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The anti-papillomavirus activity of human and bovine lactoferricin

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Abstract

Human papillomavirus (HPV) cause common warts, laryngeal papilloma and genital condylomata and is necessary for the development of cervical cancer. We have previously found that lactoferrin has antiviral activity against HPV-16 and others have demonstrated that lactoferricin, an N-terminal fragment of lactoferrin, has inhibitory activities against several viruses. Two cell lines and two virus types, HPV-5 and HPV-16, were used to study if lactoferrin and lactoferricin could inhibit HPV pseudovirus (PsV) infection. We demonstrated that bovine lactoferrin (bLf) and human lactoferrin (hLf) were both potent inhibitors of HPV-5 and -16 PsV infections. Among the four lactoferricin derivatives we analyzed, a 15 amino acid peptide from bovine lactoferricin (bLfcin) 17–31 was the most potent inhibitor of both HPV-5 and HPV-16 PsV infection. Among the other derivatives, the human lactoferricin (hLfcin) 1–49 showed some antiviral activity against HPV PsV infection while bLfcin 17–42 inhibited only HPV-5 PsV infection in one of the cell lines. When we studied initial attachment of HPV-16, only bLfcin 17–42 and hLfcin 1–49 had an antiviral effect. This is the first time that lactoferricin was demonstrated to have an inhibitory effect on HPV infection and the antiviral activity differed depending on size, charge and structures of the lactoferricin.

Keywords: Lactoferrin; Lactoferricin; Human papillomavirus; Antiviral activity

1. Introduction

Human papillomavirus (HPV) is an 8 kb naked DNA virus belonging to the family of *Papillomaviridae*. HPV infect basal cells in mucosa or skin, possibly through micro lesions, and is strongly dependent on the differential status of the epithelium for its viral life cycle and is necessary for the development of cervical cancer. HPV-16 belongs to the α-papillomavirus genus, species 9 (de Villiers et al., 2004) and is the most common type found in cervical cancer but does also cause condyloma and other infections of the genital and respiratory tracts (Stubenrauch and Laimins, 1999). HPV-5 is the most common type found on normal skin all around the world (Antonsson et al., 2003). It belongs to species 1 of the β-papillomavirus genus (de Villiers et al., 2004) and is associated with skin cancers in patients diagnosed with e*pidermodysplasia verruciformis* (Berkhout et al., 1995). Propagating HPV has been very difficult but the development of virus like particles (VLP) produced in recombinant expression systems (Zhou et al., 1992) and pseudovirus (PsV) produced inside cells (Buck et al., 2004; Roden et al., 1996; Unckell et al., 1997; Zhao et al., 1998) have simplified the studies of the papillomavirus life cycle. VLP and PsV have been used in several studies of HPV binding and entry (Day et al., 2004; Drobni et al., 2003; Evander et al., 1997; Giroglou et al., 2001; Joyce et al., 1999) and PsV have also been used for high-throughput screening of compounds with the potential to block papillomavirus infectivity *in vitro* (Buck et al., 2006b). The first step of HPV infection is believed to be binding of the major capsid protein L1 to the cell surface. The cell surface glycosaminoglycan (GAG) heparan sulfate is important for the initial attachment to the cell surface of certain α-papillomaviruses found more frequently in mucosal lesions (Combita et al., 2001; Drobni et al., 2003; Giroglou et al., 2001; Joyce et al., 1999; Shafti-Keramat et al., 2003). Heparan sulfate is also a receptor for several other viruses such as herpesvirus, norovirus, hepatitis C virus and foot-mouth disease virus (Spillmann, 2001).

Lactoferrin is an 80 kDa monomeric glycoprotein present in secretions such as breast milk, saliva, semen and tears. The highest concentration of lactoferrin is found in colostrums (Cohen et al., 1987). Lactoferrin plays important and multifunctional

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roles in host defence and some of these functions are to inhibit infection of bacteria, fungi and viruses (Bellamy et al., 1993). Lactoferrin acts as an antiviral protein against herpes simplex virus (HSV), human cytomegalovirus (HCMV), hepatitis C virus (HCV), poliovirus, enterovirus 71 (EV71), BK polyomavirus, HIV and HPV (Andersen et al., 2003; Andersen et al., 2004; Andersen et al., 2001; Drobni et al., 2004; Harmsen et al., 1995; Ikeda et al., 1998; Jenssen, 2005; Lin et al., 2002; Longhi et al., 2006; Marchetti et al., 1996; Marchetti et al., 1999). Lactoferrin exhibits its antiviral activity early in the infection cycle and for HSV (Andersen et al., 2004; Hasegawa et al., 1994; Marchetti et al., 2004), HPV-16 (Drobni et al., 2004) and hepatitis B virus (Hara et al., 2002) the lactoferrin interaction with heparan sulfate on the cell surface seem to block the attachment of the virus. The antiviral activity could also be mediated by a direct interaction between the virus and lactoferrin as seen for poliovirus (Marchetti et al., 1999), rotavirus (Superti et al., 1997), HIV (Swart et al., 1996), HCV (Yi et al., 1997), EV71 (Ammendolia et al., 2007; Weng et al., 2005), and BK polyomavirus (Longhi et al., 2006).

Lactoferricin is generated by proteolytic cleavage of the N-terminal part of lactoferrin by pepsin. Bovine lactoferricin (bLfcin) fragments have been described to be composed of either amino acid 17-41 (Bellamy et al., 1992a) or 17–42 (Dionysius and Milne, 1997). One cysteine–cysteine disulfide bond between residue 19 and 36, creates a loop structure (Hwang et al., 1998), though this loop structure is not essential for antibacterial activity (Bellamy et al., 1992a). In the homologus human lactoferricin (hLfcin), composed of amino acid 1–49, a loop is created with two disulfide bridges between residue 20–37, and 10–46 respectively (Hunter et al., 2005). Lactoferricin has broad host defense properties against bacteria, fungi, parasites and viruses (Gifford et al., 2005) and some of the antimicrobial properties of lactoferricin can be explained by its ability to form amphipathic structures with clear hydrophobic and positively charged faces. Other peptides with antimicrobial activity also display these characteristics (Epand and Vogel, 1999). Different lactoferricin types have antiviral effects against viruses such as HSV-1 and -2 (Andersen et al., 2003), HCMV (Andersen et al., 2001), HIV (Berkhout et al., 2002), HCV (Ikeda et al., 2000) and echovirus 6 (Pietrantoni et al., 2006), but have also been shown to have tumor inhibitory effects (Eliassen et al., 2002; Yoo et al., 1997). We have previously demonstrated the antiviral effect of lactoferrin on HPV internalization and binding to HaCaT cells in vitro using HPV VLPs (Drobni et al., 2004). In this study we wanted to better understand the inhibitory effect of human and bovine lactoferrin and four derived peptides on HPV-5 and HPV-16 PsV infection.

2. Materials and methods

2.1. Virus-like particle production

The HPV VLPs were produced using a baculovirus expression system. Sf-21 insect cells were infected with recombinant baculovirus expressing the HPV-16 L1 capsid protein under the control of the polyhedrin promoter. The recombinant baculovirus was a kind gift from Ian Frazer, CICR, Brisbane, Australia. The VLPs were produced as previously described (Drobni et al., 2003). Briefly, the infected cells were grown until 80% cell death, and nuclei were prepared using different steps of mechanical disruption, sonication and centrifugations. The VLPs were extracted by sucrose gradients and CsCl gradients before dialysis against extensive amounts of PBS. The integrity of the particles was assessed using electron microscopy.

2.2. Pseudovirus production

Plasmids and 293TT cells (Buck et al., 2005) used for pseudovirus production were a kind gift from Chris Buck (National Cancer Institute, Bethesda, Maryland, USA) and Martin Müller (German Cancer Research Center, Heidelberg, Germany). Detailed protocols are available at the website (http://home.ccr.cancer.gov/lco/default.asp). HPV-5 and HPV-16 GFP-expressing pseudoviruses were produced according to previously described methods (Buck et al., 2005). Briefly, 293TT cells engineered to express high levels of SV40 large T antigen were transfected with plasmids expressing the papillomavirus major and minor capsid proteins, L1 and L2, together with a green fluorescent protein (GFP)-expressing reporter plasmid, pfwB. All PsV were produced using codon-modified L1 and L2 genes. HPV16 PsV was produced using a bicistronic L1/L2 expression plasmid, p16sheLL and HPV-5 PsV was produced using the p5L1w and p5L2w plasmids. Capsids were allowed to mature overnight in cell lysate and were then purified using OptiPrep® gradients (Axis-Shield).

2.3. Proteins and peptides

Lactoferrin of bovine origin was purchased from Sigma Chemical Co. (St. Louis, MO). BLfcin and hLfcin was purchased from Centre for Food Technology (Brisbane, Australia), except for hLfcin 18–42, which was provided by MedProbe (California, USA). Four different lactoferricin peptides were used to investigate the possible differences between human and bovine peptides. BLfcin 17–41 (FKC_1RRWQWRMKKLGA-$PSITC_1$VRRAF). BLfcinB 17–42 (FKC_1RRWQWRMKKL-GAPSITC_1VRRAFA). BLfcin 17–31 (FKCRRWQWRMKK-LGA) with acetamidomethyl thiol protecting group on the cysteine. HLFcin 1–49 (GRRRRSVQWC_1AVSQPEATKC_2-FQWQRNMRKVRGPPVSC_2IKRDSPIQC_1IQA). HLfcin 18–42 (TKC_1FQWQRNMRKVRGPPVSC_1IKRDS). Cysteines forming disulfide bonds are numbered with subscript numbers to indicate their pairing.

2.4. Internalization assay

Lactoferrin and lactoferricin were tested using a previously described PsV-based papillomavirus inhibition assay (Buck et al., 2006b). Briefly, the human epithelial cell line HaCaT, from adult trunk skin (Boukamp et al., 1988) and the human cervical cell line C33A (Crook et al., 1991) were plated at 5×10^4 cells/well in 400 μl of DMEM supplemented with 10% FCS (Life technologies, Inc., Gaitersburg, MD) in 24-well

plates. Lactoferrin and lactoferricin were dissolved in sterile water and diluted to concentrations covering an appropriate range. Diluted lactoferrin and lactoferricin were added to pre-plated cells, followed by 2–5 μl of PsV stock and incubation for 44–52 h at 37 °C. The flow cytometric analysis was fixed to count 10,000 live cells and a marker region was set so that <0.1% of the negative control corresponded to GFP positive cells. The negative control was considered as background and subtracted from the GFP positive cells. PsV doses were calibrated such that between 5% and 25% of cells scored as GFP positive when no inhibitors were added, which corresponded to 1.3×10^5 transducing units/ml (about 1 ng/ml L1). The fluorescence in HPV PsV infected cells with no inhibitors added was set to 100% and the relative values were calculated. Relative to HaCaT cells the appropriate infection of C33A cells required a 1.5–2 fold higher capsid dose for HPV-5 and a 1.5–2 fold lower capsid dose for HPV-16. The 50% inhibitory concentration (IC_{50}) was defined as the compound concentrations that protect 50% of the cells against virus-induced fluorescence and the IC_{50} values was calculated using linear interpolation.

2.5. Binding assay

The assay was performed as previously described (Drobni et al., 2003), In short, HaCaT cells were grown to 80% confluence in a 75 cm^2 bottle in DMEM supplemented with 10% FCS (Life technologies, Inc., Gaitersburg, MD). The cells were removed by trypsination, resuspended in DMEM + 10% FCS and grown in suspension for 2 h to allow re-expression of surface proteins. The cells were then counted and washed once in binding medium (DMEM + 0.2% bovine serum albumin). VLPs (100 ng) were incubated with different concentrations of lactoferricin for 30 min on ice and thereafter incubated on ice with 1×10^5 cells in 200 μl binding medium for 30 min. The cells were washed twice with ice cold PBS and thereafter resuspended in 20 μl SDS-PAGE sample buffer. A western blot was then performed using monoclonal mouse anti HPV-16 L1 antibody 885 directed against the HPV L1 protein (Chemicon International Inc.) and a secondary goat antibody directed against mouse IgG conjugated to horse radish peroxidase (HRP) (Dakopatts). The bands were analyzed using the Gel-Pro Analyzer® software from Media Cybernetics (Silver Spring, MD). The IC_{50} was defined as the compound concentrations that protect 50% of the cells against virus binding detected by western blot and IC_{50} values was calculated using linear interpolation.

2.6. ELISA

A microtiter plate was coated over night with 4 μg of HPV-16 VLPs in PBS as a control and 4 μg of bLf or bLfcin 17–42 in sodium carbonate–bicarbonate buffer pH 9.6. The wells were washed twice with PBS and blocked for 1 h with PBS + 4% BSA and then washed twice with PBS + 4% BSA. Then, 4 μg of HPV-16 VLPs was added to the bLf/bLfcin wells for 1 h at 37 °C. The wells were washed twice with PBS and polyclonal conformational anti HPV-16 L1 VLP antibodies produced in rabbit by AgriSera (Vännäs, Sweden), were added in 1:100,000 dilution and incubated for 2 h at 37 °C. After washing a secondary anti-rabbit IgG conjugated to HRP (Dakopatts) was added for 1 h as above and washed. Finally 100 μl TM Blue substrate was added and incubated 15 min in dark before analysis. The results were expressed as percentage of VLP binding to wells and antibody binding to HPV-16 L1 VLP coated wells (control) was set to 100%. Data are means of three separate experiments performed in duplicates.

2.7. Analysis of protease activity

HPV-16 PsV (5 μl) diluted in DMEM + 10% FCS was mixed with hLf (80 μg/ml), bLf (80 μg/ml), bromelain protease (20 U and 200 U) (Sigma) or V8 protease (20 U and 200 U) (Sigma) in a total volume of 50 μl. The samples were incubated at 37 °C for 18 h and then 30 μl of each sample was mixed with SDS-PAGE sample buffer. A Western blot was then performed as described in Section 2.5.

2.8. Statistical analysis

For analyzing the difference between the effects of the compounds, the area under the curve for each experiment was calculated with the GraphPad Prism® v4 software (San Diego, California). A 2-tailed student *T*-test was then used to compare the means of the area under the curve from the studies and *P*-values of <0.05 were considered significant. To determine a dose-dependent inhibition a sigmoid dose–response (variable slope) curve fitting was chosen and $R^2 > 0.8$ was considered as a dose-dependent inhibition.

3. Results

3.1. Lactoferrin inhibited both HPV-5 and HPV-16 PsV infection

Previously we found that internalization and binding of carboxy-fluorescein diacetate, succinimidyl ester labeled HPV-16 L1 VLPs was inhibited by lactoferrin (Drobni et al., 2004). We now extended these studies by using an HPV infection assay where we could determine if lactoferrin affected HPV PsV infection. This assay is similar to a natural HPV infection since the PsV consists of a GFP marker plasmid encapsidated by the virion consisting of both the L1 and L2 capsid proteins. After PsV binding to and entry into the cell, the PsV, containing the GFP marker plasmid, is transported to the nucleus for expression and detection of fluorescence (Buck et al., 2004). For analysis of the lactoferrin antiviral activity we used two HPV types, the α-papillomavirus HPV-16 and the β-papillomavirus HPV-5. Furthermore, two cell lines with different origin, HaCaT from skin and C33A from cervical mucosa were infected with HPV PsV. There was no cell toxicity detected at the highest lactoferrin concentrations used. When we added lactoferrin we found that both bLf and hLf reduced the number of fluorescent cells in a dose-dependent manner ($R^2 > 0.8$) in both cell lines. In C33A cells we were surprised to find a biphasic shape of the curve at low concentrations of lactoferrin (Fig. 1B), although this phe-

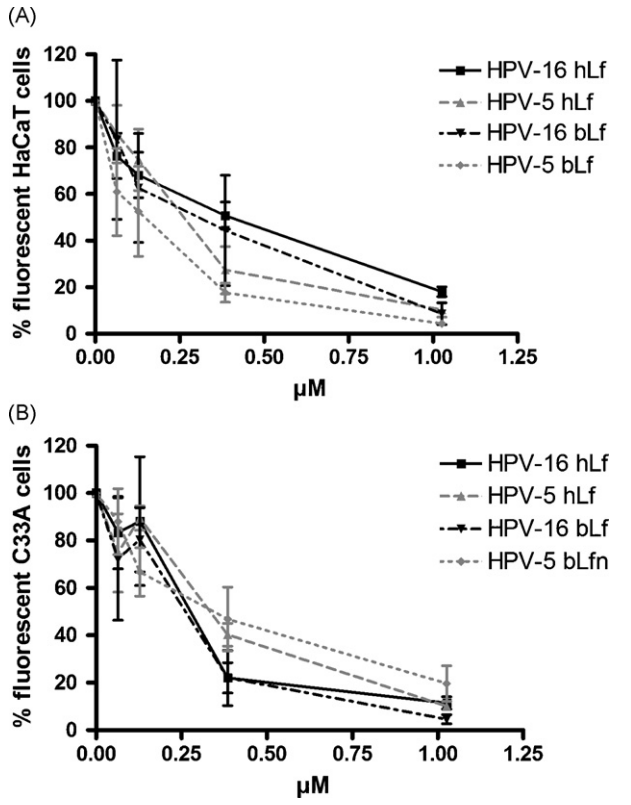

Fig. 1. Dose-dependent inhibition of HPV PsV infection by lactoferrin. HaCaT (A) and C33A (B) cells were incubated with different doses of bLf or hLf and then HPV-5 or HPV-16 PsV was added. The GFP expression was measured after 2 days by flow cytometry and the fluorescence in untreated cells was set to 100% and the relative values were calculated. Data are mean ± S.D. from three separate experiments performed in duplicates.

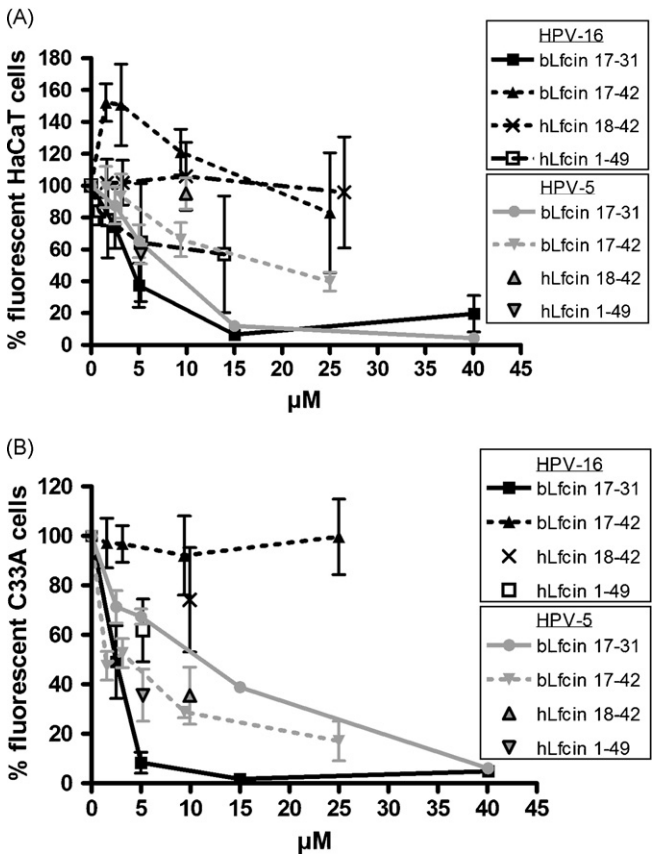

Fig. 2. Inhibition of HPV-16 PsV infection by lactoferricin. HaCaT (A) and C33A (B) cells were incubated with different doses of bLfcin or hLfcin and then HPV-5 or -16 PsV was added. The GFP expression was measured after 2 days by flow cytometry and the fluorescence in untreated cells was set to 100% and the relative values were calculated. Data are mean ± S.D. from three separate experiments performed in duplicates.

nomenon was within the standard deviations. Both human and bovine lactoferrin had a very strong inhibitory effect on HPV PsV infection in both cell lines at the highest concentration we tested, 1.0 μM (Fig. 1). On average, the IC_{50} value of bLf and hLf for inhibition of HPV PsV infection was respectively, 0.25 ± 0.24 μM and 0.32 ± 0.18 μM. To analyze for significant differences we used the area under curve as an endpoint and compared the antiviral effect of bLf and hLf on different HPV types and cell lines by using a 2-tailed student *T*-test and no significant differences were found. In conclusion both human and bovine lactoferrin were potent inhibitors of HPV-5 and HPV-16 PsV infection.

3.2. Inhibition of HPV PsV infection by lactoferricin

The small antimicrobial peptide lactoferricin has antiviral activity against several viruses (see above) and the antiviral activities of a set of defined bLfcin and hLfcin derivatives have been tested against HSV-1 and HSV-2 (Jenssen et al., 2004b). We used some of these derivatives for studying their inhibitory effect against HPV infection and no cell toxicity was detected at the highest lactoferricin concentrations used. The bLfcin 17–31 had a dose-dependent inhibitory effect ($R^2 > 0.8$) on HPV-5 and -16 PsV infections in both HaCaT and C33A cells (Fig. 2). The IC_{50} value of bLfcin 17–31 for inhibition of HPV-5 and -16 PsV infections in both cell lines was on average 9.05 ± 3.0 μM and 3.3 ± 0.7 μM, respectively. BLfcin 17–42 did not have an antiviral effect on HPV-16 PsV infection, while there was dose-dependent inhibition ($R^2 > 0.8$) of HPV-5 PsV infection in C33A cells using this peptide ($IC_{50} = 3.4 \pm 2.3$ μM) (Fig. 2). Surprisingly, we could detect increased infection by HPV-16 PsV in HaCaT cells using low concentrations of bLfcin 17–42, but with higher concentrations this phenomenon disappeared (Fig. 2A). The human homologue hLfcin 18–42 did only inhibit HPV-5 PsV infection to 50% in C33A cells at 5 μM, but no other antiviral activity was detected (Fig. 2). The hLfcin 1–49 inhibited HPV-16 PsV infection of HaCaT cells but not in a dose-dependent manner ($R^2 < 0.8$). For HPV-5 PsV infection of HaCaT cells and HPV-5 and -16 PsV infections of C33A cells we could demonstrate 40–65% inhibition at 5 μM of the hLfcin 1–49 peptide inhibitor, but unfortunately we were only able to perform those experiments at one concentration (Fig. 2). By analyzing the area under curve as an endpoint we could determine that bLfcin 17–31 was significantly better than bLfcin 17–42 for inhibiting HPV-16 PsV infection in HaCaT cells ($P < 0.00005$) and in C33A cells ($P < 0.00002$). Furthermore, bLfcin 17–31 was also a better inhibitor that bLfcin 17–42 for HPV-5 PsV infection in HaCaT cells ($P < 0.03$), while in

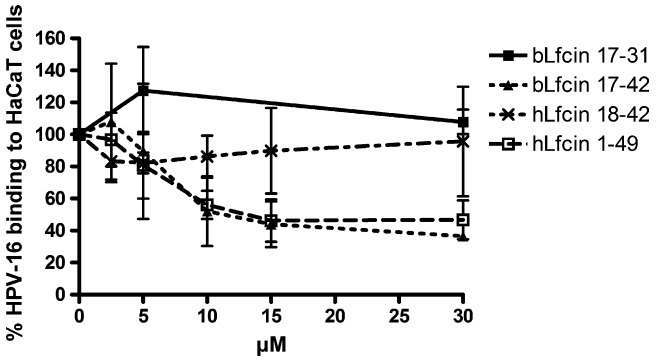

Fig. 3. Inhibition of HPV-16 VLP binding to HaCaT cells by lactoferricin at 0 °C. Binding was detected with antibodies against the L1 capsid protein. The amount of bound HPV-16 VLPs in untreated cells was set to 100% and the relative values were calculated. Data are mean ± S.D. from three separate experiments.

C33A cells bLfcin 17–42 was a better inhibitor of HPV-5 PsV infection than bLfcin 17–31 ($P < 0.00009$). In conclusion, bLfcin 17–31 was the most potent anti-HPV peptide of the derivatives analyzed.

3.3. Inhibition of binding by lactoferricin

To study if lactoferricin could inhibit initial binding of HPV to the cell surface we analyzed the effect of lactoferricin on HPV-16 L1 VLP binding to HaCaT cells. We know that lactoferrin inhibits HPV-16 VLP binding, probably due to blocking of cell surface heparan sulfate used by the virus for initial attachment (Drobni et al., 2004). To analyze the effect of different derivatives of lactoferricin we incubated HPV-16 VLPs with potential inhibitors prior to addition of cells and found that bLfcin 17–31 had no inhibitory activity and instead bLfcin 17–42 ($IC_{50} = 9.7\ \mu M \pm 5.0$) and hLfcin 1–49 ($IC_{50} = 16\ \mu M \pm 7.4$) inhibited binding at 0 °C (Fig. 3). Both bLfcin 17–42 ($P < 0.002$) and hLfcin 1–49 ($P < 0.005$) were significantly better than bLfcin 17–31 for inhibition of HPV-16 VLP binding.

3.4. Direct interaction between bLf/bLFcin and HPV-16 VLPs

To study if there was any interaction between lactoferrin or lactoferricin and HPV-16 L1 VLPs, we coated a microtiter plate with bLf and bLfcin 17–42 and bound VLPs were detected in three separate experiments using a polyclonal antibody directed against HPV-16 VLPs. We found that HPV-16 VLPs could bind to both bLf and bLfcin 17–42 but only to 40% (bLf) and 25% (bLfcin 17–42) in comparison to control (data not shown). Thus, bLf could to some extent interact with HPV-16 while bLfcin 17–42 demonstrated a much weaker interaction. Lactoferrin has been reported to have some proteolytic activity (Hendrixson et al., 2003) and we analyzed if the anti-papillomavirus activity of lactoferrin could be due to destruction of the viral capsid. When we analyzed HPV capsid degradation by protein gel and Western blot, we found that bromelain protease and V8 protease were able to digest the HPV-16 PsV while incubation with bLf and hLf for 18 h did not result in any cleavage of the HPV capsid proteins (data not shown).

4. Discussion

Lactoferrin of both human and bovine origin was a potent inhibitor of HPV-5 and -16 PsV infections. Previously we have shown that bLf inhibits HPV-16 VLP internalization in HaCaT cells (Drobni et al., 2004) and bLf has also been shown to inhibit HPV-16 PsV infection of HeLa cells at $IC_{50} = 13\ \mu g/ml$ (Buck et al., 2006b). In our study, HPV-16 PsV infection was inhibited at similar lactoferrin concentration in C33A and HaCaT cells, $IC_{50} = 20\ \mu g/ml$ (0.26 μM). The hLf inhibitory activity we demonstrated against HPV-16 PsV infection in HaCaT cells ($IC_{50} = 33\ \mu g/ml$, corresponding to 0.41 μM) and in C33A cells ($IC_{50} = 20\ \mu g/ml$, corresponding to 0.26 μM) was only slightly stronger than inhibition of HPV-16 PsV infection in HeLa cells ($IC_{50} = 62\ \mu g/ml$) (Buck et al., 2006a). Several viruses are inhibited by lactoferrin and it seems as it exhibits its antiviral activity early in infection, most probably by preventing virus entry by interacting with the viral attachment receptor heparan sulfate (Andersen et al., 2004; Drobni et al., 2004; Hara et al., 2002; Hasegawa et al., 1994; Marchetti et al., 2004). However, the competition for viral binding sites on the cell surface cannot fully explain the antiviral activity of lactoferrin (Seganti et al., 2001). Its antiviral activity can also be mediated by a direct interaction with the viral particle (Ammendolia et al., 2007; Marchetti et al., 1999; Superti et al., 1997; Swart et al., 1996; Weng et al., 2005; Yi et al., 1997). In light of the interaction we observed between bLf and HPV-16 VLPs, it is possible that lactoferrin inhibits HPV infection by binding both to the heparan sulfate on the cell surface and to the viral capsid. However, even though lactoferrin has been reported to have some proteolytic activity (Hendrixson et al., 2003) we could not detect any degradation of the viral capsid.

In this study we were for the first time able to show inhibition of HPV infection after incubation with lactoferricin, the cleaved N-terminal part of lactoferrin. HPV is a naked virus and most studies demonstrating an antiviral effect of lactoferricin have focused on enveloped viruses such as HSV-1 and -2 (Andersen et al., 2003; Jenssen et al., 2004b), HCMV (Andersen et al., 2001), HIV (Berkhout et al., 2002) and HCV (Ikeda et al., 2000). Naked viruses reported to be inhibited by lactoferricin are echovirus-6 (Pietrantoni et al., 2006) and feline calicivirus (McCann et al., 2003). In these two studies, the bLfcin was isolated by digestion of lactoferrin and probably represented the bLfcin 17–41 or 17–42 fragments. The authors suggest that both these naked viruses were inhibited during the early binding step (McCann et al., 2003; Pietrantoni et al., 2006). Similar to these results we demonstrated that bLfcin 17–42 and hLfcin 1–49 could inhibit HPV-16 VLP binding at 0 °C. BLfcin 17–42 did not show any antiviral activity against HPV-16 PsV infection at 37 °C, while for HPV-5 PsV infection of C33A cells, there was an inhibitory effect. We cannot explain the differences in lactoferricin antiviral activity against the α-papillomavirus HPV-16 and the β-papillomavirus HPV-5. However, there seem to be differences regarding their use of

heparan sulfate as a receptor (Buck et al., 2006b) and it would have been interesting to study inhibition of HPV-5 VLP binding at 0 °C, but unfortunately we did not have an appropriate antibody.

Overall, the most potent lactoferricin peptide in our study was the linear peptide bLfcin 17–31. The bLfcin 17–42 and the human homologue hLfcin 18–42 both have cyclic structures with cysteines forming disulfide bonds, while in bLfcin 17–31 eleven C-terminal residues have been removed, deleting two cationic residues Arg 22 and 23 and resulting in a reduced net positive charge (Jenssen et al., 2004b). This results in a large decrease in affinity for heparan sulfate (Jenssen et al., 2004b) and could partly explain why bLfcin 17–31 did not inhibit HPV initial binding at 0 °C in comparison to bLfcin 17–42. The bLfcin 17–31 had strong inhibitory effect against HPV PsV infection and previously it has been demonstrated that this shorter bLfcin derived peptide possess most of the properties contributing to the antibacterial activity of bLfcin 17–42 (Vorland et al., 1999). Removal of the eleven C-terminal residues in bLfcin 17–42 resulted in loss of all detectable antiviral activity against HSV (Jenssen et al., 2004b) but an increased antiviral activity against HPV-16 in HaCaT and C33A cells and HPV-5 in HaCaT cells. It has been reported that the two adjacent tryptophan residues in bLfcin 17–31 play a critical role in its antibacterial activity (Jing et al., 2006; Strom et al., 2000) but the importance of these residues regarding anti-HPV activity still has to be determined. Interestingly, a peptide encompassing amino acids 20–30 within the bLfcin 17–31 peptide was analyzed for its effect against HPV-16 PsV infection of HeLa cells, with no inhibitory effect at concentrations up to >15 μg/ml (Buck et al., 2006b). We demonstrated that the bLfcin 17–31 had an average IC_{50} of 6.5 μg/ml (3.3 μM) for HPV-16 PsV infection of C33A and HaCaT cells. The 11 amino acid peptide used by Buck et al. (2006b) contains the two tryptophan residues in bLfcin 17–31, thus it seems as additional factors such as size, three dimensional structures, charge and amino acid composition influences the antiviral activity. The hLfcin 1–49 also has a loop structure (Hunter et al., 2005) and this peptide showed inhibitory activity against HPV-5 and HPV-16 PsV infection in both cell lines, while the hLfcin 18–42 did not show any inhibitory activity. Similar activity patterns between hLfcin 1–49 and hLfcin 18–42 have been demonstrated against HSV (Jenssen et al., 2004b). The heparan sulfate binding site is located in the lactoferricin peptide and it could, similar to lactoferrin, block virus binding and entry (Andersen et al., 2004; Di Biase et al., 2003). The strong inhibitory activity of bLfcin 17–31 against HPV infection we found could not be explained by binding of the lactoferricin to cell surface heparan sulfate since bLfcin 17–31 did not block HPV binding. However, since there is evidence that lactoferrin and lactoferricin partly act through different mechanisms (Jenssen et al., 2004b) other explanations are also plausible, such as a direct interaction with the virus, although bLfcin 17–42 only interacted weakly with HPV-16 VLPs. Lactoferricin could also exert its effects inside the host cell since it is known that lactoferrin can bind nuclear DNA and act as an transcription factor (He and Furmanski, 1995), and lactoferricin can also bind to DNA (van Berkel et al., 1997) and perhaps up regulate host cell defence in response to virus infection.

Several sulfated polysaccharides have been described as inhibitors against HPV infection, such as heparin (Drobni et al., 2003; Giroglou et al., 2001; Joyce et al., 1999), dextran sulfate (Buck et al., 2006b; Drobni et al., 2003; Joyce et al., 1999), cellulose sulfate (Buck et al., 2006b; Christensen et al., 2001), chondroitin 6-sulfate, fucoidan and carrageenan (Buck et al., 2006b). Synthetic peptides corresponding to positively charged sequences of the L1 and L2 HPV capsid proteins also inhibited infectivity of HPV-31 PsV with up to 80% (Bousarghin et al., 2004). Also, several α-defensins were shown to inhibit HPV PsV transduction of HeLa cells at low inhibitory levels (Buck et al., 2006a) comparable to bLfcin 17–31 in our study. Podophyllotoxin and imiquimod are used to treat warts caused by HPV and the short-term clearance rate for podophyllotoxin is 60–80%, but unfortunately 40–60% of recurrences are reported. For imiquimod the clearance rate is 50% for women and recurrences occur in up to 20% of patients (Scheinfeld and Lehman, 2006). Several other antiproliferative and antiviral agents have been tested against warts, e.g. 5-fluorouracil, cidofovir, indol-3-carbinol and HAMLET (human α-lactalbumin made lethal to tumor cells) (Snoeck, 2006), but further studies are needed for extended use of these compounds. In addition other treatments of anogenital warts act by directly killing the infected cells, inducing apoptosis or surgically excising the lesion. Lactoferrin is expressed in human endocervix and by most of the endocervical glands (Farley et al., 1997) and has so far not been detected in normal skin (Mason and Taylor, 1978) but it has been found in pigmented warts (Tuccari et al., 2005). It was recently reported that lactoferrin levels in women infected with HPV were similar to levels in uninfected women (Bard et al., 2004). However, the majority of the women in the study were using oral contraceptives, which were earlier shown to suppress lactoferrin levels and keep it at constant low levels, similar to those before menses (Cohen et al., 1987). Lactoferricin levels in the vaginal fluids are not known. However, gastric juice lactoferricin levels are high after ingestion of lactoferrin (Kuwata et al., 1998) and lactoferricin is produced at infection sites by either bacterial or mammalian proteases (Bellamy et al., 1992b; Britigan et al., 1993). The application of lactoferricin as an antiviral treatment against HPV infections has not been studied, but lactoferricin has been shown to reduce and prevent bacterial and parasite infections in mice (Haversen et al., 2000; Isamida et al., 1998). Moreover, lactoferrin and lactoferricin act in synergy with acyclovir in reducing HSV-1 and -2 infection *in vitro* and have antiviral activity towards acyclovir resistant clinical isolates *in vitro* (Andersen et al., 2003; Jenssen et al., 2004a). Here we demonstrated that lactoferricin could inhibit HPV-5 and HPV-16 PsV entry into epithelial cells and both the size, charge, amino acid composition and the three dimensional structure could be important for the anti-HPV activity of lactoferricin. We now know more about the lactoferricin *in vitro* effects on HPV infection, but further studies are needed to analyze the *in vivo* effects of the peptide and the potential synergy effect of lactoferrin or lactoferricin with the wart treatments currently in use today.

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