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Glycosyl conjugates of biotinylated diaminopyridine applied for study of carbohydrate-to-carbohydrate interaction

Natalia Utkina · Seon-Joo Yoon · Sen-itiroh Hakomori

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Abstract Previous studies by us and others established that cell-cell adhesion is mediated by specific carbohydrate-to-carbohydrate interaction (CCI). Those previous studies were based on various biochemical and biophysical approaches, including the use of labeled glycosyl epitopes with fluorescent tag. However, these methods ideally require that the glycosyl epitope must be fixed to a solid phase molecule, preferably with multivalency. The purpose of the present study is to establish a CCI process using specific glycosyl residues conjugated to biotinylated diaminopyridine (BAP), and to observe: (i) clear occurrence of homotypic CCI between "Os Fr.B" having 5–6 GlcNAc termini, *vs*. absence of such homotypic CCI between "Os Fr.1" having 2 GlcNAc termini; (ii) occurrence of heterotypic CCI between GM3 ganglioside and Os Fr.B, *vs*. absence of such heterotypic CCI between GM3 and Os Fr.1. Interaction between Os Fr.B-BAP conjugate and Os Fr.B-ceramide mimetic (Os Fr.B-mCer) was demonstrated based on two experiments: (i) dose-dependent binding of Os Fr.B-BAP conjugate to polystyrene plates coated with Os Fr.B-mCer was observed in the presence of bivalent cation, a prerequisite for all CCI processes, and such binding was abolished by EDTA; (ii) binding between equal nanomolar Os Fr.B-BAP and Os Fr.B-mCer was inhibited by mM concentration Os Fr.B without conjugate, in dose-dependent manner. Thus, cell adhesion processes based on homotypic CCI between N-linked glycans having multiple GlcNAc termini, and heterotypic CCI between GM3 and such glycans, were clearly observed using BAP conjugates of glycosyl epitopes.

Keywords Biotinylated diaminopyridine (BAP) conjugate · N-linked oligosaccharide · Carbohydrate-to-carbohydrate interaction (CCI) · GM3 · Os Fr.B

Abbreviations

ABTs	2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
BAP	Biotinylated diaminopyridine
BSA	Bovine serum albumin
Cer	Ceramide derivative
DAP	2,6-diaminopyridine
DMF	*N,N*-dimethylformamide
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
EDTA	Ethylenediaminetetraacetic acid
GM3	NeuAcα3Galβ4Glcβ1Cer
HPLC	High performance liquid chromatography
mCer	Ceramide mimetic, based on 2-N-

Natalia Utkina and Seon-Joo Yoon contributed equally to this study.

N. Utkina · S.-J. Yoon · S. Hakomori (✉)
Division of Biomembrane Research,
Pacific Northwest Research Institute,
720 Broadway, Seattle, WA 98122-4302, USA
e-mail: hakomori@u.washington.edu

N. Utkina · S.-J. Yoon · S. Hakomori
Depts. of Microbiology and Global Health,
University of Washington,
Seattle, WA 98122, USA

N. Utkina
N.D. Zelinsky Institute of Organic Chemistry,
Russian Academy of Science,
Leninsky Prospect 47, Moscow 119991, Russia

Present Address:
S.-J. Yoon
Translational Research Center for Protein Function Control,
Yonsei University,
Seoul 120-749, Republic of Korea

	tetradecylhexadecanoyl structure
Os	Oligosaccharide
TBS	Tris-buffered saline (10 mM Tris-HCl, pH 8.0/ 140 mM NaCl)
T-TBS	TBS containing 0.05% Tween 20
T-TBS (+)	T-TBS containing 0.9 mM $CaCl_2$/ 0.5 mM $MgSO_4$/ 0.1 mM $MnCl_2$

Introduction

Our previous studies indicate that stage-specific embryonic antigen-1 (SSEA-1) mediates embryonic stem cell adhesion during the compaction process, based on Le^x-to-Le^x interaction [1, 2]. Another process based on carbohydrate-to-carbohydrate interaction (CCI) was found to mediate species-specific autoaggregation of sponge cells [3, 4], based on self-interaction of specific glycan with GlcNAcβ1-3Fucα1-Ser/Thr as core structure [5, 6]. Since then, many studies have shown that CCI not only mediates cell-cell interaction, but also provides an important basis for signal transduction, through control of growth factor receptor tyrosine kinases [7]; for review see [8].

In order to further study the functional mechanism of CCI processes, the oligosaccharide (Os) involved must be affixed in clustered form to the surface of a certain matrix. For this purpose, Os have been conjugated to polyacrylamide [9], polystyrene beads [10, 11], phosphatidylethanolamine [12], aminoceramide [13], or gold nanoparticles [6, 14], followed by analysis of Os interaction with defined proteins or carbohydrates. Along this line of study, Os conjugates to biotinylated diaminopyridine (BAP) have the advantages of high fluorescence and being readily affixed on avidin [15]. BAP was synthesized previously by two steps: (i) esterification of biotin carboxyl group with *N*-hydroxysuccinimide (NHS); (ii) coupling of the ester with diaminopyridine. The procedure was subsequently simplified [16], and applied for various studies, *e.g.* [17, 18].

Here we describe application of BAP conjugates of various Os from N-linked glycans, for study of homotypic and heterotypic CCI. Previously, various N-linked Os as above were conjugated to aminoceramide mimetics through reductive amination, to obtain Os-ceramide mimetics (Os-mCer) [13]. While Os-mCer form extensive clusters or liposomes, and were found to be useful for study of CCI process, they are not fluorescent. We therefore used BAP conjugates of specific Os in addition to mCer derivatives, and demonstrated various types of CCI process, or their inhibition.

Materials & methods

Materials

Biotin, 2,6-diaminopyridine (DAP), *N,N*-dimethylformamide (DMF), borane-dimethylamine complex, ovalbumin, cellulose powder (20 μm mesh size), ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA), Sephadex G-25, $CaCl_2$, $MnCl_2$, and $MgSO_4$ were from Sigma Chemical Co. (St. Louis, MO). C_{18}-cartridge was from Varian (Harbor City, CA). Silica gel TLC plates were from Merck (Darmstadt, Germany). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), Reacti-Vial™ Small Reaction Vials, and Reacti-Bind™ NeutrAvidin coated High Binding Capacity Clear 96-well plates with Super-Block Blocking Buffer were from Pierce Biotechnology/ Thermo Fisher (Rockford, IL). Antibody J1 (mouse IgM), originally selected based on reactivity with differentiation marker of mouse testicular cells, was found to be directed to GlcNAc termini of glycolipids and N-linked glycans [19]. Solvents (HPLC grade) were from Thermo Fisher. Other reagents were from Sigma unless described otherwise.

Synthesis of 2-amino-(6-amidobiotinyl)pyridine (BAP)

BAP was synthesized by coupling of diaminopyridine to biotin in the presence of EDC, by a procedure similar to, but simpler than, that of Varki and colleagues [15, 16]. Briefly, biotin (200 mg, 0.82 mmol) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (130 mg, 0.68 mmol) in round flask "A", and 2,6-diaminopyridine (DAP) (150 mg, 1.38 mmol) in flask "B", were dried under vacuum by oil pump for 40 min. 2 ml dimethylformamide (DMF) was added in flask A and 3 ml DMF was added in flask B. After vortexing, the atmosphere in both flasks was replaced with argon gas, and flasks were sealed with Rubber Sleeve Stopper (Fisher). The two flasks were connected by a well-dried steel needle (diameter 1 mm), through which the solution in flask A was transferred to flask B, under vacuum applied to flask B. The mixture was stirred for 4 h in darkness under argon at room temp, and dried completely under vacuum pump for 8 h. Distilled water (7 ml) was added to the reaction mixture, vortexed, and the mixture was kept at 4°C overnight. Precipitate and supernatant were separated by centrifugation at 2,000× g. The precipitate was washed with distilled water (5 ml, twice) and dried, yielding 30 mg of essentially pure BAP (see Results).

Washings by distilled water of precipitate, and supernatant by centrifugation, were combined and applied to C18-cartridge chromatography (0.8×5 cm, Vb=2.5 ml), as described previously [16].

BAP was eluted with 10% acetonitrile and dried (104 mg). The total yield of BAP obtained by precipitation and chromatography was 134 mg (50%; MP 118°C). BAP synthesis and purification were monitored on silica gel TLC plates developed in 85% ethanol, with the following R_f: BAP 0.59; DAP 0.4; biotin 0.64.

Characterization of structure of BAP by mass spectrometry and by NMR spectroscopy

Structure of BAP was determined by mass spectrometry using an LCQ DECA XP (Finnigan MAT) instrument. BAP (1 mg) was suspended in 1 mL of 30% acetonitrile/ 1% acetic acid, and diluted 100-fold in acetonitrile/ water/ acetic acid solution. This solution was directly infused into the ion source at a flow rate of 1 μl/min.

NMR spectra were recorded on a Bruker Avance 600 MHz spectrometer (Germany) in deuterated dimethyl sulfoxide, and chemical shifts (δ) were expressed in parts per million (ppm). Two-dimensional correlated spectra $^{1}H,^{1}H$ COSY and $^{1}H,^{13}C$ HSQC were recorded by standard methods. In the HMBC spectra, a 60-ms delay was used for evaluation of long-range couplings. Spectral data: ^{1}H-NMR (CD_3SOCD_3): δ 9.74 (s, 1H, NH-6′), 7.31 (t, 1H, *J*=8.0 Hz, H-3′), 7.21 (d, 1H, *J*=8.0 Hz, H-2′), 6.40 (s, 1H, NH), 6.33 (s, 1H, NH), 6.15 (d, 1H, *J*=8.0 Hz, H-4′), 5.67 (s, 2H, NH_2), 4.30 (m, 1H, H-4), 4,13 ((m, 1H, H-3), 3,11 (m, 1H, H-2), 2,82 (m, 1H, H-5a), 2,58 (m, 1H, $J_{5a,5b}$=10.8 Hz, H-5b), 2,32 (m, 1H, H-α), 1,64 (m, 1H, H-δa), 1.56 (m, 1H, H-β), 1, 48 (m, 1H, H-δb), 1.34 (m, 1H, H-γ). ^{13}C-NMR (CD_3SOCD_3): δ 171.57 (CO-7), 162.67 (CO-7′), 158.37 (C-1′), 150.41 (C-5′), 138.72 (C-3′), 103.14 (C-4′), 100.86 (C-2′), 60.99 (C-3), 59.16 (C-4), 55.33 (C-2), 39.79 (C-5), 35.87 (C-α), 28.11 (C-γ), 27.97 (C-δ), 25.01 (C-β).

Conjugation of N-linked Os from ovalbumin to BAP, and purification of product

Various N-linked glycans were prepared from ovalbumin, and their structures were identified, as described previously [11]. Three Os (Os Fr.1, Os Fr.B, and a mixture of five N-linked components, termed "Os mixture") were conjugated to BAP as follows. Completely dried 3 mg Os, 2 mg BAP, 200 μl DMF, and 300 μl pyridine/ acetic acid (8:1, pH 6.0) were added to Reacti-Vial, tightly sealed with the cap, and heated at 85°C for 20 h with stirring. After cooling, 50 mg of borane-dimethylamine complex dissolved in 400 μl pyridine/ acetic acid (8:1, pH 6.0) was added to the reaction mixture, and further incubated at 85°C for 30 h. Reaction mixture was completely dried under vacuum, added with 1 ml benzene, and kept at 4°C overnight. After careful removal of supernatant, the precipitate was dissolved in 50% acetonitrile and subjected to HPTLC (1-propanol/ n-butylacetate/ Sol B, 8:1:10, v/v/v) (Sol B=0.2 M acetic acid/ triethylamine, pH 7.2: acetonitrile, 7:3, v/v). Os-BAP conjugates separated by HPTLC were detected under UV lamp (366 nm) (Blak-Ray model UVL-56, UVP, LLC, Upland, CA), followed by staining with 0.5% orcinol in 1 M sulfuric acid to visualize glycolipids. The UV- and orcinol-positive zone (R_f 0.37) in HPTLC was scraped and transferred to a tube. For extraction of Os-BAP conjugates, 2 ml of 50% acetonitrile was added to the tube, vortexed, and centrifuged at 2,000 rpm for 5 min. The extraction procedure was repeated three times. The supernatant containing Os-BAP was dried under N_2 stream, and used for mass spectrometry. Os-BAP conjugate was dissolved in 50% acetonitrile, and the concentration was measured by spectrophotometry (λ=211 nm) based on standard curve using serially diluted BAP (10 μg/ml). Aliquots of suitable quantity were used for assay for binding with antibody J1 and GM3 in a 96-well plate.

Characterization of the structure of N-linked Os conjugates with BAP by ESI mass spectrometry

Structure of Os-BAP conjugates were confirmed by ion trap mass spectrometer EsquireLC (Bruker Daltonics, Billerica, MA) with electrospray ionization source, as described previously [11]. Samples were diluted 10-fold in spectral grade methanol/ 10% water/ 1% formic acid to approximate micromolar concentration. This solution was directly infused into the ion source at a flow rate of 1 μl/min. Spectra were collected in the positive ionization mode.

Binding of Os-BAP conjugates to antibody J1

Various quantities (0.001 to 1.0 nmol per well) of Os Fr. B-BAP, Os Fr.1-BAP, or Os mixture-BAP, in T-TBS (10 mM Tris-HCl, pH 8.0/ 140 mM NaCl/ 0.05% Tween 20) were added to Reacti-Bind™ NeutrAvidin™ coated 96-well plates (Thermo-Fisher Scientific) with Super-Block Blocking Buffer, incubated 3 h at room temp, and washed 3× with 200 μl T-TBS. After incubation with 100 μl antibody J1 (14 μg/ml) overnight, each well was rinsed 3× with 200 μl T-TBS, incubated with goat anti-mouse IgM-HRP (1:1,000 diluted in 0.1% BSA/ T-TBS) for 1 h at room temp, and washed 3× with 200 μl TBS (10 mM Tris-HCl, pH 8.0/ 140 mM NaCl). Binding of Os-BAP conjugate to J1 was measured by adding 50 μl of ABTs substrate solution (0.5% ABTs/ 0.03% H_2O_2/ 50 mM citrate buffer pH 4.0) to each well, followed by reading absorbance (λ1=405, λ2=630).

Binding of Os-BAP conjugates to GM3

GM3 (1 nmol per well) in 50 μl of 50% aqueous ethanol was added to each well of 96-well flat-bottom polystyrene plates

(Costar # 9017; Corning, Acton, MA), dried for 5 h at 37°C, blocked with fresh solution of 1% BSA in TBS for 1 h, and washed 3× with 200 μl T-TBS (+) [T-TBS containing 0.9 mM $CaCl_2$/ 0.5 mM $MgSO_4$/ 0.1 mM $MnCl_2$]. Various quantities (0.25 to 1 nmol per well) of Os Fr.B-BAP or Os Fr.1-BAP in 100 μl T-TBS (+) were added, and plate was covered by lid and incubated for 16 h at room temp with shaking (Red Rocker PR50). Each well was washed 3× with 150 μl T-TBS (+) using the tip of a thin pipette placed at the edge, and washing medium was repeatedly sucked out by peristaltic pump (1 ml/min, LKB Bromma). Os-BAP conjugate bound to GM3 was extracted twice with 200 μl of 10 mM NH_4COOH (pH 4.4) in 50% aqueous acetonitrile, and monitored at excitation wavelength 341 nm and emission wavelength 385 nm (Fluorolog, Horiba Jobin Yvon, Horiba Scientific, Edison, NJ, USA).

Binding of Os Fr.B-BAP to Os Fr.B-mCer

Os Fr.B-mCer was synthesized and dissolved in 50% aqueous ethanol as described previously [13]. A defined quantity (1 nmol per well) was added to each well of 96-well flat-bottom polystyrene plates (Costar # 9017; Corning), dried for 5 h at 37°C, blocked with fresh solution of 1% BSA in TBS for 1 h, and washed 3× with 200 μl T-TBS (+).

Various quantities (0, 0.5, 0.75, 1.0 nmol per well) of Os Fr.B-BAP conjugate in 100 μl T-TBS (+) were added, and the plate was covered by lid and incubated for 16 h at room temp with shaking (Red Rocker PR50). Each well was washed 3× with 150 μl T-TBS (+) very carefully to remove unbound Os Fr.B-BAP. Os Fr.B-BAP conjugate bound to Os Fr.B-mCer coated on plate was extracted and monitored as described for Os-BAP/ GM3 in the preceding section.

Control binding of Os Fr.B-BAP to Os Fr.B-mCer was determined in the presence of 5 mM EDTA, which abolishes CCI between Os Fr.B-BAP and Os Fr.B-mCer. Os Fr.B-BAP was pre-mixed with EDTA before adding to a plate coated with Os Fr.B-mCer.

Inhibitory effect of Os Fr.B on CCI based on binding of Os Fr.B-BAP to Os Fr.B-mCer coated plates

To confirm self-binding between Os Fr.B, the inhibitory effect of Os Fr.B on binding of Os Fr.B-BAP to Os Fr.B-mCer coated plates was assessed based on our previous method [7], with slight modification. A defined quantity (1 nmol/ well) was added to each well of 96-well flat-bottom polystyrene plates, dried for 5 h at 37°C, blocked with fresh solution of 1% BSA in TBS for 1 h, and washed 3× with 200 μl T-TBS (+). Various concentrations of Os Fr. B (0 to 1 mM) in 20 μl/ well, and/ or 20 μl T-TBS (+), were then added to plates coated with Os Fr.B-mCer (1 nmol/ well), followed by preincubation for 1 h at room temp with shaking. Each well was washed with 100 μl T-TBS (+) to remove unbound Os Fr.B, and incubated with Os Fr.B-BAP conjugate (1 nmol/ well) for 16 h at room temp with shaking. Each well was washed 3× with 150 μl T-TBS (+) carefully to remove unbound Os Fr.B-BAP. Os Fr.B-BAP conjugate bound to Os Fr.B-mCer coated on plate was extracted and monitored as described for Os-BAP/ GM3 in the preceding section. Control binding of Os Fr.B-BAP to Os Fr.B-mCer was determined in the absence of Os Fr.B.

Results

Characterization of biotinylated diaminopyridine (BAP)

BAP was synthesized using biotin and DAP, and the structure and purity were determined by LCQ DECA XP mass spectrometry, and by NMR spectroscopy, as described in M&M. BAP showed a major mass signal with m/z 336.20, identified as $[M+H]^+$ of BAP (Fig. 1a).

Since fragment of BAP contains an amide bond similar to that of peptide, if MS/MS fragmentation takes place between the carbonyl carbon and the nitrogen in the linker portion of the BAP as shown in Fig. 1b, the fragment containing biotin should have m/z 227.3, while the fragment containing 2,6-DAP should have m/z 110.1. Both of these fragments were observed in the tandem mass spectrum (Fig. 1c), supporting the proposed structure of BAP, in which the three major peaks with m/z 110.10, 227.12, and 336.14, corresponding to DAP, biotin, and BAP, respectively, were shown by MS/MS of m/z 336.20. These data clearly indicate that the proposed structure is correct, and that purity of BAP synthesized is very high.

The structure and purity of BAP were also confirmed by NMR spectroscopy. The ^1H- (Fig. 2a) and ^{13}C (Fig. 2b) NMR spectra of BAP were assigned using 2D ^1H,^1H COSY, ^1H,^{13}C HSQC, and HMBC experiments (spectra not shown). The ^1H and ^{13}C NMR chemical shifts corresponded to structures of the biotin and 2,6-DAP moieties (Fig. 2c), and further confirmed the purity of BAP.

Characterization of BAP-oligosaccharide conjugates

Three types of oligosaccharide (Os) purified from ovalbumin, Os Fr.1 (Fig. 3a), Os Fr.B (Fig. 3b), and Os mixture (Fig. 3c-1 to -5), were conjugated to amino group of BAP by reductive amination through Schiff base (Fig. 4), as described in M&M. Os-BAP conjugates were separated by HPTLC, and detected under UV followed by orcinol/ sulfuric acid reaction (Fig. 5a), in which Os-BAP conjugates showed different mobility in HPTLC from BAP-non-conjugated Os. Structures of Os-BAP conjugates were confirmed by ESI-MS spectroscopy (Fig. 5b).

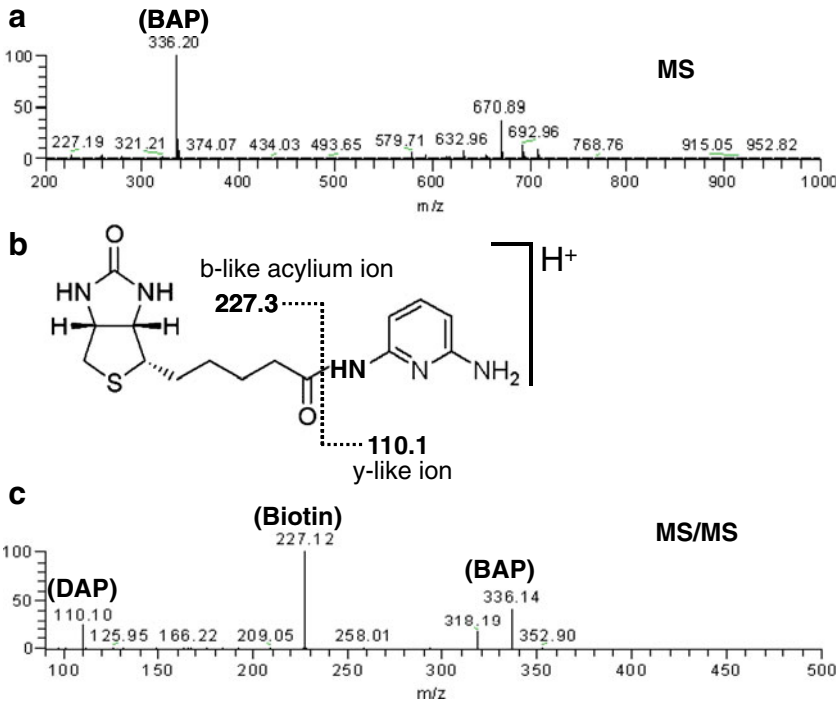

Fig. 1 LCQ DECA XP spectra of biotinylated diaminopyridine (BAP), predictive scheme of fragmentation in MS/MS, and MS/MS data for BAP. **a** MS of BAP. Note the presence of one major ion, with mass number 336.20, and corresponding $[M+H]^+$ major ions with ($C_{15}H_{21}N_5O_2S$, Mw 335.42). **b** Fragmentation scheme of BAP. Fragmentation occurring between carbonyl carbon and nitrogen in the linker portion of BAP produces b-like acylium ion and y-like ion. **c** MS/MS spectrum m/z 336.20 corresponding to BAP ($C_{15}H_{21}N_5O_2S$), of which product ions of m/z based on the scheme in (**b**) are m/z 110.10 and m/z 227.12, corresponding to DAP ($C_5H_7N_3$) and biotin ($C_{10}H_{16}N_2O_3S$), respectively

Interaction of Os-BAP with antibody J1

Various quantities (0.001–1.0 nmol/well, shown on abscissa of Fig. 6) of Os Fr.1-BAP, Os mixture-BAP, or Os Fr.B-BAP were placed on each well of Reacti-Bind™ NeutrAvidin™ coated 96-well plates (Thermo-Fisher Scientific). Each well was incubated with antibody J1, and binding of Os-BAP conjugate to J1 was measured using ABTs substrate as described in M&M. Os Fr.B-BAP showed significantly higher binding to J1 than did Os Fr.1-BAP or Os mixture-BAP (Fig. 6). The results indicate that Os Fr.B-BAP, having 5–6 GlcNAc termini, affixed on avidin, is the site to which J1 binds, and also causes homotypic CCI between Os Fr.B. Os Fr.1-BAP, which has only 2 GlcNAc termini, affixed similarly on avidin, showed no detectable binding to J1.

Interaction of Os-BAP with GM3

Various quantities (0.25–1.0 nmol/well; shown on abscissa of Fig. 7) of Os Fr.B-BAP or Os Fr.1-BAP were incubated with 1 nmol GM3 coated on multi-well plates, and binding of Os-BAP conjugate to GM3 was monitored as described in M&M. Binding of Os Fr.B-BAP conjugate to GM3 was much higher than that of Os Fr.1-BAP, while BAP by itself showed no binding to GM3 (Fig. 7). GM3 was shown to interact with N-linked glycans having 5–6 GlcNAc termini, but much less with those having 2 GlcNAc termini (see Discussion).

Interaction of Os Fr.B-BAP with Os Fr.B-mCer in the presence *vs*. absence of EDTA

Various quantities (0, 0.5, 0.75, 1.0 nmol/well; shown on abscissa of Fig. 8) of Os Fr.B-BAP conjugate with or without 5 mM EDTA were incubated with 1 nmol Os Fr.B-mCer coated on multi-well plates, and binding of Os-BAP conjugate to Os Fr.B-mCer was monitored as described in M&M. Os Fr.B-BAP (0.75 or 1.0 nmol/ well) showed strong binding to Os Fr.B-mCer, and this binding was completely inhibited in the presence of 5 mM EDTA (Fig. 8).

Interaction of Os Fr.B-BAP with Os Fr.B-mCer in the presence of Os Fr.B

Various concentrations (0 to 1.0 mM; shown on abscissa of Fig. 9) of Os Fr.B (free Os; not bound to BAP or mCer) were pre-incubated with 1 nmol Os Fr.B-mCer coated on multi-well plates. 1 nmol Os Fr.B-BAP conjugate was added to each well, incubated, and the binding was monitored as described in M&M. Os Fr.B-BAP binding to Os Fr.B-mCer was inhibited by Os Fr.B in concentration-dependent manner (Fig. 9).

Discussion

Protein-to-protein interaction (PPI) has long been considered as a basic process in all cell biological phenomena,

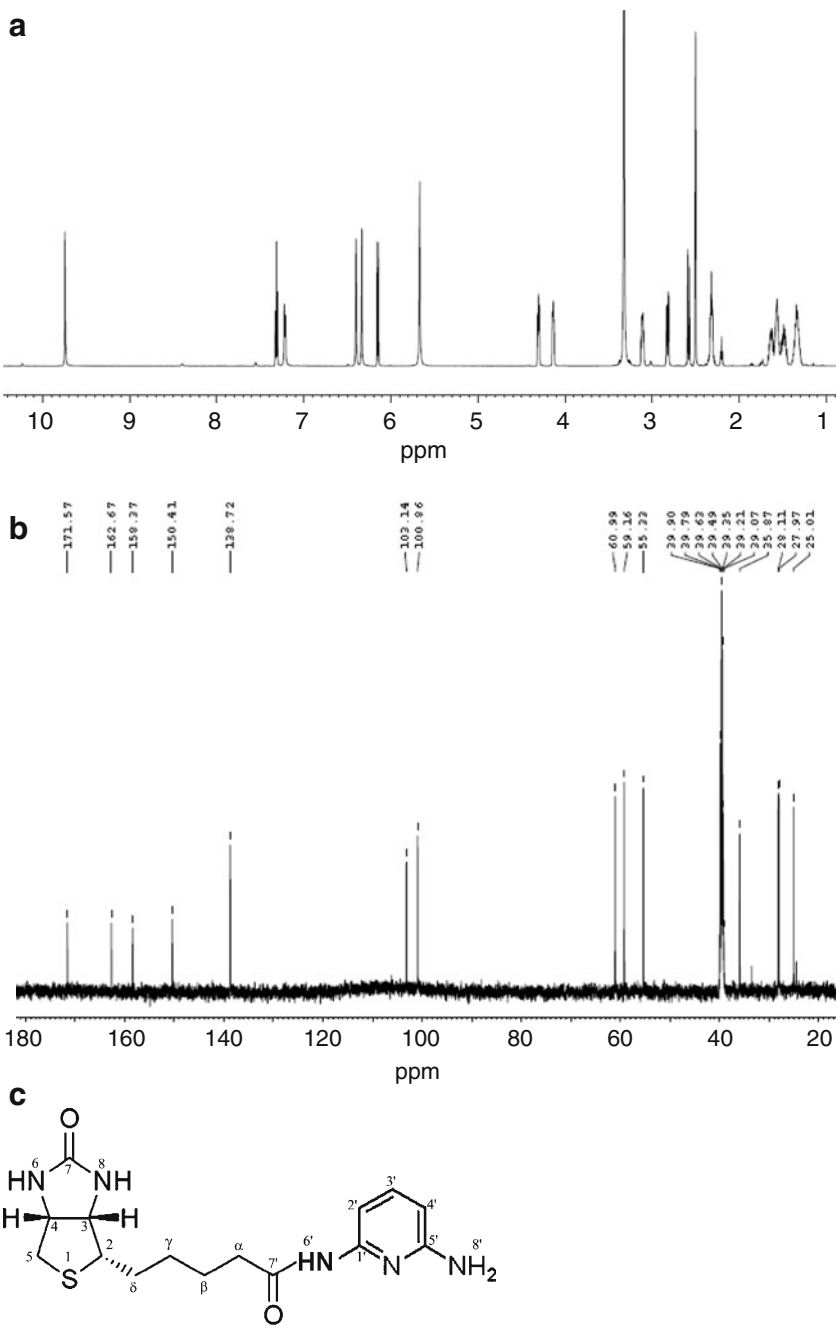

Fig. 2 NMR spectroscopy of BAP. **a** ^{1}H-NMR spectra. **b** ^{13}C-NMR spectra. **c** Atoms of biotin and DAP, composing BAP structure

including cell growth, adhesion, motility, apoptosis, differentiation, disease development, *etc*. More recently, specific carbohydrate-binding proteins, such as galectins, selectins, and siglecs, have been shown to play important roles in defining specific types of cell adhesion and cell recognition (for review see [20].

A series of our studies demonstrated the role of stage-specific embryonic antigen-1 (SSEA-1) as mediator of embryonic stem cell and carcinoma cell adhesion, based on Le^{x}-to-Le^{x} interaction [1, 2]. Another series of studies demonstrated species-specific autoaggregation of sponge cells [3, 4], for which the mechanism was found later as self-interaction of specific glycan with GlcNAcβ1-3Fucα1-Ser/Thr as core structure [5, 6]. Since then, carbohydrate-to-carbohydrate interaction (CCI) processes have been found, not only involved in cell-cell interaction, but also providing an important basis for signal transduction, through control of growth factor receptor tyrosine kinases [7]; for review see [8].

Various fluorescent labels, such as naphthalene derivatives [21], 4-aminobenzoic acid [22], 2-aminobenzamide [23], and 2-aminopyridine [24], have been used for

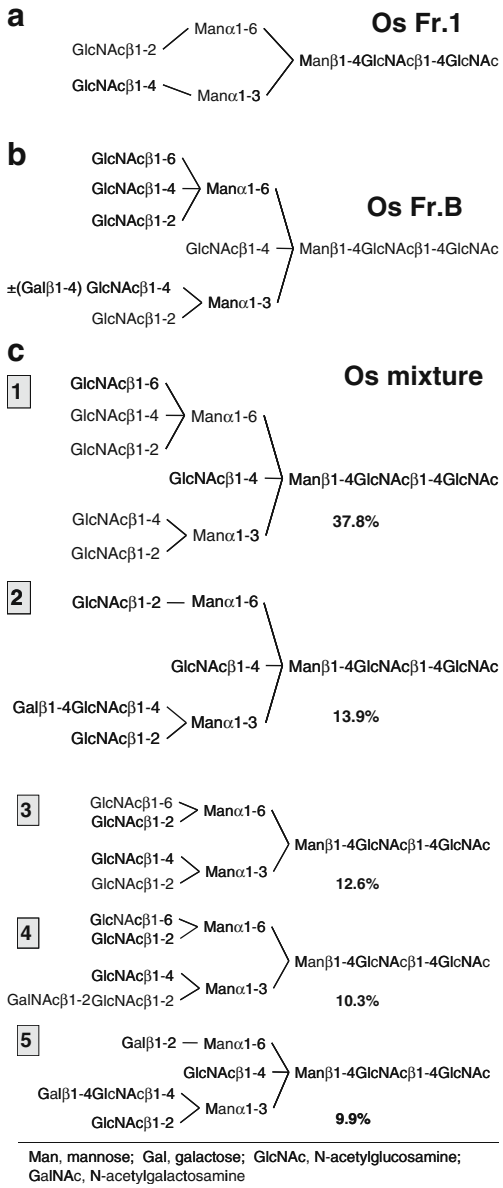

Fig. 3 Structure of Os Fr.1, Os Fr.B, and Os mixture. Three oligosaccharides were purified from ovalbumin by hydrazinolysis, acetylation, cellulose-chromatography, ConA-affinity chromatography, and HPLC. Structural assignment of Os was based on HPLC ("GALAXY"), and some were confirmed by MALDI-TOF analysis, as described in M&M. **a** Os Fr.1. **b** Os Fr.B. **c** Os mixture

determination of carbohydrate-to-protein interaction (CPI) or CCI. Separation pattern and retention time of fluorescence-labeled Os with 2-aminopyridine by reductive amination have been widely used for separation and characterization of Os. The method has been applied for identification of ~520 different Os of N-linked structures [25–28]. Since this labeling procedure could not be applied for fixation of Os on solid phase, one of the amino groups of 2,6-diaminopyridine was biotinylated and the other amino group was conjugated to Os by reductive amination, thus providing biotinyl-diaminopyridine (BAP) conjugate to Os, which was useful for fixation and separation of various Os [15]. Subsequently, the procedure for making this compound was simplified, and preparation of BAP was made easier [16]. In the present study, we further improved the procedure for preparation of BAP by omitting NHS catalyst and by using anhydrous conditions, allowing a decrease in amount of reagents, 1.6 eq DAP, and 0.85 eq EDC per 1 eq biotin, in which 25% of product (30 mg) was obtained by precipitation, 75% (104 mg) was isolated using C18 cartridge chromatography, and BAP was eluted with 10% acetonitrile. In contrast, the Varki *et al*. procedure used NHS catalyst, with amounts of 3 eq DAP, and 1.5 eq EDC plus 0.5 eq NHS per 1 eq biotin, and BAP was eluted with 50% acetonitrile.

We found previously that Os can also be well affixed to solid phase through Os conjugation to 1-aminoceramide by reductive amination (Os-mCer). Through this method, we synthesized Os Fr.B-mCer, liposomes of which displayed strong binding to Os Fr.B-mCer-coated plate; *i.e.*, self-interaction between Os Fr.B, one affixed through mCer on plate, the other incorporated in liposomes. In general, glycosyl residues linked to Cer or mCer form clusters in the presence of appropriate quantity of cholesterol and phospholipid, whereby clustered glycosyl residues are exposed to lipid layer, and display strong binding to proteins, through CPI, or to other glycosyl residues, through CCI.

We observed previously that Os Fr.B-mCer coated on plate interacted only with GM3-liposomes, but not with other glycolipid-liposomes such as those containing Forssman antigen, GalCer, or LacCer [13]. This is consistent with our observation that GM3 binds to N-linked glycan Os with 5–6 GlcNAc termini, but not to those with 2 GlcNAc termini [11].

Possible CCI was studied using BAP conjugates with purified Os Fr.B, purified Os Fr.1, and a mixture of 5 N-linked glycans, termed "Os mixture", in comparison with clear CCI observed previously for Os-mCer with the same N-linked glycan. Homotypic CCI was observed based on dose-dependent binding of Os Fr.B-BAP to plates coated with constant 1 nmole GM3. The binding of Os Fr.B-BAP to 1 nmole Os Fr.B-mCer was increased depending on the amount of Os Fr.B-BAP added to wells coated with GM3 in plate. The binding was abolished in the presence of 5 mM EDTA, in agreement with previous findings that essentially all CCI requires bivalent cation, particularly Ca^{2+} or Mg^{2+}, which are eliminated by EDTA [8, 29, 30]. The amount of Os-BAP showing highest binding to 1 nmol GM3 coated on plate was 0.75 rather than 1 nmol, whereas 1 nmol of added Os Fr.B-BAP showed maximal binding to 1 nmol Os Fr.B-mCer coated on plate. These findings suggest that not all of the 1 mol GM3 coated on plate was able to participate

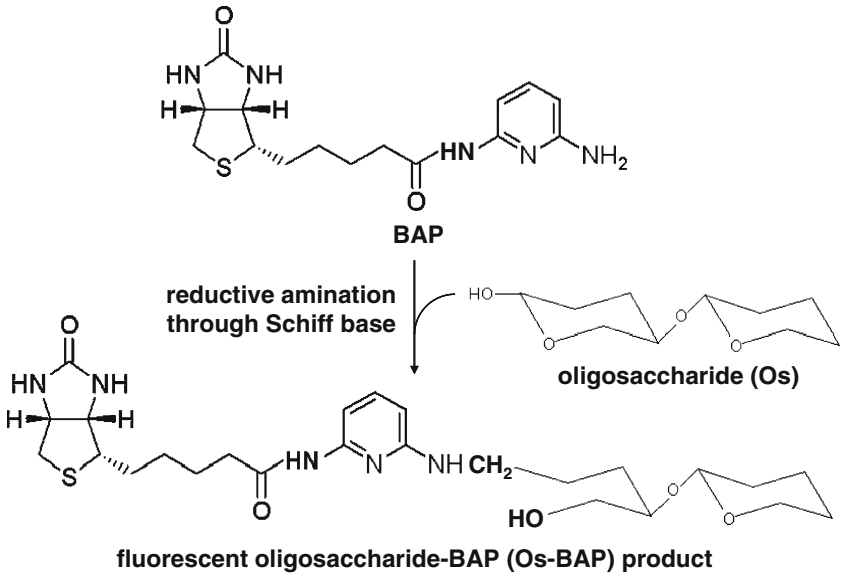

Fig. 4 Coupling reaction of BAP to oligosaccharide. Os Fr.1, Os Fr.B, and Os mixture were conjugated with BAP in the presence of ethyl carbodiimide hydrochloride (EDC) and dimethylformamide (DMF), as described in M&M. Amino terminus of pyridine group in BAP reacts with aldehyde group in reducing terminus of *N*-GlcNAc of Os, producing Os Fr.1-BAP, Os Fr.B-BAP, and Os mixture-BAP conjugates

in binding to BAP derivative of glycan, perhaps due to bulk size of the glycan, but all of the 1 nmol of Os Fr.B derivatives of mCer and BAP did interact.

Heterotypic CCI between GM3 and Os Fr.B-BAP, but not between GM3 and Os Fr.1-BAP, was demonstrated. Throughout these studies, Os-BAP was found to be useful to demonstrate CCI, either homotypic or heterotypic, since Os-BAP can be affixed on avidin, and can be quantitatively determined based on degree of fluorescence, with four units of biotin bound to a single avidin [31]. The procedure used in this report is basically similar to an ELISA assay, and the binding target molecule is determined by fluorescence. However, the procedure is not designed to determine quantity of binding force, which can be performed by a few biophysical methods. *E.g.*: (i) atomic force microscopy, by which Le^x-to-Le^x binding strength was found to be 24.5 picoNewtons (pN), whereas binding strength of Le^x-to-lactose was 0 pN [32]. (ii) surface plasmon resonance (SPR) spectroscopy, for interaction of Gg3 (linked to polymeric styrene) with GM3 as Langmuir monolayer (water/ air interface), was performed. GM3-Gg3 interaction $Ka=1.1\times10^8\ M^{-1}$; GM3-lactose interaction $Ka=7.7\times10^4\ M^{-1}$ [33]. (iii) CCI between membrane vesicular glycan was determined by change of membrane contact angle θ_c.

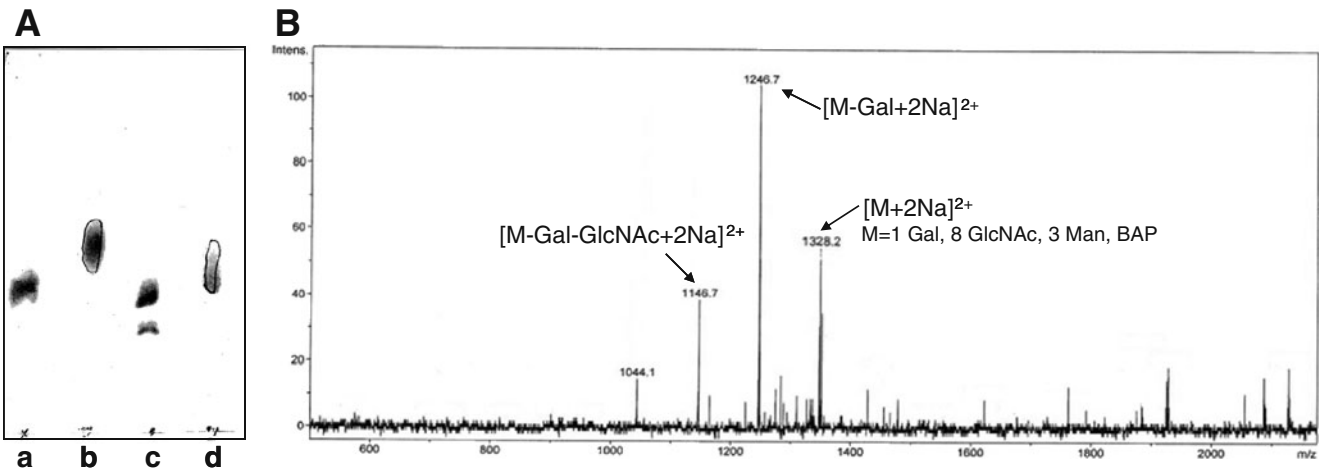

Fig. 5 **A** TLC patterns of Os Fr.1-BAP and Os Fr.B-BAP derivatives (pattern of Os mixture-BAP not shown) and ESI-MS spectra of Os Fr. B-BAP. **a** Os was detected by orcinol/ sulfuric acid reaction, and BAP was detected by UV illumination, marked with pencil line. TLC solvent: 1-propanol/ n-butylacetate/ Sol B, 8:1:10, v/v/v. Sol B: 0.2 M acetic acid/ triethylamine, pH 7.2/ acetonitrile, 7:3, v/v. *a*, Os Fr.1. *b*, Os Fr.1-BAP. *c*, Os Fr.B. *d*, Os Fr.B-BAP. **B** Positive-ion ESI-MS spectra of BAP conjugate of Os Fr.B. Note the presence of two major ions, with mass number 1328.2 and 1246.7, corresponding respectively to doubly-charged $(M+2Na)^{2+}$ major ions with (1 Gal, 8 GlcNAc, 3 Man, BAP) and (8 GlcNAc, 3 Man, BAP). A minor ion, with mass number 1146.7, is doubly-charged product ion by loss of 1 GlcNAc from mass number 1246.7

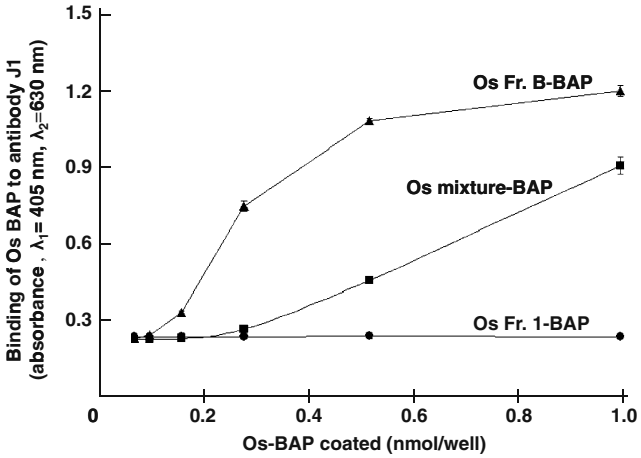

Fig. 6 Binding of antibody J1 to Os-BAP derivatives affixed on multi-well avidin-coated plate. Various quantities (0.001–1.0 nmol/ well) of Os-BAP derivatives as shown on abscissa were affixed to avidin-coated plate, and Os-BAP binding was quantified using antibody J1 as described in M&M. Data shown are typical results from a triplicate experiment. Similar results were obtained in two other triplicate experiments. *Bars* indicate standard deviation

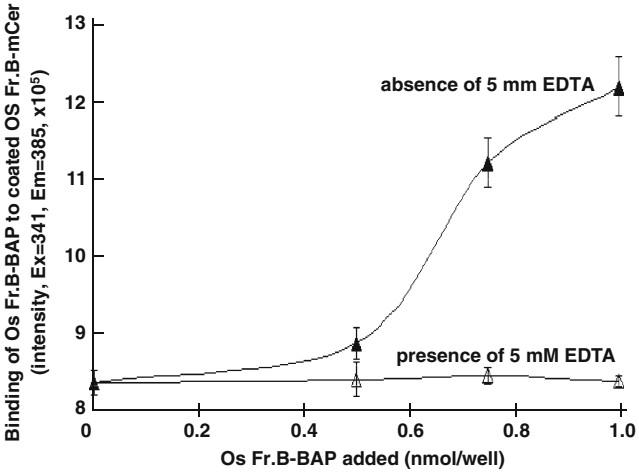

Fig. 8 Homotypic binding of Os Fr.B-BAP to Os Fr.B-mCer in the absence of EDTA, and absence of such binding in the presence of EDTA. Os Fr.B-mCer (1 nmol/ well) was coated and dried on multi-well polystyrene plates. Each well was added with 100 μl Os Fr.B-BAP pre-incubated with or without 5 mM EDTA as shown on abscissa, incubated for 16 h, and degree of binding was determined as described in M&M. Clear binding of Os Fr.B-BAP to Os Fr.B-mCer was observed, and was completely inhibited in the presence of 5 mM EDTA. Data shown are typical results from one out of three triplicate experiments. *Bars* indicate standard deviation

Value for two vesicles both containing Le^{x}Cer was much higher than for two vesicles both containing Le^{a}Cer [34]. In procedures (i) and (ii), Os Fr.B or Os Fr.1 can be appropriately assembled. In procedure (iii), Os-mCer derivative can be assembled.

There is increasing evidence for occurrence of CCI process as a basis for a large variety of cellular phenotypes. It is important to develop improved methodology for clarifying this phenomenon. This report is focused on the use of BAP conjugates of N-linked glycans of membrane glycoprotein receptors such as growth factor receptors and integrin receptors, whose interaction with gangliosides through CCI may define important cellular phenotype such as ontogenic development, oncogenic transformation, and cell death.

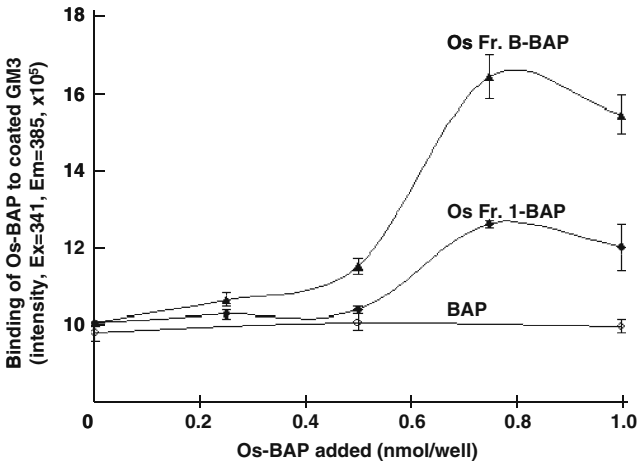

Fig. 7 Binding of Os-BAP conjugates to GM3. Various quantities (0 to 1.0 nmol in 100 μl per well) of Os Fr.B-BAP or Os Fr.1-BAP were added to each well of multi-well polystyrene plates (Costar # 9017; Corning). Each well was dried, and then GM3 (1 nmol/ well) was added, and degree of binding was determined as described in M&M. Clear GM3 binding was observed for Os Fr.B-BAP; this binding was significantly stronger than that to Os Fr.1-BAP. Similar results were obtained in three triplicate experiments; typical data from one of the three experiments are shown. *Bars* indicate standard deviation

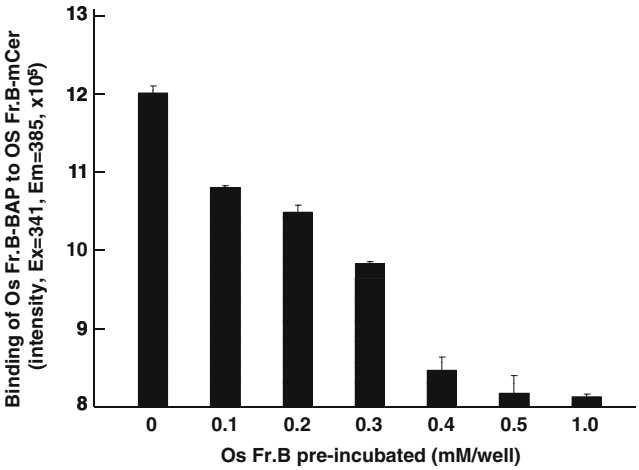

Fig. 9 Binding of Os Fr.B-BAP to Os Fr.B-mCer in the presence of various concentrations of Os Fr.B. Os Fr.B-mCer (1 nmol/ well) was coated and dried on multi-well polystyrene plates. Each well was added with 0 to 1.0 mM concentration of Os Fr.B (not Os Fr.B-BAP or Os Fr.B-mCer), as shown on abscissa, and pre-incubated for 1 h at room temp. After washing, Os Fr.B-BAP (1 nmol) was added to each well. Degree of binding of Os Fr.B-BAP to Os Fr.B-mCer was determined as described in M&M

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