

In vitro polyubiquitination assay

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INTRODUCTION

An *in vitro* polyubiquitination assay of proteins is a powerful tool to prove the *in vivo* function of proteins in ubiquitination. The *in vitro* system allows to find components of the ubiquitination machinery. We have used the assay to prove ubiquitin ligase activity of a subunit of the Gid complex, Gid2/RMD5 (Santt et al., 2008). As shown in (Regelmann et al., 2003), GID2/RMD5 deletion prevents polyubiquitination of FBPase. Alignments of the Gid2/Rmd5 protein sequence with known RING-finger E3's revealed that it bears a so-called degenerated RING-finger where Zn²⁺ coordination residues are missing (Santt et al., 2008). A complete cysteine and histidine pattern in a RING domain is not necessarily critical for the E3 function, as the U-box domain family shows (Ohi et al., 2003). The canonical RING domain encompasses eight residues coordinating two Zn cations. The first Zn²⁺ ion is coordinated by the first, second, fifth and sixth residue; the second by the remaining residues three, four, seven and eight (Fang and Weissman, 2004; Lorick et al., 1999). In Gid2 besides the first cysteine, the four residues coordinating the second Zn²⁺ ion are conserved, which strongly suggests that one Zn²⁺ is retained in this degenerated RING domain. This prompted us to suspect that Gid2 may bear an ubiquitin ligase activity.

MATERIALS

Buffer A

- 50mM Tris-HCl pH 7.5
- 250mM NaCl
- 5mM DTT
- 2mM PMSF
- 1% Triton-X100

10x Ubiquitin reaction buffer

- 500mM Tris/HCl pH 7.5
- 500mM NaCl
- 100mM MgCl₂
- 10mM DTT
- 250μM ZnCl₂

20μl ubiquitination reaction

- 0.25μg E1 (yeast)
- 0.6μg UBch5b
- 10μg HA-ubiquitin
- 1μl energy regeneration solution
- ERS (all Boston Biochemicals)
- 2μl "10x ubiquitin reaction buffer" and 8μl of Gid2 or gp78 cell lysate

METHODS

1. A GST-fusion protein is expressed in the *E. coli* BL21
2. *E. coli* cells are grown to OD₆₀₀ 0.8-1 in 2x Yeast Tryptone
3. Expression of the foreign proteins is induced at 16°C with 0.5mM IPTG and cells are grown between 6 and 12h.
4. Cells are resuspended in buffer A and lysed with a French press.
5. A typical 20µl ubiquitination reaction is carried out.
6. The reactions are incubated for 2h at 30°C
7. Ubiquitination of proteins is monitored by Western blotting using monoclonal HA antibody.

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