Mimicry of Tandem Repeat Peptides against Cell Surface Carbohydrates

Supporting Information

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General. Chemicals and reagents used the experiments described below were commercial items and were used without further purification.

Biopanning Procedure. The procedure is a modification of that described in the instruction manual of the Ph.D.-12™ phage display peptide library kit (New England Biolabs). Solutions of 10 μg/mL of sLeX-BSA (Oxford GlycoSystems) and 10 μg/mL of BSA in 0.1 M $NaHCO_3$ (pH 8.6) were added to a 96-well microplate (Costar). Each well was brought to a volume of 100 μL and incubated overnight at 4°C. After removal of the supernatant, 150 μL of blocking buffer (0.1 M $NaHCO_3$, pH 8.6, 5 mg/mL BSA, 0.02% NaN_3) was added to each well. After incubation for 2 h at 4°C, each well was washed 3 times with 200 μL of TBST (50 mM tris(hydroxymethyl)aminomethane, 150 mM NaCl, 0.1% v/v Tween 20, pH 7.5). Phage (1×10^{11} pfu) solution in 100 μL of TBST was added to the BSA coated well and stirred gently for 1 h at room temperature. Then, the resulting phage solution was transferred to the sLeX-BSA coated well and incubated for 1 h at room temperature. Non-bound phage was removed by washing 10 times with 200 μL of TBST. Bound phage was eluted with 100 μL of elution buffer (0.2 M Glycine-HCl, pH 2.2) for 10 min, followed by neutralizing with 15 μL of neutralizing buffer (1 M tris(hydroxymethyl)aminomethane-HCl, pH 9.1). Ten μL of the phage solution was diluted and was titered by using the known method.[1] The remaining eluted phage was amplified to a 20 mL of grown culture (early-log phase) of E. coli (ER2537) with vigorous shaking for 4.5 h at 37°C. The resulting suspension was centrifuged for 10 min at 10,000 rpm and 4°C. The supernatant was mixed with 1/6 volume of PEG/NaCl solution (20% w/v polyethylene glycol-8000, 2.5 M NaCl) and stored overnight at 4°C. The stored solution was centrifuged for 15 min at 10,000 rpm at

4°C to isolate the amplified phage precipitant as a pellet. The pellet was re-suspended on 1 mL of TBS (50 mM tris(hydroxymethyl)aminonethane, 150 mM NaCl, pH 7.5). Reprecipitation was accomplished by addition of 185 μL of PEG/NaCl solution at 0°C for 1 h. The phage pellet, obtained by centrifugation was re-suspended in 200 μL TBS (containing 0.02% NaN_3) and the amplified phage was titered.

A second and third round of biopanning was performed by using 5 x 10^{10} pfu as of the initial input phage. In the washing steps, the concentration of Tween 20 was raised to 0.5% v/v in order to remove weak binders. The eluted phage obtained from the 3rd round was titered and plaques were used for sequencing.[2]

Peptide Synthesis and Purification. **Preparation of 1 and 2.** Solid-phase peptide synthesis was carried out by using fluorenylmethoxycarbonyl (Fmoc) protected amino acids and a Rink amide MBHA resin (Novabiochem), as described previously.[3] The peptides were synthesized by using Pioneer peptide synthesizing system (PerSeptive Biosystems) with 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), N-hydroxybenzotriazole (HOBT) and diisopropylethylamine (DIEA) as coupling reagents. Cleavage of each the peptide from the resin was achieved by treatment with a mixture of trifluoroacetic acid (TFA)/thioanisole/water (95/2.5/2.5 by volume) for 2 h at room temperature. The filtrate obtained during removal of resin, was treated with a nitrogen stream to remove excess TFA. The residue obtained in this way was triturated with diethyl ether (20 mL). The resulting suspension was centrifuged at 3000 g for 10 min at -20°C giving the solid

crude peptide, which was purified by using a semi-preparative HPLC (Waters 7.8x300mm 125Å C18 Bondapak® column; linear gradient of 5% to 95% aqueous CH_3CN (0.1% TFA). MALDI-TOF MS was used for the identification of the purified peptides. **1-peptide**: MS ($C_{64}H_{93}O_{16}N_{19}$) Calculated 1383.7, Found 1384.7 (M+H). **2-peptide**: MS ($C_{64}H_{97}O_{16}N_{21}$) Calculated 1415.7, Found 1416.8 (M+H).

Preparation of 1-Dimer and 1-Tetramer. Synthesis of the peptides **1-dimer** and **1-tetramer** were carried out by using Fmoc-lysine (Fmoc)-OH as a branch core. An attachment of Fmoc-lysine (Fmoc)-OH to a resin, followed by sequential addition of amino acid residues resulted in **1-dimer**. Attachments of two layers of Fmoc-lysine (Fmoc)-OH, followed by sequential addition of amino acid residues resulted in **1-tetramer**. Purification and characterization of the peptides were accomplished by using the procedures described above. **1-Dimer**: MS ($C_{134}H_{196}O_{33}N_{39}$) Calculated 2879.5, Found 2880.5 (M+H) **1-Tetramer**: MS ($C_{274}H_{401}O_{67}N_{79}$) Calculated 5870.0, Found 5871.3 (M+H).

ELISA Experiment. One row of a 96-well plate (high binding, Costar) was coated with 100 μL of 5 μg/mL of sLeX-BSA in 0.1 M $NaHCO_3$ at pH 8.6 and then incubated at 4°C overnight. After removal of the sLeX-BSA solution, each well was treated with 200 μL of blocking buffer (1 mg/mL of BSA in 0.1 M $NaHCO_3$ at pH 8.6) and allowed to stand for 1 h at 4°C. Additionally, one row of uncoated wells was treated with the same blocking buffer and allowed to stand for 1 h at 4°C as the control set. After removal of blocking buffer, the wells were washed 6 times with 200 μL of TBST with slapping face-down onto a clean paper towel in each washing cycle. Amplified

phage solutions (1 x 10^{10} pfu) in 200 μL of TBST were transferred to the sLeX-BSA or BSA coated wells. After incubation for 1 h at room temperature with gentle stirring, the plate was washed 6 times with 200 μL of TBST. Two hundred μL of diluted horseradish peroxidase-conjugated anti-M13 antibody (Phamacia; diluted 5000 times with the blocking buffer) was added and incubated for 1 h at room temperature. After washing 6 times with 200 μL of TBST, 100 μL of o-phenylenediamine solution (1 mg/mL in stable peroxide buffer, Pierce) was added. The absorbance of the resulting solution was measured at 490 nm. Usually, phages with a a peptide that tightly binds sLeX show 4-5 times higher absorbance (0.4-0.5) than that of the control (< 0.1).

Biacore Analysis. The procedure used was a minor modification of the standard protocol.[4] Assays was carried out at room temperature.

Immobilization of Sugar Conjugated BSA on a F1 Sensor Chip. Sugar-BSA conjugates were purchased from Oxford GlycoSystems. The epitope densities of the carbohydrates were 12.6, 13, and 19 for sLeX, LeX and 3'-sialyl-3-fucosyllactose immobilized BSA, respectively. The carboxymethyl groups in a F1 sensor chip were cleaved by equilibration with HBS-EP buffer (Biacore: 10 mM N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid, pH 7.4, 150 mM NaCl, 3.0 mM ethylendiaminetetraacetic acid, 0.005% Surfactant P20) and then the free carboxyl groups were activated by treatment with 40 μL of the coupling solution (200 mM N-ethyl-N'-(dimethylaminopropyl)carbo-diimide, 50 mM N-hydroxysuccinimide). Forty μL of sugar conjugated BSA solution (0.1 mg/mL in 10 mM NaOAc, pH 4.0) was then injected onto the sensor chip at a flow rate of 5 μL/min. After an appropriate amount of

sugar conjugated BSA was immobilized, 40 μL of 1.0 M ethanolamine was added in order to block the remaining carboxyl groups on the surface of the sensor chip. The resulting flow cell was stabilized by injection with 5 μL of 10 mM NaOH and equilibrated with HBS-EP buffer. The same amount of BSA was immobilized onto another flow cell to serve as a control.

Binding Assay of Peptides. Each peptide was dissolved in buffer (HBS-EP) to make 2.0 mM of stock solution. Injection samples were prepared by serial dilution of the peptide stock solution with the buffer to make appropriate concentrations: **1, 2** and **1-dimer**, 1000, 500, 250, 125, 62.5, 31.3, 15.6, 7.8 μM; **1-tetramer**, 8, 4, 2, 1 μM, 500, 250, 125, 62.5 nM). All the samples were injected by using the serial automated method which is comprised of (1) sample injection (60 μL), (2) dissociation (240 s), and (3) regeneration (30 μL of 20 mM NaCl, 1 mM NaOH) by using a BIAcore 3000. The signals from the reference surface (BSA immobilized) were subtracted from those of the sugar conjugated-BSA immobilized surfaces. Each sample was injected at a flow rate of 30 μL/min using the KINECT command. The equilibrium-binding constant (K_d) of each peptide to immobilized sugar-conjugated BSA was calculated by using the steady state affinity data from 8 different peptide concentrations. K_d values of **1** and **2** were calculated, but they are not in the common ranges of BIAcore values (< 1.0 mM). Binding affinities of **1-dimer** (or **1-tetramer**) to carbohydrates were calculated by using a steady state kinetic method.

Immobilization of 1-Tetramer Peptide on Sensor Chip F1. Equilibration and activation of sensor chip F1 were performed by using the same method as used for the

sugar-conjugated BSA (see above). **1-Tetramer** (0.05 mM, 10 mM NaOAc, pH 4.5) was immobilized to the flow cell by injecting 50 μL of the peptide solution with a flow rate of 5 μL/min.

Binding Assay of Various Sugar-PAA-Biotin. A variety of sugar-polyacrylamides conjugates (20 mol % of sugar, 5 mol % of biotin) were purchased from Syntesome.[5] Solutions of various sugar-PAA (polyacrylamide)-biotin were prepared in buffer at a concentration of 0.3 mg/mL. Samples were injected by using the same automated method as described above.

[^{125}I] Labeling 1-tetramer. Iodo Beads® (Pierce) were washed with 500 μL of reaction buffer (0.1 M sodium phosphate at pH 7.4) and were dried over an air atmosphere. To a mixture of **1-tetramer** (500 μg) and 0.25 mCi (9.2 mBq) of Na[^{125}I] (PerkinElmer) in 200 μL of reaction buffer, two of the pre-washed beads were added and incubated for 15 min at room temperature. From the resulting mixture, all of the solution was removed and was applied to the D-Salt™ polyacrylamide plastic desalting column (Pierce), which was pre-equilibrated with 25 mL of reaction buffer. After removing first 1.75 mL of eluant, 0.5 mL sizes of 10 fractions were collected by applying 6.75 mL of reaction buffer. Most of the labeled peptides were collected at fraction 3 and 4. This purification provided 50 μCi (1.8 mMq) of [^{125}I] **1-tetramer**. The labeled tracer (fraction 3; 87 μg/mL, 15 μM) was diluted with appropriate buffer and used for equilibrium dialysis.

Equilibrium Dialysis and Determination of Dissociation Constant.

Equilibrium dialyzers (Spectrum) containing two 250 μL chambers separated by a 12,000-14,000 Dalton molecular mass cutoff dialysis membrane (regenerated cellulose membrane; Spectrum) were used. One chamber contained sLeX-BSA (80 nM) in dialysis buffer (PBS at pH 7.4) and the other chamber contained [^{125}I] **1-tetramer** (20 different concentrations from 0.63 nM to 120 nM; from 790 cpm to 160,000 cpm) in buffer. A 24 h incubation at room temperature was required for the equilibrium. Two 100 μL samples were drawn from each side of the dialyzer and counted with a gamma-counter (Packard). Control experiments without sLeX-BSA were carried out at the same condition to correct concentrations of [^{125}I] **1-tetramer** in solution. In all experiments, approximately 80% of [^{125}I] **1-tetramer** was found in solution after 24 h incubation, owing to non-specific binding to the dialysis membrane. The reductions of [^{125}I] **1-tetramer** by non-specific bindings to the membrane were corrected in calculations. Concentrations of bound [^{125}I] **1-tetramer** were determined by subtracting concentrations of [^{125}I] **1-tetramer** in sLeX-BSA-free compartment from those of the tracer in sLeX-BSA compartment. The concentration dependency and the saturation of bound [^{125}I] **1-tetramer** to sLeX-BSA were shown in Figure S1. Dissociation constant between [^{125}I] **1-tetramer** and sLeX-BSA was determined by a nonlinear regression (One site binding equation) in Prism (GraphPad).[6]

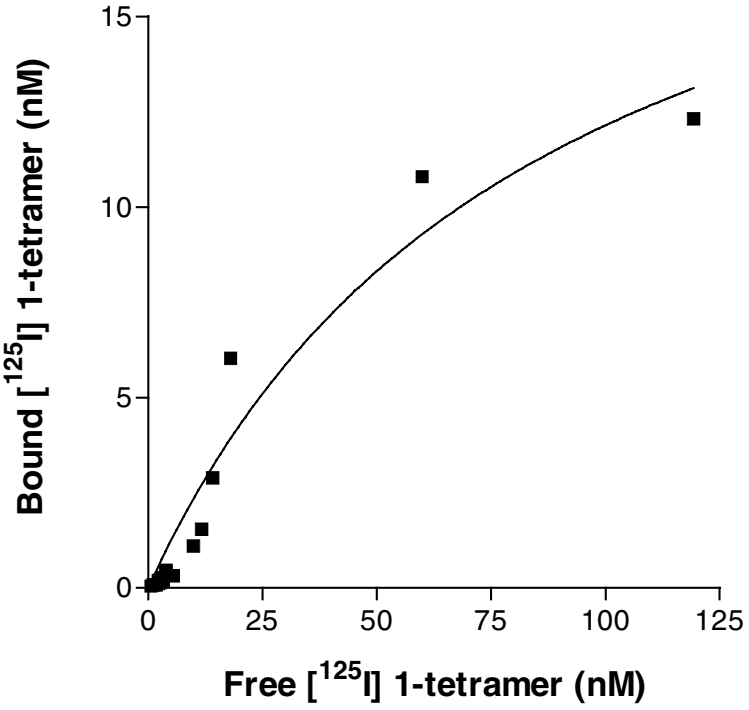
Binding Assay of 1-Tetramer to sLeX-Specific HL60 Cells. HL60 cells were maintained at 37^{o}C in RPMI1640 (Gibco BRL) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Anti-sLeX antibody (KM93) and the goat anti-mouse IgM-rhodamine antibody were purchased from Santacruz. HL60 cells (3 x 10^5 cells per well in 700 μL of RPMI1640 media) were added to a well (12-well plate)

containing a cover slip (1-cm diameter and pre-coated with 1% gelatin) at the bottom. The cells were allowed to adhere on the cover slip over a 48 h period under a 5% CO_2 atmosphere at 37°C. After removal of the remaining suspension, cells were fixed by addition of 1 mL of 4% paraformaldehyde in PBS, followed by incubation for 15 min at room temperature. Wells were then gently rinsed with 1 mL of washing buffer (PBS with 1.0 mM $CaCl_2$, 0.5 mM $MgCl_2$, followed by treatment with 1 mL of blocking buffer (10% BSA in PBS) for 30 min at room temperature. After removal of blocking buffer, wells were gently washed three times with 1 mL of washing buffer again. To measure specific binding of **1-tetramer** to HL60 cells, mixtures of anti-sLeX antibody (KM93; 1.0 nM) and **1-tetramer** (various concentrations) in PBS with 1% BSA were added to each well and incubated for 1 h at room temperature. Wells were washed three times with 1 mL of washing buffer. The goat anti-mouse IgM-rhodamine antibody (4 μg/mL) was added to each well and the resulting wells were incubated for 50 min at room temperature. Wells were washed three times with 1 mL of washing buffer. Each cover slip in the wells was then placed on a slide glass and treated with 5 μL of mounting solution (0.1% p-phenylenediamine, 10% PBS at pH 9.0, 90% glycerol). The fluorescence-labeled cells were counted and averaged from three different microscopic fields.

Competition Assay of E-selectin and sLeX-Specific HL60 Cells to Immobilized 1-Tetramer. HL60 cells (0.9 x 10^7 cells/mL) were incubated with E-selectin (1.0 μg/mL and 10.0 μg/mL) in HBS (10 mM HEPES; N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid), 150 mM NaCl, pH 7.2) for 15 min at room temperature. The resulting cell suspensions (50 μL) were injected onto the **1-**

tetramer-coated-F1 sensor chip. Dose-dependent reductions of (17% and 43%, respectively) binding level (RU) were observed in two E-selectin containing cell suspensions, as compared with a reference (without selectin). Injection of cell suspension with 10.0 μg/mL of sLeX-antibodies showed about 52% reduction of binding level as a positive control.

Figure S1. [^{125}I] **1-Tetramer** binding to sLeX-BSA conjugate by equilibrium dialysis. Equilibrium dialysis experiments was performed as described in supporting information.



References an Notes

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[2] According to the manufacturer's *instruction manual*. Sequencing was carried out by ABI PRISM 377 DNA sequencer.

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