Supporting Information for

Control of guanine-rich DNA secondary structures depending on the protease activity using a designed PNA peptide

Kenji Usui,§*[ab] Arisa Okada,§[a] Keita Kobayashi [a] and Naoki Sugimoto* [ab]

[a] FIRST (Faculty of Frontiers of Innovative Research in Science and Technology) and [b]FIBER (Frontier Institute for Biomolecular Engineering Research), Konan University, 7-1-20 Minatojima-minamimachi, Chuo-ku, Kobe 650-0047, Japan,

[*] Faculty of Frontiers of Innovative Research in Science and Technology (FIRST), and Frontier Institute for Biomolecular Engineering Research (FIBER), Konan University, 7-1-20 Minatojima-Minamimachi, Chuo-ku, Kobe 650-0047 (Japan)
E-mail: kusui@center.konan-u.ac.jp (K. Usui), sugimoto@konan-u.ac.jp (N. Sugimoto)

[§] These authors contributed equally to this work.

Experimental methods

General remarks

All chemicals and solvents were of reagent or HPLC grade and were used without further purification. Oligodeoxynucleotide samples purified by HPLC were purchased from Hokkaido System Science (Sapporo, Japan) for MYC and from FASMAC Co., Ltd. (Atsugi, Japan) for FD-MYC. HPLC was performed on a GL-7400 HPLC system (GL Sciences, Tokyo, Japan) using an Inertsil ODS-3 column (10 x 250 mm; GL Science) for preparative purification with a linear acetonitrile/0.1% trifluoroacetic acid (TFA) gradient at a flow rate of 3.0 ml/min. Peptides were analyzed using MALDI-TOF MS on an Autoflex III (Bruker Daltonics, Billerica, MA, USA) mass spectrometer with 3,5-dimethoxy-4-hydroxycinnamic acid as the matrix. Amino acid analysis was carried out using an Inertsil ODS-2 column (4.6 x 200 mm; GL Science) after hydrolysis in 6 M HCl at 110 °C for 24 h in a sealed tube, followed by phenyl isothiocyanate labeling.

Synthesis of designed PNA peptides

The designed peptides, except for dcalmyc 1 and 3, were synthesized manually on Fmoc-NH-SAL-PEG Resin (Watanabe Chemical Industries, Hiroshima, Japan) with Fmoc chemistry[1] using Fmoc-AA-OH (4 eq., Watanabe Chemical Industries) and Fmoc PNA monomers (4 eq., Panagene, Daejeon, Korea) according to the *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU, Watanabe Chemical) method. Dcalmyc 1 and 3 were synthesized with Fmoc chemistry on Wang resin (Watanabe Chemical Industries); the first coupled amino acids (Fmoc-Tyr(tBu)-OH for dcalmyc 1 and Fmoc-Leu-OH) for dcalmyc 1) were attached by the 1,3-diisopropylcarbodiimide (DIC) and N,N-dimethyl-aminopyridine (DMAP) method. Side chain protection was as follows: *t*-butyl (tBu) for Tyr, *t*-butyloxycarbonyl (Boc) for Lys, and benzhydryloxycarbonyl (Bhoc) for guanine PNA monomers. The peptides were cleaved from the resins and the side chain protection was removed by incubating the peptides for 2 h in TFA (Watanabe Chemical Industries)/H_2O/triisopropylsilane (Wako Pure Chemical Industries, Tokyo, Japan) (20:1:1, v/v). The peptides were precipitated by addition of cold diethyl ether and then collected by centrifugation. The peptides were purified by RP-HPLC and characterized by amino acid analysis and MALDI-TOF MS: calmyc, m/z 2380.0 ($[M+H]^+$ calcd. 2381.4); dcalmyc 1, m/z 1382.5 ($[M+H]^+$ calcd. 1381.5); dcalmyc 2, m/z 1019.0 ($[M+H]^+$ calcd. 1019.0); dcalmyc 3, m/z 1118.1 ($[M+H]^+$ calcd. 1119.1); dcalmyc 4, m/z 1281.1 ($[M+H]^+$ calcd. 1281.3). Purified peptides were dissolved in MilliQ water to about 300 μM and their concentrations were measured by amino acid analysis, then they were stored at 4°C.

Gel electrophoresis

Prior to analysis, each sample in 10 mM Tris-HCl (pH 7.5) buffer containing 0.1 mM EDTA was heated at 85 °C for 5 min, then gently cooled to room temperature at a rate of 1.0°C min^{-1}. Native gel electrophoresis was performed using nondenaturing gels containing 13% polyacrylamide.

Loading buffer (2 μL) was mixed with 2 μL of 5 μM MYC with 0-10 μM calmyc in 10 mM Tris-HCl (pH 7.5) buffer containing 0.1 mM EDTA. A 4 μL aliquot of each sample was loaded onto the gel and electrophoresed at 10.0 V cm^{-1} for 2 h at room temperature. Gels were stained with SYBR Gold Nucleic Acid Gel Stain (Invitrogen, Carlsbad, CA, USA) and imaged using a FLA-7000 imager (Fuji Film, Tokyo, Japan). Band intensities were quantified using Multi Gauge software (V.3.2) for Windows. The bands were cut from the gel, put into 1.5 mL tubes, and 50-100 μL of buffer containing 10 mM Tris (pH 7.5), 1 mM EDTA and 250 mM NaCl was added. After 24-48 h incubation with constant rotary shaking at 4°C, the gel was removed using an Ultrafree-MC Centrifugal Filter (Merck & Co., Inc, Whitehouse Station, NJ, USA) and the buffer solvents were removed by using a ZipTip (Merck & Co., Inc). The samples were analyzed using MALDI-TOF MS.

Fluorescence measurements

The fluorescence measurements were performed in 10 mM Tris-HCl containing 0.1 mM EDTA (pH 7.5). Fluorescence spectra were recorded on a FP-6200 fluorescence spectrophotometer (JASCO, Hachioji, Japan) with a thermoregulator, at 25˚C using a quartz cell with a 10 mm pathlength. The excitation wavelength was 495 nm. Fluorescence intensities at 515 nm were detected and the relative fluorescence intensity changes were calculated by dividing the fluorescence intensity changes from the fluorescence intensity of DNA alone by the saturated fluorescence intensity change value (assuming that 0 = no change from DNA alone, and that 1.0 = maximum change with an excess of calmyc).

Circular dichroism (CD) spectroscopy

Circular dichroism (CD) spectroscopy was performed at 25°C using DNA (1 μM) and the peptide (0 or 2 μM) in 20 mM Tris-HCl (pH 7.5) containing 0.1 mM EDTA with/without 0.1 mM KCl. A J-820 spectropolarimeter (JASCO) with a thermoregulator, and a quartz cell with a 1 cm path length, was used. Prior to analysis, each sample was heated at 85°C for 5 min, then gently cooled to room temperature at a rate of 1.0°C min^{-1}.

Thermodynamic Analysis

UV absorbance was measured using a Shimadzu 1800 spectrophotometer equipped with a temperature controller (Shimadzu, Kyoto, Japan). Melting curves for the G-quadruplex structures were obtained by measuring the UV absorbance at 295 nm in 10 mM Tris-HCl (pH 7.5) containing various concentrations of KCl and 0.1 mM EDTA at a heating rate of 1.0 °C min^{-1}. To calculate the thermodynamic parameters, including the melting temperature (T_m), the enthalpy change (ΔH°), the entropy change (ΔS°), and the free energy change at 37°C ($\Delta G°_{37}$) for intramolecular G-quadruplex formation and PNA-DNA hybrid quadruplex formation, the melting curves for intramolecular G-quadruplex formation and PNA-DNA hybrid quadruplex formation were fit[2] to the theoretical

equation for an intramolecular association and a 2:1 association, respectively.

Calpain I digestion experiments

For the HPLC experiments, calmyc (100 μM) and calpain I (50 pmol, 10 unit) were prepared in a buffer containing 10 mM Tris-HCl (pH 7.5) and 10 mM $CaCl_2$ (total volume 13 μL). After 24 h reaction at 37°C, RP-HPLC was performed using an Inertsil ODS-3 column (4.6 x 150 mm; GL Science) and a linear acetonitrile/0.1% trifluoroacetic acid (TFA) gradient at a flow rate of 1.0 mL/min. The collected fractions were characterized by MALDI-TOF MS.

For fluorescence measurements, calmyc (0.2 μM), MYC (0.4 μM) and calpain I (100 pmol, 20 unit) were prepared in a buffer containing 10 mM Tris-HCl (pH 7.5) and 1 mM $CaCl_2$ (total volume 1 mL) at 37°C (tested solution). MYC and calpain I solution (Ref. 1 solution) and MYC and calmyc solution (Ref. 2 solution) were also prepared, then the fluorescence measurements were performed. The relative fluorescence intensity changes were calculated by dividing the fluorescence intensity changes of the tested solution from the fluorescence intensity of the Ref. 2 solution by the fluorescence intensity changes of Ref. 1 solution from the fluorescence intensity of the Ref. 2 solution [assuming that 0 = no change in fluorescence intensity of the MYC and calmyc solution (solution of MYC and undigested calmyc), and that 1.0 = fluorescence intensity of MYC and calpain I (solution of MYC and calmyc completely digested by calpain I).

For the UV measurements, calmyc (10 μM), MYC (5 μM) and calpain I (0.5 pmol, 0.1 unit) were prepared in a buffer containing 10 mM Tris-HCl (pH 7.5) and 10 mM $CaCl_2$ (total volume 13 μL). A MYC and calpain I solution and a MYC and calmyc solution were also prepared. After 24 h reaction at 37°C, all the solutions were diluted 10 times with Tris-HCl buffer and Li_2EDTA was added (final concentrations: 10 mM Tris-HCl, 1 mM $CaCl_2$, 50 mM Li_2EDTA). The melting curves for the G-quadruplex structures were then obtained by measuring the UV absorbance at 295 nm at a heating rate of 1.0 °C min^{-1}. The melting temperature (T_m) values were obtained from UV melting curves, as described previously.

References

1 W. C. Chan and P. D. White, *Fmoc solid phase peptide synthesis: A practical approach* **2000**, Oxford University Press, New York.
2 L. A. Marky and K. J. Breslauer, *Biopolymers* **1987**, *26*, 1601.

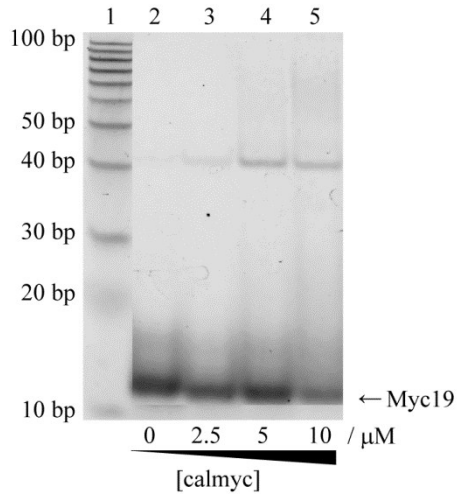

Figure S1. Native PAGE of 5 μM MYC with/without calmyc (lane 2: 0 μM, lane 3: 2.5 μM, lane 4: 5 μM, lane 5: 10 μM) in a buffer containing 10 mM Tris-HCl (pH 7.5) at 25 °C (lane 1: size marker).

Table S1. Thermodynamic parameters derived from fitting melting curves of 5 μM MYC and 5 μM MYC + 10 μM calmyc at 0.1 mM KCl.

	MYC	MYC+calmyc
ΔH° (kcal/mol)	-40.2±3.4	-79.9±15.7
ΔS° (cal/mol/K)	-128.6±10.9	-195.3±45.2
$T\Delta S^\circ$(kcal/mol)	-39.9±3.3	-60.5±14.0
ΔG°_{37}(kcal/mol)	-0.3±0.03	-19.4±1.8

Table S2. T_m measurements of MYC with the synthetic peptides.

	T_m (°C)
MYC	40.3±0.6
MYC + calmyc	54.3±3.6
MYC + dcalmyc1	38.6±0.6
MYC + dcalmyc2	41.3±1.2
MYC + dcalmyc3	42.3±0.6
MYC + dcalmyc4	43.3±0.6

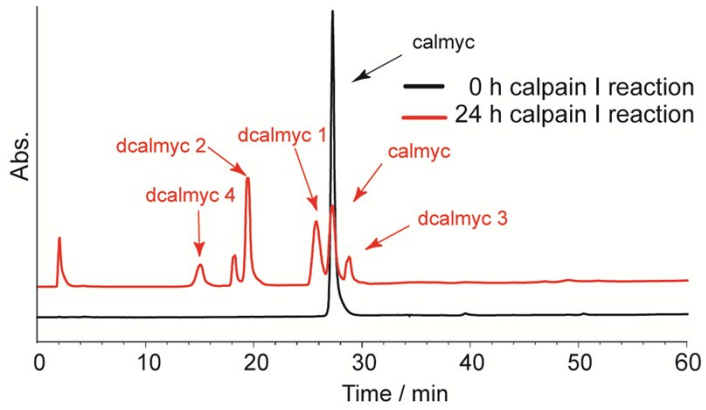

Figure S2. HPLC for calmyc + calpain I incubated for 0 h (black line) or 24 h (red line), then separated on an ODS column (150×4.6 mm 5 mm) with MilliQ water (containing 0.1% TFA) using a gradient from 0% to 60% acetonitrile (containing 0.08% TFA) over 60 min after a 5 min-flow of 0% acetonitrile, 1.0 mL/min; detection at 254 nm.

Table S3. T_m measurements from the calpain I digestion experiments in the presence of Li^+

	T_m (°C)
MYC + calpain I	n.d.
MYC + calmyc + calpain I (0h)	38.8 ± 0.2
MYC + calmyc + calpain I (24h)	n.d.

n.d.: No melting transition was observed. The quadruplex structure could not form under these conditions.