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

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Complete List of Authors:	Usui, Kenji; Konan University, Faculty of Frontiers of Innovative Research in Science and Technology (FIRST) Okada, Arisa; Konan University, Faculty of Frontiers of Innovative Research in Science and Technology Kobayashi, Keita; Konan University, Faculty of Frontiers of Innovative Research in Science and Technology Sugimoto, Naoki; Konan University, Faculty of Frontiers of Innovative Research in Science and Technology

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

We constructed a regulation system for DNA secondary structure formation of G-rich sequences using a designed PNA peptide exhibiting an on-to-off switching functionality, depending on protease activity. This study introduces the new concept of a simple and powerful system for regulating quadruplex-related important biological events.

Recent research has revealed that guanine-rich (G-rich) DNA sequences can fold into a secondary structure, the G-quadruplex structure,[1] and that this structure regulates cellular events such as transcription and telomerase elongation, which play roles in various serious diseases and cellular aging.[2] Systems capable of controlling the formation of secondary structure by G-rich DNA would therefore be useful for modulating these cellular events with beneficial biological effects. From this point of view, numerous G-rich sequence targeting ligands[3] have been described, including small compounds such as phthalocyanine derivatives,[4] porphyrin derivatives,[5] peptides,[6] and others.[7] However, the next generation of ligands will need ability of altering (switching) DNA secondary structures, more G-rich sequence specificity and a greater degree of functionality such as delivering ability to cellular organelles such as nucleus or mitochondria.

Designed peptides incorporating peptide nucleic acids (PNAs)[8] are promising next generation candidates targeting G-rich sequences because they offer several advantages: (1) peptides with PNAs are easier to design and synthesise than antibodies or recombinant proteins; (2) G-rich PNA peptides can form hetero-quadruplexes with homologous DNA oligomers;[9] (3) in addition to naturally occurring amino acids, various functional moieties can be employed as building blocks in the designed peptides;[10] and (4) because certain peptide sequences may exhibit transmembrane[11] or hormonal properties, combining peptides with these functional sequences with G-quadruplex-binding peptides can produce multifunctional molecules useful in cell and tissue engineering. Inspired by these advantages and some excellent reports regarding G-rich PNA peptides,[9] we developed a peptide ligand with a simple and powerful switching system for on-to-off switching the secondary structures of G-rich DNAs depending on protease activity which is a new concept in the development of small ligands for controlling G-quadruplex structures. Substitution of a series of key protease substrate sequences for the model protease substrate sequence used in this study could lead to promising modulators for various important biological events.

First, the peptide with PNA (calmyc) was designed (Fig. 1a). The molecule was composed of two parts. One part was composed of guanine PNA-rich sequences designed to form hetero-quadruplexes with homologous DNA oligomers (Fig. 1b). PNAs have several advantages: not only can they specifically bind to DNAs, but they are resistant to degradation by enzymes, and functions such as cell-penetration[12] or switching[13] can be introduced. In this study, a switching module, in which the on-off switch was a particular cellular environment, was incorporated as the other part into the peptide-PNA. A particular protease concentration was chosen as the cellular environment switch. We demonstrated that the designed peptide could bind to DNA and form a hetero-quadruplex structure when the protease was not expressed in cells, whereas when the protease was expressed, the conjugates were digested and simultaneously lost their binding ability, resulting in the collapse of the DNA-PNA hetero-quadruplex structure (Fig. 1c). In this study, calpain I, which is involved in

[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, K. U. E-mail: kusui@center.konan-u.ac.jp; Fax: +81 78 303 1495; Tel: +81 78 303 1418, N. S. E-mail: sugimoto@konan-u.ac.jp; Fax: +81 78 303 1495; Tel: +81 78 303 1416*
§These authors contributed equally to this work.
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serious diseases such as dystrophia, Alzheimer's disease,[14] and cancer,[15] was chosen as the model protease. The calpain I substrate sequences are relatively short (2-4 residues), so we expected the substrate sequence could be easily introduced to the PNA peptide. Thus, the calpain I substrate sequence was put into the center of the small molecule designed to act as the on-to-off switching module.

The peptides were synthesised by Fmoc chemistry. The binding of the calmyc peptide and a G-rich DNA sequence from the promoter region of human proto-oncogenes (MYC from c-MYC[16], Fig. 1b) was assayed using electrophoresis. The MYC band became thin and other migrating bands emerged as the concentration of calmyc increased (Fig. S1, ESI†). The main migrating band was located at 40 kb, which was different from what we expected, but MALDI-TOF MS showed that this band comprised MYC and calmyc. This indicated that calmyc could bind to MYC, resulting in the cationic PNA peptide causing the bands to migrate with a higher range of apparent molecular weights than expected. The binding stoichiometry was evaluated using FD-MYC (Fig. 1b), which has a fluorescent moiety and a quencher, using the quenching FRET technique. The fluorescence spectrum of FD-MYC and the change upon addition of calmyc were measured (Fig. 2a). The apparent formation of a 2:1 complex (calmyc:MYC) indicates that the PNA binds to both the peripheral G-rich sites, consistent with previous papers.[9] Additionally, the sharpness of the transition observed in the plot indicates high affinity for the 2:1 complex interaction.

We also examined the formation of the hetero-quadruplex structure by far-UV CD spectroscopy using calmyc and MYC (Fig. 2b). The spectrum of MYC:calmyc (1:2 complex) showed a similar spectrum to that of other PNA-DNA parallel G-quadruplexes previously described, including a positive maximum ellipticity at 260 nm.[9] Additionally, the presence of DNA with calmyc did not cause significant changes in the positions of the peaks or the shape of the DNA-alone spectrum at 0.1 mM KCl concentration. The CD results indicate that the hybrid calmyc-MYC structure, even in the absence of K^+, has a similar structure to the MYC DNA quadruplex structure. These results implied that the amine group of calmyc bound with MYC might act to stabilise G-quadruplex formation, similar to the action of ammonium ions.[17]

We investigated the effect of peptide binding on the thermodynamic stability of the DNA G-quadruplex. In UV melting experiments, quadruplexes in particular give rise to a hypochromic transition when absorbance is measured at 295 nm as a function of temperature.[18] To determine the relative stabilities of the species involved, we first characterised the UV melting behavior of MYC alone. This sequence demonstrated typical hypochromic transitions, and the melting temperature (T_m values) was dependent on the KCl concentration, similar to DNA quadruplex behavior. On the other hand, upon addition of calmyc in 2-fold excess over MYC, the T_m values were much higher at each KCl concentration and increased as the KCl concentration increased. Fig. 2c compares the increase in stability of the MYC quadruplex and the calmyc-MYC hybrid quadruplex as a function of K^+ concentration. The slopes of the two lines were much different than expected. The smaller slope for the hybrid quadruplex is explained by the fact that amine groups may stabilise the structure instead of K^+, which is consistent with the CD results. Furthermore, the melting curves were fitted to determine the thermodynamic parameters for formation of the MYC quadruplex and the calmyc-MYC hybrid quadruplex (2:1 complex) (Table S1, ESI†). The free energy of formation of the hybrid quadruplex is greater than that of the DNA alone by ca. -20 kcal mol^{-1} at 0.1 mM KCl, which emphasises the fact that a very high affinity complex is formed between calmyc and MYC. This is consistent with previous

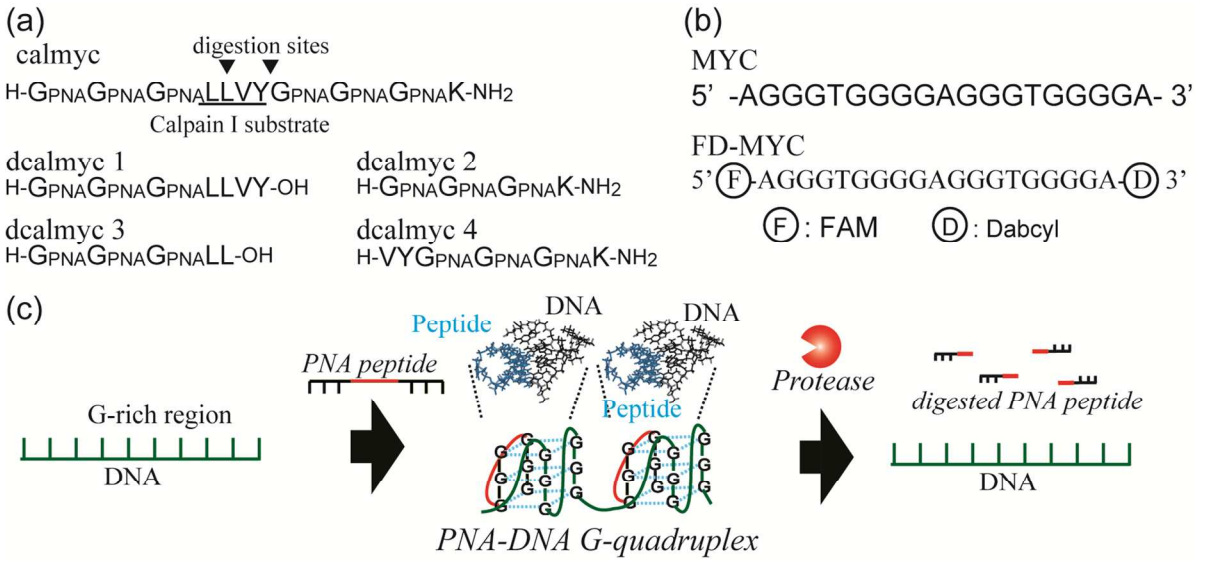

Fig 1. a) Sequences of the designed peptides. b) Sequences of the target DNAs. c) Scheme showing switching DNA secondary structure formation by the designed peptide and a specific protease.

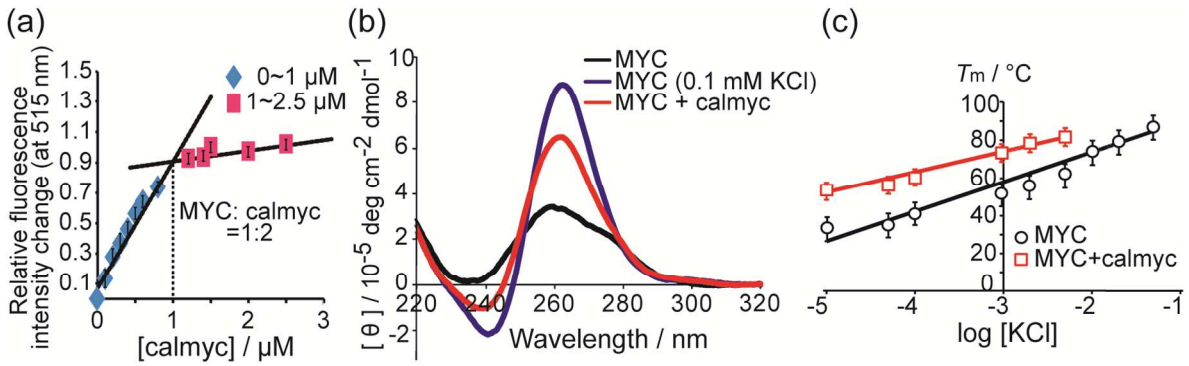

Fig. 2. a) Plots of change in relative fluorescence intensity of 0.5 μM FD-MYC upon the addition of calmyc at various concentrations. ex. 495 nm. b) CD spectra of 1 μM MYC with/without 2 μM calmyc (red and black), 1 μM MYC with 0.1 mM KCl (blue). c) Plots of melting temperatures as a function of K^+ concentration for MYC (5 μM) and MYC (5 μM) + calmyc (10 μM) solution.

work by the Armitage group, which showed hybrid quadruplex formation between a G-rich PNA and MYC.[9]

Putting these experimental results together, it is likely that calmyc invades the MYC quadruplex to form two 3-tetrad PNA-DNA hybrid quadruplexes as shown in Fig. 1c, as Armitage's group proposed. Additionally, in the stoichiometry experiment, FRET under quenching conditions was diminished upon the addition of calmyc, which indicates a transition from rather flexible to rigid and bulky structures (from unstructured DNA to PNA-DNA hybrid quadruplexes), thereby providing the lower FRET effect. This also supplementarily supports the binding model presented in Fig. 1c.

We then checked whether calmyc peptides digested by calpain I could or could not form PNA-DNA hybrid quadruplexes. There are two sites for calpain I digestion in calmyc, so we synthesised 4 sequences corresponding to digested calmyc (dcalmyc 1-4, Fig. 1a) and characterised the UV melting behavior of MYC with each of these fragment calmyc peptides. Addition of each dcalmyc 1-4, even in a 4-fold excess over MYC, provided melting curves and T_m values very similar to MYC alone (Table S2, ESI†). This indicated that digestion of calmyc abolished its ability to bind to MYC, resulting in collapse of the DNA-PNA hetero-quadruplex structure, as expected.

Thus, we successfully designed an artificial peptide as a ligand exhibiting an on-to-off switching module for quadruplex

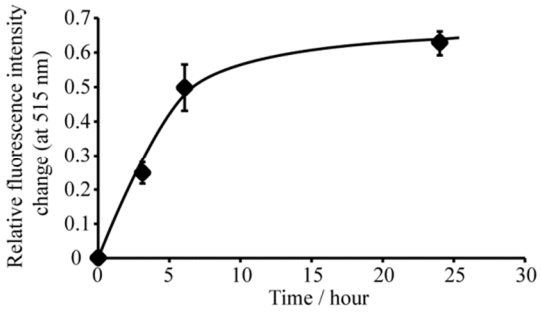

Fig. 3. Time course plots of change in relative fluorescence intensity of 0.2 μM FD-MYC and 0.4 μM calmyc after the addition of calpain I. ex. 495 nm.

forming. Further, we demonstrated that the switch function depends on a specific protease activity. We first checked by HPLC and MS whether calpain I could effectively digest calmyc (Fig. S2, ESI†), then digestion experiments using calmyc-MYC quadruplex and calpain I were conducted. Using FD-MYC, we monitored the conformational switch dependant on calpain I activity. The fluorescence intensity when calmyc bound to FD-MYC in the presence of Ca^{2+} was lower than that of FD-MYC alone. Upon the addition of calpain I, the fluorescence intensity increased with time (Fig. 3). Although the fluorescence behavior was different from that in the stoichiometry assay (in the absence of Ca^{2+}), the fluorescence indicated that we had successfully monitored the transition of DNA secondary structure formation, from PNA-DNA hybrid quadruplex to DNA G-quadruplex. Furthermore, UV melting experiments were also used to monitor this transition. To determine the relative stabilities of the species involved, we characterised the UV melting behavior of MYC alone in 50 mM Li_2EDTA. No melting curve was observed because Li^+ is known to destabilise the quadruplex structure. On the other hand, upon addition of calmyc in a 2-fold excess over MYC, although the melting transition was much lower, a T_m value could be determined because hybrid quadruplex could form in the absence of K^+, as described above. After addition of calpain I and incubation for 24 h, MYC with calmyc provided no melting curve, similar to the MYC-alone condition (Table S3, ESI†). These results indicated that we could demonstrate the on-to-off switch function using two different techniques.

In conclusion, we designed an enzyme-responsive ligand for a DNA G-rich sequence for controlling DNA secondary structure formation, and demonstrated the enzymatic switch from a PNA-DNA hybrid quadruplex structure to a DNA quadruplex structure. Despite a small-size of this peptide ligand, it not only binds tightly to the DNA G-rich sequence, but also provides a simple and powerful on-to-off switching system for secondary structures of G-rich DNAs depending on protease activity. This is new concept in the development of small ligands. Substitution with key protease substrate sequences for the calpain I substrate sequence, improvement of specificity with adding more PNAs for a target G-rich sequence and stability of the peptide in cell extracts or serum, may allow the application

of this concept to control systems for transcription and protein expression. Additionally, reversible switching system could be achieved with some enzymes such as kinases and phosphatases using this enzyme-responsive ligand concept. Throughout this study, the PNA-peptide conjugates will be a promising tool for the eventual regulation of important cellular events by cell engineering and tissue engineering.

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Notes and references

1 M. Gellert, M. N. Lipsett and D. R. Davies, *Proc. Natl. Acad. Sci. USA*, 1962, **48**, 2013; S. Neidle and S. Balasubramanian, *Quadruplex nucleic acids,* 2006, Royal Society of Chemistry Publications, Cambridge, UK.

2 E. Henderson, C. C. Hardin, S. K. Walk, I. Tinoco Jr. and E. H. Blackburn, *Cell*, 1987, **51**, 899; A. Siddiqui-Jain, C. L. Grand, D. J. Bearss and L. H. Hurley, *Proc. Natl. Acad. Sci. USA,* 2002, **99**, 11593; D. Miyoshi, S. Matsumura, S. Nakano and N. Sugimoto, *J. Am. Chem. Soc.*, 2004, **126**, 165; S. Rankin, A. P. Reszka, J. Huppert, M. Zloh, G. N. Parkinson, A. K. Todd, S. Ladame, S. Balasubramanian and S. Neidle, *J. Am. Chem. Soc.,* 2005, **127**, 10584; H. Q. Yu, D. Miyoshi and N. Sugimoto, *J. Am. Chem. Soc.,* 2006, **128**, 15461; T. Endoh, Y. Kawasaki and N. Sugimoto, *Angew. Chem. Int. Ed.,* 2013, **52**, 5522; S. Takahashi and N. Sugimoto, *Angew. Chem. Int. Ed.*, 2013, **52**, 13774.

3 L. H. Hurley, *Nat. Rev. Cancer* **2002**, *2*, 188; A. De Cian, L. Lacroix, C. Douarre, N. Temime-Smaali, C. Trentesaux, J. F. Riou and J. L. Mergny, *Biochimie*, **2008**, *90*, 131.

4 H. Yaku, T. Murashima, D. Miyoshi and N. Sugimoto, *Chem. Commun.* **2010**, *46*, 5740.

5 J. Seenisamy, E. M. Rezler, T. J. Powell, D. Tye, V. Gokhale, C. S. Joshi, A. Siddiqui-Jain and L. H. Hurley, *J. Am. Chem. Soc*. **2004**, *126*, 8702.

6 T. K. Chakraborty, A. Arora, S. Roy, N. Kumar and S. Maiti, *J. Med. Chem.* **2007**, *50*, 5539; K. Kobayashi, N. Matsui and K. Usui, *J. Nucleic Acids* **2011**, 2011:572873, doi: 10.4061/2011/572873.; A. Okada and K. Usui, *Chemical Biology of Nucleic Acids:Fundamentals and Clinical Applications*, **2014**, 459.

7 N. Kumar and S. Maiti, *J. Phys. Chem. B* **2007**, *111*, 12328; A. Arola and R. Vilar, *Curr. Top. Med. Chem.* **2008**, *15*, 1405; W. O. Tucker, K. T. Shum and J. A. Tanner, *Curr. Pharm. Des.* **2012**, *18*, 2014.

8 P. E. Nielsen, M. Egholm, R. H. Berg and O. Buchardt, *Science* **1991**, *254*, 1497.

9 B. Datta, C. Schmitt and B. A. Armitage, *J. Am. Chem. Soc.*, 2003, **125**, 4111; S. Lusvarghi, C. T. Murphy, S. Roy, F. A. Tanious, I. Sacui, W. D. Wilson, D. H. Ly and B. A. Armitage, *J. Am. Chem. Soc.* **2009**, *131*, 18415; A. Paul, P. Sengupta, Y. Krishnan and S. Ladame, *Chem. Eur. J.* **2008**, *14*, 8682; S. Roy, F. A. Tanious, W. D. Wilson, D. H. Ly and B. A. Armitage, Biochemistry **2007**, *46*, 10433.

10 S. Sano, K.-Y. Tomizaki, K. Usui and H. Mihara, *Bioorg. Med. Chem. Lett.* **2006**, *16*, 503.

11 F. Madani, S. Lindberg, U. Langel, S. Futaki and A. Graslund, *J. Biophys.* **2011**, 414729, doi: 10.1155/2011/414729

12 P. J. Deuss, A. A. Arzumanov, D. L. Williams and M. J. Gait, *Org. Biomol. Chem.* **2013**, *11*, 7621.

13 T. Stafforst and D. Hilvert, *Angew. Chem. Int. Ed.* **2010**, *49*, 9998.

14 M. Higuchi, N. Iwata, Y. Matsuba, J. Takano, T. Suemoto, J. Maeda, B. Ji, M. Ono, M. Staufenbiel, T. Suhara and T. C. Saido, *FASEB J.* **2012**, *26*, 1204.

15 M. Niapour, Y. Yu and S. A. Berger, *J. Biol. Chem.* **2008**, *283*, 21371.

16 T. M. Ou, Y. J. Lu, C. Zhang, Z. S. Huang, X. D. Wang, J. H. Tan, Y. Chen, D. L. Ma, K. Y. Wong, J. C. Tang, A. S. Chan and L. Q. Gu, *J. Med. Chem.* **2007**, 50, 1465; S. Balasubramanian, L. H. Hurley and S. Neidle, *Nat. Rev. Drug. Discov.* **2011**, *10*, 261.

17 P. Podbevsek, N. V. Hud and J. Plavec, *Nucleic Acids Res.* **2007**, *35*, 2554.

18 J. L. Mergny, A. T. Phan and L. Lacroix, *FEBS Lett.* **1998**, *435*, 74.

