Isolation of ubiquitin–E2 (ubiquitin-conjugating enzyme) complexes from erythroleukaemia cells using immunoaffinity techniques

Koji TAKADA^{*1}, Tae HIRAKAWA^{*}, Hideyoshi YOKOSAWA[†], Yutaka OKAWA^{*‡}, Hideki TAGUCHI[§] and Kiyoshi OHKAWA^{*}

^{*}Department of Biochemistry 1, Jikei University School of Medicine, Nishishinbashi 3-25-8, Minato-ku, Tokyo 105-8461, Japan, [†]Department of Biochemistry, Graduate School of Pharmaceutical Science, Hokkaido University, Sapporo 060-0812, Japan, [‡]Department of Internal Medicine, Jikei University School of Medicine, Nishishinbashi 3-25-8, Minato-ku, Tokyo 105-8461, Japan, and [§]Chemical Resources Laboratory, Tokyo Institute of Technology, Nagatsuta 4259, Midori-ku, Yokohama, 226-8503, Japan

A variety of ubiquitin-associated (or conjugated) proteins, including substrates and enzymes for the ubiquitin system, are present in eukaryotic cells. In the present study we developed a simple method for their isolation, consisting of immunoaffinity chromatography using the monoclonal antibody FK2, which recognizes the conjugated ubiquitin molecule. Using this method followed by gel filtration, we isolated multi-ubiquitinated proteins with high molecular masses (> 30 kDa) and also ubiquitin thioester-linked and mono-ubiquitinated forms of ubiquitin-conjugating (E2) enzymes, UbcH7 and UBE2N, together with mono-, di- and tri-ubiquitin molecules, from the cytoplasmic extract of heat-shock-treated K562 erythroleukaemia cells. We

also demonstrated that the FK2 antibody was capable of precipitating a ubiquitin–UbcH7 thioester, but not free UbcH7, which enabled the measurement of the respective cellular levels separately. The immunoprecipitable ubiquitin–UbcH7 thioester was found only when the cells were treated with heat-shock. These results suggest the usefulness of the immunoaffinity techniques for identifying and analysing the cellular enzyme/protein–ubiquitin complexes.

Key words: affinity chromatography, heat-shock, immuno-precipitation, thioester.

INTRODUCTION

Extensive studies [1–3] have revealed that a variety of cellular proteins are targeted for degradation via covalent ligation of ubiquitin chains and that ubiquitin-dependent proteolysis plays important roles in diverse biological phenomena and diseases. It has been found that members of the families of ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin-protein ligase (E3) function in a ubiquitin-conjugating pathway [4,5], and substrate selectivity of this pathway is thought to be mediated by collaboration of the members of E2 and E3. Therefore identification of these enzymes and their target proteins is essential to elucidate the role of the ubiquitin system in cellular events.

The protein substrates of the ubiquitin-dependent pathway have been characterized by various biochemical and genetic approaches [1,6,7]. However, there have been few attempts to isolate them from biological materials, because such experiments have been limited by the difficulties encountered in isolating ubiquitin–protein conjugates using conventional methods. On the other hand, it has been reported that ubiquitin tagged at its N-terminus with Myc epitope or polyhistidine was used to isolate the ubiquitin conjugates [8–11]. However, these applications are also limited only to cells expressing the tagged ubiquitin, and the Myc-tagged ubiquitin does not completely function as wild ubiquitin [8]. Hence, a simpler method is required for the isolation of proteins (or enzymes) associated/conjugated with ubiquitin, particularly in mammalian cells.

A monoclonal antibody, FK2, developed by immunizing a mouse with ubiquitinated lysozyme, binds with high affinity to the ubiquitin moiety of the ubiquitin–protein conjugate [12], and has been applied to ELISA for the quantification of multi-

ubiquitin chains [13]. In the present study, using the FK2 antibody, we established an immunoaffinity method to isolate ubiquitin-associated molecules. From heat-shocked K562 erythroleukaemia cells, we isolated two E2 enzymes ligated to ubiquitin through thioester and/or isopeptide bonds, and found that immunoprecipitation with this antibody is applicable for analysing the cellular ubiquitin–E2 thioester.

EXPERIMENTAL

Cell culture and cytoplasmic extraction

K562 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum in a humidified atmosphere containing 5% CO₂ at 37 °C. Cells seeded at a density of approx. 0.3 × 10⁶ cells/ml were grown for 3 days and then sequentially exposed to the proteasome inhibitor, MG132, for 1 h and heat-shock-treated at 45 °C for 10 min. After 30 min of the heat-shock treatment, approx. 2 × 10⁷ cells were washed with PBS and lysed with 1 ml of 10 mM Tris/HCl, pH 7.4, containing 0.05% Nonidet P40, 150 mM NaCl, 3 mM MgCl₂, 0.5 mM PMSF, 1 mM EDTA, 0.5 μM antipain, 1 μM aprotinin, 0.1 μM leupeptin, 35 μM chymostatin, 1 μM pepstatin A, 3 μM *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane, 0.5 mM benzamidin, 30 μM phosphoramidon and 0.1 mM bestatin. Nuclei and insoluble debris were removed by centrifuging at 70 000 *g* for 1 h, yielding a cytoplasmic fraction.

Antibodies, ELISA and immunoblotting

The monoclonal antibody FK2 [12], purified as previously described [13], was covalently coupled to Sepharose using *N*-hydroxysuccinimide ester-activated Sepharose 4FF (Amersham

Abbreviations used: E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin-protein ligase; MUCRP1, multi-ubiquitin chain reference preparation 1; DTT, dithiothreitol; MALDI-TOF-MS, matrix-assisted laser-desorption ionization-time-of-flight MS.

¹ To whom correspondence should be addressed (e-mail takada@jikei.ac.jp).

Pharmacia Biotech) in accordance with the manufacturer's instructions, yielding approx. 7 mg of IgG₁/ml gel. The FK2-immobilized Sepharose was employed as a matrix for affinity chromatography and immunoprecipitation. This antibody was also used to perform the ELISA for multi-ubiquitin chains, and measured values were expressed in terms of the multi-ubiquitin chain reference preparation 1 (MUCRP1) [13]. Anti-ubiquitin monoclonal antibody Ub5F9 was used for immunoblotting to detect free and conjugated ubiquitin molecules [14].

A polyclonal mouse serum was prepared in our laboratory against a synthetic peptide corresponding to residues 122–137 of Ub_{CH7} [15], with a cysteine added to the C-terminus. This peptide was coupled to keyhole-limpet haemocyanin using 3-maleimidobenzoic acid *N*-hydroxysuccinimide ester (Pierce, Rockford, IL, U.S.A.) and then injected into Balb/c mice. The first intradermal injection was performed with 50 µg of peptide coupled to 800 µg of haemocyanin and emulsified with complete Freund's adjuvant, and similar injections of the peptide in incomplete Freund's adjuvant were administered every 2 weeks. Serum was taken 7 days after the fourth injection, and its cross-reactivity with Ub_{CH7} was confirmed by immunoblotting. The diluted antiserum (1:500) is capable of detecting Ub_{CH7} in the immunoblots of K562 cytoplasmic extracts.

SDS/PAGE and protein determination

Protein samples dissolved in SDS/PAGE sample buffer, containing 50 mM Tris/HCl, pH 6.8, 2% SDS, 10% glycerol, and 0.005% Bromophenol Blue in the presence or absence of 50 mM dithiothreitol (DTT), were analysed by SDS/PAGE on 10–25% and 8–25% acrylamide gradient gels in Tris/glycine buffer [14] and in PhastSystem (Amersham Pharmacia Biotech) respectively. Proteins were visualized by methods of conventional silver nitrate [16] or sensitive silver diammine staining [17]. Molecular mass estimates of bands were determined by comparing their electrophoretic mobilities with those of the 15 molecular-mass-marker proteins ranging from 2500 to 116000 Da (Sigma). Protein concentration was estimated by the DC protein assay (Bio-Rad Laboratories) standardized with BSA.

Purification of ubiquitin-associated proteins

Approx. 100 ml of the cytoplasmic fraction was applied to a FK2-Sepharose HiTrap column (5 ml) equilibrated with buffer A that contained 10 mM phosphate, pH 7.2, 137 mM NaCl, 2.7 mM KCl, 1 mM EDTA, 0.5 mM PMSF and 0.1% CHAPS, at 0.3 ml/min. After washing with approx. 25 ml of buffer A, the column was developed with 10 ml of 10 mM Tris/HCl, pH 7.4, containing 3.5 M MgCl₂ and 0.1% CHAPS. Fractions of 1 ml were collected and dialysed against buffer A. The fraction containing the largest amount of multi-ubiquitin chains was concentrated by ultrafiltration using Centricon SR3 (Millipore) to a volume of approx. 0.25 ml. The sample was then applied to a Superdex 75 HR10/30 column (Amersham Pharmacia Biotech) equilibrated in buffer A at the flow rate of 0.5 ml/min. Fractions of 0.5 ml were collected and stored at –80 °C. Fractions containing ubiquitin-associated proteins were subjected to reverse-phase capillary HPLC (173A Microblotter system; Applied Biosystems, Tokyo, Japan), with or without deposition of the proteins on a PVDF membrane, after passage through the detector cell or with fractionation of the eluate into polypropylene microcentrifuge tubes. The HPLC separation was performed using a Capillary LC column (C18, 5 µm, 300 Å, 150 × 0.5 mm; Brownlee Columns, Perkin–Elmer) for the separation of the

proteins, using solvent A (0.1% trifluoroacetic acid) and solvent B (0.085% trifluoroacetic acid with 80% acetonitrile) at the flow rate of 5 µl/min under the following elution conditions: 0–10 min, 18% B; 10–85 min, 18–78% B; and 85–95 min, 78% B.

Digestion of purified proteins with lysyl endopeptidase and separation of digested peptides by HPLC

The HPLC-purified proteins immobilized on PVDF membranes were stained with the cLC staining dye (Applied Biosystems). Each spot was reduced and S-carboxymethylated, and then digested with the lysyl endopeptidase, *Achromobacter* protease I (Sigma), as described by Iwamatsu [18]. Alternatively, approx. 30 pmol of the purified proteins in the HPLC eluate was dried in a SpeedVac, dissolved in 50 µl of 20 mM Tris/HCl, pH 9.0, and incubated at 37 °C for 15 h with 0.3 pmol of lysyl endopeptidase. These digests were acidified by adding trifluoroacetic acid at a final concentration of 0.1%, and applied to the reverse-phase capillary HPLC with the deposition of the peptides on a PVDF membrane as described above, but with the following elution conditions: 0–10 min, 6% B; 10–150 min, 6–56% B; 150–180 min, 56% B.

Protein sequence and MS analyses

Each of the digests, as well as the purified proteins, were subjected to Edman degradation using the Procise 492 protein sequencer (Applied Biosystems) through direct introduction of the PVDF membrane and use of the 'pulsed liquid PVDF peptide method', according to the manufacturer's instructions. For matrix-assisted laser-desorption ionization–time-of-flight mass spectrometry (MALDI–TOF–MS) analysis, samples were prepared by depositing 1 µl of a matrix solution of sinapinic acid (10 mg/ml in water) on the sample target surface prior to the addition of a 1 µl sample. The solvent was then removed by drying under air. All mass spectra were recorded using a Finnigan MAT Vision 2000 MALDI–TOF–MS instrument (Thermoquest, Palm Beach, FL, U.S.A.).

Immunoprecipitation of ubiquitin–enzyme thioesters

Ubiquitin–enzyme thioesters were prepared *in vitro* under the following two conditions: (i) 30 ng of partially purified Ub_{CH7} (fraction 24 in the gel filtration; see Figure 2) and 2 pmol of E1 (MBL, Nagoya, Japan) were incubated at 37 °C for 2.5 h with 40 pmol of biotinylated ubiquitin, prepared using sulphosuccinimidyl-6-(biotinamido)hexanoate (Pierce), in a reaction mixture (final volume 15 µl) containing 50 mM Tris/HCl, pH 7.5, 5 mM MgCl₂, 1 mM DTT, 0.1% CHAPS, 2.7 mM ATP and 40 m-units of PP_i. (ii) Ub_{CH7} (180 ng) and 2 pmol of E1 were incubated with 120 pmol of ubiquitin in the above reaction mixture at 37 °C for 1 h. These mixtures were then incubated with 4 µl of either FK2- or 5F9-immobilized Sepharose in PBS containing 0.1% CHAPS (PBS-CHAPS) at 4 °C for 12 h with constant shaking. Immunoprecipitates were washed three times with PBS-CHAPS and suspended in the SDS/PAGE sample buffer. They were then subjected to SDS/PAGE and transferred to nitrocellulose membrane (BA83; Schleicher and Schuell, Dassel, Germany) by electroblotting [14]. These blots were blocked with 10 mM Tris/HCl, pH 7.5, containing 140 mM NaCl, 0.1% gelatin and 0.1% casein. Blots in the experiment (i) were incubated with alkaline phosphatase-conjugated strept-avidin, and biotinylated ubiquitin was visualized with Nitro Blue Tetrazolium/5-bromo-4-chloroindol-3-yl phosphate. The blots in the experiment (ii) were incubated with the anti-Ub_{CH7}

peptide_{122–137} antiserum, followed by alkaline phosphatase-conjugated anti-mouse IgG, and UbCH7-immunoreactive proteins were stained with Nitro Blue Tetrazolium/5-bromo-4-chloroindol-3-yl phosphate.

RESULTS

Isolation of ubiquitin-conjugated molecules from K562 cells

Immunoaffinity chromatography was performed to isolate proteins recognized by FK2 antibody from the cytoplasmic fraction of heat-shocked K562 cells (2×10^9 cells) (Figure 1A). The starting material contained 300 mg of proteins and 91 μ g of

Figure 1 Immunoaffinity chromatography using FK2 antibody-immobilized Sepharose

(A) The cytoplasmic extract of K562 cells was applied to the FK2-Sepharose column. Multi-ubiquitin chain levels in each fraction were estimated by ELISA and expressed in terms of MUCRP1. After unadsorbed materials were eluted between 3 ml and 160 ml, adsorbed materials were eluted with 3.5 M MgCl_2 . A portion of the samples were dissolved in SDS/PAGE sample buffer with DTT, heated (100 °C for 3 min), and subjected to SDS/PAGE. (B) SDS/PAGE in the PhastSystem. Proteins in the cytoplasmic extract (800 ng in lanes 1 and 4), the unadsorbed materials (800 ng in lane 2), and the adsorbed materials (27 and 420 ng in lanes 3 and 5 respectively) were visualized by silver nitrate stain. (C) Immunoblotting with anti-ubiquitin Ub5F9 antibody. Proteins in the cytoplasmic extract (30 μ g in lane 1), the unadsorbed materials (30 μ g in lane 2) and the adsorbed materials (1 μ g in lane 3) were analysed. Molecular mass estimates are indicated in the centre of (B) and to the right of (C).

Figure 2 Gel filtration of immunoaffinity-purified proteins

(A) The adsorbed materials obtained from the FK2-Sepharose column were fractionated by gel filtration on the Superdex 75 column. Multi-ubiquitin chain levels estimated by the ELISA were expressed in terms of MUCRP1. Small aliquots of the eluates were dissolved in SDS/PAGE sample buffer with (+) or without (–) DTT and heated. (B, C) SDS/PAGE in PhastSystem. Proteins in each fraction (1.5 μ l) were visualized by silver diamine stain. (D) Immunoblotting with the Ub5F9 antibody. Eluates (1.5 μ l) in each fraction were analysed. Molecular mass estimates are indicated to the right in (B)–(D).

the ELISA-estimated multi-ubiquitin chains in terms of MUCRP1. The great majority of the proteins did not bind to FK2-immobilized Sepharose, whereas a small amount (1.8 mg), approx. 0.7% of the initial proteins, bound to FK2-Sepharose and was then dissociated from it by 3.5 M MgCl_2 . On the other hand, most of the multi-ubiquitin chains were adsorbed to the FK2-Sepharose, and 91 μ g of the multi-ubiquitin chains, in terms of MUCRP1, which accounts for over 99% of the initial amount, were recovered in the MgCl_2 eluate (Figure 1A). SDS/PAGE followed by silver staining revealed several distinct bands with molecular masses ranging from 5.5 to 30 kDa and a smear with a molecular mass of greater than 40 kDa in the MgCl_2 eluate (Figure 1B). Similar bands were immunostained with the Ub5F9 antibody to ubiquitin (Figure 1C).

The MgCl_2 eluate (0.96 mg of protein) was further fractionated using the Superdex 75 gel-filtration column (Figure 2). Multi-ubiquitin chains detected by the ELISA were mostly eluted in a void volume fraction (fractions 16–18; Figure 2A). Several proteins ranging from 5.5 to 30 kDa on SDS/PAGE were eluted according to their sizes at fractions 23–28. In fraction 28, two

Figure 3 Reverse-phase HPLC of immunoaffinity/gel filtration-purified proteins

Small amounts (1–20 μ l) of fractions 23–28 in the gel filtration (see Figure 2) were applied to a reverse-phase capillary HPLC column. Typical chromatograms of the fractions, whose numbers are indicated in the right corner, are shown. In order to define each peak chromatographically, small aliquots of each fraction were mixed in various combinations and analysed by this HPLC (results not shown). The peaks were then subjected to SDS/PAGE (see Figure 4), and the eight peak fractions (A–H) indicated were characterized.

bands of 5.5 kDa and 8 kDa (α and β respectively; Figure 2B) were detected, both of which were ubiquitin-immunoreactive (Figure 2D). Incomplete denaturation of mono-ubiquitin may yield these two bands, since only mono-ubiquitin was obtained from this fraction. In fraction 23 (the result of treatment with DTT), the γ -band (27 kDa) and δ -band (26 kDa) disappeared, and the ϵ -band (18 kDa), ζ -band (17 kDa) and 5.5 kDa bands appeared (Figures 2B and 2C). Each of the gel-filtration fractions 23–28 was further subjected to separation by reverse-phase HPLC. The typical elution profiles are shown in Figure 3, and eight distinct peak fractions (A–H) were obtained.

Identification of ubiquitin-ubiquitinated molecules isolated from K562 cells

The ELISA-reactive multi-ubiquitin chains were enriched in the void volume in the gel filtration (fractions 16–18 in Figure 2A), and immunoblotting showed a ubiquitin-immunoreactive smear with a molecular mass > 30 kDa (Figure 2D). Similar smears were also detected by protein staining (Figures 2B and 2C). Total proteins (300 ng) in fraction 17 were directly sequenced by automated Edman degradation, giving a major sequence of MQIFVKTLTGKTITL, the N-terminal sequence of human ubiquitin [19]. These results indicate that a substantial amount of multi-ubiquitinated proteins are present in this fraction.

On SDS/PAGE, major bands of fractions A, B and C had molecular masses of 5.5 kDa, 16 kDa and 25 kDa respectively under either reducing or non-reducing conditions (Figure 4 and Table 1). Their N-terminal amino acid sequences were determined to be MQIFVKTLTGK, the N-terminal sequence of human

Figure 4 SDS/PAGE of proteins in fractions of reverse-phase HPLC

Small aliquots (1–2 μ l) of the peak fractions A–G obtained from the reverse-phase HPLC column (see Figure 3) were dissolved in SDS/PAGE sample buffer with (+) or without (–) DTT, heated, and analysed by SDS/PAGE in the PhastSystem. Proteins were visualized by the silver diamine stain. Molecular mass estimates are indicated in the centre of the upper panel and to the right of the middle and lower panel.

ubiquitin. Their molecular mass values determined by MALDI-TOF-MS (Table 1) were in good agreement with the calculated masses of mono-, di- and tri-ubiquitin (8565, 17112 and 25659 Da respectively). In addition, they have nearly identical reverse-phase peptide maps (results not shown). From these results, proteins in fractions A, B and C are identified as monomer, dimer and trimer of ubiquitin respectively.

When fractions D, F and G were analysed by Edman degradation, all showed the N-terminal sequence of human ubiquitin (MQIFVKTLTGK). On the other hand, analyses of fractions E and H showed no detectable significant sequences. Proteins of fractions E, F and H on PVDF membranes, and those of fraction D and G in solution, were digested by lysyl endopeptidase. Reverse-phase peptide maps of fractions E and D are shown in Figures 5(A) and 5(B) respectively (results from fractions F, G and H are not shown). Results of amino acid sequence analyses for the peptide digests of fractions D–H are summarized in Figure 6. The fraction E digests showed complete sequence identity with those derived from Ubch7 [15]. The sequences of the fraction D digests were identical with those derived from either ubiquitin or Ubch7. Similarly, sequences of the fraction H digests were completely identical with those derived from UBE2N (Ubch-ben) [20], whereas the sequences of the fraction F digests were identical with those derived from either ubiquitin or UBE2N (Figure 6). MALDI-TOF-MS analysis showed that the molecular mass values of fractions E (17850 Da), D (26470 Da), H (17190 Da) and F (25740 Da) were in good agreement with the calculated masses of Ubch7 (17861 Da), thioester- (or isopeptide-) linked ubiquitin–Ubch7 complex (26408 Da), UBE2N (17138 Da) and thioester- (or isopeptide-) linked ubiquitin–UBE2N complex (25685 Da) respectively (see Table 1). In addition, 27 kDa and 26 kDa bands of fractions D and F respectively appeared to be dissociated into 5.5 kDa and 18 kDa or 17 kDa bands under the reducing conditions (Figure 4 and

Table 1 Molecular masses determined by SDS/PAGE and MALDI–TOF–MS analysis, and proposed structures

Each HPLC eluate (see Figure 3) was concentrated with adsorptive C₁₈ pipette tips (ZipTip_{C18}; Millipore) and then submitted to MALDI–TOF–MS analysis. Ub, ubiquitin.

Fraction	SDS/PAGE under non-reducing conditions (kDa)*	SDS/PAGE under reducing conditions (kDa)*	Mass by MALDI–TOF–MS (Da)	Proposed structure
A	5.5	5.5	8610	Ub
B	16	16	17260	(Ub) ₂
C	25	25	25850	(Ub) ₃
D	28, 27	28, 18, 5.5	26470	Ub–UbcH7†
E	18	18	17850	UbcH7
F	26	26, 17, 5.5	25740	Ub–UBE2N†
G	26	26	25730	Ub–UBE2N‡
H	17	17	17190	UBE2N

* Estimates for minor bands were excluded.

† Linkage through thioester and isopeptide bonds.

‡ Linkage through isopeptide bond.

Figure 5 HPLC of lysyl endopeptidase-digested peptide fragments obtained from fractions E and D

Each protein (approx. 500 ng) of fraction E spotted on a PVDF membrane and fraction D in solution was digested with lysyl endopeptidase. Each digest was applied to the reverse-phase capillary column and the absorbance at 210 nm was monitored. Peaks were numbered in the order of elution as E1–8 of the fraction E digests (A) and D1–14 of the fraction D digests (B).

Table 1) due to reductive breakdown of the thioester bonds. Therefore we concluded that proteins of fractions E and H were UbcH7 and UBE2N, respectively, and that those of D and F were their thioesters with ubiquitin. In addition, the presence of residual undissociated proteins, 28 kDa and 26 kDa bands in fractions D and F, respectively, under the reducing conditions (Figure 4 and Table 1), suggests that mono-ubiquitinated E2 enzymes are also present in both fractions.

The digests of fraction G also showed a complete sequence identity with those derived from either ubiquitin or UBE2N (Figure 6), and its molecular mass value (25730 Da) by MALDI–TOF–MS analysis is virtually identical with that of fraction F (25740 Da). However, the 26 kDa band of fraction G was completely resistant to DTT treatment (Figure 4 and Table 1). Thus it can be deduced that this fraction contains isopeptide-

Ubiquitin		1	10	20	30	40	50	60	70
MQIFVKLTGKTTITLEVEPSDTIENVKAKIQDQKGGIPPDQRLIFAGK QLEDGRTLSDYNIQKESTLHLVLRGG									
D-8 D-11 D-6									
MQIFVK. TITLEVEPSDTIENVK. IQDK.									
F-6 F-9 F-7 F-4									
MQIFVK. TT-LEVEPSDTIENVK. EGIPPDQRLIFAGK QLEDGRTLSDYNIQK.									
G-6 G-9 G-7 G-4									
MQIFVK. TITLEVEPSDTIE. EGIPPDQRLI. QLEDGRTLSDYNIQK.									

UbcH7		1	10	20	30	40	50	60	70	80
MAASRLMKELKEIRKCMKNFRNIQVDEANLLTWQGLIVDPNPYPDKGAFRIETNFPAPYFPKPKITFTKIYHPNID										
E-1 E-7 E-6 E-2 E-3										
ELEETRK. NFRNIQVDEANLLT-Q. -AFRIETNFP. ITFK. IYHPNIDEK.										
D-2 D-13 D-12 D-3 D-4										
ELEETRK. NFRNIQVDEANLLT-Q. GAFRIET. ITFK. IYHPNIDEK.										

UBE2N		1	10	20	30	40	50	60	70	80
MAGLPRIIKETQRLLAEPVPGIKAEPPDESARYFHVVIAGPDSPFEGGTFKLELFLPEEYXMAAPKVRFMTK. IYHPNV										
NF1 NF2 NF3 NF4 NF5 NF6										
H-4 H-1 H-2										
ETQRLLAEPVPGIK. VRFMTK. IYHPNVDK.										
F-8 F-1 F-2										
ETQRLLAEPVPGIK. VRFMTK. IYHPNVDK.										
G-8 G-11 G-13 G-1 G-2										
ETQRLLAEPVP. AEPDESARYFH. LELFLPEEYXMAA. VRFMT. IYHPN.										

Ubiquitin		1	10	20	30	40	50	60	70	80
DKLGRICLDILKDKWSPALQIRTVLLSIQALLSAPNPDDPLANDVAEQWKTNEAQAIETARAWTRLYAMNNI										
NF7 NF8 NF9 NF10										
H-6 H-14										
TNEAQAIETARA. TNEAQAIETARAXTRLYAMNN.										

Figure 6 Sequences determined in fractions D–H

Sequences determined by Edman degradation of lysine endopeptidase digests of proteins in fractions D–H. In the case of fractions E, F and H, each protein spot on the PVDF membranes was digested *in situ*, whereas proteins in the eluates of fractions D and G were digested in solution. Each digest was subjected to reverse-phase HPLC, and the peaks obtained were numbered in the order of elution as indicated in Figures 5(A) and 5(B) for fractions E (E1–8) and D (D1–14) respectively. In the case of fractions F, G and H, 10, 15 and 6 peaks, designated as F1–10, G1–15, H1–6 respectively, were obtained (results not shown). The names of the peptide peaks are shown in *italics*, and those given no significant sequences are excluded. Sequences are given in one-letter codes below the sequences of ubiquitin, UbcH7 and UBE2N. Sequences not identified are indicated by dashes, and the dot at the end of the sequence indicates that the amino acid has not been determined from this point. Expected 10 fragments of UBE2N generated by the complete lysyl endopeptidase digestion are indicated as NF1–10.

linked ubiquitin–UBE2N. Among the 10 theoretical digests of UBE2N, six fragments of NF2–6 and NF10 were identified in fraction G (Figure 6). Consequently, lysine-10, lysine-24,

Figure 7 Immunoprecipitation of ubiquitin-enzyme thioesters with FK2 antibody

Ubiquitin-enzyme thioester were prepared *in vitro* under the two conditions (i) and (ii) as described in the Experimental section. **(A)** Condition (i): biotinylated ubiquitin was incubated in the presence (+) or absence (–) of E1, partially purified UbcH7 (fraction 24 of the gel filtration shown in Figure 2) and ATP as indicated. **(B)** Condition (ii): the partially purified UbcH7 was incubated with (+) or without (–) E1, ubiquitin (Ub) and ATP as indicated. In both cases, the reaction mixtures (15 μ l) were mixed with the 5 $\times$ SDS/PAGE sample buffer without DTT (4:1, v/v) and subjected to SDS/PAGE followed by blotting (lanes 1 and 2). Alternatively, the reaction mixture was subjected to immunoprecipitation with the FK2 antibody (lanes 3–7) or the Ub5F9 antibody (lane 8), and the resulting precipitates were solubilized in 20 μ l of SDS/PAGE sample buffer with (lane 7) or without (lanes 3–6 and 8) DTT and subjected to SDS/PAGE and blotting. **(A)** Biotinylated ubiquitin molecules were detected using enzyme-labelled streptavidin, and ubiquitin thioester with E1 (black arrowheads) and UbcH7 (white arrowheads) are shown. Location of mono-ubiquitin is indicated as Ub. **(B)** Immunoreactive UbcH7 was detected with the antibody against the anti-UbcH7 peptide_{122–137} antiserum, and ubiquitin-UbcH7 thioester (white arrowheads) and free UbcH7 (black arrowheads) are shown. #Light chains of the antibodies leaked from the antibody Sepharose beads and detected by the enzyme-labelled anti-mouse IgG. The Ub5F9 antibody can detect both free and conjugated ubiquitin in immunoblotting, but cannot recognize them in solution.

lysine-53, lysine-68, lysine-74 and lysine-130 are not sites of ubiquitination, since lysyl endopeptidase is unable to cleave at the carboxyl side of the lysine residue if its ϵ -amino group is modified. By sequencing the G-2 fragment (Nf6), we could not find a sequence of a ubiquitin C-terminal fragment ligated to UBE2N, which suggests that lysine-82 also does not seem to be a ubiquitination site. In addition, a peptide obtained by digestion with endoproteinase Asp-N was found to contain simultaneously two sequences of 'DXXSPALXIRLT' identical with residues 93–103 of UBE2N and 'DYNIQESTLH' identical with the ubiquitin C-terminal region (results not shown). Therefore, in fraction G, ubiquitin may conjugate to lysine-94 and/or lysine-92 of UBE2N. The proposed structures of proteins in fractions A–H are summarized in Table 1.

Figure 8 Levels of UbH7-immunoreactive proteins in K562 cells

The cells were cultivated with (+) or without (–) treatment with 3 μ M MG132 (1 h) and/or heat-shock at 45 $^{\circ}$ C (10 min), and collected after 30 min of heat-shock. The cytoplasmic extracts (400 μ l) derived from 1×10^7 cells were incubated with the FK2 antibody Sepharose (20 μ l beads) at 4 $^{\circ}$ C for 12 h under constant shaking, and centrifuged at 10000 *g* for 5 min. The precipitates were solubilized in 60 μ l of the SDS/PAGE sample buffer with DTT and subjected to SDS/PAGE (25 μ l/lane). The initial extracts were also mixed with 5 $\times$ SDS/PAGE sample buffer with DTT (4:1, v/v) and subjected to SDS/PAGE (30 μ l/lane). Separated proteins were then transblotted to nitrocellulose membranes and immunostained with the anti-UbcH7 peptide_{122–137} antiserum. 'Thioester' and 'Total' indicate 18 kDa UbcH7 immunoreactive proteins in the precipitates and the initial extracts respectively.

Immunoprecipitation of ubiquitin-enzyme thioesters

In order to determine the cross-reactivity with the FK2 antibody to ubiquitin-UbcH7 thioester, we subjected the reaction mixtures, containing ubiquitin or biotinylated ubiquitin, E1, and partially purified UbcH7, to immunoprecipitation with the FK2 antibody. The 26 kDa bands containing biotinylated ubiquitin (Figure 7A, white arrowheads), which were detectable only in the presence of ATP, were immunoprecipitated with the FK2 antibody (lane 6) and were DTT-labile (lane 7). This band was also immunoreactive to the anti-UbcH7 antiserum (white arrowheads in Figure 7B), and transformed into free UbcH7 by treatment with DTT (lane 7 of Figure 7B, indicated by a black arrow), thus identified as a ubiquitin-UbcH7 thioester. Therefore the FK2 antibody was immunoreactive to this ubiquitin-E2 thioester, but not to free UbcH7. This thioester in the reaction mixtures was almost completely immunoprecipitated, since it was not detectable in the remained supernatants (results not shown). Additionally, this antibody also precipitated mono-ubiquitin, unknown ubiquitin conjugates, and a putative ubiquitin-E1 thioester with 125 kDa (Figure 7A, black arrowheads). These results indicated that this method is applicable to the analysis of ubiquitin-enzyme thioesters, as well as free and conjugated ubiquitin.

Levels of ubiquitin-UbcH7 thioesters in K562 cells

Thioester-linked UbcH7 in the cytoplasm of K562 cells was analysed by immunoprecipitation with the FK2 antibody. The brief treatment with MG132, which has been employed to increase ubiquitin-protein conjugates, showed no effect on the levels of either total or thioester-linked UbcH7 molecules, whereas heat-shock-treatment at 45 $^{\circ}$ C for 10 min resulted in an increase in the level of the ubiquitin-UbcH7 thioesters (Figure 8).

DISCUSSION

In the present paper, we demonstrated the usefulness of immunoaffinity techniques using FK2 antibody-immobilized Sepharose for the isolation and analysis of thioester-linked ubiquitin-E2 intermediates as well as ubiquitin-protein conjugates. From the cytoplasm of heat-shock-treated K562 cells, we also isolated two

free E2 enzymes, UbcH7 and UBE2N, together with their derivatives, e.g. thioester- and isopeptide-linked ubiquitin–E2 complexes. We predict that free E2 enzymes may be dissociated from the respective ubiquitin–E2 complexes, which are bound to the immunoaffinity column, by reduction and/or de-ubiquitination during the step of de-salting and/or concentration of the eluate, since free UbcH7 did not immunoreact with the FK2 antibody (Figure 7B). In addition, a putative E1 thioester (Figure 7A) and distinct ubiquitin thioesters in a rabbit reticulocyte lysate (results not shown) were also immunoprecipitated with the FK2 antibody. Thus this antibody probably recognizes most of the ubiquitin–enzyme complexes, and the ubiquitin complexes with UbcH7 and UBE2N may be particularly abundant in the heat-shock-treated K562 cells.

The FK2 antibody also recognized mono-ubiquitin (Figures 1 and 7), although this antibody displayed almost no cross-reactivity with mono-ubiquitin in the immunoblotting and the ELISA [12,13]. These apparently contrasting results suggest that this antibody recognizes both free and conjugated ubiquitin in solution; however, when mono-ubiquitin is immobilized on to a nitrocellulose membrane (in the immunoblotting) or a microtitre well (in the ELISA) an FK2-recognizing epitope becomes less accessible.

In budding yeast, Ubc4 and Ubc5 are essential for tolerance to heat-stress [21]; however, such E2 enzymes have not been determined in mammalian cells. The presence of substantial amounts of the ubiquitin thioesters of UbcH7 and UBE2N in heat-shock-treated K562 cells suggests a possible involvement of these two E2 enzymes in stress-related phenomena in mammals. This speculation is supported by the heat-shock-induced increase in ubiquitin–UbcH7 thioester (Figure 8). UbcH7 is known to participate in the ubiquitination of several proteins, including p53, c-Fos, the precursor of nuclear factor κ B, Myc and epidermal growth factor receptor [15,22–25]. It is ubiquitously expressed [26,27] and indispensable for mouse embryogenesis [28]. Little is yet known about UBE2N (human homologue of yeast UBC13 and the *Drosophila bendless* gene product) [20]; however, Ubc13 itself is known to function in assembly of multi-ubiquitin chains through lysine-63 for DNA repair [29] and I κ B kinase activation [30]. Identification of target proteins for UbcH7 and UBE2N during the heat-shock response is necessary to define the roles of these two E2 enzymes in the stress response.

We also isolated DTT-insensitive ubiquitin–E2 complexes, which are mono-ubiquitinated E2 enzymes. Since these were not inducible by heat-shock (results not shown), it can be predicted that they are always present in K562 cells. Auto-ubiquitinating activities have been reported in yeast Ubc3 (Cdc34) and Ubc4, a *Caenorhabditis elegans* RAD6 homologue, and human E2_{EFF} [31–34]. However, we could not detect any self-ubiquitination activity of UbcH7 in our experimental system (Figure 7). The fact that the proposed ubiquitination site(s) of UBE2N, lysine-92 and/or lysine-94, are close to the active site, cysteine-87, implies that the mono-ubiquitinated UBE2N may not retain enzyme activity. Further studies are necessary to determine the regulatory roles of mono-ubiquitination in the expression of E2 enzyme activity.

Heat-shock leads to a marked increase in levels of multi-ubiquitinated proteins [35–37]. In HeLa cells, heat-shock accelerates ubiquitin-conjugating activity estimated by using denatured lysozyme as a substrate [36], suggesting that it involves in the accumulation of multi-ubiquitinated proteins. Oxidative stress has similar effects on bovine lens epithelial cells, which may be due to activation of E1 [38]. However, in K562 cells, we could not find any heat-shock effects upon either free or thioester E1 (results not shown). It is believed that heat-shock brings about

intracellular protein denaturation, which is targeted by the ubiquitination machinery [3,39]. However, to our knowledge, these damaged proteins have not been well characterized. In response to heat-shock treatment, the levels of multi-ubiquitin chains in the cytoplasm of K562 cells increased severalfold (results not shown). Therefore the void volume fraction in gel filtration (Figure 2A) may contain stress-induced damaged and ubiquitinated proteins. Identification of such ubiquitinated proteins is now in progress in our laboratory. The present immunoaffinity techniques are also expected to be applicable to the isolation of ubiquitinated proteins observed in neurodegenerative disorders [40] and in forebrain ischaemia [41].

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