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The epitope for the polyol-responsive monoclonal antibody 8RB13 is in the flap-domain of the beta-subunit of bacterial RNA polymerase and can be used as an epitope tag for immunoaffinity chromatography

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ABSTRACT

Polyol-responsive monoclonal antibodies (PR-mAbs) are useful for the purification of proteins in an easy, one step immunoaffinity step. These antibodies allow for gentle purification of proteins and protein complexes using a combination of a low molecular weight polyhydroxylated compound (polyol) and a non-chaotrophic salt in the eluting buffer. mAb 8RB13 has been characterized as one of these PR-mAbs and has been used to purify RNA polymerase from five species of bacteria. Here the epitope for 8RB13 has been identified as PEEKLLRAIFGEKAS, a sequence that is highly conserved in the β-subunit of bacterial RNA polymerase. This sequence is located in the "beta-flap" domain of RNA polymerase (and essentially comprises the "flap-tip helix"), an important binding site for sigma70. This location explains why only the core RNAP is purified using this mAb. This amino acid sequence has been developed into an epitope tag that can be used to purify a target protein from either bacterial or eukaryotic cells when genetically fused to a protein of interest.

Introduction

Polyol-responsive monoclonal antibodies (PR-mAbs)[1] are extremely useful in the gentle purification of proteins and protein complexes in their native form while allowing the proteins and protein complexes to retain activity. Many immunoaffinity chromatography procedures use harsh conditions (e.g., low pH or chaotropic reagents) to release the antigen from the antibody. These conditions can result in the protein of interest being denatured. The PR-mAb allows for a simple, gentle elution of the protein of interest by using buffers containing low molecular weight polyhydroxylated compounds (polyols) and non-chaotropic salt [1–3]. It is currently unknown why some mAbs are polyol-responsive and some are not, but the epitopes for PR-mAbs (designated "softags") can be used as a way to tag and purify proteins of interest without affecting the protein's function [4,5].

In bacterial cells, the RNA polymerase (RNAP) can be in either the core enzyme form or in one of the holoenzyme forms. Core RNAP consists of five subunits (β′, β, α_2, and ω). A holoenzyme contains the core subunits plus a sigma factor (β′, β, α_2, ω, and σ); the sigma factor lends specificity to the RNAP to bind to and initiate transcription from different classes of promoters. In *Escherichia coli*, there are seven sigma factors; thus, there are seven potential holoenzymes. We have isolated many mAbs that react with the β-subunit, but mAb 8RB13 has proven to be particularly interesting because it is polyol-responsive, and it reacts with the β-subunit of RNAP from many bacterial species [6]. Using these properties, we have purified core RNAP from *E. coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Streptomyces coelicolor* [6], *Shewanella oneidensis* [7], and *Clostridium difficile* (our unpublished data). However, immunoaffinity chromatography with mAb 8RB13 isolates only the core form of RNAP from all of these bacteria despite the presence of a holoenzyme.

High-resolution crystal structures of core RNA polymerase, the holoenzyme, and enzyme/DNA complexes have become available (reviewed in [8]). This study sought to identify the epitope for mAb 8RB13 to understand why the holoenzyme cannot be purified using this immunoaffinity column. In addition, we were able to demonstrate that fusing this epitope tag to a target protein allows the target protein to be purified from either bacterial extracts or from extracts from mammalian cell culture. By developing a novel epitope tag (designated "softag4") from the 8RB13 epitope we increase the flexibility of this system to include purification of proteins expressed in eukaryotic cell lines.

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[1] *Abbreviations used*: PR-mAbs, polyol-responsive monoclonal antibodies; RNAP, RNA polymerase; IPTG, isopropylthiogalactoside; ELISA, enzyme-linked immunosorbent assay; PEI, polyethyleneimine; PG, propylene glycol; TFIIB, human transcription factor IIB; PCR, polymerase chain reaction.

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Materials and methods

Plasmids and constructs

The plasmids used in this study are listed in Table 1. Plasmids TA501 and TA502, containing the coding regions for the *E. coli* β-subunit in the pET28b vector (Novagen, Madison, WI), have been described previously [9]. Plasmid pTA501 contained a N-terminal His_6-tag and pTA502 contained a C-terminal His_6-tag [9]. Plasmid pLN06 was constructed by amplifying nucleotides 2364–2870 of the β-subunit by polymerase chain reaction (PCR), using plasmid TA501 as a template. An oligo (5′-cat gcc atg gca tgt gtg tct ctg ggt gaa ccg) containing a *Nco*I site (underlined) on the 5′-end and 15 nucleotides specific to nucleotides 2364–2379 served as the forward primer; an oligo (5′-cgc gga tcc gcc atc gcg agt aaa gac) containing a *Bam*H1 site (underlined) and 15 nucleotides specific to nucleotides 2855-2870 served as the reverse primer. The amplified product was then ligated in frame at the *Nco*1 site contained in the N-terminal domain of human transcription factor IIB (TFIIB), and the *Bam*H1 site contained in vector pET11a. This created a fusion protein, containing amino acids 1–124 of TFIIB and amino acids 770–938 of the β-subunit (Fig. 2A). Plasmid pLH07 was created by subcloning the fusion protein from pLN06 (*Nde1*/*BamH1*) into pET33b. Plasmids containing epitope-tagged GFP and YFP for over-expression and purification are described below.

Protein expression

Prokaryotic

Plasmids were transformed into *E. coli* BL21(DE3) *pLysS* (Novagen). Bacteria were cultured in LB broth containing 30 μg/ml chloramphenicol and either 100 μg/ml ampicillin (pET11a vector) or 35 μg/ml kanamycin (pET28b and pET33b vectors) at 37 °C with shaking. Protein expression was induced when the culture reached an O.D. (600 nm) of 0.6 by the addition of 1 mM isopropylthiogalactoside (IPTG) and the cells were harvested 2.5–3.0 h later. For epitope-tagged GFP, *E. coli* BL21(DE3) *pLysS* containing over-expressed GroEL and GroES [10] was transformed with the epitope-tagged GFP contained in the pET11a plasmid. The bacteria were cultured in LB broth containing ampicillin at 26 °C until the optical density (600 nm) reached 0.3; a 10% solution of arabinose was added to achieve a 0.1% solution to induce the GroEL/GroES chaperones. The bacteria were then cultured until the O.D. reached 0.6, and the epitope-tagged GFP was induced by the addition of 1 mM IPTG. The cells were then cultured at 26 °C for 3 h, harvested by centrifugation and the pellet was frozen at −80 °C until use.

Eukaryotic

Plasmids were transfected into HEK293 cells using TransIT-LT1 transfection reagent (MirusBio, Madison, WI) using the manufacturer's recommended protocol, and harvested after 48 h. HEK293 cells were cultured in DMEM with 10% FBS and 1% Pen/Strep + L-glutamine. Cells were then resuspended in lysis buffer (50 mM Tris–HCl pH 7.4, 0.5 M NaCl, 1 mM EDTA, 10% glycerol, 1 mM DTT, and complete protease inhibitor cocktail from Roche) and placed in an ethanol/dry ice bath for 5 min; the cells were returned to the 37 °C water bath for 1 min. The freeze–thaw procedure was performed for a total of three cycles.

Antibodies

The preparation of mAb 8RB13 has been described [6]. mAb IIB8, that reacts with amino acids 61–68 of human TFIIB, has been described [5].

ELISA-elution assay

The polyol-responsive properties of mAb 8RB13 were observed by using a modified enzyme-linked immunosorbent assay (ELISA), termed ELISA-elution assay as described previously [1,2]. Briefly, antigen was immobilized on the wells of a polystyrene microtiter plate and the plates were blocked. After incubation with the mAb, the wells were treated with 100 μl of TE buffer (50 mM Tris–HCl and 0.1 mM EDTA, pH 7.9) containing varying amounts of ammonium sulfate (0–0.75 M) and propylene glycol (0–40%). After washing, the enzyme-labeled secondary antibody was applied, and then, after incubating and extensive washing, the substrate was applied. A polyol-responsive signal is defined as a reduction in signal of at least 50% in wells treated with buffer containing polyol and salt compared to those treated with TE buffer alone.

Antibody production, purification, and conjugation

mAb 8RB13 was produced in continuous culture, using INTEGRA CELLine CL350 Flasks (distributed by Argo, Elgin, IL) as described [3]. Antibody was harvested every 3–4 days by removing the contents of the cell compartment, centrifuging the contents at 1500 rpm, removing the supernatant, and storing it at −20 °C. The cells were then diluted 1:4 with medium, and 5 ml of the cells were returned to the growth chamber. The chamber was maintained for about 30–40 days.

Supernatant samples collected from the CELLine flasks were tested for 8RB13 antibody production using an ELISA and *E. coli* core RNAP as an antigen [1]. Samples that titered out to at least 1:12,000 were pooled for purification. A typical purification used about 20 ml of the pooled cell culture supernatant. A saturated solution of ammonium sulfate was added to the supernatant to achieve 40% saturation (at 4 °C) and stirred for 20 min. The precipitate was collected by centrifugation (20 min at 7000 rpm at 4 °C). The supernatant was removed, and the pellet was resuspended in 5 ml of antibody buffer (50 mM Tris–HCl, pH 6.9, containing

Table 1
Plasmids used in this study.

Plasmid designation	Construct description	Description of protein produced	Reference
pTA501	Nucleotides 1–1342 of the β-subunit of *E. coli* RNAP in pET28b	Full-length β-subunit with N-terminal His_6-tag	9
pTA502	Nucleotides 1–1342 of the β-subunit of *E. coli* RNAP in pET28b	Full-length β-subunit with C-terminal His_6-tag	9
pLN06	Nucleotides 2364–2870 of the β-subunit inserted into the *Nco*1 site of human TFIIB and the *Bam*H1 site in pET11a	Fuses aa 770–938 of the β-subunit to aa 1–24 of human TFIIB	This study
pLN07	*Nde*1/*Bam*H1 (complete fusion construct from pLN06) cloned into pET33b	Fusion protein from pLN06, making it amenable to Exo III digestion	This study
pNT103	Oligo encoding mAb 8RB13 epitope inserted 3′ to GFP in pET11a	Fuses aa 897–911 of the β-subunit to C-terminus of GFP	This study
pBS1	Oligo encoding mAb 8RB13 epitope inserted 3′ to YFP in pcDNA3.1(+)	Fuses aa 897–911 of the β-subunit to the C-terminus of YFP	This study