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Preparation of ubiquitin-conjugated proteins using an insect cell-free protein synthesis system

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ABSTRACT

Ubiquitination is one of the most significant posttranslational modifications (PTMs). To evaluate the ability of an insect cell-free protein synthesis system to carry out ubiquitin (Ub) conjugation to *in vitro* translated proteins, poly-Ub chain formation was studied in an insect cell-free protein synthesis system. Poly-Ub was generated in the presence of Ub aldehyde (UA), a de-ubiquitinating enzyme inhibitor. *In vitro* ubiquitination of the p53 tumor suppressor protein was also analyzed, and p53 was poly-ubiquitinated when Ub, UA, and Mdm2, an E3 Ub ligase (E3) for p53, were added to the *in vitro* reaction mixture. These results suggest that the insect cell-free protein synthesis system contains enzymatic activities capable of carrying out ubiquitination. CBB-detectable ubiquitinated p53 was easily purified from the insect cell-free protein synthesis system, allowing analysis of the Ub-conjugated proteins by mass spectrometry (MS). Lys 305 of p53 was identified as one of the Ub acceptor sites using this strategy. Thus, we conclude that the insect cell-free protein synthesis system is a powerful tool for studying various PTMs of eukaryotic proteins including ubiqutination presented here.

1. Introduction

There is increasing interest in analyzing PTMs of proteins. Cell-free protein synthesis systems are potentially powerful tools for post-genomic studies including analyses of PTMs, because they can not only be used to synthesize desired proteins, including those toxic to cells (Sakurai et al., 2007), but they can also carry out various PTMs on these proteins. A cell-free protein synthesis system from *Spodoptera frugiperda* 21 (Sf21) insect cells (Ezure et al., 2006), which are widely used as the host for baculovirus expression systems, was developed previously, and it was demonstrated that this system could generate various eukaryotic-specific protein modifications, such as *N*-myristoylation (Suzuki et al., 2006b) and prenylation (Suzuki et al., 2007).

Ubiquitination is one of the most significant PTMs because it plays central roles in the regulation of many cellular processes, such as targeting for proteasome degradation, cell cycle progression, signal transduction, DNA repair, and so on (Ciechanover, 1998; Hershko and Ciechanover, 1998). Therefore, techniques to prepare Ub-conjugated proteins are extremely important to understand these processes in detail. Some methodologies for the purification of ubiquitinated proteins have been developed (Tomlinson et al., 2007), and large-scale MS analyses have been performed (Peng et al., 2003; Gururaja et al., 2003). However, it is still challenging to identify ubiquitinated proteins and Ub-conjugation sites.

A rabbit reticulocyte lysate system has often been utilized for *in vitro* ubiquitination assays of target proteins, because it possesses enzymatic activities involved in the ubiquitination reaction (Ciechanover et al., 1991; Etlinger and Goldberg, 1977). However, a serious drawback of this system is that only radio-isotope labeling or an immunoblotting strategy may be used to detect ubiquitinated proteins, because of the low expression levels.

In order to evaluate whether the insect cell-free protein synthesis system contains enzymes capable of carrying out ubiquitination reactions, poly-Ub chain formation was investigated using the insect cell-free protein synthesis system and FLAG-tagged Ub. The p53 tumor suppresor protein was chosen as a model protein because it is highly regulated by the ubiquitin–proteasome pathway (Haupt et al., 1997), and *in vitro* ubiquitination of p53 occurred.

Abbreviations: PTMs, posttranslational modifications; Ub, ubiquitin; UA, ubiquitin aldehyde; E3, E3 ubiquitin ligase; MS, mass spectrometry; Sf21, *Spodoptera frugiperda* 21; Me-Ub, methylated ubiquitin; MALDI-TOF MS, matrix assisted laser desorption/ionization time-of-flight mass spectrometry; QIT, quadrupole ion trap; MS/MS, tandem mass spectrometry; *m/z*, mass-to-charge ratio.

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The present study describes a simple and robust strategy to prepare ubiquitin-conjugated proteins using this cell-free protein synthesis system and the identification of exact location of Ub-conjugation sites by mass spectrometry.

2. Materials and methods

2.1. Materials

Transdirect *insect cell*, which is based on the Sf21 extract, is a commercial product of Shimadzu (Kyoto, Japan). Restriction endonucleases and DNA modifying enzymes were purchased from Toyobo (Osaka, Japan) and New England Biolabs (Ipswich, MA, USA). Human cDNA clone p53 (GenBank accession no. **NM_000546**) and Mdm2 (GenBank accession no. **BT007258**) were obtained from Toyobo and Open Biosystems (Huntsville, AL, USA), respectively. Caspase-3 Inhibitor I (Ac-DEVD-CHO) and MG-132 (Z-LLL-CHO) were obtained from Calbiochem (Darmstadt, Germany). Ubiquitin and FLAG-tagged ubiquitin were purchased from Sigma (St. Louis, MO, USA). Ubiquitin aldehyde and methylated ubiquitin were obtained from Peptide Institute (Osaka, Japan) and BostonBiochem (Cambridge, MA, USA), respectively.

2.2. Construction of expression clones for in vitro translation

The protein coding regions of human p53 were amplified by PCR and inserted into the multiple cloning site of the pTD1-strep vector, which is an expression vector for synthesizing C-terminal *Strep*-tagged target proteins using the insect cell-free protein synthesis system (Ezure et al., 2007). The resultant plasmid was named pTD1-strep-p53. The ORF of the human Mdm2 gene was amplified by PCR. The amplified DNA fragment was ligated into the pTD1-vector (Suzuki et al., 2006a), and the resulting plasmid was designated pTD1-Mdm2. N-terminal or C-terminal GST-tagged Mdm2 constructs were also constructed using conventional cloning techniques. The resultant plasmids were named pTD1-NGST-Mdm2 and pTD1-CGST-Mdm2, respectively. The DNA sequences of these recombinant constructs were confirmed by the dideoxynucleotide chain termination method.

2.3. In vitro transcription and translation

mRNAs were synthesized with the T7 RiboMAX Express Large Scale RNA Production System (Promega, Madison, WI, USA) using linearized expression clones as the template. Purification of *in vitro* transcribed mRNAs was performed as described previously (Suzuki et al., 2006b). *In vitro* translation was carried out using an insect cell-free protein synthesis system according to the instruction manual. In the case of Mdm2, translation was performed with or without the addition of a caspase-3 inhibitor I at a final concentration of 1.0 μM.

2.4. Detection of synthesized proteins by fluorescent labeling

For the synthesis of fluorescently labeled proteins, 1 μL of FluoroTect $Green_{Lys}$ tRNA (Promega) was added to 50 μL of the *in vitro* translation reaction mixture. The sample was resolved by SDS-PAGE. The fluorescently labeled proteins were detected using a laser-based fluorescent scanner, Molecular Imager FX (Bio-Rad Laboratories, Hercules, CA, USA).

2.5. Poly-Ub chain formation

The Sf21 cell-free extract for the insect cell-free protein synthesis system (75 μL) and the N-terminal FLAG-tagged Ub (50 μg) were mixed and incubated at 25 °C for 2 h in a 250 μL reaction mixture that included ubiquitination buffer (50 mM Tris–HCl, pH 7.5, containing 5 mM $MgCl_2$, 1 mM ATP, 10 mM creatine phosphate, 4 U mL^{-1} creatine kinase and 2.5 mM dithiothreitol) and 5 μg of ubiquitin aldehyde (UA).

2.6. In vitro ubiquitination assay

In vitro translated p53 (6 μL) and Mdm2 (4 μL) were mixed and incubated at 30 °C for 2 h in a 25 μL reaction mixture that included the ubiquitination buffer, 0.63 μg of UA, and 6.3 μg of Ub. Methylated Ub (Me-Ub) was added instead of Ub to the *in vitro* ubiquitination reaction mixture to suppress poly-Ub chain formation.

2.7. Affinity purification

Affinity purification of FLAG-tagged or strep-tagged proteins was performed as described previously (Suzuki et al., 2006b, 2007). In the case of GST-tagged proteins, a GST purification module was used (GE Healthcare, Piscataway, USA).

2.8. Analysis of N-terminal amino acid sequence

The affinity-purified protein was separated by SDS-PAGE and then transferred to a PVDF membrane, then stained with CBB R-250. The protein band was sequenced with a PPSQ-33A protein sequencer (Shimadzu).

2.9. Mass spectrometry

The tryptic digests from affinity-purified proteins were analyzed with an AXIMA-CFR-plus MALDI-TOF MS (matrix assisted laser desorption/ionization time-of-flight mass spectrometry) instrument and an AXIMA-QIT MALDI-QIT (quadrupole IT)-TOF hybrid mass spectrometer (Shimadzu/Kratos, Manchester, UK) as described previously (Suzuki et al., 2006b).

3. Results

3.1. Poly-Ub chain formation using the insect cell-free extract

To evaluate the ability of the insect cell-free protein synthesis system to conjugate Ub to target proteins synthesized *in vitro*, generation of poly-Ub chains was analyzed after adding Ub to the cell-free extract of the insect cell-free protein synthesis system. FLAG-tagged Ub and the extract were incubated in the ubiquitination buffer, and then FLAG-tagged Ub was collected by affinity purification. The reaction was performed in the presence or absence of UA, a de-ubiquitinating enzyme inhibitor. Ladder bands at around 15–27 kDa, which probably corresponded to poly-Ubs, were observed upon SDS-PAGE of the affinity-purified sample after UA was added to the reaction mixture (Fig. 1). A protein band detected around 50 kDa was identified as β-tubulin by peptide mass fingerprinting (data not shown). This is probably a non-specific protein band because β-tubulin has been sometimes coeluted in the affinity purification step (Suzuki et al., 2007). On the other hand, when the reaction was carried out without adding UA, only a predominant 10 kDa band was detected (Fig. 1). The 10 kDa band and slowly migrating bands were excised individually and digested with trypsin, and the digests were analyzed by MALDI-TOF MS. The spectra produced from these samples were almost identical, and these MS spectra corresponded to tryptic digests of Ub (Fig. 2). Trypsin digestion of ubiquitinated proteins produces peptides with internal lysine residues harboring a di-glycine remnant (GG-tag)