A Probe for Capture and Fe³⁺-Induced Conformational Change of Lactoferrin Selected from Phage Displayed Peptide Libraries

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ABSTRACT

Linear pentadecamer and cyclic hexamer peptide phage libraries were used to isolate phage clones with binding affinity toward lactoferrins purified from human and bovine milk. Phage clones with high specificity toward lactoferrin were selected with different binding strengths depending on the sequence of the peptide displayed by the phage. Phages coated to a microtiterplate were able to capture lactoferrin from crude milk samples without prior treatment. One of the selected sequences, EGKQRR, failed to bind to lactoferrin. In contrast, a branched tree-peptide bearing 4 EGKQRR sequences did bind to lactoferrin (K_d–29 μM) and was also capable of inhibiting the binding of the phage to lactoferrin (IC₅₀–17 μM), indicating that avidity was important. Unexpectedly, the affinity of the phage for lactoferrin was influenced by the amount of bound Fe³⁺, with a much lower affinity when lactoferrin was saturated with Fe³⁺ as compared with the iron-depleted or partially saturated (natural) lactoferrin. As the phage does not bind to the Fe³⁺-binding site, the difference in binding affinity is due to differences in conformation of lactoferrin induced by Fe³⁺. These results demonstrate that avidity or multipoint attachment and Fe³⁺-induced conformational changes play an important role in the binding of the selected phage to lactoferrin. Thus, we could demonstrate that, by the use of selected phage clones, we are able not only to detect lactoferrin, but also to capture lactoferrin from crude milk samples. Furthermore, the extent of phage binding provides additional information about the iron content and the concomitant conformation of lactoferrin.

(**Key words:** lactoferrin, phage display, iron binding, avidity)

Abbreviation key: apo-lactoferrin = iron-depleted lactoferrin, BovLF = bovine lactoferrin, HRP = horseradish peroxidase, HuLF = human lactoferrin, TPBS = 0.1% Tween-20 in phosphate-buffered saline.

INTRODUCTION

Lactoferrin is an iron-binding 80 kDa protein appearing in many physiological fluids, such as milk, tear fluid, saliva, seminal plasma, and synovial fluid (Steijns and Hooijdonk, 2000). The N- and C-terminal halves of the protein form 2 separate lobes connected by a short α-helix, resulting in a 2-fold internal homology. Each lobe consists of 2 domains that form a cleft, where the Fe³⁺ ion is bound in synergistic cooperation with a bicarbonate anion (Anderson et al., 1987; Moore et al., 1997).

Initial research on the lactoferrin function was mainly directed toward its iron-binding properties, iron metabolism, and related antimicrobial activity. However, more intensive research revealed several noniron-associated lactoferrin functions such as antiviral, antiparasitic, and immunomodulatory activity, and effects on inflammation, tumorigenesis, and enzymatic activity (Brock, 2002). Furthermore, specific receptors for lactoferrin have been identified on a wide variety of mammalian cells including macrophages, lymphocytes, hepatocytes, epithelial cells, intestinal cells, and platelets (Suzuki and Lönnnerdal, 2002). These receptors are considered to function as mediators for some functions of lactoferrin, such as iron turnover (macrophages, intestinal cells) and incorporation of lactoferrin (lymphocytes). Binding of lactoferrin to its platelet receptor inhibits platelet aggregation (Leveugle et al., 1993). Binding or release of an Fe³⁺ ion induces conformational changes in the 3-D structure of lactoferrin, which influence the binding of lactoferrin to its membrane receptor, important for the assimilation of iron in microorgan-

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isms (Schryvers et al., 1998). Similar effects were observed with transferrin, also an Fe^{3+} -binding protein belonging to the same family as lactoferrin: the binding of transferrin to its receptor is Fe^{3+} -dependent, and Fe^{3+} -saturated transferrin showed at least 100-fold greater affinity for the receptor as compared with the iron-depleted transferrin (apo-transferrin) (Retzer et al., 1998). These findings on the Fe^{3+} dependency of the binding of lactoferrin to its receptor confirms that the Fe^{3+} -induced conformational state of lactoferrin determines the protein-receptor interaction. In this respect, it is important to know how exactly Fe^{3+} influences structure and conformation of lactoferrin.

In this study, we isolated specific lactoferrin binding phages, which allows detection and capture of lactoferrin in milk. In addition and surprisingly, we found that the phage binding to lactoferrin was dependent on the Fe^{3+} saturation, such that phage binding in addition can be used as a probe for Fe^{3+} -induced conformational changes of lactoferrin.

MATERIALS AND METHODS

Materials

The linear pentadecamer phage display peptide was a gift of G. P. Smith (University of Missouri, Colombia). The library with random hexapeptides flanked by cysteine residues was from Corvas (Gent, Belgium). *Escherichia coli* strain TG-1(*K12*, Δ [*lac-pro*], *supE*, *thi*, *hsdD5*, *F'**TraD36*, *proA*⁺*B*⁺, *LacI*^q, *LacZ*, Δ *M15*) was from Stratagene (La Jolla, CA). Monoclonal antiM13 horseradish peroxidase (**HRP**) conjugate (HRP/antiM13), PD-10 desalting columns, Hi Prep 26/10 desalting column, Streamline SP-XL, Superdex 200 prep grade, and Superdex 75 prep grade were from Amersham Pharmacia Biosciences (Uppsala, Sweden) and Poros-50 HS from Perseptive Biosystems (Framingham, MA). Rabbit antihuman lactoferrin was from Sigma (St. Louis, MO). Swine antirabbit immunoglobulin-HRP conjugate was from Dako (Glostrup, Denmark) and streptavidin-HRP conjugate from Roche (Indianapolis, IN). Skimmed bovine milk powder (Nestlé, Switzerland) used as blocking reagents (4% solution in PBS) contained less than 0.5 ng/mL lactoferrin as determined in ELISA. Also, on SDS-PAGE no lactoferrin was observed in the milk powder. The SDS-PAGE was performed on Novex 10 to 20% gradient Tris-Tricine gel (San Diego, CA) under reducing and denaturing conditions. Linear 15-mer HuH6 and cyclic 6-mer peptides, HuN and HuL, corresponding to peptides exposed on selected phages, were custom synthesized at Sigma-Genosys Ltd. (Pampisford, UK). Branched tree-peptide HuN was custom synthesized at Ansynth (Roosendaal,

The Netherlands). All peptides had a purity of >70% and were used without further purification.

Purification of Lactoferrin

Crude milk lactoferrin was purified with Expanded Bed chromatography as described earlier (Noppe et al., 1996) with some modifications. Briefly, 1.3 L of human or bovine milk was defatted by centrifugation at $7500 \times g$ for 10 min at 4°C. The purification involved cation-exchange chromatography on Streamline SP-XL in expanded bed. Skimmed milk was diluted 5 times with Tris buffer to a final concentration of 25 mM Tris, and the pH was adjusted to 8.5 with NH_3 . After column expansion, the diluted milk sample was applied to the column at 300 cm/h in upward flow. The column was washed with buffer in upward flow, the flow was interrupted, allowing the bed to settle, and the adaptor was lowered until a packed bed was obtained. The bound fraction was eluted in packed bed mode with 25 mM Tris-HCl, pH 8.5, containing 1 M NaCl. Sodium dodecyl-sulfate-PAGE and enzymatic tests for lysozyme (Kikuchi et al., 1988) and lactoperoxidase (Morrison et al., 1970), showed that the fraction contained lactoferrin, lactoperoxidase, and lysozyme. After dialysis against 25 mM NaH_2PO_4 , pH 6.0, the lactoferrin-containing fraction was applied to a Poros 50-HS column equilibrated with 25 mM NaH_2PO_4 , pH 6.0, and eluted with an NaCl step gradient (0.3 to 0.4 to 1 M). The fraction eluted with 1 M NaCl was identified as lactoferrin, as it did not contain lysozyme (lytic activity) nor lactoperoxidase (peroxidase activity). Finally, the lactoferrin-containing fraction was polished on a Superdex 200 prep grade column. After dialysis and freeze-drying, the purity was checked on SDS-PAGE and showed one homogeneous band estimated at 76,000 Da. Furthermore, lactoferrin was detected in ELISA by a polyclonal rabbit antilactoferrin antibody. Lactoferrin iron content was measured by atomic absorption spectroscopy at 248.3 nm and absorption at 462 nm. The final yield was ± 2.5 g/L of human lactoferrin (**HuLF**) and ± 0.9 g/L of bovine lactoferrin (**BovLF**).

Preparation of Apo and Fe^{3+} -Saturated Lactoferrin

Apo-lactoferrin. Forty milligrams of purified, partially Fe^{3+} -saturated lactoferrin was dissolved in 3 mL of 10 mM HCl, pH 2.0, and applied to a Hi-Prep 26/10 desalting column. The protein fraction was pooled, and residual iron in the protein was measured by atomic absorption spectroscopy at 248.3 nm.

Fe^{3+} -saturated lactoferrin. Five milligrams of lactoferrin was dissolved in 2 mL of 5 mM FeCl_3 and 50 mM sodium bicarbonate, pH 8.5, and then incubated

overnight at 4°C to saturate the lactoferrin completely with iron. The protein sample was applied to a PD-10 column and eluted with Tris-buffered saline at pH 7.5 to remove unbound iron. This step was repeated twice. The iron content of the saturated protein was measured using atomic adsorption spectroscopy at 248.3 nm.

Selection of Lactoferrin Binding Phages

Isolation of HuLF or BovLF binding phages was performed as described elsewhere with minor modifications (Smith and Scott, 1993). Polystyrene tubes (Immunotubes, Nunc, Denmark) were coated with lactoferrin (20 to 400 μg) in coating buffer (PBS, pH 7.4) and incubated overnight at 4°C on a rotator. Tubes were washed 3 times with 0.1% Tween-20 in PBS (TPBS), and postcoated with 4 mL of 4% skimmed milk powder (Nestlé, Switzerland) in PBS for at least 2 h at room temperature. After brief washing in TPBS, 2×10^{12} or 1.1×10^{11} phage particles in 0.4% skimmed milk powder in PBS were added in the first and second panning rounds, respectively, and incubated overnight at 4°C on a rotator. After 3 washes with TPBS, the phages were eluted with 0.1 M glycine, pH 2.0, the eluate was neutralized with 1 M Tris, pH 8.0, and used to infect TG1-*E. coli* cells. The cell suspension was plated on Luria agar plates containing 10 $\mu\text{g}/\text{mL}$ tetracycline and incubated overnight at 37°C. Phages were rescued from the plates and precipitated with 2.5 M NaCl + 20% PEG-6000. After washing, the phage pellet was finally dissolved in 0.5 mL of PBS. Phage concentration was determined by measuring absorbance at 260 nm (1 absorbance unit = 2.214×10^{11} phage particles). Depending on the different panning conditions, 2 to 4 panning rounds were performed, and single colonies were isolated as described (Smith and Scott, 1993). Identification of lactoferrin binding phages was performed in ELISA (cfr. below). Single stranded DNA of phage from single colonies was extracted using the Tris-Phenol method.

The DNA sequence was determined by Genomex (Grenoble, France) using the 5' TGAATTTTCTGTATGAGG 3' primer for the cyclic hexamer peptides and 5'AGCATTCACAGACAGCCCTCATAGTT 3' primer for the linear pentadecamer peptides.

Detection of Lactoferrin Binding Phages

The wells of a Maxisorb microtiter plate (Nunc, Denmark) were coated with 10 $\mu\text{g}/\text{mL}$ HuLF or BovLF in PBS and incubated overnight at 4°C. The wells were postcoated with 4% skimmed milk powder solution for at least 2 h at room temperature. Next, a 2-fold serial phage dilution starting from 1×10^{11} phages/mL in 0.4%

skimmed milk powder in PBS was added and incubated for 2 h at room temperature. Bound phages were detected with HRP/antiM13 (1/5000 dilution in 0.4% skimmed milk powder in PBS). Visualization was performed with o-phenylenediamine-dihydrochloride (OPD) and H_2O_2 . The reaction was stopped with 4 M H_2SO_4 , and the absorbance was measured at 490 nm. Wells were washed 3 times after coating and blocking, and 12 times in other cases.

Competition ELISA

Wells of a microtiter plates were coated with HuLF and blocked as described above. Next, dilution series of peptide were added for at least 30 min, followed by the addition of a constant amount of phages for another 75 min. Bound phages were detected as described above.

A similar procedure was followed in the ELISA for determination of phage binding sites. After coating and blocking, a dilution series of phage was added for at least 30 min, followed by a constant concentration of biotinylated phage for another 75 min. Bound phages were detected with streptavidin-HRP conjugate and visualized as described above.

Capture of Lactoferrin

The wells of a Maxisorb microtiter plate were coated with 5×10^{10} lactoferrin binding phages in PBS and incubated overnight at 4°C. The wells were postcoated with 4% skimmed milk powder solution for at least 2 h at room temperature. Next, a 2-fold serial dilution of a milk sample in PBS was added and incubated for 2 h at room temperature. A reference sample of purified lactoferrin was started at a concentration of 10 $\mu\text{g}/\text{mL}$ in PBS and diluted in the same way as the milk sample. Bound lactoferrin was detected with rabbit antihuman lactoferrin (1/5000 in 0.4% skimmed milk powder in PBS) and incubated for 2 h at room temperature. After extensive washing, a second antibody, swine antirabbit IgG-HRP (1/5000 in 0.4% skimmed milk powder in PBS), was added and incubated for 1.5 h at room temperature. Visualization was performed as described above.

Determination of Affinity Binding Constants Using Surface Plasmon Resonance

Human lactoferrin-peptide binding experiments were performed on a BIAcore 1000 (BIAcore, Uppsala, Sweden) as described earlier (Andersson et al., 1999) with minor modifications.

Briefly, HuLF was immobilized on a CM5 sensor chip. The binding phase with peptide was performed at 5 $\mu\text{L}/$

Table 1. Deduced amino acid sequences of peptides displayed on phages after panning experiments against HuLF and BovLF. Linear pentadecamer and cyclic hexamer libraries were used under different panning conditions. All hexamer peptides are flanked by a Cys residue at the N- and C-terminal site (CxxxxxxC).

HuLF		BovLF	
HuB9	GGVCFRVLGSIVCNF	BovC12	IPASKA
HuE6	ALVFFFSSGDFYGV	BovD4	GLTGLA
HuH6	GSCRAFLSGVVCSP	BovD10	DRPNAK
HuA	APRQPP	BovE4	PKHRNK
HuD	RTSLKV	BovE6	PRSQQE
HuI	DTNREQ	BovE11	QWLQHP
HuK	DQDQDT	BovF5	PYTYWT
HuL	DQVAKG	BovF8	TAPRRA
HuM	EKGQRR	BovF11	RKSVHS
HuN	EGKQRR	BovG4	SKVKNR
Hu1	TKGNQP	BovG7	SFRGQS
Hu5	TRSGTR	BovG11	YQSQNG
Hu6	SATEHG	BovG12	HGKSAN
Hu7	PGGYTK	BovH4	GKFSYQ
Hu9	TDNAKE	BovH7	PKTERR
Hu11	DVGQNK		
Hu14	HQHRQR		

min for 10 min in HEPES buffer (HBS buffer, BIAcore), followed by a dissociation phase for another 3 min in the same buffer. A washing step of 5 μ L of 50 mM NaOH/2.5 M NaCl at 30 μ L/min was included between every run.

RESULTS

Isolation of HuLF and BovLF Binding Phages

Binding phages for HuLF were isolated after 4 or 2 rounds of biopanning from a linear pentadecamer and cyclic hexamer library, respectively. Eleven individual clones selected from the linear pentadecamer library were sequenced and revealed the presence of only 3 different sequences (Table 1). All these peptides contain a high amount of aromatic/aliphatic noncharged amino acids. Fourteen individual clones of the cyclic hexamer library were sequenced, and all 14 peptides had a different amino acid composition, with no consensus motifs. As compared with the pentadecamer peptides, the cyclic peptides contained only a small amount of aromatic amino acids.

After 2 rounds of biopanning with the cyclic hexamer library on BovLF, 15 individual clones were isolated and sequenced. Also here, all selected phage clones displayed peptides with different sequences (Table 1) and with no consensus motifs. Sequence comparison between the BovLF and HuLF binding peptides did not reveal any consensus in amino acid sequence, net charge, or hydrophobic amino acid content.

Specificity of the Lactoferrin Binding Phages

To determine whether the lactoferrin binding phages showed cross-reactivity with other milk proteins,

Table 2. Cross-reactivity of human lactoferrin (HuLF) and bovine lactoferrin (BovLF) binding phages. ELISA plates were coated with HuLF, BovLF, bovine lactoperoxidase (BovLPO), bovine lactalbumin (Bov LA), bovine serum albumin (BSA), bovine caseins (Bov Caseins), and β -lactoglobulin at 10 μ g/mL and dilution series of HuLF or BovLF binding phages were added. Phage binding was detected using HRP/antiM13. Binding of HuLF or BovLF binding phages to HuLF or BovLF, respectively, was set as 100%.

Coat protein	% Phage binding				
	HuB9	HuE6	HuL	HuN	Hu5
HuLF	100	100	100	100	100
BovLF	171.8	158.5	171.0	120.0	104.0
BovLPO	27.7	25.5	10.8	21.0	9.1
BSA	5.2	5.6	2.5	3.7	0.9
Coat protein	BovF5		BovD10		BovG11
BovLF	100		100		100
HuLF	87.8		81.9		64.4
BovLPO	12.9		11.0		10.6
BovLA	4.5		5.5		4.5
BSA	4.4		5.4		4.3
Bov Caseins	3.3		4.9		3.7
β -Lactoglobulin	4.1		4.6		3.4

phages were analyzed for binding to α -lactalbumin, serum albumin, lactoperoxidase, β -lactoglobulin, and caseins (total casein mixture; Table 2).

The HuLF binding phages did not cross react (<10%) with the other milk proteins, except for lactoperoxidase, which is expected in view of the sequence homology between the 2 proteins (Table 2). Interestingly, phages selected on HuLF bound better to BovLF. Cross-reactivity of BovLF binding phages with other milk proteins was the same as observed for HuLF (Table 2). The reactivity toward HuLF is high, as expected, but lower than with BovLF. Both HuLF and BovLF binding phages also reacted to a lesser extent with goat-, dog-, and horse-milk lactoferrin (data not shown).

Study of the Binding of Selected Peptides to HuLF

The custom synthesized peptide HuH6 (GSCRAFLSGVVCSP, Table 1) displayed on the HuLF binding phage isolated from the linear pentadecamer library, however, did not bind lactoferrin, as detected with Surface Plasmon Resonance, nor did it inhibit the phage binding to lactoferrin.

Also, the peptides HuN (EGKQRR, Table 1) and HuL (DQVAKG, Table 1) displayed on the HuLF binding phages selected from the cyclic hexamer library, custom synthesized with flanking cysteines to obtain the constrained form, did neither bind nor inhibit. Furthermore, reducing both peptides to obtain a linear form did not change this.

As each peptide is displayed in 5 copies on the phage, a branched tree-peptide was synthesized to better

Figure 1. Structure of the custom synthesized branched tree-HuN peptide (EGKQRR). The branched tree-HuN (TAM-HuN) peptide consists of a spacer part containing 6-amino-caproic acid (1), a (Lys)₃ core (2): On the free amino group and amino group of the side chain of the first Lys, 2 Lys were chemically attached, resulting in the presence of 4 free amino groups available for attachment of peptide HuN (3).

mimic the displayed N-terminal site of the pIII protein complexes on the phage. The branched tree-peptide, HuN, was composed of a lysine core, a spacer, and 4 HuN peptides (Figure 1).

Binding experiments performed with Surface Plasmon Resonance (Figure 2 inset) confirmed the binding of the branched tree-peptide HuN to HuLF. A K_D of $29 \mu\text{M} \pm 2 \mu\text{M}$ was determined. The branched tree-peptide, HuN, also inhibited the binding of phage HuN to lactoferrin with an IC_{50} of $17 \mu\text{M} \pm 3 \mu\text{M}$ (Figure 2).

Capture of Lactoferrin

As the above results showed that lactoferrin could be detected through binding of either the phages or the corresponding synthesized peptide complex, we next determined whether phages are able to capture HuLF. Coated phages indeed could retain lactoferrin not only from a purified lactoferrin sample but also from a complex protein sample such as human milk (Figure 3). Similar results were obtained with BovLF binding phages. The ability of the phage to capture lactoferrin

was demonstrated both in an ELISA format and in a dot blot experiment.

Influence of Fe^{3+} on Binding of Phages to Lactoferrin

We next investigated the effect of the level of Fe^{3+} saturation of lactoferrin on the binding of the selected phages.

Depending on the clone used, phage binding to iron-depleted lactoferrin (**apo-lactoferrin**) was 110 to 200% of the binding to lactoferrin isolated from milk that is partially saturated with iron (15 to 25%).

Fully saturated lactoferrin ($2 \text{ Fe}^{3+}/\text{lactoferrin}$), on the other hand, had an even lower binding affinity for phages. Indeed, in the presence of $>1 \text{ mM Fe}^{3+}$, phages no longer bound to lactoferrin (Figure 4a). A clear dose-dependent effect on the binding of phage could be observed when apo-HuLF was titrated with Fe^{3+} (Figure 4b). Similar results were obtained with apo-BovLF. Furthermore, we observed that once the phage was bound to the apo-lactoferrin, the consequent addition of an excess of Fe^{3+} did not displace the bound phage.

Metal ions like Cu^{2+} , Mn^{2+} , or Co^{2+} had no inhibitory effect on the binding between apo-lactoferrin and phages, whereas Zn^{2+} , at high concentrations, also decreased the binding of phage to apo-lactoferrin. This result is in agreement with the finding that lactoferrin is able to bind Zn^{2+} even though with much lower affinity than Fe^{3+} (Zhang and Allan, 1995).

Binding Site of Phages on Lactoferrin

In view of the similar effects of the Fe^{3+} load on the binding of the different phages, despite the high diversity of sequences displayed, we investigated whether the different phages might bind in each other's proximity on lactoferrin.

A competition ELISA was performed between biotinylated and nonbiotinylated phages. The binding of biotinylated Hu5 phage to HuLF decreased in function of the concentration of nonbiotinylated phages (data not shown), suggesting that all phages tested indeed bind in each other's neighborhood.

DISCUSSION

Selecting phages that bind to HuLF or BovLF, from both linear pentadecamer and cyclic hexamer libraries, readily resulted in the identification of several interacting phages. The phages were quite specific for lactoferrin, as they only cross-reacted with lactoferrin from other species and to some minor extent with lactoperoxidase, which was not completely unexpected as lactofer-

Figure 2. Inhibition by the branched tree-peptide, HuN, of phage binding to HuLF. Human lactoferrin was coated and a serial dilution of the branched tree-peptide HuN was added and preincubated for 45 min, followed by the addition of a constant concentration of phage clone HuN. Bound phages were detected with antiM13-HRP. Error bars represent SEM (triplicate result). Inset: Binding of the branched tree-peptide HuN to HuLF using Surface Plasmon Resonance on BIAcore 1000. Human lactoferrin was bound on a CM-5 chip. Different peptide concentrations were added at 10 μ L/min during 10 min in HBS buffer, followed by a dissociation phase for another 3 min. K_D value was calculated by BIAcore evaluation software.

rin and lactoperoxidase are both proteins of approximately 700 amino acid residues, with some 11% identity and 17% similarity in amino acid composition. Specific fragments, AA 195 to 690 of lactoferrin and AA 1 to 465 of lactoperoxidase, even have 19% identity and 30% similarity. Nevertheless, as the concentration of lactoferrin in human milk is at least 50 to 100 times higher than the concentration of lactoperoxidase (Langbakk et al., 1989; Steijns et al., 2000; Shin et al., 2001), the cross-reactivity of lactoferrin-binding phages toward lactoperoxidase will not hamper possible further application such as affinity purification.

Coated phages were indeed able to capture lactoferrin from whole milk as detected both by ELISA and by dot blot. No appreciable cross-reactivity with other milk proteins was measured. Indeed, we could even use skimmed milk powder as postcoat reagents, where surprisingly milk proteins gave the best postcoating, resulting in the least background interferences when compared with soy milk proteins, gelatin + Tween-20, or ovalbumin for postcoating. The skimmed milk powder

consisted mainly of casein and β -lactoglobulin, for which no cross-reactivity was detected.

The different phages competed with each other for binding to lactoferrin; however, the peptide sequences that they are displaying showed wide variety and did not allow identification of relevant features or consensus sequences, as the different sequences did not occur more than once. Barrett (1992) reported a similar observation when using 3E7 Fab as a target and suspected that the library was more extended than expected and therefore resulted in a greater diversity of sequences, whereas Sato (2002) suggested that the diversity might be partially due to conformational changes of the target protein on binding to the support. At present, therefore, it is not clear whether the phages directly compete with each other for their binding site or sterically hinder each other. To circumvent this problem, 3 peptides displayed on the strongest binding phages were synthesized: 1 pentadecamer peptide (HuH6) and 2 cyclic hexamer (HuN, HuL) peptides. However, none of the individual peptides seem to bind to lactoferrin, nor could

Figure 3. Capture of lactoferrin. ELISA plates were coated with $5 \cdot 10^{10}$ phages, and a serial dilution of lactoferrin (A) or milk (B) was added. Lactoferrin binding was detected by a sandwich of rabbit-antiHuLF and swine-antirabbit immunoglobulin-HRP. (A) (■) HuN; (○) Hu1; (▲) Hu5 (B) (■) HuN; (△) Human milk. These results each present the result of one phage clone and are representative for 10 different phage clones.

they inhibit the binding of the parent phage. Binding of synthesized peptides has been reported with success, however, mainly of peptides interacting with the binding site of antibodies (Cwirla et al., 1990; Scott and Smith 1990; Ferrières et al., 2000; Moshitch-Moshkovitz et al., 2000; Zhu et al., 2001). A few authors also reported binding of phage derived peptides with other proteins (Krook et al., 1995; Gaskin et al., 2001; Ulrichs et al., 2001).

We suspect that the lack of detectable binding of the peptides to lactoferrin might be due to low affinity of the individual peptide in contrast to what happens on the phage, where the same peptide is displayed simultaneously on 5 gpIII proteins located in close proximity to each other at the end of the phage. All 5 gpIII proteins

Figure 4. Influence of Fe^{3+} on binding of phages to lactoferrin (A). Natural HuLF (□), apo-HuLF (■), and apo-HuLF + 0.5 mM Fe^{3+} (■) were used for coating, and phages were applied. Bound phages were detected with HRP/antiM13. These results each present the result of one phage clone and are representative for 10 different phage clones. (B) Dose-dependent binding effect. Apo-HuLF was titrated with Fe^{3+} and coated, after which phage clone HuN was incubated, and bound phages were measured, as described above. These results present the result of one phage clone.

could interact simultaneously with the lactoferrin molecule, resulting in a marked gain in avidity. Indeed, a tree-peptide presenting 4 copies of the peptide did bind and inhibit the binding of the parent phage, proving the important role avidity plays in the binding configuration. Cwirla (1990) stated that even with low affinity interactions, multivalent binding leads to high avidity and tenacious adherence of phage. A similar effect was observed by Molenaar (2002).

Lactoferrin has a 2-fold internal homology consisting of a N- (AA 1 to 332) and C- globe (AA 344 to 703) connected by a short α -helix (AA 333 to 343), both hav-

ing a Fe^{3+} -binding site. Anderson et al. (1987) stated that the iron atom is buried, but its immediate environment is quite hydrophilic. Also, the binding of Fe^{3+} induces a significant conformational change, with the protein structure becoming markedly more compact. For example, Fe^{3+} binding results in the contraction of the one globule from ~65 to ~55 Å (Jameson et al., 1998; Sun et al., 1999). As we found a dose-dependent increase of selected phage binding to lactoferrin, when the latter was less saturated with Fe^{3+} ions, one could expect that phage binding takes place somewhere at or near the site where large conformational changes occur upon Fe^{3+} binding. The phage diameter is less than 100 Å, with 5 copies of gpIII protein located at the butt end of the filamentous phage (Sidhu, 2001). The peptide library was expressed at the N-terminus of the N1 domain of the gpIII protein with a diameter of ~30 Å (Lubkowski et al., 1998). With 5 N1 domains of gpIII protein molecules packed closely together in a pentagon-like manner, the centers of these domains will be located on a circle with a diameter ~40 Å, meaning that the peptides expressed at the gpIII proteins could easily interact simultaneously with the protein molecule of 50 to 60 Å in size, for example, of the size of the lactoferrin molecule. The multisite attachment looks to be a prerequisite for the efficient binding of the phage to the lactoferrin molecule. An extensive conformational change in the lactoferrin molecule taking place after Fe^{3+} binding could disturb the finely arranged multisite binding, resulting in a drastic decrease in the efficiency of phage binding to Fe^{3+} -bound lactoferrin, as the binding of individual peptides to lactoferrin is weak. However, as no competition between phages and Fe^{3+} has been demonstrated, there is no direct evidence for the phage binding at the Fe^{3+} -binding site.

In conclusion, we selected and identified phages that bind to lactoferrin in a Fe^{3+} -dependent manner and that are able to capture lactoferrin from crude samples. With these we have created a new set of tools to detect and purify lactoferrin and to further study the conformational changes within lactoferrin induced by Fe^{3+} binding.

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REFERENCES

- Anderson, B. F., H. M. Baker, E. J. Dodson, G. E. Norris, S. V. Rumball, J. M. Waters, and E. N. Baker. 1987. Structure of human lactoferrin at 3.2-Å resolution. *Proc. Natl. Acad. Sci. USA* 84:1769–1773.
- Andersson, K., M. Hämäläinen, and M. Malmqvist. 1999. Identification and optimization of regeneration conditions for affinity-based biosensor assays. A multivariate cocktail approach. *Anal. Chem.* 71:2475–2481.
- Barrett, R. W., S. E. Cwirla, M. S. Ackerman, A. M. Olson, E. A. Peters, and W. J. Dower. 1992. Selective enrichment and characterization of high affinity ligands from collections of random peptides on filamentous phage. *Anal. Biochem.* 204:357–364.
- Brock, J. H. 2002. The physiology of lactoferrin. *Biochem. Cell Biol.* 80:1–6.
- Cwirla, S. E., E. A. Peters, R. W. Barrett, and W. J. Dower. 1990. Peptides on phage: A vast library of peptides for identifying ligands. *Proc. Natl. Acad. Sci. USA* 87:6378–6382.
- Ferrières, G., S. Villard, M. Pugnère, J.-C. Mani, I. Navarro-Teulon, F. Rharbaouil, D. Laune, E. Loret, B. Pau, and C. Granier. 2000. Affinity for the cognate monoclonal antibody of synthetic peptides derived from selection by phage display. *Eur. J. Biochem.* 267:1819–1829.
- Gaskin, D. J. H., K. Starck, N. A. Turner, and E. N. Vulfson. 2001. Phage display combinatorial libraries of short peptides: ligand selection for protein purification. *Enzyme Microb. Technol.* 28:766–772.
- Jameson, G. B., B. F. Anderson, G. E. Norris, D. H. Thomas, and E. N. Baker. 1998. Structure of human apolactoferrin at 2.0 Å resolution. Refinement and analysis of ligand-induced conformational change. *Acta Crystallogr. D Biol. Crystallogr.* 54:1319–1335.
- Kikuchi, M., Y. Yamamoto, Y. Taniyama, K. Ishimaru, W. Yoshikawa, Y. Kaisho, and M. Ikehara. 1988. Secretion in yeast of human lysozymes with different specific activities created by replacing valine-110 with proline by site-directed mutagenesis. *Proc. Natl. Acad. Sci. USA* 85:9411–9415.
- Krook, M., C. Lindblad, S. Birnbaum, H. Naess, J. A. Eriksen, and K. Mosbach. 1995. Selection of peptides with surface affinity for α chymotrypsin using a phage display library. *J. Chromatogr.* 711:119–128.
- Langbakk, B., and T. Flatmark. 1989. Lactoperoxidase from human colostrum. *Biochem. J.* 259:627–631.
- Leveugle, B., J. Mazurier, D. Legrand, C. Mazurier, J. Montreuil, and G. Spik. 1993. Lactotransferrin binding to its platelet receptor inhibits platelet aggregation. *Eur. J. Biochem.* 213:1205–1211.
- Lubkowski, J., F. Hennecke, A. Plückthun, and A. Wlodawer. 1998. The structural basis of phage display elucidated by the crystal structure of N-terminal domains of gpIII. *Nat. Struct. Biol.* 5:140–147.
- Molenaar, T. J. M., C. C. M. Appeldoorn, S. A. M. de Haas, I. N. Michon, A. Bonnefoy, M. F. Hoylaerts, H. Pannekoek, T. J. C. van Berkel, J. Kuiper, and E. A. L. Biessen. 2002. Specific inhibition of P-selectin-mediated cell adhesion by phage display-derived peptide antagonists. *Blood* 100:3570–3577.
- Moore, S. A., B. F. Anderson, C. R. Groom, M. Haridas, and E. N. Baker. 1997. Three-dimensional structure of diferric bovine lactoferrin at 2.8 Å resolution. *J. Mol. Biol.* 274:222–236.
- Morrison, M., and G. Bayse. 1970. Catalysis of iodination by lactoperoxidase. *Biochemistry* 9:2995–3000.
- Moshitch-Moshkovitz, S., Y. Heldman, A. Yayon, and E. Katchalski-Katzir. 2000. Sorting polyclonal antibodies into functionally distinct fractions using peptide phage display a library on top of a library. *J. Immunol. Methods* 242:183–191.
- Noppe, W., I. Hanssens, and M. De Cuyper. 1996. Simple two-step procedure for the preparation of highly active pure equine milk lysozyme. *J. Chromatogr. A* 719:327–331.
- Retzer, M. D., R.-H. Yu, Y. Zhang, G. C. Gonzalez, and A. B. Schryvers. 1998. Discrimination between apo and iron-loaded forms of transferrin by transferrin binding protein B and its N-terminal subfragment. *Microb. Pathog.* 25:175–180.

- Sato, A. K., D. J. Sexton, L. A. Morganelli, E. H. Cohen, Q. L. Wu, G. P. Conley, Z. Streltsova, S. W. Lee, M. Devlin, D. B. DeOliveira, J. Enright, R. B. Kent, C. R. Wescott, T. C. Ransohoff, A. C. Ley, and R. C. Ladner. 2002. Development of mammalian serum albumin affinity purification media by peptide phage display. *Biotechnol. Prog.* 18:182–192.
- Schryvers, A. B., R. Bonnah, R. H. Yu, H. Wong, and M. Retzer. 1998. Bacterial lactoferrin receptors. *Adv. Exp. Med. Biol.* 443:123–133.
- Scott, J. K., and G. P. Smith. 1990. Searching for peptide ligands with an epitope library. *Science* 249:386–390.
- Shin, K., H. Hayasawa, and B. Lönnerdal. 2001. Purification and quantification of lactoperoxidase in human milk with use of immunoadsorbents with antibodies against recombinant human lactoperoxidase. *Am. J. Clin. Nutr.* 73:984–989.
- Sidhu, S. S. 2001. Engineering M13 for phage display. *Biomol. Eng.* 18:57–63.
- Smith, G. P., and J. K. Scott. 1993. Libraries of peptides and proteins displayed on filamentous phage. *Methods Enzymol.* 217:228–257.
- Steijns, J. M., and A. C. Hooijdonk. 2000. Occurrence, structure, biochemical properties and technological characteristics of lactoferrin. *Br. J. Nutr.* 84:S11–S17.
- Sun, X. L., H. M. Baker, S. C. Shewry, and G. B. Jameson. 1999. Structure of recombinant human lactoferrin expressed in *Aspergillus awamori*. *Acta Crystallogr. D Bio. Crystallogr.* 55:403–407.
- Suzuki, Y. A., and B. Lönnerdal. 2002. Characterization of mammalian receptors for lactoferrin. *Biochem. Cell Biol.* 80:75–80.
- Ulrichs, H., H. Depraetere, J. Harsfalvi, and H. Deckmyn. 2001. Selection of phages that inhibit vWF interaction with collagen under both static and flow conditions. *Thromb. Haemost.* 86:630–635.
- Zhang, P., and J. Allan. 1995. Free zinc concentration in bovine milk measurements by analytical affinity chromatography with immobilised metallothionein. *Biol. Trace Elem. Res.* 50:135–148.
- Zhu, Z., Y. Ming, and B. Sun. 2001. Identification of epitopes of trichosanthin by phage display peptide library. *Biochem. Biophys. Res. Commun.* 282:921–927.