

Histatin 5-Derived Peptide with Improved Fungicidal Properties Enhances Human Immunodeficiency Virus Type 1 Replication by Promoting Viral Entry

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Antimicrobial peptides are found in a number of body compartments and are secreted at mucosal surfaces, where they form part of the innate immune system. Many of these small peptides have a broad spectrum of inhibitory activity against bacteria, fungi, parasites, and viruses. Generally, the peptide's mode of action is binding and disruption of membranes due to its amphipathic properties. Histatin 5 is a salivary peptide that inhibits *Candida albicans*, an opportunistic fungus that causes oropharyngeal candidiasis in a majority of human immunodeficiency virus type 1 (HIV-1)-infected patients progressing towards AIDS. Previously, we increased the fungicidal properties of histatin 5 by replacing amino acids in the active domain of histatin 5 (Dh-5) (A. L. Ruissen, J. Groenink, E. J. Helmerhorst, E. Walgreen-Weterings, W. van't Hof, E. C. Veerman, and A. V. Nieuw Amerongen, *Biochem. J.* 356:361–368, 2001). In the current study, we tested the anti-HIV-1 activity of Dh-5 and its derivatives. Although Dh-5 inhibited HIV-1 replication, none of the peptide variants were more effective in this respect. In contrast, one of the derivatives, Dhvar2, significantly increased HIV-1 replication by promoting the envelope-mediated cell entry process. Most likely, Dhvar2 affects membranes, thereby facilitating fusion of viral and cellular membranes. This study shows that modification of antimicrobial peptides in order to improve their activity against a pathogen may have unpredictable and unwanted side effects on other pathogens.

Antimicrobial peptides have been identified in all species investigated, where they form part of the innate and possibly adaptive immune system. Many of these peptides have a broad spectrum of activity against bacteria, fungi, parasites, and viruses. Several different types of antimicrobial peptides have been found in humans (37, 42). Although very heterogeneous in amino acid sequence, they share some characteristics, like a low molecular mass (1 to 5 kDa), a positively charged domain of 10 to 25 amino acids, and the tendency to form amphipathic structures (28, 37). Generally, the peptide's mode of action is binding and disruption of membranes, but some studies indicate that these peptides also act intracellularly (12, 13, 31).

Three modes of action of antimicrobial peptides have been described with respect to viruses and human immunodeficiency virus type 1 (HIV-1) in particular: direct virolysis, inhibition of transcription from the long terminal repeat (LTR) promoter, and block of cell entry by binding to cell surface receptors (5, 23, 39, 43). In this study, we investigated the anti-HIV-1 activity of derivatives of histatin 5, an antimicrobial peptide present in human saliva. Histatins form a group of electrophoretically distinct histidine-rich polypeptides with microbicidal activity that are present in human parotid and submandibular gland secretions (35). Histatin 5 is active against the yeast *Candida albicans*, an opportunistic fungus that causes oropharyngeal candidiasis in a majority of HIV-1-infected patients (6). Inter-

nalization of histatin 5 and subsequent targeting of the mitochondria leads to release of vital components and cell death of the yeast (7). To increase the activity of this peptide, amino acids in the active domain (Dh-5) of histatin 5 have been substituted. The resulting peptides (Dhvar2 to Dhvar5; Table 1) have previously been shown to possess increased fungicidal activity, and all but one (Dhvar5) are more amphipathic than Dh-5 (14, 25). In the present study, we tested the anti-HIV-1 activity of Dh-5 and its derivatives. Dh-5 inhibited HIV-1 replication, but none of the derivatives was a more efficient inhibitor. Surprisingly, one of the peptides (Dhvar2) dramatically increased HIV-1 replication by promoting the envelope (Env)-mediated entry process. Dhvar2 probably destabilizes membranes and thereby facilitates fusion of viral and cellular membranes. This study shows that the development of more potent antimicrobial agents against certain pathogens like *Candida* may have unexpected and dangerous side effects if they are used in the context of other pathogens like HIV-1.

MATERIALS AND METHODS

Peptide synthesis and purification. The active domain of histatin 5 (Dh-5, KRKFHEKHSHRGY) and variants (Dhvar2, KRLFKELLSLRKY; Dhvar3, KRLFKKLFSLRKY; Dhvar4, KRLFKKLLFSLRKY; Dhvar5, LLLFLLKKRKKRKY) were synthesized by F-moc chemistry with a MilliGen 9050 peptide synthesizer (MilliGen/Biosearch, Bedford, MA) and purified to at least 95% by reverse-phase high-performance liquid chromatography (RP-HPLC; Jasco Corporation, Tokyo, Japan). The authenticity of the peptides was confirmed by ion trap mass spectrometry with an LCQ Deca XP (Thermo Finnigan, San Jose, CA). During synthesis, Dh-5 and Dhvar2 were labeled with fluorescein isothiocyanate (FITC) for FACScan analysis as described previously (7). Labeling was achieved by addition of 25 μ l FITC (1 mg/ml) in dimethyl sulfoxide to 1 ml of a peptide solution (0.66 mM) in water, adjusted to pH 9.7 with Na₂CO₃. After

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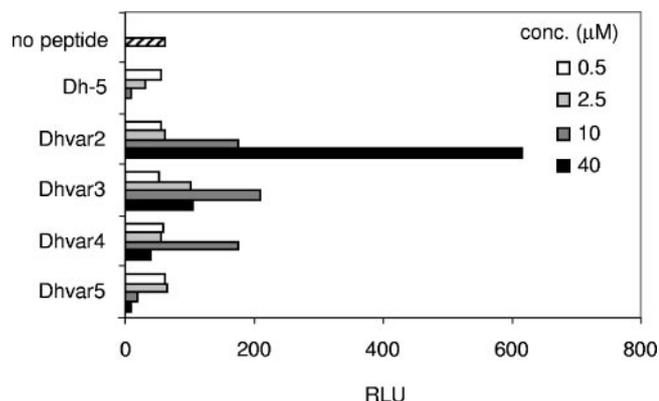


FIG. 1. Modified antimicrobial peptides with improved antifungal properties enhance HIV-1 infectivity. The active domain (Dh-5) of salivary peptide histatin 5 was modified in order to increase the antifungal activity. Dh-5 and derivatives were tested for their anti-HIV-1 activity in a single-cycle replication assay. HIV-1 was incubated with the peptides and subsequently cocultured with LuSIV cells. These reporter cells express luciferase after HIV-1 infection, which was detected 24 h later. RLU, relative light units; conc., concentration.

of the samples was accomplished at 200 nm. Run time of the separation was 10 min. RP-HPLC separations were performed by HPLC (Jasco, Tokyo, Japan) on a reversed-phase C_{18} column (Vydac 218TP54; 24 cm and 4.6 mm ID; 5 μ m particle size) developed with a linear gradient of acetonitrile containing 0.1% trifluoroacetic acid. On-line UV detection of the samples was accomplished at 214 nm. Run time of the separation was 20 min. Relative peptide concentrations in the samples were determined based on the relevant peak areas.

RESULTS

Dh-5 inhibits, but derivatives enhance, HIV-1 replication.

Amino acids in the active domain (Dh-5) of salivary peptide histatin 5 have previously been replaced to increase the fungicidal activity of this peptide against *Candida albicans* (Table 1). Dhvar2 to Dhvar4 are more amphipathic, while Dhvar5 is less amphipathic than Dh-5 (14, 25). Because *Candida* is associated with immune-compromised patients like HIV-1-infected persons progressing towards AIDS (6), we wanted to explore the antiviral properties of Dh-5. We furthermore investigated whether the variant peptides displayed enhanced antiviral activity, which was expected due to their increased antifungal properties. We incubated HIV-1 with these peptides and tested viral infectivity in a single-cycle replication assay with LuSIV reporter cells. These reporter cells contain the firefly luciferase gene downstream of the LTR promoter, resulting in Tat-mediated luciferase expression, which is a measure of infectivity (24). Dh-5 was able to reduce the infectivity of HIV-1, but modification of Dh-5 did not enhance the anti-HIV-1 properties of this peptide: none of the peptide variants decreased luciferase production more than Dh-5 (Fig. 1). Surprisingly, the Dhvar2, Dhvar3, and Dhvar4 peptides with increased amphipathicity stimulated HIV-1 infection. In the absence of HIV-1, none of these peptides influenced the background luciferase levels of the LuSIV cells, excluding that the peptides trigger signaling pathways in the cell that lead to LTR activation and luciferase production (data not shown). Dhvar2 showed the highest enhancing effect by increasing luciferase production 10-fold, on average.

Dhvar2 also stimulated HIV-1 replication in T cells over a

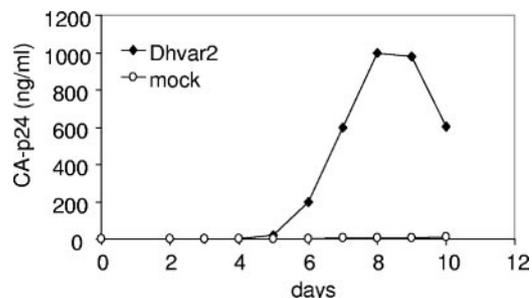


FIG. 2. Dhvar2 can rescue HIV-1 replication at low viral inputs. A noninfectious HIV-1 dose of 0.4 ng CA-p24/ml was mock treated or incubated with Dhvar2, followed by coculture with PM1 T cells. Viral replication was subsequently followed by measuring CA-p24 production in the supernatant by ELISA.

prolonged period of time. PM1 T cells were infected with Dhvar2-treated or mock-incubated HIV-1, and viral replication was followed by CA-p24 ELISA on the supernatant. The results are very obvious when a noninfectious dose of HIV-1 was used, in which case Dhvar2 rescued HIV-1 replication (Fig. 2). When a high viral input was used, we found no stimulation by Dhvar2 in comparison to the mock-treated sample (results not shown). Cell toxicity was observed at higher concentrations of Dhvar2 ($>60 \mu$ M), which obviously decreased viral replication (data not shown).

Dhvar2 binds to cells. To elucidate the mechanism by which Dhvar2 enhances HIV-1 infection, we preincubated the peptide (or medium as a mock treatment) either with HIV-1 or the LuSIV cells for 30 min at 37°C. Both treatments yielded the approximately 10-fold enhancement (Fig. 3). Preincubation of virus and cells prior to addition of Dhvar2 yielded the same level of stimulation. This experiment therefore does not identify the target for Dhvar2 stimulation, but it does show that the peptide has a broad time span in which it can stimulate HIV-1 infection.

We next investigated whether Dhvar2 (or Dh-5 as a control) affects the binding of a broad range of reagents to the HIV-1 envelope (Env) gp120 protein in a gp120 ELISA (Fig. 4A). We focused on reagents that bind to the CD4 and coreceptor

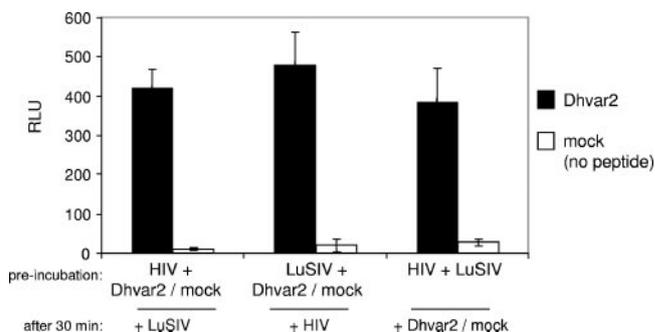


FIG. 3. Dhvar2 preincubation experiments. (Black bars) HIV-1, LuSIV, and Dhvar2 were preincubated in different pairs, as indicated on the x axis. After 30 min, all were incubated together for an additional 24 h. (White bars) The same experiment was also performed with medium instead of Dhvar2 (mock treatment). Luciferase was measured after 24 h. Error bars represent standard deviations. RLU, relative light units.

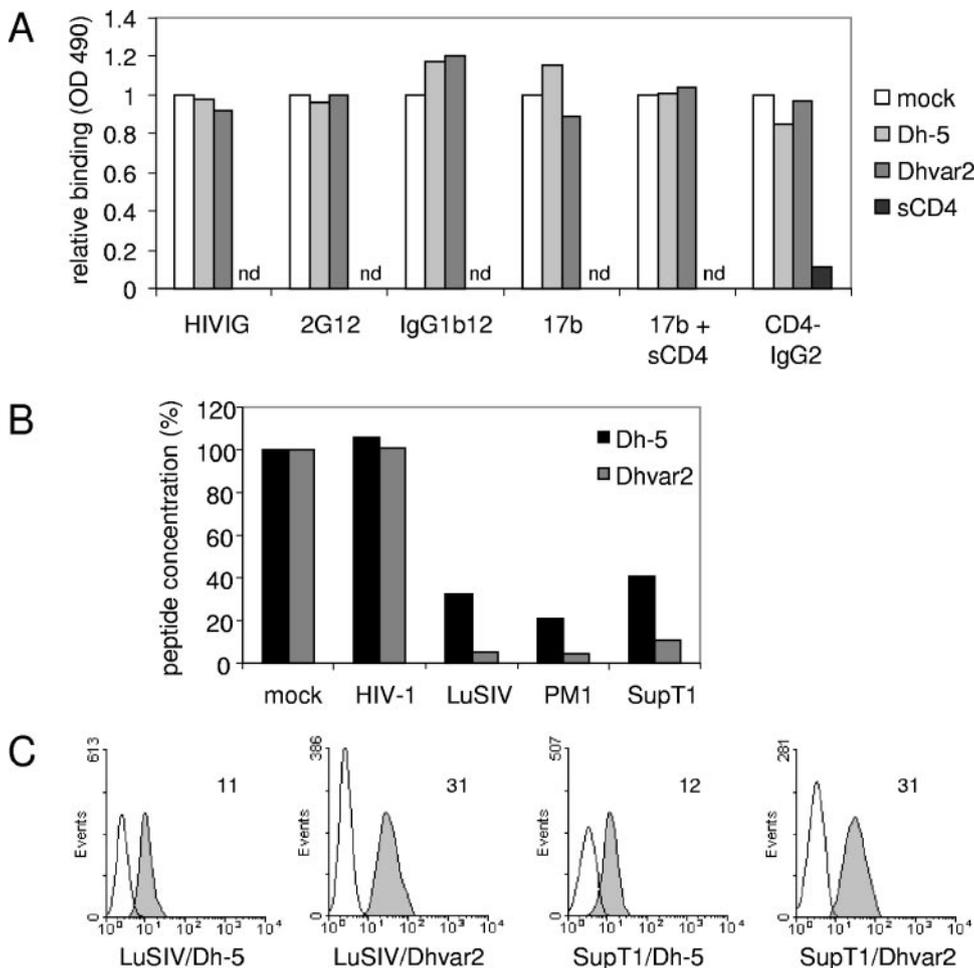


FIG. 4. Dhvar2 binds T cells. (A) gp120 ELISA. Increasing amounts of peptide (Dh-5 or Dhvar2) were allowed to compete with several reagents for binding to captured gp120. The reagents (HIVIg, 2G12, IgGb12, 17b [with or without sCD4], and CD4-IgG2) target the CD4 or coreceptor binding sites on gp120 and were used at their predetermined half-maximal binding capacity. Competition of sCD4 with CD4-IgG2 was performed in parallel to provide a control for competition efficiency. Results obtained with the highest concentration (100 μ M) of peptide are depicted. nd, not done. (B) Peptide depletion assay. HIV-1 or cells (LuSIV, PM1, SupT1) were added to a stock of Dh-5 or Dhvar2 in PBS. After incubation for 1 h, virus and cells were spun down, and the remaining concentration of peptide was determined. (C) FACS analysis. Dh-5 and Dhvar2 were labeled with FITC. After incubation with LuSIV or SupT1 T cells and subsequent washing, cells were analyzed by FACS. The mean fluorescence intensity is indicated. OD, optical density.

binding sites, since binding to these sites might provide a plausible mechanism for stimulation of infectivity by Dhvar2. Purified Ig from HIV-1-infected individuals (HIVIg) was used as well as several monoclonal antibodies. 2G12 binds to a glycan-dependent epitope on the outer domain of gp120 (27), IgGb12 targets the CD4 binding site (4), and 17b is directed to a CD4-induced site overlapping the coreceptor binding site (17). 17b was therefore tested with and without soluble CD4 (sCD4). Furthermore, we tested CD4-IgG2, which is a tetrameric CD4-based molecule that is directed to the CD4 binding site (1). Increasing amounts of peptide were allowed to compete with these respective reagents for binding to gp120, which was captured on an ELISA plate. The reagents were used at the predetermined half-maximal binding concentrations. CD4-IgG2 binding was also performed in the presence of sCD4 to provide a control for competition efficiency. Although we observed efficient inhibition of CD4-IgG2 binding to gp120 by sCD4, we did not observe an effect of Dh-5 or Dhvar2 on

binding of the reagents to gp120 (Fig. 4A). We tested a wide range of concentrations of the peptides (0, 3.7, 11.1, 33.3, and 100 μ M), and 100 μ M is twice the concentration that stimulates virus infectivity. Even this amount of peptide did not influence virus binding of the various reagents to gp120, indicating that the peptide's mode of action does not involve binding to the CD4 or coreceptor binding sites on gp120.

To further identify the target of Dhvar2, we tested whether Dhvar2 or Dh-5 could be depleted from a PBS solution by virus or T cells. High concentrations of virus (950 ng CA-p24/ml) did not deplete the peptides from the solution by virus centrifugation. In contrast, T cells (LuSIV, PM1, or SupT1) could deplete both peptides from the solution (Fig. 4B). The amount of Dh-5 that remained in solution was, on average, five times higher than that of Dhvar2, indicating that Dhvar2 binds more efficiently to T cells than Dh-5. This observation was confirmed with FITC-labeled peptides that can be detected by FACS analysis. The amount of FITC-labeled Dhvar2 that bound to

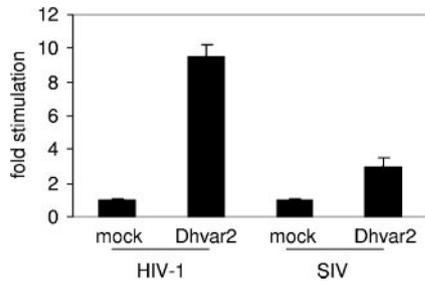


FIG. 5. Dhvar2 enhances infectivity of both HIV-1 and SIV. LuSIV cells were infected with HIV-1 or SIV-mac239 in the presence of 40 μ M Dhvar2. Luciferase was determined after 24 h. Error bars represent standard deviations.

LuSIV or SupT1 T cells was, on average, three times higher than the amount of Dh-5 (Fig. 4C). Combined, these experiments suggest that the target of Dhvar2 is the T cell rather than the virus.

Stimulation by Dhvar2 is dependent on HIV-1 Env. To investigate whether the stimulatory properties of Dhvar2 are also applicable to other viruses, we studied whether the peptide could enhance LuSIV infection by CCR5-using SIV-mac239. Although not as efficient as the stimulation observed with CXCR4-using HIV-1, Dhvar2 enhanced SIV infection by a factor of 3.5 (Fig. 5).

To study whether Dhvar2 stimulates viruses that use other entry mechanisms, we compared a lentiviral vector that is pseudotyped with either the vesicular stomatitis virus glycoprotein (VSV-G) or HIV-1 Env. We used a standard lentiviral vector with the green fluorescent protein (GFP) reporter gene (29). After entry of this pseudovirus into the cell and subsequent reverse transcription, GFP will be produced, which can be detected by FACS 3 days later. Dhvar2 could elevate the transduction efficiency of the HIV-1 Env-based vector twofold (Fig. 6A, upper row). In contrast, the percentage of GFP-positive SupT1 T cells was not elevated by Dhvar2 when the vector was bearing VSV-G (Fig. 6A, second row). This result

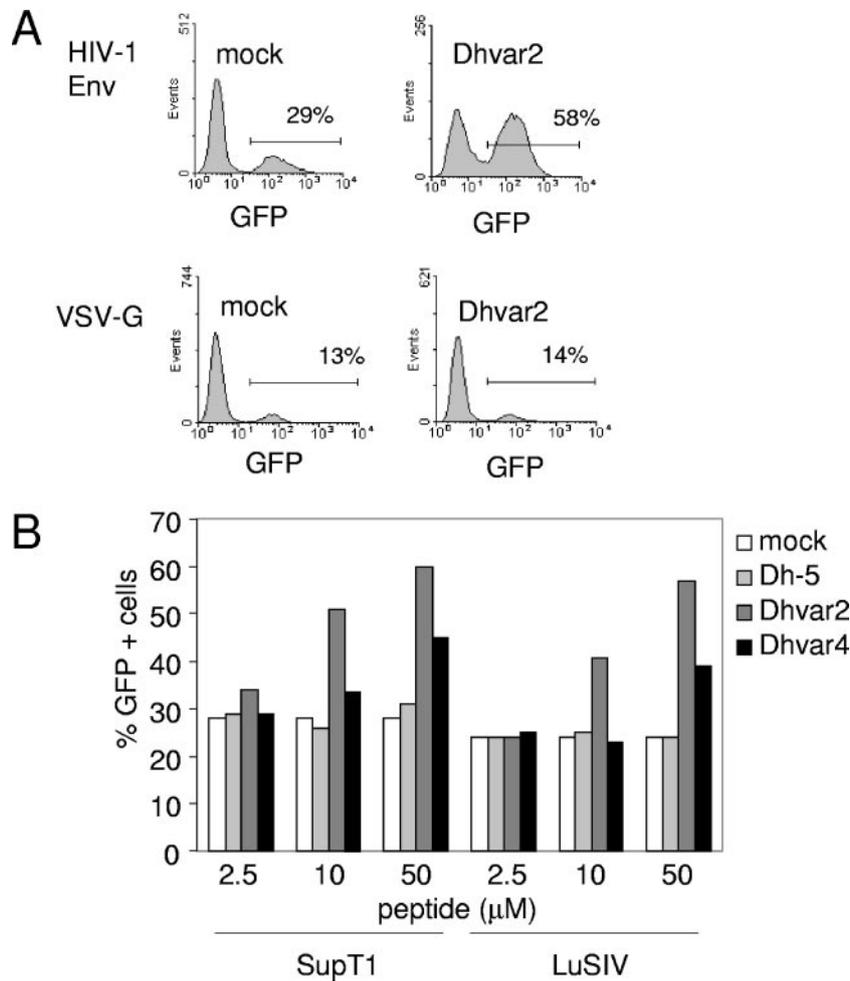


FIG. 6. Stimulation by Dhvar2 is dependent on HIV-1 Env. (A) SupT1 T cells were infected with a lentiviral vector in the presence of Dhvar2. The vector with the GFP gene was pseudotyped with either HIV-1 Env or VSV-G. After 3 days, the percentage of GFP-positive cells was determined by FACS. Representative FACS plots are shown, with the percentage of GFP-positive cells indicated. (B) SupT1 or LuSIV cells were infected with the lentiviral vector bearing HIV-1 Env, which was mock treated or incubated with Dh-5, Dhvar2, or Dhvar4. Three days later, the percentage of GFP-positive cells was determined.

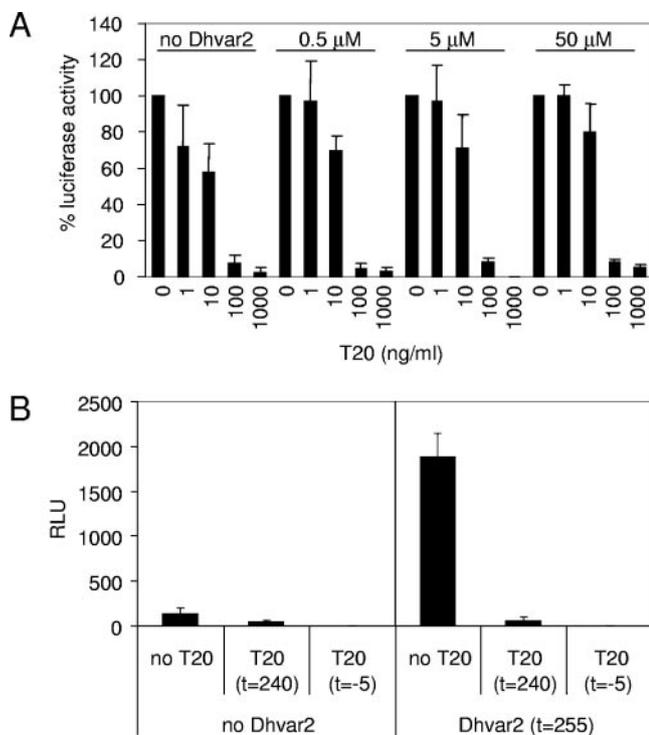


FIG. 7. Dhvar2 promotes HIV-1 Env-mediated cell entry. (A) The 90% inhibitory concentration of fusion inhibitor T20 is unaffected by Dhvar2. LuSIV cells were infected with HIV-1 in the presence of varying concentrations of Dhvar2 and T20. Luciferase production was determined 24 h later. (B) In the left panel, LuSIV cells were HIV-1 infected, followed by T20 addition 240 min later to stop the ongoing infection process. In the right panel, Dhvar2 was added at $t = 255$ min, with or without prior ($t = 240$ min) T20 application. As a control, T20 was added 5 min prior to HIV-1 infection. Error bars represent standard deviations. RLU, relative light units.

clearly demonstrates that the enhancing effect of Dhvar2 is dependent on the HIV-1 Env protein and excludes that the peptide affects other steps of the retroviral replication cycle shared by both vectors, which includes reverse transcription, integration, transcription, and translation. The FACS results with SupT1 T cells are quantified in Fig. 6B. Similar results were obtained when the vector was used to infect LuSIV cells (Fig. 6B). As a control, we included Dh-5 and Dhvar4. No stimulation was observed with Dh-5, and a slight stimulation was observed with Dhvar4.

Dhvar2 promotes HIV-1 Env-mediated entry. VSV and HIV-1 use different routes of cellular entry: VSV enters through endocytosis, whereas HIV-1 enters through receptor-mediated fusion of viral and cellular membranes (19, 20). The experiment using VSV-G and HIV-1 Env-pseudotyped lentiviral vectors indicates that the effect of Dhvar2 is HIV-1 Env dependent (Fig. 6). From these results, one cannot discriminate between effects on Env-mediated membrane fusion or postentry steps preceding reverse transcription (e.g., uncoating). To make this distinction, we employed the entry inhibitor T20. This peptide stabilizes a transient structural Env intermediate, thereby blocking subsequent conformational changes and the fusion of viral and cellular membranes (2, 9). We first investigated whether Dhvar2 interferes with the inhibitory

properties of T20 by titrating both peptides on LuSIV cells that were subsequently infected with HIV-1. Addition of Dhvar2 did not alter the inhibition profile of T20 (Fig. 7A). This result demonstrates that T20 inhibition is dominant over Dhvar2 stimulation, justifying further usage of T20 in these assays. We next infected LuSIV cells with HIV-1 and added T20 after 240 min to stop the ongoing infection process. Compared to the control infection, T20 partially reduced the luciferase production, demonstrating that a substantial amount of virus, but not all of it, had already entered the cells within 240 min (Fig. 7B, left panel). As a control, T20 was added 5 min before HIV-1 infection ($t = -5$ min), which totally abolished luciferase production. When Dhvar2 was added at $t = 255$ min, this expectedly led to a considerable increase in luciferase production. However, this stimulation was not observed when T20 was supplied 15 min prior to Dhvar2 addition (Fig. 7B, right panel). If Dhvar2 stimulated a postentry step, one would expect an increase in luciferase production despite the presence of T20, because this drug inhibits new entry events but not virus that has already been fused with the cell at the time of T20 addition. We therefore conclude that Dhvar2 promotes the Env-mediated entry step.

DISCUSSION

Vaginal, rectal, or oral transmission of HIV-1 involves crossing of mucosal tissue, a process that is not yet entirely understood (30, 40). One of the complicating factors for the virus is antimicrobial peptides that are secreted at mucosal surfaces as part of the innate immune system. Their use as candidate forms of prophylaxis against HIV-1 has therefore been proposed (5, 36, 38). There are several studies on antimicrobial peptides and inhibition of HIV-1 replication, and their mode of action is diverse, including disruption of the viral membrane, inhibition of reverse transcription, or prevention of viral entry (5, 18, 23, 32, 33, 39, 43). Previously, we reported that human saliva contains several compounds that inhibit HIV-1 at different stages of the replication cycle (3). We have now investigated the active domain of one salivary peptide (histatin 5) and derivatives thereof. Amino acids in the active domain (Dh-5) were substituted, yielding peptide variants (Dhvar2 to Dhvar5) with increased fungicidal activity (14, 25). We report that Dh-5 decreases the infectivity of HIV-1 in a single-cycle replication assay. Unexpectedly, none of the peptide variants were more potent antivirals. Interestingly, the Dhvar2 variant significantly stimulated HIV-1 up to 30-fold in the single-cycle replication assay.

We found that Dhvar2 binds to T cells and that it enhances Env-mediated viral entry. Stimulation was observed with CCR5-using SIV, CXCR4-using HIV-1, and pseudotyped viruses, but not when this pseudovirus was bearing the VSV-G protein. VSV and HIV-1 have different routes of entry: VSV enters a cell through endocytosis, whereas HIV-1 enters through receptor-mediated fusion of viral and cellular membranes (19, 20). In a pulse-chase experiment with the T20 entry inhibitor, we could demonstrate that Dhvar2 acts early at the entry step (Fig. 7B). Membrane interaction is a very general property of antimicrobial peptides (37). Possibly, Dhvar2 facilitates fusion of the viral and cellular membrane due to the fact that this amphipathic peptide inserts in membranes and

weakens the structure of the lipid bilayer. We observed a strong correlation between amphipathicity and the stimulatory or inhibitory properties of the different peptides (Table 1). We therefore do not think that Dhvar2 has a specific protein-based receptor on T cells or the virus.

The difference between Dh-5 and the variants is the predicted 3-dimensional α -helical conformation: Dhvar2, Dhvar3, and Dhvar4 have a more pronounced separation between the hydrophilic and hydrophobic amino acids than Dh-5, leading to an optimal amphipathicity in the α -helical conformation (Table 1). While in the context of *Candida* the derivatives have an increased fungicidal activity, this apparently does not apply to the HIV-1–human cell interaction. At the concentrations used in our study, Dhvar2 seems to affect the membrane such that Env-mediated membrane fusion and subsequent viral entry is promoted. This also applies to Dhvar3 and Dhvar4 to a lesser extent.

Interestingly, one of the Dh-5 variants (Dhvar5) shows sequence similarity to the C terminus of a recently described papillomavirus capsid protein (15). This peptide mediates escape of viral genome degradation by destabilizing the membrane of the endocytic compartment. Remarkably, Dhvar5 is the only Dh-5 variant that did not stimulate HIV-1 infection. Furthermore, Dhvar5 is the only variant that is less amphipathic than Dh-5. These results demonstrate that the stimulatory properties of a peptide on viruses is context dependent and therefore difficult to predict. This papillomavirus study, combined with our results, suggests that minor membrane destabilization may be beneficial to a broad range of viruses.

Recently, another study showed that antimicrobial peptides with comparable bactericidal effects have differential effects on HIV-1 replication. One peptide, cryptdin 3 (Crp3), stimulated HIV-1 in an unidentified step preceding reverse transcription (33). Crp3 is a member of the α -defensin family and has been described to form anion-selective pores in mammalian cell membranes (41). It is therefore possible that the mode of action is comparable to that of Dhvar2. We are the first to show that peptides with minor amino acid substitutions can have an unexpected reverse effect on HIV-1 replication.

Our findings are relevant for the design and clinical application of antimicrobial peptides. *Candida albicans* is the predominant species of yeast isolated from patients with oral candidiasis, which is a frequent symptom of HIV-1 infection and a criterion for staging and progression of AIDS (6). Treating candidiasis patients with the peptide variants with increased antifungal activity seems tempting but would be unadvisable and potentially dangerous if the patient is HIV-1 infected. In the absence of HIV-1 infection, people who receive treatment for oral candidiasis with the variant peptides should be aware of the increased susceptibility to HIV-1 infection.

In conclusion, our study shows that modification of antimicrobial peptides in order to improve their activity may have unwanted and potentially dangerous side effects on other pathogens. Since these effects are difficult to predict, this should be considered in the development of antimicrobial peptides for clinical application.

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