Chapter 9  Structure-activity of histatin, a potent wound healing peptide from human saliva: cyclization of histatin potentiates molar activity 1,000 fold

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ABSTRACT

Wounds in the mouth heal faster and with less scarification and inflammation than those in skin. Saliva is thought to be essential for the superior oral wound healing, but the involved mechanism is still unclear. We have previously discovered that a human-specific peptide, histatin, might be implicated in the wound-healing properties of saliva. Here we report that histatin enhances re-epithelialization in a human full-skin wound model closely resembling normal skin. The peptide does not stimulate proliferation, but induces cell spreading and migration, two key initiating steps in re-epithelialization. Activation of cells by histatin requires a G-protein-coupled receptor that activates the ERK1/2 pathway. Using a stepwise-truncation method, we determined the minimal domain (SHREFPFYGDYG) of the 38-mer-parent peptide which is required for activity. Strikingly, N-to-C terminal cyclization of Histatin-1 potentiates the molar activity ~1,000-fold, indicating that the recognition of histatin by its cognate receptor requires a specific spatial conformation of the peptide. Our results emphasize the importance of histatin in human saliva for tissue protection and recovery, and establish the experimental basis for the development of synthetic histatins as novel skin wound-healing agents.
INTRODUCTION

It is generally assumed that saliva plays a critical role in maintaining oral health. The importance of saliva is best recognized by patients that have reduced salivary flow, for example after radiation of head and neck cancer or patients with Sjögren syndrome. Many of these patients suffer from conditions such as mucositis, xerostomia, they have increased risk for oral infections as well as trouble eating, tasting, and sometimes even talking (1). Saliva is a very versatile fluid secreted by the different salivary glands and, according to the latest count, it contains over one thousand different proteins (2). Saliva is thought to be essential for the phenomenon of superior oral wound healing as compared to skin (3-5). The wound-healing effects of human saliva have been attributed to the presence of growth factors such as EGF and nerve growth factor (6,7). However, this finding was based on rodent studies that found particularly high concentrations of these two growth factors in saliva (17-899 µg/ml, depending on type of stimulation) (8,9). We have demonstrated that members of the salivary histatin peptide family, histatin-1 (Hst1), Hst2 (or (Hst1(12-38)), and Hst3 stimulate epithelial cell migration and might therefore be the prime agents that mediate the wound-healing activity of human saliva (10). Although histatins in saliva readily are degraded by proteases, the steady state concentrations are well within the range needed for cell activation (11). Our finding revealed a new and important activity of this histatin peptide family, which for over three decades had been primarily regarded as antimicrobial peptides implicated in the innate immunity of humans and higher primates (12,13).

The two activities of histatins, i.e. antimicrobial and cell-stimulating, are very distinct in their modes of action, structural requirements and selectivity. The antimicrobial activity occurs through disruption of the phospholipid membrane of the target cell, and is independent of the chirality of the peptide (10,14-16). This is in contrast to its stimulating activity on host cells, which involves a stereo-specific interaction with a putative membrane receptor (10). In addition, Hst1 and Hst1(12-38) are the most potent enhancers of in vitro wound closure, whereas Hst5, the most potent antimicrobial of the histatin family, is virtually inactive (10).

Building on our previous finding that Hst enhances in vitro cell migration (10), in the present study we demonstrate that Hst enhances re-epithelialization in a tissue-engineered epidermal-skin equivalent, which closely resembles the human skin (17). Rather than proliferation, Hst stimulated cell spreading and migration, which was inhibited by Pertussis Toxin (PTx). We mapped the active domain by stepwise truncation and explored the effect of cyclization of the peptide. This revealed that the minimal biologically active domain encompasses a 13-amino acid stretch of Hst1, and that cyclization of Hst1 results in a 1,000 fold increase in activity on a molar basis. The data suggest that the interaction with a G-protein-coupled receptor (GPCR) requires a specific three-dimensional conformation of the peptide. This work identifies histatin as a potent wound-healing agent, present in human saliva, which may form the basis of a novel skin wound-healing medication.
MATERIALS AND METHODS

Peptide synthesis

Linear peptides were synthesized by solid phase peptide synthesis using Fmoc chemistry with a Milligen 9050 peptide synthesizer (Milligen-Biosearch, Bedford, MA, USA), purified by RP-HPLC, and their authenticity was confirmed by MS, as described before (15). The amino-acid sequence of Hst1 is DSHEKRHG-YRKRKHFHEKHHSHREFPFYG-DYGSNYLYDN, that of the truncated versions can be deduced from this sequence (e.g. Hst1(12-38) represents the amino acids 12 until 38). The cyclic peptide was synthesized using Fmoc-Glu-NovaSyn®TGA-ODmab (NovaBiochem, Läufelfingen, Switzerland), a dedicated support, in which the first amino acid is coupled to the resin by its side-chain carboxyl group while its α-carboxyl group is protected by the semi-orthogonal Dmab group (18). After completion of the sequence the N-terminal Fmoc was removed with 20% piperidine in N-methylpyrrolidone, subsequently the C-terminal ODmab was removed by 2% hydrazine in N,N-dimethylformamide (DMF). On-resin head-to-tail cyclization was achieved by prolonged reaction (72 h) with 1 eq. PyBOP, 1 eq. HOBT, and 1 eq. DIEA in DMF, containing 20% DMSO and 2% DCM. After cleavage from the resin and purification by RP-HPLC, cyclization was confirmed by MS, which showed that the molecular mass of cHst1 was 18 Da less than that of the linear Hst1, 4830 Da instead of 4848 Da, respectively, in accordance with the formation of a lactam-bond. The recovery was 1.3%, which is a reasonable yield for on-resin synthesis of a cyclic peptide of this length.

Epidermal skin equivalent culture, wound model, and re-epithelialization measurements

Epidermal keratinocytes were isolated from neonatal foreskins, essentially as described earlier (19). The study was approved by the VUmc Medical Ethical Committee. Second-passage cultures of keratinocytes were seeded on de-epidermized dermis. After culturing for one week in medium containing DMEM (ICN Biomedicals, Irvine, CA, USA)/Ham’s F12 (ICN Biomedicals) (3:1), 1% UltroSerG (BioSepra SA, Cergy-Saint-Christophe, France), 1% penicillin/streptomycin (Gibco, Paisley, UK.), 1 μM hydrocortisone, 1 μM isoproterenol, 0.1 μM insulin, 2 ng/ml KGF and 0.5 ng/ml EGF, cultures were lifted to the air-liquid interface and cultured for a further two weeks in air-exposed culture medium (DMEM/Ham’s F12 (3:1), 0.2% UltroSerG, 1% penicillin/streptomycin, 1 μM hydrocortisone, 1 μM isoproterenol, 0.1 μM insulin, 2 ng/ml KGF, 0.5 ng/ml EGF, 10 μM L-carnitine, 10 mM L-serine, 1 μM DL-α-tocopherol acetate, with a lipid supplement containing 25 μM palmitic acid, 15 μM linoleic acid, 7 μM arachidonic acid and 24 μM bovine serum albumine). Culture medium was refreshed twice a week. Unless otherwise stated, all culture additives were obtained from Sigma (Sigma-Aldrich, St.Louis, MO, USA).
Full-thickness wounds were made in epidermal skin equivalents after 2 weeks of air-exposed culture (20). Wounds were created using freeze burning, which resulted in cell death of the entire region of the epidermis that was treated with a device cooled to –196°C in liquid nitrogen for 10 sec. During freeze wounding, the area of the device in contact with the epidermal skin equivalent was 2 cm long and 2 mm wide. Two wounds separated by at least 0.5 cm were introduced into each culture. The experiment was performed from three independent donors in duplicate.

Directly after wounding, epidermal equivalents were further cultured in air-exposed medium without EGF and KGF (see above) and supplemented with 72 µM Hst1(12-38) or D-Hst1(12-38). Medium supplemented with 10 ng/ml rhEGF was used as positive control, medium without supplementation as negative control. Medium was changed every two days. After 6 days, re-epithelialization was analyzed on haematoxylin/eosin stained paraffin sections (5μm) with the aid of a Nikon microscope and Osteomeasure software (osteometrics, Atlanta, USA). Re-epithelialization was measured as the distance the newly formed epidermis had migrated into the wound bed. Re-epithelialization after exposure to Hst1(12-38), D-Hst1(12-38), or rhEGF-supplemented medium was compared to control medium and given relative to control. As re-epithelialization occurred from both wound margins, duplicate readings were obtained for each wound.

Cell-line culture and in vitro wound-scratch assay

The human buccal epithelial cell line HO-1-N-1 was provided by the Japanese Collection of Research Bioresources (Osaka, Japan) and maintained as previously described (10). In vitro wound-scratch experiments were performed as previously described (10). In short, in a confluent layer of cells, that was serum starved in DMEM/F12 (Invitrogen, Carlsbad, CA, USA) for 6 h, a scratch was made with a sterile tip. The width of the scratch was measured at the beginning and after 18 h using microscopic images (Leica DM IL, Leica DFC320 camera). Relative closure was calculated by dividing the closure of treated wound (DMEM/F12 + peptide) by that of the untreated wound (DMEM/F12 only). Final concentration of synthesized peptides in the assays was 10 µM, except for the dose-response experiments. As a positive control 10 ng/ml rhEGF (Invitrogen) was used. Pertussis toxin (Invitrogen) concentration was applied to the cells 1 h before the scratch was made and during the experiment at a final concentration of 200 ng/ml. U0126 (5 µM, LC Laboratories, Woburn, MA, USA) was used during incubation (no pre-incubation) as a specific inhibitor of MEK, thereby inhibiting phosphorylation of ERK1/2.

Proliferation assay

Serum-starved (24h) HO-1-N-1 cells were trypsinized and seeded in 96-well culture plates (1x10^4 per well). After 6 h of adherence, medium was changed with DMEM/F12 supplemented
with 0.1, 1, 10, or 100 µM Hst1(12-38), 10 ng/ml rhEGF, or without anything (control). Medium was refreshed after 24 h. After 48 h, cells were fixed with paraformaldehyde (3.7%) (Merck) in PBS for 20 min, and permeabilized with Triton X-100 (0.25%) in PBS for 5 min. Cells were then stained with propidium iodide (10 µM) (Invitrogen), and propidium iodide fluorescence was measured at excitation and emission wavelengths of 544 and 620 nm, respectively, in a Fluostar Galaxy microplate fluorimeter (BMG Labtechnologies, Offenburg, Germany). Fluorescence was proportionally increased with cell number as was tested separately. Representative microscopic (Leica DM IL equipped with Leica DFC320 camera) images were taken to illustrate differences in cell numbers.

**Cell spreading assay**

HO-1-N-1 cells, that were serum-starved for 24 h, were trypsinized and subsequently seeded at low density so that a single-cell population prevailed. The cells were seeded with or without 10 µM Hst1(12-38), and after 16 h they were fixed with paraformaldehyde (3.7%) (Merck) in PBS for 20 min, and permeabilized with Triton X-100 (0.25%) in PBS for 5 min. Then the cells were incubated with ALEXA Fluor® 488 phalloidin (Invitrogen) 1 h for F-actin staining, and DAPI for 10 min for nuclei staining. Images were taken (Leica DM IL equipped with Leica DFC320 camera), and the area of individual cells (n = 35 per treatment, of phase-contrast images) was calculated using ImageJ software (21).

**Electric cell-substrate impedance sensing (ECIS)**

For ECIS-based cell-spreading experiments (22), ECIS electrodes (Applied Biophysics, Troy, NY, USA) were coated with L-Cysteine for 30 min, and with fibronectin (Sigma) in 0.9 % NaCl overnight at 37°C. Serum-starved (24 h) HO-1-N-1 cells were seeded (2 x 10⁵ cells per well) in DMEM/F12 (400 µl) with or without 10 µM Hst1(12-38). Immediately after seeding the chamber slide was placed in its holder. ECIS was continuously monitored for up to 6 h.

**Statistical analysis**

Data was analyzed using one-way ANOVA with additional least significant difference test to determine significance between samples. P-values are as described in the legends.
RESULTS

Histatin accelerates wound re-epithelialization in a human-skin model through a PTx-sensitive pathway

In a previous study we demonstrated that histatins stimulate cell migration in an artificial wound-scratch model (10). In this model a wound is made mechanically by applying a scratch in a monolayer of epithelial cells cultured on a plastic substrate (see also Figure 1d). In the present study, to test whether histatins have potential applicability as general wound-healing agents, their activity was examined in a tissue-engineered epidermal-skin equivalent that closely resembles the native healthy skin (17). The epidermal-skin equivalent consists of reconstructed epidermis on human a-cellular dermis. Due to the air-exposed culture method, complete epidermal differentiation occurs. This results in formation of a compact basal layer, spinous layer, granular layer and stratum corneum, which is similar to that found in native healthy skin (Figure 1a). Full thickness wounds were introduced by cold injury, as described earlier (20). The degree of re-epithelialization of the wound was studied after six days.

Representative images of Hst1(12-38) and D-Hst1(12-38) (the D-enantiomer of Hst1(12-38)) treated wounds show that re-epithelialization was enhanced when treated with Hst1(12-38) (Figure 1b). In contrast, D-Hst1(12-38) had no effect, indicating that stereospecific interactions underlie the histatin-induced enhancement of re-epithelialization. Quantification of the re-epithelialization by analyzing a series of images represented by the one shown in Figure 1b, confirmed that re-epithelialization was significantly enhanced by Hst1(12-38) with comparable rates as rhEGF, which we included as a positive control (Figure 1c). These data indicate that histatins stimulate re-epithelialization of the skin after wounding and build upon our previous work showing that histatins are key modulators of the superior wound-healing properties of the mouth (10).

Since the D-enantiomer of Hst1(12-38) is not able to activate cells (Figure 1b & c), we suspected that histatin-mediated activation of cells is a receptor-mediated process. In a first attempt to define which class of receptors may be involved in this effect, cells were treated with PTx during an in vitro wound-scratch experiment. PTx is known to specifically inhibit signaling through GPCRs that couple to heterotrimeric proteins of the Gαi subtype. We applied a scratch in a confluent layer of epithelial cells, and measured relative closure of the wound at t=0 and t=18 h (Figure 1d). Hst1(12-38) enhanced wound closure to the same extent as rhEGF, but contrary to rhEGF its activity was inhibited by PTx (Figure 1e). This implies the involvement of a Gq-linked GPCR in the activation of cell migration by histatins. Previously, we have determined that the activation of epithelial cells by histatins is not mediated by the EGF receptor (10), which is a common receptor utilized for transactivation by different cellular stimuli.
Figure 1: Histatin enhances re-epithelialization in skin equivalent and is PTx sensitive.

(A) Comparison of human skin and tissue-engineered skin model. Tissue sections were stained with Hema-toxylin and Eosin for histological analysis. Bar = 100 µm (B) Representative micrographs of D-Hst1(12-38) and Hst1(12-38) treated wounds 6 days after wounding. a = healthy epidermis; b = ingrowing epidermis; arrow = start of wound area. Bar = 250 µm (C) Wounds were introduced in the skin model. After 6 days, cultures were harvested, and re-epithelialization was studied on haematoxylin/eosin stained paraffin sections. Re-epithelialization was quantified as the distance the newly formed epidermis (in blue) had migrated into the wound bed after D-Hst1(12-38), Hst1(12-38) and rhEGF supplementation relative to unsupplemented control cultures. Two wounds were introduced in each culture and duplicate readings were obtained from each wound. Data represents means ± SD, n = 3, * = p ≤ 0.01 (D) Representative micrographs of Hst1(12-38) enhanced wound closure in the in vitro wound-scratch assay after 18 h. Dotted lines in images represent wound edges at t = 0 h. Bar = 100 µm (E) Relative closure, by measuring wound width from images shown in D, is calculated by dividing wound closure of treated wounds by that of the control. Wounds are treated with Hst1(12-38) or rhEGF, with and without PTx. Data represent means ± SD, n = 12, * p < 0.01 compared to the appropriate control.
Histatin promotes cell spreading and cell migration, but does not enhance cell proliferation

Histatin promotes cell migration (Figure 1d & e). However, since re-epithelialization is also enhanced by cell proliferation, we tested the effect of histatin on cell division. The dose-response effect of Hst1(12-38), or of rhEGF as a positive control, on proliferation was examined by quantifying the amount of DNA after 48 h of incubation. In addition, we made microscopic images of the wells that were used for DNA quantification by measuring fluorescence (Figure 2a). The results clearly show that in contrast to rhEGF, Hst1(12-38) does not enhance cell proliferation (Figure 2b).

In addition to cell migration and proliferation, cell spreading, which precedes migration, is very important for re-epithelialization. To test the effect of histatin on cell spreading, low densities of epithelial cells were seeded in the presence or absence of Hst1(12-38). After 16 h, cells were fixed and permeabilized, and subsequently stained for F-actin and nuclei. Representative fluorescent microscopic images are shown in Figure 3a. The surface of 35 individual cells was calculated using ImageJ software. The area per cell was larger in the Hst1(12-38) treated cells as compared to the control (Figure 3b) or D-Hst1(12-38)-treated cells (data...
not shown) indicating that Hst1(12-38) promotes cell spreading. To further study histatin-stimulated cell spreading we examined the effect of Hst1(12-38) on spreading with the aid of ECIS (22). This technique provides quantitative information, in real time, on cell attachment and spreading on coated golden electrodes. Serum-starved cells (2x10^5) were added to the chambers with or without Hst1(12-38), and impedance was measured online. Hst1(12-38)-treated cells already showed higher impedance after 2.5 h, as well as for the remainder of the experiment (Figure 3c). These results show that histatin does enhance cell spreading.

**Figure 3: Histatin enhances cell spreading.**

(A) Representative micrographs of HO-1-N-1 cell spreading 16 h after seeding, in the presence or absence (control) of Hst1(12-38). Actin is shown in green and nuclei are shown in blue. Bar = 20 µm. (B) Average surface area per cell was quantified from images similar to those in A. Data represent means ± SD. n = 35, * p < 0.01. (C) Hst1(12-38)-mediated cell spreading in real time by ECIS. HO-1-N-1 cells were seeded on fibronectin-coated golden ECIS electrodes and attachment (0-30 min) and spreading (1-6 h) were monitored continuously. n = 4, * p < 0.01 comparing Hst1(12-38) to the untreated control.

**The minimal active domain of Hst1 is SHREFPFYGYDYGs**

Our next step was to identify the minimal domain that mediates the biological activity of histatin. Since the *in vitro* wound-scratch properties of Hst1(12-38) are comparable to that of the parent peptide Hst1 (10), this peptide was used as a starting point for mapping the minimal active domain. We compared the activities of fragments that differ by two-residue-stepwise truncation in the C-terminus, the N-terminus, or both (Figure 4). Removal of the first six C-terminal residues had no significant effect on the cell-stimulating properties in the
in vitro wound-scratch assay (Fig. 4A). Removal of the next two residues resulted in a fragment Hst1(12-30) with a decreased activity. Further truncation resulted in a complete loss of activity (Figure 4a). Stepwise truncation of the first four residues at the N-terminus did not affect the activity (Figure 4b). Removal of amino acids 16 and 17, generating the fragment Hst1(18-38) starting with a double histidin at position 18 and 19, resulted in a response that was not statistically different from the control (P=0.07). Strikingly, upon further truncation,

Figure 4: Hst1(20-32) is the minimal active domain of Hst1 in the in vitro wound-scratch assay.

Wound-scratch experiments and relative-closure measurements of confluent HO-1-N-1 wells were done as described and shown in Figures 1D & E. (A) C-terminal-stepwise truncation of Hst1(12-38), and subsequent measuring of relative-wound-closure characteristic for each peptide. (B) N-terminal-stepwise truncation of Hst1(12-38), and subsequent measuring of relative-wound-closure characteristic for each peptide. (C) N-to-C-terminal truncation of Hst1(12-38), peptides were made based on the results shown in A and B. (A-C) Error bars represent means ± SD. n = 12, ** p<0.01, * p<0.05 compared to the control.
the activity was completely restored (Figure 4b). Similarly, when variants were prepared in which residues had been removed from both termini, the peptides starting with His\textsuperscript{18}His\textsuperscript{19} also showed decreased activity compared to the shorter, N-terminally truncated fragments. This decreased activity was statistically significant for Hst1(18-34) (Figure 4c). Our hypothesis is that these variants are less or not able to activate the putative receptor because of the presence of clustered positive charges at the N-terminus. Overall, on the basis of these results we conclude that the region between residues 20 and 32 is critical for the wound healing properties of Hst1.

Cyclization of Hst1 potentiates its activity thousand-fold

The structure-activity relationship of histatins, particularly the anomalous behavior in the N-truncated series (Figure 4), suggests that, in addition to a specific amino-acid sequence, additional structural requirements govern the activity of histatin. This prompted us to prepare a variant in which the conformational freedom was constrained by covalently linking the C-terminus to the N-terminus. The activity of this cyclic Hst1 (cHst1) was compared to that of linear Hst1 in the \textit{in vitro} wound-scratch assay. We found a dose-dependent enhancement of wound closure with both peptides (Figure 5A). However, the minimal concentration needed to enhance wound closure differed greatly, 0.001 µM for cHst1 versus 1 µM for Hst1 (Figure 5a). Thus, constraining the conformation of Hst1 by cyclization resulted in a more

![Cyclization of Hst1 potentiates its effect 1,000 times.](image)

**Figure 5:** Cyclization of Hst1 potentiates its effect 1,000 times. Wound-scratch experiments and relative closure measurements of confluent HO-1-N-1 wells were done as described in Methods section, and shown in Figure 1D & E. (A) Relative wound closure of Hst1 (■) and cHst1 (▲) compared to the control (depicted as 0 µM in the graph). cHst1 remains active until 0.001 µM, whereas Hst1 is active until 1 µM. (B) Relative closure by cHst1 (0.1 µM) and rhEGF (10 ng/ml) with and without the addition of pharmaceutical inhibitors PTx or U0126 (inhibitor of MEK, the activator of ERK1/2). (A-B) Error bar and symbols represent mean ± SD, n = 12, *p<0.01 compared to the appropriate control.
than 1,000-fold stimulation of the molar activity, likely because the affinity for the receptor was increased.

Similar to high concentrations of linear Hst1(12-38), low-concentrations of cHst1 also promoted wound healing in a PTx-sensitive and ERK1/2 dependent fashion (Figure 5b). This indicates that the two histatin variants signal through the same receptor.

DISCUSSION

The superior wound healing in the oral cavity compared to cutaneous wound healing has been an intriguing yet unexplained phenomenon. Although several mechanisms have been proposed, saliva has always been considered to be of significant beneficial relevance to the outcome of oral healing. It is also possible that the healing effects of saliva are not only limited to the oral cavity since licking of a skin wound by animals and humans is a readily acknowledged event which may also promote skin wound healing. Recently, we isolated histatin from human saliva, and identified histatin as the component that was responsible for the in vitro epithelial-cell-migration inducing properties of saliva (10). In the current study, we examined the effect of histatin on re-epithelialization of a wound created in a tissue-engineered three-dimensional skin model. Re-epithelialization of full thickness wounds is enhanced by histatin, with rates comparable to that of the gold standard rhEGF. Histatin does not enhance proliferation, but stimulates re-epithelialization by stimulating cell migration and cell spreading. We also showed that the activation by histatins is not only sequence, but also structure dependent, by using a cyclic version of Hst1. This cyclization of the peptide increased its concentration-dependent effect on wound healing 1,000 fold.

Chemokines and growth factors are generally small proteins (5-10 kDa) that have a stable conformation by sulphur bridges of their cysteine residues. Nevertheless, linear peptides that were derived from chemokines or growth factors can activate cells, but at 1,000 times higher concentrations than the original ligand (23). The binding affinities of these linear peptides are greatly enhanced upon cyclization (23-25). This seems similar to our finding that cyclization increases the molar activity of histatin. However, most studies aim to find antagonists of receptors to inhibit pathological processes such as cancer (23), or HIV-1 entry via CXCR4 (26). In contrast, to our knowledge this is the first study to show that cyclization results in improved cell-stimulating activity. The only known cyclic protein motif expressed in mammals is presented by theta defensins, which thus far have been considered as broad-spectrum antimicrobial peptides (27, 28). In view of the strong potentiating effect of cyclization on histatin activity, it is tempting to speculate that theta defensins may play also a role in the activation of host cells.

Our current data show that a GPCR is involved in the activation of cells by histatin. This is in line with our previous study in which we excluded the EGF receptor (10). The EGF recep-
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tor is commonly utilized by indirect activation of cells by various factors including UV-light, lysophosphatic acid, or other antimicrobial peptides (29-33). Thus, histatin appears to signal through a specific receptor, albeit that this as yet unidentified receptor may be shared with other ligands, similar to the situation for the chemokine receptor subgroup of GPCRs that also signal through Goi (34).

At first sight, our findings have some similarities with other proclaimed antimicrobial peptides that can affect host tissues, such as LL-37 and defensins. At a closer look however, there are substantial differences. Firstly, contrary to D-Hst1(12-38), the D-enantiomer of LL-37 is as active as the natural-occurring peptide (35). Secondly, both LL-37 and defensins have been shown to act via the EGF-receptor (30,31,33), whereas we have previously shown that histatin does not (10). Thirdly, LL-37 and defensins enhance cell proliferation (30,35), whereas in this study we show that cell proliferation is unaffected by histatins. Finally, LL-37 and defensins enhance several cellular processes, but at somewhat higher concentrations they are cytotoxic (36-38). This is in contrast to histatins, which are not cytotoxic at least up to 100 µg/ml.

As a concluding remark, we would like to note that unlike manufactured recombinant growth factors currently in clinical trials (39,40), histatins are stable molecules that can be produced easily and on a large scale. They therefore have high potential for use as novel therapeutics suited for the treatment of wounds. For clinical implementation of histatins in the treatment of wounds it is of key interest to minimize the production costs. Also, in general, the smaller the compound the better it is able to penetrate the skin. Our identification of the minimal domain might thus be very important for the development of histatin as a new therapeutic agent for wound healing. Our finding of potentiation of the molar activity by cyclization is obviously clinically relevant, but also gives valuable insight into the mode of action utilized by histatin. We think that a specific spatial conformation of histatin is required to bind and activate the receptor, which in turn triggers signal transduction and cell activation.
REFERENCES


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