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# Tissue Transglutaminase-Mediated Formation and Cleavage of Histamine-Gliadin Complexes: Biological Effects and Implications for Celiac Disease[1]

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**Celiac disease is an HLA-DQ2-associated disorder characterized by an intestinal T cell response. The disease-relevant T cells secrete IFN-γ upon recognition of gluten peptides that have been deamidated in vivo by the enzyme tissue transglutaminase (transglutaminase 2 (TG2)). The celiac intestinal mucosa contains elevated numbers of mast cells, and increased histamine secretion has been reported in celiac patients. This appears paradoxical because histamine typically biases T cell responses in the direction of Th2 instead of the Th1 pattern seen in the celiac lesions. We report that histamine is an excellent substrate for TG2, and it can be efficiently conjugated to gluten peptides through TG2-mediated transamidation. Histamine-peptide conjugates do not exert agonistic effects on histamine receptors, and scavenging of biologically active histamine by gluten peptide conjugation can have physiological implications and may contribute to the mucosal IFN-γ response in active disease. Interestingly, TG2 is able to hydrolyze the peptide-histamine conjugates when the concentrations of substrates are lowered, thereby releasing deamidated gluten peptides that are stimulatory to T cells.** *The Journal of Immunology,* **2005, 174: 1657–1663.**

Celiac disease (CD)[3] is a chronic inflammatory disease of the small intestine caused by the ingestion of proline- and glutamine-rich wheat gluten or related proteins from rye and barley. The lesion is characterized by villous atrophy, crypt hyperplasia, and infiltration of lymphoid cells both in the epithelium and in the lamina propria. CD demonstrates a strong genetic association with the genes coding for HLA-DQ2 and, to a lesser extent, -DQ8. Gluten-reactive $CD4^+$ T cells can be isolated from the small intestine of CD patients (but not from controls), and these cells predominantly recognize gluten peptides in which glutamine residues at certain positions have been converted to glutamate residues by tissue transglutaminase (transglutaminase 2 (TG2)), the very same enzyme that is the target of disease-specific serum autoantibodies in untreated celiac patients (1). The T cells specific for gluten peptides deamidated in situ by TG2 are believed to play an essential role in the pathogenesis of CD. Increased epithelial permeability and increased influx of gluten peptides to the lamina propria may be part of the anti-gluten immune response in CD (1).

TG2 belongs to a family of structurally and functionally related enzymes that catalyze $Ca^{2+}$-dependent transamidation or deamidation reactions. In the transamidation reaction, an acyl residue, derived from the γ-carboxamide group of a peptide-bound glutamine (the acyl donor), is transferred to an appropriate primary amine, either the ε-amino group of a lysine residue or a small biogenic amine molecule, such as putrescine, spermine, spermidine, or histamine (the acyl acceptor). After the initial formation of a thioester bond, either the activated acyl group is transferred to the acyl acceptor amine forming an amide bond (transamidation), or the thioester bond is hydrolyzed, leading to deamidation of glutamine into glutamate. The sequence specificity of TG2 toward acyl donor substrates was recently elucidated (2, 3), and studies on the affinity of TG2 for various gluten (e.g., gliadin) peptides suggested that TG2 is directly involved in the selection of gluten T cell epitopes in CD.

All of the naturally occurring primary amines, including histamine, have a broad range of biological functions. The effects of histamine are mediated through cell surface receptors categorized as histamine 1 receptor (H1R), H2R, H3R, and H4R. Histamine released from granulae in mast cells and basophils has long been recognized as an effector molecule in allergic and inflammatory reactions. These effects are mainly mediated by the binding of histamine to H1R on endothelial cells. More recently, it has been shown that histamine can regulate both T cell and Ab responses. These effects on adaptive immunity are regulated through differential expression of H1R and H2R on Th1- and Th2-type Th cells (4). In this respect, it is interesting that the *H1R* gene has been identified as a susceptibility locus for autoimmune murine diseases (experimental allergic encephalomyelitis and autoimmune orchitis) in which deregulated T cells are critically involved (5). Histamine receptors are also expressed by dendritic cells (DC) (6, 7), and it has been demonstrated that the cytokine secretion profiles of both monocyte-derived and plasmacytoid DC are affected by histamine. Histamine can thus, in addition to its direct effect on T cells, modulate the Th1/Th2 differentiation of T cells indirectly via effects on cytokine secretion by DC (6–8).

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[3] Abbreviations used in this paper: CD, celiac disease; TG2, transglutaminase 2 (tissue transglutaminase); H1R, histamine 1 receptor; DC, dendritic cell; GDH, glutamate dehydrogenase; CE, capillary electrophoresis; TCC, T cell clone.



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The number of mast cells, the principal cells in which histamine is stored, is increased in the intestinal mucosa of active CD patients (9). Moreover, histamine has been suggested to be a suitable substrate for TG2 (10), and histamine was found to be bound to mast cell proteins via the transglutaminase activity expressed in these cells (11). This prompted us to investigate how efficiently histamine is conjugated to gluten peptides by TG2, and to look at possible biological functions of these conjugates.

We report that histamine is an excellent acyl acceptor of the TG2-mediated transamidation of gluten peptides. This transamidation process results in the formation of histamine and gluten peptide conjugates. We find that these histamine-peptide conjugates do not stimulate either H1R or H2R. The histamine-peptide conjugation thus results in scavenging of bioactive histamine. When the glutamine residues involved in the histamine-peptide conjugate are located within the core region of T cell epitopes, we find that the conjugates lose their T cell-stimulatory effect. Interestingly, TG2 is also able to hydrolyze the isopeptide bonds of these conjugates resulting in the release of free histamine and active, deamidated T cell epitope peptides. Sustained release of bioactive histamine and deamidated T cell-stimulatory peptides may play a role in shaping of the anti-gluten immune response in CD.

## Materials and Methods

### *Reagents and peptides*

Histamine dihydrochloride (H7250), *N*-acetyl lysine methyl ester (859095), LPS (from *Escherichia coli*; 62326), hydrocortisone (H0135), and cycloheximide (R750107) were obtained from Sigma-Aldrich. Putrescine, spermine, and 5-hydroxytryptamine were from VWR Scientific, and spermidine was from Fisher Scientific. Glutamate dehydrogenase (GDH) was from Biozyme, dexchlorpheniramine was from Nycomed Pharma, and ranitidine was from GlaxoWellcome. Human rIL-4, GM-CSF, IL-1$\beta$, epidermal growth factor, DuoSet IL-12p70 ELISA set, and IL-8 ELISA reagents were obtained from R&D Systems.

Synthetic native peptides corresponding to the epitopes DQ2-$\alpha$I (LQLPF PQPQLPY, hereafter defined as $\alpha$I), DQ2-$\alpha$II and -$\alpha$III (PQPQLPYPQ PQLPY; $\alpha$II/$\alpha$III), as well as a deamidated peptide containing the DQ2-$\alpha$III epitope (PQPQLPYPQPELPY; $\alpha$II/$\alpha$III;E72) were obtained from Prof. G. Jung (University of Tübingen, Tübingen, Germany). The $\alpha$II/$\alpha$III peptide used in experiments in Figs. 3 and 6, and the Ac-PQPQLPF-$NH_2$ and Ac-PQPELPF-$NH_2$ peptides used for the amine specificity and hydrolysis reaction kinetics, were synthesized using standard Boc chemistry at the Stanford Protein and Nucleic Acid Biotechnology facility. All peptides were purified by reverse-phase HPLC, and their identities were confirmed by mass spectrometry. For fluorescein labeling of the $\alpha$I peptide, carboxyfluorescein (Sigma-Aldrich) was coupled to the free N-terminal amino group of the resin-bound peptide using diisopropylcarbodiimede as coupling reagent. Recombinant human TG2 were expressed as either GST (GST-TG2) or hexa-His ($His_6$-TG2) fusion protein in *E. coli* and purified as described previously (12, 13).

### *Kinetics of TG2-catalyzed transamidation*

The activity of the $His_6$-TG2 was measured by a coupled assay previously developed by Keillor and Day (14), where GDH couples the ammonium ion from the TG2-catalyzed deamidation to $\alpha$-ketoglutarate to yield glutamate. The concomitant oxidation of NADH to $NAD^+$ can be spectrophotometrically monitored at 340 nm:

$$\text{NADH} + NH_4^+ + \alpha\text{-ketoglutarate} \rightarrow \text{glutamate} + NAD^+ + H_2O$$

In the standard assay, the following freshly prepared stock solutions were used: 1) MOPS buffer solution: a 5× stock of 200 mM MOPS, 5 mM $CaCl_2$, 1 mM $Na_4$EDTA, and 10 mM $\alpha$-ketoglutarate (pH 7.2); 2) NADH solution: NADH (1.25 mM); and 3) GDH solution: 0.2 U/$\mu$l GDH in MOPS buffer. A volume of 25 $\mu$l of the NADH stock solution was combined with 40 $\mu$l of the GDH stock solution, 400 $\mu$M Ac-PQPQLPF-$NH_2$, the specified amount of amine, and water to a final volume of 170 $\mu$l. The reaction was started by adding $His_6$-TG2 (10 $\mu$g in a final volume of 30 $\mu$l), and NADH consumption was monitored at 22°C for 5 min. The reaction velocity was calculated from the linear portion of the progress curve, assuming a molar extinction coefficient for NADH of 6220 $M^{-1}cm^{-1}$. The parameters $k_{cat}$ and $K_M$ were calculated by fitting $v$ vs [$S$] data to the Michaelis-Menten equation assuming $v_{total} = v_{hydrolysis} + v_{transamidation}$ and $v_{hydrolysis}$ is constant. Errors in specificity, as presented, indicate the error associated with fitting data to Michaelis-Menten equation.

### *Kinetics of TG2-catalyzed hydrolysis of the conjugates*

Ac-PQP(E-Histamine)-LPF-$NH_2$ and Ac-PQP(E-Putrescine)-LPF-$NH_2$ were used to determine the kinetics of histamine and putrescine removal by TG2 treatment (i.e., the reverse transamidation reaction). The reaction was similar to above with Ac-PQPQLPF-$NH_2$ being replaced by Ac-PQP(E-Histamine)-LPF-$NH_2$ or Ac-PQP(E-Putrescine)-LPF-$NH_2$, and the velocity was calculated by measuring the disappearance of starting material using reverse-phase HPLC. Histamine was conjugated to Ac-PQPELPF-$NH_2$ at the glutamate residue by reacting 1 equivalent of Ac-PQPELPF-$NH_2$ with 1 equivalent of histamine, 1.2 equivalents of (1-(3-dimethylaminopropyl))-3-ethylcarbodiimide hydrochloride, 1.2 equivalents of 1-hydroxybenzotriazole hydrate, and 3.5 equivalents of diisopropylethylamine, all in dimethylformamide overnight at room temperature. Putrescine was conjugated to Ac-PQPQLPF-$NH_2$ by reacting with human $His_6$-TG2 and 5-fold excess putrescine at 30°C for 30 min and then purified using analytical HPLC.

### *Capillary electrophoresis (CE) analysis of histamine-peptide conjugates*

CE was performed on a Beckman MDQ CE system equipped with a laser-induced fluorescence detector (488 nm) as described in Ref. 3. Fluorescein-labeled $\alpha$I peptide (Fluo-$\alpha$I) was incubated at 100 $\mu$M with 2 $\mu$M $His_6$-TG2 in 100 mM Tris-HCl (pH 7.4), 2 mM $CaCl_2$, and three different concentrations of histamine (100, 50, or 20 $\mu$M). Fresh $His_6$-TG2 was added repeatedly every 2 h. After different time points, 1.5 $\mu$l of each sample was added to 8.5 $\mu$l of freshly prepared 40 $\mu$M iodoacetamide solution (Sigma-Aldrich I6125) to abolished enzymatic activity. Before CE analysis, the aliquots were diluted 1/300 in running buffer (80 mM sodium borate (pH 7.5)). The concentration of the conjugate was quantified by the area under the corresponding peak in the electropherogram. The presence of Fluo-$\alpha$I-histamine conjugates was furthermore confirmed by MALDI-TOF mass spectrometry (Ultraflex; Bruker-Daltonik).

### *Production and purification of histamine-peptide conjugates*

Histamine conjugates of PQPQLPYPQPQLPY ($\alpha$II/$\alpha$III peptide; the underlined glutamine residues are targeted by TG2) in Fig. 1, and histamine conjugates with the peptides LQLQPFPQPQLPYPQPQLPYPQPQLPY PQPQPF, VSFQQPQQQYPSSQ, PQQPQQSFPQQQRP, and LGQQQPF PPQQPYPQPQPF were made by reacting the specified peptide with human $His_6$-TG2 and 20-fold excess histamine for 30 min at 30°C and then purified by preparative reverse-phase HPLC. This purification method does not distinguish between conjugates with either one or several histamine molecules per peptide. As a control, PQPQLPYPQPQLPY was combined with 20-fold excess histamine without TG2 treatment and purified exactly like the histamine-conjugated peptides.

Histamine-peptide conjugates in Figs. 3*B* and 6 were generated by reacting the $\alpha$II/$\alpha$III peptide (PQPQLPYPQPQLPY) with human $His_6$-TG2 and either 5- or 20-fold excess histamine for 30 min at 30°C. The conjugates were then purified by HPLC, according to the number of histamine molecules attached to each $\alpha$II/$\alpha$III peptide. The resultant peptide-histamine conjugates have either one histamine attached per peptide (×1 hist) at either one of the TG2-targeted glutamine residues; or two histamine molecules per peptide (×2 hist), with histamine attached to both of the targeted glutamine residues. The numbers of histamine molecules conjugated to each peptide were assessed by mass spectrometry.

Histamine-peptide conjugates in Fig. 2 were generated by incubating 1 mM either $\alpha$I peptide (LQLPFPQPQLPY, glutamine residue targeted by TG2 underlined) or $\alpha$II/$\alpha$III peptide with 10 mM histamine in the presence of 10 $\mu$M GST-TG2, in TBS buffer (50 mM Tris, 150 mM NaCl (pH 7.3)) with 5 mM $Ca^{2+}$ for 90 min at 37°C. After the incubation, the samples were subjected to size exclusion fast protein liquid chromatography on a Superdex 200 (Amersham Biosciences) column. The peptide fraction (containing both the modified peptides and histamine-peptide conjugates) was collected.

### *Assessment of histamine activity by the AequoScreen assay*

Lyophilized histamine-conjugated peptides were submitted to Euroscreen for H1R agonist activity using the AequoScreen system. The peptide conjugates were suspended in 10 mM DMSO, diluted to specific concentrations, and then tested in duplicate by incubation with the ES-390-A cell line expressing rH1R. Receptor activation results in intracellular $Ca^{2+}$ release, which leads to the luminescence of mitochondrial aequorin, a calcium-sensitive photoprotein. The emission of light at 466 nm was measured by

Hamamatsu FDSS 6000 (Hamamatsu Photonics). Histamine was used as a reference.

### *Assessment of histamine activity by secretion of IL-8 in endothelial cells*

HUVECs were isolated as described (15) and cultured in MCDB 131 medium (Sigma-Aldrich) containing 7.5% FCS, 10 ng/ml epidermal growth factor, 1 $\mu$g/ml hydrocortisone, 50 $\mu$g/ml gentamicin, and 0.25 $\mu$g/ml amphotericin B. Cells were grown to confluence in 96-well trays. Cells were activated with 100 U/ml IL-1$\beta$ for 24 h, washed, and then treated with the protein synthesis inhibitor cycloheximide (1 mg/ml) for 2 h. Cycloheximide reduces the constitutive IL-8 secretion from HUVEC (16). In some wells, simultaneous with the cycloheximide treatment, cells were pretreated with various concentrations of histamine receptor blockers. Cells were washed twice and subsequently incubated in culture medium in the presence of the following: 100 $\mu$M purified histamine-peptide conjugates; or 100 $\mu$M histamine, with or without the presence of various concentrations of the H1R antagonist dexchlorpheniramine (1 or 10 $\mu$M), or the H2R antagonist ranitidine (10 or 100 $\mu$M). Cell culture supernatants were collected after 20-min incubation at 37°C, and analyzed for IL-8 in ELISA with a detection limit of at least 30 pg/ml.

### *Assessment of histamine activity by secretion of IL-12p70 in DC*

PBMC were isolated from healthy volunteers by standard density gradient centrifugation on Lymphoprep (Axis-Shield). Monocytes were purified from PBMC by positive selection using a magnetic cell separator (MACS; Miltenyi Biotec) with CD14 beads according to the manufacturer's instructions. Monocytes were cultured in RPMI 1640 supplemented with 10% FCS, 200 U/ml penicillin, 100 $\mu$g/ml streptomycin, 1000 U/ml GM-CSF, and 500 U/ml IL-4 at $1 \times 10^6$ cells/1 ml/well in 24-well tissue culture plates. On days 6–7, DC were assessed with FACS (>95% $CD11c^+$) and recultured in cytokine-depleted medium with 100 ng/ml LPS (from *E. coli*), at $10^5$ cells/100 $\mu$l/well in 96-well flat-bottom tissue culture plates. In triplicate, DC were exposed to various concentrations of histamine, purified histamine-peptide conjugates, or histamine preincubated with peptides and TG2. After overnight incubation at 37°C, supernatants were collected and assayed for IL-12p70 in ELISA with a detection limit of at least 30 pg/ml.

### *Human T cell assays*

Purified histamine-$\alpha$II/$\alpha$III peptide conjugates with either one or two histamine molecules per peptide were tested, with or without preincubation with TG2. For TG2 treatment, 120 $\mu$M purified conjugates were incubated with 1.5 $\mu$M GST-TG2 in 100 mM Tris + 2 mM $Ca^{2+}$ (pH 7.3) for 90 min at 37°C. The samples were then diluted and added to $5 \times 10^5$ APC per well for overnight incubation at 37°C in U-bottom 96-well plates, in RPMI 1640/15% pooled human serum. Irradiated (75 Gy), HLA-DQ2-positive EBV-transformed B cells (cell line RN) were used as APC. On day 1, $5 \times 10^5$ gliadin-specific T cells derived from intestinal biopsies of celiac patients were added to each well. The following T cell clones (TCC) were used: TCC387.E9, which recognizes the DQ2-$\alpha$III-epitope (9-mer core region PYPQPELPY), and TCC430.1.135, which recognizes the DQ2-$\alpha$II-epitope (9-mer core region PQPELPYPQ). T cell proliferation was measured by the uptake of [$^3$H]thymidine (1 $\mu$Ci/well; Hartmann Analytic), which was added 24 h before harvesting. Cells were harvested after 72 h onto glass fiber filter paper with an automated harvester (TomTec Mach III), and [$^3$H]thymidine incorporation was measured by liquid scintillation counting (Wallac MicroBeta TriLux 1450; PerkinElmer). Each Ag concentration was done in triplicate, and at least three independent experiments were done for each assay.

### *Statistical analysis*

Two-tailed unpaired Student's *t* test was performed with GraphPad Prism 4.01 (GraphPad Software). When pairwise comparisons were done between one control group and several nonindependent test groups, e.g., when one sample was tested in several concentrations, nonsignificant result in all tests was followed with a posttest Student's *t* test between the control group and the sum of all the tested groups, to rule out falsely negative result; whereas significant *p* values were corrected with Bonferroni corrections when necessary.

## Results

### *Histamine is an excellent acyl acceptor and is efficiently conjugated to gluten peptides in TG2-mediated transamidation*

The transamidation activity of purified $His_6$-TG2 was measured in the presence of various primary amines using the peptide Ac-PQPQLPF-$NH_2$ as acyl donor. As shown in Table I, putrescine and histamine were significantly better substrates in this system, with $k_{cat}/K_M$ values several times higher than those for the other biogenic amines tested, such as *N*-acetyl lysine methyl ester, spermine, and spermidine. Notably, we did not detect any reaction above background using serotonin as the acyl acceptor, which is surprising given the recent paper reporting transamidation of serotonin to small GTPases by TG2 (17). Histamine was efficiently conjugated to gluten peptides after coincubation with TG2. After only 30 min of incubation, two histamine molecules were attached to each $\alpha$II/$\alpha$III peptide, the theoretical number of glutamine residues that should be targeted based on TG2 motif (2, 3).

### *No agonistic or antagonistic effect of histamine after conjugation to peptides*

Because histamine is an excellent substrate for TG2 and because histamine is a potent biological mediator, we were interested in looking at the biological activity of histamine-gluten peptide conjugates. The agonistic activities of histamine-peptide conjugates were assessed in three different assays testing for activity mediated through H1R or H2R. The AequoScreen system uses an ES-390-A cell line expressing rH1R, and binding is monitored spectrophotometrically. HUVEC rapidly release IL-8 prestored in Weibel-Palade bodies by histamine stimulation mediated via H1R (16), whereas LPS-induced IL-12 secretion from monocyte-derived DC is inhibited by histamine via H2R (6, 7).

### *AequoScreen (H1R)*

In the AequoScreen system, free histamine bound to the H1R with an $EC_{50}$ value at around 2–5 nM. The $\alpha$II/$\alpha$III peptide-histamine conjugate, in comparison, did not show any binding activity at concentrations up to 100 nM (Fig. 1). When used at even higher concentrations (in micromolar range), the conjugate exhibited a slightly higher agonistic activity than the purified control sample of peptide and histamine coincubated without TG2. We believe that this difference reflects unspecific binding, or alternatively a small contamination with unbound histamine in the conjugate sample (Fig. 1). However, we cannot formally exclude the possibility that histamine-peptide conjugates may exhibit a slight low-affinity agonistic H1R activity. The binding activities of four other gluten-derived peptide-histamine conjugates tested (histamine conjugates with the peptides LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF, VSFQQP QQQYPSSQ, PQQPQQSFPQQQRP, and LGQQQPFPPQQP YPQPQPF) behaved similarly to the $\alpha$II/$\alpha$III peptide-histamine conjugate (data not shown).

Table I. *$k_{cat}$/$K_M$ values of TG2-mediated transamidation reaction between the peptide Ac-PQPQLPF-$NH_2$ and various biological amines*

| Amine | $k_{cat}/K_M$ ($mM^{-1}min^{-1}$)[a] |
|---|---|
| Putrescine | 280 ± 60 |
| Histamine | 210 ± 30 |
| *N*-Acetyl lysine methyl ester | 38 ± 2 |
| Spermine | 70 ± 5 |
| Spermidine | 76 ± 4 |
| 5-Hydroxytryptamine | NR[b] |

[a] Determined as described in Ref. 14.
[b] NR, No reaction detected above baseline.

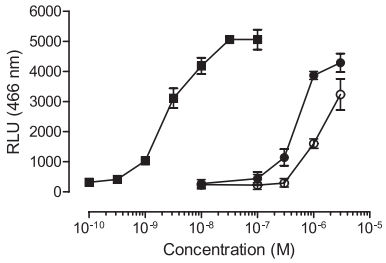


**FIGURE 1.** Purified peptide-histamine conjugates do not bind to H1R. Purified peptide-histamine conjugates were subjected to H1R activity screen in the AequoScreen system (Euroscreen). Histamine conjugates of PQPQLPYPQPQLPY were synthesized by reacting the peptide with human $His_6$-TG2 and 20-fold excess histamine and then purified by preparative HPLC (●). As a negative control, PQPQLPYPQPQLPY was combined with 20-fold excess histamine without TG2 treatment and purified exactly like the histamine-conjugated peptides (○). Histamine HCl ($EC_{50}$, 2.4 nM) was used as the positive control (■). One representative experiment of two is shown, and error bars indicate observed range within duplicates.

### *HUVEC IL-8 secretion (H1R)*

Stimulation of HUVEC with 100 $\mu$M free histamine for 20 min gave a marked increase in IL-8 secretion (Fig. 2). By contrast, coincubation with 100 $\mu$M either purified histamine-$\alpha$I peptide or histamine-$\alpha$II/$\alpha$III peptide conjugates did not result in any increase in IL-8 secretion. Coincubation of 100 $\mu$M free histamine with selective antagonist for either H1R (dexchlorpheniramine) or H2R (ranitidine) demonstrated that histamine-stimulated IL-8 release from HUVEC was inhibited by the H1R, but not the H2R antagonist. In a separate experiment, IL-8 secretion stimulated by purified peptide-histamine conjugates together with an equimolar concentration of free histamine were compared with medium and free histamine. The conjugate plus free histamine samples stimulated higher IL-8 secretion than the medium control ($p < 0.05$), but not significantly different from the IL-8 secretion stimulated by free histamine alone, demonstrating that peptide-histamine conjugates do not exert antagonistic activity on H1R in this system (data not shown).

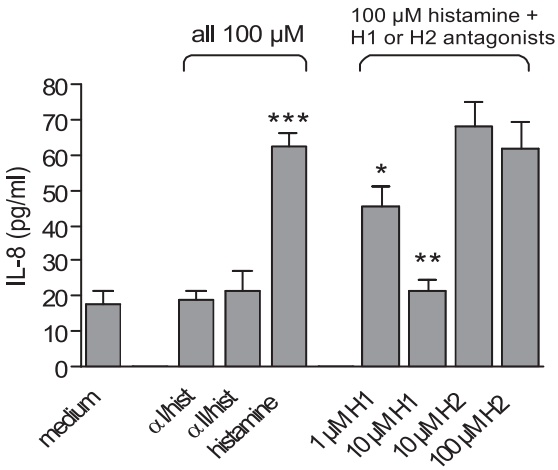


**FIGURE 2.** Free histamine, but not purified histamine-peptide conjugates, stimulates the H1R-mediated release of prestored IL-8 from HUVEC. HUVEC were grown to confluence in 96-well trays and activated overnight with IL-1$\beta$. Cells were washed and subsequently incubated at 37°C in culture medium with the following: 100 $\mu$M histamine, 100 $\mu$M purified histamine-$\alpha$I peptide ($\alpha$I/hist) or -$\alpha$II/$\alpha$III peptide ($\alpha$II/hist) conjugates, or 100 $\mu$M histamine together with variable concentrations of the H1R antagonist dexchlorpheniramine (1 or 10 $\mu$M), or the H2R antagonist ranitidine (10 or 100 $\mu$M). Cell culture supernatants were analyzed for IL-8 with an ELISA kit. One representative experiment of three is shown, and error bars indicate observed range within triplicates. Two-tailed unpaired Student's *t* test was performed with Bonferroni corrections when necessary. ***, Free histamine-stimulated IL-8 secretion was significantly higher than medium control ($p < 0.001$ after correction), and this secretion was lowered by both 1 $\mu$M (*, $p < 0.05$ compared with 100 $\mu$M free histamine, NS after correction) and 10 $\mu$M (**, $p < 0.01$, $p < 0.05$ after correction) H1R antagonist. Neither 10 nor 100 $\mu$M H2R antagonist alone nor both concentrations collectively gave significantly different secretion than 100 $\mu$M free histamine alone.

### *DC IL-12 inhibition (H2R)*

Secretion of IL-12p70 from LPS-matured DC was efficiently inhibited by the presence of free histamine in a dose-dependent fashion as shown in Fig. 3*A*. The purified histamine-$\alpha$II/$\alpha$III peptide conjugate was not able to exert this inhibitory effect because none of these samples led to significantly different IL-12p70 secretion compared with the medium control (Fig. 3*B*). When 10 $\mu$M free histamine was coincubated together with 10 $\mu$M purified histamine-peptide conjugate, the inhibition on IL-12p70 secretion was

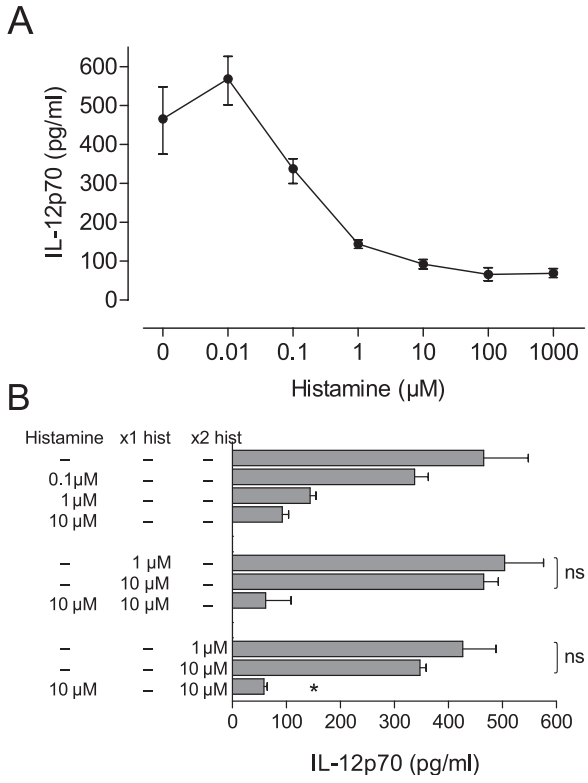


**FIGURE 3.** No agonistic or antagonistic effects of histamine on DC after TG2-mediated conjugation to gluten peptides. Day 6–7 monocyte-derived DC were matured overnight with 100 ng/ml LPS. Supernatants were assessed for IL-12p70 with ELISA. *A*, Free histamine was added at different concentrations together with LPS. *B*, Purified 1 or 10 $\mu$M histamine $\alpha$II/$\alpha$III peptide conjugates with either one histamine ($\times$1 hist) or two histamine ($\times$2 hist) molecules attached per peptide were added together with LPS. As controls, 10 $\mu$M free histamine was tested together with 10 $\mu$M of either $\times$1 hist or $\times$2 hist peptide conjugates. One representative experiment of three is shown. Error bars indicate observed range within triplicates. Two-tailed unpaired Student's *t* test was performed with Bonferroni corrections when necessary. ns, Nonsignificant difference compared with medium control, either each concentration tested alone, or collected data from both concentrations. *, $p = 0.01$ compared with 10 $\mu$M free histamine alone, $p = 0.02$ after correction.

either similar to (for ×1 hist) or slightly stronger (for ×2 hist, $p = 0.01$) than the inhibition seen with 10 μM free histamine alone (Fig. 3*B*), showing that histamine-peptide conjugates did not exert any antagonistic effect on H2R. However, we cannot formally exclude the possibility that these conjugates may have a slight agonistic effect on H2R. To note, the presence of free histamine during the LPS maturation did not alter the cellular expression of DC surface markers such as HLA class II, CD11c, CD83, and CD86 molecules (data not shown).

### *Scavenging of biological active histamine by TG2-mediated peptide conjugation*

To further assess how the histaminergic activities of histamine were affected by coincubation with gliadin peptides and TG2, we used the DC IL-12p70 secretion assay to test histamine and peptide coincubation samples at various concentrations. Stock samples with 6 μM histamine and either 6 or 60 μM αI peptide were coincubated with 2 μM GST-TG2, in 100 mM Tris (pH 7.3) buffer, containing 2 mM $Ca^{2+}$, for 80 min at 37°C. In some samples, either TG2 or $Ca^{2+}$ was omitted from the sample. After incubation, the samples were diluted either 1/6 (final 1 μM histamine) or 1/60 (final 0.1 μM histamine) and added to immature DC for overnight incubation during LPS maturation. As shown in Fig. 4, DC secreted large amounts of IL-12p70 upon LPS maturation, and this secretion was inhibited by 0.1 μM and 1 μM histamine. When histamine had been subjected to coincubation with equimolar ratio of αI peptide in the presence of TG2 and $Ca^{2+}$, its histaminergic effect on DC IL-12p70 inhibition was reduced, evidenced by the increase in IL-12p70 secretion compared with free histamine stimulation at both 0.1 μM ($p < 0.01$) and 1 μM ($p < 0.05$). This reduction in the histaminergic effect was even more pronounced when histamine had been subjected to coincubation with 10× molar excess of αI peptide, demonstrated at both 0.1 μM and 1 μM histamine (both $p < 0.01$) in Fig. 4. Moreover, as shown with control samples depleted of either TG2 or $Ca^{2+}$, the reduction of the histaminergic effect was dependent on the presence of both TG2 and $Ca^{2+}$ during the coincubation of histamine and the αI peptide.

### *Hydrolysis of amine-peptide conjugates by TG2*

It has been reported that TG2 may also catalyze the cleavage of isopeptide bonds similar to those formed by TG2-mediated transamidation (18). This backward reaction releases the acyl acceptor from the peptide, while the specific glutamine residues in the peptide that were involved in isopeptide bond formation are deamidated. We tested for this possibility and measured the specificity of TG2 for peptide-conjugated putrescine and histamine (Table II). The results indicate that the histamine-conjugated peptide has a $k_{cat}/K_M$ similar to that of the parent peptide (72 $mM^{-1}min^{-1}$) (12). In contrast, the specificity of human TG2 for the putrescine-conjugated peptide is significantly lower, suggesting that the identity of the leaving group can influence the molecular recognition features associated with the hydrolytic reaction.

The kinetics of the transamidation and the subsequent reverse reaction were further studied by quantitative measurement of the peptide-histamine conjugate concentration assessed by CE. As seen in Fig. 5, the transamidation reaction was fast, because the maximum concentrations of peptide-histamine conjugates were reached within 20 min of incubation for all three histamine concentrations tested. Between 20 and 120 min, the conjugate concentration remained relatively stable, reflecting the net result of ongoing conjugate formation and hydrolysis. After 120 min, the conjugate formation became insignificant because the native peptide concentration had decreased to <10% (data not shown). In contrast, the hydrolysis of conjugate continued, resulting in its decrease mirrored by an increase in the concentration of deamidated peptides as observed in the electropherograms (data not shown). The hydrolysis kinetics was slower compared with the conjugate formation, as predicted by the lower $k_{cat}/K_M$ value.

Finally, the immunological relevancy of the hydrolysis reaction was studied using the histamine-conjugated peptide with a deamidation-sensitive T cell readout. In Fig. 6, we used one TCC, TCC387.E9, which recognizes the αI epitope (9-mer core region PFPQPELPY) and the closely related αIII epitope (9-mer core region PYPQPELPY) derived from α-gliadin. The T cell recognition is dependent on the deamidation of one specific glutamine residue within the epitope to glutamate (underlined). The native sequence of the αIII epitope is contained within the αII/αIII peptide PQP**Q**LPYPQP**Q**LPY (underlined). We incubated 120 μM purified histamine-αII/αIII peptide conjugates with either one histamine (×1 hist) or two histamine molecules (×2 hist) per peptide with 1.5 μM GST-TG2 in the presence of $Ca^{2+}$ for 90 min at 37°C, and tested the samples in a T cell proliferation assay for the detection of deamidated peptides. The αII/αIII peptide has two glutamine residues that can serve as acyl donors for TG2 (in bold), and histamine can be conjugated to either of these through isopeptide bonds. Histamine conjugation to the targeted glutamine residue within the αIII T cell epitope could potentially abrogate T cell recognition due to an altered peptide structure. This is indeed the case as demonstrated in Fig. 6. As predicted, the purified peptide-histamine conjugate with two histamine residues per peptide (×2 hist) gave no T cell stimulation. However, T cell stimulation comparable with the synthetic αIII control peptide was observed when the same conjugate had been treated with TG2, indicating that TG2 hydrolyzed the conjugate and released deamidated peptides. Similar observations were made with the single histamine per peptide conjugate. Although the purified conjugates alone elicited good T

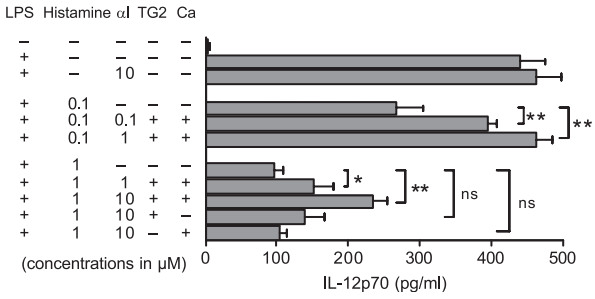


**FIGURE 4.** Preincubation of histamine with peptides and TG2 decrease its histaminergic effect. Using the same DC assay system as described in Fig. 3, 6 μM histamine was preincubated with either 6 or 60 μM αI peptide in the presence of GST-TG2 and $Ca^{2+}$, and subsequently diluted to either final 0.1 μM or 1 μM histamine and tested. In some samples, GST-TG2 or $Ca^{2+}$ was omitted from the incubations. One representative experiment of three is shown. Error bars indicate observed range within triplicates. Two-tailed unpaired Student's *t* test was performed with Bonferroni corrections when necessary. *, $p < 0.05$ uncorrected, NS after correction; **, $p < 0.01$ both before and after correction.

Table II. $k_{cat}/K_M$ *values of TG2-mediated hydrolysis reaction for amines conjugated to Ac-PQPELPF-NH*$_2$

| Amine | $k_{cat}/K_M$ ($mM^{-1}min^{-1}$)[a] |
|---|---|
| Putrescine | 15 ± 5 |
| Histamine | 90 ± 10 |

[a] Measured by HPLC analysis.

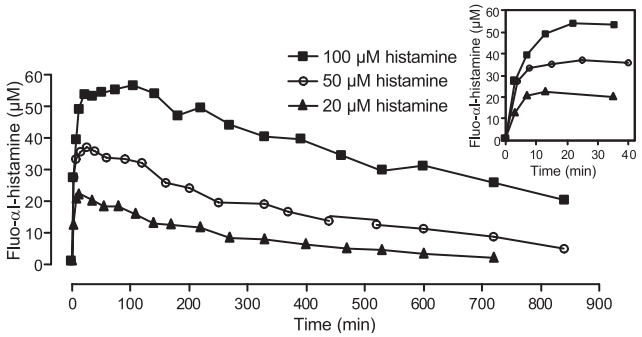


**FIGURE 5.** TG2 mediates first the formation and then also the hydrolysis of histamine-peptide conjugates. One hundred micromolar fluorescein-labeled αI peptide QLQPFPQPQLPY was incubated with 20, 50, or 100 μM histamine, with 2 μM $His_6$-TG2 added freshly every 2 h, in 100 mM Tris and 2 mM $CaCl_2$. An aliquot was analyzed quantitatively by laser-induced fluorescence-CE after different incubation times. The histamine-peptide conjugate concentration reaches maximum after 20 min, and decreases after a plateau phase between 20 and 120 min. The *inset* shows the fast formation of conjugates in the early phase.

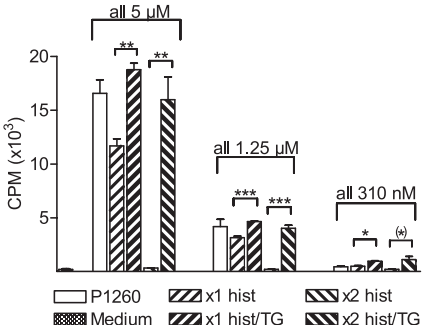


**FIGURE 6.** TG2 mediates the hydrolysis of histamine-peptide isopeptide bonds and thereby release deamidated peptide, recognized by an epitope-specific and deamidation-sensitive TCC. Purified histamine and αII/αIII peptide conjugates, with either one histamine (×1 hist) or two histamine (×2 hist) molecules per peptide, were incubated overnight with irradiated $DQ2^+$ EBV cell line, before the TCC387.E9 recognizing the αIII epitope (9-mer core region PYPQPELPY) was added. Some conjugate samples were pretreated with GST-TG2 for 90 min. The synthetic αIII peptide (PQPQLPYPQPELPY) was used as a control peptide. All Ags were tested at three different concentrations (5 μM, 1.25 μM, and 310 nM). One representative experiment of three is shown. Error bars indicate observed range within triplicates. Two-tailed unpaired Student's *t* test was performed with Bonferroni corrections when necessary. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ all after corrections. (*), $p < 0.05$ uncorrected, NS after correction.

cell response without TG2 treatment, the response was substantially increased after treatment with TG2 (Fig. 6). Similar results were obtained using another TCC (TCC430.1.135), which recognized the DQ2-αII-epitope (9-mer core region PQPELPYPQ) (data not shown). The native sequence of this epitope is contained within the first part of the αII/αIII peptide PQPQLPYPQPQLPY (underlined).

## Discussion

In this paper, we report that histamine is an excellent acyl acceptor in the TG2-mediated transamidation reaction using gluten peptides as acyl donors. The purified histamine-gliadin peptide conjugates resulting from this reaction demonstrate no significant agonistic activity for histamine receptors as assessed with three different biological cellular assays covering both the H1R and H2R. The preincubation of histamine with gluten peptides in the presence of TG2 efficiently eliminates the agonistic activity of histamine. We also find that the conjugation of histamine to glutamine residues within gliadin T cell epitopes destroys its T cell-stimulatory properties. However, T cell-stimulatory peptides may be obtained from histamine-gliadin peptide conjugates by (extended) incubation with TG2. The reason for this is that the isopeptide bonds between histamine and the reactive glutamine can be cleaved by TG2 in a reversed transamidation reaction, resulting in the release of deamidated peptides.

Histamine is efficiently conjugated to gluten peptides after coincubation with TG2. Interestingly, the agonistic activity of histamine to both H1R and H2R was lost after its peptide conjugation. This finding can be reconciled with the proposed interaction models of histamine with H1R and H2R. In these models, the protonated amine group of the free histamine molecules interacts with an aspartate residue in the third transmembrane domain of the receptor, an interaction that is a highly conserved feature of all aminergic receptors (19, 20). TG2-mediated conjugation of histamine to glutamine residues in peptides involves the amine group of histamine and hence eliminates the possibility for protonation. In addition to the disturbance of an important binding group of the histamine molecule, it is also possible that the peptide conjugation introduces a steric hindrance to the histamine-receptor interaction.

In the gut, in addition to the histamine secreted by mast cells within the mucosa, intestinal histamine may also come from food, such as fish, dairy products, and red wine (21). Because the antigluten immune response in CD is so clearly biased toward a Th1-type response, it is puzzling that celiac lesions in the gut contain elevated numbers of mast cells (9). Even more so is the observation that an in vitro gluten challenge of small intestinal biopsies from celiac patients activates the eosinophil and mast cells that are present within the lamina propria (22). In an effort to explain these paradoxical findings, we show in the present work that in vitro, biological active histamine can be scavenged by TG2-mediated conjugation to gluten-derived peptides. This conjugation renders the histamine inactive, thus potentially preventing high levels of bioactive histamine in the intestinal mucosa exposed to dietary gluten peptides.

The involvement of histamine in Th2-dominated immune reactions such as allergic and atopic reactions is well established, but there is also increasing evidence that histamine may affect the Th1/Th2 differentiation of T cells, either via direct binding to histamine receptors expressed by T cells, or via influencing the cytokine secretion profiles of professional APC. The role of histamine in the regulation of Th1/Th2 differentiation is still not entirely clear (reviewed in Refs. 23–25). Jutel et al. (4) reported that histamine in certain situations may activate the Th1 cell subset by triggering H1R. This is in contrast to an array of other data that supports a pro-Th2 effect of histamine. In murine models, histamine enhances IL-5, IL-13, and IL-10 production by Th2 cells (26–29), and suppresses IFN-γ production from Th1 cells (27, 29). Similar Th2-deviating effects of histamine on cytokine secretion profiles were also found in PBMC and mouse splenocytes stimulated by anti-CD3 Ab (30–33). In addition, histamine may also deviate T cells toward Th2 via inhibiting IL-12 and IFN-α, and enhancing IL-10 production by APC (6, 8, 32). Interestingly, recent data from H2R-deficient mice showed that macrophages had significantly higher spontaneous secretion of IL-12p70 compared with both wild-type and H1R-deleted mice (34). These data support the pro-Th2 and anti-Th1 effect of histamine, and are in keeping with our and previous results (6) showing H2R mediated inhibition of IL-12p70 secretion from APC by histamine. Gluten-reactive T cells of CD patients have a Th0/Th1 profile (35) and lamina propria $CD4^+$ cells and intraepithelial lymphocytes of the celiac lesion produce

vast amounts of IFN-γ (36, 37). The mechanism(s) responsible for this cytokine profile is not known. It is conceivable that scavenging of bioactive histamine by TG2-mediated conjugation to gluten peptides in the intestinal mucosa can be contributing to the dominant IFN-γ response in the celiac mucosa. However, no direct evidence for this notion, or any other proposed in vivo effect of histamine-gliadin conjugate formation, can be given before an animal model for CD is developed.

Parameswaran et al. (18) demonstrated that TG2 may also exhibit isopeptidase activity by catalyzing the hydrolysis of isopeptide bonds in synthetic model compounds. In the present work, we demonstrate that human TG2 is able to catalyze the hydrolysis of the isopeptide bonds between gluten-derived peptides and primary amine (histamine or putrescine) to release deamidated peptide. Notably, the gliadin-histamine conjugate was more efficiently hydrolyzed than the gliadin-putrescine conjugate. These results may be relevant for the generation of deamidated T cell epitopes from gluten protein. In the excess of biogenic amines, gluten peptides may be conjugated and thereby lose their T cell stimulatory capacity, but when the concentration of either amines or gluten peptides decrease, the reversed transamidation reaction might prevail as demonstrated by quantitative kinetics studies in Fig. 5. The isopeptide bonds in the conjugates will then be hydrolyzed by TG2, and deamidated gluten peptides that are able to trigger intestinal T cells are released.

In conclusion, we have demonstrated that histamine is an excellent substrate for TG2 and is efficiently conjugated to gluten peptide in vitro through TG2-mediated transamidation. Histamine-peptide conjugates do not exert agonistic effects on histamine receptors, but scavenging of bioactive histamine by gluten peptide conjugation can have physiological implications and may contribute to the dominant IFN-γ response seen in the intestinal mucosa of CD patients. Interestingly, TG2 is able to hydrolyze the peptide-histamine conjugates when the concentration of substrates (histamine or gluten peptides) is lowered, thereby releasing deamidated gluten peptides that are stimulatory to T cells. These findings give new insights on how gluten peptides involved in CD may become deamidated via a primary transamidation to histamine.

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