C-termini of the PKs suggest that CASP3 cleavage may regulate the activity level and/or cellular localization of the PKs, rather than simply inactivate the kinases.

Discussion

In 1995, PITSLRE,[29] PKCδ,[30] and DNA-PKcs[31] were reported as the first PK-type substrates of CASP3. During the next 15 years, 36 additional PKs that can be cleaved by CASP3 were found.[23,24,26] Notably, these authors showed that CASP3-cleaved PKs abrogate survival signals and accelerate apoptosis. In this study, we identified an additional 30 PKs that can be cleaved by CASP3. In addition, many of the cleavage sites were found in regulatory elements or in the regions near the N- and C-termini, rather than the kinase domain itself. Some of the newly identified CASP3-substrate PKs may be involved in apoptotic signal cascades. Sixteen PKs were shown to be cleaved *in vitro* near their N- or C-termini and at least five of them were also cleaved near their N- or C-termini in apoptotic cells (Figure 5). Using standard immunoblotting, proteins that are cleaved into a large and a small fragment may be overlooked because the mobilities of the large fragment and the intact protein will be nearly identical. Most of the PKs that had been previously reported to be cleaved by CASP3 were identified because the cleaved fragments had very different molecular weights than did the intact PK and were therefore easily detected by SDS-PAGE. Consequently, cleavages near the termini may have been overlooked. Taken together, our results suggest that CASP3 cleavage of some of the members of the CASP3-substrate kinome alters the function of the PKs and thereby signals apoptosis.

For the study reported herein, 304 out of 518 known PKs, synthesized as NCtagged PKs, were subjected to the *in vitro* cleavage assay (Supplementary Table S1). The relative number of PKs that were cleaved was ~14%. A total of 69 PKs that are CASP3 substrates are now known, which suggests that at least ~13% of the PKs in the human kinome are targets of CASP3. As ~200 PKs have yet be tested as CASP3 substrates, an additional 26 PKs (13% of the 200) may be CASP3 substrates. The human genome contains 518 annotated PKs, which have been divided into 10 groups on the basis of their sequence homologies.[3] Interestingly, the groups differ in terms of their susceptibilities to CASP3 cleavage (Table 2). Approximately 30% of the PKs in the AGC group are known CASP3 substrates, for example, AKT2, S6K, MSK, PKC, and PDK1. Many of the AGC-type PKs are commonly found in mammalian tissues,[32] and their cleavage sites are located in their regulatory domains (Figure 4 and Table 1). Therefore, these abundant PKs may be activated when CASP3 cleaves them and then act as intracellular apoptosis signals. Conversely, CASP3 cleaved only a relatively small number (6~8%) of the PKs in the CMGC group, which includes the kinases of the CDK and CDKL families, and the tyrosine kinase groups. Therefore, most members of these groups may only act indirectly as apoptosis signals after CASP3 activation.

Such ROCK1 and MST1, certain caspase cleavage products, work as apoptosis signaling.[23,24] In this study, we found at least six new CASP3 cleavage products, derived from AKT2, CaMKK1, eEF2 K, MARK1, MNK2, and TRB3,

Table 2 Characteristics of the protein kinases used in this study

Groups	Total[a]	Tested clones	Cleaved clones (new[b])	Cleaved/test clones (%)
AGC	63	33	10 (6)	30
CAMK	74	52	8 (7)	15
CK1	12	8	1 (1)	13
CMGC	61	39	3 (3)	8
Other	83	46	3 (2)	7
STE	47	25	4 (1)	16
TK	90	51	3 (1)	6
TKL	43	27	6 (4)	22
RGC	5	2	0	0
Atypicals	40	21	5 (5)	24
Total	518	304	43 (30)	14

[a]Each number is corresponding to human kinome. [b]Newly PK numbers found in this study.

after 6 h from apoptosis induction (Figures 4 and 5). These cleavage products retain kinase domain, as in the case of ROCK1 and MST1. On the other hand, we could not detect any cleavage products from the other kinases *in vivo*. The reasons are not yet understood. However, recent proteomics approach has shown that the cleaved proteins displayed transient fragments in the apoptotic cells.[12] Further analysis at multiple time points during the apoptotic cascade would be required for detection of the cleavage products from the remaining PKs.

For TRIB3, full-length TRIB3 (D338A) mutant was decreased in apoptotic condition (compared TNFα lane with TNFα plus z-VAD-FMK lane in Figure 5c). However, the mutant could not produce the CASP3 cleavage product found as the shorter form in TNFα lane of WT, indicating that the mutant was not cleaved by CASP3. The mutant was also not cleaved by CASP3 *in vitro* (data not shown). As TRIB3 has been known to receive proteasomal degradation,[33] this unexpected reduction of the mutant TRIB3 in the apoptotic cells may be the effects of cycloheximide and/or caspase-inhibitor treatment on TRIB3 degradation.

Proteases often modify the activities of their targeted protein substrates. Identification of the specific substrate that is cleaved by a protease is necessary if the functions of both the protease and its substrate are to be understood. Proteomic studies have used mass spectrometry to exhaustively identify cellular proteins that have been cleaved by proteases.[11,12] However, it has been difficult to correlate specific proteases with their substrates because many proteases act at the same time *in vivo*.

Many full-length cDNAs derived from the genes of higher eukaryotes are available from many different sources. These cDNAs are potentially a great DNA template resource for *in vitro* syntheses of proteins. As a protein production system and for the functional analysis of proteins, the wheat cell-free system has many advantages: It can effectively use PCR-generated DNA templates.[14] It is easily adapted to an automated system.[15] It can be used to incorporate a single label into target proteins.[20] Its synthesized proteins do not require purification before being assayed, and it has no detectable proteasome activity.[21] In addition, the screening cost is very low (~US$1/assay), which for our study translated to 10 cents to produce each NCtagged protein and

20 cents for the beads, CASP3, and disposable hardware used in one assay.

In summary, we showed that an NCtagged PK library synthesized in a cell-free system could be used to characterize a CASP3-substrate kinome. Analysis of the CASP3-cleavage sites indicated that CASP3 cleavage of PKs depends on both primary and tertiary structure. Almost all of the PK substrates that we identified *in vitro* were also identified *in vivo*. Systems similar to that used herein could be used to screen other protease substrates.

Materials and Methods

General. The following procedures have been described:[14–16,20–22,34] wheat cell-free protein production; split-primer PCR synthesis of the DNA templates; parallel syntheses of mRNAs and their translated proteins; and measurements of the amounts of protein synthesized using densitometer scans of Coomassie brilliant blue-stained proteins or of radiolabeled proteins.

Construction of DNA templates for the expression of a PK protein library. The cloned genes encoding the PKs used in this study are listed in Supplementary Table S1. Their open-reading frames (without stop codons) were modified in two steps using PCR and the primers S1 (5′-CCACCCACCAC CACCAatg(n)$_{16}$-3′) and T1 (5′-TCCAGCACTAGCTCCAGA(n)$_{19}$-3′) (lowercase letters indicate nucleotides of the gene) for the first step, and the primers attB1-Flag-S1 (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGACTACAAG GATGACGATGACAAGCTCCACCCACCACCACCAATG-3′) and T1-bls-STOP-attB2-anti (5′-GGGGACCACTTTGTACAAGAAAGCTGGGTTTATTCGTGCCACTC GATCTTCTGGGCCTCGAAGATGTCGTTCAGGCCGCTTCCAGCACTAGCTCCA GA-3′) for the second step. The PCR-modified genes were each inserted into a pDONR221 vector using the Gateway BP Clonase II enzyme mix (Invitrogen, Carlsbad, CA, USA) to give pDONR-Flag-PK-bls vectors. *Escherichia coli* cells were transformed with these vectors and then cultured in wells of a 96-well plate that contained GYT medium (10% (v/v) glycerol, 0.125% (w/v) yeast extract, and 0.25% (w/v) tryptone) for 48 h without shaking. DNA templates for mRNA and protein expression were constructed using split-primer PCR[14] in two steps. For the first step, the pDONR221-Flag-PK-bls plasmids that had not been isolated from the *E. coli* cells, and the primers pDONR221-1st_4080 (5′-ATCTTTTCTACGGGGT CTGA-3′) and deSP6E02-Flag-S1 (5′-GGTGACACTATAGAACTCACCTATCTC TCTACACAAAACATTTCCCTACATACAACTTTCAACTTCCTATTATGGACTACAA GGATGACGATGACAAGCTCCACCCACCACCACCAATG-3′) were used, and for the second step, the amplified sequences of the first step and the primers SPu (5′-GGGTAGCATTTAGGTGACACT-3′) and pDONR221-2nd_4035 (5′-ACGTTAA GGGATTTTGGTCA-3′) were used to give SP6-E02-Flag-PK-bls DNA templates. (The E02 sequence is a translational enhancer,[35] and the SP6 sequence is an SP6 RNA polymerase promoter.)

Cell-free protein synthesis. Cell-free protein synthesis used the reagents of an ENDEXT Wheat Germ Expression S Kit according to the manufacturer's instructions (CellFree Sciences Co., Ltd.), the bilayer translation method,[15,16,34] and a robotic synthesizer (GenDecorder 1000; CellFree Sciences). Each DNA template was transcribed by SP6 RNA polymerase, then precipitated with ethanol, and collected by centrifugation (15 000 r.p.m. for 5 min., R10H rotor; Hitachi). Each mRNA ($\sim$30–35 μg) was washed with 75% ethanol, added into a translation mixture, and translated in the bilayer mode[31] with the following modifications. The translation mixture (25 μl) (bottom layer) contained 60 A_{260}/ml of ENDEXT wheat germ extract, 1 × SUB-AMIX (24 mM Hepes-KOH, pH 7.8, 1.2 mM ATP, 0.25 mM GTP, 16 mM creatine phosphate, 2.5 mM DTT, 0.4 mM spermidine, 0.3 mM each of the 20 amino acids, 2.8 mM magnesium acetate, 100 mM potassium acetate), 2 μg creatine kinase (Roche Applied Science, Indianapolis, IN, USA), 500 nM D-biotin (Nacalai Tesque, Kyoto, Japan), and 1 μl of the wheat cell-free translational mixture that expressed BirA biotin ligase ($\sim$50 ng/μl, BirA: GenBank Accession No. NP_0312927). A 1 × SUB-AMIX solution (125 μl) was placed over the translation mixture. The bilayer was incubated at 26°C for 17 h to allow for protein synthesis. All steps including construction of the DNA templates were performed in the wells of a 96-well plate.

Cleavage assay. The cell-free-synthesized PKs that had luminescent signals >500 units (in the absence of CASP3) were studied. For each PK, 10 μl of the CASP3 cleavage buffer (20 mM Tris-HCl, pH 7.5, 0.2 mM DTT, 5 mM $MgCl_2$, 3 mM ATP, 1 mg/ml BSA, 1 mU CASP3 (Sigma-Aldrich, St. Louis, MO, USA)) was mixed with 1 μl of the translation mixture that contained a Flag-PK-bls $\sim$ biotin construct, and the mixture was incubated at 30°C for 2 h in a well of a 384-well Optiplate (Perkin Elmer, Foster City, CA, USA). Using the reagents of an AlphaScreen IgG (protein A) detection kit (Perkin Elmer) according to the manufacturer's instructions, 15 μl of 20 mM Tris-HCl, pH 7.5, 0.2 mM DTT, 5 mM $MgCl_2$, 5 μg/ml anti-FLAG M2 antibody (Sigma-Aldrich), 1 mg/ml BSA, 0.1 μl of streptavidin-coated donor beads and 0.1 μl of anti-IgG acceptor beads were added to the well. The solution was incubated at 23°C for 1 h. Luminescence was analyzed using the AlphaScreen detection program (Perkin Elmer). All repetitive mechanical procedures were performed by a Biomek FX robotic workstation (Beckman Coulter, Fullerton, CA, USA). The value of a luminescent signal is reported as the mean of three independent measurements.

TD immunoblotting. A mixture of each Flag-PK-bls $\sim$ biotin construct (3 μl of a translation mixture) and 7 μl of the CASP3 cleavage solution was incubated at 30°C for 1 h in a well of a 384-well Optiplate (Perkin Elmer). Then, the proteins were separated in SDS-PAGE gels and transferred to PVDF membranes (Millipore Bedford, MA, USA). The blotted proteins were prepared for detection using the reagents of an ECL-Plus Western Blotting Detection System kit (GE Healthcare, Piscataway, NJ, USA), anti-Flag M2 antibodies (Sigma-Aldrich) for N-TD, and Alexa488-streptavidin (Invitrogen) for C-TD. The labeled proteins were visualized using a Typhoon Imager (GE Healthcare) with a 532-nm laser and a 526-nm emission filter or an ImageQuant LAS-4000mini CCD camera system (Fujifilm).

Sequencing and other purification procedures. When possible, long biotinylated C-terminal fragments produced by CASP3 cleavage were recovered attached to streptavidin beads, and then sequenced directly. When a PK construct had a low biotin-labeling efficiency and was cleaved near its C-terminus, a new construct was made by fusing the GST nucleotide sequence encoded in the pEU-E01-Gateway-GST vector to the C-terminal codon of the corresponding PK open-reading frame using the Gateway system and the pEU-E01-Gateway-GST vector. For purification, synthesized PKs (1.2 ml) were purified using Streptavidin Magnesphere Paramagnetic beads (Promega Corp., Madison, WI, USA) for the Flag-PK-bls $\sim$ biotin constructs or glutathione Sepharose 4B (GE Healthcare) for the PK-GST constructs. After washing the beads with PBS, the bound PKs were incubated with CASP3 (15 μl of total volume) as described above. The samples were boiled and the proteins separated by SDS-PAGE. After blotting and visualization (ProBlott, Applied Biosystems, Foster City, CA, USA), the membrane areas that contained the cleaved fragments were cut out and the fragments were sequenced (Applied Biosystems ABI 473A). CSK kinase (Carna Biosciences Inc., Kobe, Japan) and AMPKa1 (Cell Signaling Technology, Beverly, MA, USA) were cleaved with CASP3 (10 μl of total volume), and the cleavage products subjected to MALDI/TOF-MS (Shimazu Techno-Research Inc., Kyoto, Japan) for sequencing. D→A mutagenesis was carried out using the reagents of a PrimeSTAR Mutagenesis Basal kit (TakaraBio, Otsu, Japan) according to the manufacturer's instructions. The mutated genes were sequenced using an ABI PRISM 310 DNA sequencer (Applied Biosystems).

Construction of PK expression plasmids for the cell-based assay. Expression plasmids were produced using the Gateway method. To obtain the attB1-PK-Flag-(stop codon)-attB2 for Gateway BP Clonase II recombination, the open-reading frame products of the 30 newly identified PK substrates of CASP3 that had been produced by PCR using the S1 and T1 primers as described above were PCR amplified using the primers, attB1-S1 (5′-GGGGACAAGTTTGTA CAAAAAAGCAGGCTTCCACCCACCACCACCA-3′) and T1-Flag-stop-attB2 (5′-GGGGACCACTTTGTACAAGAAAGCTGGGTTTACTTGTCATCGTCATCCTTG TAGTCGCTTCCAGCACTAGCTCCAGA-3′). These PCR products were each inserted into a pDEST26 vector (Invitrogen) using the Gateway system for construction of the His-PK-Flag nucleotide sequences. All sequences were confirmed by DNA sequencing as described above.

Cell-based assay. HeLa cells were cultured in Dulbecco's modified Eagle's medium, 10% fetal bovine serum, penicillin (100 mg/ml), and streptomycin (50 μg/ml). Transient transfections were carried out using Lipofectamine 2000 Transfection Reagent (Invitrogen) according to the manufacturer's instructions. At 24 h after transfection, cells were harvested after apoptosis was induced. Control cells were treated with DMSO and, for apoptosis induction or inhibition, with 20 ng/ml TNFα

(Calbiochem, La Jolla, CA, USA) and 100 μM cycloheximide (Chemicon, Temecula, CA, USA) or 125 ng/ml anti-Fas antibody (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan) in the presence (inhibition) or absence (induction) of 100 μM Z-VAD-FMK (Peptide Institute Inc., Osaka, Japan) for 6 h. Cells were washed with PBS and then lysed directly by adding one volume of 2 $\times$ SDS-PAGE sample buffer (125 mM Tris-HC1, pH 6.8, 20% glycerol, 4% SDS, 10% 2-mercaptoethanol, 0.001% bromphenol blue) before subjecting the cell extracts to SDS-PAGE and immunoblotting, which used anti-His antibodies (GE Healthcare) or anti-Flag M2 antibodies (Sigma-Aldrich). The following antibodies were employed to detect endogenous proteins: anti-α-tubulin (Sigma-Aldrich); anti-AKT2, anti-eEF2K, anti-AMPKa1, and anti-TGFbR1 (Cell Signaling Technology). Chemiluminescent signals, generated by ECL-Plus reagents (GE Healthcare), or Immobilon Western HRP substrate Luminol Reagent (Millipore), were detected using an LAS-4000 mini biomolecular imager (GE Healthcare).

Conflict of interest

Dr. Endo is a founder of CellFree Sciences Co., Ltd. and a member of its scientific advisory board. Other authors declare no conflict of interest.

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1. Puente XS, Sanchez LM, Overall CM, Lopez-Otin C. Human and mouse proteases: a comparative genomic approach. *Nat Rev Genet* 2003; **4**: 544–558.
2. Hunter T. Signaling – 2000 and beyond. *Cell* 2000; **100**: 113–127.
3. Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. The protein kinase complement of the human genome. *Science* 2002; **298**: 1912–1934.
4. Matrisian LM, Hogan BL. Growth factor-regulated proteases and extracellular matrix remodeling during mammalian development. *Curr Top Dev Biol* 1990; **24**: 219–259.
5. Turgeon VL, Houenou LJ. The role of thrombin-like (serine) proteases in the development, plasticity and pathology of the nervous system. *Brain Res Brain Res Rev* 1997; **25**: 85–95.
6. van Kempen LC, de Visser KE, Coussens LM. Inflammation, proteases and cancer. *Eur J Cancer* 2006; **42**: 728–734.
7. Friedl P, Wolf K. Tube travel: the role of proteases in individual and collective cancer cell invasion. *Cancer Res* 2008; **68**: 7247–7249.
8. Abdel-Rahman HM, Kimura T, Hidaka K, Kiso A, Nezami A, Freire E *et al.* Design of inhibitors against HIV, HTLV-I, and *Plasmodium falciparum* aspartic proteases. *Biol Chem* 2004; **385**: 1035–1039.
9. Alnemri ES. Mammalian cell death proteases: a family of highly conserved aspartate specific cysteine proteases. *J Cell Biochem* 1997; **64**: 33–42.
10. Hengartner MO. The biochemistry of apoptosis. *Nature* 2000; **407**: 770–776.
11. Mahrus S, Trinidad JC, Barkan DT, Sali A, Burlingame AL, Wells JA. Global sequencing of proteolytic cleavage sites in apoptosis by specific labeling of protein N termini. *Cell* 2008; **134**: 866–876.
12. Dix MM, Simon GM, Cravatt BF. Global mapping of the topography and magnitude of proteolytic events in apoptosis. *Cell* 2008; **134**: 679–691.
13. Righetti PG, Boschetti E. Sherlock Holmes and the proteome – a detective story. *FEBS J* 2007; **274**: 897–905.
14. Sawasaki T, Ogasawara T, Morishita R, Endo Y. A cell-free protein synthesis system for high-throughput proteomics. *Proc Natl Acad Sci USA* 2002; **99**: 14652–14657.
15. Sawasaki T, Morishita R, Gouda MD, Endo Y. Methods for high-throughput materialization of genetic information based on wheat germ cell-free expression system. *Methods Mol Biol* 2007; **375**: 95–106.
16. Takai K, Sawasaki T, Endo Y. Practical cell-free protein synthesis system using purified wheat embryos. *Nat Protoc* 2010; **5**: 227–238.
17. Endo Y, Sawasaki T. Cell-free expression systems for eukaryotic protein production. *Curr Opin Biotechnol* 2006; **17**: 373–380.
18. Sawasaki T, Hasegawa Y, Morishita R, Seki M, Shinozaki K, Endo Y. Genome-scale, biochemical annotation method based on the wheat germ cell-free protein synthesis system. *Phytochemistry* 2004; **65**: 1549–1555.
19. Goshima N, Kawamura Y, Fukumoto A, Miura A, Honma R, Satoh R *et al.* Human protein factory for converting the transcriptome into an *in vitro*-expressed proteome. *Nat Methods* 2008; **5**: 1011–1017.
20. Sawasaki T, Kamura N, Matsunaga S, Saeki M, Tsuchimochi M, Morishita R *et al.* Arabidopsis HY5 protein functions as a DNA-binding tag for purification and functional immobilization of proteins on agarose/DNA microplate. *FEBS Lett* 2008; **582**: 221–228.
21. Takahashi H, Nozawa A, Seki M, Shinozaki K, Endo Y, Sawasaki T. A simple and high-sensitivity method for analysis of ubiquitination and polyubiquitination based on wheat cell-free protein synthesis. *BMC Plant Biol* 2009; **9**: 39.
22. Matsuoka K, Komori H, Nose M, Endo Y, Sawasaki T. Simple screening method for autoantigen proteins using the N-terminal biotinylated protein library produced by wheat cell-free synthesis. *J Proteome Res* 2010; **9**: 4264–4273.
23. Fischer U, Janicke RU, Schulze-Osthoff K. Many cuts to ruin: a comprehensive update of caspase substrates. *Cell Death Differ* 2003; **10**: 76–100.
24. Kurokawa M, Kornbluth S. Caspases and kinases in a death grip. *Cell* 2009; **138**: 838–854.
25. Lee KK, Murakawa M, Nishida E, Tsubuki S, Kawashima S, Sakamaki K *et al.* Proteolytic activation of MST/Krs, STE20-related protein kinase, by caspase during apoptosis. *Oncogene* 1998; **16**: 3029–3037.
26. Tomiyoshi G, Horita Y, Nishita M, Ohashi K, Mizuno K. Caspase-mediated cleavage and activation of LIM-kinase 1 and its role in apoptotic membrane blebbing. *Genes Cells* 2004; **9**: 591–600.
27. Tatsuta T, Shiraishi A, Mountz JD. The prodomain of caspase-1 enhances Fas-mediated apoptosis through facilitation of caspase-8 activation. *J Biol Chem* 2000; **275**: 14248–14254.
28. Packard BZ, Komoriya A, Brotz TM, Henkart PA. Caspase 8 activity in membrane blebs after anti-Fas ligation. *J Immunol* 2001; **167**: 5061–5066.
29. Lahti JM, Xiang J, Heath LS, Campana D, Kidd VJ. PITSLRE protein kinase activity is associated with apoptosis. *Mol Cell Biol* 1995; **15**: 1–11.
30. Emoto Y, Manome Y, Meinhardt G, Kisaki H, Kharbanda S, Robertson M *et al.* Proteolytic activation of protein kinase C delta by an ICE-like protease in apoptotic cells. *EMBO J* 1995; **14**: 6148–6156.
31. Casciola-Rosen LA, Anhalt GJ, Rosen A. DNA-dependent protein kinase is one of a subset of autoantigens specifically cleaved early during apoptosis. *J Exp Med* 1995; **182**: 1625–1634.
32. Pearce LR, Komander D, Alessi DR. The nuts and bolts of AGC protein kinases. *Nat Rev Mol Cell Biol* 2010; **11**: 9–22.
33. Ohoka N, Sakai S, Onozaki K, Nakanishi M, Hayashi H. Anaphase-promoting complex/ cyclosome-cdh1 mediates the ubiquitination and degradation of TRB3. *Biochem Biophys Res Commun* 2010; **392**: 289–294.
34. Sawasaki T, Hasegawa Y, Tsuchimochi M, Kamura N, Ogasawara T, Kuroita T *et al.* A bilayer cell-free protein synthesis system for high-throughput screening of gene products. *FEBS Lett* 2002; **514**: 102–105.
35. Kamura N, Sawasaki T, Kasahara Y, Takai K, Endo Y. Selection of 5′-untranslated sequences that enhance initiation of translation in a cell-free protein synthesis system from wheat embryos. *Bioorg Med Chem Lett* 2005; **15**: 5402–5406.
36. Crooks GE, Hon G, Chandonia JM, Brenner SE. WebLogo: a sequence logo generator. *Genome Res* 2004; **14**: 1188–1190.

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METHODOLOGY ARTICLE Open Access

Biotinylated-sortase self-cleavage purification (BISOP) method for cell-free produced proteins

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Abstract

Background: Technology used for the purification of recombinant proteins is a key issue for the biochemical and structural analyses of proteins. In general, affinity tags, such as glutathione-S-transferase or six-histidines, are used to purify recombinant proteins. Since such affinity tags often interfere negatively with the structural and functional analyses of proteins, they are usually removed by treatment with proteases. Previously, Dr. H. Mao reported self-cleavage purification of a target protein by fusing the sortase protein to its N-terminal end, and subsequently obtained tag-free recombinant protein following expression in *Escherichia coli*. This method, however, is yet to be applied to the cell-free based protein production.

Results: The histidine tag-based self-cleavage method for purifying proteins produced by the wheat cell-free protein synthesis system showed high background, low recovery, and unexpected cleavage between the N-terminally fused sortase and target protein during the protein synthesis. Addition of calcium chelator BAPTA to the cell-free reaction inhibited the cleavage. In order to adapt the sortase-based purification method to the cell-free system, we next used biotin as the affinity tag. The biotinylated sortase self-cleavage purification (BISOP) method provided tag-free, highly purified proteins due to improved recovery of proteins from the resin. The N-terminal sequence analysis of the GFP produced by the BISOP method revealed that the cleavage indeed occurred at the right cleavage site. Using this method, we also successfully purified the E2 heterocomplex of USE2N and USE2v1. The c-terminal src kinase (CSK) obtained by the BISOP method showed high activity in phosphorylating the Src protein. Furthermore, we demonstrated that this method is suitable for automatically synthesizing and purifying proteins using robots.

Conclusion: We demonstrated that the newly developed BISOP method is very useful for obtaining high quality, tag-free recombinant proteins, produced using the cell-free system, for biochemical and structural analyses.

Background

Technology used for purifying a recombinant protein has a significant impact on its biochemical function, structural properties, and other aspects, such as generating an antibody against the protein. Currently available, established purification methods generally attach an affinity tag to the N-terminus or C-terminus end of the target protein, and then recover the target protein by affinity chromatography [1]. Purification tags used today are classified into peptide-tags and protein-tags based on their nature and form. His-tag, a typical and globally the most used peptide-tag, is highly effective in purifying the tagged protein by using immobilized metal affinity chromatography, such as nickel sepharose [2]. The glutathione-S-transferase (GST) tag, a typical protein-tag, has a high specific binding capacity for glutathione, and is, generally, known to have little or no effect on the activity of the fused recombinant protein [3]. In both cases, however, production of tag-free recombinant protein requires treatment with a protease, such as PreScission or TEV protease. Therefore, in order to purify a tag-free recombinant protein multiple chromatography steps are necessary.

Currently, there are only a few recombinant protein purification methods that combine affinity purification, cleavage, and separation of the fusion partner in one-step. One such one-step purification method was reported by Mao [4], in which the catalytic core of the transpeptidase

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sortase A (srtA, amino acid residues from 60-206), found in the cell envelope of *Staphylococcus aureus* [5,6], was used for fusion with the target protein. The SrtA enzyme is known to catalytically cleave the Thr-Gly bond of its recognition motif LPXTG (X is any amino acid) in the presence of calcium and triglycine [6-9]. Thus, the purified target protein eluted off the affinity column has only an extra Gly residue on the N-terminus end. This excellent approach was designed for the purification of recombinant proteins expressed in *Escherichia coli* cells. However, as discussed in that report [4], the fusion protein was partially self-cleaved during the expression, probably because of the difficulty in controlling the concentration of calcium in the living cells. Inability to suppress the srtA activity during the expression of the fused recombinant protein in *E. coli* was, therefore, a major limiting factor for using this otherwise excellent approach as a general tool for the production and purification of recombinant proteins.

At present, several types of cell-free protein production systems have been reported as alternative methods for obtaining recombinant proteins [see 10 and 11 for reviews]. In this regard, it is noteworthy that we are also developing wheat embryo based cell-free system for in vitro protein production [12-14]. Cell-free protein production is very flexible because it utilizes only the translational machinery of the cell without other factors, such as DNA replication and metabolic pathways, of the living system. The cell-free system, thus, could simply be modified by the addition or subtraction of reagents. In this study, we have adopted the self-cleavage activity of srtA in the wheat cell-free system for the production of tag-free recombinant proteins, and demonstrated an improved self-cleavage purification method by incorporating biotinylation reagents and calcium chelates in the cell-free synthesis system.

Results and Discussion

Self-cleavage activity of srtA during the cell-free protein synthesis

First, based on the previous report, we constructed the expression vector pEU-His-srtA-GW by inserting the DNA fragment required for the Gateway (GW) recombination technology into the previously described wheat germ cell-free expression vector pEU-E01 [13]. The Gateway system allows easy recombination of the targeted genes. The srtA cleavage site, LPETG, was introduced in the forward PCR primer according to the instructions provided with the Gateway system (Figure 1A). To test the self-cleavage purification system, we selected the human protein kinases and malaria vaccine candidate Pfs25 *(Plasmodium falciparum* 25 kDa ookinete surface antigen precursor) as candidate proteins because they are very important proteins for practical use. PCR products of the coding regions of Pfs25 and six protein kinases were amplified, and each PCR amplified fragment was individually inserted into the pDONR221 vector by BP recombination reaction. Subsequently, the inserted LPETG-gene fragment was cloned into the pEU-His-srtA-GW vector by LR recombination reaction, resulting in a pEU-His-srtA-LPETG-gene plasmid. These pEU-His-srtA-LPETG-gene plasmids were then used for ^{14}C-Leu-labeled protein synthesis using the wheat cell-free system. Unfortunately, in all cases, 20 to 40% of the synthesized proteins were cleaved during the cell-free synthesis (Figure 1B). The cleavage rate was dependent on the type of the gene used in creating the plasmid construct. For example, cleavage of Pfs25 (S25 lane in Figure 1B) and GFP (Figure 1C) during the protein synthesis process were very low, whereas almost 40% of the synthesized SGK495 protein was cleaved during the cell-free synthesis. Since calcium supplementation is known to induce the srtA activity [4,9], the cell-free system was treated with the calcium chelator, BAPTA (Figure 1C). Treatment with more than 4 mM BAPTA dramatically blocked the inexpedient cleavage of SGK495. However, protein synthesis was inhibited as the BAPTA concentration was increased (Figure 1D). For example, levels of SGK495 and GFP proteins produced in the presence of 5 mM BAPTA were approximately 20% and 30%, respectively, of their respective levels in the absence of BAPTA. In other proteins, the synthesis conditions in the presence of 1 and 3 mM BAPTA were investigated (Figure 1E and 1F). By comparing the results of the BAPTA-concentration dependent blocking of the srtA activity and inhibition of the protein synthesis, we concluded that 1 mM BAPTA is optimal for the cell-free synthesis of srtA-fusion proteins, because at this concentration of BAPTA the recovery of all full-length srtA fusion proteins improved by approximately 10-20% without any major inhibition of the protein synthesis.

Self-cleavage purification of His-srtA-fusion proteins

A total of eight plasmid constructs, each containing a different gene (generalized here as pEU-His-srtA-LPETG-gene), were used for the cell-free protein production and self-cleavage purification studies, and the results are shown in Figure 1G. Out of eight proteins, three proteins, GFP, p38g and Pfs25, clearly eluted from a nickel-nitrilotriacetic acid (Ni-NTA) sepharose column, whereas other five proteins could not be recovered in the eluted fraction (Figure 1G, left panel). To confirm protein synthesis and self-cleavage, column resins of all samples were analyzed by SDS-PAGE after boiling with SDS-sample buffer (Figure 1G, right panel). Surprisingly, even though AMPKa1, CSK, SGK and SGK495 were synthesized and self-cleaved on the column resin, their cleaved forms were not eluted off the resin. We could not find the RIPK3 protein in the

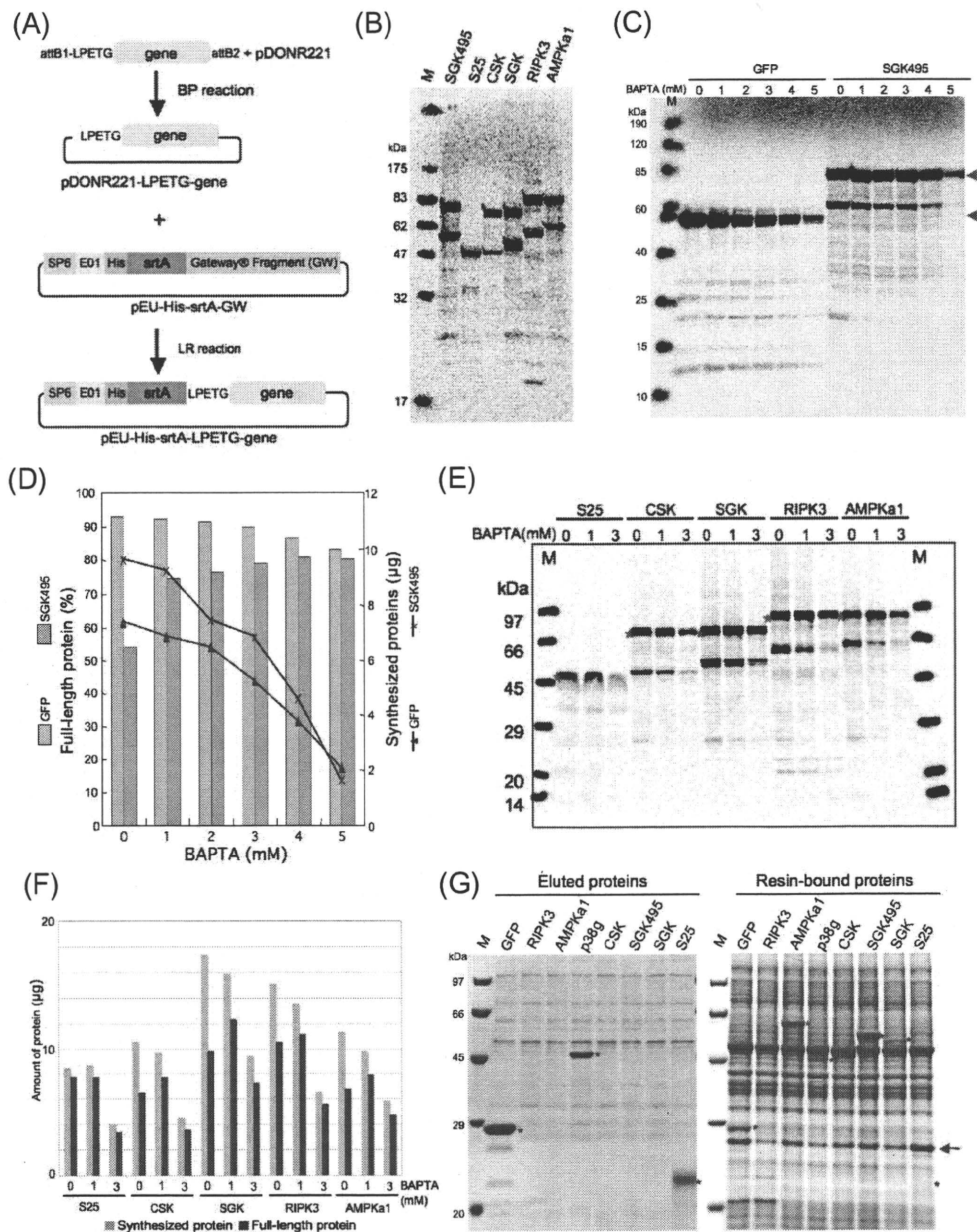

Figure 1 Synthesis of srtA-fusion proteins using the wheat germ cell-free system. A. Schematic representation of the pEU-His-srtA-LPETG-Gene plasmids created using the Gateway system. **B**. Autoradiogram of SDS-PAGE of proteins synthesized using the cell-free system in the presence of [^{14}C] Leu. Lane M, Protein MW standards labeled by using [^{14}C]-containing felt pen. **C**. Autoradiogram of [^{14}C] Leu incorporated GFP and SGK495 proteins synthesized by the wheat cell-free system in the presence of the Ca^{2}+ chelating reagent BAPTA. The number represents concentration (mM) of BAPTA used in the protein synthesis reaction. Arrowheads denote the sizes of the full-length proteins. **D**. Rate of synthesis of the full-length protein and productivity of GFP (pink-colored bar and red-colored line) and SGK495 (purple-colored bar and blue-colored line) in the presence of different concentrations of BAPTA. **E**. Autoradiogram of [^{14}C]-Leu incorporated proteins synthesized by the cell-free system in the presence of BAPTA. Asterisk denotes the sizes of the full-length proteins. **F**. Rate of synthesis of the full-length protein and productivity of proteins in the presence of different concentrations of BAPTA. Productivities of total synthesized and full-length proteins indicated as blue and red bars respectively. **G**. Purifications of proteins by the cell-free synthesis using the pEU-His-srtA-LPETG-Gene plasmid constructs. CBB-stained protein bands on the SDS-PAGE gel of the eluted (left panel) and resin-bound (right) target proteins are indicated using asterisk. Arrow represents the cleaved His-tagged srtA. Lane M (both panels): Protein MW standards.

eluted or in the resin-bound fraction, suggesting that this protein was expressed at a very low level. In addition, purity of the protein in the eluted fraction was not high, as there were several similar protein contaminants in every lane. Furthermore, many proteins bound to the Ni-NTA resin (right panel in Figure 1G). These results suggested that further technical improvements were necessary to achieve high quality purified proteins with high-efficiency from the cell-free based His-srtA system.

Biotinylated sortase self-cleavage purification (BISOP)

Recently we successfully adapted the biotin-labeling system of *E. coli* biotin ligase and biotin to the wheat cell-free protein synthesis system [15]. The biotinylation reaction modifies a specific lysine residue at the biotin ligation site (bls: GLNDIFEAQKIEWHE, the underline is the ligation site). The biotinylated proteins could be directly used for an assay without further purification because of very low biotin concentration. Since the His-tag based approach showed many contaminated proteins in the eluted fraction of the Ni-NTA column, we next used the biotin-labeled tag for protein purification. For this purpose, we constructed the pEU-BISOP-LPETG-GFP plasmid based on the pEU-His-srtA-LPETG-GFP vector as shown in Figure 1A and 2A. Next, we compared whether the His-tagged or the biotin-tagged protein could be better purified by the sortase self-cleavage method, processed either manually or using automated robots to eliminate any human error. Clearly, on the CBB-stained SDS-PAGE, a single major protein band (with low background) was found in the eluted fraction following the sortase self-cleavage of the biotin-tagged srtA fusion protein; in contrast, contaminating proteins were found along with the major protein band in the eluted fraction of the self-cleaved His-tagged srtA fusion protein (Figure 2B). Reaction performance of robot for His-srtA-LPETG-GFP or BISOP-LPETG-GFP was 63 or 58% of recovery, 52 or 88% of purity, and 68 or 62 μg of yield respectively. These results suggest that the BISOP method is better suited for producing tag-free purified proteins by the cell-free system.

Next, to examine whether the number of bls has any effect on the purification, two vectors, one having a single bls and the other having double bls, were constructed (Figure 2A). Coding regions of GFP, Pfs25 and CSK were individually cloned into each one of these two vectors, and then the resultant recombinant plasmids were used for the protein synthesis using the cell-free system. Staining with Alexa488-labeled streptavidin revealed slight mobility shifts for proteins containing double bls, as compared to those containing single bls (Figure 2C). We did not observe any difference between the single and double bls containing proteins with respect to biotinylation, elution and resin binding characteristics. Similar to the self-cleavage purification of GFP (shown in Figure 2B), all three eluted proteins were highly purified (left panel in Figure 2D). The higher purity of proteins obtained using the BISOP method might be due to the presence of very few contaminating proteins on the streptavidin-conjugated resin (compare right panel in Figure 2D with Figure 1G). Interestingly, the CSK protein, prepared by the BISOP method was eluted of the resin, whereas with the His-tag based method it was not found in the eluted fraction (Figure 1G). Total amounts of purified GFP, S25 and CSK by the BISOP method were 44, 37, and 55 μg per reaction respectively. Also full-length GFP, S25 and CSK proteins remaining on the column were approximately 6, 3 and 3 μg respectively, and cleaved S25 and CSK proteins on the column were 3 and 3 μg respectively. These data means that rate of target proteins remaining on the column was approximately 15%. Next, the BISOP method was used for purifying several other proteins. For this purpose, four protein kinase genes were individually inserted into the Gateway system vector pEU-BISOP-GW following the procedures described above, and the results are shown in Figure 2E. Both SGK495 and AMPKa1, which were not recovered earlier from the resin when the His-tag based method was used (Fgiure 1G), were also purified by the BISOP method (Figure 2E).

Total amounts of purified CSK, SGK495, AMPKa1 and p38g by the BISOP method were 55, 42, 24 and 57 μg per reaction respectively. In addition, analysis of the N-terminal sequence of the GFP protein purified by the BISOP revealed the expected cleavage of the Thr-Gly bond of the inserted LPETG sortase-recognition site (Figure 2F). These results suggested that the BISOP method would be suitable for the purification of the cell-free produced proteins with high efficiency and purity.

Purification of E2 heterocomplex by BISOP

Analysis of protein complex is one of important targets for their structural and biochemical analysis. Thus, next we examined whether a protein heterocomplex, co-expressed using the wheat cell-free system, could be purified by the BISOP method. To test this notion, we next co-expressed UBE2N and UBE2v1, two proteins forming the heterodimer complex of the ubiquitin-conjugate (E2) enzyme [16], using the BISOP method. Specific complex formation between these two proteins produced by the cell-free system has already been reported [15]. At first, we confirmed co-expression of the biotinylated srtA-LPETG-UBE2N and UBE2v1 (tag-free form) by incorporating ^{14}C-Leu during the cell-free synthesis (Figure 3A). Notably, we recovered the E2 heterocomplex consisting of UBE2N and UBE2v1 when both UBE2v1 and srtA-LPETG-UBE2N were co-expressed using the cell-free system and then purified by the BISOP method (Figures 3B and 3C). The protein band corresponding to UBE2v1

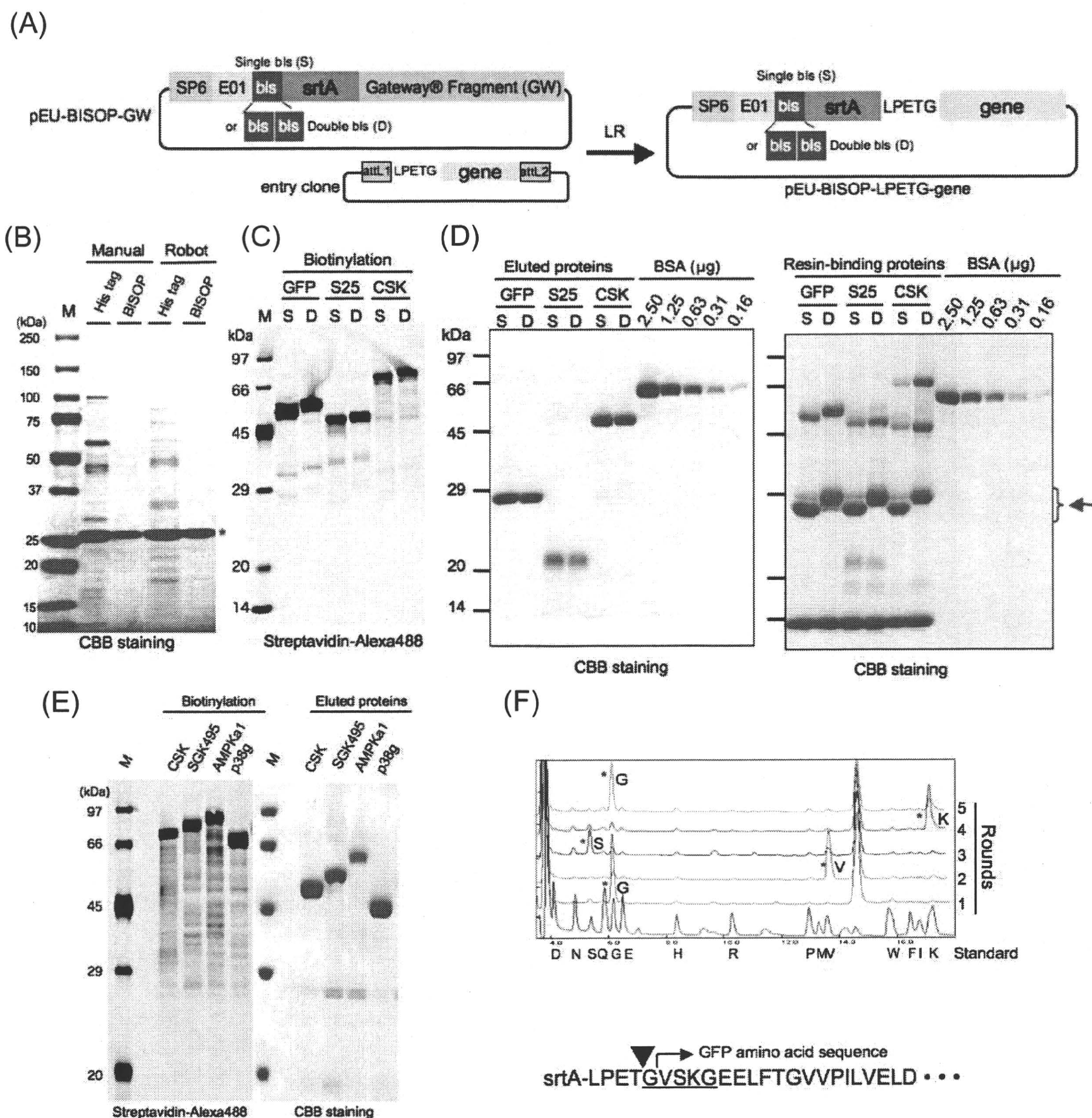

Figure 2 Preparation of proteins by the biotinylated-sortase self-cleavage purification (BISOP) method. A. Scheme for creating the pEU-BISOP-LPETG-gene plasmids using the Gateway recombination method. The LPETG-fused target gene containing entry clone plasmid was recombined with the pEU-BISOP-GW vector by using the Gateway LR reaction. **B**. CBB-stained SDS-PAGE comparing the His- or biotin-tagged GFP protein (indicated using an asterisk) purified manually or by using an automated robot. Lane M: Protein MW standards. **C**. Biotinylation of GFP, Pfs25 (S25) and CSK proteins synthesized by the cell-free system with vectors having a single (S) or a double (D) biotin ligation site. Biotinylated proteins were detected by labeling the separated protein bands with streaptavidin-Alexa488 as described in the text, followed by scanning using the Typhoon Imager. **D**. CBB-stained eluted (left panel) and resin-bound (right panel) proteins. Arrow indicates the cleaved biotinylated srtA protease left on the resin. Samples (5 or 10 μL in left or right panel respectively) were loaded on the gel. **E**. Left panel: Biotinylated proteins were detected by streaptavidin-Alexa488; Right panel: CBB-stained proteins purified by the BISOP method. Samples (5 μL) were loaded on the gel. **F**. Amino acid sequences from the N-terminal end of GFP purified by the BISOP method were determined by using an amino acid sequencer (asterisks, upper panel). Rounds indicate the number of Edman degradation cycle. Underlined amino acid sequence in the lower panel shows the determined sequence. Arrowhead indicates the site cleaved by the sortase enzyme.

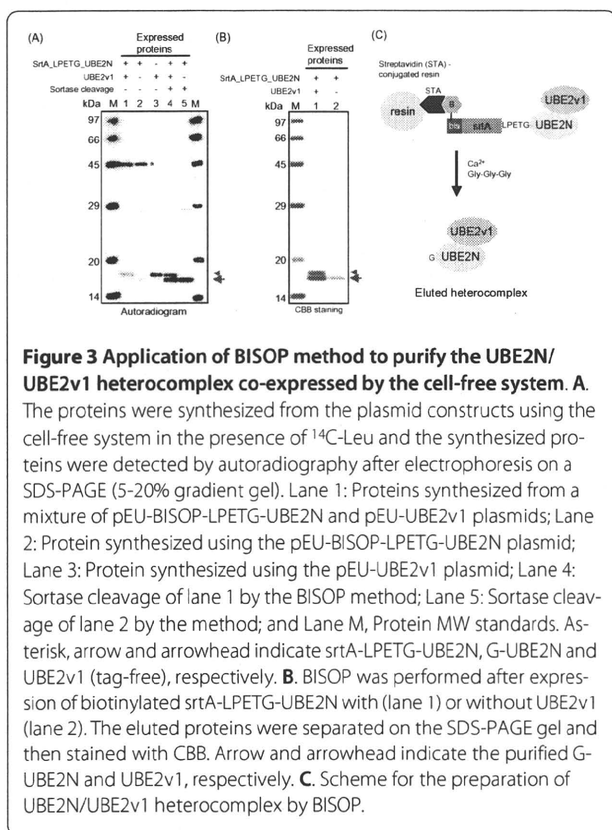

Figure 3 Application of BISOP method to purify the UBE2N/UBE2v1 heterocomplex co-expressed by the cell-free system. A. The proteins were synthesized from the plasmid constructs using the cell-free system in the presence of ^{14}C-Leu and the synthesized proteins were detected by autoradiography after electrophoresis on a SDS-PAGE (5-20% gradient gel). Lane 1: Proteins synthesized from a mixture of pEU-BISOP-LPETG-UBE2N and pEU-UBE2v1 plasmids; Lane 2: Protein synthesized using the pEU-BISOP-LPETG-UBE2N plasmid; Lane 3: Protein synthesized using the pEU-UBE2v1 plasmid; Lane 4: Sortase cleavage of lane 1 by the BISOP method; Lane 5: Sortase cleavage of lane 2 by the method; and Lane M, Protein MW standards. Asterisk, arrow and arrowhead indicate srtA-LPETG-UBE2N, G-UBE2N and UBE2v1 (tag-free), respectively. **B.** BISOP was performed after expression of biotinylated srtA-LPETG-UBE2N with (lane 1) or without UBE2v1 (lane 2). The eluted proteins were separated on the SDS-PAGE gel and then stained with CBB. Arrow and arrowhead indicate the purified G-UBE2N and UBE2v1, respectively. **C.** Scheme for the preparation of UBE2N/UBE2v1 heterocomplex by BISOP.

was however not found when the biotinylated srtA-LPETG-UBE2N was expressed alone. Therefore, this result suggests that the BISOP method would be useful for purification of protein complexes produced by the cell-free system.

Activity of CSK purified by the BISOP method

It is very important that the purification method provide functionally active protein. We, therefore, investigated whether the CSK protein purified using the BISOP method could specifically phosphorylate the Tyr-530 residue of human Src protein (Swiss-Prot no. P12931) [17]. For this purpose, biotinylated Src was synthesized by the cell-free system and the synthesized protein was partially purified using the magnetic streptavidin-conjugated beads. The bead-bound Src was then treated with [^{32}P]-labeled or unlabeled ATP and the CSK protein that was purified by the BISOP method. Both autoradiogram of the SDS-PAGE separated proteins from the reaction mix containing [^{32}P]-labeled ATP and immunoblot analysis of the SDS-PAGE separated proteins from the reaction mix containing the unlabeled ATP using the specific anti-phospho-Src antibody showed specific phosphorylation of the Y530 residue of Src by CSK (Figure 4). These results suggest that BISOP would be suitable for the in vitro synthesis of active proteins.

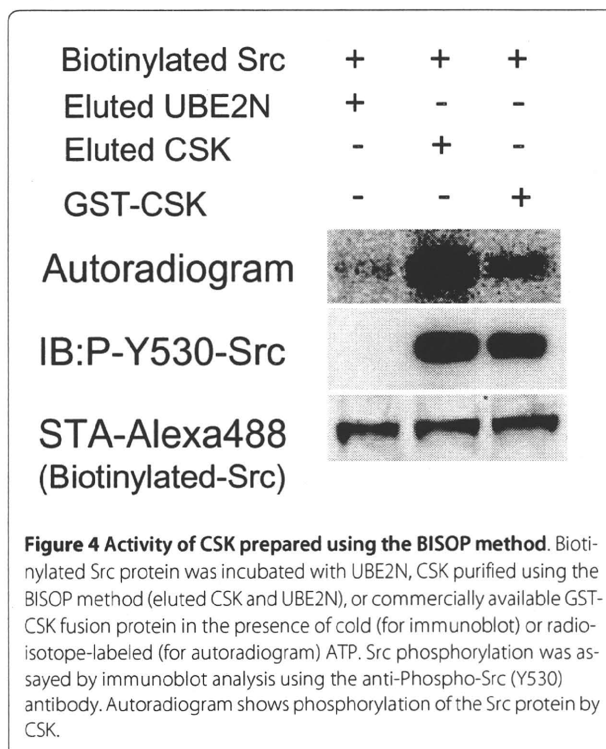

Figure 4 Activity of CSK prepared using the BISOP method. Biotinylated Src protein was incubated with UBE2N, CSK purified using the BISOP method (eluted CSK and UBE2N), or commercially available GST-CSK fusion protein in the presence of cold (for immunoblot) or radioisotope-labeled (for autoradiogram) ATP. Src phosphorylation was assayed by immunoblot analysis using the anti-Phospho-Src (Y530) antibody. Autoradiogram shows phosphorylation of the Src protein by CSK.

Conclusion

The cell-free system is an easy to use method for synthesizing recombinant proteins. In this study, we have developed a new biotinylated-sortase self-cleavage purification (BISOP) method to achieve high quality purification of several proteins, including a protein heterocomplex, without any affinity tag. In addition, we showed that this method could be automated using robots. Results of this study indicate that the combination of the cell-free protein synthesis system and BISOP could provide a simple and easy method for the preparation of high quality recombinant proteins without any affinity tag. Since the cell-free system is suitable for high-throughput protein production, this combined method could also be utilized at the genome-wide level.

Methods

General

The following procedures have been either described in detail or cited [13,15,18,19]: isolation of the wheat germs and preparation of the extract, generation of the DNA template by polymerase chain reaction (PCR) using split-primers, parallel synthesis of mRNA and protein, estimation of the amount of protein synthesized by densitometric scanning of the Coomassie brilliant blue (CBB)-stained band and autoradiogram of radiolabeled-proteins, and detection of biotinylated proteins using Alexa488-conjugated streptavidin (Invitrogen) and the

Typhoon Imager (GE Healthcare) fitted with 532 nm laser and 526 emission filter.

Template genes

The cDNA clone of the malarial parasite *Plasmodium falciparum* 25 kDa ookinete surface antigen precursor (Pfs25) was kindly provided by Dr. Tsuboi (Cell-free Research and Technology Center, Ehime University). cDNAs of GFP, UBE2N (GenBank accession no. BC003365), and UBE2v1 (GenBank accession no. BC000468) were described in our previous reports [15,18]. Mammalian gene collection (MGC) cDNA clones of CSK (BC104875), SGK (BC001263), SGK495 (BC007835), AMPKa1 (BC048980), p38g (BC015741), RIPK3 (BC062584) and Src (BC011566) were also used in this study.

Construction of the srtA-based self-cleavage vector

DNA fragment encoding the mature-form of srtA (corresponding to amino acids 60-206, GenBank accession no. AF162687) was artificially synthesized and inserted into the pUC57 to create the plasmid pUC57-srtA-EcoRV-SpeI by the GenScript Corporation (Boston). The pUC57-srtA-EcoRV-SpeI plasmid as used as a template to amplify the mature srtA fragment (DraI-His or bls-srtA-EcoRV-SpeI) by PCR using the following pair of primers: M13(M3) (5'-GTAAAACGACGGCCAGT) and DraI-A-His-srtA (5'-GAGATTTAAATGGCCAGCAGC CATCACCATCACCATCATAGCAGCGGCCTGGTGC CGC) or M13(M3) and Dra1-A-bls-srtA (5'-GAGAT TTAAATGGCCAGCAGCGGCCTGAACGACATCTTC GAGGCCCAGAAGATCGAGTGGCACGAAAGCAGC G GCCTGGTGCCGC). The DraI-A-His-srtA primer included 6 × His-tag codons and a DraI restriction enzyme site, and the DraI-A-bls-srtA primer included a bls (biotin ligase site) recognition sequence and a DraI restriction enzyme site. After digestion with DraI and SpeI enzymes each fragment was inserted into the EcoRV and SpeI sites in pEU-E01-MCS provided by CellFree Sciences, Ltd http://www.cfsciences.com/eg/index.html to create pEU-His-srtA-MCS and pEU-BISOP-MCS plasmids, respectively. To create the Gateway®-based plasmids pEU-His-srtA-GW and pEU-BISOP-GW, DNA fragment needed for Gateway® recombination cloning technology (Invitorogen) was inserted into the EcoRV site of the pEU-His-srtA-MCS and pEU-BISOP-MCS plasmids. PCR reaction was performed by PrimeStar enzyme according to instruction (Takara Bio, Otsu, Japan). Nucleotide sequences of the DNA inserts in all plasmid constructs were subsequently confirmed by using the ABI PRISM 310 Genetic Analyzer using the BigDye terminator v1.1 Cycle sequence kit (Applied Biosystems, Foster City, CA).

Plasmid construction for the cell-free protein production

We introduced the srtA self-cleavage site (DNA encoding for the amino acid sequence LPETG) into the recombinant plasmid construct for the cell-free production of proteins. The DNA fragments coding the respective protein were amplified by PCR using two gene specific primers: forward primer attB1-LPETG-Gene (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTC-**CTGCCCGAGACCGGC**atg(n)$_{19}$; uppercase, lowercased and bold sequences indicated common, gene specific and LPETG sequences, respectively; n represent gene specific sequence) and reverse primer attB2-Gene (5'-GGGGACCACTTTGTACAAGAAAGCTG-GGTCxxxnnnnnnnnnnnnnnnn; xxx is the complementary sequence of the stop codon; n represent gene specific sequence). PCR reaction was performed using the PrimeStar enzyme (Takara Bio, Otsu, Japan) and following the supplier's instructions. The amplified attB1-LPETG-Gene-attB2 fragments were inserted into the donor vector pDONR221 by BP reaction to generate the entry plasmids. The LPETG-fusion Gene in the entry plasmid was transferred to the pEU-His-srtA-GW or pEU-BISOP-GW by LR reaction to generate the pEU based-plasmid clones. BP and LR reactions were carried out according to the instructions provided by the supplier of the reagents (Invitrogen, Carlsbad, CA). Nucleotide sequences of the DNA inserts in all the plasmid constructs were subsequently confirmed by using the ABI PRISM 310 Genetic Analyzer described method above.

Cell-free protein production

For the cell-free protein production, we employed the wheat germ cell-free protein expression system using the bilayer translation method described previously [18,19]. Cell-free protein production was carried out using the ENDEXT® Wheat Germ Expression S Kit and according to the instructions provided by the supplier (CellFree Sciences Co., Ltd., Matsuyama, Japan). Briefly, 250 μL of transcriptional mixture [80 mM HEPES-KOH, pH 7.8, 16 mM magnesium acetate, 2 mM spermidine, 10 mM DTT, 2.5 mM NTP mix, 1 U/μL SP6 RNA polymerase (Promega, Madison, WI), 1 U/μL RNase inhibitor, RNasin (Promega), and 100 ng/μL undigested plasmid DNA] was incubated at 37°C for 6 h, and then mixed with 250 μL of wheat embryo extract (120 A 260/mL, CellFree Sciences Co., Ltd.) and 1 μL of 20 mg/mL creatine kinase (Roche Applied Science, Indianapolis, IN). This mixture, called the translational mixture, was then carefully transferred to the bottom of the well of a 6-well tissue culture plate (Whatman Inc., Clifton, NJ) that already contained 5.5 mL of TSB (30 mM HEPES-KOH, pH 7.8, 100 mM potassium acetate, 2.7 mM magnesium acetate, 0.4 mM spermidine, 2.5 mM DTT, 0.3 mM amino acid mix, 1.2 mM ATP, 0.25 mM GTP and 16 mM creatine phosphate) by

inserting the pipette tip down to the bottom of the well, thereby creating two distinct layers. The plate was then covered with the sealing film, and was incubated at 17°C for 18 hr without shaking. For the calcium chelating experiment, 1 mM BAPTA (1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid) (Sigma-Aldrich, St Louis, MO) was added to both the translational mixture and TSB. For biotin labeling of proteins [15,20], 2 μg of biotin protein ligase (BirA, GenBank accession no. NP_0312927) produced by the wheat cell-free system and 6 μM D-biotin (Nacalai Tesque, Kyoto, Japan) were added to the bottom translational mixture.

For co-expression of the biotinylated srtA-LPETG-UBE2N and UBE2v1, the cell-free production method was slightly modified. Each translation mixture was prepared independently and pre-incubated at 26°C for 30 min, following which they were mixed, and were subsequently used in the bilayer translation reaction as described above.

Self-cleavage purification of proteins produced by the cell-free system

Reaction mixture (6 mL) from the cell-free expression system described above was mixed with 100 μL of Ni-NTA sepharose (GE Healthcare) or 100 μL of streptavidin-sepharose (GE helthcase). The sepharose beads were pre-equilibrated with phosphate buffered saline (PBS) for 4 to 6 hours (His tag) or 30 min (BISOP) at 4°C. Sepharose bead-captured srtA-fusion proteins were collected by centrifugation (3,000 × g) and the beads were washed three times with PBS buffer. Self-cleavage purification of the target protein was performed by incubation of the beads with 100 μL of Elution buffer [20 mM Tris-HCl (pH 7.5), 5 mM Tri-Gly (Sigma-Aldrich), 5 mM CaCb, 150 mM NaCl, 1 mM DTT and 2% glycerol] for 4 hours at 16°C. The buffer-bead mixture was then transferred into a micro spin-column (GE Healthcare) and the eluted fraction was recovered by flash centrifuge (3,000 × g) at 4°C. The N-terminal end of the eluted GFP was determined by amino acid sequence analysis using the Applied Biosystems ABI 473A protein sequencer and according to the instructions provided by Applied Biosystems.

The cell-free protein production and self-cleavage purification were also carried out using an automatic robot, Protemist DTII (CellFree Sciences Co., Ltd.), basically according to manufacturer's instructions. Addition of biotinylation reagents and BAPTA were also carried out as mentioned above.

Phosphorylation assay

The phosphorylation assay was mainly performed according to the previous published methods [13,21]. To assay for phosphorylation of the biotinylated Src by CSK, 40 μl of the reaction mixture was mixed with 15 μl of biotin magnetic beads (Promega, MI), and was washed twice with PBS buffer and once with protein kinase (PK) buffer [50 mM Tris-HCl (pH 7.6), 500 mM potassium acetate, 50 mM $MgCl_2$, 0.1 mM DTT]. Beads were suspended in 10 μl PK buffer supplemented with cold ATP (for immunoblot) or [γ-^{32}P]-ATP (for autoradiogram), and CSK purified using the BISOP method, UBE2N or commercially available GST-CSK fusion protein (purchased from Carna Biosciences Inc., Kobe) was added in the reaction mixture. The mixtures were incubated at 37°C for 30 min, following which they were boiled in the SDS-denaturing buffer and the proteins were separated on 12.5% SDS-polyacrylamide gel. Autoradiogram of Src phosphorylation was analyzed by BAS-2500 (FUJIFILM, Tokyo, Japan). For immunoblot analysis, proteins were transferred from the SDS-PAGE gel to PVDF membrane (Millipore Bedford, MA, USA) following standard procedures. The blots were then processed using the Immobilon Western detection reagents (Millipore) and antibody against phosphorylated Src (Y527) or Src (Cell Signaling Technology, Beverly, MA) according to the manufacturer's procedure. The anti-phospho-Src (Y527) antibody recognizes the phosphorylated Y530 residue in human Src.

Abbreviations

srtA: sortase SrtA; GFP: green fluorescent protein; SGK: serum/glucocorticoid regulated kinase 1; CSK: c-src tyrosine kinase; UBE2N: ubiquitin-conjugating enzyme E2N; UBE2v1: ubiquitin-conjugating enzyme E2 variant 1; AMPKa1: 5'-AMP-activated protein kinase alpha 1 catalytic subunit; PRKAA1: protein kinase, AMP-activated: alpha 1 catalytic subunit; MAPK12: mitogen-activated protein kinase 12; p38g: p38 gamma; Pfs25: *Plasmodium falciparum* 25 kDa ookinete surface antigen precursor; BAPTA 1: 2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; PBS: phosphate buffered saline.

Authors' contributions

SM conceived the study and performed some of the experiments; KM and KS performed also participated in performing the experiments; YE conceived the study and supervised the work; TS conceived and designed the study, supervised the work and contributed to writing the manuscript. All authors read and approved the final manuscript.

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References

1. Nygren PA, Stahl S, Uhlen M: **Engineering proteins to facilitate bioprocessing.** *Trends Biotechnol* 1994, **12(5)**:184-188.

2. Janknecht R, de Martynoff G, Lou J, Hipskind RA, Nordheim A, Stunnenberg HG: **Rapid and efficient purification of native histidine-tagged protein expressed by recombinant vaccinia virus.** *Proc Natl Acad Sci USA* 1991, **88(20)**:8972-8976.
3. Smith DB, Johnson KS: **Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione S-transferase.** *Gene* 1988, **67(1)**:31-40.
4. Mao H: **A self-cleavable sortase fusion for one-step purification of free recombinant proteins.** *Protein Expr Purif* 2004, **37(1)**:253-263.
5. Mazmanian SK, Liu G, Ton-That H, Schneewind O: **Staphylococcus aureus sortase, an enzyme that anchors surface proteins to the cell wall.** *Science* 1999, **285(5428)**:760-763.
6. Ton-That H, Mazmanian SK, Faull KF, Schneewind O: **Anchoring of surface proteins to the cell wall of Staphylococcus aureus. Sortase catalyzed in vitro transpeptidation reaction using LPXTG peptide and NH(2)-Gly(3) substrates.** *J Biol Chem* 2000, **275(13)**:9876-9881.
7. Ilangovan U, Ton-That H, Iwahara J, Schneewind O, Clubb RT: **Structure of sortase, the transpeptidase that anchors proteins to the cell wall of Staphylococcus aureus.** *Proc Natl Acad Sci USA* 2001, **98(11)**:6056-6061.
8. Ton-That H, Mazmanian SK, Alksne L, Schneewind O: **Anchoring of surface proteins to the cell wall of Staphylococcus aureus. Cysteine 184 and histidine 120 of sortase form a thiolate-imidazolium ion pair for catalysis.** *J Biol Chem* 2002, **277(9)**:7447-7452.
9. Mao H, Hart SA, Schink A, Pollok BA: **Sortase-mediated protein ligation: a new method for protein engineering.** *J Am Chem Soc* 2004, **126(9)**:2670-2671.
10. Endo Y, Sawasaki T: **Cell-free expression systems for eukaryotic protein production.** *Curr Opin Biotechnol* 2006, **17(4)**:373-380.
11. He M: **Cell-free protein synthesis: applications in proteomics and biotechnology.** *N Biotechnol* 2008, **25(2-3)**:126-132.
12. Madin K, Sawasaki T, Ogasawara T, Endo Y: **A highly efficient and robust cell-free protein synthesis system prepared from wheat embryos: plants apparently contain a suicide system directed at ribosomes.** *Proc Natl Acad Sci USA* 2000, **97(2)**:559-564.
13. Sawasaki T, Ogasawara T, Morishita R, Endo Y: **A cell-free protein synthesis system for high-throughput proteomics.** *Proc Natl Acad Sci USA* 2002, **99(23)**:14652-14657.
14. Madin K, Sawasaki T, Kamura N, Takai K, Ogasawara T, Yazaki K, Takei T, Miura K, Endo Y: **Formation of circular polyribosomes in wheat germ cell-free protein synthesis system.** *FEBS Lett* 2004, **562(1-3)**:155-159.
15. Sawasaki T, Kamura N, Matsunaga S, Saeki M, Tsuchimochi M, Morishita R, Endo Y: **Arabidopsis HY5 protein functions as a DNA-binding tag for purification and functional immobilization of proteins on agarose/DNA microplate.** *FEBS Lett* 2008, **582(2)**:221-228.
16. Zhang M, Windheim M, Roe SM, Peggie M, Cohen P, Prodromou C, Pearl LH: **Chaperoned ubiquitylation--crystal structures of the CHIP U box E3 ubiquitin ligase and a CHIP-Ubc13-Uev1a complex.** *Mol Cell* 2005, **20(4)**:525-538.
17. Nada S, Okada M, MacAuley A, Cooper JA, Nakagawa H: **Cloning of a complementary DNA for a protein-tyrosine kinase that specifically phosphorylates a negative regulatory site of p60c-src.** *Nature* 1991, **351(6321)**:69-72.
18. Sawasaki T, Hasegawa Y, Tsuchimochi M, Kamura N, Ogasawara T, Kuroita T, Endo Y: **A bilayer cell-free protein synthesis system for high-throughput screening of gene products.** *FEBS Lett* 2002, **514(1)**:102-105.
19. Sawasaki T, Morishita R, Gouda MD, Endo Y: **Methods for high-throughput materialization of genetic information based on wheat germ cell-free expression system.** *Methods Mol Biol* 2007, **375**:95-106.
20. Takahashi H, Nozawa A, Seki M, Shinozaki K, Endo Y, Sawasaki T: **A simple and high-sensitivity method for analysis of ubiquitination and polyubiquitination based on wheat cell-free protein synthesis.** *BMC Plant Biol* 2009, **9**:39.
21. Sawasaki T, Hasegawa Y, Morishita R, Seki M, Shinozaki K, Endo Y: **Genome-scale, biochemical annotation method based on the wheat germ cell-free protein synthesis system.** *Phytochemistry* 2004, **65(11)**:1549-1555.

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Methodology article

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A simple and high-sensitivity method for analysis of ubiquitination and polyubiquitination based on wheat cell-free protein synthesis

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Abstract

Background: Ubiquitination is mediated by the sequential action of at least three enzymes: the E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme) and E3 (ubiquitin ligase) proteins. Polyubiquitination of target proteins is also implicated in several critical cellular processes. Although Arabidopsis genome research has estimated more than 1,300 proteins involved in ubiquitination, little is known about the biochemical functions of these proteins. Here we demonstrate a novel, simple and high-sensitive method for *in vitro* analysis of ubiquitination and polyubiquitination based on wheat cell-free protein synthesis and luminescent detection.

Results: Using wheat cell-free synthesis, 11 E3 proteins from Arabidopsis full-length cDNA templates were produced. These proteins were analyzed either in the translation mixture or purified recombinant protein from the translation mixture. In our luminescent method using FLAG- or His-tagged and biotinylated ubiquitins, the polyubiquitin chain on AtUBC22, UPL5 and UPL7 (HECT) and CIP8 (RING) was detected. Also, binding of ubiquitin to these proteins was detected using biotinylated ubiquitin and FLAG-tagged recombinant protein. Furthermore, screening of the RING 6 subgroup demonstrated that At1g55530 was capable of polyubiquitin chain formation like CIP8. Interestingly, these ubiquitinations were carried out without the addition of exogenous E1 and/or E2 proteins, indicating that these enzymes were endogenous to the wheat cell-free system. The amount of polyubiquitinated proteins in the crude translation reaction mixture was unaffected by treatment with MG132, suggesting that our system does not contain 26S proteasome-dependent protein degradation activity.

Conclusion: In this study, we developed a simple wheat cell-free based luminescence method that could be a powerful tool for comprehensive ubiquitination analysis.

Background

Protein ubiquitination plays a crucial role in numerous cellular processes such as cell growth, regulation of diverse signal transduction and disease [1-3]. The covalent attachment of ubiquitin to protein substrates requires a stepwise cascade of enzymatic reactions. First, ubiquitin is activated by E1 (ubiquitin-activating enzyme, UBA) in an ATP-dependent manner by forming a high-energy thioester-bond between the carboxyl-terminal glycine residue of ubiquitin and a cysteine residue of E1. The activated ubiquitin is then transferred to the core-cysteine residue of E2 (ubiquitin-conjugating enzyme, UBC). Together with an E3 ligase enzyme, ubiquitin is attached via its carboxyl-terminus to an e-amino group of a lysine residue in the target protein. Since E3 binds to both E2 and the target protein, and acts as scaffold between E2 and the substrate protein, the E3 ligase is the major determinant for selecting target proteins for ubiquitination. There is large number of genes encoding E3 ligases in all eukaryotes, and the diversity of E3s is thought to contribute to the substrate specificity of numerous target proteins. E3 ligases are structurally divided into three groups: HECT, RING and U-box [4]. The HECT-type E3 ligase is distinct from the other two ligases in that it forms a thioester-bond with ubiquitin prior to the transfer of ubiquitin to target proteins. The RING-type E3 ligase contains a unique domain similar to the zinc finger motif that mediates protein-protein interactions [5] and is further divided into two classes: one that can function alone and another that forms a complex with other E3 components [4].

Recent studies have shown that attachment of polyubiquitin chains on target proteins linked via lysine-48 of ubiquitin typically leads to degradation by the 26S proteasome [6], whereas linkage via lysine-63 mediates different pathways such as internalization of membrane proteins, activation of signal transduction and DNA damage repair [7]. The formation of lysyl-63-linked polyubiquitin chains is generated by specific combinations of E2s and E2 variants, which are similar to E2s except that they lack core cysteine residues required for E2 activity [8,9]. In addition, ubiquitination of substrates without polymerization, mono-ubiquitination, acts as a sorting signal for protein endocytosis and as a regulation factor for diverse proteins, including histones and transcription factors [10].

In plant, genomic research of the model plant *Arabidopsis thaliana* showed that there are two E1s, 37 E2s and more than 1,300 predicted E3s [11]. Although little is known about protein ubiquitination in plants compared with yeast and mammals, recent studies revealed that the plant ubiquitination pathway is involved in the regulation of morphogenesis, the circadian clock and responding to hormone or pathogen signal molecules [12-15]. Despite the importance of ubiquitination in plants, much of the plant ubiquitination cascade is still unknown because of its complexity and the issues inherent to the use of Arabidopsis plants for biochemical analysis. Although several interactions between E2s and RING type E3s have been demonstrated *in vitro* using recombinant proteins expressed in *Escherichia coli*, these efforts are hampered by the inability to obtain functional protein using conventional methods [16].

With this in mind, we sought to develop a novel *in vitro* method to analyze the ubiquitin pathway genome-wide. The two major obstacles hindering the development of an *in vitro* assay for genome-wide screening are the difficulty of efficiently producing recombinant protein and the inability to detect ubiquitination in a high-throughput fashion. To address the first problem we used the wheat cell-free protein synthesis system, which has been previously reported to produce a wide range of functional Arabidopsis and human proteins [17-19]. Moreover, a collection of RIKEN Arabidopsis Full Length (RAFL) cDNA clones covering about 70% of Arabidopsis genes is available [20]. Using these RAFL clones as templates, recombinant proteins involved in the ubiquitination pathway were expressed in the wheat cell-free system and used for several functional analyses. For screening, conventional detection methods such as immunoblot analysis or radioisotope-labeled proteins are not suitable for the detection of a large number of ubiquitination reactions. Recently, a high-throughput luminescence method to detect protein ubiquitination was reported [21], however this method requires purified protein and creation of specialized vectors to produce proteins. In this study, a novel *in vitro* assay to detect polyubiquitin chain formation was developed using wheat cell-free synthesis and a modified luminescence-based detection method. We demonstrate (1) creation of a simple *in vitro* method to detect polyubiquitination using crude recombinant E3s, (2) discovery of the activity of At1g55530 by screening a RING subgroup in the reported assay, and (3) the polyubiquitination assay in the presence of MG132 demonstrated the absence of 26S proteasome-dependent protein degradation activity in wheat cell-free system.

Results

Detection of Polyubiquitin Chains on AtUBC22 E2 enzyme

Recently, AtUBC22 (At5g05080) E2 protein has been shown to catalyze polyubiquitin chain formation without an E3 ligase, although AtUBC35 (At1g78870) E3-independent polyubiquitination activity could not be detected [16]. We employed AtUBC22 and AtUBC35 as model E2 proteins to develop a novel polyubiquitination assay. We have also demonstrated that addition of biotin ligase (BirA) and biotin to the wheat cell-free protein production system yields a single biotinylation on a target pro-

tein containing a biotin ligation site [22]. Using this method, biotinylated recombinant AtUBC22 and AtUBC35 were synthesized and, without purification from the translation mixture, the polyubiquitination reaction was performed on the crude recombinant protein. After the reaction, biotinylated AtUBC22 and AtUBC35 were purified using streptavidin-conjugated magnetic beads and the polyubiquitin chain was detected by immunoblot analysis. As shown in Fig 1A, AtUBC22 showed polyubiquitination, whereas AtUBC35 showed mainly monoubiquitination. Interestingly, both E2s still had activity in absence of exogenous E1 in polyubiquitin reaction mixture (Fig. 1A, middle lanes), suggesting that wheat cell-free system has high endogenous E1 activity.

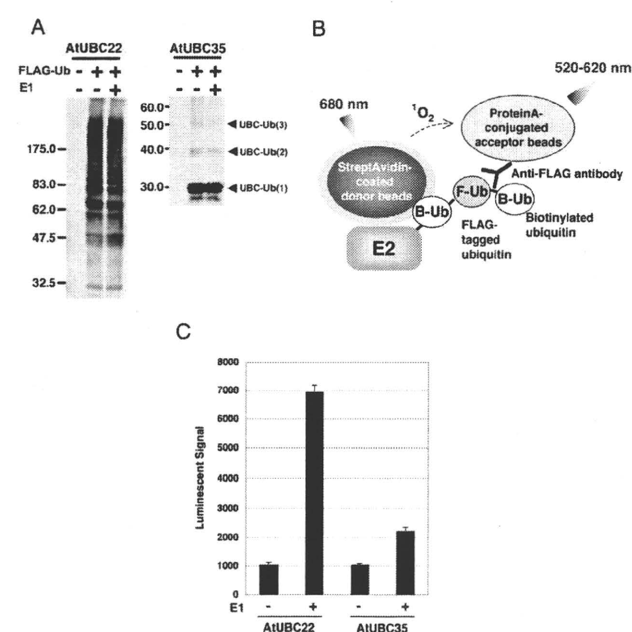

Figure 1
Detection of E3-independent polyubiquitination of AtUBC22 by luminescent analysis. A, Polyubiquitin chain on AtUBC22 but not on AtUBC35 was detected by immunoblot analysis. In this assay, polyubiquitination reaction was carried out with FLAG-tagged ubiquitin, and detected by immunoblot analysis using anti-FLAG antibody. B, Schematic diagram of detection of polyubiquitin chains by luminescent analysis. Protein A-conjugated acceptor beads and streptavidin-coated donor beads are bound to anti-FLAG antibody bound to FLAG-tagged ubiquitin and biotinylated E2, respectively, and these two beads are in closed proximity when polyubiquitin chain formed. Upon excitation 680 nm, a singlet oxygen is generated from the donor beads, and then transferred to the acceptor beads within 200 nm, and the singlet oxygen reacts the acceptor beads which in turn emits light at 520–620 nm. This light is measured by AlphaScreen kit and change to signal value. C, Polyubiquitin chain on purified recombinant E2 was detected by luminescent analysis in the presence (E1 +) or absence (E1 -) of exogenous E1. Error bars represent standard deviations from three independent experiments.

While immunoblot analysis is an excellent detection method, it is not suitable for high-throughput detection of numerous polyubiquitination reactions. Initially, we attempted to use luminescent analysis, based on the AlphaScreen technology, to detect the polyubiquitination activity of AtUBC22 and AtUBC35. In principle, if a polyubiquitin chain is formed by FLAG-tagged and biotinylated ubiquitins, it will bring into proximity the streptavidin-coated donor bead (bound to biotin) and the protein A-conjugated acceptor bead (bound to anti-FLAG IgG), producing a luminescent signal (Fig. 1B). Considering that the wheat cell-free system has high endogenous E1 activity (Fig. 1A), it may also have endogenous E2 and E3 activity. In order to avoid formation of polyubiquitin chains by an endogenous wheat germ ubiquitin pathway, purified E2s were used in this assay. As shown in Fig 1C, high luminescent signal was observed in the presence of AtUBC22 in E1-dependent manner. In contrast, AtUBC35 showed low signal. The two luminescent signals were approximately consistent with immunoblot data that AtUBC22 and AtUBC35 have high and low polyubiquitination activities respectively, as demonstrated in Fig 1A. These results indicate that the luminescent method can detect polyubiquitin chain formation by using the two types of ubiquitins.

Ubiquitination and Polyubiquitination Analyses of HECT-TypeE3 Ligases

Polyubiquitination activity of E3 ligases activated by the step-wise E1 to E3 cascade is well documented [3]. We next attempted to reconstruct this cascade *in vitro* and to detect the E3-formed polyubiquitin chains using our luminescent method. Due to the size of HECT-type E3 ligases, ranging from 100 to 428 kDa in Arabidopsis, production of active protein by traditional expression methods may not be easy and biochemical analysis using only truncated recombinant protein has been carried out previously [23]. We attempted to produce full-length Arabidopsis HECT-type E3 ligase proteins using the wheat cell-free system and monitored ubiquitin-conjugation and polyubiquitination by luminescence. Two genes that encode Arabidopsis HECT-type E3 ligase, *UPL5* and *UPL7* [24], were analyzed in this study. We obtained UPL5 and UPL7 cDNA from the RAFL library and produced FLAG-tagged protein in the wheat cell-free system. Ubiquitination of FLAG-labeled UPLs (UPL-FLAGs) was investigated by both the luminescent and immunoblot methods. The successful production of the two recombinant HECT proteins was observed by immunoblot analysis (Fig. 2A) and used in the luminescence assay without purification. To detect ubiquitination of the HECT proteins, UPL-FLAGs

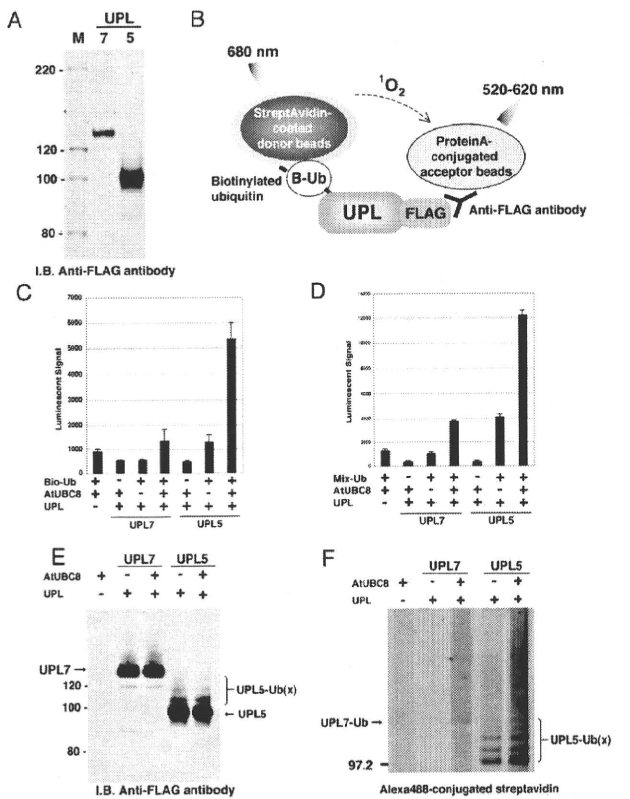

Figure 2
Analysis of recombinant Arabidopsis HECT-type E3 ligases (UPL7 and UPL5). A, Production of FLAG-tagged recombinant UPL proteins was detected by immunoblot analysis. For analysis, 5 μl of crude recombinant UPL proteins were loaded, and detected by immunoblot analysis using anti-FLAG antibody. B, Schematic diagram of detection of ubiquitin-conjugation of UPLs by luminescent analysis. Protein A-conjugated acceptor beads and streptavidin-coated donor beads were bound to anti-FLAG antibody bound to FLAG-tagged recombinant UPLs and biotinylated ubiquitin, respectively, and detected by same principle and procedure described in Figure 1B. C, The ubiquitination of crude recombinant UPL7 and UPL5 was detected by luminescent analysis described in B. Bio-Ub means biotinylated ubiquitin. D, polyubiquitination of crude recombinant UPL7 and UPL5 was detected by luminescent analysis with anti-His antibody. Mix-Ub indicated the mixture of His-tagged and biotinylated ubiquitin. E and F, Mobility shift of UPLs (E) and formation of polyubiquitin chains (F) were detected by immunoblot using anti-FLAG antibody and Alexa488-conjugated streptavidin, respectively. The polyubiquitination reaction was done with FLAG-tagged recombinant UPLs in presence or absence of crude AtUBC8, and then recombinant UPLs were purified by anti-FLAG antibody-conjugated agarose. Error bars represent standard deviations from three independent experiments.

and biotinylated ubiquitin were used. When biotinylated ubiquitin is conjugated to the UPL-FLAG, a high luminescent signal is obtained (Fig. 2B). As a result of the analysis, ubiquitin-conjugation of UPL5 was observed (Fig. 2C). In addition, polyubiquitin chains formed by UPLs were detected with the luminescence assay using His-tagged and biotinylated ubiquitin. To subtract polyubiquitin chain formation from endogenous E2 and E3 in wheat cell-free system, the assay was performed without recombinant UPL and only low signal was detected (Fig. 2D, "UPL-" lane). As expected, luminescent signal was observed in recombinant UPL5 and UPL7 (Fig. 2D). Although the luminescent signal of UPL7 was lower than that of UPL5, the signal was still two-fold higher than the endogenous background signal. These results were confirmed by immunoblot analysis that showed distinct mobility shifts of UPL5 (Fig. 2E) when detecting FLAG-tagged UPLs, and polyubiquitin chain formation of UPL5 monitoring Alexa488-conjugated streptavidin (Fig. 2F). Comparing the amount of polyubiquitin chain formation in absence of UPLs (Fig. 2F, "UPL-" lane), UPL7 formed weak but distinct polyubiquitin chains in presence of AtUBC8. These luminescent signals were consistent with immunoblot data. Interestingly, polyubiquitin chains were formed by UPL5 without supplementing exogenous E2 protein (Fig. 2D and 2F, "AtUBC8-" lane), suggesting that wheat germ extract has endogenous E2 activity as well as endogenous E1 activity. These data indicate that the wheat cell-free production system is able to produce high molecular weight proteins in functional forms and that our luminescence method can detect activity of HECT-type E3 ligases without purification. This is the first data showing that full length recombinant HECT-type E3s have ubiquitin-conjugating and polyubiquitination activity. Taken together, the luminescent method based on the wheat cell-free system could be useful for biochemical analysis of HECT-type E3 ligases.

Detection of Polyubiquitin Chains by RING-Type CIP8 E3 Ligase

It is reported that at least 469 predicted RING-type E3 ligases are encoded in the Arabidopsis genome [25]. Like the HECT-type E3, we attempted to express and carry out the functional analysis of the RING-type E3 ligases. In this study, we selected CIP8 as a model RING-type E3 ligase, which is reported to possess a RING finger motif and have typical features of an E3 ligase [26]. At first, polyubiquitination activity of purified CIP8 in presence or absence of exogenous E1 and purified E2 (AtUBC8) was investigated by luminescence. As shown in Fig 3A, luminescence analysis using His-tagged and biotinylated ubiquitin showed the polyubiquitination of purified CIP8 only when exogenous E1 and purified E2 were added to the reaction mixture. The CIP8-dependent polyubiquitination was

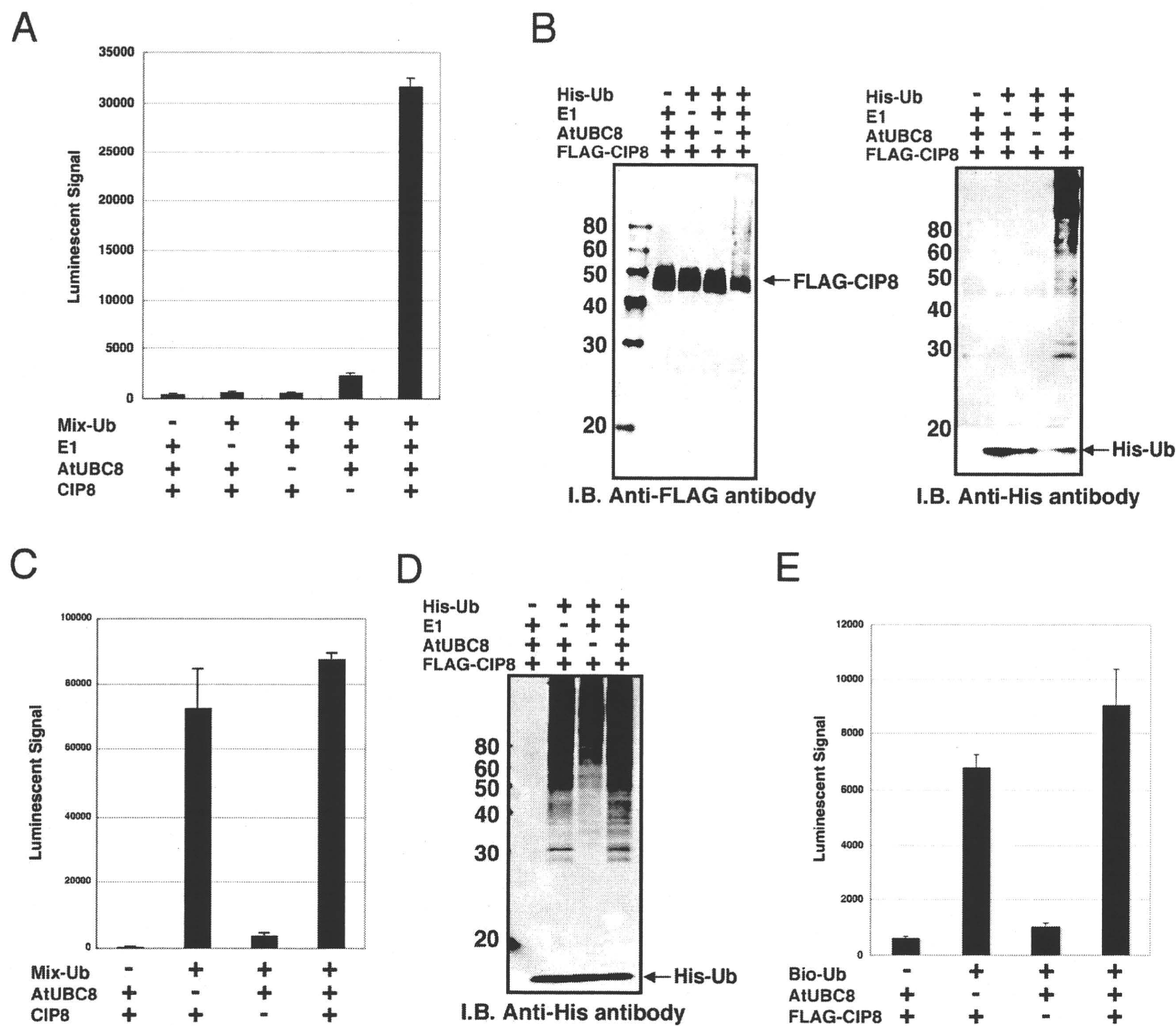

Figure 3
Detection of polyubiquitination and self-ubiquitination of CIP8. A to D, The polyubiquitination assay was carried out with purified (A and B) or crude recombinant CIP8 (C and D) and detected by luminescent analysis with anti-FLAG antibody (A and C) and immunoblot analysis (B and D). His-Ub or Mix-Ub indicate His-tagged ubiquitin or the mixture of FLAG-tagged and biotinylated ubiquitin, respectively. The polyubiquitination assay using luminescent analysis was carried out with recombinant CIP8 without tag in the presence or absence of ubiquitin related components indicated below each graph. E, Ubiquitination of crude recombinant CIP8 was observed by luminescent analysis with anti-FLAG antibody. The assay was carried out with or without biotinylated ubiquitin and crude AtUBC8 recombinant protein. Bio-Ub means biotinylated ubiquitin. Error bars represent standard deviations from three independent experiments.

confirmed by immunoblot analyses detecting both FLAG-CIP8 and His-tagged ubiquitin (Fig. 3B). On the other hand, luminescent analysis with crude CIP8 protein showed high polyubiquitination activity both in the presence or absence of purified E2 (Fig. 3C), and was confirmed by immunoblot analysis with crude protein (Fig. 3D). These data indicated that, like recombinant UPL5, crude CIP8 also utilized endogenous wheat extract E1 and E2 proteins, and therefore we could carry out the simple polyubiquitination analysis of E3 without addition of exogenous E1 and E2 proteins. Furthermore, immunoblot analysis detecting purified CIP8 (Fig. 3B) showed a mobility shift of FLAG-tagged CIP8 to higher molecular weights due to ubiquitination, whereas the mobility of the E2 was

not altered (data not shown). This result indicates that the CIP8-dependent polyubiquitin chains might be elongated on CIP8 itself. This data is consistent with a recent report showing that TRIM5a, a typical RING-type E3 ligase in human, also undergoes self-ubiquitination, forming polyubiquitin chains on itself [27]. To clarify whether the mobility shift of CIP8 was concomitant with polyubiquitin chain formation resulting from self-ubiquitination, we tried to detect ubiquitination of CIP8 by the luminescent method using crude FLAG-CIP8 protein and biotinylated ubiquitin. The luminescent method clearly detected the binding of biotinylated ubiquitin to FLAG-tagged CIP8 both in the presence and absence of exogenous E2 (Fig. 3E). Similar to polyubiquitin formation, the ubiquitination of CIP8 also occurred without the addition of exogenous E2 protein (Fig. 3E, "AtUBC8-" lane). Taken together, these data demonstrate that the luminescent method could detect formation of RING-type CIP8-dependent polyubiquitin chains and self-ubiquitination of crude CIP8.

Screening of RING-Type E3 Ligases Having Polyubiquitination Activity

Recent papers have reported that the polyubiquitin chain is an important biological regulator. Identification of activity and features of E3 ligases offers important information about the ubiquitin-dependent regulation system. Our luminescent method based on the wheat cell-free system produced a simple and high-sensitivity detection of CIP8-dependent polyubiquitin chains without any purification (Fig. 3C). Using these tools, we screened new E3 ligases for the ability to form polyubiquitin chains like CIP8.

The RING-type E3 ligases in Arabidopsis were divided into 30 subgroups based on domain structure, and CIP8 is categorized into subgroup 6 as it contains a coiled-coil domain [25]. Eight other RING-type E3s from subgroup 6 were selected for screening, and the simple polyubiquitination assay was carried out with FLAG-tagged and biotinylated ubiquitins, and the crude recombinant RING-type E3s without addition of exogenous E1 and E2. The screening result showed significant polyubiquitination activity of At1g55530, whereas other RING-E3 proteins were not active (Fig. 4A). Immunoblot analysis of purified recombinant At1g55530 confirmed the polyubiquitination activity and indicated that At1g55530 was self-ubiquitinated (Fig. 4B). The polyubiquitination activity of At1g55530 suggests that it may have a biological role for proteasome-mediated degradation like CIP8 [26]. These results show that the wheat cell-free protein expression system and the luminescent ubiquitination detection method could support functional high-throughput screening of E3 proteins.

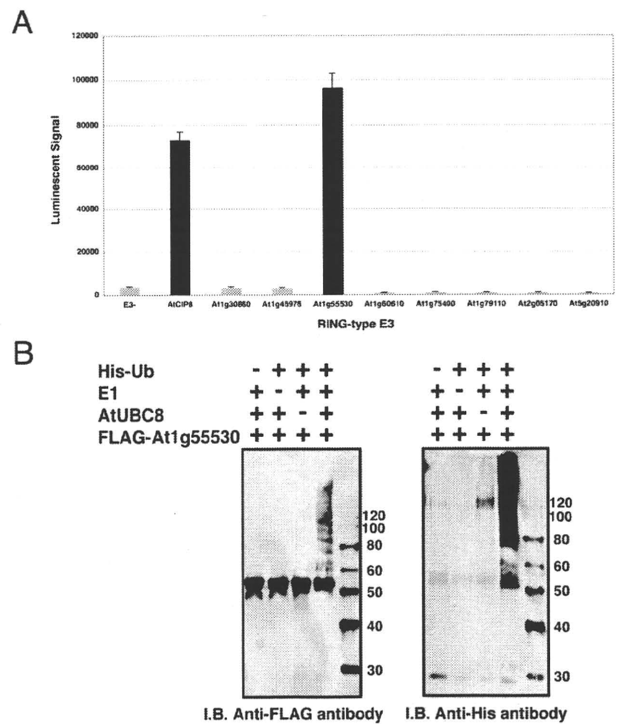

Figure 4
Screening of RING-type E3 ligases having polyubiquitination activity. A, Polyubiquitination reaction of crude recombinant E3 proteins was carried out with mixture of FLAG-tagged and biotinylated ubiquitins, and investigated by luminescent analysis with anti-FLAG antibody. B, Polyubiquitination activity of At1g55530 was confirmed by immunoblot analysis. The assay was carried out using purified recombinant AtUBC8 and At1g55530, and mobility shift of FLAG-tagged At1g55530 and polymer of His-ubiquitin were detected by immunoblot analysis using anti-FLAG and anti-His antibodies, respectively. Error bars represent standard deviations from three independent experiments.

Analysis of the Wheat Cell-free Based Ubiquitination in the Presence of Proteasome Inhibitor

It is known that some cell extracts, such as rabbit reticulocyte or HeLa S-100 fraction, have 26S proteasome-dependent proteolytic activity [28,29]. Based on the presence of endogenous E1 and E2 ubiquitination and polyubiquitination in the wheat cell-free system, it is expected that the 26S proteasome activity will be very low (Fig. 2, 3 and 4). It was previously reported that the wheat germ extract had little 26S proteasome-dependent protein degradation activity [30]. Thus, we determined whether the wheat cell-free system contains active 26S proteasome. Using the crude recombinant proteins that formed polyubiquitin chains in this study, the polyubiquitination reaction was carried out in presence or absence of MG132, and accrual of the polyubiquitinated recombinant pro-

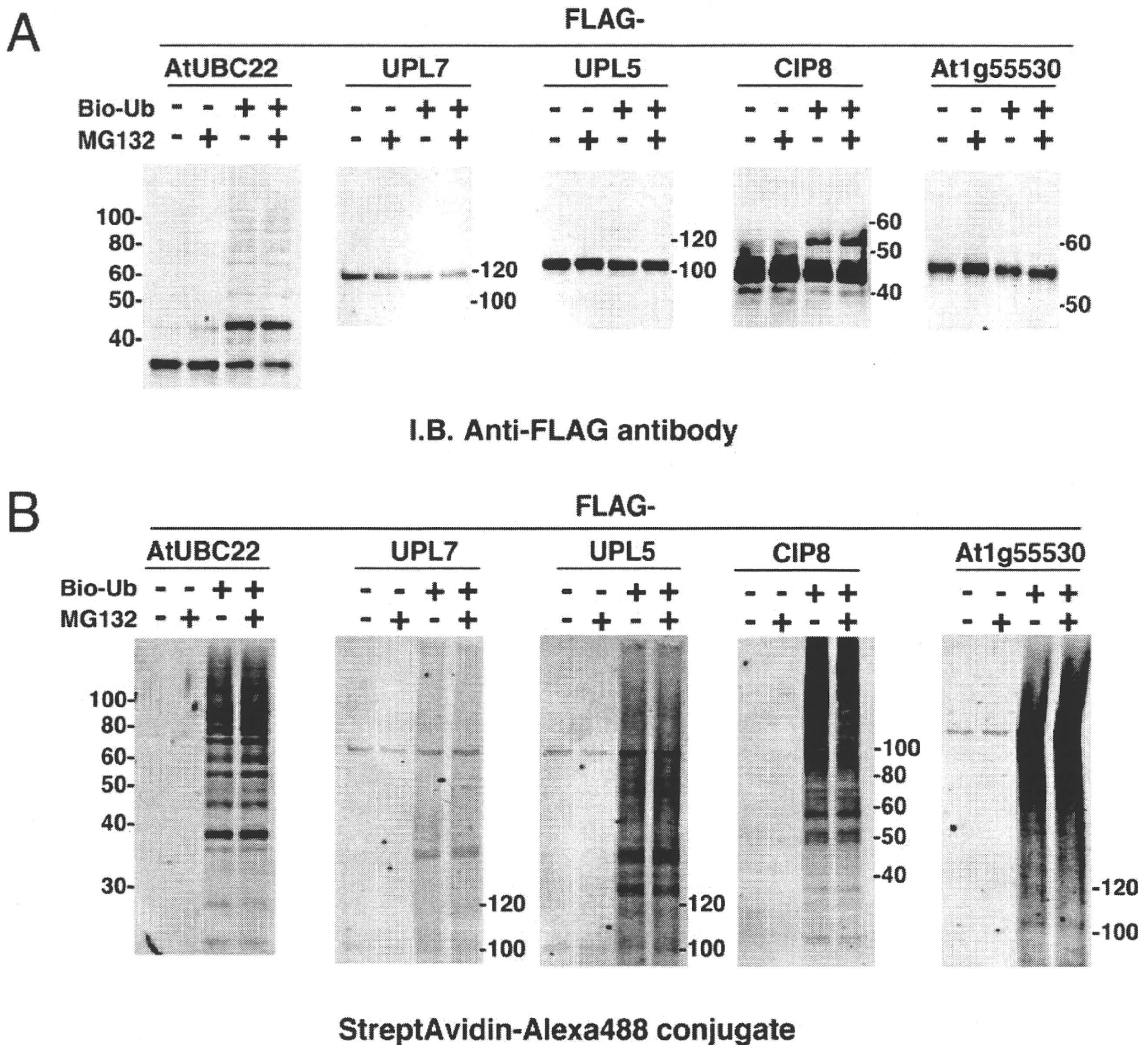

Figure 5

Effect of proteasome inhibitor on stability of polyubiquitinated proteins. Polyubiquitination assays of crude FLAG-tagged E2s and E3s were carried out in the presence or absence of biotinylated ubiquitin and 20 μM MG132. A, FLAG-tagged recombinant proteins were detected by immunoblot analysis using anti-FLAG antibody. B, Polyubiquitination chain formed by each recombinant protein was detected by Alexa488-conjugated streptavidin.

teins and its polyubiquitin chain was estimated. As shown in Fig 5, the amounts of UBC22, UPL5, UPL7 and At1g55530 (Fig. 5A) and of its polyubiquitin chains (Fig. 5B) were hardly altered by MG132 treatment. This result indicates that the proteolytic activity of the 26S proteasome in the wheat cell-free system was below the detection level. Thus, the wheat cell-free system could be suitable for ubiquitination analysis.

Discussion

The ubiquitin signal is an important protein modification in eukaryotes. Binding of a single ubiquitin to a target protein, mono-ubiquitination, is essential for membrane trafficking, protein functions and protein-protein interaction [7]. As for polyubiquitination, both Lys-48- and Lys-63-linked polyubiquitin chains have been well characterized in mammals and yeast. Lys-48 linked chains cause proteolysis of target proteins [6], and Lys-63 linked chains reg-

ulate signal transduction such as cellular localization of protein or protein-protein interactions [7]. In mammals, the multi-functional activities of NF-κB are regulated by the Lys-63 linked chain [31]. In plants, the function of the Lys-63 linked chain is still obscure. However, Arabidopsis E2 and its variants promote formation of the Lys-63 linked chain [32], suggesting that the Lys-63 linked chain in plant cells might also function similar to animal cells. Hence, comprehensive analysis of the ubiquitin-related plant proteins would open a door for elucidation of the plant ubiquitin pathway. In this study, we developed a simple and highly sensitive ubiquitination assay method by combination of the wheat cell-free protein synthesis system and luminescent detection. In general, *in vivo* protein production requires many time-consuming steps such as vector construction, cell culture and purification to obtain the recombinant protein. In contrast, this cell-free based luminescence method could analyze a large amount of ubiquitin reactions without these steps.

Using this method, we conveniently detected polyubiquitin chain formation of E2 and E3s by using two tagged ubiquitins (Fig. 1, 2, 3 and 4). The result of polyubiquitination analysis of the E2s obtained from luminescent-based detection method was verified by immunoblot analysis (Fig. 1). Our analysis also produced recombinant protein of HECT-type E3 ligases without truncation and detected their ubiquitin-conjugation and polyubiquitination activity by luminescent analysis (Fig. 2C and 2D). The ubiquitin-conjugation of UPL5 was not observed when a reductant was added to the reaction (data not shown), suggesting that UPL5 formed a thioester bond with ubiquitin. In addition, the model RING-type E3 CIP8 possessed high polyubiquitin formation activity without substrate, consistent with what was reported previously [26]. Crude recombinant CIP8 formed polyubiquitin chains in the absence of exogenous E1 and E2 (Fig. 3C and 3D), suggesting that the wheat cell-free system might include enough endogenous E1 and E2 activity. It was reported that wheat germ extracts have only a partial ubiquitin pathway [30]. Although the process to isolate wheat germ extract is different from the conventional methods [33], this report strongly supports the existence of endogenous ubiquitin pathway in our wheat cell-free system. Indeed, luminescent analysis using crude recombinant protein showed slight polyubiquitin chain formation even in absence of recombinant E3 (Fig. 2D, Fig. 3C and Fig. 4A, "E3-" lane), indicating that wheat cell-free system might include not only E1 and E2, but E3s or other factors that accelerates the polyubiquitin chain formation. Further, quantitative immunoblot analysis using anti-ubiquitin antibody showed that free ubiquitin was also present in wheat germ extract at a concentration of at least 10 nM (data not shown). This is similar to the ubiquitin concentration supplied in the *in vitro* assay. Although we developed a convenient screening method to detect E3 activity in this study, removal of the endogenous ubiquitin and ubiquitin related components such as E1, E2 and E3, would yield a more sensitive assay. However, wheat cell-free system does not have 26S proteasome proteolytic activity (Fig. 5), indicating that using crude recombinant protein is sufficient for *in vitro* ubiquitination assays.

By using this method, we found that a previously uncharacterized RING type E3, At1g55530, possessed high polyubiquitination activity without exogenous E1 and E2 proteins (Fig. 4). This result suggested that the method developed here is expected to find the activity of other unknown E3 ligases such as At1g55530. Despite having only 32% sequence similarity, the E3s CIP8 and At1g55530 showed similar biochemical functions. Polyubiquitin chains formed by CIP8 and At1g55530 elongated on themselves, while another report showed that polyubiquitin chains were formed on E2 before transferring them to substrates [34]. This reflects that the pattern of polyubiquitin chain formation differs between individual E3s and that the detailed mechanisms are still unknown. These studies suggest the importance of functional analysis using active recombinant proteins. Although we developed a simple screen using crude recombinant E3s in absence of exogenous E1 and E2 (Fig. 4), this method could not detect the activity of some E3 ligases that were unable to utilize endogenous ubiquitination components in wheat cell-free system. The polyubiquitination activity of At5g20910 recombinant protein, expressed in *E. coli* in the presence of AtUBC8 [25], was not active in our *in vitro* system (Fig. 4A), suggesting that in some cases exogenous E2 and/or other components are necessary additions. Such modifications to the ubiquitination assays detailed here would help elucidate the biochemical features of E3s (e.g., addition of recombinant E2s to reaction mixture could give us further information about the E2–E3 specificity, and of other E3 components would lead to the elucidation of structure of complex type E3 ligase such as SCF).

Conclusion

In this study, we found that the wheat cell-free system was an excellent expression system to produce recombinant protein efficiently and to carry out *in vitro* ubiquitination assays without the interference of proteolytic activity. Coupled with luminescent analysis, detection of these ubiquitin reactions in the crude translation reaction mixture was possible. Thus, this method should be helpful for solving the complicated ubiquitin pathway in plant.

Methods

Construction of DNA Templates for Transcription

We used RAFL as templates. DNA templates of E2s and E3s for transcription were constructed by "Split-Primer"