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Production and characterization of monoclonal antibodies specific to multi-ubiquitin chains of polyubiquitinated proteins

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Abstract

Polyubiquitinated proteins tagged with multi-ubiquitin chains are substrates preferred by the 26 S proteasome (a ubiquitin/ATP-dependent proteolytic complex). Here, we developed a simple method for the efficient preparation of polyubiquitinated proteins which are degraded by the 26 S proteasome in an ATP-dependent manner. Our efficient method enabled us to produce ten monoclonal antibodies that recognized the multi-ubiquitin chains of the polyubiquitinated proteins, but not free ubiquitin or the protein moieties. Eight of the antibodies recognized only the multi-ubiquitin chains of the polyubiquitinated proteins, while the other two antibodies cross-reacted with mono-ubiquitin and methyl-ubiquitin, both of which are linked to proteins via an isopeptide bond, as well as with the multi-ubiquitin chains. Thus these antibodies are novel and useful tools for the identification and quantification of polyubiquitinated proteins in various cells and tissues under physiological and pathological conditions.

Key words: Ubiquitin; Proteasome; Multicatalytic; Protease; ATP; Monoclonal antibody

1. Introduction

Ubiquitin (Ub), a polypeptide of 76 amino acid residues, is the highly conserved and widely distributed protein in eukaryotic cells [1]. It has been proposed that this protein plays important roles in various biological processes such as the regulation of cell cycles [2–4], DNA replication [5], DNA repair [6], stress response [7,8], apoptosis [9], signal transduction [10–12], biogenesis of ribosome [13,14], nucleosome [15], peroxisome [16] and myofibril [17]. In these processes, the Ub functions as both a signal for protein degradation and as a chaperone promoting the formation of organelles. It has also been reported that Ub–protein conjugates accumulate in the brain under certain pathological conditions, for example, Alzheimer's disease [18,19] and Parkinson's disease [20], and also ischemic damage [21,22].

Several previous reports have suggested that intracellular abnormal and short-lived proteins are degraded through the Ub-dependent proteolytic pathway (for reviews, see [23–26]). In the Ub-dependent pathway, a target protein is tagged with poly-Ub molecules (referred to as the multi-Ub chain in this report) via isopeptide bonds which are formed between the carboxyl-termini of the Ub molecules and either of the ε-amino groups of the lysine residues in the protein or in the Ub molecules themselves [1,23,24]. This reaction is catalyzed by sequential actions of E1 (Ub-activating enzyme), E2 (Ub-conjugating enzyme), and occasionally E3 (N-recognin) with the aid of energy derived from ATP hydrolysis. Thus formed, polyubiquitinated proteins tagged with the multi-Ub chains (poly-Ub–protein conjugates) are destined to be degraded by the 26 S proteasome, a Ub/ATP-dependent proteinase complex, in an ATP-dependent manner [23–26].

While the poly-Ub–protein conjugates are substrates preferred by the 26 S proteasome [23–29], the previous use of the partially purified preparation of the above Ub-ligating system, which could be contaminated by the 26 S proteasome and/or isopeptidase, did not lead to the production of a large amount of the poly-Ub–protein conjugate. Recently, Tamura et al. [30] improved the previous methods of Hough et al. [29] and Hershko et al. [31] for the preparation of the poly-Ub–protein conjugates. We have slightly modified the method of Tamura et al. [30] and performed ultracentrifugation as an additional purification procedure in order to remove the possible contaminating 26 S proteasome and/or isopeptidase. Our modified method permitted us to prepare the poly-Ub–protein conjugates more efficiently. The preparation of poly-Ub–protein conjugates appeared very stable during a prolonged incubation without proteasome, and was rapidly degraded in an ATP-dependent manner by adding the 26 S proteasome.

The improvement in the preparation of a large amount of the poly-Ub–protein conjugate using lysozyme enabled us to produce 10 monoclonal antibodies that recognized the poly-Ub–lysozyme conjugates but not free Ub or lysozyme. Two of the established monoclonal antibodies were found to recognize mono-Ub and methyl-Ub linked via isopeptide bonds to lysozyme in addition to the multi-Ub chains of the poly-Ub–lysozyme conjugates, while the other eight antibodies were highly specific to the multi-Ub chains but not the mono- or methyl-

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Abbreviations: Ub, ubiquitin; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; IgG, immunoglobulin G; ELISA, enzyme-linked immuno-sorbent assay.

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Ub linked to lysozyme. To our knowledge, this is the first report on such specific monoclonal antibodies directed against the poly-Ub–protein conjugates. These antibodies are expected to be useful tools for identifying and quantifying the poly-Ub–protein conjugates and also for discriminating the multi-Ub chains from the protein-linked mono-Ub and free Ub in various cells and tissues under certain physiological and pathological conditions.

2. Materials and methods

2.1. Materials

Bovine Ub, ATP, yeast inorganic pyrophosphatase, rabbit creatine phosphokinase (Type I), phosphocreatine and molecular mass standard proteins were purchased from Sigma Chemical Co. Affi-Gel 10 was obtained from Bio-Rad Laboratories. Bovine chymotrypsinogen A, chicken egg white lysozyme and DEAE-cellulose (DE-32) were purchased from Worthington Biochemical Corp., Seikagaku Kogyo Co. and Whatman, respectively. [^{125}I]NaI was obtained from New England Nuclear. Rabbit polyclonal antibody raised against chicken lysozyme was prepared by Dr. F. Arisaka in our laboratory.

2.2. Preparation of Ub-ligating enzymes (E1, E2 and E3)

E1, E2 and E3 were prepared from the reticulocyte lysate according to the methods described by Tamura et al. [30] and Hershko et al. [31] with slight modifications. The reticulocyte lysate was ultracentrifuged at 100,000 × g for 6 h in order to precipitate the 26 S proteasome. The resulting supernatant was successively subjected to DEAE-cellulose chromatography, concentration using an Amicon PM-10 membrane and chromatography on Ub–Affi-Gel 10 (prepared according to the manufacture's protocol). A mixture of E1 and E2, and E3, separately isolated from the Ub–Affi-Gel 10 column, were dialyzed against 50 mM Tris-HCl (pH 7.5), 1 mM DTT, 0.2 mM ATP and 20% glycerol, concentrated by ultrafiltration using PM-10 membrane and then frozen in liquid N_2. Protein concentration was determined by the method of Bradford [32].

2.3. Preparation of poly-Ub–[^{125}I]lysozyme and methyl-Ub–[^{125}I]lysozyme

[^{125}I]Lysozyme was prepared with [^{125}I]NaI and chloramine-T essentially according to the standard method [33]. Methyl-Ub was prepared according to the method of Hershko and Heller [34]. Poly-Ub–protein conjugates were prepared as described by Tamura et al. [30] with slight modifications. Usually, the reaction mixture contained 100 mM Tris-HCl (pH 9.0), 5 mM $MgCl_2$, 1 mM DTT, 2 mM ATP, 20 μg/ml of heat-denatured [^{125}I]lysozyme (100°C, 20 min), 6 mg/ml of Ub (or 0.1 mg/ml of methyl-Ub), 0.15 mg/ml of E1/E2 mixture, 0.45 mg/ml of E3, 2.4 unit/ml of inorganic pyrophosphatase, 10 μg/ml of creatine phosphokinase and 10 mM phosphocreatine. This mixture was incubated at 37°C for 1–3 h, and the amounts of poly-Ub– and methyl-Ub–lysozymes were quantified by SDS-PAGE followed by autoradiography.

2.4. Degradation of poly-Ub–protein conjugates

ATP-dependent degradation of poly-Ub–protein conjugates by the 26 S proteasome was measured as follows; a reaction mixture (200 μl), which contained 50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 2 mM ATP, 1 mM DTT, 0.2 μg of [^{125}I]proteins (18,000 cpm) tagged with multi-Ub chains and 3 μg of the 26 S proteasome purified from rabbit skeletal muscle as described previously [35], was incubated at 37°C for 2 h, and the reaction was stopped by the addition of 25 μl of cold 10% bovine serum albumin and 600 μl of cold 10% trichloroacetic acid. After standing for more than 10 min on ice, the acid-soluble radioactivity was measured by a γ-counter.

The ATP-independent degradation was measured as described above except for 5 mM EDTA instead of 10 mM $MgCl_2$ and 2 mM ATP.

2.5. Electrophoreses and Western blot analysis

SDS-PAGE (12.5%) was carried out according to the method of Laemmli [36]. After electrophoresis, proteins were stained with Coomassie brilliant blue R-250 and dried in vacuo followed by autoradiography using Fiji X-ray film New RX. Western blot analysis was performed by the method of Towbin et al. [37] with peroxidase-conjugated anti-mouse or anti-rabbit IgG as a secondary antibody and both H_2O_2 and 3,3′-diaminobenzidine as peroxidase substrates.

2.6. Immunization and screening of hybridoma cells

A crude preparation of the poly-Ub–lysozyme, which contained lysozyme, free Ub, Ub-ligating system and the ATP-regenerating system, was used as an immunogen (50 μg of lysozyme/mouse) and the fusion of the spleen cells with myeloma cells was performed according to the standard polyethylene glycol method [38].

Antibody production was screened by the sandwich ELISA method as described below. Each well of ELISA plate (96-wells) was coated with 100 μl of anti-Ub polyclonal antibody (5 μg/ml PBS) which had been produced according to the method of Haas and Bright [39] and subsequently affinity-purified using the Ub–Affi-Gel 10 (prepared as described above). After washing with PBS and blocking with 200 μl of 3% skim milk in PBS, a crude preparation of poly-Ub–lysozyme (100 μl) diluted with PBS (300-fold) was added to each well, and the plate was incubated at room temperature for 2 h to allow the poly-Ub–lysozyme to be adsorbed on the anti-Ub polyclonal antibody. After the wells were extensively washed with PBS, a culture medium of hybridoma cells was added to each well and the plate was incubated at room temperature for 2 h to allow antibodies in the culture medium to be adsorbed on the poly-Ub–lysozyme bound to the anti-Ub polyclonal antibody. After extensive washing with PBS, biotin-conjugated anti-mouse IgG (Vector) and then avidin-biotinated peroxidase complex were added to each well and the plate was incubated at room temperature for 30 min. Color was developed using 0.02% H_2O_2 and 0.2 mg/ml 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) as peroxidase substrates. Positive culture media of hybridoma cells thus tested were further subjected to negative screening by ELISA using either free Ub (50 μg/ml) or the reaction mixture for the Ub-ligation which lacked only Ub, in place of the crude poly-Ub–lysozyme preparation. Hybridoma cells producing the antibodies that were cross-reactive with only the poly-Ub–lysozyme but not with free Ub or the above negative control reaction mixture were cloned by the method of limiting dilution, and ten antibody-producing clones were established.

3. Results

3.1. Preparation and characterization of poly-Ub–protein conjugates

Ub-ligating enzymes, E1, E2 and E3, were isolated from rabbit reticulocyte lysate, as described by Tamura et al. [30] with slight modifications, by a procedure including ultracentrifugation and chromatography on DEAE-cellulose and Ub–Affi-Gel 10 columns. Approximately 5 mg each of a fraction containing E1 and E2, and of an E3 fraction was obtained from 300 ml of reticulocyte lysate.

The Ub-ligating reaction was mediated by our preparations of E1, E2 and E3 using heat-denatured lysozyme and Ub as substrates, one of which was labeled with ^{125}I. The extent of the reaction was analyzed by SDS-PAGE (12.5% gel) followed by autoradiography (Fig. 1). Strong bands of molecular masses higher than 66 kDa in both cases of [^{125}I]lysozyme and [^{125}I]Ub were detected within a period of 30-min reaction, indicating that these newly-formed bands correspond to the poly-Ub–lysozyme conjugates. Within 60 min, approximately 70% of the total [^{125}I]lysozyme was found to be polyubiquitinated by quantitative analysis using Fuji Bioimaging Analyzer

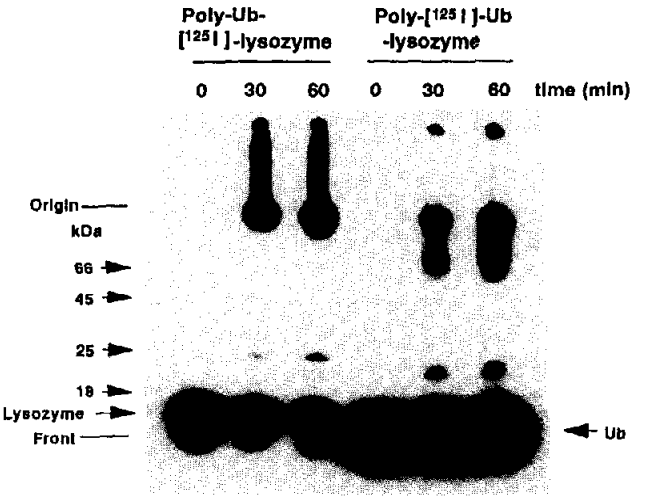

Fig. 1. Polyubiquitination of lysozyme with E1, E2 and E3 using either radioiodinated lysozyme or radioiodinated Ub. Poly-Ub-[^{125}I]lysozyme conjugates were prepared as described in section 2. Poly-[^{125}I]Ub–lysozyme conjugates were prepared by the same method except for 0.1 mg/ml of [^{125}I]Ub and 3.9 mg/ml of cold Ub instead of 6 mg/ml of Ub. The reaction was carried out for 0, 30 and 60 min and then stopped by the addition of a sample buffer containing SDS. A portion (30 μl) of the reaction mixture was subjected to SDS-PAGE and analyzed by autoradiography. Amounts of radioactivities of the poly-Ub-[^{125}I]lysozyme conjugates were quantified by Fuji Bio-Imaging Analyzer BAS 2000. Molecular mass standards used were bovine serum albumin (66 kDa), chicken ovalbumin (45 kDa), bovine chymotrypsinogen A (25 kDa) and sperm whale myoglobin (18 kDa). The migration points of lysozyme and Ub are indicated by arrows.

Bas 2000. The poly-Ub–lysozyme conjugates thus formed remained stable during a prolonged incubation (at least 3 h). Since the poly-Ub–protein conjugates are known to be substrates which are highly susceptible to the 26 S proteasome, we next examined the susceptibility of our preparation of poly-Ub-[^{125}I]lysozyme conjugates to the 26 S proteasome purified from rabbit skeletal muscle. As shown in Fig. 2, poly-Ub-[^{125}I]lysozyme conjugates, detected as main bands of about 200 kDa on SDS-PAGE, were rapidly degraded by the 26 S proteasome in an ATP-dependent manner.

In order to elucidate whether proteins other than lysozyme can also be polyubiquitinated and the polyubiquitinated proteins can serve as substrates for the 26 S proteasome, polyubiquitinations of oxidized [^{125}I]ribonuclease A and [^{125}I]α-lactalbumin were performed. It was found that our preparation of Ub ligation system (E1, E2 and E3) was capable of catalyzing polyubiquitination of either protein (see autoradiography in Fig. 4). In addition, these poly-Ub–protein conjugates were degraded by the 26 S proteasome in an ATP-dependent fashion as efficiently as the poly-Ub–lysozyme conjugates (Table 1).

All of these results indicate that our preparations of E1, E2 and E3 are very useful for efficient preparation of poly-Ub–protein conjugates. These results enabled us to produce monoclonal antibodies raised against the poly-Ub–lysozyme conjugates.

3.2. *Production and characterization of monoclonal antibodies specific to poly-Ub–protein conjugates*

Eight hybridoma clones called FK1, 169H, 1E, 31A, 12H, 99F, 9A and 58D were established, which produced monoclonal antibody cross-reactive only with the poly-Ub–lysozyme conjugates but not with free Ub, the plain lysozyme or mono-Ub linked to the lysozyme, as revealed by Western blotting (Fig. 3a in the cases of FK1 and 1E, see Table 2). Free Ub and mono-Ub linked to the lysozyme were detectable by Western blotting with anti-Ub polyclonal antibody, while the lysozyme molecule was detected by blotting with anti-lysozyme polyclonal antibody. We also established two hybridoma clones called FK2 and 135C, both of which produced monoclonal antibody specific to the mono-Ub linked to lysozyme as well as to the poly-Ub–protein conjugates, but not free Ub or free lysozyme (Fig. 3a in the case of FK2, see Table 2).

In order to prove that the latter two antibodies (FK2 and 135C) but not the former eight ones recognized the mono-Ub linked to lysozyme, we prepared the methyl-Ub–[^{125}I]lysozyme using methyl-Ub in place of unmodified Ub and tested the cross-reactivity of each antibody

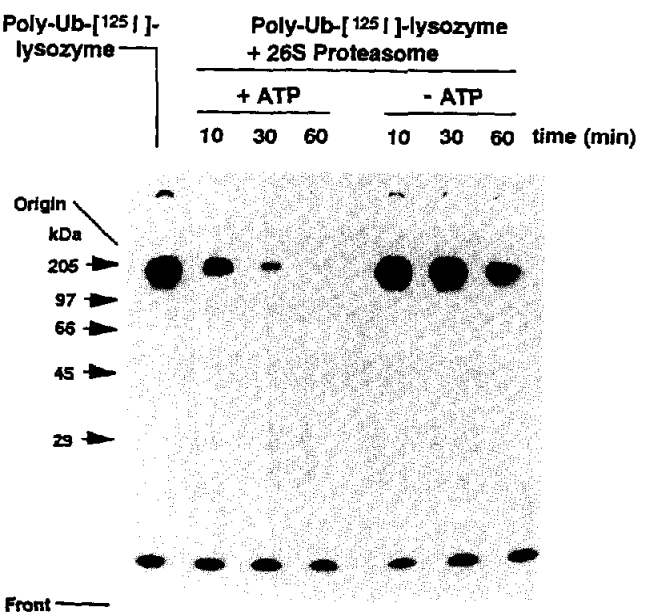

Fig. 2. Degradation of poly-Ub-[^{125}I]lysozyme conjugates by the 26S proteasome. The enzymatic reaction with 6 μg of the purified 26S proteasome from rabbit skeletal muscle in the presence or absence of 2 mM ATP was carried out for the indicated time using 0.2 μg of poly-Ub-[^{125}I]lysozyme conjugates as substrate under the conditions described in secton 2. The reaction was terminated by adding the SDS sample buffer. The extent of degradation was monitored by SDS-PAGE followed by autoradiography. Molecular mass standards used were rabbit myosin light chain (205 kDa), rabbit phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), chicken ovalbumin (45 kDa) and bovine carbonic anhydrase (29 kDa).

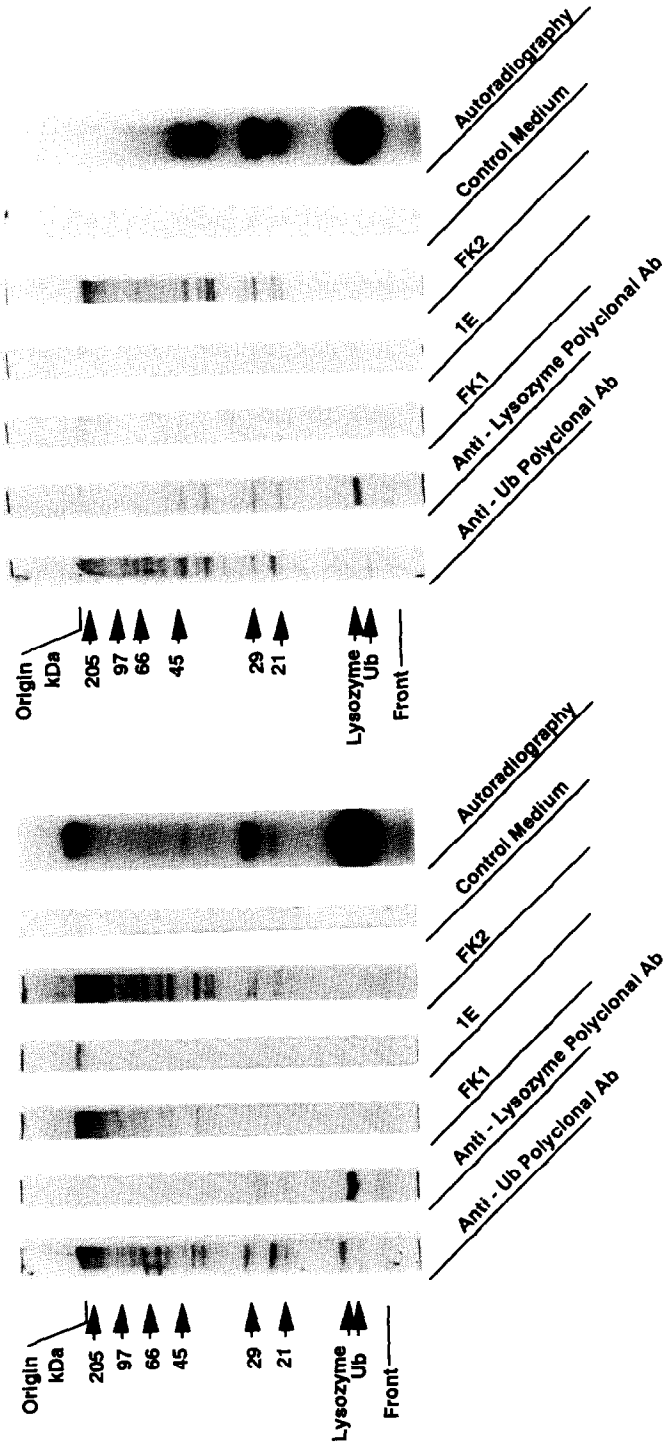

Fig. 3. Western blotting of poly-Ub–lysozyme conjugates (*a*) and methyl-Ub–lysozyme (b) with monoclonal antibodies of FK1, 1E and FK2. Poly-Ub–lysozyme conjugates and methyl-Ub–lysozyme were prepared using either cold or radioiodinated lysozyme as described in section 2 except for 0.1 mg/ml of Ub and methyl-Ub, respectively. After conjugation, aliquots (30 μl) of the reaction mixture were subjected to SDS-PAGE. After electrophoresis, Western blotting was carried out as in [37] with non-diluted cultured medium of each hybridoma clone (FK1, 1E or FK2) as the primary antibody. Culture medium obtained in culture of myeloma cells (control medium) and rabbit anti-Ub polyclonal antibody (Ab) and anti-lysozyme polyclonal Ab were used as controls. Autoradiogram of poly-Ub– or methyl-Ub–[^{125}I]lysozyme is shown in the right side of each panel of (a) and (b). Molecular mass standards used were rabbit myosin light chain (205 kDa), rabbit phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), chicken ovalbumin (45 kDa), bovine carbonic anhydrase (29 kDa), and soybean trypsin inhibitor (21 kDa).

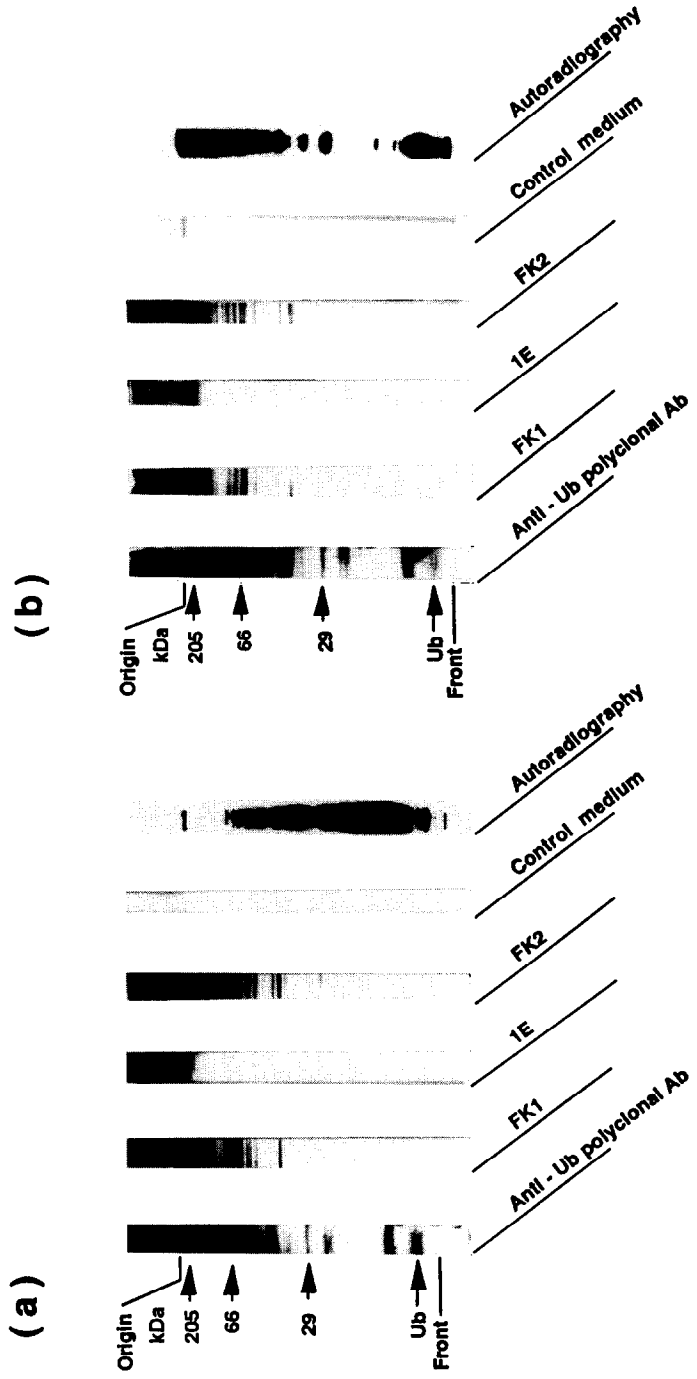

Fig. 4. Western blotting of poly-Ub–ribonuclease A conjugates (a) and poly-Ub–α-lactalbumin conjugates (b) with monoclonal antibodies of FK1, 1E and FK2. Cold and ^{125}I-labeled poly-Ub–ribonuclease A and poly-Ub–α-lactalbumin were prepared as in section 2. SDS-PAGE and Western blotting were performed as in Fig. 3. Autoradiograms of poly-Ub–[^{125}I]protein conjugates are shown in the right sides of (a) and (b). Molecular mass standards used were the same as in Fig. 3.

Table 1
Degradations of [^{125}I]proteins and poly-Ub-[^{125}I]protein conjugates by the 26 S proteasome in the presence or absence of ATP

[^{125}I]Protein		Degradation (%)		Ratio
		+ATP	−ATP	(+ATP/−ATP)
Heat-denatured lysozyme	−Ub[a]	11.9	18.9	0.6
	+Ub[b]	54.8	23.3	2.4
Ribonuclease A	−Ub[a]	27.8	28.1	1.0
	+Ub[b]	57.6	11.5	5.0
α-Lactalbumin	−Ub[a]	15.1	12.8	1.2
	+Ub[b]	64.8	28.6	2.3

[^{125}I]Proteins (0.2 μg, about 20,000 cpm) or poly-Ub-[^{125}I]protein conjugates (0.2 μg, about 20,000 cpm) were hydrolyzed by the purified 26 S proteasome (6 μg) in the presence or absence of 2 mM ATP.
[a] Original [^{125}I]protein.
[b] Poly-Ub-protein conjugates.

with this modified protein by Western blotting (Table 2). The representative results with FK1, 1E and FK2 antibodies are shown in Fig. 3b. The results actually showed that FK2 antibody, as well as 135C antibody, reacted with the methyl-Ub–lysozyme and also with the poly-Ub–lysozyme conjugates. In contrast, FK1 and 1E antibodies did not react with the methyl-Ub–lysozyme. The same was true for other antibodies of 31A, 12H, 99F, 169H, 9A and 58D. These results indicate that our ten antibodies can be classified into at least two groups. Furthermore, close investigation of band patterns on Western blots suggests that the former eight antibodies may be further divided into two subgroups (see Fig. 3a):

Table 2
Cross-reactivities and classes of monoclonal antibodies raised against poly-Ub–lysozyme conjugates

Clone	Subclass (light chain)		Cross-reactivity				
			Free Ub		Poly-Ub[a]		Methyl- Ub[b]
			WB[c]	ELISA	WB[c]	ELISA	WB[c]
FK1	IgM	(κ)	−	−	+++[d]	+++	−
169H	IgG2b	(κ)	−	−	++[d]	++	−
1E	IgM	(κ)	−	−	+[d]	+++	−
31A	IgG1	(κ)	−	−	+[d]	++	−
12H	IgG1	(κ)	−	−	+[d]	+	−
99F	IgG1	(κ)	−	−	+[d]	+++	−
9A	IgM	(κ)	−	−	+[d]	++	−
58D	IgM	(κ)	−	−	±[d]	++	−
FK2	IgG1	(κ)	−	−	+++	+++	+++
135C	IgM	(κ)	−	−	+++	+++	+++

[a] Poly-Ub–lysozyme conjugates.
[b] Methyl-Ub–lysozyme.
[c] Western blotting.
[d] Note that band patterns on Western blots are different between a group of FK1 and 169 H and a group of 1E, 31A, 12H, 99H, 9A and 58D.

one is an FK1 type (FK1 and 169H) and the other is a 1E type (1E, 31A, 12H, 99F, 9A and 58D).

Almost the same results by Western blot analysis were obtained with polyubiquitinated oxidized [^{125}I]ribonuclease A (Fig. 4a) and polyubiquitinated [^{125}I]α-lactalbumin (Fig. 4b). These results including those with polyubiquitinated [^{125}I]lysozyme indicate that our monoclonal antibodies never recognize the substrate protein moiety of the poly-Ub–protein conjugates and do recognize the Ub moiety (either the mono-Ub form or the multi-Ub chain, dependent on the antibodies) of the conjugates. So far, such specific monoclonal antibodies have not yet been produced. Thus, our monoclonal antibodies are very useful for identification and quantification of the multi-Ub chains of poly-Ub–protein conjugates and also for discrimination between the multi-Ub chain and the mono-Ub linked to proteins ubiquitinated.

4. Discussion

We succeeded in developing a modified method to prepare large amounts of poly-Ub–protein conjugates efficiently [30]. Ultracentrifugation (100,000 × *g*, 6 h) utilized as a new procedure in this study was found to be an effective step for removing the 26 S proteasome and its associating isopeptidase, contamination of which could make the prepared poly-Ub–protein conjugates unstable through degradation or de-ubiquitination. Thus, this step appeared essential for efficient preparation of the poly-Ub–protein conjugates.

It has been reported that a crude preparation of the poly-Ub–[^{125}I]lysozyme conjugates is not suitable for a substrate of the 26 S proteasome due to their insusceptibility [30]. In our experiments, however, our preparation of the poly-Ub–[^{125}I]lysozyme conjugates was found to be a very useful substrate for assay of Ub-conjugate degrading activity of the 26 S proteasome: the poly-Ub–[^{125}I]lysozyme conjugates prepared by our method were reasonably stable in the absence of the 26 S proteasome but were very rapidly degraded by the enzyme in an ATP-dependent manner. These properties of our poly-Ub–protein conjugates give strong support to our preparation being a natural (i.e. 26 S proteasome-susceptible) form of poly-Ub–protein conjugates.

Using such natural poly-Ub–lysozyme conjugates as immunogen, we established ten hybridoma clones producing monoclonal antibodies that recognized the poly-Ub–protein conjugates. We found that there are three different band patterns detected by our ten monoclonal antibodies on Western blots of both the crude preparations of the poly-Ub–lysozyme conjugates and the methyl-Ub–lysozyme. One is reactive with both the poly-Ub–protein conjugates and the methyl-Ub–lysozyme, while the other two are reactive with only the poly-Ub–protein conjugates. In the latter case there are two differ-

ent band patterns on the blots of the poly-Ub–protein conjugates. It can be imagined that the latter two groups of the antibodies recognize the molecular structure of the isopeptide bond formed between a carboxyl group of the C-terminal glycine residue of one Ub molecule and an ε-NH_2 group of the lysine residue of the other Ub molecule. On the other hand, the former group could recognize the sequence around the isopeptide bond formed between the C-terminus of Ub and the lysine residue of lysozyme. These considerations imply there is a difference in molecular structure of isopeptide bond formed between the Ub molecules themselves from that formed between the Ub molecule and the protein moiety ubiquitinated. Furthermore, different band patterns on Western blots between the latter two groups also implies the presence of different types of isopeptide bonds formed between the Ub molecules themselves. Such a situation would occur if an isopeptide bond could be formed between the C-terminus of one Ub molecule and one of the Lys^6, Lys^{11}, Lys^{27}, Lys^{29}, Lys^{33}, Lys^{48}, or Lys^{63} residue of the other Ub molecule. Further detailed studies are necessary for elucidation of the epitopes recognized by our monoclonal antibodies at a molecular level.

Several immunocytochemical reports on the localization of Ub in the brain of neurodegenerative diseases have been presented (for review, see [40]), suggesting that ubiquitinated proteins accumulate under these pathological conditions. Since polyclonal antibody raised against Ub molecule was always used in these studies, the extent of polyubiquitination of putative target proteins cannot be assigned. On the other hand, Western blot analysis using the conventional anti-Ub polyclonal antibody indicates that highly-polyubiquitinated proteins accumulate in ischemic damage [21,22] or in response to heat shock [7]. Furthermore, it has been reported that the multi-Ub chains are linked via isopeptide bonds to the paired helical filaments in Alzheimer's disease [18,19]. Even under certain physiological conditions, intracellular regulatory proteins functioning in the progression of cell cycles have been reported to be polyubiquitinated at restricted points and then degraded by the 26 S proteasome [2–4], and the Ub molecule linked via peptide bond in a monoubiquitinated form or linked via isopeptide bond in mono- or oligo-ubiquitinated form has been proposed to promote the biogenesis of organelles [13–17]. These reported data indicate that the Ub-ligation reaction is a crucial event for cellular activities. Our monoclonal antibodies produced in this study can quantify the extent of polyubiquitination in the above circumstances and their combinations can discriminate the states among polyubiquitination leading to the formation of the multi-Ub chains, monoubiquitination and free Ub. Actually, in our experiments, we found that the amount of polyubiquitinated proteins in plasma was measurable by sandwich ELISA using our monoclonal antibodies. We also found that Western blot analysis of the Alzheimer's paired helical filaments using our antibodies gave different patterns among the monoclonal antibodies, and also between our antibodies and the conventional anti-Ub antibody (to be published). Thus, we consider the monoclonal antibodies produced in this study to be useful tools for the analysis of the Ub-ligation system in various cellular events.

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References

[1] Rechsteiner, M. (1988) Ubiquitin, Plenum Press, New York.
[2] Glotzer, M., Murray, A.W. and Kirschner, M.W. (1991) Nature 349, 132–138.
[3] Rechsteiner, M. (1991) Cell 66, 615–618.
[4] Nishizawa, M., Furuno, N., Okazaki, K., Tanaka, H., Ogawa, Y. and Sagata, N. (1993) EMBO J. 12, 4021–4027.
[5] Mori, M., Eki, T., Takahashi-Kudo, M., Hanaoka, F., Ui, M. and Enomoto, T. (1993) J. Biol. Chem. 268, 16803–16809.
[6] Jentsch, S., McGrath, J.P. and Varshavsky, A. (1987) Nature 329, 131–134.
[7] Carlson, N., Rogers, S. and Rechsteiner, M. (1987) J. Cell Biol. 104, 547–555.
[8] Finley, D., Ozkaynak, E. and Varshavsky, A. (1987) Cell 48, 1035–1046.
[9] Delic, J., Morange, M. and Magdelenat, H. (1993) Mol. Cell. Biol. 13, 4875–4883.
[10] Cenciarelli, C., Hou, D., Hus, K.-C., Rellahan, B.L., Wiest, D.L., Smith. H.T., Fried, V.A. and Weissman, A.M. (1992) Science 257, 795–797.
[11] Mori, S., Heldin, C.-H. and Claesson-Welsh, L. (1992) J. Biol. Chem. 267, 6429–6434.
[12] Paolini, R. and Kinet, J.-P. (1993) EMBO J. 12, 779–786.
[13] Finley, D., Bartel, B. and Varshavsky, A. (1989) Nature 338, 394–401.
[14] Redman, K.L. and Rechsteiner, M. (1989) Nature 338, 438–440.
[15] Li, W., Nagaraja, S., Delcuve, G.P., Hendzel, M.J. and Davie, J.R. (1993) Biochem. J. 296, 737–744.
[16] Wiebel, F.F. and Kunau, W.-H. (1992) Nature 359, 73–76.
[17] Ball, E., Karlik, C.C., Beall, C.J., Saville, D.L., Sparrow, J.C., Bullard, B. and Fyrberg, E.A. (1987) Cell 51, 221–228.
[18] Mori, H., Kondo, J. and Ihara, Y. (1987) Science 235, 1641–1644.
[19] Morishima-Kawashima, M., Hasegawa, M., Takio, K., Suzuki, M., Titani, K. and Ihara, Y. (1993) Neuron 10, 1151–1160.
[20] Kuzuhara, S., Mori, H., Izumiyama, N., Yoshimura, M. and Ihara, Y. (1988) Acta Neuropathol. 75, 345–353.
[21] Hayashi, T., Takada, K. and Matsuda, M. (1992) J. Neurosci. Res. 31, 561–564.
[22] Hayashi, T., Tanaka, J., Kamikubo, T., Takada, K. and Matsuda, M. (1993) Brain Res. 620, 171–173.
[23] Goldberg, A.L. (1992) Eur. J. Biochem. 203, 9–23.
[24] Hershko, A. and Ciechanover, A. (1992) Annu. Rev. Biochem. 61, 761–807.
[25] Rechsteiner, M., Hoffman, L. and Dubiel, W. (1993) J. Biol. Chem. 268, 6065–6068.
[26] Tanaka, K., Tamura, T., Yoshimura, T. and Ichihara, A. (1992) New Biol. 4, 173–187.
[27] Hough, R. and Rechsteiner, M. (1986) J. Biol. Chem. 261, 2391–2399.

[28] Hough, R., Pratt, G. and Rechsteiner, M. (1986) J. Biol. Chem. 261, 2400–2408.
[29] Hough, R., Pratt, G. and Rechsteiner, M. (1987) J. Biol. Chem. 262, 8303–8313.
[30] Tamura, T., Tanaka, K., Tanahashi, N. and Ichihara, A. (1991) FEBS Lett. 292, 154–158.
[31] Hershko, A., Heller, H., Elias, S. and Ciechanover, A. (1983) J. Biol. Chem. 258, 8216–8214.
[32] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[33] Parker, C.W. (1990) in: Methods in Enzymology (Deutscher, M.P. ed.) vol. 182, pp. 721–737, Academic Press, New York.
[34] Hershko, A. and Heller, H. (1985) Biochem. Biophys. Res. Commun. 128, 1079–1086.
[35] Sawada, H., Muto, K., Fujimuro, M., Akaishi, T., Takagi Sawada, M., Yokosawa, H. and Goldberg, A.L. (1993) FEBS Lett. 335, 207–212.
[36] Laemmli, U.K. (1970) Nature 227, 680–385.
[37] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[38] Kohler, G. and Milstein, C. (1976) Eur. J. Immunol. 6, 511–519.
[39] Haas, A.L. and Bright, P.M. (1985) J. Biol. Chem. 260, 12464–12473.
[40] Mayer, R.J., Arnold, J., Laszio, L., Landon, M. and Lowe, J. (1991) Biochim. Biophys. Acta 1089, 141–157.