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Inhibition of HSV cell-to-cell spread by lactoferrin and lactoferricin

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ABSTRACT

The milk protein lactoferrin (Lf) has multiple functions, including immune stimulation and antiviral activity towards herpes simplex virus 1 and 2 (HSV-1 and HSV-2); antiviral activity has also been reported for the N-terminal pepsin-derived fragment lactoferricin (Lfcin). The anti-HSV mode of action of Lf and Lfcin is assumed to involve, in part, their interaction with the cell surface glycosaminoglycan heparan sulfate, thereby blocking of viral entry. In this study we investigated the ability of human and bovine Lf and Lfcin to inhibit viral cell-to-cell spread as well as the involvement of cell surface glycosaminoglycans during viral cell-to-cell spread. Lf and Lfcin from both human and bovine origin, inhibited cell-to-cell spread of both HSV-1 and HSV-2. Inhibition of cell-to-cell spread by bovine Lfcin involved cell surface chondroitin sulfate. Based on transmission electron microscopy studies, human Lfcin, like bovine Lfcin, was randomly distributed intracellularly, thus differences in their antiviral activity could not be explained by differences in their distribution. In contrast, the cellular localization of iron-saturated (holo)-Lf appeared to differ from that of apo-Lf, indicating that holo- and apo-Lf may exhibit different antiviral mechanisms.

1. Introduction

Herpes simplex viruses 1 and 2 (HSV-1 and HSV-2) establish life-long latent infections in the host and can re-emerge periodically throughout life, primarily causing facial and genital herpetic lesions, respectively. Antiviral therapy with nucleoside analogues, like acyclovir, is commonly used to reduce the infection period. However, resistance development has lately provided new incentives for researchers to look for new viral interventions.

The initial step in HSV infection is attachment of the viral particle to the host cell surface, through interactions between viral glycoproteins (gD) and the cell surface glycosaminoglycan, heparan sulfate (Shieh et al., 1992; WuDunn and Spear, 1989). In the absence of heparan sulfate, chondroitin sulfate may assist in viral attachment to the host cell surface (Banfield et al., 1995; Gruenheid et al., 1993; Shukla and Spear, 2001; Mårdberg et al., 2002). Fusion of the cell membrane with the viral envelope involves viral glycoprotein and one or more specific cellular viral entry receptors (Spear, 2004). One of theses receptors is 3-*O*-sulfated heparan sulfate, which mediates primary entry of HSV-1 (Shukla et al., 1999) as well as HSV-1 transfer across cellular junctions, referred to as cell-to-cell spread (Cocchi et al., 2000). HSV remains cell associated at cell junctions throughout this transfer (Johnson et al., 2001).

Several natural antimicrobial proteins and peptides have demonstrated the ability to inhibit viral infection and either block viral entry into the host cell or influence later stages of viral growth (Jenssen et al., 2006). The milk protein lactoferrin (Lf) has multiple functions, including antiviral activity and immune stimulation (Brock, 2002; Jenssen, 2005; Weinberg, 2001). Studies have shown that metal complexes of bovine Lf inhibit *in vitro* replication of both HSV-1 and HSV-2 (Marchetti et al., 1998). It has been shown that the apo (iron free) forms of bovine and human Lf interact with cell surface heparan sulfate and block HSV-1 entry (Andersen et al., 2004).

Lactoferricin (Lfcin) is a peptide generated by pepsin cleavage from the N-terminal part of Lf (Tomita et al., 1991). Bovine Lfcin is 25 amino acids long (residues 17–41) (Bellamy et al., 1992) while the human Lfcin equivalent comprises amino acids 1–49 (Hunter et al., 2005). Both bovine and human Lfcin have been shown to exert antiviral activity against HSV-1 and HSV-2 (Andersen et al., 2003, 2004; Jenssen et al., 2004). Lfcin also binds heparan sulfate and exhibits antiviral activity both at the cell surface and at later stages after internalization.

The aim of this study was to explore the anti-HSV activity of bovine and human Lf and Lfcin. An infectious center assay was used to further describe the antiviral effect of Lf and Lfcin dur-

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ing cell-to-cell spread. The importance of heparan- and chondroitin sulfate in viral cell-to-cell spread was investigated by assessing the influence of the enzymatic removal of the glycosaminoglycans in a plaque reduction assay. Using transmission electron microscopy, the cellular localization of human Lfcin was investigated and compared with previously performed localization studies on bovine Lfcin (Andersen et al., 2004), in an attempt to explain differences in antiviral activity between the two peptides. Cellular localization of iron-saturated (holo) human Lf was investigated in addition the affinity of interaction between holo-Lf and heparan sulfate.

2. Materials and methods

2.1. Reagents

Human iron-saturated Lf, antibody against human Lf, bovine Lf, heparinase III, chondroitinase-ABC and bovine serum albumin were purchased from Sigma (St. Louis, MO). Heparan sulfate (bovine kidney) was from Seikagaku Corporation Inc. (Rockville, MD). CNBr-activated Sepharose was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Bovine and human Lfcin, human apo-Lf and polyclonal sheep IgG against human Lfcin were purchased from the Center for Food Technology (Hamilton, Queensland, Australia). Gamma-globulin (normal human immunoglobulin, Ig) was purchased from Pharmacia & Upjohn AS (Oslo, Norway). Bovine anti-Lfcin was purchased from Eurogentec, Belgium. Minimum essential medium (MEM) and the antibiotic G418 were purchased from Gibco BRL, Life Technology Ltd. (Paisley, Scotland). Primary rabbit antibodies against HSV-1 and HSV-2, secondary antibody (peroxidase-conjugated goat anti-rabbit Ig) and 1,2-*O*-phenylenediamine dihydrochloride (OPD) were purchased from DAKO (Glostrup, Denmark).

2.2. Virus and cell cultures

The viral strains used in this study were HSV-1 (ATCC VR-539, MacIntyre) and HSV-2 (ATCC VR-734, strain G), both purchased from Medprobe (Lund, Sweden). Several different cell lines were used in this study as illustrated in Table 1. MRC-5 and VERO cells permissive for HSV-1 and HSV-2 infection were obtained from BioWhittaker (Verviers, Belgium). Chinese Hamster Ovary (CHO) cells stably expressing the HSV co-receptor HveA (CHO-HveA) and CHO cells carrying a control plasmid (CHO-C8) were obtained from Dr. P. Spear (Northwestern University, Chicago) (Montgomery et al., 1996). The murine fibroblast, LMtk-, clone 1D (L), and its mutant variants, gro2C, sog9 and sog9 EXT-1, were obtained from Dr. F. Tufaro (University of Göteborg, Göteborg, Sweden) (Banfield et al., 1995; Gruenheid et al., 1993; McCormick et al., 1998). MRC-5 and VERO cells were grown in minimum essential medium supplemented with Ultroser G or Fetal Bovine Serum (FBS) (10%) (Gibco BRL, Life Technology Ltd., Paisley, Scotland). The CHO-HveA and CHO-C8 cells were cultivated in Ham's F12 medium supplemented with FBS (10%) and G418 (700 μg/ml). The murine cell line LMtk-clone 1D, and its mutant variants gro2C and sog9 were grown in Dulbecco's modified Eagle's Medium with high glucose supplemented with 10% FBS (D-MEM). Sog9-EXT-1 cells were grown in D-MEM supplemented with 10% FBS and 700 μg/ml G418.

2.3. Infectious center assays

Infectious center assays were designed using previously described principles (Roller and Herold, 1997; Sinha et al., 2003). Vero cells plated in six-well plates at 50% confluence were exposed to HSV-1 at a multiplicity of infection of 5, at 37 °C to allow entry. After 90 min of incubation, the cells were washed once with PBS, once with 0.1 M citrate buffer (pH 3.0), and then resuspended and incubated in citrate buffer for 1 min to inactivate residual virus particles. The monolayer was then washed twice in PBS to remove the low-pH buffer, and the cells were placed in growth medium supplemented with human IgG to neutralize any extracellular virus. After a total incubation of 5.5 h, the infected cells were detached with trypsin-EDTA, resuspended in growth medium, and ~125 cells were plated onto 50% confluent monolayers of uninfected Vero cells in medium containing IgG. Human and bovine Lf/Lfcin (at IC_{50} concentrations) were added in quadruplicate and the experiment repeated four times, to evaluate the effect of these agents on cell-to-cell spread. Plaques were counted and compared with untreated controls after 48 h incubation at 37 °C.

2.4. Enzymatic digestion of glycosaminoglycan molecules during the plaque reduction assay

To investigate the importance of cellular glycosaminoglycan molecules during cell-to-cell spread, the cells were treated with either heparinase III or chondroitinase-ABC, to remove extracellular heparan- or chondroitin sulfate, respectively. Both glycosaminoglycans are known to participate in HSV infection and spread. The enzymatic treatment was done after viral entry and bovine Lfcin treatment, and the enzymes where present throughout the plaque assay. Vero cells (1×10^5 cells/well) were treated with heparinase III (0.4 U) or chondroitinase-ABC (0.1 U) in a buffer containing 10% (v/v) 200 mM Tris–HCl, pH 7.5, with 1 mg/ml BSA and 40 mM $CaCl_2$ and 90% (v/v) MEM supplemented with 3.2% (v/v) IgG (Mårdberg et al., 2004), after HSV-1 infection. The effects of serial dilutions (2- and 10-fold) of the enzymes were also investigated.

The plaque reduction assay was performed as previously described (Andersen et al., 2004). Briefly, 1×10^5 Vero cells were incubated with 100–200 plaque forming units of HSV-1 for 1 h at 37 °C to allow viral entry. Excessive virus was washed away with PBS, before the bovine Lfcin (at its IC_{50}: 15 μM) was added and incubated for 1 h at 37 °C. Additional incubation was performed

Table 1
Cell types and their HSV receptor molecules

Cells	Heparan sulfate	Chondroitin sulfate[a]	Co-receptor(s)[a]	Cellular distribution of holo-Lf[b]
Vero	+	+	++	Evenly spread on surface
MRC-5	+	+	++	Largely in surface clusters; small amount intracellular
CHO-HveA	+	+	HveA	Largely in surface clusters; small amount intracellular
CHO-C8	+	+		Surface clusters with frequent intracellular location
L	+	+	++	Largely in surface clusters; small amount intracellular
Gro2C	–	+	++	Largely found intracellularly
Sog9	–	–	++	Only very small amounts observed
Sog9 EXT-1	+	–	++	Largely in surface clusters; small amount intracellular

[a] Cell surface expression virus receptors and co-receptors: + indicates that the cells express this molecule, while ++ indicates that these cells express co-receptor(s) for HSV.

[b] Data were taken from electron micrographs such as those shown in Fig. 5.

with MEM supplemented with IgG, or a mix of MEM, heparinase III or chondroitinase-ABC, enzyme buffer and IgG as outlined above. Plaques were counted 48 h after viral addition and compared with plaque counts of controls not treated with bovine Lfcin. Statistical differences were verified with a paired two-tailed *t*-test using a confidence interval of 95% (PRISM 3.0® GraphPad Software Inc., San Diego, CA).

2.5. Localization of human holo-Lf and Lfcin in different cell types

Different cell types (1×10^5) were exposed to 250 μg/ml (3.2 μM) of human holo-Lf or human Lfcin at 37 °C for 15 min. To visualize the localization of holo-Lf and Lfcin, immunogold-labeling and transmission electron microscopy studies were performed. Localization of human holo-Lf was performed in eight cell types (Table 1), while localization of human Lfcin was done in MRC-5 cells. Briefly the cells were fixed with 4% paraformaldehyde and pelleted. The pellet was incubated with 2.3 M sucrose, frozen on aluminium specimen pins by immersion in liquid nitrogen, and sectioned using an RMC MT-7 ultramicrotome (65–70 nm thick sections). The sections were submerged overnight in 1% cold water fish skin gelatin. The sections were labeled with primary antibody against human Lf/Lfcin and then with protein-A gold. The sections were contrasted with a mixture of uranyl acetate and methylcellulose and examined by transmission electron microscope. To exclude the possibility of non-specific binding during the labeling procedure, cells unexposed to human Lf/Lfcin were immunolabeled. By omitting the primary antibody in the labeling procedure, the possibility of non-specific binding of protein-A gold was checked.

2.6. Heparan sulfate affinity assay

Glycosaminoglycan affinity assays were performed as described earlier (Jenssen et al., 2004). Heparan sulfate was linked to a Sepharose column and the apo- and holo-Lf of bovine and human origin were eluted with a gradient of NaCl (0–1 M) at a flow of 1.0 ml/min, and detected at 214 nm.

3. Results

3.1. Lf and LFcin effects on HSV cell-to-cell spread

Plaque reduction assays following pre-attachment of HSV-1 to the cell surface previously demonstrated that both bovine Lf and Lfcin caused a decreased number of plaques compared to the positive control (Andersen et al., 2004). This could have been due to inhibition of intracellular growth of virus or to inhibition of cell-to-cell spread. To investigate the role of Lf and Lfcin in inhibiting cell-to-cell spread of the virus, an infectious center assay was utilized. In this assay infected cells were seeded on a monolayer of healthy cells plated to 50% percent confluence, prior to exposure to bovine or human Lf/Lfcin. This led to a direct correlation between the inhibition of plaque formation and the inhibition of viral cell-to-cell spread. The results illustrated that bovine Lf strongly inhibited plaque formation by both HSV-1 and HSV-2, while human Lf showed a somewhat lesser reduction in plaque formation of 20–30% (Fig. 1). Plaque size was also significantly reduced, compared to the untreated positive control (data not shown), consistent with an interference with the viral cell-to-cell spread. Both bovine and human Lfcin moderately reduced the number of plaques in HSV-1-infected cells, while no effect was observed towards HSV-2 (Fig. 1). In this case, the plaque size was not influenced by the presence of the peptides.

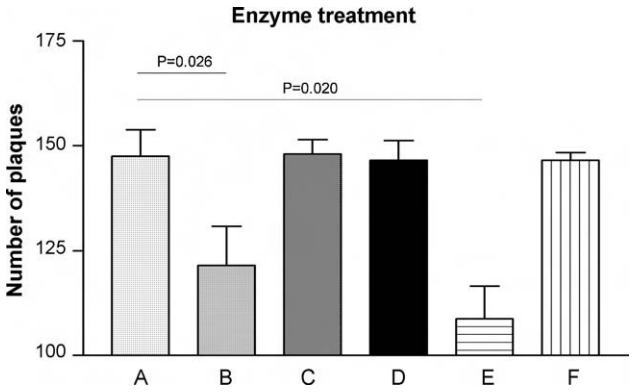

Fig. 2. Number of HSV-1 plaques observed on monolayers of Vero cells after a total of 48 h of incubation in the presence or absence of bovine Lfcin and enzymatic treatments (means and standard deviations of four independent experiments). All cells were exposed to HSV-1 for 1 h at 37 °C followed by treatments to neutralize any residual extracellular virus and inhibit further extracellular viral infections. (A) Positive control without any subsequent treatment; (B) 1 h bovine Lfcin exposure following HSV-1 entry; (C) 1 h with bovine Lfcin followed by a 46-h chondroitinase-ABC treatment. (D) One hour with medium followed by 46-h chondroitinase-ABC treatment; (E) 1 h with bovine Lfcin, followed by 46-h heparinase-III treatment; (F) 1 h with medium followed by a 46-h heparinase-III treatment. NB only chondroitinase-ABC treatment prevented the inhibition by bovine Lfcin of cell-to-cell spread.

3.2. Glycosaminoglycan involvement of Lfcin inhibition of HSV-1 cell-to-cell spread

As a positive control, Vero cells were exposed to HSV-1 for 1 h, treated with acidic citrate buffer to kill extracellular virus and then incubated in the presence of Ig for a further 47 h to block any addi-

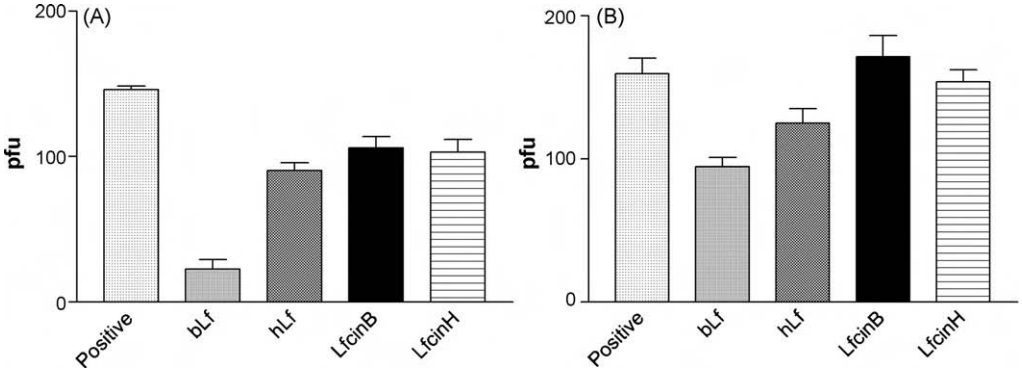

Fig. 1. Number of plaques observed in an infectious center assay after 48 h incubation in the presence of bovine/human Lf (bLf/hLf) and bovine/human Lfcin (LfcinB/LfcinH), after infection with (A) HSV-1 or (B) HSV-2.

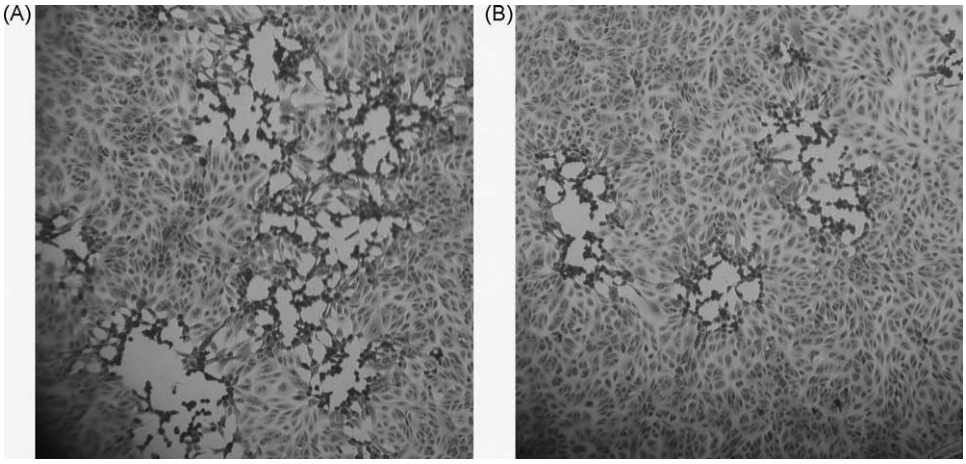

Fig. 3. Plaque morphology of HSV-1 on a monolayer of Vero cells after treatment with (A) heparinase-III (0.2 U) alone and (B) bovine Lfcin (15 μM) prior to heparinase-III (0.2 U) treatment. Experimental conditions were the same as for Fig. 2F and E, respectively, and the result shown is representative of multiple plaques observed in four independent experiments.

tional infection by extracellular HSV-1 that was released by the cells during the incubation period. As observed by several others, a high plaque count was recorded in four independent experiments each repeated in quadruplicate due to direct cell-to-cell spread of virus. When bovine Lfcin was added after the initial viral entry period, a significant (paired two-tailed *t*-test, $P = 0.0259$), but modest plaque inhibition was observed compared to the positive control (Fig. 2). To reveal if this effect could be linked to the presence of heparan- or chondroitin sulfate, these glycosaminoglycans were separately removed by enzymatic treatement after initial infection. When infected cells were treated with chondroitinase-ABC or heparinase-III after viral entry, in the absence of bovine Lfcin, no plaque reduction activity was observed, either in terms of total plaque counts or plaque size. This indicates that enzymatic removal of either of the glycosaminoglycans alone had no effect on the plaque formation. However, when the infected cells were exposed to bovine Lfcin, and then incubated with 0.005 U chondroitinase-ABC for 2 days, to remove chondroitin sulfate from the cell surface, the antiviral activity of bovine Lfcin was abolished, resulting in no significant change in plaque formation when compared to the positive control. When the same experiment was done with 0.2 U heparinase-III, to remove heparan sulfate from the cell surface, the number of plaques still decreased significantly (paired two-tailed *t*-test, $P = 0.0203$) compared to the positive control. In addition to reducing the number of plaques, bovine Lfcin also reduced the plaque size in the absence of heparan sulfate (Fig. 3), confirming that the peptide continued to have an effect on the viral cell-to-cell spread mechanism.

3.3. Cellular localization of Lfcin and Lf

Bovine Lfcin is known to demonstrate superior antiviral activity against HSV-1 and HSV-2, compared to human Lfcin (Andersen et al., 2003) (e.g. Fig. 1). Consequently the cellular localization of human Lfcin was investigated and compared to the previously described distribution of bovine Lfcin (Andersen et al., 2004). The electron micrograph in Fig. 4A demonstrated that human Lfcin was taken up into MRC-5 cells and adopted a random intracellular distribution (Fig. 4A). The electron micrographs of the cells not exposed to Lfcin demonstrated that non-specific labeling did not occur (Fig. 4B).

The cellular localization of human holo-Lf was investigated and compared with the earlier described localization of apo-Lf (Andersen et al., 2004). The influence of glycosaminoglycan molecules and viral entry receptors on the distribution of humane holo-Lf was also investigated using eight different cell lines (Table 1). The different cells were exposed to 250 μg/ml (3.2 μM) of holo-Lf at 37 °C for 15 min, before immunogold-labeling and transmission electron microscopy were performed. The electron micrographs showed that holo-Lf was evenly spread on the cell

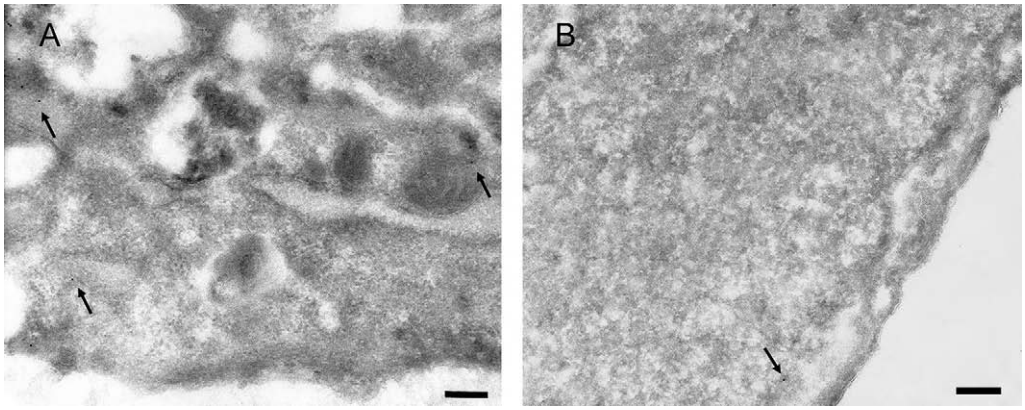

Fig. 4. Uptake and intracellular distribution of human Lfcin as observed by transmission electron microscopy. (A) MRC-5 cells were exposed at 37 °C for 15 min to 250 μg/ml of human Lfcin that was subsequently visualized using anti-human Lf antibody and protein-A-gold particles (circled black dots) and (B) negative control without added Lfcin. Bar = 200 nm.

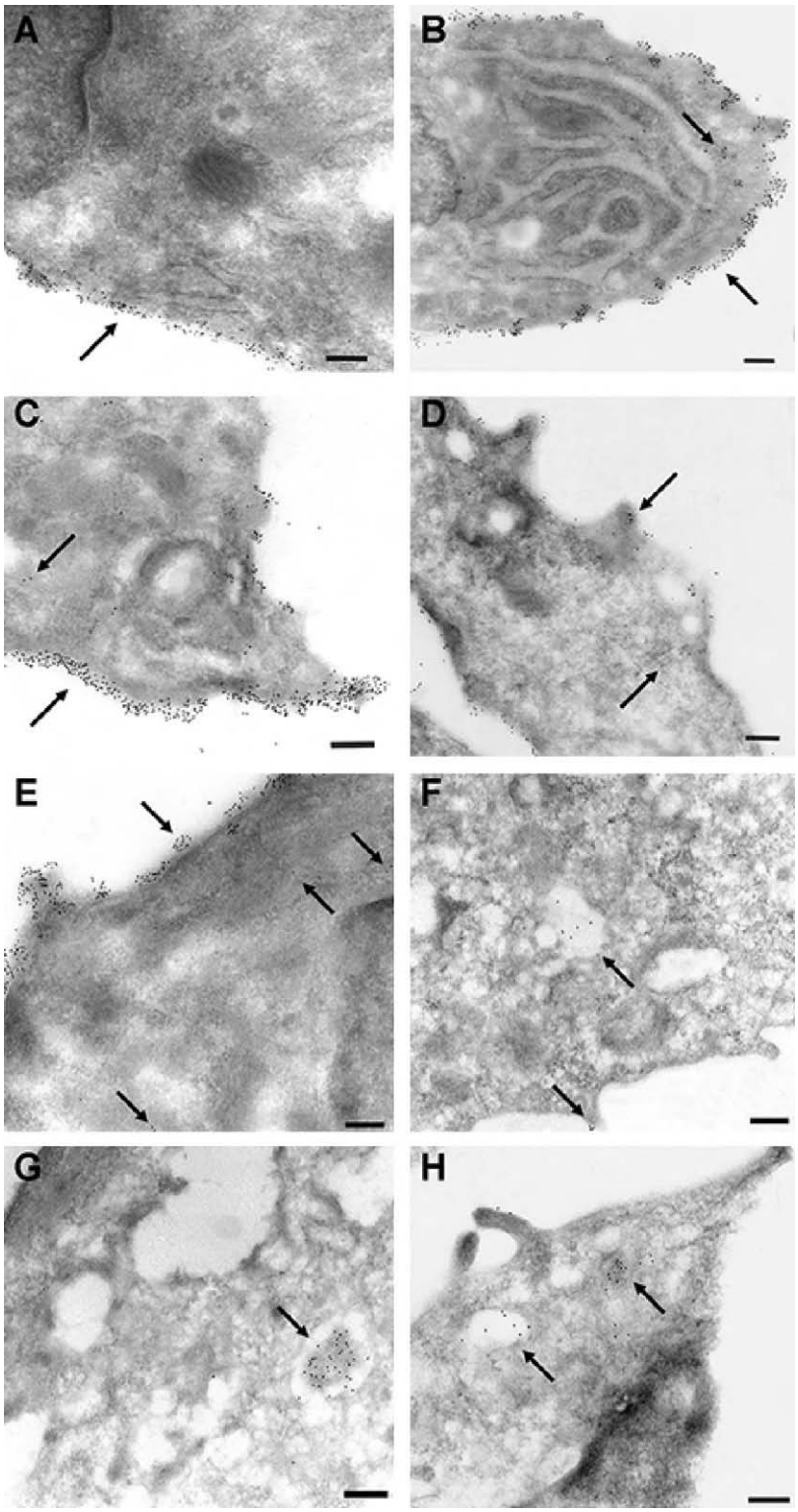

Fig. 5. Transmission electron micrographs of Vero (A), MRC-5 (B), L (C), CHO-HveA (D), CHO-C8 (E), Sog9-EXT1 (F), Sog9 (G) and Gro2C (H) cells exposed to human holo-Lf for 15 min at 37 °C. Lf was visualized using anti-human Lf antibody and protein-A-gold particles. Bar = 200 nm.

surface of Vero cells, and was only occasionally found intracellularly (Fig. 5A). Holo-Lf was mainly localized in clusters at the cell surface of the MRC-5, L- and CHO-HveA cells, although minor amounts were also observed intracellularly in some cells (Fig. 5B–D).

In CHO-C8 cells holo-Lf appeared in clusters on the cell surface as in the CHO-HveA cells, however, intracellular clusters were also found quite frequently (Fig. 5E). Holo-Lf was mostly distributed at the cell surface of Sog9 EXT-1 cells expressing HS at the cell surface, but the protein also appeared in intracellular clusters (Fig. 5F). In the

Sog9 cells, completely deficient of heparan- and chondroitin sulfate, only small amounts of holo-Lf were detected, and it appeared at the cell surface or as intracellular clusters (Fig. 5G). Almost all of the holo-Lf was localized intracellularly in vacuole-like compartments in the Gro2C cells lacking heparan sulfate, but expressing chondroitin sulfate (Fig. 5H).

3.4. Holo/apo-Lf affinity for heparan sulfate

The major importance of heparan sulfate affinity for the antiviral activity of human Lf against extracellular virus was previously illustrated (Andersen et al., 2004). For that reason the heparan sulfate affinity of both iron-saturated bovine and human Lf was investigated. Holo-Lf showed the same affinity for heparan sulfate as reported earlier for its apo-form (Jenssen et al., 2004).

4. Discussion

A plaque reduction assay conducted after the pre-attachment of HSV-1 to host cells previously indicated that bovine Lf and Lfcin both inhibit plaque formation (Andersen et al., 2004). Although plaque reduction can result from both inhibition of the primary infection and cell-to-cell spread, the reduction of plaque size provides good evidence for inhibition of cell-to-cell spread. This was further reinforced here by the neutralization of extracellular virus during plaque reduction assays. Overall the results presented here demonstrate that both bovine and human Lf were able to inhibit cell-to-cell spread of HSV-1 and HSV-2, resulting in both decreased numbers of plaques (Figs. 1 and 2) and their size compared to the positive control not exposed to Lf (Fig. 4). Bovine and human Lfcin were able to inhibit cell-to-cell spread of HSV-1, but not HSV-2.

Primary infection can be distinguished from cell-to-cell spread by the involvement of the viral glycoprotein complex (gE–gI), indicating that Lfcin may be able to interfere with the mechanism by which this complex assists cell-to-cell spread. The results are not completely consistent with the assumption that homologous viral glycoproteins play identical roles for HSV-1 and HSV-2. Similar viral specificity and diverse ability to inhibit cell-to-cell spread have been reported earlier for an essential oil (De Logu et al., 2000) and other polycationic compounds (Nyberg et al., 2004). Despite the claim that Lf and Lfcin inhibit cell-to-cell spread, it should be kept in mind that an intracellular antiviral mechanism/target, e.g. use of a nucleoside analogue, potentially could give a similar reduction in total plaque number and plaque size. Consequently, a new assay removing cell surface glycoproteins was employed to support the hypothesis of Lf/Lfcin inhibiting cell-to-cell spread.

Lfcin is known to bind to heparan sulfate and block initial viral entry (Andersen et al., 2004). As expected, the number of plaques decreased only moderately when bovine Lfcin was added after viral entry, compared to the untreated positive control, indicating that this agent is capable, but to a lesser extent, of inhibiting an on-going infection and cell-to-cell spread. To investigate if this effect could be linked to the presence of glycosaminglycans on the cell surface, we designed an experiment were heparan- and chondroitin sulfate where separately removed enzymatically from the cell surface after viral infection. Inhibition of plaque formation, and consequently cell-to-cell spread, was still observed when the cells were exposed to bovine Lfcin prior to enzymatic removal of heparan sulfate. In contrast, bovine Lfcin was unable to inhibit plaque formation when cell surface chondroitin sulfate was removed. This clearly illustrates that Lfcin is able to inhibit plaque formation in the absence of heparan sulfate, probably by interacting with chondroitin sulfate, indicating that inhibition of cell-to-cell spread by Lfcin is dependent on a completely different glycosaminoglycan to that used in inhibition of initial infection. Enzyme treatment alone, in the absence of Lf or Lfcin did not influence viral plaque formation, indicating that in the absence of heparan sulfate, plaque formation and cell-to-cell spread may involve chondroitin sulfate, and vice versa.

Both viral glycoproteins gB and gC have lower affinity for chondroitin sulfate compared to heparan sulfate (Mårdberg et al., 2002; Williams and Straus, 1997). We propose that bovine Lfcin interaction with chondroitin sulfate might block the HSV interaction with the glycosaminoglycan, thus inhibiting plaque formation in chondroitin sulfate-expressing cells that lack heparan sulfate.

We did not confirm here that the cells were totally free of heparan- or chondroitin sulfate after the treatment with the respective enzymes. When higher enzyme concentrations (0.4 U heparinase-III and 0.1 U chondroitinase-ABC) were utilized, they resulted in substantial cell debris in strong contrast to lower enzyme concentrations, at which debris was absent and the cells seemed healthy. Nevertheless, a reduction in the amounts of cellular glycosaminoglycan molecules was sufficient to provide an indication of the involvement of chondroitin sulfate during cell-to-cell spread.

This study revealed a random intracellular distribution of human Lfcin in the MRC-5 fibroblast cell line, consistent with previous results for bovine Lfcin (Andersen et al., 2004), and very limited amounts of Lfcin was detected on the cell surface. Both, bovine and human Lfcin show high affinity towards heparan sulfate (Jenssen et al., 2004), and it seems unlikely that an interaction with cell surface heparan sulfate could be avoided during sample preparation. Detection of Lfcin by the antibody has been shown to be specific (data not shown); however, the relative amount of observed Lfcin was considerably lower than that of Lf (Andersen et al., 2004), and perhaps the detection of Lfcin with antibody was disrupted under the experimental conditions utilized here. The relative amount of bovine Lfcin observed was significantly higher after incubation at 4 °C than at 37 °C (Andersen et al., 2004), indicating that the differences may be the result of either rapid proteolytic cleavage or rapid energy-dependent uptake into cells of Lfcin.

Holo- and apo-Lf have similar activity towards HSV (Marchetti et al., 1998); thus, the cellular localization of apo- and holo-hLf should be similar, as long as they both exhibit antiviral activity. Earlier studies showed that heparan sulfate on the cell surface is essential for Lf to exert its antiviral activity (Andersen et al., 2004). In this study, holo-Lf was found to possess equal affinity for heparan sulfate to that previously described for apo-Lf (Jenssen et al., 2004). Holo-Lf was mainly found on the cell surface of cells expressing heparan sulfate. Our results also illustrate that holo-Lf is more abundant than the apo form on the cell surface of Vero- and CHO-HveA cells. This may result from interactions with a highly specific receptor for Lf, characterized on several mammalian cell types and tissues (Birgens et al., 1983; Kawakami and Lönnerdal, 1991; Mazurier et al., 1989; McAbee and Esbensen, 1991; Retegui et al., 1984; Ziere et al., 1992). This receptor has been linked to the cellular uptake of iron in small intestine (Suzuki et al., 2001), indicating that it may have a higher affinity for holo- than apo-Lf. The observation that apo- and holo-Lf have similar activity against HSV infections in Vero cells (Marchetti et al., 1998), in addition to our observation that there is significantly more holo- than apo-Lf on the cell surface of Vero, implies that cell surface localization of Lf might be less important for its ability to execute its antiviral activity than earlier anticipated.

More apo-Lf was found intracellularly in the Sog9 cells, compared to holo-Lf. Internalization of Lf in Sog9 cells probably due to the nuclear localization sequence in the N-terminal region (G_1RRRR_5) of the protein (Penco et al., 2001). Large conformational changes in the Lf molecule as a result of iron binding have been

documented (Grossmann et al., 1992). The difference in amount of internalized apo- (Andersen et al., 2004) and holo-Lf may indicate that nuclear localization sequence internalization mechanisms are influenced by the secondary structure of the protein.

Low-density-lipoprotein receptor-related protein (Ji and Mahley, 1994) and nucleolin (Legrand et al., 2004), have both been shown to facilitate internalization of human Lf. Whether their effects on holo- and apo-Lf are similar cannot be easily determined since the proteins experience similar localization pattern in the rest of the cells (Andersen et al., 2004). Several internalization processes have been proposed and confirmed for Lf, and one process does not exclude the others.

5. Conclusion

Lf is more active than Lfcin in inhibiting viral cell-to-cell spread. Inhibition of cell-to-cell spread by bovine Lfcin, appears to involve chondroitin sulfate. Human Lfcin is localized intracellularly in the same manner as bovine Lfcin; thus, localization of these peptides cannot explain their differences in antiviral activity. There is a slight difference in the cellular localization of holo- and apo-Lf despite their similar activities, creating additional evidence that Lf may have a more diverse antiviral mode of action than simply inhibition of viral attachment.

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