Enteric infections have been implicated in the pathogenesis of several pathological conditions, including allergic, autoimmune, and inflammatory diseases, by causing impairment of the intestinal barrier. We have generated evidence that small intestines exposed to enteric bacteria secreted zonulin (53). This secretion was independent of either the animal species from which the small intestines were isolated or the virulence of the microorganisms tested, occurred only on

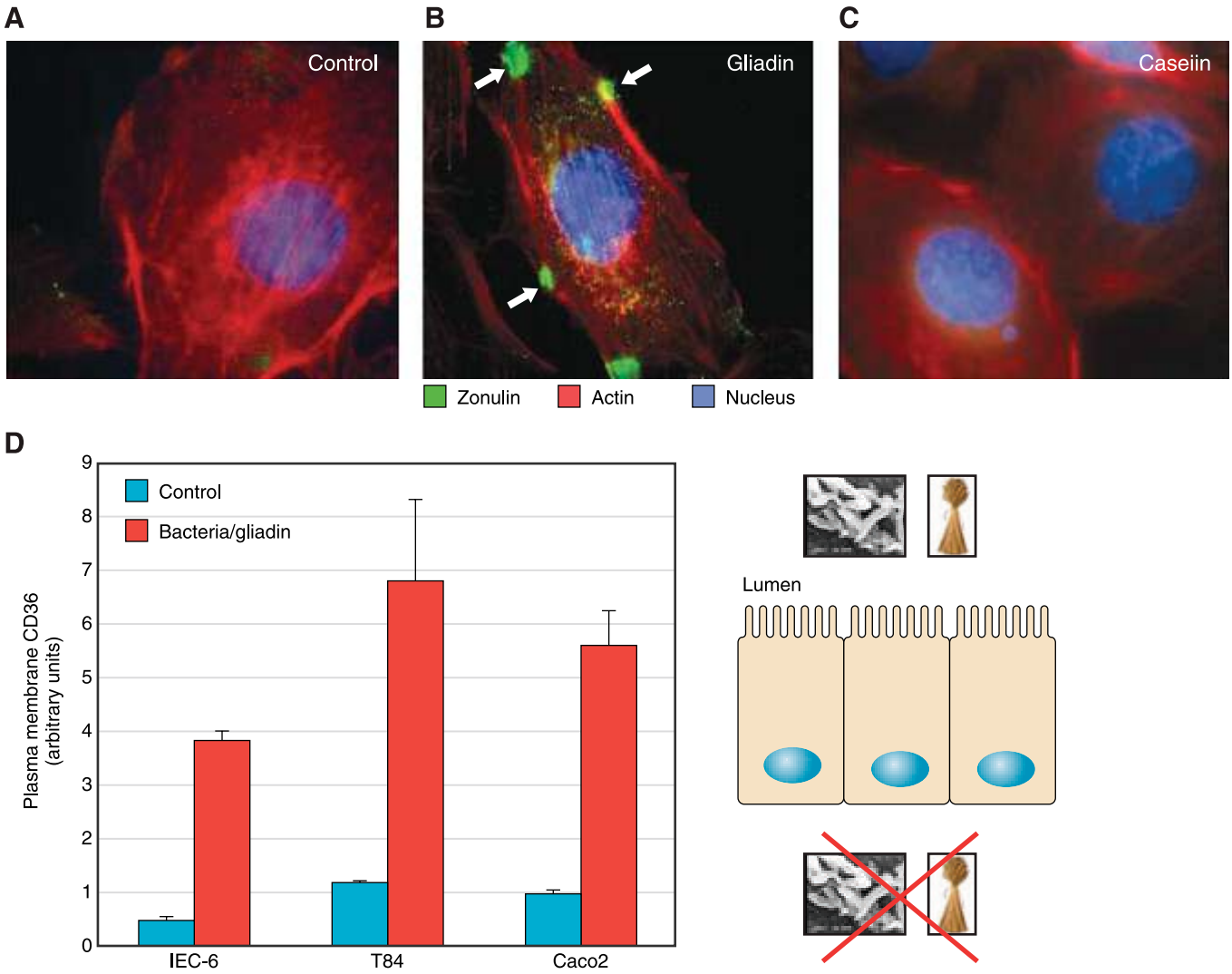

FIG. 7. Stimuli causing polarized zonulin release from intestinal epithelial cells. *A–C*: anti-zonulin immunoflourescence staining of human intestinal Caco2 cells. Cells exposed to gliadin/PT-gliadin (*B*) react by packaging preformed zonulin in vesicles (arrows) that gradually approached the cell membrane and then released their zonulin content in the cell medium within a few minutes of the exposure to gliadin. No packaging was detected in nonstimulated cells (control, *A*) or cells incubated with PT-casein (*C*). The nucleus is in blue (DAPI), cytoskeleton in red (RITC), and zonulin in green (FITC). Magnification ×100. *D*: polarized zonulin secretion of intestinal cells exposed to either bacteria or PT-gliadin. Both rat (IEC6) and human (Caco2 and T84) intestinal epithelial cells exposed to either nonpathogenic bacteria or gliadin secrete large amounts of zonulin in the bath medium compared with the amount of zonulin measured in media of cells exposed to control. This secretion was detected only when the triggers were added to the luminal (apical) aspect of the cell monolayers.

the luminal aspect of the bacteria-exposed small intestinal mucosa, and was followed by an increase in intestinal permeability coincident with the disengagement of the protein ZO-1 from the tight junctional complex (53). This zonulin-driven opening of the paracellular pathway may represent a defensive mechanism which flushes out microorganisms so contributing to the innate immune response of the host against bacterial colonization of the small intestine.

In addition to bacterial exposure, we have shown that gliadin also affects the intestinal barrier function by releasing zonulin (35). This effect of gliadin is polarized, i.e., gliadin increases intestinal permeability only when administered on the luminal side of the intestinal tissue (35) (Fig. 7). This observation led us to the identification of the chemokine receptor CXCR3 as the target intestinal receptor for gliadin (92). Our data demonstrate that in the intestinal epithelium, CXCR3 is expressed at the luminal level, is overexpressed in CD patients (Fig. 8), and colocalizes with gliadin and that this interaction coincides with recruitment of the adapter protein MyD88 to the receptor (92). We also demonstrated that binding of gliadin to CXCR3 is crucial for the release of zonulin and subsequent increase of intestinal permeability, since CXCR3-deficient mice failed to respond to gliadin challenge in terms of zonulin release and TJ disassembly (92). Using a α-gliadin synthetic peptide library, we identified two α-gliadin 20-mers (QVLQQSTYQLLQELCCQHLW and

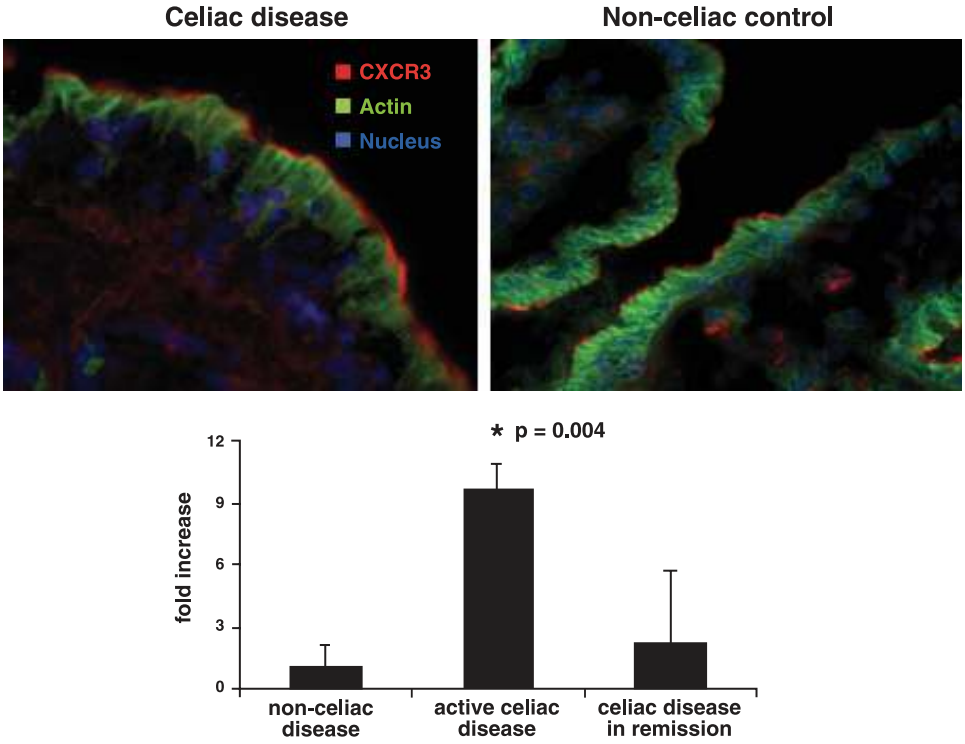

FIG. 8. *Top:* in situ immunofluorescence microscopy of CXCR3 in human small intestinal biopsies obtained from either celiac patients or nonceliac controls. CXCR3 staining in red (RITC) is homogeneously visible on the apical side of intestinal epithelial cells of biopsies from celiac disease patients, while the CXCR3 staining is patchy in nonceliac controls. *Bottom:* quantitative real-time PCR of the CXCR3 gene confirmed an increased expression of the receptor compared with nonceliac controls that decreased after treatment with a gluten-free diet. Note the increased infiltrate of CXCR3-expressing immune cells in celiac disease biopsies compared with nonceliac controls. The nucleus is in blue (DAPI) and the cytoskeleton in green (FITC). Magnification ×60.

QQQQQQQQQQQQILQQILQQ) that bind to CXCR3 and release zonulin (92).

G. Zonulin and Immunoglobulins Have a Common Ancestor But Are Distinct Molecules

Our previously reported zonulin NH_2-terminal amino acid sequence showed striking similarities with the light chain of immunoglobulins (Ig) (172). Similarities between the primary structures of HP and of Ig light chains have been previously reported (79). They were supported by a common evolutionary origin (10) and by functional homologies, since both form complexes with specific proteins. Clustal W dendogram analysis showed a region in the zonulin β-chain just upstream from the C163 binding site with the following Ig consensus motif: QLVE—V—P. To confirm that zonulin and Ig are two distinct moieties, Western immunoblot analysis of whole serum, serum depleted of immunoglobulin, and serum immunoglobulin fraction from subjects either positive or negative to zonulin ELISA was performed. The whole serum from a zonulin-negative subject showed the expected single α1-subunit (HP1–1 homozygous), while the whole serum from a zonulin-positive subject showed the expected single α2-subunit (HP2–2 homozygous). Sera depleted of albumin (Fig. 9, *lanes 1* and *4*) or albumin + Ig (Fig. 9, *lanes 3* and *6*) retained the immunoreactive α-bands. The zonulin band was also visible at the expected 47-kDa size in the HP2–2 sera (Fig. 9). Conversely, the sera Ig fractions of both HP1–1 and HP2–2 sera showed no immunoreactivity (Fig. 9, *lanes 2* and *5*) and did not cause any changes in TEER when tested on mouse small intestine mounted in microsnapwell (A. Fasano, personal communication), confirming that zonulin and Ig are structurally related yet functionally distinct molecules. If the differences between

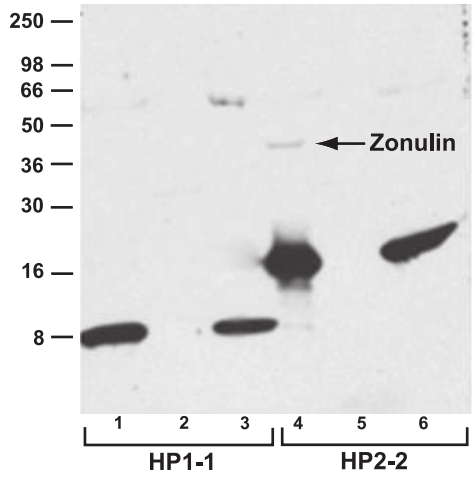

FIG. 9. Western blotting using zonulin cross-reacting anti-Zot polyclonal antibodies on ELISA zonulin-negative and zonulin-positive sera. Sera depleted of albumin (*lanes 1* and *4*) or albumin + Ig (*lanes 3* and *6*) from both a zonulin-negative subject (HP1–1 homozygous, *lanes 1–3*) and a zonulin-positive subject (HP2–2 homozygous, *lanes 4–6*) showed the expected single α1- and α2-subunits, respectively. Conversely, the sera Ig fractions of both subjects (*lanes 2* and *5*) showed no immunoreactivity. The zonulin band was also visible at the expected 47-kDa size in the HP2–2 sera (arrow).

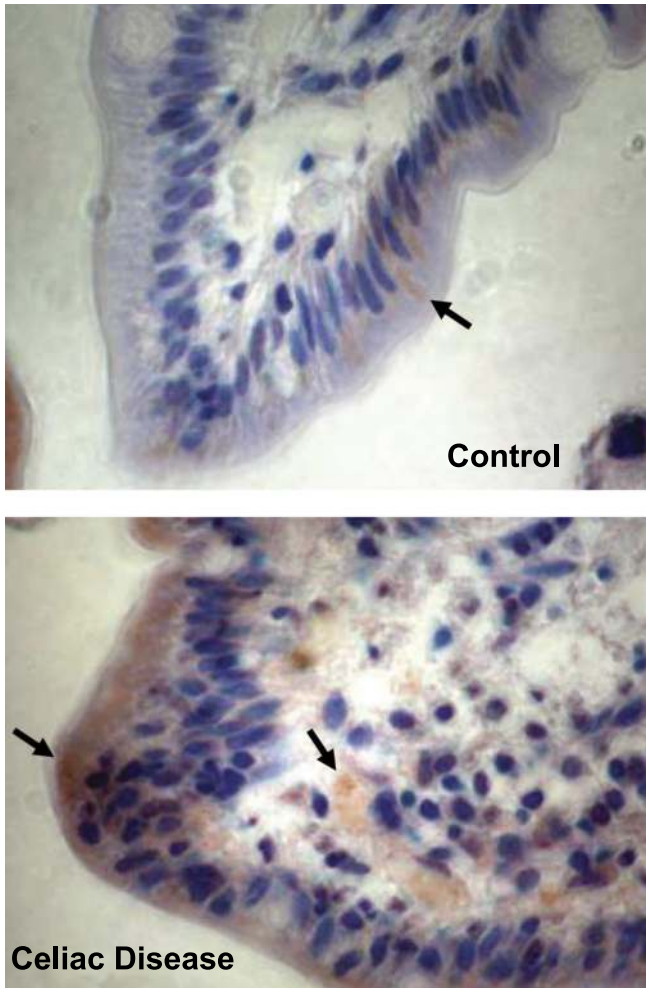

FIG. 10. Photomicrographs of immunohistochemistry on small intestinal tissues from a healthy control and an active CD patient stained with zonulin cross-reacting anti-Zot antibodies. Zonulin is visualized both in enterocytes and in cells of the lamina propria (arrows) and is overexpressed in active CD patients compared with controls.

the reported zonulin sequence and the pre-HP2 sequence are due to intraspecies variability related to high zonulin mutation rate or to our sequence error remains to be established.

H. Zonulin Is Upregulated in the Intestinal Mucosa of Celiac Disease Patients

We have previously reported that zonulin is upregulated during the acute phase of CD (62, 50) and that zonulin is released from intestinal mucosa following exposure to either gluten or microorganisms (50, 53, 157, 172). However, HPs have never been reported to be expressed in the intestine. Using specific HP primers, we have shown for the first time the expression of both HP2 and HPR mRNA in human intestine (159). RT-PCR experiments showed that zonulin was significantly higher in CD patients compared with controls, while CD patients on a gluten-free diet showed intermediate mean values (159). We have confirmed zonulin overexpression in both intestinal epithelial cells and lamina propria cells by in situ immunohistochemistry studies (Fig. 10). Interestingly, Papp et al. (127) have recently reported that HP polymorphism represents a novel genetic risk factor for CD development and its clinical manifestations. The authors found that the phenotype HP2–1 was associated with a significant risk of CD. Conversely, HP2–2 was less frequent in CD patients than in controls, but patients having this phenotype were at an increased risk for severe malabsorption (127). Therefore, it is tempting to hypothesize that one copy of the zonulin gene increases the risk of CD because of its permeating effect on the intestinal barrier, while two copies of this gene, causing a severe malabsorption secondary to a more profound intestinal barrier dysfunction, led to high mortality and, therefore, was negatively selected during evolution. Interestingly, our proteomic analysis of sera from CD patients revealed a higher percentage of HP2 haplotype (either homozygote or heterozygote) compared with healthy controls (see Table 1) (159). These data suggest that only 7% of CD patients do not possess the zonulin gene.

IV. INTESTINAL PERMEABILITY AND DISEASE

A fast-growing number of diseases are recognized to involve alterations in intestinal permeability related to changes in TJ competency. These comprise autoimmune diseases, including T1D (107, 117, 149, 163), CD (45, 46, 51, 126), multiple sclerosis (124, 176, 184), and rheumatoid arthritis (52), in which intestinal TJs allow the passage of antigens from the intestinal milieau, challenging the immune system to produce an immune response that can target any organ or tissue in genetically predisposed individuals (1, 13, 57, 58, 70, 151). TJs are also involved in cancer development, infections, and allergies (32, 55, 57, 147).

It is generally accepted that it is the interplay between environmental factors and specific susceptibility genes that underlies the aberrant immune response responsible for the onset of these diseases. Less than 10% of

TABLE 1. *HP phenotype distribution among celiac disease patients and the general population*

HP phenotype	CD Patients, %	Healthy Population in the United States, %	Healthy Population in Europe, %
HP 1-1	7.1	20.6	13.1
HP 1-2	35.7	43.5	50.4
HP 2-2	57.2	35.9	36.5

HP, haptoglobin; CD, celiac disease.

those with increased genetic susceptibility progress to clinical disease, suggesting a strong environmental trigger in the predisease state (75, 152). Environmental factors are also likely affecting the outcome of the process and the rate of progression to disease in those who develop pathological outcomes. One theory is that antigens absorbed through the gut may be involved. The intestinal epithelium is the largest mucosal surface and provides an interface between the external environment and the mammalian host. Healthy, mature gut mucosa with its intact TJ serves as the main barrier to the passage of macromolecules. In a healthy state, quantitatively small, but immunologically active antigens may cross the mucosal barrier. These antigens are absorbed across the mucosa via two functional pathways. The vast majority of absorbed proteins (up to 90%) cross the intestinal barrier through the transcellular pathway (22, 113, 119, 142, 155), followed by lysosomal degradation that converts proteins into smaller, nonimmunogenic peptides. The remaining portion of peptides is transported as intact proteins, resulting in antigen-specific immune responses. This latter phenomenon utilizes the paracellular pathway that involves a subtle but sophisticated regulation of intercellular TJ that leads to antigenic tolerance (57, 58). When the integrity of the TJ system is compromised, as is seen during prematurity or exposure to radiation, chemotherapy, and/or toxins, an immune response to environmental antigens may develop (32, 55, 58, 147). The specific cells that are key for this immune response [i.e., antigen presenting cells (APC), T and T killer lymphocytes, B lymphocytes, and plasma cells] lie in close proximity to the intestinal epithelial barrier (23, 24).

Another critical factor for intestinal immunological responsiveness is the major histocompatibility complex (MHC). Human leukocyte antigen (HLA) class I and II genes encode APC glycoprotein receptors, which bind peptides, and this HLA-peptide complex is recognized by certain T-cell receptors in the intestinal mucosa (20, 21, 39). Susceptibility to at least 50 diseases has been associated with specific HLA class I or class II alleles. A common denominator of these diseases is the presence of several preexisting conditions that lead to a pathological process. The first is a genetic susceptibility for the host immune system to recognize, and potentially misinterpret, an environmental antigen presented within the gastrointestinal tract. Second, the host must be exposed to the antigen. Finally, the antigen must be presented to the gastrointestinal mucosal immune system following its paracellular passage (normally prevented by TJ competency) from the intestinal lumen to access the gut submucosa (18, 19, 57, 58, 175). In most cases, increased permeability precedes disease and causes an abnormality in antigen delivery that triggers the multiorgan process leading to systemic diseases (58).

V. ROLE OF ZONULIN IN AUTOIMMUNE, INFLAMMATORY, AND NEOPLASTIC DISEASES

A. Specific Diseases in Which Zonulin Involvement Has Been Proven

1. CD

CD is an immune-mediated chronic enteropathy with a wide range of presenting manifestations of variable severity. It is triggered by the ingestion of gliadin fraction of wheat gluten and similar alcohol-soluble proteins (prolamines) of barley and rye in genetically susceptible subjects with subsequent immune reaction leading to small bowel inflammation and normalization of the villous architecture in response to a gluten-free diet (25). CD not only affects the gut, but it is a systemic disease that may cause injury to any organ. It is a complex genetic disorder, and HLA status appears to be the strongest genetic determinant of risk for celiac autoimmunity. There is a propensity for individuals with CD to carry specific HLA class II alleles, which has been estimated to account for up to 40% of the genetic load (15). In affected individuals, 95% have either DQ2 (*HLA-DQA1*05-DQB1*02*) or DQ8 (*HLA-DQA1*03-DQB1*0302*), compared with the general population in which 39.5% have either DQ2 or DQ8 (77).

CD is a unique model of autoimmunity in which, in contrast to most other autoimmune diseases, a close genetic association with HLA genes, a highly specific humoral autoimmune response against tissue transglutaminase auto-antigen, and, most importantly, the triggering environmental factor (gliadin), are all known. It is the interplay between genes (both HLA and non-HLA associated) and environment (i.e., gluten) that leads to the intestinal damage typical of the disease (131). Under physiological circumstances, this interplay is prevented by competent intercellular TJ. Early in CD, TJs are opened (50, 101, 154, 159, 180) and severe intestinal damage ensues (154).

Gluten is a complex molecule made of gliadin and glutenins, both toxic for CD patients (Fig. 12). The repertoire of gluten peptides involved in the disease pathogenesis is greater than appreciated previously and may differ between children and adult patients (4). There are at least 50 toxic epitopes in gluten peptides exerting cytotoxic, immunomodulatory, and gut-permeating activities (122). These activities have been partially mapped to specific domains in α-gliadin (Fig. 13): the cytotoxic peptide 31–43 (106, 132, 166), the immunomodulatory peptide 57–89 (33-mer) (29, 146), the CXCR3-binding zonulin-releasing (gut-permeating) peptides 111–130 and 151–170 (92), and the interleukin (IL)-8-releasing peptide 261–277 (91). The 33-mer gliadin fragment is the most immuno-

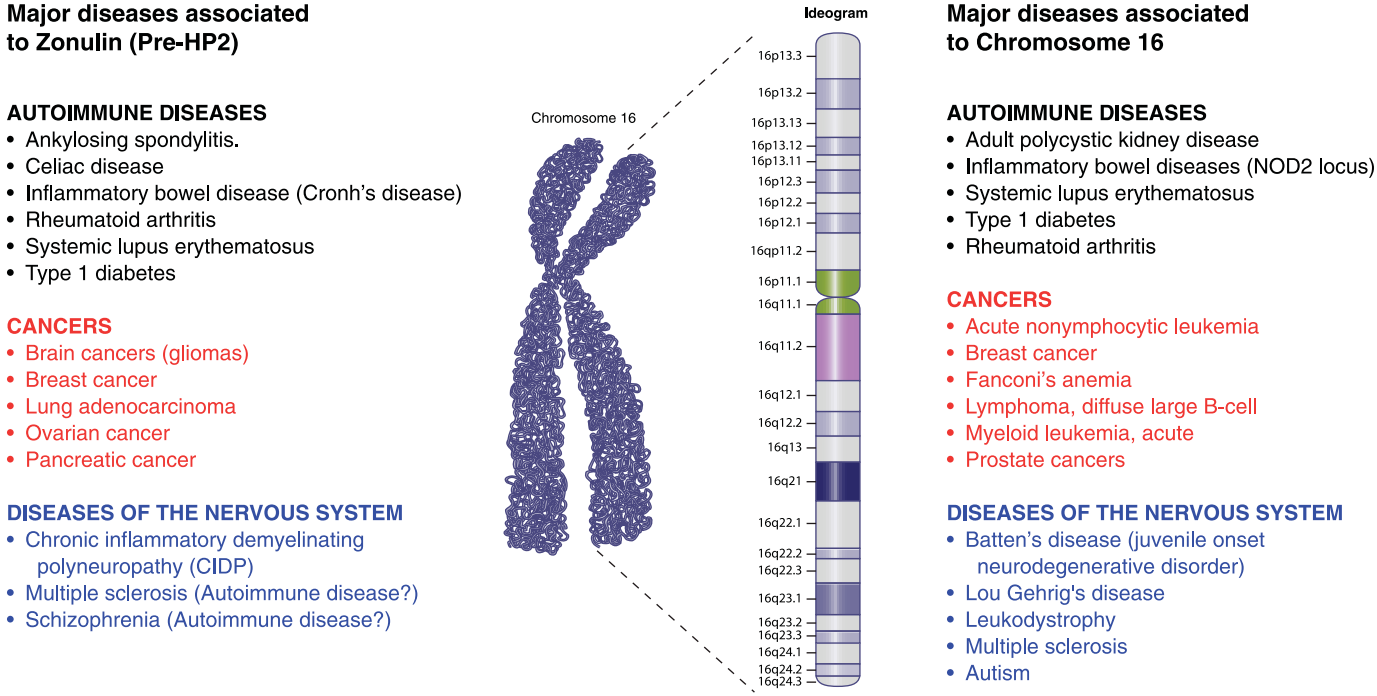

FIG. 11. Diseases associated with zonulin and chromosome 16. Diseases that have been proven, suspected, or related to zonulin whose gene is located on chromosome 16, as a biomarker include autoimmune diseases, cancers, and diseases of the nervous system. The same categories of diseases have been related to other genes located on chromosome 16.

genic peptide because it harbors 6 overlapping epitopes. Moreover, it is resistant to the enzymatic degradation by gastric acidity and pancreatic and brush-border peptidases. This peptide might reach the immune districts of intestinal mucosa in an intact and stimulatory form (146). Furthermore, the 33-mer peptide does not require further processing in antigen-presenting cells for T-cell stimulation because it binds to DQ2 molecules with a pH profile that promotes extracellular binding (134). The effect of the permeating gliadin peptides in vivo was confirmed by the analysis of intestinal tissues from patients with active CD and non-CD controls probed for zonulin expression (50). Quantitative immunoblotting of intestinal tissue lysates from active CD patients confirmed

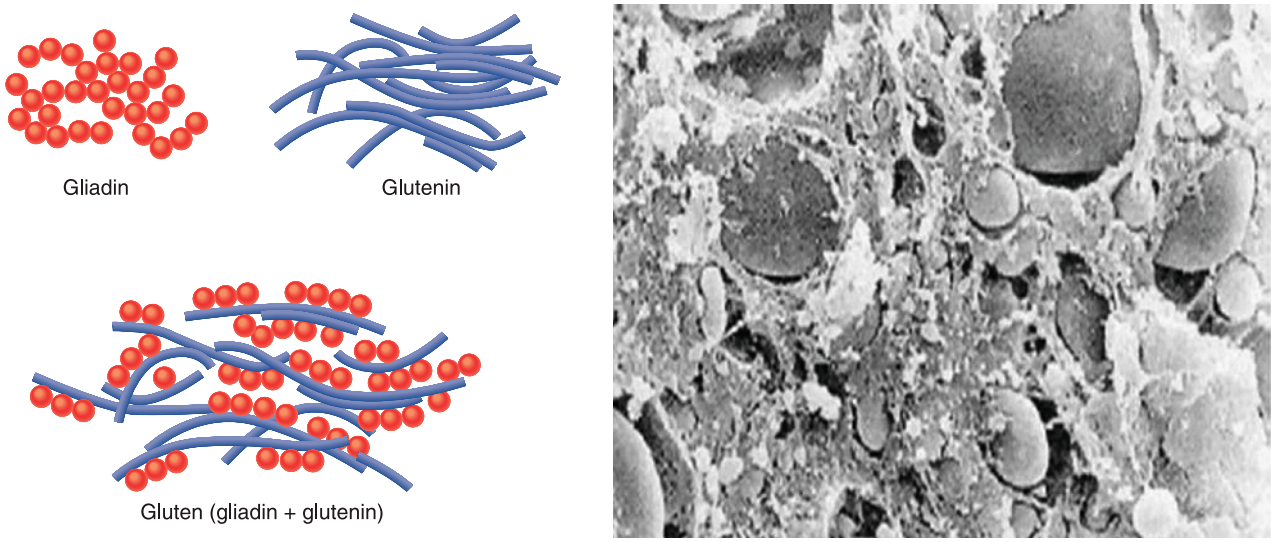

FIG. 12. Gluten structure. Gluten is composed by a mixture of two main proteins, gliadins and glutenins, both being toxic for celiac disease patients. Glutenin forms a meshwork of fibers in which globular gliadins are entrapped. On the *left*, cartoons show both class of proteins and how they interact. On the *right*, a scan electron micrograph shows the structural interaction between gliadins and glutenins.

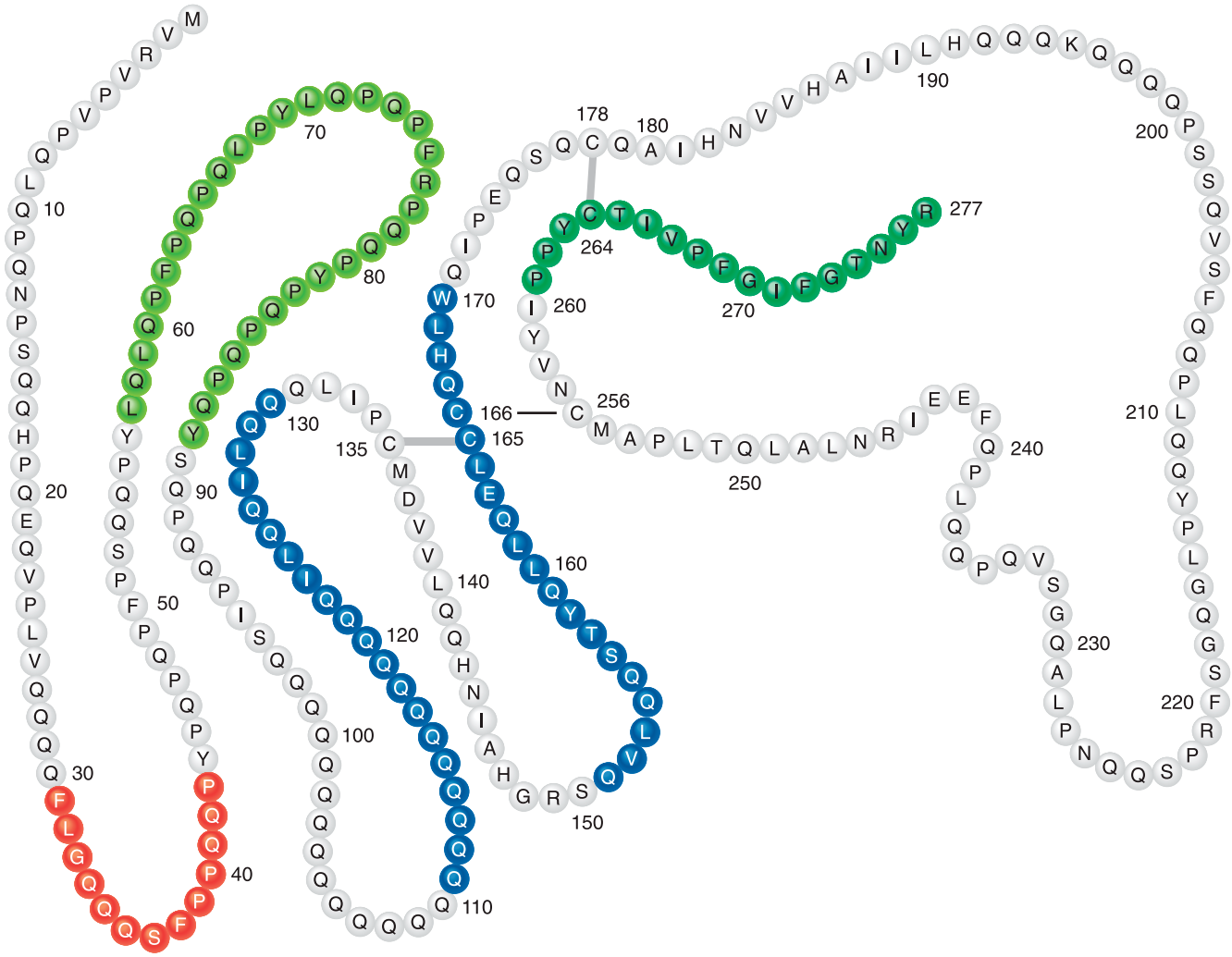

FIG. 13. Gliadin motifs. Mapping of α-gliadin motifs exerting cytotoxic activity (red), immunomodulatory activity (light green), zonulin release and gut-permeating activity (blue), and CXCR3-IL-8 release in CD patients (dark green).

the increase in zonulin protein compared with control tissues (50). Zonulin upregulation during the acute phase of CD was confirmed by measuring zonulin concentration in sera of 189 CD patients using a sandwich ELISA. Compared with healthy controls, CD subjects had higher zonulin serum concentrations ($P < 0.000001$) during the acute phase of the disease that decreased following a gluten-free diet (62).

Current data suggest that altered processing by intraluminal enzymes, changes in intestinal permeability, and activation of innate immunity mechanisms precede the activation of the adaptive immune response (56) (Fig. 14). Based on these data and on the gliadin epitope mapping described above, it is conceivable to hypothesize the following sequence of events: after oral ingestion, gliadin interacts with the small intestinal mucosa causing IL-8 release from enterocytes (peptide 261–277), so leading to immediate recruitment of neutrophils in the lamina propria. At the same time, gliadin-permeating peptides 111–130 and 151–170 initiate intestinal permeability through a MyD88-dependent release of zonulin (as we have recently confirmed by identifying CXCR3 as the receptor that releases zonulin in a MyD88-dependent manner, see Ref. 92) that enables paracellular translocation of gliadin and its subsequent interaction with macrophages (through 33-mer and other immunomodulatory peptides) within the intestinal submucosa (157). This interaction initiates signaling through a MyD88-dependent but TLR4- and TLR2-independent pathway, resulting in the establishment of a proinflammmatory (Th1-type) cytokine milieu (157) that results in mononuclear cell infiltration into the submucosa. The persistent presence of inflammatory mediators such as tumor necrosis factor (TNF)-α and interferon (IFN)-γ causes further increase in permeability across the endothelial and epithelial layers (161, 186), suggesting that the initial breach of the intestinal barrier function caused by zonulin can be perpetuated by

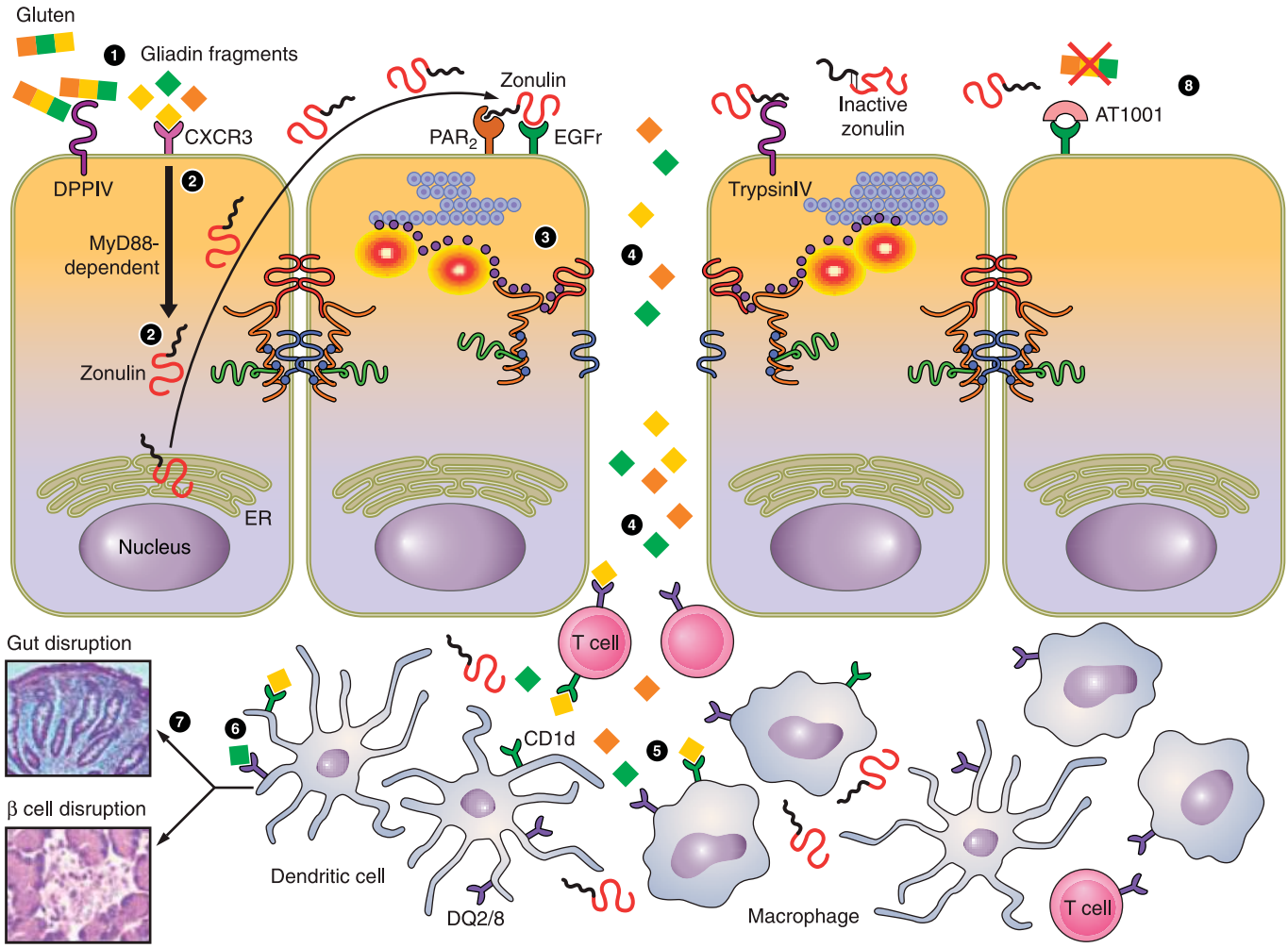

FIG. 14. Mechanisms of gliadin-induced zonulin release, increased intestinal permeability, and onset of autoimmunity. The production of specific gliadin-derived peptides by digestive enzymes causes CXCR3-mediated, MyD88-dependent zonulin release (*2*) and subsequent transactivation of EGFR by PAR_2 leading to small intestine TJ disassembly (*3*). The increased intestinal permeability allows non-self antigens (including gliadin) to enter the lamina propria (*4*), where they are presented by HLA-DQ, -DR molecules (*5*). The presentation of one or more gliadin peptides leads to abrogation of oral tolerance (switch to Th1/Th17 response) and a marked increase in peripheral immune responses to gliadin. Furthermore, gliadin-loaded dendritic cells migrate from the small intestine to mesenteric and/or pancreatic lymph nodes (*6*) where they present gliadin-derived antigens. This presentation leads to migration of $CD4^-CD8^-$ $\gamma\delta$ and $CD4^-CD8^+$ $\alpha\beta$ T cells to the target organ (gut and/or pancreas) where they cause inflammation (*7*). Implementation of a gluten-free diet or treatment with the zonulin inhibitor AT1001 (*8*) prevents the activation of the zonulin pathway and, therefore, of the autoimmune process targeting the gut or pancreatic β-cells.

the inflammatory process after the access of gliadin to the submucosa. In genetically predisposed individuals, this, in turn, may permit the interaction of T cells with antigen-presenting cells, including macrophages, leading ultimately to the antigen-specific adaptive immune response causing the autoimmune insult of the intestinal mucosa seen in patients with CD (81, 130, 143).

Once gluten is removed from the diet, serum zonulin levels decrease, the intestine resumes its baseline barrier function, the autoantibody titers are normalized, the autoimmune process shuts off and, consequently, the intestinal damage (that represents the biological outcome of the autoimmune process) heals completely.

2. T1D

The trigger of the autoimmune destruction of pancreatic β-cells in T1D is unknown. T1D has the same pathogenic challenges as other autoimmune diseases: what are the environmental triggers, and how do these triggers cross the intestinal barrier to interact with the immune system (57, 58)? Certain HLA class II alleles account for 40% of the genetic susceptibility to T1D in Caucasians (26, 158); however, the majority of individuals with these HLA alleles do not develop T1D. This supports the concept that reaction to some environmental products triggers autoimmune destruction of β-cells and leads to T1D. T1D is sometimes associated with other autoimmune diseases that are characterized by marked immunologic features,

such as CD and thyroiditis (36, 108). Gastrointestinal symptoms in T1D have been generally ascribed to altered intestinal motility (65) secondary to autonomic neuropathy (54). However, more recent studies have shown that altered intestinal permeability occurs in T1D prior to the onset of complications (40, 30), which is not the case in type 2 diabetes (145). This has led to the suggestion that an increased intestinal permeability due to alteration in intestinal TJ is responsible for the onset of T1D (40, 37, 118). This hypothesis is supported by studies performed in BioBreeding diabetic-prone (BBDP) rats that develop T1D spontaneously. In this animal model, an increased permeability of the small intestine (but not of the colon) preceded the onset of diabetes by at least a month (115). Furthermore, histological evidence of pancreatic islet destruction was absent at the time of increased permeability but was clearly present at a later time (115). Therefore, these studies provided evidence that increased permeability occurred before either histological or overt manifestation of diabetes in this animal model. We confirmed these data by reporting in the same rat model that zonulin-dependent increase in intestinal permeability precedes the onset of T1D by 2–3 wk (173). Oral administration of the zonulin inhibitor AT1001 to BBDP rats blocked autoantibody formation and zonulin-induced increases in intestinal permeability, so reducing the incidence of diabetes (173). These studies suggest that the zonulin-dependent loss of intestinal barrier function is one of the initial steps in the pathogenesis of T1D in the BBDP animal model of the disease. The involvement of zonulin in T1D pathogenesis was corroborated by our studies in humans showing that ~50% of T1D patients have elevated serum zonulin levels that correlated with increased intestinal permeability (138). We also provided preliminary evidence suggesting that, as in the BBDP rat model of the disease, zonulin upregulation precedes the onset of diabetes in T1D patients (138). Interestingly, a smaller percentage (~25%) of unaffected family members of probands with T1D have also been found to have increased serum zonulin levels and increased gut permeability (138), suggesting that loss of intestinal barrier function is necessary but not sufficient for the onset of the autoimmune process.

Several reports have linked gliadin (the environmental trigger of CD autoimmunity that also causes zonulin release from the gut, see Refs. 6 and 57) to T1D autoimmunity both in animal models and in human studies. Findings from studies using nonobese diabetic (NOD) mice and BBDP rats have implicated wheat gliadin as a dietary diabetogen (67, 144, 167, 168). In BBDP rats, gliadin exposure is accompanied by increased intestinal permeability (115) and zonulin release (168, 173), presumably allowing food antigens to come in contact with the underlying lamina propria. Feeding NOD mice and BBDP rats a gluten-free hydrolyzed casein diet resulted in a delay and reduction of T1D development (27, 28, 144, 167). Interestingly, in these animal models of T1D, the moment of exposure to wheat proteins was shown to be important for the development of T1D. Delaying the exposure to diabetogenic wheat proteins by prolonging the breastfeeding period reduced T1D development in the BBDP rats (167). Conversely, exposing neonatal rats or mice to diabetogenic wheat components or bacterial antigens reduced T1D incidence, which is probably due to the induction of immunological tolerance (67, 144).

Studies in humans showed that gliadin-specific, lamina propria-derived T cells play an important role in the pathogenesis of CD (97). The same class II MHC antigen, DQ (α 1*0501, β1*0201), that is associated with gliadin peptides in CD, is also one of two HLA class haplotypes inherited most frequently by people with T1D (2). There is also evidence of immunological activity in the small intestine of T1D patients: jejunal specimens from T1D patients have been found to contain significantly greater concentrations of IFN-γ- and TNF-α-positive cells than those of healthy controls, suggesting an inflammatory response (177). A second study found significantly greater expression of HLA-DR and HLA-DP molecules on intestinal villi of jejunal specimens from T1D patients than in specimens from healthy controls (140). A more recent report confirmed these findings by studying the mucosal immune response to gliadin in the jejunum of patients with T1D (7). Small intestinal biopsies from children with T1D were cultured with gliadin and examined for epithelial infiltration and lamina propria T-cell activation. The density of intraepithelial $CD3^+$ cells and of lamina propria $CD25^+$ mononuclear cells was higher in jejunal biopsies from T1D patients versus control subjects. In the patients' biopsies cultured with enzimatically treated gliadin, there was epithelial infiltration by CD3 cells, a significant increase in lamina propria $CD25^+$ and $CD80^+$ cells, enhanced expression of lamina propria cells positive for ligand and receptor molecules $\alpha4/\beta7$ and intracellular adhesion molecule (ICAM)-1, and increased expression of CD54 and crypt HLA-DR (7). $\alpha4$ Positive T cells were recovered from the pancreatic islets of a T1D patient (72), providing circumstantial evidence to support the hypothesis that $\alpha4+$T cells are involved in the destruction of the pancreatic islet cells.

More recently, we reported a direct link between antibodies to Glo-3a (a wheat-related protein), zonulin upregulation, and islet autoimmunity (IA) in children at increased risk for T1D (148). Sera from 91 IA positive cases and 82 controls were analyzed. Adjusting for age, family history of T1D, and HLA-DR4 positivity, Glo-3A antibody levels were inversely associated with breastfeeding duration and directly associated with current intake of foods containing gluten in IA cases but not in controls (148). Furthermore, zonulin was directly associated with Glo-3A antibody levels in cases but not in

controls, suggesting that the presence of Glo-3A antibodies and zonulin upregulation in IA cases are related to an underlying difference in mucosal immune response compared with controls.

3. Proof of the pathogenic role of zonulin-mediated intestinal barrier defect in CD and T1D

CD and T1D autoimmune models suggest that when the finely tuned trafficking of macromolecules is deregulated in genetically susceptible individuals, autoimmune disorders can occur (56). This new paradigm subverts traditional theories underlying the development of autoimmunity, which are based on molecular mimicry and/or the bystander effect, and suggests that the autoimmune process can be arrested if the interplay between genes and environmental triggers is prevented by reestablishing the intestinal barrier function. To challenge this hypothesis, zonulin inhibitor AT1001 was used with encouraging results in the BBDP rat model of autoimmunity (173) (see also sect. V*A2* above). In addition to preventing the loss of intestinal barrier function, the appearance of autoantibodies, and the onset of disease, pretreatment with AT1001 protected against the insult of pancreatic islets and, therefore, of the insulitis responsible for the onset of T1D (see Fig. 15).

This proof-of-concept in an animal model of autoimmunity provided the rationale to design human clinical trials in which AT1001 was initially tested in an inpatient, double-blind, randomized placebo-controlled trial to determine its safety, tolerability, and preliminary efficacy in CD patients (129). No increase in adverse events was recorded among patients exposed to AT1001 compared with placebo. Following acute gluten exposure, a 70% increase in intestinal permeability was detected in the

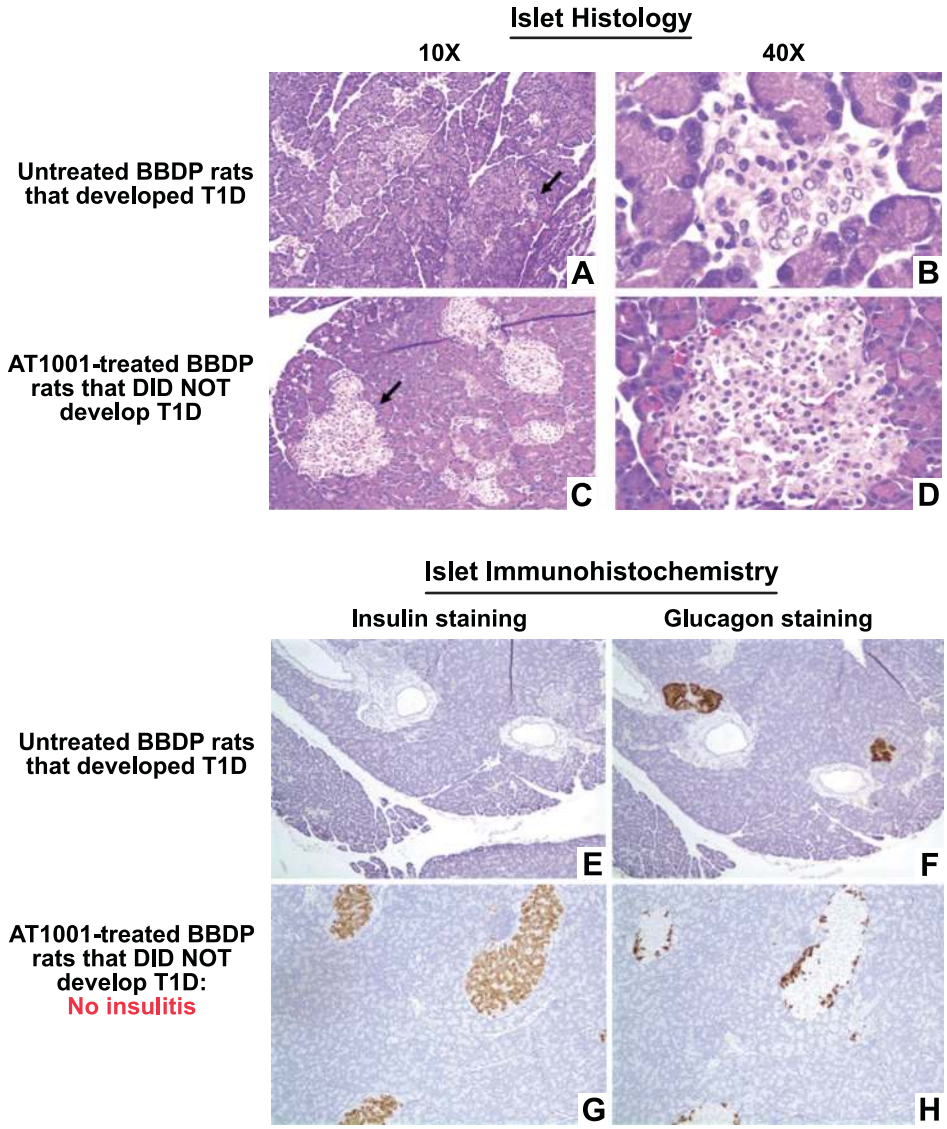

FIG. 15. AT1001 protects against insulitis in BB-Wor diabetes-prone rats. Histological analysis (*A–D*) and immunohistochemistry (*E–H*) of the pancreata isolated from both untreated BB-Wor diabetes-prone rats that developed T1D (*A*, *B*, *E*, and *F*) and AT1001-treated rats that did not develop T1D (*C*, *D*, *G*, and *H*). The islets indicated by the arrows in *A* and *C* (magnification ×10) are shown at higher magnification (×40) in *B* and *D*. Islets from rats that developed T1D showed the typical collapsed aspect with no insulin staining (*E*) and clusters of preserved glucagon-producing α-cells (*F*). Conversely, AT1001-treated animals showed undamaged islets producing both insulin (*G*) and glucagon (*H*).

placebo group, while no changes were seen in the AT1001 group (129). After gluten exposure, IFN-γ levels increased in 4 of 7 patients (57.1%) of the placebo group, but only in 4 of 14 patients (28.6%) of the AT1001 group. Gastrointestinal symptoms were significantly more frequent among CD patients of the placebo group compared with the AT1001 group (129). Combined, these data suggest that AT1001 is well tolerated and appears to reduce proinflammatory cytokine production and gastrointestinal symptoms in CD patients. AT1001 has now been tested in ~500 subjects with excellent safety profile and promising efficacy as concern protection against symptoms caused by gluten exposure in CD patients (85).

B. Other Possible Roles for Zonulin

1. Asthma

Asthma is a complex clinical syndrome characterized by airflow obstruction, airway hyperresponsive, and inflammation. The mechanisms by which airway inflammation and alterations in airway function are maintained remain incompletely understood. Because wheezing can also be triggered by food challenges in some asthmatic children, increased intestinal permeability of asthmatics (14, 76) may play a role in susceptibility to environmental allergens (87). Intestinal permeability can be altered as a result of exposure to antigens (82, 87). Therefore, correction of the gut barrier defect may be an additional novel approach for asthma treatment.

We have generated preliminary data suggesting that serum zonulin levels are high in a subset of subjects affected by asthma and that ~40% of asthmatic patients have an increased intestinal permeability (C. Blaisdell and A. Fasano, personal communication). This preliminary observation suggests that, beside inhalation, an alternative route for the presentation of specific antigens or irritants may occur through the gastrointestinal mucosal immune system following their paracellular passage (normally prevented by the intercellular TJ) (17, 18, 87).

2. Multiple sclerosis

In addition to an increase in blood-brain barrier permeability (38, 120), multiple sclerosis (MS) patients may also experience an increased permeability of intestinal TJ. Yacyshyn et al. (182) have demonstrated that 25% of MS patients studied had an increased intestinal permeability. The fact that patients with MS (182) and Crohn's disease (183) both present an increased number of peripheral B cells exhibiting CD45RO, a marker of antigen exposure, further supports the concept of preexisting, genetically determined small intestinal permeability abnormalities with subsequent altered antigen exposure as a pathogenic factor common to these diseases.

To challenge this hypothesis, we measured serum levels of zonulin in MS patients with different subtypes: relapsing-remitting (RRMS) versus secondary-progressive (SPMS) and activities to ascertain whether expression of zonulin into peripheral circulation can differentiate these two groups. Serum from 44 patients with RRMS (30 in relapse and 14 in remission), 18 patients with SPMS, and 171 controls were studied. The average age of MS patients was 35 years, and 65% of patients were female. All patients underwent neurological examination as well as brain MRI with contrast. Approximately 29% of patients with either relapsing RRMS or SPMS had elevated serum zonulin levels (a percentage similar to increased intestinal permeability in MS patients reported by Yacyshyn et al., see Ref. 182), with overall average serum levels ~2.0-fold higher than in controls (Fig. 16). Interestingly, patients with RRMS in remission showed serum zonulin levels comparable to controls (Fig. 16). Only in patients with RRMS were the highest levels of serum zonulin associated with the presence of gadolinium-enhancing lesions (A. Minagar and A. Fasano, personal communication).

3. Glioma

We have previously reported that the zonulin pathway is operative not only in the intestine but in other epithelial and endothelial districts, including airways

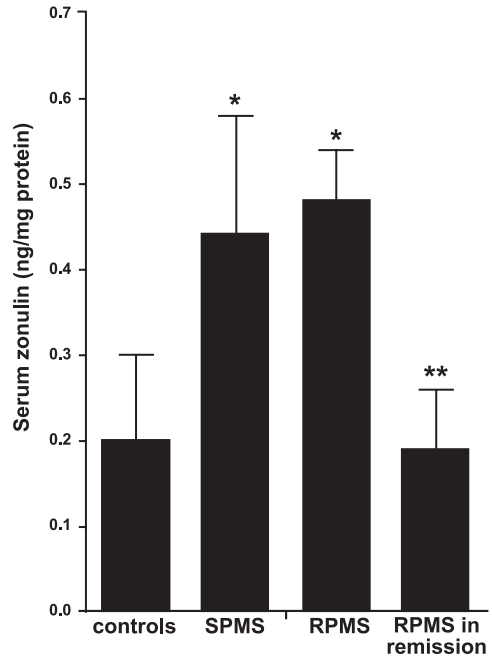

FIG. 16. Serum zonulin levels in subjects affected by different types of MS. Serum zonulin levels were assessed in MS subjects affected by different subtypes: relapsing-remitting (RRMS) during exacerbation of the disease ($n = 30$), RRMS in remission ($n = 14$), secondary-progressive (SPMS) ($n = 18$) and healthy controls ($n = 171$). Data are presented as means ± SE. *$P = 0.04$ compared with controls; **$P = 0.03$ compared with RRMS. These data were partially presented at the American Academy of Neurology 2004 annual meeting.

(110, 111, 137) and the blood-brain barrier (96, 84, 116). Because of its ubiquitous distribution and its function, we hypothesized that dysregulation of the zonulin pathway may contribute to disease states that involve disordered intercellular communication, including malignant transformation and metastasis. This theory has been recently corroborated by Skardelly et al. (150) that reported an increase in zonulin expression in gliomas. The authors reported that the increased expression of c-*kit*, a cancer and degeneration marker of gliomas, was associated with an increase of zonulin expression, and both correlated with the degree of malignancy of human brain cancers (150). The expression of zonulin also correlated with the degradation of the blood-brain barrier that paralleled the severity of the neoplastic (150).

4. Inflammation

A) ANIMAL MODELS OF GUT INFLAMMATION. In some models of inflammatory bowel diseases (IBD), increased permeability appears to be a very early event. The senescence accelerated prone mouse (SAMP) (125) and the mouse downregulated in adenoma (mdra) deficient mouse (135) show increased small intestinal permeability well before disease expression. The IL-10 gene-deficient mouse spontaneously develops colitis after 12 wk of age (102). Similar to the SAMP and mdra mice, IL-10$^{-/-}$ mice have been shown to have increased small intestinal permeability that appears early in life (102). Furthermore, the development of colitis is dependent on luminal microorganisms, as animals do not develop disease if raised under germ-free conditions. Arrietta et al. (5) recently demonstrated that a zonulin-dependent break in small intestinal barrier function is necessary for colitis to occur in the IL-10$^{-/-}$ mouse. Animals showed an increase in small intestinal permeability as early as 4 wk of age that was corrected by treatment with the zonulin inhibitor AT1001 (5). At 8 wk of age, treated animals showed a significant reduction of colonic mucosal permeability, and by 17 wk of age, secretion of neoplastic TNF-α from a colonic explant was significantly lower in AT1001-treated animals compared with untreated controls (5). All other markers also demonstrated a clear reduction of colitis in the AT1001-treated animals. Combined, these data demonstrate that a zonulin-dependent break in small intestinal barrier function is necessary for colitis to occur in the IL-10$^{-/-}$ mouse. This study extends previous reports in an important manner by demonstrating that reversal of the zonulin-dependent barrier defect in the small intestine can attenuate the inflammation in the colon, implying that the increased permeability is not simply an epiphenomenon but rather is an important etiological event that can cause inflammation in a district distant from where the breach in intestinal barrier occurs.

B) INFLAMMATORY BOWEL DISEASES. The pathogenesis of IBD remains unknown, although in recent years there is convincing evidence to implicate genetic, immunological, and environmental factors in initiating the autoimmune process. Several lines of evidence, however, suggest that an increased intestinal permeability plays a central role in the pathogenesis of IBD (57, 58, 161, 174, 181). Like CD, IBD may be related to an innate immune deficiency, leading to the inappropriate access of non-self antigens to the gut-associated lymphoid tissue (GALT). In clinically asymptomatic Crohn's disease patients, increased intestinal epithelial permeability precedes clinical relapse by as much as 1 yr, suggesting that a permeability defect is an early event in disease exacerbation (160, 174). The hypothesis that abnormal intestinal barrier function is a genetic trait involved in the pathogenesis of IBD is further supported by the observation that clinically asymptomatic first-degree relatives of Crohn's disease patients may have increased intestinal permeability (174, 181). We have recently generated evidence suggesting that zonulin upregulation is detectable in the acute phase of IBD and that its serum levels decrease (but still are higher than normal) once the inflammatory process subsides following specific treatment (J. Bai and A. Fasano, personal communication). While a primary defect of the intestinal barrier function (possibly secondary to activation of the zonulin pathway) may be involved in the early steps of the pathogenesis of IBD, the production of cytokines, including IFN-γ and TNF-α, secondary to the inflammatory process serve to perpetuate the increased intestinal permeability by reorganizing TJ proteins ZO-1, junctional adhesion molecule 1, occludin, claudin-1, and claudin-4 (3, 170, 171). In this manner, a vicious cycle is created in which barrier dysfunction allows further leakage of luminal contents, thereby triggering an immune response that in turn promotes further leakiness.

C. Diseases in Which Zonulin Has Been Identified as a Biomarker

A sytematic review of the literature revealed that HP precursor [that we identified as zonulin (159) and, therefore, the two terms can be used interchangeably] has been reported as a biomarker of several pathological conditions, including autoimmune diseases, diseases of the nervous system, and neoplastic conditions. Interestingly, genes related to these three classes of pathological states have been mapped on chromosome 16 (8, 33, 48, 69, 80, 86, 112, 121, 165), the chromosome where zonulin gene is located (Fig. 11).

1. Autoimmune diseases

Using a proteomic approach, Liu et al. (95) have identified HP as an ankylosing spondylitis-associated pro-

tein. The authors investigated the serum protein profiles of ankylosing spondylitis patients and healthy controls from a large Chinese ankylosing spondylitis family using two-dimensional electrophoresis analysis. A group of four highly expressed protein spots was observed in all ankylosing spondylitis patients' profiles and subsequently identified as isoforms of HP by ESI-Q-TOF MS/MS (95). Increased expression of HP was also observed in sera of sporadic ankylosing spondylitis patients. Moreover, bioinformatics analysis revealed epitopes derived from HP with high-affinity binding to HLA-B(*)2705, a primary subtype associated with ankylosing spondylitis. Based on their results, the authors speculated that HP may be involved in the pathogenesis of ankylosing spondylitis. More recently, Li et al. (94) obtained similar results by analyzing sera from rheumatoid arthritis patients.

The association of HP polymorphism with the risk and clinical course of different inflammatory diseases prompted Papp et al. (128) to investigate the HP distribution among patients affected by inflammatory diseases. Their finding suggests that HP is more frequently expressed in patients affected by sclerosing cholangitis.

2. Diseases of the nervous system

In a study focused on detecting schizophrenia-related changes of plasma proteins, Wan et al. (169) used proteomic technology to examine the relation between schizophrenia and HP genotype. The authors investigated plasma proteins from schizophrenic subjects and healthy controls by two-dimensional gel electrophoresis in combination with mass spectrometry. To further reveal the genetic relationship between acute phase proteins and schizophrenia disease, they tested HP α1/HP α2 (i.e., zonulin) polymorphism and two single nucleotide polymorphisms (SNPs) of HP, rs2070937 and rs5473, for associations with schizophrenia. The authors found that four proteins in the family of positive acute phase proteins were all upregulated in patients. In a genetic association study, the authors found significant associations existing between schizophrenia and polymorphisms related to the HP gene (169). Schizophrenia is accompanied by both an altered expression of HP and a different genotype distribution of HP gene, demonstrating that HP is associated with schizophrenia. The authors concluded that their results from proteomic and genomic aspects both indicate that acute phase reaction is likely to be an etiological agent in the pathophysiology of schizophrenia, rather than just an accompanying symptom. Similar results were previously reported by Maes et al. (104) that examined HP phenotypic and genotypic frequencies in 98 Northwestern Italian schizophrenic patients compared with healthy controls. The frequency of the HP gene resulted significantly higher in schizophrenic patients compared with controls (104). Alterations in HP phenotypic and genotypic distribution in favor of HP genotype were more pronounced in the schizo-affective, disorganized, undifferentiated, and residual schizophrenic patients than in paranoid schizophrenic patients (104).

To better understand the pathophysiology of the mechanisms underlying neuromyelitis optica, Bai et al. (9) developed a proteomics platform for biomarker discovery in the cerebrospinal fluid of patients affected by this clinical condition. Two-dimensional electrophoresis and matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) were used to compare the cerebrospinal fluid proteome of patients affected by neuromyelitis optica with that of controls. Subsequent ELISA and western blot analyses were performed to verify the results of the proteomic analysis. HP was identified as one of the four proteins that the authors found to be enhanced in the neuromyelitis optica group compared with controls. Increased levels of HP were also detected in the cerebrospinal fluid of patients affected by MS (156) and in the serum of Guillain Barré syndrome subjects (71).

3. Neoplastic diseases

Using a combination of a mouse model of oral squamous cell carcinoma and human plasma samples obtained from 52 subjects affected by the disease, Lai et al. (90) searched for specific biomarkers for this type of cancer. The authors applied a proteomics approach, immunoblot and immunohistochemical analysis, and ELISA to validate the expressed biomarkers in mice and patients affected by oral squamous cell carcinoma (90). Proteomic profiling of mouse plasma samples indicated that HP was upregulated in mice with the disease. Immunoblotting of plasma samples and immunohistochemical analysis of oral tissues showed a significantly higher level of HP in oral squamous cell carcinoma mice compared with control mice (90). The expression of HP in human plasma samples from 52 patients with oral squamous cell carcinoma indicated a strong correlation between the increasing levels of HP and the clinical stages of oral squamous cell carcinoma (90).

A proteomic approach was also used by Dowling et al. (49) to search for specific biomarkers for lung cancers. Most lung cancers are diagnosed too late for curative treatment to be possible; therefore, early detection is crucial. Serum proteins have the potential to be used as diagnostic and prognostic indicators for lung cancer. To examine differences in serum levels of specific proteins associated with human lung squamous carcinoma, the authors performed a two-dimensional difference gel electrophoresis analysis and subsequent mass spectroscopic identification to generate a panel of proteins found to be differentially expressed between the cancer and normal samples. Among others, they identified HP as one of the

protein biomarkers upregulated in lung cancer patients (49). Similar results were obtained by Heo et al. (74) that compared the serum glycoproteome of healthy and lung adenocarcinoma individuals, so identifying several cancer-selective proteins that have been previously characterized as potential indicators of lung cancer in serum or plasma, including HP.

Identification of new biomarkers for pancreatic carcinoma was the goal of Sun et al. (153) that applied a proteomic approach to compare serum protein expression patterns of pancreatic carcinoma patients with that of gastric cancer patients, other pancreatic disease patients, and healthy volunteers. By two-dimensional gel electrophoresis analyses and mass spectroscopic identification, they found five proteins, including HP, to be significantly changed in pancreatic carcinoma (153).

Finally, Kotaka et al. (88) carried out suppression subtractive hybridization to identify variable expression of genes linked to hepatocellular carcinoma with Hepatitis C virus (HCV) infection. The authors isolated RNA from both cancerous (tester) and noncancerous (driver) liver tissues and subjected the cDNA clones to MegaBACE PCR sequencing to identify those that hybridized to the subtracted library with preference. Nucleic acid sequences generated were searched against the human UniGene database. Among 576 clones screened in the neoplasticous liver tissue, the authors identified 30 genes and 28 expressed sequence tags. Among 30 genes detected, 23 were with known functions including gene previously known to be cancer-related, and those most frequently appearing were HP and HP-related protein (88). Based on these results, the authors concluded these genes may contribute to carcinogenesis caused by DNA-damaged agents.

VI. CONCLUSIONS

The gastrointestinal tract has been extensively studied for its digestive and absorptive functions. A more attentive analysis of its anatomo-functional characteristics, however, clearly indicates that its functions go well beyond the handling of nutrients and electrolytes. The exquisite regional-specific anatomical arrangements of cell subtypes and the finely regulated cross-talk between epithelial, neuroendocrine, and immune cells highlights other less-studied, yet extremely important, functions of the gastrointestinal tract. Of particular interest is the regulation of antigen trafficking by the zonulin pathway and its activation by intestinal mucosa-microbiota/gluten interactions. These functions dictate the switch from tolerance to immunity and are likely integral mechanisms involved in the pathogenesis of inflammatory and neoplastical processes.

The classical paradigm of inflammatory pathogenesis involving specific genetic makeup and exposure to environmental triggers has been challenged recently by the addition of a third element, the loss of intestinal barrier function. Genetic predisposition, miscommunication between innate and adaptive immunity, exposure to environmental triggers, and loss of intestinal barrier function secondary to the activation of the zonulin pathway by food-derived environmental triggers or changes in gut microbiota all seem to be key ingredients involved in the pathogenesis of inflammation, autoimmunity, and cancer. This new theory implies that once the pathological process is activated, it is not auto-perpetuating. Rather, it can be modulated or even reversed by preventing the continuous interplay between genes and the environment. Since zonulin-dependent TJ dysfunction allows such interactions, new therapeutic strategies aimed at reestablishing the intestinal barrier function by downregulating the zonulin pathway offer innovative and not-yet-explored approaches for the management of these debilitating chronic diseases.

ACKNOWLEDGMENTS

Address for reprint requests and other correspondence: A. Fasano, Univ. of Maryland School of Medicine, Mucosal Biology Research Center, Health Science Facility II, Rm. S345, 20 Penn St., Baltimore, MD 21201 (e-mail: afasano@mbrc.umaryland.edu).

GRANTS

Some of the data presented in this review were generated with the partial supports of the National Institutes of Health Grants DK048373, DK078699, and AT004089 (to A. Fasano).

DISCLOSURES

A. Fasano has a financial interest in Alba Therapeutics.

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Alessio Fasano

Physiol Rev 91:151-175, 2011. doi:10.1152/physrev.00003.2008