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Observations on different resin strategies for affinity purification mass spectrometry of a tagged protein

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ABSTRACT

Co-affinity purification mass spectrometry (CoAP-MS) is a highly effective method for identifying protein complexes from a biological sample and inferring important interactions, but the impact of the solid support is usually not considered in design of such experiments. Affinity purification (AP) experiments typically utilize a bait protein expressing a peptide tag such as FLAG, c-Myc, HA or V5 and high affinity antibodies to these peptide sequences to facilitate isolation of a bait protein to co-purify interacting proteins. We observed significant variability for isolation of tagged bait proteins between Protein A/G Agarose, Protein G Dynabeads, and AminoLink resins. While previous research identified the importance of tag sequence and their location, crosslinking procedures, reagents, dilution, and detergent concentrations, the effect of the resin itself has not been considered. Our data suggest the type of solid support is important and, under the conditions of our experiments, AminoLink resin provided a more robust solid-support platform for AP-MS.

1. Introduction

Co-affinity purification mass spectrometry (CoAP-MS) is a highly effective method for isolating and identifying protein interactions from a complex biological sample [1–7]. CoAP-MS uses a solid-support affinity system to selectively bind the protein of interest [8,9]. Successful CoAP-MS experiments require affinity purification (AP) of a bait protein, which is often achieved using well documented high-affinity antibodies to unique peptide sequence tags such as FLAG (DYKDDDDK) [10,11], c-Myc (EQKLISEEDL) [12], HA (YPYDVPDYA) [13,14,15] or V5 (GKPIPNPLLGLDST) [16]. Protein expression with these tags enables selective isolation without requiring an antibody specific for the bait protein [3,13,17]. Evaluation of the AP of the bait protein by mass spectrometry is useful to identify factors influencing the AP procedure, which is the focus of this article. For clarity, we denote AP-MS to be consistent with the alternative IP and CoIP terminology.

Immobilization of the antibody to a support resin enables selective enrichment of the tagged protein through noncovalent [18,19,20] or covalent methods [21,22]. Noncovalent immobilization usually uses Protein A and G covalently attached to a resin matrix (agarose, sepharose, or paramagnetic bead) [23,24]. Unfortunately, elution conditions that destabilize the antibody-antigen interaction often destabilize the Protein A/G-antibody interaction, resulting in co-elution of the antibody. Diverse covalent antibody-immobilization strategies include prelinked resin-antibody or resin-protein conjugate beads (for example, EZ View and Streptavadin-Sepharose), use of a bifunctional crosslinking agent such as dimethyl pimelimidate (DMP) or bis(sulfosuccinimidyl) suberate (BS^3) to couple the antibody to Protein A and G, or beads with functionally derivatized surfaces (acids, amines, maleimides, succiminidyl esters, epoxy, or aldehydes) [25–29]. Each strategy has unique advantages and challenges that changes antibody immobilization and subsequent affinity purification of the bait protein.

The impact of the solid-support resin is often overlooked. In this article, we evaluated Protein A/G Agarose, Protein G Dynabeads and AminoLink aldehyde-functionalized resins under different crosslinking conditions (Fig. 1) using two protein tag strategies: Anti-FLAG M2 Clone antibody/N-terminal tagged FLAG-p53 (N-FLAG-p53) and Anti-HA antibody/N-terminal tagged HA-PTEN (N-HA-PTEN), both expressed in HEK293 cells. PTEN and p53 are tumor suppressor proteins mutated in many cancer types and have several known protein interactions. Confining our experiments to these

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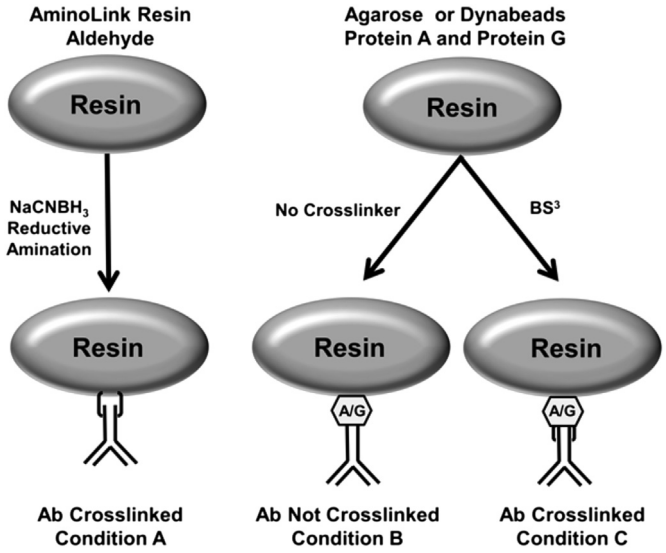

Fig. 1. Schematic representation of AminoLink (Left), Agarose, and Dynabead (Right) resins and conditions used in these experiments. AminoLink resin uses reductive amination between resin-linked aldehyde and antibody amine functional groups to immobilize antibodies (Condition A). Agarose and Dynabeads were covalently linked to Protein A and Protein G, which enables non-covalent attachment to antibodies (Condition B) or the addition of crosslinkers including BS^3 for covalent attachment through Protein A and G (Condition C). The antibody-coupled resins were used for affinity purification of either FLAG-tagged p53 or HA-tagged PTEN proteins under identical conditions. Conditions A, B, and C and affinity purification conditions are described in Materials and Methods.

specific antibody/labeled protein pairs, we were able to compare widely-used commercial solid-supports under similar conditions. Our initial AP-MS experiments used widely available epoxy-functionalized Dynabeads. However, subsequent experiments over 6–8 weeks exhibited progressively reduced affinity purification, likely because of epoxide hydrolysis. This prompted us to evaluate Protein A/G Agarose, Protein G Dynabeads and AminoLink aldehyde-functionalized resins to optimize resin immobilization, protein binding, and elution conditions using N-FLAG-p53 as a model. We then used N-HA-PTEN for more detailed comparisons.

We note that all tested resins can perform adequately for AP-MS under compatible conditions, but require optimization of tag, crosslinking strategies, lysis buffers, and binding and elution conditions [6,7]. However, under the conditions of our experiments, AminoLink resin provided a more robust platform for affinity purification of tagged proteins. The objective of this article is to provide insight into our experimental observations and discuss the impact of solid-support resins on AP-MS experiments.

2. Material and methods

2.1. Antibodies and reagents

PTEN and p53 antibodies were purchased from Cell Signaling Technology (p53 #9282 Rabbit Ab, PTEN (D4.3) XP (R) #9188 Rabbit mAb). Anti-FLAG antibodies were purchased from Cell Signaling Technology (DYKDDDDK Tag 9A3 #8146 Mouse Ab) or from Sigma Aldrich (FLAG Ab #F1804 Mouse M2 Clone). HA and V5 antibodies were purchased from Sigma Aldrich (HA #H3663 Mouse HA-7 Clone, V5 #V8012 Mouse V5-10 Clone). Mouse monoclonal His.H8 antibody was purchased from Abcam. Secondary HRP conjugated anti-rabbit and anti-mouse antibodies were purchased from Cell Signaling Technology (Anti-Mouse IgG HRP-linked Antibody #7076S, Anti-Rabbit IgG HRP-linked Antibody #7074S). Buffers, reagents, chemicals were purchased from Sigma Aldrich, Fisher, or VWR and were ACS certified reagent grade or better. Solvents were purchased from Fisher and were HPLC grade except for solvents used in mass spectrometry, which were LC/MS Grade. The M-270 Epoxy Dynabeads Co-IP Kit 14321D, Reagents, and Protein G Dynabeads were purchased from Life Technologies. Protein A/G PLUS-Agarose resin (SC-2003) was purchased from Santa Cruz Biotechnologies. AminoLink was purchased from Thermo Scientific. Ni-NTA spin columns (300 μg protein binding capacity) were purchased from Qiagen. Spin columns were purchased from Pierce.

2.2. Cell culture and transfection

HEK293 cells were purchased from ATCC and maintained in DMEM/High Glucose media with 10% fetal bovine serum and 1% L-Glutamine-Penicillin-Streptomycin at 37 °C in 5% CO_2. Cells were seeded in T75 mm^2 flask such that it will be 70%–90% confluent after 24 h of incubation. 15–20 μg of plasmid DNA and Lipofectamine® 2000 (Invitrogen) at a ratio of 1:3 was used for transfection following manufacturer's instruction. 500 μL of Opti-MEM medium was used to resuspend both plasmid and Lipofectamine® 2000. Before transfection, the medium was changed to serum free and antibiotic free DMEM, DNA/lipid complex was added and further incubated for 24 h.

2.3. Cell lysate preparation and preclearing for AP-MS

After transfection, cells were trypsinized, washed two times with Phosphate Buffered Saline (PBS), then lysed in 1 mL of 1× IP Buffer (M-270 Epoxy Dynabeads CoIP Kit 14321D, Life Technologies) to which we added 1× Halt Protease inhibitor cocktail (ThermoScientific) on ice for 30 min with occasional vortexing. Lysate was centrifuged at 13,000 rpm for 10 min at 4 °C and transferred to new LoBind Eppendorf tube. Total protein concentration was estimated using absorbance at A_{280}. The cell lysate was precleared by incubation at 4 °C for 2 h with 100 μL of Protein A/G agarose beads.

2.4. Preparation of soluble protein extracts of E. coli MG 1655 for Rho experiments

A single colony of *E. coli* MG1655 was inoculated in 100 mL starter culture of LB broth at 37 °C. The culture was collected during early stationary growth phase (1.4 O.D. at 550 nm). Cells were harvested by centrifugation at 4 °C at 14,000 rpm for 10 min and resuspended in a total of 30 mL of 50 mM Tris HCl, 0.1 M NaCl pH 7.8 with protease inhibitors. The cells were kept on ice and subjected to French press disruption followed by sonication using a probe sonicator in ice for two 10 s periods to reduce viscosity. The cell debris was removed by centrifugation at 40,000 rpm for 30 min and supernatants from the centrifugation were stored in 1.5 mL aliquots at −80 °C.

2.5. Resin loading and binding capacity standardization

Resin loading capacity for resins was first determined based on product literature for each resin: AminoLink resin at 10 μg antibody/mg of resin, Protein G Dynabeads at 8 μg antibody/mg resin, Protein A/G resin at 10 μg antibody/mg of resin, M-270 Epoxy Dynabeads at 5–7 μg antibody/mg of resin. The estimated resin loading was lowest for M-270 Epoxy Dynabeads and Protein G Dynabeads, we initially coupled 8 μg of antibody to each resin and the extent of coupling was determined by UV absorbance at 280 nm. Coupling efficiencies were measured at >60% for multiple