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Anti-HSV activity of lactoferricin analogues is only partly related to their affinity for heparan sulfate

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Abstract

Earlier studies have shown that the heparan sulfate (HS) on the cell surface acts as a receptor for herpes simplex virus (HSV). We have recently shown that bovine lactoferricin (LfcinB), a small part of the milk protein lactoferrin, inhibits HSV-1 and HSV-2 infection, probably by blocking the entry of the virus. The human homologue (18–42), which shares 36% sequence similarity with LfcinB (17–41), displayed much lower antiviral activity. In the present study, a set of cyclic and linear human and bovine Lfcin derivatives were constructed to investigate the relation between their affinity to HS and chondroitin sulfate (CS) and their antiviral activity against HSV-1 and HSV-2. The lactoferrin (LF) proteins and several of the Lfcin derivatives exhibited similar affinity for HS, but the LF proteins possess a much higher antiviral activity than the smaller peptides. Our structure–activity relationship studies on the Lfcin derivates confirmed that affinity for HS, that was correlated to the net positive charge, is an important factor, but does not well predict the antiviral activity. Structural parameters such as hydrophobicity, molecular size, spatial distribution of charged and lipophilic amino acids, and the cyclic structure of Lfcin also seem to be important factors to govern antiviral activity against HSV.

Keywords: Lactoferricin; Antiviral activity; Herpes simplex virus; Heparan sulfate; Structure–activity relation

1. Introduction

Herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) infections have a broad variation of clinical symptoms, from periodical skin lesions to encephalitis. Both viruses may develop latent infection and reactivation occurs in response to various types of physiologic stress (Whitley et al., 1998). Most of the treatment for HSV is based on acyclovir and acyclovir-like drugs. There are still relatively minor problems with drug resistance among immunocompetent patients. However, among HIV-positive patients the incidence of resistant HSV-2 isolates is increasing (Reyes et al., 2003).

The first step in viral infection is the attachment to the host cell that enables HSV-1 and HSV-2 to penetrate into the cell. This initial step in HSV infection involves glycosaminoglycans (GAG) on the cell surface and glycoproteins in the viral envelope of both HSV-1 and HSV-2 (WuDunn and Spear, 1989). The major type of GAG chains found at the cell surface is heparan sulfate (HS; Lindahl et al., 1994). Many cell surface proteoglycans are also substituted with chondroitin sulfate (CS), another type of highly negatively charged GAG chains (Stringer and Gallagher, 1997). It is known that the viral glycoproteins gC and/or gB binds to HS on the cell surface (Tal-Singer et al., 1995). Then gD interacts with one or several other receptors on the cell surface, before the cell and viral particle can fuse, involving gB, gD, gL, and gH (Qie et al., 1999).

Lactoferrin (LF) is a 80 kDa, multi-functional (Weinberg, 2001), iron-binding glycoprotein (Kuwata et al., 1998), present in external secretions, like milk, tears, and plasma (Masson et al., 1966). Bovine (LfcinB) and human (LfcinH) lactoferricins are generated from pepsin digestion of bovine and human LF, respectively (Fig. 1).

Studies have demonstrated that the mammalian LF and Lfcin in addition to activities reported against bacteria, fungi, protozoa and tumors (Bellamy et al., 1992; Yoo et al., 1998; Ueta et al., 2001; Omata et al., 2001) exert antiviral activities against HSV and human cytomegalo virus (HCMV; Harmsen et al., 1995; Marchetti et al., 1996; Andersen et al.,

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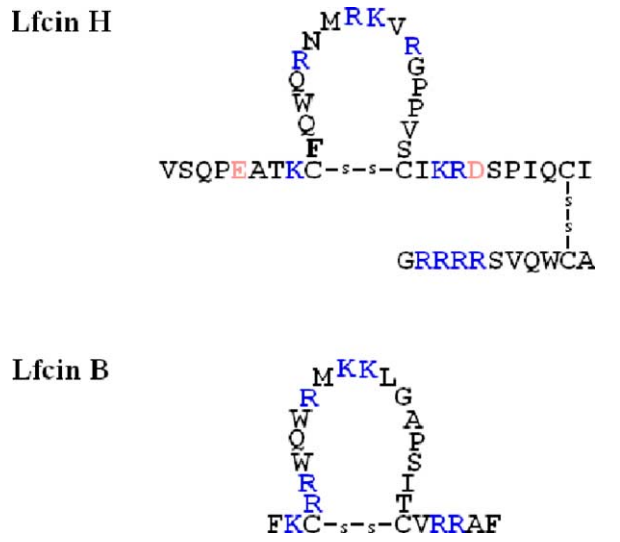

Fig. 1. Loop structure of LfcinH and LfcinB. Single letter code is used to indicate the amino acid sequence of each peptide. The blue ones are positively charged residues while the red ones are negatively charged. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2001, 2003). LF has the ability to interact with HS (Ji and Mahley, 1994; Wu et al., 1995) and bovine Lfcin (LfcinB) has been shown to exert antiviral activity against HCMV by inhibiting the entry of the virus (Andersen et al., 2001). Both LF and LfcinB, with their relative high net positive charge, may interact with negatively charged GAG chains expressed on the target cells (El Yazidi-Belkoura et al., 2001), and thereby display antiviral activities.

The aim of this study was to investigate the structural basis for the antiviral activity exerted by LfcinB and to study a possible correlation between GAG affinity and antiviral activity. LfcinB has been shown to be more effective than its corresponding human peptide analogue (LfcinH) in inhibiting the entry of HCMV into human fibroblasts (Andersen et al., 2001). A set of cyclic and linear human and bovine Lfcin derivatives was constructed in order to investigate the role of different structural features such as net positive charge, chain length, and the number of aromatic residues. The peptides were tested for their affinity to HS and CS and their antiviral activity against HSV-1 and HSV-2.

2. Material and methods

2.1. Reagents

Bovine lactoferrin, ekathiox resin, chondroitin sulfate A (CS-A; whale cartilage), chondroitin sulfate C (CS-C; shark cartilage), 3,3′-diaminobenzidine tablets (DAB) tablets and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were all purchased from Sigma Chemical Co. (St. Louis, MO). Human lactoferrin (55839) was a gift from Cappel/Organon Teknika Corporation (Durham, NC). Bovine and human lactoferricin was purchased from the Centre for Food Technology (Brisbane, Australia). Human lactoferricin 18–42 was provided by MedProbe (La Jolla, CA). PAL–PEG–PS resin was purchased from Perseptive Biosystems GmbH (Hamburg, Germany). HS (bovine kidney) was from Seikagaku Corporation Inc. (Rockville, MD). CNBr-activated sepharose was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). HSV-1 (ATCC VR-539, MacIntyre) and HSV-2 (ATCC VR-734, G) were purchased from American Type Culture Collection (Rockville, MD). MRC-5 cells (human diploid lung fibroblasts, ATCC CLL 171) were purchased from BioWittaker (Oslo, Norway). Minimum essential medium (MEM) was purchased from Gibco BRL, Life Technology Ltd. (Paisley, Scotland). Primary antibody against HSV-1 or HSV-2, secondary antibody and 1,2-*O*-phenylenediamine dihydrochloride (OPD) were purchased from DAKO (Glostrup, Denmark).

2.2. Solid phase peptide synthesis

Different lactoferricin (Lfcin) derivatives were synthesized as earlier described (Strom et al., 2000). Briefly, the peptides were synthesized automatically using Fmoc-amino acids and PAL–PEG–PS resin on a Milligen 9050 PepSynthesizer (Mildford, MA; Rekdal et al., 1999; Strom et al., 2002). Coupling reactions with Fmoc-amino acids in the presence of DIPEA were activated in situ using HBTU as a coupling reagent. Pre-activated pentafluorophenyl (Pfp)-esterified amino acids were coupled to the growing peptide chain using HOBt as a catalyst. De-protection was achieved during acid based cleavage of the peptide from the solid support.

The peptides were purified by preparative reverse-phase HPLC (Delta-Pak^{TM} C18, 100 Å, 15 μm, 25 mm × 100 mm, Waters Corporation, Milford, MA). Analytical HPLC was used to confirm that the purity of the peptide was at least 98% (C18-column, Delta-Pak^{TM} C18, 100 Å, 5 μm, 3.9 mm × 150 mm, Waters Corporation). Eluted fractions were analyzed by electron-spray interface on a VG QUATTRO quadruple mass spectrometer VG Instruments Inc. (Altrincham, UK).

Cyclic peptides were constructed using the Ekathiox resin according to the procedure described by the manufacturer (Sigma). Purified peptides were dissolved to 1 mM in methanol, and bubbled with argon gas before Ekathiox resin was added in a 15-fold excess relative to the amount of peptide (w/w). The cyclization reaction was monitored by HPLC (Lee et al., 1989) before the final product was purified by HPLC and analyzed by mass spectrometry. Both synthesized peptides and proteins were dissolved in distilled, pyrogen-free water, and stored at −70 °C.

2.3. Affinity assay

Three different sepharose affinity columns were prepared, using HS, CS-A, and CS-C as ligands. The ligands

were mixed with swollen CNBr-activated sepharose. Non-reactive groups on the medium were blocked with Tris–HCl, and the gel was washed before the column was packed. The peptides/proteins were dissolved in double distilled H_2O, and 100 μl samples were applied. A gradient of NaCl was used to elute the different peptides and proteins from the columns using a GradiFrac from Amersham Pharmacia Biotech (Uppsala, Sweden) at a flow rate of 1.0 ml/min. The peptides/proteins were detected at 214 nm using a single path monitor UV-1/214 from Amersham Pharmacia Biotech (Uppsala, Sweden).

2.4. Cell cultures

MRC-5 cells were grown at 37 °C under an atmosphere of 5% CO_2 in minimum essential medium (MEM) buffered by HEPES buffer, containing non-essential amino acids, ultroser G, and gentamicin (10 μg/ml). The cells were cultured as monolayers in 50 and 250 ml culture bottles (Nunc, Tamro MedLab, Skårer, Norway).

2.5. Infection assay

Cells were seeded in 96-well microtiter plates (Nunc, Tamro MedLab, Skårer, Norway), to obtain subconfluence over night. Serial dilutions of HSV-1 or HSV-2 were inoculated into each well to obtain an infectious dose (ID_{75}) equal to 75% infected cells. Different concentrations of the peptides/proteins (dose range 3–160 μM) were added prior to the virus infection. After 2.5 h, the inocula was removed and replaced with MEM. The plates were incubated for an additional 18–20 h. The medium were then removed, plates were washed in PBS and fixed with cold methanol for 10 min. Unspecific binding was blocked with 4% BSA in PBS, for 30 min at 37 °C. Primary rabbit immunoglobulin against HSV-1 or HSV-2 and a secondary horseradish peroxidase conjugated goat-anti-rabbit immunoglobulin were diluted after the Producers recommendations. The plates were washed with PBS between each antibody exposure. Either OPD tablets or DAB tablets were used as substrate. After OPD was added the microtiter plates were incubated in the dark for 10 min, and the reaction was stopped by adding 0.5 M H_2SO_4. Absorbance was detected at 492 nm using a Microwell Reader 510 (Organon Teknika). If DAB was added, the microtiter plate was incubated for 60 min, before the wells were washed with water. Infections rate was counted visually in the microscope. The calculations of IC_{50} values were based on the median effect principle of Chou and Talalay. This method involves the plotting of dose–effect curves for each agent and calculation of median-effect dose (D_m) analogue to IC_{50} (Chou and Talalay, 1984).

2.6. Toxicity assay

To determine the cytotoxic effect of the peptides against the MRC-5 cells the MTT reduction assay was employed (Mosmann, 1983). Cells were seeded in 96-well microtiter plates to obtain sub confluence over night, prior to the addition of peptides in serum-free medium. The cells were exposed to the peptides for 2.5 h at 37 °C in a humidified atmosphere of 5% CO_2. After 2.5 h, the inocula was removed and replaced with MEM. The plates were incubated for an additional 21.5 h. Cells in MEM medium were used as a negative control, whereas cells treated with 1% Triton X-100 were used as a positive control. After the incubation period, 10 μl MTT solution (5 mg MTT/ml PBS) per 100 microliters of the medium was added to each well and incubated for 2 h. A volume of 130 μl was removed from the cells. In order to dissolve the formazan crystals, 100 μl 0.04 M HCl in isopropanol was added and the plates were shaken for 1 h. A microtiter plate reader Thermomax Molecular Devices (Sunnyvale, CA) finally estimated the absorbance at 590 nm. The concentration of peptides required to kill 50% of the cells (IC_{50}) was determined from the dose–response curves.

2.7. Calculations

Net charge at pH 7.0 was calculated using the PROTEAN program (DNASTAR Inc., version 1.17, Madison, WI). Grand average of hydropathicity (GRAVY; Kyte and Doolittle, 1982) and aliphatic index (Ikai, 1980) of the peptides was calculated using services at ExPASy ProtParam tool provided by the Swiss Institute of Bioinformatics. General correlation between antiviral activity and other observed or calculated values was shown using PRISM program (GraphPad Software Inc., version 3.0, SanDiego, CA).

3. Results

3.1. Peptide design

LfcinH encompasses the amino acid residues 1–47 in human LF (Fig. 1). The peptide is composed of two fragments linked together by a disulfide bridge between Cys 10 and Cys 46. The sequence 18–42 of LfcinH corresponds to LfcinB (residues 17–41). LfcinB features a loop region due to a disulfide bridge between Cys 19 and Cys 36 which is also found in the homologous region of LfcinH (Cys 20 and Cys 37).

In order to identify structural parameters critical for the antiviral activity by LfcinB against HSV-1 and HSV-2, a number of cyclic and linear analogues of LfcinB, and the corresponding LfcinH 18–42 peptide, were constructed (Table 1). In the B2 peptide, the Gly 30 residue in LfcinB was replaced by arginine. This modification yielded an increased net positive charge. Further, by performing this modification, a consensus sequence for GAG binding present in LfcinH (Cardin and Weintraub, 1989) was included in the peptide. The lower antiviral activity of LfcinH compared to LfcinB could be due a lower net positive charge, i.e. 5.85 and 7.84, respectively, at pH 7.0. An analogue of LfcinH,

Table 1
Overview of the synthesized bovine and human analogue peptides

Name	Amino acid sequence (single letter code)																									Structure[a]	Net charge[b]	Molecular weight[c]
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25			
LfcinB	F	**K**	C	**R**	**R**	W	Q	W	**R**	M	**K**	**K**	L	G	A	P	S	I	T	C	V	**R**	**R**	A	F	Cyclic	7.84	3125.0
B2	–	–	–	–	–	–	–	–	–	–	–	–	–	**R**	–	–	–	–	–	–	–	–	–	–	–	Cyclic	8.84	3223.6
B3	–	–	–	–	–	–	–	–	–	–	–	–	–	← Hexa[d] →						–	–	–	–	–	–	Cyclic	7.84	2711.1
B4		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–		Cyclic	7.84	2830.4
B5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Linear	7.84	3269.4
B6	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–											Linear	5.88	2065.9
B7	**R**	F	L	V	C	–	**K**	Q	**K**	I	W	G	**K**	A	**R**	–	–	M	C	T	**R**	–	A	**R**	–	Linear	7.84	3269.5
H1[e]	T	–	–	F	Q	–	–	**R**	N	–	**R**	–	V	**R**	G	–	P	V	S	–	I	**K**	–	D	S	Cyclic	5.85	3019.9
H2	T	–	–	–	–	–	–	**R**	N	–	**R**	–	V	**R**	G	–	P	V	S	–	I	K	–	D	S	Cyclic	7.85	3058.1
H3	T	–	–	F	Q	–	–	**R**	N	–	**R**	–	V	← Hexa[d] →						–	I	**K**	–	D	S	Cyclic	4.85	2539.9
H4	T	–	–	F	Q	–	–	–	N	–	**R**	–	V	**R**	G	–	P	V	S	–	I	**K**	–	D	S	Cyclic	4.85	3049.8
H5	T	–	–	F	Q	–	–	**R**	N	–	**R**	–	V	**R**	G	–	P	V	S	–	I	**K**	–	D	S	Linear	5.85	3164.2

[a] Peptide structure, cyclic with disulfide bridges and linear with acetamidomethyl (Acm)-protecting groups on the cysteines.
[b] Net charge at pH 7.0.
[c] Observed molecular weight including the Acm-protecting group on cysteine in the linear peptides. Values differed 0.1–1.4 Da from the calculated values.
[d] Six amino acids were replaced with one hexane chain.
[e] Fragment 18–42 of LfcinH, homologue to LfcinB.

designated H2, was therefore constructed in which Phe 4 and Gln 5 were replaced by two arginine residues, identical to the two arginines residues present in positions 4 and 5 of LfcinB. These modifications gave a LfcinH analogue with an almost identical net positive charge as that of LfcinB (7.85 at pH 7.0). The role of the hydrophobic part between the charged region and the Cys residue in the C-terminal part of LfcinB and its antiviral activity was investigated by replacing the sequence encompassing residues 14–19 in LfcinB by an hexane chain (B3) (Table 1). A similar replacement was also performed in the LfcinH analogue H3. A linear peptide lacking 10 C-terminal residues compared to LfcinB was also made (B6). Previous studies has revealed that the number of aromatic amino acid residues in lactoferricin-derived peptides are important for interactions with bacterial cell membranes (Haug and Svendsen, 2001). In order to investigate whether the aromatic residues in the Lfcin peptides were critical for the antiviral activity, the bovine Lfcin analogue B4 and the human Lfcin analogue H4 were made. In B4 one aromatic amino acid residue was deleted from each terminus. Since a Trp residue is located in position 8 in LfcinB, Arg 8 in LfcinH was replaced by Trp, giving the H4 analogue. Linear LfcinB (B5) and LfcinH (H5) analogues, and a scramble peptide (B7) encompassing the same amino acids content as LfcinB, but in a random sequence, were also constructed.

3.2. Antiviral activity

Both human and bovine LF displayed much higher antiviral activity towards HSV-1 and HSV-2 than all the Lfcin derivatives (Table 2). The native LfcinB displayed a higher antiviral activity than the native LfcinH (1–47). None of the other LfcinH derivatives, including the H1 peptide which is an analogue to LfcinB, displayed antiviral activity at the concentrations tested. The B2 peptide, which has an increased positive charge compared to native LfcinB, showed an antiviral activity identical against HSV-1 to that of LfcinB. Surprisingly, the B2 peptide had 10-fold lower antiviral activity against HSV-2 compared with LfcinB. The B3 peptide, with a replacement of six residues in the C-terminal part of LfcinB by a hexane chain showed 3.5- and 16-fold decrease in antiviral activity against HSV-1 and HSV-2, respectively. Neither the B4 peptide, with two aromatic phenylalanines deleted from the LfcinB sequence, nor the linear LfcinB analogues B5 and B6 showed any antiviral activity at the concentrations tested. The random sequence peptide B7 showed low but detectable antiviral activity.

3.3. HS and CS affinity

To investigate whether the antiviral activity correlated with the GAG binding capacity, the affinity of the various peptides for HS and CS, was studied by affinity chromatography. In general, all the peptides had higher affinity for HS than CS. Since the relative affinity for CS-A and CS-C was almost equal for all the peptides, only the results from the CS-A affinity studies, are presented in Table 2. The LF proteins showed higher affinity for HS than all the Lfcin analogues. Although LfcinB had much higher antiviral activity than LfcinH (1–47), they showed similar affinity to HS. The human analogue of LfcinB, H1, had very low affinity for HS, whereas the B2 and H2 peptides, with an increased net positive charge, showed a slightly higher affinity for HS than unmodified LfcinB and LfcinH 18–42 (H1). Surprisingly, the B2 peptide showed decreased affinity for CS compared

Table 2
Affinity, toxicity, and antiviral activity

Name	Elution concentration from HS (mM NaCl)[a]	Elution concentration from CS-A (mM NaCl)[a]	Aliphatic index	Fibroblasts, IC_{50} (μM)[b]	HSV-1, IC_{50} (μM)[c]	HSV-2, IC_{50} (μM)[c]
B-LF	369	240	75.46	8.5	0.6	0.2
LfcinB	313	224	50.80	245	14.6	12.3
B2	326	80	50.80	239	13.5	122.2
B3	356	108	41.05	264	51.3	207.3
B4	309	90	55.22	311	N.D.	N.D.
B5	245	145	50.80	258	N.D.	N.D.
B6	130	70	32.67	380	N.D.	N.D.
B7	227	200	50.80	240	74.9	67.2
H-LF	344	165	70.78	10.9	1.2	0.7
LfcinH	310	117	53.83	131	42.6	30.6
H1	143	90	38.80	274	N.D.	N.D.
H2	219	105	38.80	273	N.D.	N.D.
H3	113	90	35.79	310	N.D.	N.D.
H4	150	131	38.80	249	N.D.	N.D.
H5	135	55	38.80	267	N.D.	N.D.

N.D., not detectable antiviral activity within tested concentration range (3–160 μM).

[a] Concentration of NaCl required to elute the peptide from the affinity column, mean value of three–five experiments.

[b] Peptide concentration giving 50% cell death, mean value of four experiments.

[c] Concentration required for 50% reduction in virus amplification. Values are expressed as a result of four–eight repeated experiments.

to the unmodified control. The B3 peptide, which includes a hexane chain in the C-terminal part, showed increased affinity for both HS and CS, whereas the human analogue, H3, showed decreased affinity. The effect of aromatic residues on the affinity for HS and CS was investigated. The B4 peptide, with the deletion of the aromatic residue Phe in both the C-terminus and in the N-terminus of LfcinB, showed a slightly lower affinity for HS relative to LfcinB. The H4 peptide, with Arg 8 replaced by Trp, showed a slightly higher affinity for HS. All the linear LfcinB and LfcinH analogues had lower affinity for HS than the cyclic derivatives. When 10 residues were deleted from the C-terminal end of linear LfcinB (B6), the affinity for HS was reduced two-fold relative to linear LfcinB. A low difference in relative affinity between HS and CS was also observed with the scramble peptide B7.

3.4. Fibroblasts toxicity

The toxic effect against the MRC-5 cell line were examined by treating the cells with different concentrations (5–1000 μg/ml) of each of the peptide and proteins for 24 h (Table 2). In general, both the native proteins and the peptides exerted low toxic effects against the MRC-5 cells. With regard to the compounds with high antiviral activity, much lower concentrations were needed for antiviral activity than what was needed for toxic effects against the fibroblasts.

3.5. Calculations

To correlate GRAVY with HS affinity, these values were plotted (Fig. 2), and the peptides could be separated into two groups. The group to the right in Fig. 2 contain all the peptides with antiviral activity, plus two peptides with no antiviral activity (B4 and B5), whereas the group to the left contain only peptides with no antiviral activity. Values for GRAVY (Fig. 2), aliphatic index (Table 2), and net charge at pH 7.0 (Table 1) showed all good correlation with HS affinity (Table 2), with two-tailed *P* values ranging from 0.0019 to 0.0100 in a 95% confidence interval (Table 3). There was also a good correlation between HS affinity and the antiviral activity for the two viruses, with *P* values ranging from 0.0015 to 0.0234 (Table 3). The aliphatic index correlated only with anti-HSV-2 activity and not with anti-HSV-1 activity.

Table 3
Correlation of observed and calculated properties of the peptides

Correlation[a]		*P* value (two-tailed)[b]
Net charge	HS affinity	0.0070
GRAVY	HS affinity	0.0100
Aliphatic index	HS affinity	0.0019
HS affinity	IC_{50} HSV-1	0.0015
HS affinity	IC_{50} HSV-2	0.0083
GRAVY	IC_{50} HSV-1	0.0234
GRAVY	IC_{50} HSV-2	0.0119
Aliphatic index	IC_{50} HSV-1	0.0569
Aliphatic index	IC_{50} HSV-2	0.0411
Net charge	IC_{50} HSV-1	0.0556
Net charge	IC_{50} HSV-2	0.0989

[a] Calculated or measured parameters which were correlated, with a significance level ($P < 0.05$).
[b] The two-tailed *P* value is the chance that a randomly selected samples would have as far apart as observed from a null hypothesis.

4. Discussion

The aim of the present study was to investigate the structural basis for the antiviral activity exerted by Lfcin, based on the knowledge that that Lfcin inhibits HSV infection (Hammer et al., 2000; Andersen et al., 2003). A set of human and bovine Lfcin derivatives were constructed and tested for their antiviral activity against HSV-1 and HSV-2. In addition, the antiviral activity of the constructed peptides was

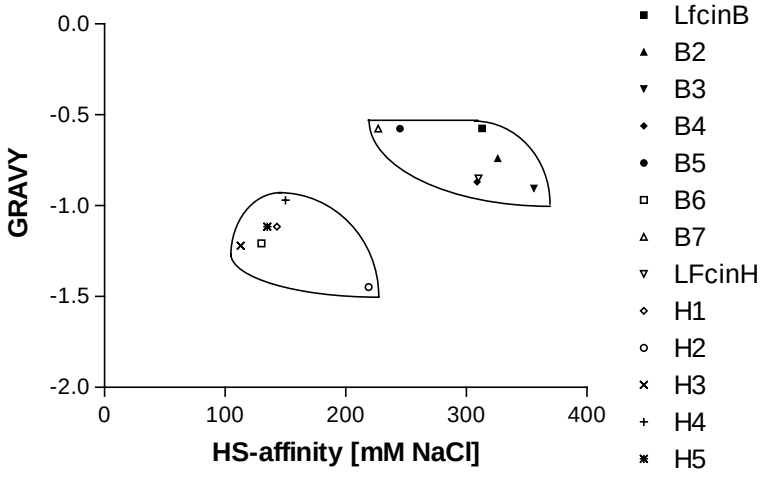

Fig. 2. Grand average of hydropathicity (GRAVY) for the peptides plotted against their affinity for HS.

compared with their ability to interact with HS, the cell surface molecules involved in the initial step of HSV infection.

Our results show that all the peptides that inhibited HSV infection did bind HS with high affinity. This is in agreement with the hypothesis that Lfcin mediates its antiviral activity by binding HS molecules on the cell surface and blocking the attachment of HSV (Marchetti et al., 1996). Native lactoferricin has been shown to inhibit the entry of HSV-1 (Andersen et al., 2002). This assay was not performed with the synthetic peptides due to the limited amounts available. However, other factors also seem to influence the activity, e.g. the LF proteins exhibited a much higher antiviral activity than smaller Lfcin peptides with similar affinities for HS. In addition, the replacement of the residues 14–19 in the C-terminal part of LfcinB with an hexane chain, as performed in the B3 peptide, resulted in an enhanced HS affinity but a significantly lower antiviral effect. It is also noteworthy that the deletion of the N- and C-terminal Phe residues in LfcinB (B4) led to a total loss of antiviral activity without a decrease in HS affinity. These results indicate that not only the affinity for HS, but also the size of the proteins/peptides seem to be of importance for the antiviral activity.

That LfcinB was found to be more efficient than LfcinH in inhibiting HSV infection is consistent with the previous studies on the inhibition of HCMV entry into human fibroblasts (Andersen et al., 2001). When the N-terminal part of LfcinH was deleted, giving the corresponding human homologue of LfcinB (H1), the affinity for HS decreased dramatically and the antiviral effect was lost. In fact, none of the human Lfcin analogues showed high HS affinity, indicating that the LfcinB sequence contain unique structural features necessary for HS binding which is not present in the LfcinH homologue. The loss of HS binding by the shorter LfcinH peptides could be due to the removal of the N-terminal sequence G_1RRRR_5, since R_4 and R_5 has been shown to be critical for the binding of LFH to heparin (Mann et al., 1994). Interestingly, this highly cationic sequence is not found in the LFB sequence (SWISS-PROT protein knowledgebase TrEMBL), suggesting that other elements in the LFB protein compensate for the absence of this sequence. Hence, some of the HS binding properties in LFB seem to reside in LfcinB, since the affinity for HS of LfcinB was only slightly lower than LFB.

It should be expected that the net positive charge of the peptides is critical for the affinity for HS since HS is highly negatively charged, mostly due to the many negatively charged sulfate groups. In fact, the B2 peptide with an increased net positive charge showed a slightly increased affinity for HS. The large decrease in affinity for HS observed when the 10 C-terminal residues 16–25 were removed in the linear peptide B6 may be due to the deletion of the cationic residues Arg 22 and 23, resulting in a reduced net positive charge. Moreover, by introducing R_4R_5, which are present in LfcinB, into the human analogue (H2), the net positive charge was increased to the same level as in LfcinB. However, even though the affinity to HS was increased, the affinity was much lower than the HS affinity of LfcinB, suggesting that the position of the charged amino acids also are critical for the peptides' ability to interact with HS.

The role of aromatic amino acid residues, and especially Trp, in the antibacterial activity excerted by LfcinB has been earlier highlighted (Strom et al., 2000), and was therefore investigated. The replacement of Arg 8 in H1 with a Trp residue (H4) and the deletion of the N- and C-terminal Phe residues in the LfcinB sequence (B4) did not lead to any change in HS affinity indicating that aromatic residues did not play an important role for HS binding. Although both LfcinB and B4 show the same affinity for HS, B4 displayed no antiviral activity. Whether this is due to the hydrophobic property of the amino acid or the size is unclear. However, the N- and C-terminal Phe residue were crucial for the antiviral activity of LfcinB. This may be a result of a reduced size of the molecule rather than the amino acids' lipophilic property, supporting that, in addition to size, charge is also an important factor for antiviral activity.

The linear analogues displayed lower affinity to HS than the cyclic analogues, and only the linear peptide B7 displayed antiviral activity. This suggest that the distribution of amino acids is not only important in the primary structure but also that their spatial position in a secondary structure is important for HS affinity and antiviral activity.

Differences in antiviral activity against HSV-1 and HSV-2 have also been reported earlier for polyanionic compounds (Hutton et al., 1973) and also for polycationic molecules (Langeland et al., 1988). Trybala et al. (2000) demonstrated that HSV-2 had higher affinity for HS-binding compared to HSV-1. This may also explain why the LfcinB analogues B2 and B3 displayed a differentiated antiviral activity against HSV-1 and HSV-2. For example, the peptide B2 showed a nine-fold lower antiviral activity against HSV-2 than against HSV-1. The small Gly residue in position 14, therefore, seems to play a crucial role in the antiviral activity by LfcinB against HSV-2 suggesting that a more flexible structure in that region of the peptide is more important for HSV-2. In addition, the results demonstrate that the antiviral activity against HSV-2 of LfcinB seems to be more sensitive to structural changes than the antiviral activity against HSV-1.

It has previously been shown that binding to HS proteoglycans at the cell surface of the host cell represents the first step in HSV infection (Laquerre et al., 1998). However, several cells surface proteoglycans are hybrid molecules, substituted with both CS and HS chains. Since CS may contribute to the HSV infection (Banfield et al., 1995; Mardberg et al., 2002), the affinity of the peptides for CS was also studied. The results revealed that the studied peptides showed a much stronger affinity to HS than to CS. This is consistent with earlier findings that HS is known to bind most molecules with higher affinity than CS, probably due to the unique sulfation pattern and a higher degree of conformational flexibility of the GAG chains (Casu et al., 1986; Sanderson et al., 1987). The nature of the GAG chains is decisive for the peptide affinity. It seems to be easier to create

peptide with high affinity for the conformational flexibility HS molecule, compared to the more rigid CS molecule. This may be an important finding since HS serves as the main attachment sight for HSV on the cell surface. B2 and B3 showed an increased affinity to HS and a decreased affinity to CS relative to LfcinB. Both peptides gave also decreased antiviral activity against HSV-2, which may be a consequence of the lower CS affinity. In contrast, the lower antiviral activity towards HSV-1 observed for B2 and B3 may not be explained by the decreased CS affinity. Previously it has been established that HSV-1 have higher affinity for HS than CS (Banfield et al., 1995; Mardberg et al., 2002).

Comparison of the aliphatic index and the antiviral activity of the peptides seem to follow somewhat the same pattern as the correlation between net charge and antiviral activity. B4 and B5 were the only two peptides with an aliphatic index $\geq$50.80 and with no antiviral activity. It seems like the GRAVY should be higher than -0.740 for the peptide to exhibit antiviral activity. B4 is the only one above this limit with no antiviral activity. It seem to be a clear correlation between hydrophobic properties and antiviral activity, except of the peptides B4 and B5.

5. Conclusion

A relative high net positive charge and the position of the cationic amino acids seem to be critical for HS affinity. Even though all the peptides with antiviral activity showed high affinity to HS, there was no direct correlation between net charge and antiviral activity. Additional structural features such as hydrophobicity, molecular size, the spatial position between the charged and hydrophobic amino acid in a stabilized secondary structure seem to be important for antiviral activity. Interestingly, the antiviral activity against HSV-2 was more affected by structural changes in LfcinB than the antiviral activity against HSV-1.

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References

Andersen, J.H., Osbakk, S.A., Vorland, L.H., Traavik, T., Gutteberg, T.J., 2001. Lactoferrin and cyclic lactoferricin inhibit the entry of human cytomegalovirus into human fibroblasts. Antiviral Res. 51, 141–149.

Andersen, J.H., Jenssen, H., Gutteberg, T.J., 2002. Lactoferricin inhibits the entry of HSV-1. In: 28th International Herpes Workshop, Cairns, Australia.

Andersen, J.H., Jenssen, H., Gutteberg, T.J., 2003. Lactoferrin and lactoferricin inhibit herpes simplex 1 and 2 infection and exhibit synergy when combined with acyclovir. Antiviral Res. 58, 209–215.

Banfield, B.W., Leduc, Y., Esford, L., Visalli, R.J., Brandt, C.R., Tufaro, F., 1995. Evidence for an interaction of herpes simplex virus with chondroitin sulfate proteoglycans during infection. Virology 208, 531–539.

Bellamy, W., Takase, M., Wakabayashi, H., Kawase, K., Tomita, M., 1992. Antibacterial spectrum of lactoferricin B, a potent bactericidal peptide derived from the N-terminal region of bovine lactoferrin. J. Appl. Bacteriol. 73, 472–479.

Cardin, A.D., Weintraub, H.J., 1989. Molecular modeling of protein-glycosaminoglycan interactions. Arteriosclerosis 9, 21–32.

Casu, B., Choay, J., Ferro, D.R., Gatti, G., Jacquinet, J.C., Petitou, M., Provasoli, A., Ragazzi, M., Sinay, P., Torri, G., 1986. Controversial glycosaminoglycan conformations. Nature 322, 215–216.

Chou, T.C., Talalay, P., 1984. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv. Enzyme Regul. 22, 27–55.

El Yazidi-Belkoura, I., Legrand, D., Nuijens, J., Slomianny, M.C., van Berkel, P., Spik, G., 2001. The binding of lactoferrin to glycosaminoglycans on enterocyte-like HT29-18-C1 cells is mediated through basic residues located in the N-terminus. Biochim. Biophys. Acta 1568, 197–204.

Hammer, J., Haaheim, H., Gutteberg, T.J., 2000. Bovine lactoferrin is more efficient than bovine lactoferricin in inhibiting HSV-I/II replication in vitro. In: Shimazaki, K. (Ed.), Lactoferrin: Structure, Functions and Application. Elsevier Science, Amsterdam, pp. 239–243.

Harmsen, M.C., Swart, P.J., de Béthune, M.P., Pauwels, R., De Clercq, E., Thé, T.H., Meijer, D.K., 1995. Antiviral effects of plasma and milk proteins: lactoferrin shows potent activity against both human immunodeficiency virus and human cytomegalovirus replication in vitro. J. Infect. Dis. 172, 380–388.

Haug, B.E., Svendsen, J.S., 2001. The role of tryptophan in the antibacterial activity of a 15-residue bovine lactoferricin peptide. J. Pept. Sci. 7, 190–196.

Hutton, R.D., Ewert, D.L., French, G.R., 1973. Differentiation of types 1 and 2 herpes simplex virus by plaque inhibition with sulfated polyanions. Proc. Soc. Exp. Biol. Med. 142, 27–29.

Ikai, A., 1980. Thermostability and aliphatic index of globular proteins. J. Biochem. (Tokyo) 88, 1895–1898.

Ji, Z.S., Mahley, R.W., 1994. Lactoferrin binding to heparan sulfate proteoglycans and the LDL receptor-related protein. Further evidence supporting the importance of direct binding of remnant lipoproteins to HSPG. Arterioscler. Thromb. 14, 2025–2031.

Kuwata, H., Yip, T.T., Yamauchi, K., Teraguchi, S., Hayasawa, H., Tomita, M., Hutchens, T.W., 1998. The survival of ingested lactoferrin in the gastrointestinal tract of adult mice. Biochem. J. 334, 321–323.

Kyte, J., Doolittle, R.F., 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. 157, 105–132.

Langeland, N., Moore, L.J., Holmsen, H., Haarr, L., 1988. Interaction of polylysine with the cellular receptor for herpes simplex virus type 1. J. Gen. Virol. 69, 1137–1145.

Laquerre, S., Argnani, R., Anderson, D.B., Zucchini, S., Manservigi, R., Glorioso, J.C., 1998. Heparan sulfate proteoglycan binding by herpes simplex virus type 1 glycoproteins B and C, which differ in their contributions to virus attachment, penetration, and cell-to-cell spread. J. Virol. 72, 6119–6130.

Lee, K.K., Black, J.A., Hodges, R.S., 1989. Air oxidation of peptides and separation of their reduced and intrachain disulfide bridged forms by reversed-phase high performance liquid chromatography (RPC). In: 11th American Peptide Symposium, San Diego, CA.

Lindahl, U., Lidholt, K., Spillmann, D., Kjellen, L., 1994. More to "heparin" than anticoagulation. Thromb. Res. 75, 1–32.

Mann, D.M., Romm, E., Migliorini, M., 1994. Delineation of the glycosaminoglycan-binding site in the human inflammatory response protein lactoferrin. J. Biol. Chem. 269, 23661–23667.

Marchetti, M., Longhi, C., Conte, M.P., Pisani, S., Valenti, P., Seganti, L., 1996. Lactoferrin inhibits herpes simplex virus type 1 adsorption to Vero cells. Antiviral Res. 29, 221–231.

Mardberg, K., Trybala, E., Tufaro, F., Bergstrom, T., 2002. Herpes simplex virus type 1 glycoprotein C is necessary for efficient infection of chondroitin sulfate-expressing gro2C cells. J. Gen. Virol. 83, 291–300.

Masson, P.L., Heremans, J.F., Dive, C., 1966. Studies of the proteins of secretions from two villous tumours of the rectum. Gastroenterologia 105, 270–282.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.

Omata, Y., Satake, M., Maeda, R., Saito, A., Shimazaki, K., Yamauchi, K., Uzuka, Y., Tanabe, S., Sarashina, T., Mikami, T., 2001. Reduction of the infectivity of Toxoplasma gondii and Eimeria stiedai sporozoites by treatment with bovine lactoferricin. J. Vet. Med. Sci. 63, 187–190.

Qie, L., Marcellino, D., Herold, B.C., 1999. Herpes simplex virus entry is associated with tyrosine phosphorylation of cellular proteins. Virology 256, 220–227.

Rekdal, O., Andersen, J., Vorland, L.H., Svendsen, J.S., 1999. Construction and synthesis of lactoferricin derivatives with enhanced antibacterial activity. J. Pept. Sci. 5, 32–45.

Reyes, M., Shaik, N.S., Graber, J.M., Nisenbaum, R., Wetherall, N.T., Fukuda, K., Reeves, W.C., 2003. Acyclovir-resistant genital herpes among persons attending sexually transmitted disease and human immunodeficiency virus clinics. Arch. Intern. Med. 163, 76–80.

Sanderson, P.N., Huckerby, T.N., Nieduszynski, I.A., 1987. Conformational equilibria of alpha-L-iduronate residues in disaccharides derived from heparin. Biochem. J. 243, 175–181.

Stringer, S.E., Gallagher, J.T., 1997. Heparan sulphate. Int. J. Biochem. Cell. Biol. 29, 709–714.

Strom, M.B., Rekdal, O., Svendsen, J.S., 2000. Antibacterial activity of 15-residue lactoferricin derivatives. J. Pept. Res. 56, 265–274.

Strom, M.B., Rekdal, O., Svendsen, J.S., 2002. The effects of charge and lipophilicity on the antibacterial activity of undecapeptides derived from bovine lactoferricin. J. Pept. Sci. 8, 36–43.

Tal-Singer, R., Peng, C., Ponce De Leon, L., Abrams, W.R., Banfield, B.W., Tufaro, F., Cohen, G.H., Eisenberg, R.J., 1995. Interaction of herpes simplex virus glycoprotein gC with mammalian cell surface molecules. J. Virol. 69, 4471–4483.

Trybala, E., Liljeqvist, J.A., Svennerholm, B., Bergstrom, T., 2000. Herpes simplex virus types 1 and 2 differ in their interaction with heparan sulfate. J. Virol. 74, 9106–9114.

Ueta, E., Tanida, T., Osaki, T., 2001. A novel bovine lactoferrin peptide, FKCRRWQWRM, suppresses *Candida* cell growth and activates neutrophils. J. Pept. Res. 57, 240–249.

Weinberg, E.D., 2001. Human lactoferrin: a novel therapeutic with broad spectrum potential. J. Pharm. Pharmacol. 53, 1303–1310.

Whitley, R.J., Kimberlin, D.W., Roizman, B., 1998. Herpes simplex viruses. Clin. Infect. Dis. 26, 541–553.

Wu, H.F., Monroe, D.M., Church, F.C., 1995. Characterization of the glycosaminoglycan-binding region of lactoferrin. Arch. Biochem. Biophys. 317, 85–92.

WuDunn, D., Spear, P.G., 1989. Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. J. Virol. 63, 52–58.

Yoo, Y.C., Watanabe, S., Watanabe, R., Hata, K., Shimazaki, K., Azuma, I., 1998. Bovine lactoferrin and lactoferricin inhibit tumor metastasis in mice. Adv. Exp. Med. Biol. 443, 285–291.