

pIKBa ubiquitination assay using purified SCF complex

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INTRODUCTION

We established a novel *in vitro* phosphorylation and ubiquitination assays of IKB α . Using these assays we have been able to identify the IKB degradation motif and to purify the ubiquitination ligase. Identification of the key enzymes in the IKB α phosphorylation ubiquitination process allowed us to replace many of the components that were initially provided by cell extract with recombinant or immunopurified proteins.

MATERIALS

Buffer A

- 50 mM Tris pH 7.6
- 1 mM DTT
- 150 mM NaCl
- 0.1% NP-40

Buffer B

- 1M KCl
- 0.5% NP-40
- 50 mM Tris buffer pH 7.6
- 1 mM DTT

Reaction Mixture

- 50 mM Tris pH 7.6
- 1 mM DTT
- 2 mM MgCl₂
- 20 nM Okadaic acid
- 20mM p-nitrophenyl phosphate (PNPP)
- 20mM β -glycerophosphate
- 1mg/ml bovine ubiquitin (Sigma)
- 5 mM ATP γ S (Sigma)

METHODS

Substrate

1. To incorporate ³⁵S-radiolabeled IKB α in the cellular NF-kB complex, *in-vitro* translated hemagglutinin-tagged IKB α (Alkalay et al., 1995) is incubated with Hela lysate in buffer A.
2. IKB α /NF-kB complexes are immunoprecipitated with anti NF-kB p65 beads (Santa Cruz SC-109 AC).
3. The slurry is agitated for 2 h at 4°C
4. The immune complexes are washed extensively 3 times with buffer B and once with 50 mM Tris pH 7.6, 1 mM DTT

5. Incubated with constitutively active recombinant IKK- β , IKK2-EE, in the presence of 2 mM ATP for 30 min at 37°C (Mercurio et al., 1997).
6. The beads are washed with 50 mM Tris pH 7.6, 1 mM DTT.

Conjugation assay

1. The immobilized phosphorylated substrate is agitated for 90 min at 37°C in the reaction mixture containing purified His tag E1, recombinant UBC5C and E3RS protein complex (Yaron et al., 1998)
 - The E3RS complex is immunopurified from 293T cells transfected with FLAG-Roc and Myc-Cullin1, using anti FLAG-beads (A220 SIGMA) and eluted with 1mg/ml FLAG peptide.
2. The beads were boiled in SDS-buffer and the sample was separated on SDS-PAGE and analyzed using a phospho-imager (Fujifilm).

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