In vitro histone deubiquitination assay

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Method Article

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Abstract

Introduction

Posttranslational histone modifications play important roles in regulating chromatin structure and function. One example of such modifications is histone ubiquitination, which occurs predominately on H2A and H2B. Although the recent identification of the ubiquitin ligase for histone H2A has revealed important roles for H2A ubiquitination in Hox gene silencing as well as in X inactivation, the enzyme(s) involved in H2A deubiquitination and the function of H2A deubiquitination are not known. In order to identify the deubiquitinase for histone H2A, we developed an in vitro deubiquitination assay employing uH2A (ubiquitinated H2A)-containing mononucleosomes as substrates.

Reagents

HeLa cells expressing Flag-H2A and HA-ubiquitin, micrococcal nuclease (Sigma), Anti-Flag M2 beads (Sigma); Anti-HA antibody, Flag and HA peptides, Hydroxyapatite column (Bio-Rad), Tris-HCl, Hepes-KOH, sucrose, KCl, NaCl, MES, MgCl₂, CaCl₂, Triton X-100, DTT, PMSF, peptidesaprotinin, 1 µg/mL leupeptin and 1 µg/mL pepstatin A, glycerol.

Equipment

SDS-PAGE apparatus, semiphor transphor unit, power supply, and benchtop microcentrifuge.

Procedure

1. Incubate stable cells expressing Flag-H2A and HA-ubiquitin with buffer A (0.25 M sucrose, 60 mM KCl, 15 mM NaCl, 10 mM MES, pH 6.5, 5 mM MgCl₂, 1 mM CaCl₂, 0.5% Triton X-100, 1 mM DTT, 0.1 mM PMSF) to release nuclei.
2. Subject the released nuclei to micrococcal nuclease (Sigma) digestion in buffer B (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2 mM MgCl₂, 0.3 M sucrose, 1 mM PMSF, 5 mM CaCl₂) to generate mononucleosomes.
3. Extract these nucleosomes from nuclei with buffer containing 20 mM Tris-HCl, pH 7.9, 10 mM EDTA, and 0.5 M NaCl.
4. After dialysis against histone storage buffer (10 mM Hepes-KOH, pH 7.5, 1 mM EDTA, 10 mM KCl, 0.1 mM PMSF, 0.05% NP40, 10 % glycerol), subject these nucleosomes to immunoprecipitation (IP) with anti-Flag antibody and subject the eluate from Flag IP to IP with anti-HA antibody.
5. Dialyze the eluate from the anti-HA IP, which contains the uH2A-containing nucleosomes, against histone storage buffer.
6. To obtain uH2A-containing core histones, mononucleosomes, and oligonucleosomes, micrococcal nuclease (Sigma) controll digestion to generate nucleosomal arrays ranging from 1 to 15 nucleosomes.
7. Separate mononucleosomes and oligonucleosomes (5-10) by a sucrose gradient (5-30%) and verify by measuring the length of DNA fragments extracted from these nucleosomes.
8. Prepare uH2A-containing core histones by removing DNA components from these nucleosomes with a hydroxyapatite column. ****Deubiquitination reactions****
9. Incubate 1.5 microgram of nucleosomes with protein fractions derived from HeLa cells in

deubiquitination reaction buffer (100 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.1 mM PMSF, 1 mM DTT, 1 µg/mL aprotinin, 1 µg/mL leupeptin and 1 µg/mL pepstatin A) at 37°C for 45 min or as indicated. 10. Terminate the reaction by addition of SDS-PAGE sample loading buffer and proteins were resolved on SDS-PAGE and blotted with the anti-HA antibody.

Timing

1-2 weeks