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Short communication

Improvement of a puromycin-linker to extend the selection target varieties in cDNA display method

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ABSTRACT

cDNA display using a puromycin-linker to covalently bridge a protein and its coding cDNA is a stable and efficient in vitro protein selection method. The optimal design of the often-used puromycin-linker is vital for effective selection. In this report, an improved puromycin-linker containing deoxyinosine bases as cleavage sites, which are recognized by endonuclease V, was introduced to extend the variety of the selection targets to molecules such as RNA. The introduction of this linker enables efficient in vitro protein selection without contamination from RNase T1, which is used for the conventional linker containing ribonucleotide G bases. In addition, mRNA–protein fusion efficiency was found to not depend on the length of the flexible poly (ethylene glycol) (PEG) region of the linker. These findings will allow practical and easy-to-use in vitro protein selection by cDNA display.

In vitro display technology is a powerful tool used to search for functional peptides/proteins from a combinatorial protein library (Grönwall and Ståhl, 2009; Ullman et al., 2011). In particular, in vitro display methods using a cell-free translation system (e.g., ribosome display (Hanes and Plückthun, 1997); mRNA display (Nemoto et al., 1997; Roberts and Szostak, 1997)) represent promising approaches since the library size used in these methods is huge. However, the selection conditions used in these methods are restrictive because of the lability of the mRNA template molecules. To overcome this problem, the cDNA display method has been developed by covalently linking a protein with its coding cDNA via a puromycin-linker (Yamaguchi et al., 2009). Furthermore, the manipulability of the cDNA display (i.e., one-pot preparation of the cDNA display) has been dramatically improved by redesigning the puromycin-linker (Mochizuki et al., 2011). However, the RNA moiety for the RNase T1 cleavage site in the puromycin-linker may be labile during the preparation process. Moreover, RNase T1 contamination, which is used in the release of cDNA display molecules from the beads, would limit the potential applications of cDNA display; for example, target RNA molecules are easily digested by the RNase T1 contamination in the selection of RNA binding proteins. Since RNA–protein interactions are important in molecular biology, especially in functional genomics, exploration and analyses of RNA binding proteins are crucial for applications involving cDNA display.

To resolve this difficulty, ribonucleotide G (rG) has been replaced with deoxyinosine (dI) at the cleavage sites in the linker, and RNase T1 has been changed to a repair enzyme "Endonuclease V (Endo V)" as the digestion enzyme (Fig. 1A). Initially, we examined the performance of the new designed puromycin-linker termed SBP(dI) at each process: (a) ligation efficiency between an mRNA and the new linker; (b) mRNA–protein fusion efficiency; (c) reverse transcription efficiency; and (d) enzymatic cleavage efficiency (Fig. 1B). These efficiencies were found to be almost the same as the conventional puromycin-linker termed SBP(rG) (Mochizuki et al., 2011) (Fig. 2). We next investigated the stability of the puromycin-linkers during the preparation process of the cDNA display. In general, the wheat germ extract has high RNase activity when compared with the rabbit reticulocyte lysate used for cell-free translation (Hino et al., 2008); thus, we examined the stability of the puromycin-linkers themselves against RNase. Although SBP(rG) was readily digested by RNase, SBP(dI) was stable under the same conditions (Fig. 3A). SBP(rG) and SBP(dI) were then incubated with the rabbit reticulocyte lysate and the wheat germ extract. SBP(rG) was observed to be slightly degraded when incubated with the wheat germ extract. In contrast, the degradation of SBP(dI) was negligible under the same conditions (Fig. 3B). These results indicate that cDNA display using SBP(dI) can be prepared

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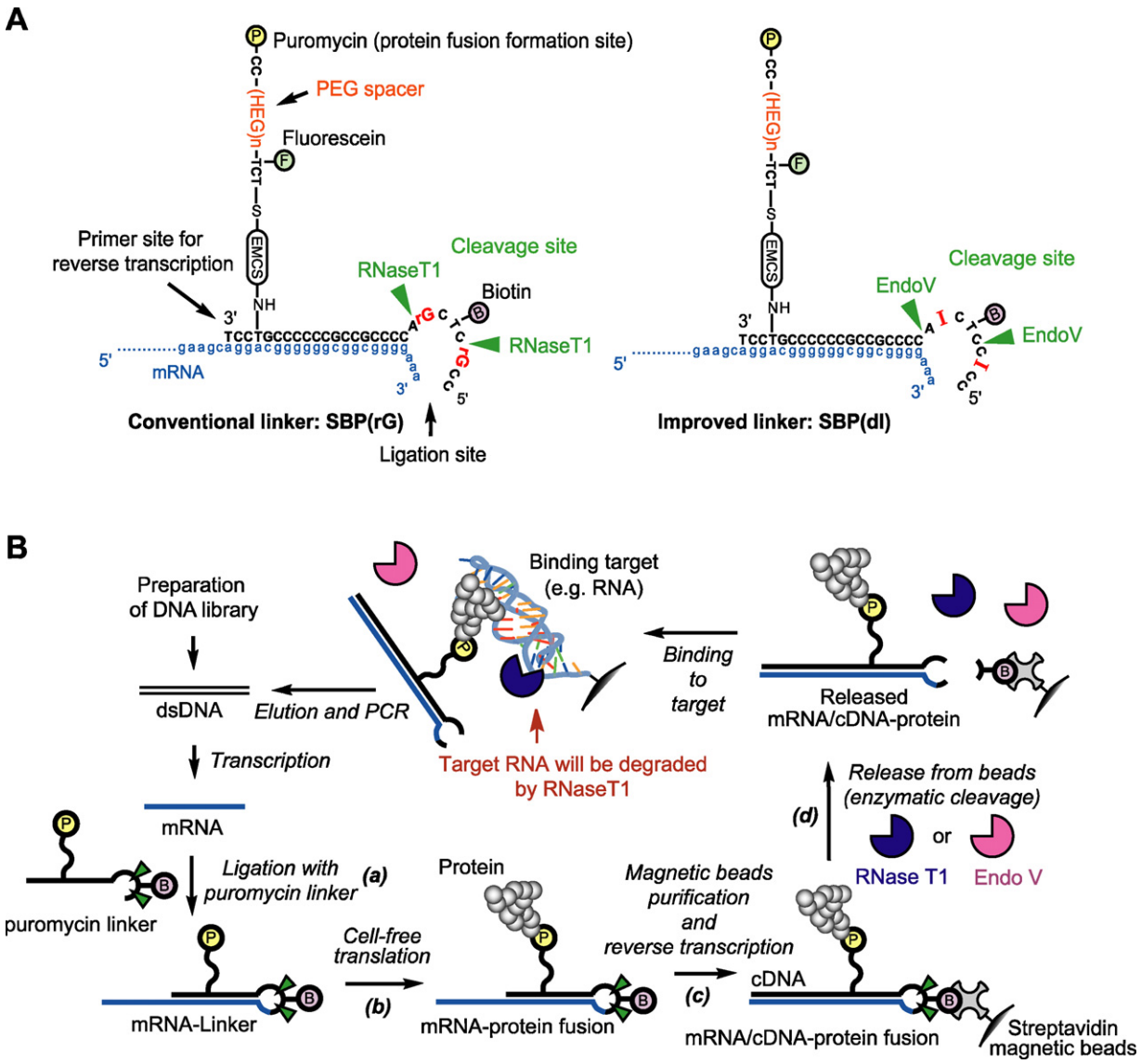

Fig. 1. (A) Schematic representation of conventional and improved puromycin-linkers, termed SBP(rG) and SBP(dI), respectively. These puromycin-linkers are composed of puromycin, a poly (ethylene glycol) (PEG)-spacer consisting of multiple units of hexa (ethylene glycol) (HEG), a fluorescein-derivative and a DNA oligomer containing the primer site for reverse transcription, a biotin moiety and the enzyme-cleavage sites. SBP(rG) contains two riboguanosines (rG) as cleavage sites recognized by RNase T1. SBP(dI) contains two deoxyinosines (dI) as cleavage sites recognized by Endo V. Lower-case characters are RNA sequences that hybridize with the linkers. [*N*-(6-maleimidocaproyloxy)succinimide] (EMCS) is a bifunctional cross-linker used in the preparation of the puromycin-linker. (B) Schematic representation of the selection cycle of cDNA display with enzyme-cleavable linkers, SBP(rG) and SBP(dI). An initial dsDNA library was transcribed into an mRNA library and ligated with the puromycin-linkers (a). The ligated mRNA library was translated in a cell-free translation system and converted to an mRNA–protein fusion library (b). The mRNA–protein fusion library was captured on streptavidin magnetic beads and reverse transcribed to make an mRNA/cDNA–protein fusion library (c). The captured protein-fusion library was released from the beads via enzymatic digestion by RNase T1 or Endo V (d). If RNase T1 was used, undesired digestion of RNA molecules by contaminated RNase T1 would occur following this procedure. On the other hand, undesired digestion of RNA and DNA molecules by Endo V rarely occurred, because the enzyme has base-specific digestion activity. The released mRNA/cDNA–protein fusion library was screened according to the binding properties against target molecules and the selected molecules were PCR amplified to generate the next round of the dsDNA library. Parenthetic letters are discussed in the text.

without the loss of the linker by nuclease degradation. The contamination of RNase T1 into the successive selection process has been another critical problem for cDNA display using SBP(rG). This is particularly the case when the target molecule is an RNA or RNA-related molecule. Thus, the degradations of RNA molecules by RNase T1 and Endo V were examined. The results in Fig. 3C show that RNase T1 degraded the majority of the mRNA. In contrast, Endo V was found to not degrade mRNAs under the same conditions. Additionally, a model experiment of in vitro selection using SBP(dI) against RNA molecules was performed. The cDNA–protein fusion products which were composed of SBP(rG) or SBP(dI) treated with RNase T1 or Endo V, respectively, were applied to RNA targets on the magnetic beads as a typical selection process. As a result, when the cDNA–protein fusion products composed of SBP(rG) was used, the RNA molecules were degraded. On the other hand, in the case of SBP(dI), the RNA molecules were not degraded (Fig. 3D). Therefore, cDNA display using SBP(dI) will extend the repertoire of target molecules including RNAs and RNA–protein complexes (e.g., ribosomes). In directed evolution, exploring novel enzymes is another challenging field (Seelig and Szostak, 2007). In the application of cDNA display to the field, the length of the PEG-region in the puromycin-linker is expected to affect the activity of the displayed enzyme. The length of the PEG-region is also related to the efficiency of the mRNA–protein fusion reaction on the ribosome. In a previous report, the length of the flexible linker region composed of oligo-dA and tri (ethylene glycol) (TEG) equivalent to 25–40