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Photocleavable linkage between genotype and phenotype for rapid and efficient recovery of nucleic acids encoding affinity-selected proteins

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Abstract

In vitro display technologies, such as mRNA display and DNA display are powerful tools to screen peptides and proteins with desired functions from combinatorial libraries in the fields of directed protein evolution and proteomics. When screening combinatorial libraries of polypeptides (phenotype), each of which is displayed on its gene (genotype), the problem remains, how best to recover the genotype moiety whose phenotype moiety has bound to the desired target. Here, we describe the use of a photocleavable 2-nitrobenzyl linker between genotype (DNA or mRNA) and phenotype (protein) in our DNA and mRNA display systems. This technique allows rapid and efficient recovery of selected nucleic acids by simple UV irradiation at 4 °C for 15 min. Further, we confirmed that the photocleavable DNA display and mRNA display systems are useful for *in vitro* selection of epitope peptides, recombinant antibodies, and drug–receptor interactions. Thus, these improved methods should be useful in therapeutics and diagnostics, *e.g.*, for screening high-affinity binders, such as enzyme inhibitors and recombinant antibodies from random peptide and antibody libraries, as well as for screening drug–protein interactions from cDNA libraries.

Keywords: Directed evolution; Drug receptor; Protein engineering; Proteomics; Puromycin

1. Introduction

Screening peptides and proteins with desired functions from combinatorial libraries, such as random peptide libraries and cDNA libraries, is important in the fields of directed protein evolution and proteomics. To permit multiple rounds of selection of polypeptides, the establishment of a linkage between each polypeptide (phenotype) and its encoding gene (genotype) is required, and various display technologies have been developed for this purpose (Doi and Yanagawa, 2001; Dower and Mattheakis, 2002; Lipovsek and Plückthun, 2004; Leemhuis et al., 2005). For example, phage display (Smith, 1985) allows physical association of a peptide phenotype displayed on the surface of a phage particle with the corresponding encapsulated DNA genotype. Ribosome display (Mattheakis et al., 1994; Hanes and Plückthun, 1997; He and Taussig, 1997) and mRNA display (Nemoto et al., 1997; Roberts and Szostak, 1997) permit totally *in vitro* construction of larger libraries by linking mRNA with its nascent polypeptide during cell-free translation. DNA display (Doi and Yanagawa, 1999; Yonezawa et al., 2003; Bertschinger and Neri, 2004; Reiersen et al., 2005), as well as phage display, utilizes DNA as a genetic material and thus would be suited for screening of peptides under conditions where RNA is easily degraded, *e.g.*, in the selection of peptide ligands for receptors that are localized on membranes of cell lysates or on whole cells.

When screening combinatorial libraries of polypeptides (phenotype) displayed on their genes (genotype), it is important to establish how best to recover the genotype moiety whose phenotype moiety has bound to the desired target molecules (bait) immobilized on beads or expressed on cells. Most frequently, this is achieved by elution of the whole phenotype-genotype linking molecules with appropriate buffers containing an excess amount of free target, or a suitable pH or salt gradient. However, such methods may fail to release the most interesting binders,

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such as antibodies and peptide inhibitors with very high affinity to the target antigens or enzymes. In addition, it is impossible to prepare elution buffer containing the free competitor when screening endogenous peptide ligands of orphan receptors whose native ligand is unknown. In phage display, several alternative methods using enzymatic cleavage have been developed, either by providing an enzymatic cleavage site between the phenotype and genotype (Orum et al., 1993; Ward et al., 1996), or between the target and its carrier (beads) (Santala and Saviranta, 2004). However, enzymatic reactions need a rather long incubation time and relatively high temperature, and there could be a problem of steric constraints in access of the enzyme to the cleavage site. A disulfide bond between the phenotype and genotype (Löhning, 2001) or between the target and its carrier (Griffiths et al., 1994) has been used for selection of high-affinity antibodies, since it can easily be cleaved by reducing agents. While this approach is attractive, unexpected disulfide bridges may be formed when the displayed proteins contain cysteine, and selection in a reducing environment is impossible. In ribosome display, genotype (mRNA) and phenotype (antibody) are linked with the ribosome and thus the linkage can be easily broken by using an excess of EDTA to remove Mg^{2+}, which is required for stabilization of the ribosome. This represents an advantage of ribosome display for totally *in vitro* selection of high-affinity antibodies (Jermutus et al., 2001; Zahnd et al., 2004), though it remains difficult to select high-stability proteins at high temperature or in the presence of denaturants of ribosomes.

We have developed a DNA display system "STABLE" (Doi and Yanagawa, 1999) and an mRNA display system "*in vitro* virus (IVV)" (Nemoto et al., 1997), and applied them to the epitope mapping of an antibody (Yonezawa et al., 2003), directed evolution of a single-chain antibody (Fukuda et al., 2006) and proteomic studies of protein–protein and protein-DNA interactions (Horisawa et al., 2004; Miyamoto-Sato et al., 2005; Tateyama et al., 2006). In the mRNA display and DNA display technologies, the genotype-phenotype linkage *via* a covalent bond or streptavidin–biotin complex is very strong, even in the presence of heat or denaturants, and thus recovery of the genotypes from selected molecules is difficult. Here, we describe the use of a photocleavable (PC) 2-nitrobenzyl linker (Olejnik et al., 1995, 1996; Bai et al., 2004) between genotype (DNA or mRNA) and phenotype (protein) in our DNA and mRNA display systems, and we show that this technique allows rapid and efficient recovery of selected DNAs or mRNAs (Fig. 1). We confirmed that these photocleavable DNA display and mRNA display systems can be used for *in vitro* selection of an epitope peptide, a recombinant antibody and a drug–receptor interaction from a cDNA library.

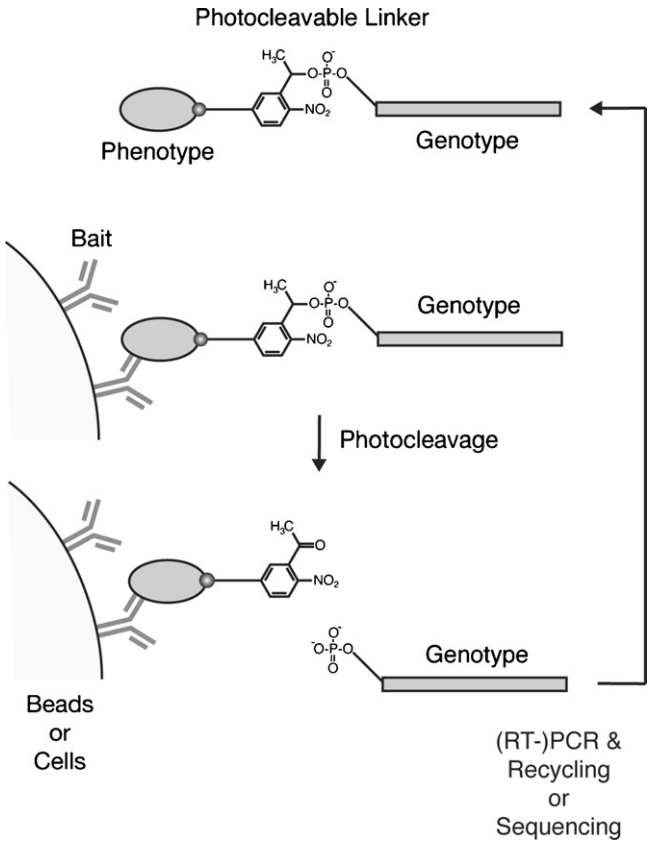

Fig. 1. Physical linkage of phenotype (peptide or protein) and genotype (DNA or mRNA) through a photocleavable 2-nitrobenzyl linker. A library of the phenotype-genotype linking molecules is captured on bait-immobilized beads or cells, and the selected genotype molecules can be easily recovered by photocleavage of the linker from the phenotype molecules that are specifically bound to the beads after washing. The eluted genotype molecules can be amplified by (RT-)PCR and used as the template of protein synthesis for the next round of selection or identified by DNA sequencing.

2. Materials and methods

2.1. Photocleavable reagents

For photocleavable DNA display, a photocleavable biotin phosphoramidite (PCB-phosphoramidite; Glen Research) (Olejnik et al., 1995) was used to synthesize the 5′-PCB-labeled oligonucleotides by usual phosphoramidite chemistry. The synthesis was performed by Tsukuba Oligo Service.

A photocleavable spacer for mRNA display was prepared as follows. Controlled-pore glass support (CPG)-bound protected polyethylene glycol (PEG)-trinucleotide [HO-PEGp-$(dCp)_2$-puromycin-CPG] was synthesized as described previously (Miyamoto-Sato et al., 2003). The PEG-trinucleotide was reacted with photocleavable spacer phosphoramidite (PCp; Glen Research) followed by reaction with fluorescein-labeled thymidine phosphoramidite [T(Fluor)p; Glen Research], deoxycytidine phosphoramidite (dCp), and chemical phosphorylation reagent II (Glen Research). Deprotection and reverse-phase HPLC purification afforded the PC-PEG-Puro spacer [p$(dCp)_2$-T(Fluor)p-PCp-PEGp-$(dCp)_2$-puromycin].

2.2. DNA preparation

A double-stranded DNA fragment encoding the C-terminal 13-aa of streptavidin (STA), a TEV protease cleavage site (ENLYFQG) and the FLAG peptide (DYKDDDDK) was prepared by annealing of SA-T-FLAG-F and SA-T-FLAG-R (Table 1), and inserted into the HincII-XhoI site of pSta4