QIAGEN News

Interactions of 6xHis-tagged protein kinase A catalytic subunit examined using Ni-NTA Magnetic Agarose Beads

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The cAMP-dependent protein kinase A (PKA) phosphorylates key cell proteins thereby mediating the majority of the known effects of cAMP in eukaryotic cells. As a heterotetramer consisting of a regulatory (R) dimer and two monomeric catalytic (C) subunits, the enzyme is inactive. In response to nanomolar concentrations of the second messenger cAMP, the R-subunits dissociate and the C-subunits become enzymatically active. It has been shown previously that the R-subunits (1, 2) as well as the heat-stable protein kinase inhibitor PKI (3) bind to the C-subunit of PKA. Recently, we have established a fast and easy-to-use method for screening physiological binding partners using Ni-NTA Magnetic Agarose Beads together with a 6xHis-tagged C-subunit of PKA. Here we demonstrate the interaction of the C-subunit with three physiological inhibitors: the R-subunits type I and II (RI and RII), and the heat-stable protein kinase inhibitor (PKI).

Materials and methods

Protein purification

The C-subunit was overexpressed in *E. coli* as a 6xHis-tagged fusion protein and purified by affinity chromatography with Ni-NTA Agarose (4). R-subunits were overexpressed in *E. coli* and purified without a fusion tag by ion-exchange chromatography (5). PKI was purified as a GST-fusion protein on glutathione agarose.

Magnetocapture assay

6xHis-tagged C-subunit was diluted in Binding Buffer A (50 mM Na_2HPO_4, 300 mM NaCl, 20 mM imidazole, 0.05% Tween® 20; pH 8.0) or B (Binding Buffer A, 1 mM ATP, 10 mM $MgCl_2$) to a final concentration of 120 nM (2 µg in 400 µl buffer). A two-fold molar excess of the inhibitors, RI-subunit, RII-subunit, or PKI was added. Since binding of the C-subunit to the RI-subunit and PKI strongly depends on the presence of MgATP (1), 1 mM ATP and 10 mM $MgCl_2$ were included in binding and wash buffers for assays with RI and PKI. The R-subunits were added in the presence or absence of 100 µM cAMP. After addition of 10 µl Ni-NTA Magnetic Agarose Bead suspension, the tubes were incubated for 5 min at room temperature with gentle mixing. After binding, the tubes were placed on a magnetic separator to collect the magnetic beads on the sides of the tubes. This allowed efficient removal of the supernatants without any loss of material. A wash step was performed with 200 µl binding buffer and gentle mixing. This significantly reduced non-specific binding of nontagged proteins to the Ni-NTA Magnetic Agarose Beads. Another wash step with 100 µl Buffer C (20 mM MOPS, pH 7.0; 150 mM KCl) for the RII subunit, or Buffer D (Buffer C, 1 mM ATP, 10 mM $MgCl_2$) for the RI-subunit and PKI, reduced the high salt concentration which can cause diffused bands in SDS-PAGE. The magnetic beads with the bound proteins were boiled in 15 µl SDS-PAGE sample buffer for 2 min at 95°C and the proteins were analyzed by SDS-PAGE. ▶

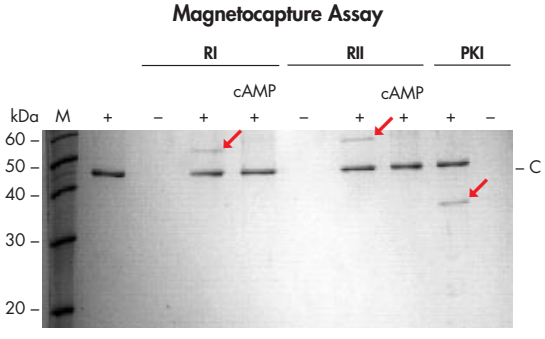

Figure 1 *The magnetocapture assay was performed as described in materials and methods. The inhibitors indicated: RI-subunit (**RI**), RII-subunit (**RII**), or protein kinase inhibitor (**PKI**) were incubated with Ni-NTA Magnetic Agarose Beads with (+) or without (–) captured 6xHis-tagged C-subunit (**C**). cAMP was included where indicated (**cAMP**). Specifically bound inhibitors are indicated by arrows. Sizes of marker proteins (**M**) are indicated.*

References

1. *Herberg, F.W. and Taylor, S.S. (1993) Physiological inhibitors of the catalytic subunit of cAMP-dependent protein kinase: effect of MgATP on protein–protein interactions. Biochemistry* ***32****, 14015.*
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3. *Walsh, D.A., Angelos, K.L., Van Patten, S.M., Glass, D.B., and Garetto, L.P. (1990) The inhibitor protein of the cAMP-dependent protein kinase. In: Kemp, B.E. ed. Peptides and Protein Phosphorylation, Boca Raton: CRC Press, p. 43.*
4. *Narayana, N., Cox, S., Shaltiel, S., Taylor, S.S., and Xuong, N. (1997) Crystal structure of a polyhistidine-tagged recombinant catalytic subunit of cAMP-dependent protein kinase complexed with the peptide inhibitor PKI (5–24) and adenosine. Biochemistry* ***36****, 4438.*
5. *Saraswat, L.D., Filutowicz, M., and Taylor, S.S. (1986) Expression of the type I regulatory subunit of cAMP-dependent protein kinase in* Escherichia coli. *J. Biol. Chem.* ***261****, 11091.*
6. *Roskoski, R., Jr. (1983) Assays of protein kinase. Methods Enzymol.* ***99****, 3.*

Kinase activity assay

Phosphotransferase activity of the 6xHis-tagged C-subunit was assayed as described by Roskoski (6) using the phosphate-acceptor heptapeptide, Kemptide (LRRASLG), as substrate. After capture of the C-subunit, Ni-NTA Magnetic Agarose Beads were resuspended in 30 µl Buffer D. A sample of the suspension (10 µl) was transferred to assay mix to give a reaction volume of 100 µl (final composition: 20 mM MOPS, pH 7.0; 50 mM KCl; 1 mM ATP; 10 mM $MgCl_2$; 250 µM Kemptide; 65 cpm/pmol γ-^{32}P-ATP) and activity was determined at 30°C. Aliquots were transferred to phosphocellulose filters to bind the Kemptide, washed three times with 0.5% phosphoric acid to remove unincorporated γ-^{32}P-ATP, and dried before scintillation counting to determine the amount of incorporated ^{32}P.

Results and discussion

Initially, experiments were performed using physiological buffer conditions (20 mM MOPS, pH 7.0; 150 mM KCl). Under these conditions, all three inhibitors bound nonspecifically to the magnetic beads, even in the absence of the 6xHis-tagged C-subunit. Therefore, in further experiments, buffers containing imidazole, which were recommended by QIAGEN, were used during binding and washing procedures. Using these buffers, the physiological inhibitors bound to the immobilized catalytic subunit and no nonspecific binding was detected (Figure 1). After the addition of cAMP, binding of the R-subunits was abolished and no protein band corresponding to the R-subunits was detected. This clearly demonstrated dissociation of the immobilized complex and showed physiological functionality of the protein immobilized on the beads. In order to further test the functionality of the immobilized 6xHis-tagged C-subunit, a kinase activity assay was performed in the presence and absence of PKI. The presence of PKI resulted in 94% inhibition of Kemptide phosphorylation activity of PKA (Figure 2).

Adhesion of the magnetic beads to the walls of the tubes induced by the magnetic separator occurred within seconds and facilitated pipetting without any loss of material. The use of Ni-NTA Magnetic Agarose Beads to detect protein–protein interactions is an easy-to-use and rapid method for screening a number of different putative binding partners, for examining the influence of cofactors that affect binding, and for optimization of binding conditions. ■

For ordering information, see page 8.

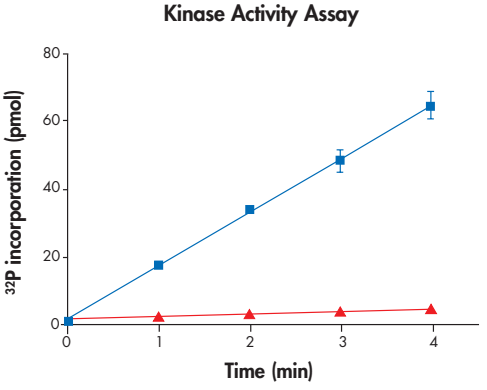

Figure 2 *The assay was performed with 6xHis-tagged C-subunit bound to Ni-NTA Magnetic Agarose Beads as described in materials and methods with (▲) or without (■) the inhibitor PKI.*

Ni-NTA Magnetic Agarose Beads and 96-Well Magnet with 96-Well Microplate FB

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Improved protein-interaction assays

The QIA*express* System is based on the superior properties of the patented NTA ligand, available exclusively from QIAGEN. Nickel ions immobilized on NTA have high affinity for a tag of six consecutive histidine residues. 6xHis-tagged proteins are immobilized to Ni-NTA Magnetic Agarose Beads in a spatially directed manner and remain conformationally active, providing optimal accessibility of interacting regions. This makes Ni-NTA Magnetic Agarose Beads ideal for all kinds of magnetocapture assays involving biomolecular interactions (Figure 2). Ni-NTA Magnetic Agarose Beads can be used for assays without the need for prior protein purification. Interacting biomolecules can either be eluted alone or as a complex with the 6xHis-tagged interacting partner by using the mild conditions for elution. Alternatively, the interacting biomolecules can be detected directly. Ni-NTA Magnetic Agarose Beads can be used in single tubes, or in 96-well microplates.

The power of Ni-NTA

The stable Ni–NTA interaction prevents dissociation of nickel ions from the NTA ligand, which results in high binding capacity and specificity for 6xHis-tagged proteins. In addition, the high-affinity interaction between Ni-NTA and the 6xHis tag is unaffected by a variety of reagents, such as strong denaturants and many detergents, which allows binding in a wide variety of buffer systems and under native or denaturing conditions. ■

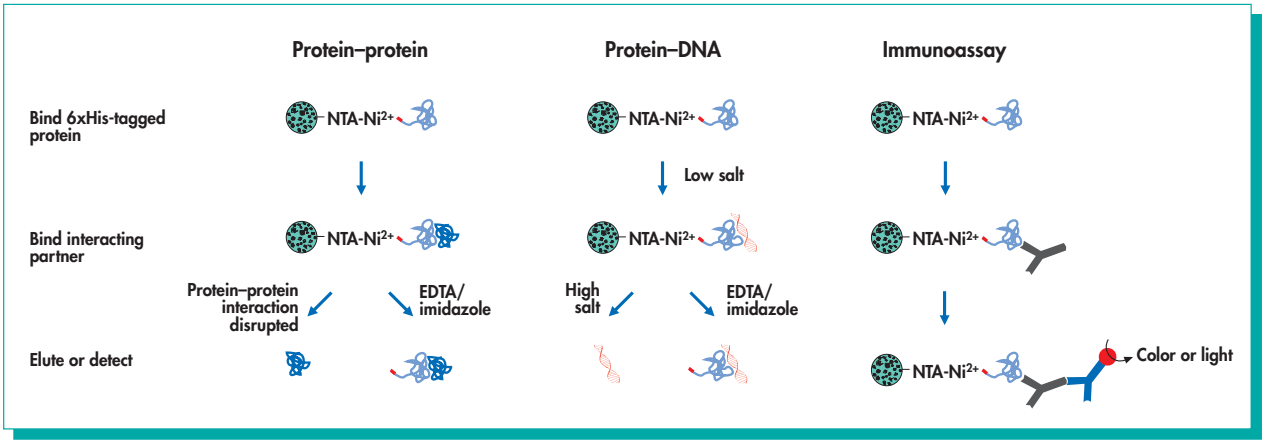

Figure 2 *Magnetocapture assays with Ni-NTA Magnetic Agarose Beads.*

Ordering Information

Product	Contents	Cat. No.
Ni-NTA Magnetic Agarose Beads (2 x 1 ml)	2 x 1 ml nickel-charged magnetic agarose beads (5% suspension)	36111
Ni-NTA Magnetic Agarose Beads (6 x 1 ml)	6 x 1 ml nickel-charged magnetic agarose beads (5% suspension)	36113
96-Well Magnet	Magnet for separating magnetic beads in wells of 96-well microplates, 2 x 96-Well Microplates FB	36915
96-Well Microplates FB (24)	96-well microplates with flat-bottom wells, 24 per case, for use with the 96-Well Magnet	36985