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Phage display aided improvement of a unique prostate-specific antigen (PSA) antibody unreactive with Lys^{145}–Lys^{146} internally cleaved forms

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ABSTRACT

Prostate specific antigen (PSA) is a commonly used marker of prostate cancer. A panel of four kallikrein immunoassays has been reported to improve the prediction of prostate biopsy outcome (cancer vs benign) in men with elevated PSA in the circulation. Assay of one of the kallikrein forms, intact free PSA (fPSA-I), is based on a unique monoclonal antibody (4D4), which is specific for PSA without the internal cleavage at Lys^{145}–Lys^{146}. Due to high dissociation rate the 4D4 antibody is less than optimal for achieving a highly sensitive robust assay. In this study, we cloned the 4D4 Mab into a recombinant fragment (Fab) format and constructed three mutant libraries with the aim to increase its binding affinity. The libraries contained targeted mutations either in the CDR-H1, CDR-H2 or CDR-L3 region. PSA-I specific antibodies were enriched from the libraries by phage display technology. We identified fourteen unique clones with 1–5 mutated amino acids showing reduced dissociation of the PSA conjugate compared to the wt-4D4 Fab. Five of these mutant antibodies had 2–6 times higher binding affinity compared to the wt-4D4 Fab yet retaining the original specificity for PSA-I. The analytical sensitivity of fPSA-I assay with mutant L3-2 Fab was 0.12 μg/L compared to 4.46 μg/L with the original wt-4D4 Fab. In the method comparison study, the developed assay showed an excellent correlation to the existing fPSA-I assay. The high affinity and specificity of these mutant antibodies have potential to provide sensitive and robust detection of intact and nicked PSA from patient samples in different test formats.

1. Introduction

Prostate specific antigen (PSA) is a member of the kallikrein-related peptidase family and a commonly used marker for the detection of prostate cancer. It is an androgen-regulated serine protease and its main function is to cleave biological substrates in the seminal coagulum (Lilja, 1985). In blood, PSA can be found in complexes with the protease inhibitors, mainly α1-antichymotrypsin and α2-macroglobulin, or in a non-complexed free form (Lilja et al., 1991; Stenman et al., 1991). Several subforms of free PSA (fPSA) have been identified, such as the inactive precursor of PSA, proPSA, free intact PSA (fPSA-I) and free nicked PSA (fPSA-N), which are associated with either prostate cancer or benign prostate hyperplasia (BPH) (Nurmikko et al., 2001; Mikolajczyk et al., 2002). The most common site for internal nicking lies between lysines 145 and 146 (Christensson et al., 1990; Noldus et al., 1997). A unique monoclonal antibody (Mab) 4D4 is specific for fPSA-I and does not recognize the nicked fPSA-N form that is internally cleaved at Lys^{145}–Lys^{146}. This antibody has been used in an immunoassay to measure fPSA-I (Nurmikko et al., 2000, 2001). The measured concentrations of fPSA-I and calculated fPSA-N concentration (by subtracting the fPSA-I from the total fPSA concentration) have provided statistically significant discrimination between BPH and prostate cancer in earlier studies (Nurmikko et al., 2001; Steuber et al., 2002). fPSA-N is more closely associated with BPH and prostate volume, whereas fPSA-I correlates with the total tumor volume in prostate cancer lesions (Steuber et al., 2005). Importantly, in a panel of four immunoassays of kallikrein forms, fPSA-I plays a central role in predicting the biopsy outcome in men with moderately elevated PSA (Vickers et al., 2008).

Affinity is a central determinant of immunoassay performance as it together with unspecific binding and the specific activity of the label sets the limit for assay sensitivity (Ekins, 1989). Due to the moderate binding affinity of the 4D4 Mab (Nurmikko et al., 2000) and its fast dissociation rate, it cannot capture the target antigen (PSA-I) tightly, or in the indirect fPSA-N assay, cannot block all PSA-I present in the sample (Peltola et al., 2011). Phage display provides an efficient tool to improve the binding properties of an antibody by isolating the

Abbreviations: PSA, prostate-specific antigen; fPSA, free prostate-specific antigen; fPSA-I, intact free prostate-specific antigen; fPSA-N, internally cleaved "nicked" free prostate-specific antigen; proPSA, pro-form of prostate-specific antigen; BPH, benign prostate hyperplasia; Mab, monoclonal antibody; Fab, fragment antigen-binding; BSA, bovine serum albumin; CDR, complementarity determining region; RAM, rabbit anti-mouse IgG; EDTA, ethylenediaminetetraacetic acid; DTPA, diethylene triamine pentaacetic acid.

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antibody fragments with desired characteristics from large libraries of variants generated from the parental antibody by mutagenesis (Winter et al., 1994; Saviranta et al., 1998; Korpimäki et al., 2004a). In this study, phage display was applied to improve the affinity of the antibody 4D4 to fPSA-I.

2. Materials and methods

2.1. Reagents

2.1.1. Bacterial strains and helper phage

Used *Escherichia coli* strains were XL1-Blue (Stratagene, La Jolla, CA, USA), SS320 also known as MC1061 F′ (Sidhu et al., 2000) and BL21 (New England Biolabs, Ipswich, MA, USA). Helper phage VCSM13 (Stratagene, La Jolla, CA, USA) was used in the phage production.

2.1.2. Panning buffers and culture media

Buffers used in phage display selections were TBS (Tris–HCl, pH 7.5, 150 mM NaCl), TBT-0.1 (TBS, 1% BSA and 0.1% Tween-20) and TBT-0.05 (TBS, 1% BSA and 0.05% Tween-20). The Luria-broth agar (LA) plate, Super broth (SB) medium and SOC medium were prepared as described (Sambrook et al., 1989).

2.1.3. Biotinylated reagents

Anti PSA monoclonal antibodies (Mab) H117 (Abbott Laboratories, Abbott Park, IL, USA) and 4D4 (Nurmikko et al., 2000) were biotinylated with biotin isothiocyanate synthesized at the Department of Biotechnology, University of Turku, Finland, using procedure described earlier (Eriksson et al., 2003). Recombinant 5A10 Fab (Eriksson et al., 2000) and 4D4 Fab fragments were biotinylated site-specifically at an unpaired cysteine residue genetically introduced to the C-terminal end of the Fd chain (Eriksson et al., 2000). The cysteine residue was reacted with maleimide-PEG_2-biotin (Thermo Scientific, Rockford, IL, USA) during purification in the Ni-NTA column using procedure described earlier (Korpimäki et al., 2004b).

2.1.4. Labeled reagents

Anti-phage Mab 9E7 (Department of Biotechnology, University of Turku), 5A10 Mab (Lilja et al., 1991), wild-type pro-PSA (wt-PSA) (Bowman et al., 2000), rabbit anti-mouse IgG (DAKO, Glostrup, Denmark) and wt-4D4 Mab and Fab were labeled with Eu (N1)-chelate using procedure described earlier (Väisänen et al., 2006).

2.1.5. Microtiter plates and buffers for immunoassays

Immunoassays were carried out on rabbit anti-mouse IgG (RAM) and streptavidin-coated microtiter plates (Kaivogen, Turku, Finland) in 200 μl volume, with slow shaking at room temperature unless otherwise stated. Plates were washed in Delfia plate washer (Perkin-Elmer Life Sciences, Turku, Finland) with wash solution (Kaivogen, Turku, Finland). Time-resolved fluorescence signal from Europium was measured with Victor 1420 multilabel counter (Perkin-Elmer Life Sciences, Turku, Finland) after 15 min incubation at room temperature with enhancement solution (200 μl/well). Enhancement solution (1.4 g/L potassium hydrogen phthalate, 0.02 g/L 4,4,4-trifluoro 1-(2-naphthyl)-1,3-butanedione, 0.11 g/L trioctylphosphine oxide, 5.9 g/L Triton X-100, 5.6 ml/L ethanol and 5.8 ml/L acetic acid) was prepared at the Department of Biotechnology, University of Turku, Finland. Bovine serum albumin (BSA) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Buffers used in immunoassays were: 1) Kaivogen (KG) assay buffer, pH 7.75 (Kaivogen, Turku, Finland). 2) MES assay buffer: 50 mM 2-(N-morpholino) ethanesulfonic acid (MES), pH 6.75, 0.9% NaCl, 0.01% Tween-40, 0.5% BSA. Denatured mouse IgG (25 μg/ml) was added as a blocking component in the Kaivogen assay buffer and MES assay buffer for correlation assay. Mouse IgG (native) was obtained from Meridian Life Science (Memphis, TN, USA) and denaturation was performed by heating to 63 °C for 30 min.

Buffers for pH optimization were: 1) MES buffer, pH 6.0–6.75: 50 mM MES (pH 6.0, 6.63, and 6.75), 0.9% NaCl, 0.01% Tween-40, 0.05% BSA. 2) Tris buffer, pH 7.0–8.5: 50 mM Tris (pH 7.0, 7.25, 7.5, 8.0, 8.25 and 8.5), 0.9% NaCl, 0.01% Tween-40, 0.05% BSA.

Unless otherwise stated, incubations in microtiter plate immunoassays were performed in Kaivogen assay buffer (Kaivogen, Turku, Finland).

2.2. Construction of mutated 4D4 Fab libraries

Recombinant 4D4 Fab fragment was cloned from the monoclonal antibody 4D4 producing hybridoma cell line (Nurmikko et al., 2000) by reverse transcription-PCR and cloned into bacterial expression vector pAK400 (Krebber et al., 1997). Three Fab libraries, each containing mutations in one complementarity determining region (CDR), CDR-L3, -H1 or -H2, were constructed by oligonucleotide-directed mutagenesis. For this, the cloned 4D4 Fab was PCR amplified in two parts: 1) Using Acc65Ifor and the 1st randomizing oligo (EB173_4D4_H2REV, EB175_4D4_H1REV or EB179_4D4_L3REV for CDR-H2, -H1 and -L3 libraries, respectively). 2) Using the 2nd randomizing oligo (EB172_4D4_H2FOR, EB174_4D4_H1FOR or EB179_4D4_L3REV for CDR-H2, -H1 and -L3 libraries, respectively) and pAK400rev (Table 1). The PCR products were digested with *Bsp*QI and joined by ligation to complete Fab gene. The Fab cassette was PCR amplified from the ligation mixture using primers Acc65Ifor and pAK400rev, digested with *Sfi*I and ligated with 1.3 μg of *Sfi*I digested vector pEB32x (Huovinen et al., 2013). The ligation product was electroporated into *E. coli* SS320 cells, which were then infected with VCS M13 helper phage and induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) to produce phage display libraries using procedures described earlier (Brockmann et al., 2011).

2.3. Panning of mutant libraries

The libraries were subjected to three rounds of phage display selection against wt-PSA. First the libraries ($0.5 - 1 \times 10^{11}$ phages in 1 ml of TBT-0.05) were negatively selected against streptavidin and H117 Mab by incubating 1 h on streptavidin-coated plate and subsequently on streptavidin microtiter plate bound with biotinylated H117 Mab, after which native mouse IgG (20 ng/ml) and biotin-blocked streptavidin (250 ng/ml) were added as blockers. The preselected libraries were then incubated for 1 h with wt-PSA (40 ng/ml) and PSA bound phages were captured for 1 h by M-280 streptavidin beads (Dynal, Oslo, Norway) immobilized with 130 ng of biotinylated H117 Mab. Beads were collected with magnet and washed two (rounds 1 and 2) or three times (round 3) with 1 ml of TBT-0.1 and subsequently once with TBS. Phages were eluted with 100 mM HCl and neutralized with Tris. XL1-Blue cells were infected with the eluted phages and new phage stocks were produced as described previously (Brockmann et al., 2011). Panning output was calculated by plating aliquots of the infected cells. Background was checked from an identical panning without PSA.

2.4. Screening of soluble Fabs for affinity

For screening, individual clones were picked up from the output plates and cultured on a 96-well culture plate (Sarstedt, Newton, NC, USA) in 200 μl of SB medium containing 0.05% glucose, 10 μg/ml tetracycline and 25 μg/ml chloramphenicol. After 3 h at 37 °C, 700 rpm, 100 μM IPTG was added and Fab was produced overnight at 26 °C, 700 rpm. Periplasmic Fab was released from the cells by a freeze–thaw cycle and 30 min incubation at room temperature after adding 70 mM Tris, pH 8.0, 2 mg/ml lysozyme and 2 mM EDTA. Cell debris was removed by centrifugation. Fab containing supernatant was diluted