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"Cleavable" hapten–biotin conjugates: Preparation and use for the generation of anti-steroid single-domain antibody fragments

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ABSTRACT

Antibody engineering technology has the potential to provide artificial antibodies with higher performance than conventional antibodies. Filamentous phage particles are often used to express a vast diversity of mutated antibody fragments from which clones displaying improved fragments can be isolated. We recently showed that hapten–biotin conjugates, combined via a linker involving a reductively cleavable disulfide bond, are useful for isolating phage clones displaying high-affinity anti-hapten antibody fragments. Here we prepare cleavable hapten–biotin conjugates and use them to isolate anti-hapten antibody fragments with relatively low affinities. Three diagnostically important steroids (estradiol-17β [E_2], cortisol, and 17α-hydroxyprogesterone) were each coupled with a biotin derivative containing a disulfide bond. These conjugates could be bound simultaneously by their relevant anti-steroid antibody and NeutrAvidin, and their linkers were easily cleaved by dithiothreitol (DTT) treatment. The E_2–biotin conjugate was used to generate anti-E_2 single-domain antibody fragments (sdAbs). Random point mutations were introduced by error-prone PCR into the gene fragment encoding the V_H domain of a mouse anti-E_2 antibody, and these products were expressed as phagemid particles that were reacted with the E_2–biotin conjugates that had already been immobilized on a solid-phase via NeutrAvidin. Thorough washing off of nonspecific phages and subsequent DTT treatment provided a phagemid clone that displayed a mutated sdAb with improved binding properties.

In many biochemical and biomedical research fields, antibodies are essential tools for monitoring, determining, isolating, and/or inactivating particular molecules. Recent advances in genetic engineering have offered attractive ways in which to generate various types of artificial antibody fragments (e.g., Fabs, single-chain Fvs [scFvs],[1] single-domain antibodies [sdAbs]) that have practical antigen-binding properties and bypass the immunization of animals [1–3]. The smaller molecular mass of these artificial antibody fragments (compared with native immunoglobulin G [IgG] molecules) allows deeper penetration into tissues and faster clearance from the body. Thus, these fragments could be useful not only as analytical tools but also as therapeutic agents. In the most widely used antibody engineering strategies, the gene encoding an antibody fragment is artificially randomized and expressed on filamentous phage particles to construct a library of mutated fragments [4–7]. From this library of antibody fragment-displaying phage or phagemid particles [4,5] (i.e., phage antibodies), clones with desirable binding properties can be selected and isolated using a procedure called biopanning. Antigen-specific phage antibodies are first adsorbed on a solid-phase (where the target antigens have been immobilized) and then, after thoroughly washing off nonspecific phages, are eluted by dissociating the antigen–antibody complexes with a change in pH or the addition of organic solvents or chaotropic agents.

Previously, we reported a new selection method (illustrated in Fig. 1A) that is suitable for isolating phage antibodies with high-affinity against haptens [8]. The background for this study was

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[1] *Abbreviations used:* scFv, single-chain Fv fragment; sdAb, single-domain antibody fragment; IgG, immunoglobulin G; 11-DC, 11-deoxycortisol; E_2, estradiol-17β; CS, cortisol; 17-HP, 17α-hydroxyprogesterone; SIMS, secondary ion mass spectra; FAB–MS, fast atom bombardment mass spectra; THF, tetrahydrofuran; TFA, trifluoroacetic acid; DMF, *N,N*-dimethylformamide; E_2–SS-bio, cleavable estradiol-17β–biotin conjugate; 17-HP–SS-bio, cleavable 17 α -hydroxyprogesterone–biotin conjugate; CS–SS-bio, cleavable cortisol–biotin conjugate; IgM, immunoglobulin M; T-PBS, phosphate-buffered saline containing Tween 20; G-PBS, PBS containing gelatin; DTT, dithiothreitol; sdAb–wt, wild-type sdAb; 2×YT–AG, 2×YT medium containing ampicillin and glucose; IPTG, isopropyl 1-thio-β-D-galactopyranoside; 2×YT–ATG, 2×YT medium containing ampicillin, tetracycline, and glucose; PEG, polyethylene glycol; cfu, colony-forming unit; BSA, bovine serum albumin; M-PBS, PBS containing skim milk; BOC, *tert*-butoxycarbonyl; ELISA, enzyme-linked immunosorbent assay; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; MALDI–TOF, matrix-assisted laser desorption/ionization time-of-flight; CDR, complementarity-determining region; DNP, 2,4-dinitrophenyl; FR, framework region.

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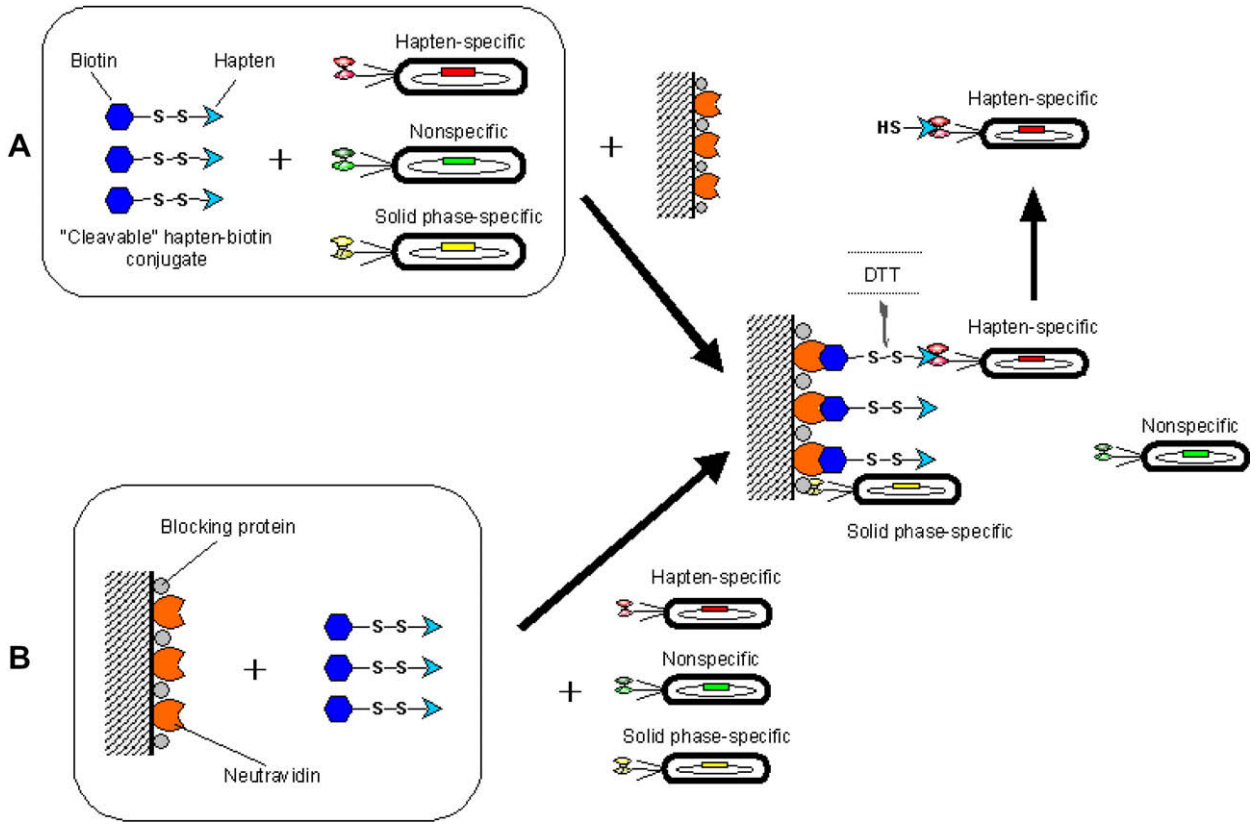

Fig. 1. Schematic representation of the selection procedures (A and B) for the phage clones displaying anti-hapten antibody fragments using cleavable hapten–biotin conjugates.

the need for antibodies against various haptens (e.g., steroids, thyroids, synthetic drugs) that would be available for high-throughput immunoassay systems directed toward clinical diagnosis. 11-Deoxycortisol (11-DC) was chosen as a model hapten and conjugated with biotin via a linker containing a cleavable disulfide bond. Using this "cleavable" hapten–biotin conjugate, we isolated specific phagemids displaying high-affinity anti-11-DC scFv molecules ($K_a = 1.3 \times 10^{10}\ M^{-1}$) from a 100,000-fold excess of nonspecific phagemids [8]. The success of this procedure prompted us to examine whether such conjugates are generally useful for creating other types of mutated anti-hapten antibody fragments.

In the current study, we synthesized three novel cleavable conjugates containing estradiol-17β (E_2), cortisol (CS), or 17α-hydroxyprogesterone (17-HP) as the hapten moiety. These conjugates had suitable biochemical properties to be used as the selecting reagents for antibody engineering. Thus, the E_2–biotin conjugate was used to create E_2-specific sdAbs. sdAbs are antibody fragments containing a single V domain that can be expected to have a molecular recognition unit with much smaller molecular mass than scFvs. Random point mutations were introduced into the gene fragment encoding the V_H domain of a mouse anti-E_2 antibody with error-prone PCR and expressed as phagemid particles. From this mutated sdAb library, despite the fact that anti-hapten sdAbs have an inherently lower affinity ($K_a \sim 10^7\ M^{-1}$) than that of scFvs and IgGs, the cleavable conjugate provided a phagemid clone that displays a mutated sdAb with stronger binding than the wild-type V_H protein.

Materials and methods

Structure identification

^{1}H NMR (500 MHz) spectra were recorded on a Varian VXR-500 FT-NMR spectrometer using tetramethylsilane as the internal reference (δ: 0.00). Secondary ion mass spectra (SIMS) and fast atom bombardment mass spectra (FAB–MS) were obtained on Hitachi M-4100 and JEOL JMSDX303 mass spectrometers, respectively. UV spectra were determined on an Ultrospec 2100 pro spectrophotometer (Amersham Biosciences).

Synthesis of a cleavable estradiol-17β–biotin conjugate

6-Oxoestradiol 6-(*O*-carboxymethyl) oxime (Sigma) (**1**) (Fig. 2A) was converted to its *N*-succinimidyl ester (**2**) according to the previously reported method [9]. *N*-(2-Aminoethyl)carbamic acid *tert*-butyl ester (0.64 ml) was added to crude ester **2** (31 mg) dissolved in tetrahydrofuran (THF)/pyridine (1:1, 1.5 ml) and stirred at room temperature for 1 h. The solution was diluted with AcOEt, washed (chilled 1.7 M HCl, 50 g/L $NaHCO_3$, water, and half-brine), and dried (Na_2SO_4), and then the solvent was evaporated. The residue was chromatographed on silica gel ($CHCl_3$/MeOH, 10:1) to give carbamate (**3**) (40 mg) as an oil. ^{1}H NMR ($C_5D_5N + D_2O$) δ: 0.93 (3H, s, 18-CH_3), 1.47 [9H,s,$COOC(CH_3)_3$], 3.58 and 3.70 (each 2H, m, —$NHCH_2CH_2NH$—), 3.93 (1H, t, J = 8.5 Hz, 17-H), 4.98 (2H, s, —OCH_2CO—), 7.28 (1H, d, J = 9.0 Hz, 1-H), 7.35 (1H, dd, J = 2.8, 9.0 Hz, 2-H), 8.11 (1H, d, J = 2.8 Hz, 4-H); MS (SIMS, m/z): 502 $[M + H]^+$. Trifluoroacetic acid (TFA, 51.4 µl) was added to a solution of compound **3** (35 mg) in CH_3CN (1.0 ml), and the mixture was stirred at 60 °C for 40 min. The reaction mixture was neutralized with 1 M Na_2CO_3, diluted with half-brine, and washed with $CHCl_3$. The aqueous solution was run over a column packed with Supelpak-2 (Supelco). The column was washed with water, and then the adsorbed substances were eluted with MeOH. After removal of the solvent, the residue was chromatographed on silica gel ($CHCl_3$/MeOH/28% NH_3, 4:1:0.3) to give amine (**4**) as an amorphous solid (13 mg). ^{1}H NMR ($C_5D_5N + D_2O$) δ: 0.91 (3H, s, 18-CH_3), 3.91 (1H, t, J = 8.5 Hz, 17-H), 5.00 (2H, s, —OCH_2CO—), 7.29 (1H, dd,