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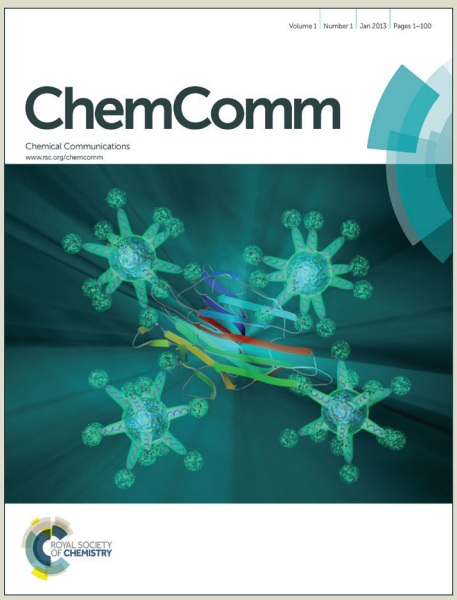

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Controlling leucine-zipper partner recognition in cells through modifications of *a-g* interactions†

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By focusing on the *a-g* interaction, successful design and selection were accomplished to obtain a leucine-zipper segment that discriminates the appropriate partner over another that provides very similar patterns of electrostatic interactions.

The establishment of peptides that specifically inhibit protein interactions in our body has been one of the major challenges in chemical biology. These peptides could be useful tools for the understanding of biological significances of protein interactions and eventually pave the way for lead molecules in drug discovery.[1-3] Selection of peptides from sophisticatedly designed libraries is one of the promising approaches in order to obtain peptides that strongly bind the target proteins, where improvement of specificity is also being pursued.[3] We have focused on the inhibition of a protein from the human T-lymphotropic virus type I (HTLV-1) that is known to cause adult T- cell leukemia (ATL).

ATL is a peripheral T cell neoplasm associated with infection by the HTLV-1.[4] The viral genome encodes the HTLV-1 bZIP factor (HBZ).[5] HBZ is expressed in all ATL patients and is known to promote T cell proliferation and systemic inflammation *in vivo*. HBZ has a domain containing a cFos-like leucine-zipper segment, which confers interaction of HBZ to cJun and related leucine-zipper proteins (JunB, JunD and so on).[6] Peptides that specifically block the interaction of HBZ with Jun related proteins could be powerful tools in order to sufficiently elucidate the role of the interaction in the onset of ATL. These inhibitory peptides may also provide a novel therapeutic approach to ATL. The inhibition must be accomplished without hampering endogenous Fos-Jun interaction since these proteins are involved in regulation of various genes and inhibition of the Fos-Jun interaction would cause considerable dysfunction in cells (***Fig. 1a***).[7] However, the design of HBZ specific inhibitory peptides is complicated by the sequence similarity between HBZ and Fos in the leucine-zipper segments. From the viewpoint of protein and peptide engineering, it has been always a challenge to design peptides that recognise small differences on protein surfaces.

The interaction of HBZ and cFos with cJun related-proteins is based on coiled-coil helix dimer formation between the leucine-zipper segments in these proteins (***Fig. 1b***). In the heterodimer formation among leucine-zipper segments, electrostatic interaction between the positions ***e*** and ***g*** has been considered most critical and employed as a strategy for primary designs of artificial heterodimer-forming leucine-zipper segments.[8] However, both HBZ and cFos have very similar charge distributions in the positions ***e*** and ***g***; four Glu at ***g*** positions and two at ***e*** position together with neutral amino acids at this position (***Fig. 1b***). On the other hand, X-ray structural analyses of the cJun/cFos complex as well as computational design of coiled coil peptides suggested the potential importance of the interactions between a and g positions.[9,10] Here, by utilizing this additional interaction, together with employing the Hitchhiker Translocation (HiT) *in vivo* selection system,[11] we have succeeded in acquiring a mutated cJun-derived leucine-zipper segment (cJun winner peptide for HBZ, JWH) that shows significantly higher affinity towards leucine-zipper of HBZ than that of cFos.

The HiT selection system employs the bacterial twin-arginine translocation (TAT) pathway that secretes properly folded proteins bearing a TorA translocation signal peptide (see Supporting Information ***Fig. S1***).[11] In this system, a leucine-zipper segment bearing the TorA signal peptide and the partner fused to β-lactamase lacking its original lead sequence are co-expressed in bacteria. Successful formation of a stable coiled-coil structure by the two leucine-zipper segments leads to periplasmic secretion of the β-lactamase, where it confers ampicillin resistance. Colonies observed on ampicillin-containing plates should represent the bacteria expressing a suitable pair of leucine-zipper segments.

A peptide library of mutated cJun leucine-zipper sequences was constructed to obtain HBZ-targeting peptides that specifically bind to HBZ but not to cFos. The substitutions were designed for key

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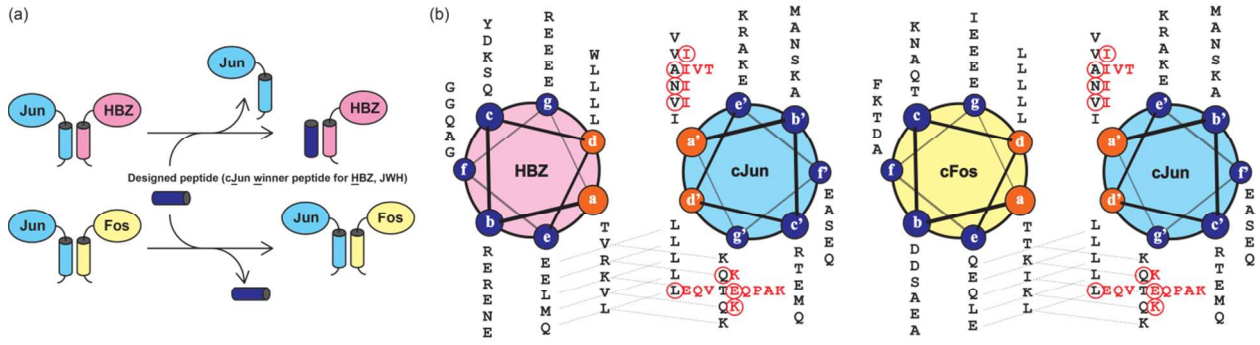

Fig. 1. (a) The goal of this study; obtaining an HBZ specific peptide without interfering with Jun-Fos recognition in cell (JWH, cJun winner peptide for HBZ). (b) Helical wheel projections of the HBZ/cJun and cFos/cJun coiled coils. The a-g denotes the heptad positions in leucine-zipper. Amino acid residues given in black show the wild-type sequence. Residues shown in red are amino acids implemented in the cJun-based library. The red circles indicate amino acids selected in the winner peptide JWH.

amino acids at specific positions to minimise library size and to facilitate the library selection, while obtaining higher affinity of the winner peptide to the target. Modification of the hydrophobic or electrostatic interactions at the dimeric interface was thus considered (***Fig. 1b***). Namely, since the leucine zippers of HBZ and cFos have positively charged Lys and Arg at ***a*** positions (Arg(***a3***) and Lys(***a4***) in HBZ and Lys(***a3***) and Lys(***a5***) in cFos), positively charged Lys was offered in the library design at ***g'2*** and ***g'4*** in addition to wild-type Gln to facilitate potential repulsions in the cFos leucine-zipper.‡ At position ***g'3*** Glu was offered in addition to wild-type Thr for potential interaction with Lys at ***a4*** in the HBZ leucine zipper. Since β-branched Ile is favoured at ***a*** positions in dimeric coiled coils,[12] Ile was offered in addition to wild-type residues at positions ***a'2***, ***a'3***, ***a'4***, and ***a'5*** in the library design. Glu was offered together with wild-type Leu at ***d'5*** in the library as it promises electrostatic repulsion with Glu at position ***e5*** of cFos but should be less destabilizing with Gln at ***e5*** of HBZ. Use of genetic codes to express wild-type and designed amino acids at the specified positions was in some cases accompanied by the simultaneous incorporation of other amino acids as shown in ***Fig. 1b***.

The plasmids encoding HBZ leucine-zippper–β-lactamase fusion protein and the library of mutated cJun-derived leucine-zipper peptides bearing the TorA sequence were prepared and simultaneously transformed into *E. coli* BL21.¶ After three rounds of selection by reducing the isopropyl β-D-1-thiogalactopyranoside (IPTG) concentration (from 1 mM to 0.3 mM) to fine-tune the induction of protein complexes, a peptide sequence (JWH: ASIARLEEKVKTLKAQNYELASEANMLREKIAQLKQKVMNG AP) was finally obtained (***Fig. 2a***) (see Supporting Information ***Table S1*** about the details of the selection). The winner peptides has three amino acid mutations compared to the cJun leucine-zipper peptide; a Val-to-Ile substitution at ***a'5***, a Gln-to-Lys substitution at ***g'2*** and a Thr-to-Glu substitution at ***g'4***, respectively. To characterise the winner peptide, the JWH peptide with acetylated N-terminus and amidated C-terminus (denoted hereafter as JWH-ZIP) were prepared using Fmoc-solid-phase peptide synthesis, together with leucine-zipper peptides derived from HBZ, cFos, and cJun (HBZ-ZIP, cFos-ZIP, and cJun-ZIP, respectively) (***Fig. 2a***).§

Heterodimer formation of JWH-ZIP with HBZ-ZIP was validated through circular dichroism (CD) (***Fig. 2***), analytical ultracentrifugation (AUC) (***Fig. S2***), and size-exclusion chromatography with on-line light-scattering (SEC-LS) (***Fig. S3***). Both AUC and SEC-LS data suggest the stable dimer formation of the HBZ-ZIP/JWH-ZIP mixture (***Figs. S2*** *and* ***S3***). The CD spectrum of a mixture of HBZ-ZIP and JWH-ZIP shows double minima around 208 nm and 222 nm (***Fig. 2b***), indicating the formation of a helical structure (molar ellipticity at 222 nm ($[\theta]_{222}$): -3.4×10^4 deg cm^2 dmol^{-1} and $[\theta]_{222}/[\theta]_{208}$: 1.02) (***Fig. 2b***). $[\theta]_{222}$ and $[\theta]_{222}/[\theta]_{208}$ values, which have been employed as an indicator of helical contents[13] and stranded formation[8c], respectively, suggested that the peptides fully (~100%) adopted a helical, particularly, coiled-coil structure. Although JWH-ZIP and HBZ-ZIP alone yielded spectra of a helical structure, the estimated helical contents of these peptides were lower than that of the HBZ-ZIP/JWH-ZIP mixture ($[\theta]_{222}$ ($[\theta]_{222}/[\theta]_{208}$) for JWH-ZIP and HBZ-ZIP: -2.5×10^4 (0.94) and -1.7×10^4 (0.72) deg cm^2 dmol^{-1}, respectively). The raw CD spectrum of a 1:1 mixture of HBZ-ZIP and JWH-ZIP (25 μM each) showed twice as much helical content than the added spectra of HBZ-ZIP and JWH-ZIP (25 μM each) (***Fig. S4a***). The CD analysis, together with the AUC and SEC-LS data, clearly showed that HBZ-ZIP and JWH-ZIP form a heterodimeric coiled-coil.∫

The stability of HBZ-ZIP/cJun-ZIP was next compared with that of HBZ-ZIP/JWH-ZIP. A CD spectrum of cJun-ZIP/HBZ-ZIP showed that cJun-ZIP forms heterodimeric helical structure with HBZ-ZIP (***Fig. S4c***)∅ as reported previously.[6e] Thermal denaturing of both heterodimeric complexes revealed that JWH-ZIP forms a more stable dimer with HBZ-ZIP than cJun-ZIP with HBZ-ZIP (***Fig. 2d***, left). Melting temperature (T_m) of HBZ-ZIP/JWH-ZIP is 7 °C higher than that of HBZ-ZIP/cJun-ZIP (47 °C) (***Table 1***). Isothermal titration calorimetry (ITC) study also showed that the dissociation constant (K_d) for HBZ-ZIP/JWH-ZIP complex at 37 °C (0.31 μM) is almost one-order lower than that for HBZ-ZIP/cJun-ZIP (2.12 μM) (***Table 1*** and ***Fig. S5a,b***). The obtained K_d value of JWH-ZIP for HBZ-ZIP in submicromolar range at 37 °C should be low enough to allow JWH-ZIP to specifically recognise the target in cytoplasm, which is supported by the similar K_d value for cFos-ZIP/cJun-ZIP complex (0.29 μM) (***Table 1***). cFos-ZIP also forms heterodimers with JWH-ZIP as well as with cJun-ZIP as seen by CD, AUC and SEC-LS analysis (***Figs. S2d, S3d,*** and ***S4d***). Preference of cFos-ZIP in heterodimer formation to cJun-ZIP over JWH-ZIP was confirmed by thermal denaturation analysis (T_m of cFos-ZIP/cJun-ZIP and

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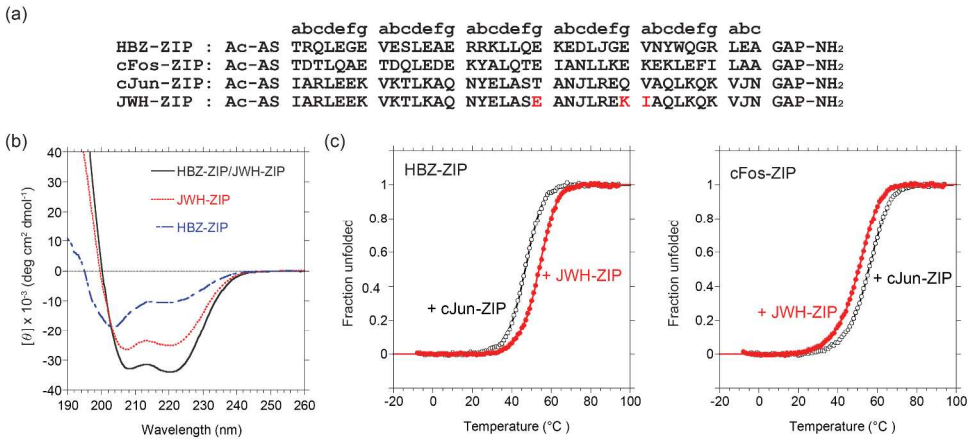

Fig. 2. (a) Sequences of the peptides used in this study methionine residues in the original sequences were altered to norleucine (shown as "J") to prevent undesired oxidation. (b) CD spectra of HBZ-ZIP (dashed-dotted line, blue), JWH-ZIP (dotted line, red), and the mixture (continuous line, black). (c) Thermal denaturation curves for the heterodimers of HBZ-ZIP (left) and cFos-ZIP (right) with JWH (closed circles, red) and cJun-ZIP (open circles, black), respectively.

Table 1. T_m and K_d values of the heterodimers

peptide 1	peptide 2	Tm (ºC)[a]	K_d (μM)[b]
HBZ-ZIP	JWH-ZIP	54	0.31
	cJun-ZIP	47	2.12
cFos-ZIP	JWH-ZIP	51	0.83
	cJun-ZIP	56	0.29

[a]determined by CD; [b]determined by ITC[#]

cFos-ZIP/JWH-ZIP are 56 and 51 °C, respectively) (***Fig. 2d,*** right and ***Table 1***), together with dissociation constant obtained by ITC (K_d of cFos-ZIP/JWH-ZIP is 0.83 μM, almost 3-fold higher than that of cFos-ZIP/cJun-ZIP) (***Table 1*** and ***Fig. S5c,d***).

The ability of the JWH segment to bind HBZ protein within the cellular context was analysed next. For immunodetection, JWH and HBZ were tagged with a peptide derived from human influenza hemagglutinin (HA) and the C-terminal region of human c-myc (Myc), respectively (HA-JWH and Myc-HBZ). Since it is known that HBZ is mainly localised in nucleus,[14] the nuclear localisationsignal peptide derived from simian virus 40 (SV40) was also attached to the HA-JWH to allow JWH facilitate interaction with HBZ. The plasmids encoding HA-JWH and Myc-HBZ were co-transfected into HeLa cells. Immunostaining with anti-HA and anti-Myc antibodies showed that HA-JWH and Myc-HBZ were well co-localised in the cells, particularly in nucleus (***Fig. S6***; see Supporting Information for experimental details).

Marked JWH interference with HBZ/c-Jun interaction in cells was confirmed by immunoprecipitation. HA-tagged cJun (HA-cJun) and Myc-HBZ proteins were co-expressed in HeLa cells. Proteins that bind HBZ in the cells were then precipitated by the anti-Myc antibody. A band for HA-cJun was detected in the precipitate with Myc-HBZ by western blotting using anti-HA antibody, indicative for the HBZ association with cJun in the cells (***Fig. 3a***, second band from the left). Alternatively, only the band for HA-JWH was observed in western blotting when both HA-cJun and HA-JWH were expressed in the cells together with Myc-HBZ (***Fig. 3a***, right). These results suggested that HBZ preferentially binds HA-JWH over HA-cJun in the cells, thus JWH effectively inhibits the HBZ/cJun interaction. No significant bands neither for HA-Jun nor HA-JWH were observed in the absence of Myc-HBZ (***Fig. 3a***, second band from the right).

An AP1-luciferase reporter assay[15] also showed that an HBZ-induced suppression on AP1 pathway is completely abolished by JWH (***Fig. 3b***). When cJun was overexpressed in Jurkat cells, a 6.7-fold increase of luciferase activity was observed (***Fig. 3b***, second bar from the left). This result suggested that the overexpressed cJun protein forms dimer with endogenous cFos and related cellular

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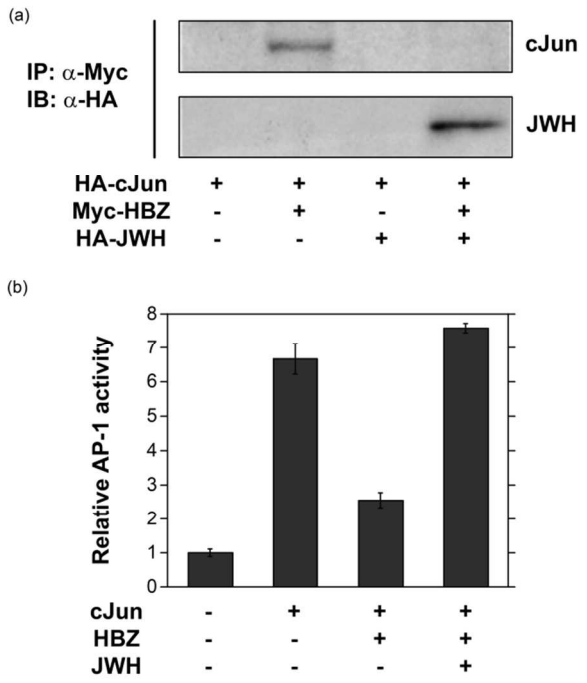

Fig. 3. (a) Immunoprecipitation for the analysis of HBZ-binding protein/peptide in cells. (b) AP-1 reporter assay for the assessment of HBZ inhibition for the binding of cJun with endogenous cFos to the promoter.

proteins and then binds to the AP1 site, which results in an enhanced luciferase expression. Co-expression of HBZ revealed a significant decrease in the luciferase activity (***Fig. 3b***, second bar from the right), suggesting that HBZ inhibited the binding of cJun with cFos. When JWH was additionally expressed in cells, this diminishment was not observed (***Fig. 3b***, right), suggesting that preferential binding of JWH to HBZ liberated cJun from the trap by HBZ.

In summary, we demonstrated a promising approach to design a peptide that specifically inhibits a disease related coiled-coil interaction. Combining the HiT selection system with a minimized library harbouring defined randomisation of amino acids in ***a'*** and ***g'*** positions, we have succeeded in establishing a leucine-zipper segment (JWH) that specifically discriminates subtle differences in interaction surfaces of HBZ over cFos. The selection endowed JWH with the Thr-to-Glu and Gln-to-Lys substitutions at ***g'*** position of cJun leucine-zipper segment. These additional positive and negative charges at ***g'*** position may contribute to the preferential recognition of the ***a*** position of HBZ leucine-zipper segment over that of cFos. The obtained submicromolar range of dissociation constant at body temperature should be practically sufficient to control cell functions. The detailed study in the control of HBZ using JWH is ongoing in our laboratory.

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Notes and references

† Electronic Supplementary Information (ESI) available: Materials and Methods, Supporting Figures and Table. See DOI: 10.1039/b000000x/

‡ Positions for the library of mutated cJun-ZIP peptides and cJun leucine-zipper are denoted with a prime, and heptad positions are numbered.

¶ The Asp-Ser and Gly-Ala-Pro sequences were added on at the N- and C-termini of these leucine-zipper segments for the terminus capping to stabilize their helical structures and to facilitate the cloning. The amino acid at solvent-exposed ***b'*** position in the third heptad repeat of cJun was also mutated to tyrosine to determine the peptide concentration by UV absorption.

§ To unify the structure with that of JWH, the AS and GAP sequences were also added in these peptides and their N- and C-termini were acetylated and amidated, respectively.

CD spectrum of JWH-ZIP (10 μM) at 37°C under the condition used for ITC showed little helical structure (data not shown) and thus data were analysed using the two-state model between monomers and heterodimers.

∫ Thermal denaturation analyses revealed that both HBZ-ZIP and cFos-ZIP homodimers are highly unstable (***Fig. S4b***). T_m values of HBZ-ZIP and cFos-ZIP are 7 and -1°C. It is reported that cFos derived leucine-zipper exclusively forms heterodimer with cJun derived leucine-zipper which also forms stable homodimer, and thermodynamic instability of the cFos homodimer shifts the equilibrium toward more stable heterodimer formation with cJun.[8b] In the same manner, instability of HBZ-ZIP homodimer would lead to the heterodimer formation with JWH-ZIP. This mechanism is also supported by the fact that the T_m of JWH-ZIP homodimer (35°C) and HBZ-ZIP/JWH-ZIP heterodimer (54°C) are similar to that of cJun-ZIP homodimer (34°C) and cFos-ZIP/cJun-ZIP homodimer (56°C), respectively (***Fig. 2c, S4b***). T_m of an equimolar mixture of HBZ-ZIP and cFos-ZIP was also estimated to be below zero (data not shown).

∅ AUC analysis suggested that a 1:1 mixture of HBZ-ZIP and cJun-ZIP forms a dimer (***Fig. S2e***).

 See also ***Fig. S7*** about expression of HA-cJun and HA-JWH in the cells.

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