Human proteasomes analysed with monoclonal antibodies

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The proteasome or multicatalytic endopeptidase from eukaryotic cells consists of at least 14 subunits that fall into two families, α and β. Subunit-specific monoclonal antibodies against ten different subunits of human proteasomes have been produced, together with an antibody that reacts with a motif (prosbox 1), common to α-type subunits. Four of the subunit-specific antibodies were able to precipitate proteasomes. The subunit composition of HeLa-cell proteasomes precipitated with these four different antibodies were identical, as judged from two-dimensional electrophoresis. One of the four antibodies was used to obtain proteasomes from cell lines (HeLa, Daudi, IMR90 and BSC-1) and human tissues (placenta, kidney, and liver). Electrophoretic analysis of these proteasomes, combined with peptide mapping of some subunits, suggests that they all contain 14 types of subunits as their major constituents. However, one subunit was present in two isoelectric isoforms in all cells examined. Two other subunits occurred in two or three isoelectric isoforms in placenta, liver and kidney, but not in the cell cultures. Extracts of human cells (HeLa, IMR90, Daudi and erythrocytes) were analysed by non-denaturing electrophoresis and immunoblotting. All of the 11 subunits detected by antibodies were present in a pair of ATP-stabilized protein complexes, presumed to be the 26 S proteinase, and in a doublet of complexes which migrated more slowly than purified proteasomes. Besides being present in proteasomes, one subunit was also found to occur in the free state in cell extracts.

INTRODUCTION

Nuclei and cytoplasm in eukaryotic cells contain cylinder-shaped particles with an M_r of about 740000. The particles are called proteasomes or multicatalytic endopeptidase (EC 3.4.99.46), since they posses at least five distinct endopeptidase activities [reviewed by Rivett (1993) and Rechsteiner et al. (1993)]. Being the catalytic core of the larger 26 S proteinase complex, proteasomes are involved in the ATP-dependent degradation of proteins conjugated to ubiquitin (Hershko and Ciechanover, 1992). This ubiquitin-dependent pathway of protein degradation is important for the turnover of cellular proteins and for the generation of antigen fragments in the major histocompatibility complex class I-dependent pathway of antigen presentation (Monaco, 1992; Michalek et al., 1993).

Proteasomes consist of subunits of M_r 21000–32000. The number of subunits has not been agreed upon, but is reported to vary between 12 and more than 20, perhaps depending on the source of enzyme (Rivett, 1993). In most cells, two of the subunits, lmp2 and lmp7, are found in only small amounts. Their synthesis is induced by interferon-γ and they replace two other subunits in the proteasome (Aki et al., 1994; Belich et al., 1994).

A detailed knowledge of the structure of the proteasome is a prerequisite for understanding its function. We have produced monoclonal antibodies to most of the proteasome subunits and used them in an analysis of proteasomes from different cell types. Rivett and Sweeney (1991) showed that several of the proteasome subunits are antigenically distinct. The outcome of our analysis has led us to conclude that the proteasome is made up of 14 different subunits in addition to small amounts of lmp2 and lmp7. Some of the subunits appear to exist as several isoforms, thus accounting for the reported presence of subpopulations of proteasomes.

Two of the antibodies have been described previously (Kaltoft et al., 1992), and two of them have been used in immunoelectron-microscopical studies of the quaternary structure of proteasomes (Kopp et al., 1993).

MATERIALS AND METHODS

Materials

Cell-culture media were purchased from Gibco (Paisley, Renfrewshire, Scotland, U.K.). Chemicals for PAGE were obtained from Bio-Rad (Richmond, CA, U.S.A.). *Staphylococcus aureus* V8 proteinase (endoproteinase glu-C) was purchased from Boehringer (Mannhein, Germany), DEAE-Sephacel from Pharmacia (Uppsala, Sweden), BCG (Bacille Calmette–Guerin) tuberculosis vaccine and tuberculin purified protein derivative (PPD) from the State Serum Institute (Copenhagen) and other chemicals were from Sigma (St. Louis, MO, U.S.A.) unless otherwise stated.

Proteasomes were purified from human placenta by conventional chromatography or by immunoaffinity chromatography on immobilized antibody MCP21 (Hendil and Uerkvitz, 1991). For immunizations, proteasomes were coupled to tuberculin PPD with glutardialdehyde (Lachmann et al., 1986) or dinitrophenylated with dinitrobenzenesulphonic acid (Hudson and Hay, 1989) to 2.4 mol of dinitrophenol/mol of proteasomes.

Antibodies

Antibody IB5 (Grossi de Sa et al., 1988) was kindly donated by Dr. Klaus Scherrer (Institut Jacques Monod, Université Paris VII, Paris, France) or purchased from Organon Teknika (Turnhout, Belgium). Rabbit antisera to lmp7 (Glynne et al., 1993) and lmp2 were generously provided by Dr. John Trowsdale (Imperial Cancer Research Fund, London, U.K.).

For production of hybridomas, Balb/c or Balc/c × CF1 hybrid mice were immunized with repeated intraperitoneal injections, each time with 20–50 μg of proteasomes emulsified in Freund's adjuvant. Freund's complete adjuvant was used for initial immunizations with dinitrophenylated or native proteasomes and incomplete adjuvant was used for all other immunizations. Mice immunized with proteasome–tuberculin PPD complexes

Abbreviations used: 2D, two-dimensional; NP40, Nonidet P40; PPD, purified protein derivative.

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were primed with BCG vaccine for 1 month before the first injection with proteasomes (Lachmann et al., 1986).

The mice were bled and screened for proper immunoreactive sera on SDS/PAGE blots with proteasomes. Spleen cells from selected mice were fused with Sp2/0 myeloma cells according to standard methods (Hudson and Hay, 1989). The hybridomas from each fusion were distributed into 12 microtitre plates (Nunc, Roskilde, Denmark) and screened by e.l.i.s.a. with purified proteasomes. Cell cultures from positive wells were expanded and screened on immunoblots from SDS/PAGE gels with proteasomes and then on blots from O'Farrell-type two-dimensional (2D) PAGE gels. Eventually the hybridomas were cloned at least twice by limiting dilution in microtitre plates.

Monoclonal antibodies were purified from peritoneal fluid on Protein A–agarose (Goding, 1980). Antibody MCP444 was unstable at low pH and was instead purified by $(NH_4)_2SO_4$ precipitation followed by chromatography on DEAE-Sephacel (Goding, 1980). For isotyping, the culture supernatants were incubated in microtitre plates with adsorbed proteasomes. Bound antibodies were then detected with isotype-specific goat antibodies to mouse immunoglobulin (Zymed, South San Francisco, CA, U.S.A.) followed by peroxidase-conjugated rabbit antibody to goat immunoglobulin (Dako, Copenhagen, Denmark) and *o*-phenylenediamine/H_2O_2.

Cells

Erythrocytes were obtained from freshly drawn blood, stabilized with heparin. HeLa and IMR90 cells were grown in Eagle's minimal essential medium with 10% newborn-calf serum. Daudi cells were grown in RPMI1640 with 10% foetal-calf serum.

Immunoprecipitations

Monoclonal antibodies were immobilized on CNBr-activated Sepharose CL 4B to a concentration of 2–3 mg of immunoglobulin/ml of gel. Cells were homogenized by sonication for 5 s in 5 vol. of 50 mM Tris/HCl/17% glycerol, pH 7.4. Tissues were homogenized in the same buffer, but in a Potter–Elvehjem homogenizer. Homogenates were cleared by centrifugation at 12000 *g* for 5 min, and 400 μl of supernatant was incubated on a rocking platform for 1 h with 20–60 μl of Sepharose with immobilized antibody. The Sepharose beads were washed in 3 × 15 ml of 20 mM Tris/HCl/20 mM NaCl/0.1 mM Na_2EDTA/1 mM $MgCl_2$/0.5% Nonidet P40 (NP40)/0.1% SDS/17% glycerol, pH 7.5, with intervening centrifugations at 40 *g* for 5 min and then in 15 ml of 50 mM ammonium acetate/acetic acid, pH 7.0. The final pellet was suspended into 3 ml of distilled water, freeze-dried, and suspended into 100 μl of sample buffer for isoelectric focusing.

Electrophoresis

Proteasomes or immunoprecipitates were dissolved in a sample buffer with 9 M urea, 2% NP40, 5% mercaptoethanol, 1.5% 2D Pharmalyte pH 3–10, 1% Ampholine 5–8 and 0.5% Ampholine 9–11 (Pharmacia, Uppsala, Sweden). The subunits, in 10 μl of sample buffer with 20 μg of proteasomes, were separated in a Hoefer (San Franscisco, CA, U.S.A.) Mighty Small unit by 2D PAGE as described by O'Farrell et al. (1977). The first dimension was non-equilibrium pH-gradient electrophoresis towards the cathode for 3 h at 400 V. The second-dimensions runs were in Laemmli (1970) SDS/PAGE gels with 12.5% acrylamide.

For non-denaturing electrophoresis, cells were brought into suspension and washed in 137 mM NaCl/2.7 mM KCl/4.1 mM Na_2HPO_4/0.73 mM KH_2PO_4, pH 7.4, with centrifugations. The cells were homogenized by sonication for 5 s at 0 °C in 5 vol. of 50 mM Tris/HCl (pH 7.4)/17% glycerol and centrifuged at 13000 *g* for 5 min. Samples (10 μl) of supernatant were resolved by electrophoresis in 0.15 cm × 7 cm × 8 cm gels with 4.5% acrylamide and Tris/borate buffer (Hoffman et al., 1992). With 10 mA/slab gel, separation was attained in about 80 min. For estimation of M_r, other samples were electrophoresed for 6 h at 12 mA/gel in 7 cm × 8 cm non-denaturing gels with a linear gradient of 5–30% (w/v) acrylamide (Slater, 1969). The M_r standards were human α_2-macroglobulin, horse ferritin, ox liver catalase, BSA (monomer and dimer) and β-lactoglobulin. Alternatively, the supernatant was mixed with SDS sample buffer with mercaptoethanol and electrophoresed in Laemmli (1970) gels with 12.5% acrylamide. Proteins were blotted on to BA83 nitrocellulose sheets (Schleicher und Schüll, Dassel, Germany) in a semi-dry blotter with 48 mM Tris/39 mM glycine/0.01% SDS in 20% (v/v) methanol. The blots were stained reversibly with Ponceau S, blocked with 5% fat-free dried milk and incubated overnight with monoclonal antibody in 50 mM Tris/HCl/150 mM NaCl/5 mM NaN_3/0.01% Tween-20, pH 7.4. Antibodies bound to blots were detected with peroxidase-coupled rabbit antibody to mouse immunoglobulins (Dako) and tetramethylbenzidine/H_2O_2 as substrates.

RESULTS

Antibody specificities

A total of about 400 antibodies to human proteasomes were found in the first turn when hybridomas from 23 fusions were screened by e.l.i.s.a. About half of the antibodies also reacted with proteasome subunits blotted from SDS/PAGE gels and were further characterized.

Human placenta proteasomes separate on 2D PAGE gels into about 20 spots, some of which have been indicated by numbers in Figure 1. Placenta proteasomes purified by conventional chromatography and by immunoaffinity chromatography as in Figure 1 had identical subunit patterns (results not shown). The same spots were found when the pH-gradient electrophoresis was run for shorter times or in the opposite direction (towards

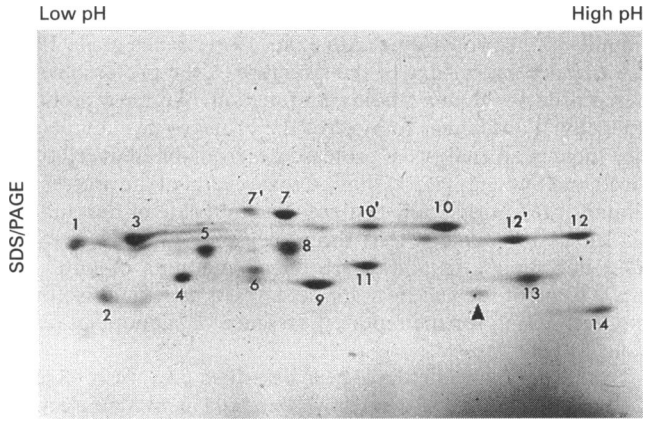

Figure 1 Two-dimensional PAGE of proteasomes from human placenta

The proteasomes were purified by immunoaffinity chromatography. Coomassie Blue-stained subunits are designated by numbers, except the minor subunit Imp7, which is indicated by an arrowhead.

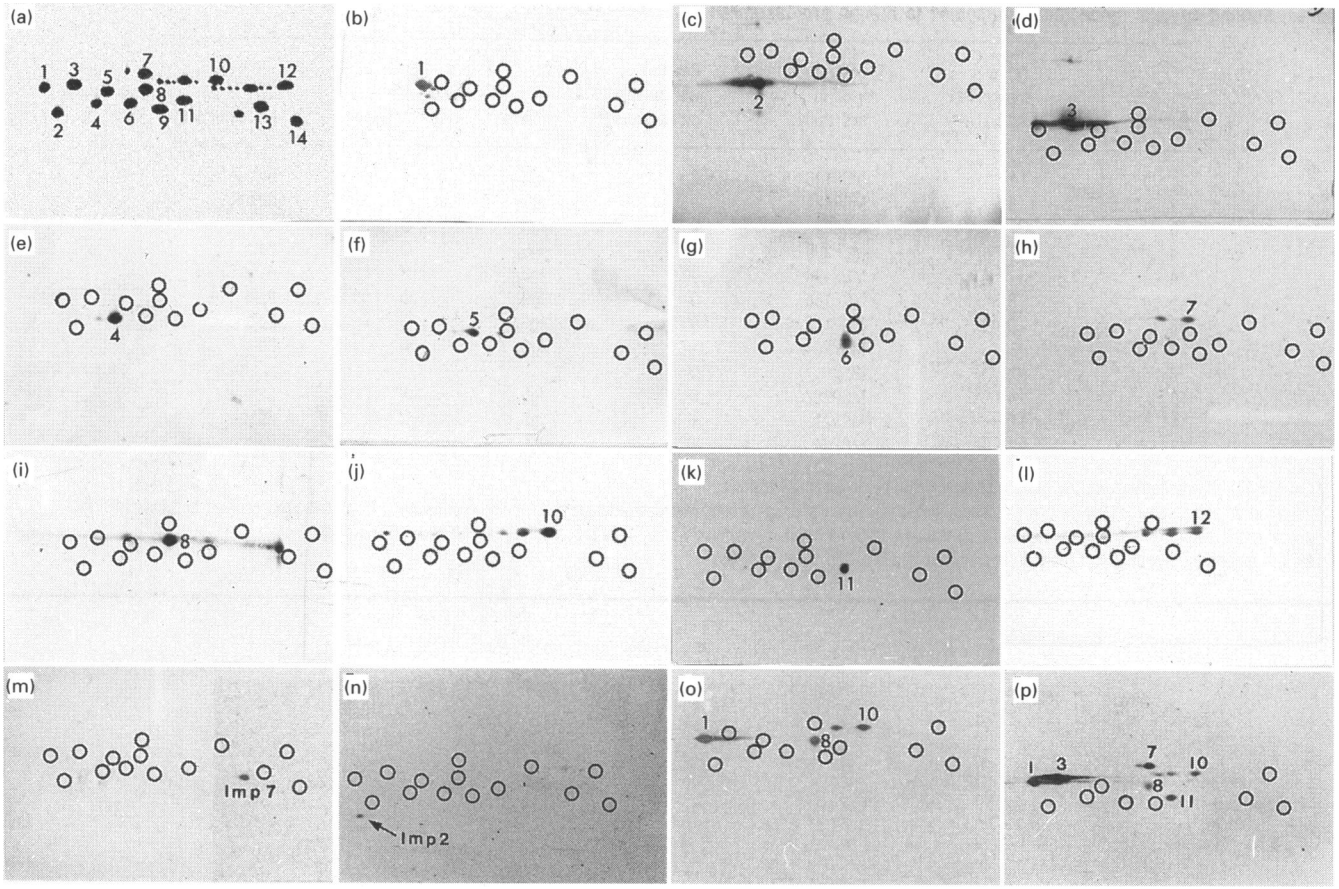

Figure 2 Reactivities of antibodies with subunits from human proteasomes

Proteins from gels like that shown in Figure 1 were blotted on to nitrocellulose filters, stained reversibly with Ponceau S and probed with antibodies. (**a**) is a diagram of the gel shown in Figure 1. The antibodies were: (**b**) MCP196; (**c**) MCP421; (**d**) MCP72; (**e**) MCP205; (**f**) MCP168; (**g**) MCP102; (**h**) MCP20; (**i**) antibody IB5 (Grossi de Sa et al.,1988); (**j**) MCP257; (**k**) MCP21; (**l**) MCP34; (**m**) anti-Imp7 antibody (Glynne et al., 1993); (**n**) anti-Imp2 antibody (kindly given by Dr. John Trowsdale); (**o**) MCP222; (**p**) MCP231. Positions of unreactive subunits on the blots are indicated by circles and reactive ones are numbered as in Figure 1.

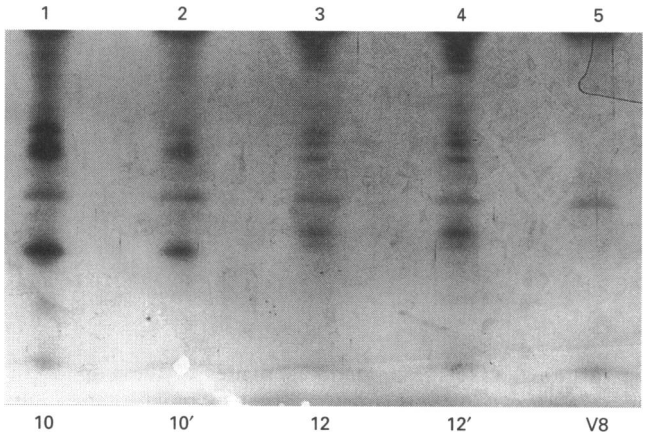

Figure 3 Peptide fingerprints of proteasome subunits

Subunits were excised from two Coomassie Blue-stained gels as in Figure 1 and digested with 0.2 μg of V8 proteinase (Cleveland et al., 1977). The SDS/PAGE gel was silver-stained. Lane 1, subunit 10; lane 2, subunit 10′; lane 3 subunit 12; lane 4, subunit 12′; lane 5, V8 proteinase alone.

the anode). This makes it unlikely that any subunits could have been lost during electrophoresis.

Blots of gels like the one in Figure 1 were used to characterize the monoclonal antibodies. Many of the antibodies reacted with only one protein spot (Figures 2b–2g and 2k), but several of them gave two or more spots. Spots 1 and 2 (Figures 2b and 2c) correspond to the subunits zeta and delta respectively (Lee et al., 1990; DeMartino et al., 1991). Gerards et al. (1994) have identified spot 4 as subunit HSB, also called HsN3 (Nothwang et al., 1994). Figure 2(i) shows the reaction of subunit iota (Bey et al., 1993) with the monoclonal antibody IB5, produced by Grossi de Sa et al. (1988). The minor spots in Figure 2(i) contained too little protein to allow detection by Coomassie Blue staining (Figure 1). They probably contain a fraction of the protein from the major spot that has been chemically modified, either in the tissue or during enzyme purification. The pattern seen in Figure 2(h) was found with 43 different hybridomas, whereas no antibody reacted with only one of the two spots. Thus subunit 7 and the protein in the minor spot with lower pI (7 in Figure 1) are structurally closely related. Weitman and Etlinger (1992) have produced a monoclonal antibody to subunit 7 that also reacted with a putative precursor of M_r 41000. This reaction could not be detected with any of our antibodies. Proteasome preparations containing degradation products, particularly of

Table 1 Subunit-specific monoclonal antibodies to human proteasomes

Subunit	No. of monoclonal antibodies*	Antibodies used in this study	Reactive with soluble proteasomes	Isotype	Antigen†	Mouse strain‡
1	14	MCP196	0	IgG_{2a}	D	Balb
2	8	MCP421	0	IgG_1	P	BalbXCF
3	7	MCP72	0	IgG_1	D	Balb
4	22	MCP205	0	IgG_1	D	Balb
4		MCP444	+	IgG_1	P	BalbXCF
5	8	MCP168	0	IgG_1	D	Balb
6	5	MCP102	0	IgG_1	D	Balb
7	43	MCP20	+	IgG_1	N	Balb
8	(0)	IB5§	0			
10	4	MCP257	0	IgG_{2b}	N	Balb
11	17	MCP21	+	IgG_1	N	Balb
12	29	MCP34	+	IgG_1	N	Balb

* Total number of hybridomas with the designated specificity, produced in 23 fusions.
† Antigen used when obtaining the particular antibody listed in column 3: D, dinitrophenylated proteasomes; N, native proteasomes; P, proteasomes coupled to tuberculin PPD.
‡ Mouse strain refers to the parent mouse of the particular hybridoma listed in column 3; Balb = Balb/c; BalbXCF are Balb/c × CF1 hybrids.
§ IB5 is a monoclonal antiboby to subunit iota, produced by Grossi de Sa et al.(1988).

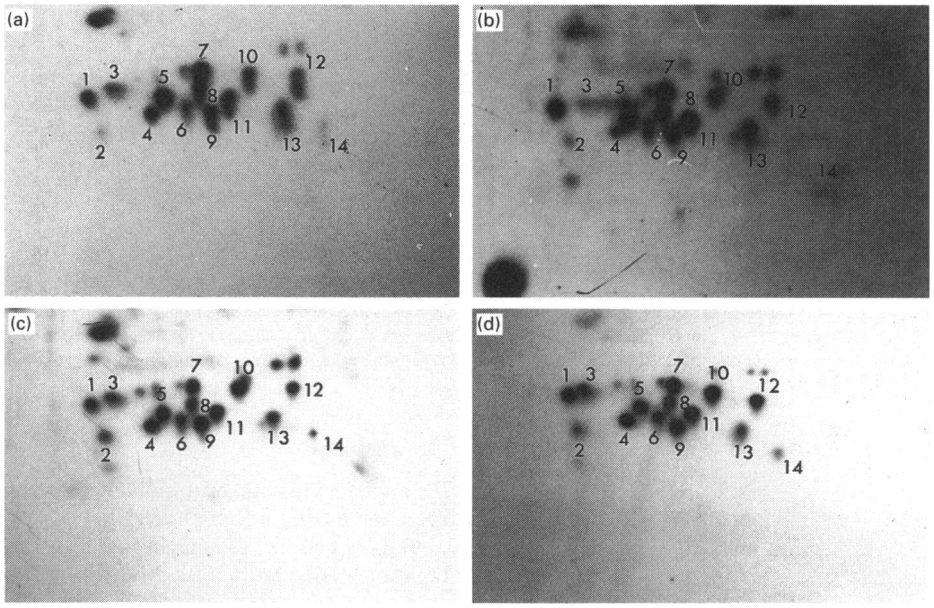

Figure 4 Proteasomes precipitated with antibodies to four different subunits

Antibodies MCP444 (**a**), MCP20 (**b**), MCP21 (**c**) and MCP34 (**d**) were immobilized on CNBr-activated Sepharose CL-4B and used to precipitate proteasomes from extracts of HeLa cells that had been labelled for 18 h in medium with 183 kBq of [^{3}H]leucine (Amersham)/ml. Cell proteins were separated by 2D PAGE and detected by fluorography (Chamberlain, 1979). Subunits are designated as in Figure 1.

subunit 7, were sometimes encountered. Such preparations gave reaction patterns with several more spots than those shown in Figure 2.

Figure 2(j) shows that an antibody reacting with subunit 10 also stained two other proteins with the same M_r but different pI. Three other antibodies with the same reactivity were found. Similarly, subunit 12 was stained by 29 antibodies that all reacted with three proteins with the same M_r (Figure 2l and Table 1). For both subunits 10 and 12 the amount of protein in the spots decreased with their pI. The two most alkaline isoforms in each family were sufficiently abundant to be analysed by proteolytic fingerprinting. Like the immunoreactivities of these spots, the peptide mapping suggests (Figure 3) that subunits 10 and 12 are distinct from each other but that each occur in closely related isoforms.

In Figures 2(m) and 2(n) the subunits lmp7 and lmp2 were identified with polyclonal rabbit anti-peptide antibodies (Glynne et al., 1993). So far we have not been able to produce stable antibodies to these subunits, nor to subunits 8, 9, 13 and 14. lmp2 was present in amounts too low to appear on Figure 1. However, the subunit was discernible on overloaded gels, and the position determined here agrees with that found previously (Früh et al.,

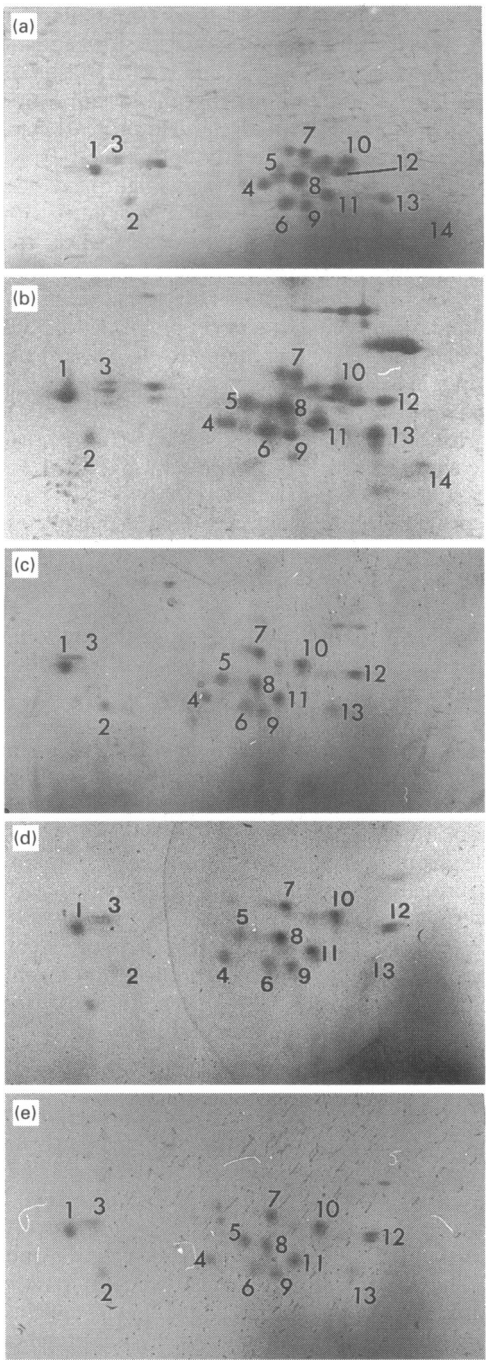

Figure 5 Proteasomes precipitated from tissues and cell lines with antibody MCP21

Proteasomes were precipitated from (**a**) liver, (**b**) kidney, (**c**) IMR90 human fibroblasts, (**d**) Daudi human B-lymphoma cells or (**e**) BSC-1 African-green-monkey epithelial cells. The proteins were separated by 2D-PAGE and stained with Coomassie Brilliant Blue.

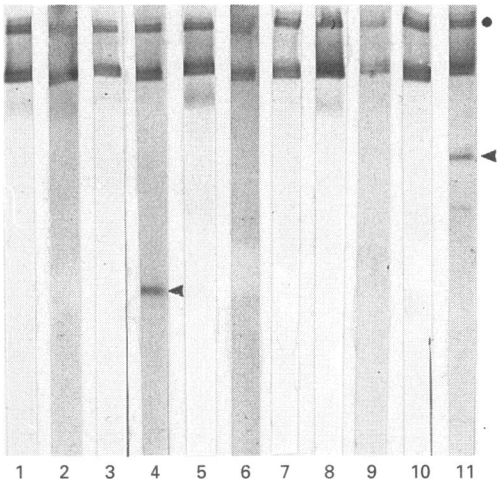

Figure 6 Proteasome subunits in cell extracts

Supernatants from HeLa extracts were subjected to non-denaturing PAGE, blotted on to nitrocellulose and incubated with the following monoclonal antibodies to proteasomes: (lanes) 1, MCP20; 2, MCP257; 3, MCP72; 4, MCP196; 5, MCP34; 6, IB5; 7, MCP168; 8, MCP21; 9, MCP102; 10, MCP205; and 11, MCP421. The uppermost doublet of bands (26 S proteinase) is marked by ●. The unmarked doublet is proteasomes. Fast migrating bands are marked by arrowheads in lanes 4 and 11. That in lane 11 is not reproducible and is probably an artifact. The one in lane 4 is free subunit 1 (zeta).

1992; Frentzel et al., 1993; Brown et al., 1993; Aki et al., 1994, Patel et al., 1994).

The proteasome subunits are structurally related and fall into two families, α and β. Antibodies reacting with more than one subunit are therefore expected and were indeed found (Figures 2o and 2p). Hybridomas with such antibody reactivities were usually discarded, since it is difficult to discriminate multisubunit reactivity from simple low specificity. However, Figures 2(o) and 2(p) show some extremes, antibody MCP231 (Figure 2p) binding to six different subunits. In e.l.i.s.a. the antibody also reacted with the peptide TVWSPQGRLHQVEYAMEA, generously supplied by Dr. P.-M. Kloetzel. This peptide sequence is found in *Drosophila* proteasome subunit Pros-35 (Haass et al., 1989) and encompasses the prosbox I motif common to α-type subunits (Haass et al., 1990). The subunits stained by MCP231 are therefore α-type subunits. On the other hand not all α subunits need react with antibody MCP231, because the prosbox motif is not completely identical in all subunits. In general, however, the prosbox I motif is phylogenetically preserved and the antibody reacts with proteasomes from several animal species and even from higher plants, when tested on immunoblots from SDS/PAGE gels (results not shown).

The panel of antibodies was screened in order to find antibodies that react with native soluble proteasomes. Only four different specificities were found, so the vast majority of antibodies react only with denatured proteasomes. An overview of the subunit-specific antibodies and their reactivities is given in Table 1.

Composition of proteasomes from cells and tissues

Proteasomes from ^{3}H-labelled HeLa cells were precipitated with the four antibodies reacting with different native proteasome subunits. Figure 4 shows that proteasomes that bind to the four different antibodies have the same subunit pattern as seen in placenta (Figure 1). The relative amounts of some subunits differ between the parts of Figure 4. However, the relative abundances varied somewhat from experiment to experiment, and this variability may result from a partial loss of some of the subunits during electrophoresis. The results show that at least proteasomes containing the subunits reacting with the precipitating antibodies, namely 4, 7, 11 and 12, all contain the same complement of 14 subunits. However, some differences are

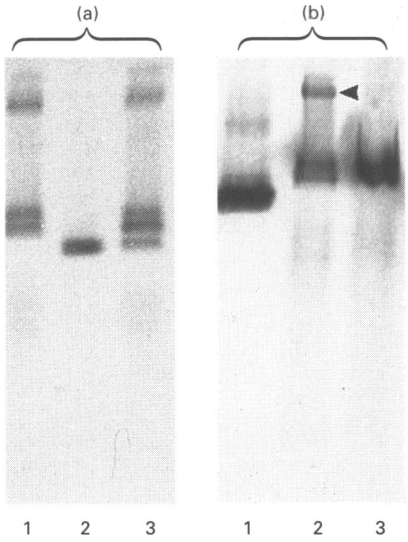

Figure 7 Proteasome complexes in cell extracts

Electrophoresis and blotting was done as described in Figure 6. Proteasomes were detected with antibody MCP20. (**a**) Lane 1 contained HeLa cell extracts as in Figure 6. Lanes 2 and 3 contained 0.1 μg of purified proteasomes electrophoresed either alone (lane 2) or after mixing with cell extract (lane 3). (**b**) Supernatants from cell extracts in 50 mM Tris/HCl (pH 6.8)/17% glycerol/2 mM $MgCl_2$/2 mM $CaCl_2$ were incubated for 20 min at 37 °C after addition of either an ATP-generating system [lane 2; 1 mM ATP, 10 mM phosphocreatine and creatine kinase (50 μg/ml] or apyrase [lane 3; Sigma type VIII (20 units/ml)]. Lane 1 contained 0.1 μg of purified proteasomes. The 26 S proteinase in lane 2 is labelled by an arrowhead.

apparent between placental proteasomes and those from HeLa cells: subunits 10 and 12 are each present in the three isoelectric variants in placenta (Figures 1 and 2), whereas only the one with the highest pI is present in HeLa cells (Figure 4).

In order to analyse the variability of subunits 10 and 12 more closely, antibody MCP21 was used to precipitate proteasomes from a number of tissues and cell lines. Figure 5 shows that the 14 subunits from placenta proteasomes (Figure 1) are also present in liver and kidney, again with subunits 10 and 12 appearing as isoelectric variants. The two most alkaline isoforms of subunit 12 are apparently absent in liver (Figure 5a). In contrast, proteasomes from cell lines derived from human fibroblasts (IMR90; Figure 5c), B-lymphoma cells (Daudi; Figure 5d), and monkey epithelial cells (BSC-1; Figure 5e) show 2D PAGE patterns similar to that of HeLa (human cervical carcinoma cell) proteasomes (Figure 4), i.e., with only the most alkaline isoform of subunits 10 and 12. Subunit 14 is discernible on the original gels in Figures 5(c)–5(d), but is not well reproduced, mainly because of the background of ampholines in this part of the gel.

In summary, the same 14 subunits are found in four different cell lines and three tissues. Subunit 7 is found as two isoforms in all the tissues and cells. Isoelectric variants of subunits 10 and 12 were found in the tissues, but not in cell cultures.

Proteasome complexes in cell extracts

The anti-proteasome antibodies did not react with any other protein in blots from SDS/PAGE gels with extracts of HeLa cells (results not shown). They could therefore be used to screen cell extracts for the presence of assemblies containing proteasome subunits. Figure 6 shows that all the 11 subunits, detected by antibodies, were present in a closely spaced slowly migrating pair of bands and in two closely spaced bands with a faster migration. Figure 7(a), lanes 1 and 2, shows that the lowermost doublet of bands migrates more slowly than purified proteasomes and that proteasomes as such are apparently not present. This apparent absence of proteasomes in cell extracts might have been caused by differences in electrophoretic conditions in lanes with purified proteasomes and in lanes with cell extracts where the protein load was much higher. However, purified proteasomes had the same migration when electrophoresed alone as when added as an internal standard to cell extracts (Figure 7a). Identical results were obtained with HeLa carcinoma cells, IMR90 fibroblasts, Daudi lymphoma cells and with erythrocytes (results not shown).

The protein in the uppermost bands (Figure 6, lanes 1–11) had the same electrophoretic mobility as the S and F forms of the 26 S proteinase (Hoffman et al., 1992; Ugai et al., 1993). As expected for the 26 S proteinase (Hershko and Ciechanover, 1992) the protein disassembled in cell extracts depleted of ATP (Figure 7b). Proteasomes generated by decay of the 26 S proteinase also had a lower electrophoretic mobility than purified proteasomes (Figure 7b, lane 3).

With antibodies to subunit 1 (zeta) a fast-migrating protein was stained, in addition to the two doublets of bands that reacted with all the antibodies (Figure 6, lane 4). The M_r of native proteins can be estimated by electrophoresis in polyacrylamide gradient gels provided that the proteins reach near stationarity (Slater, 1969). An immunoblot of such a gel with HeLa cell extract showed that the rapidly migrating protein had an apparent M_r of 37000 (results not shown). Determinations of M_r from electrophoresis in non-denaturing gradient gels are not precise, so this is probably not significantly different from the M_r of 28000 determined for the free subunit by SDS/PAGE (results not shown). The fast-migrating protein therefore seems to be a free proteasome subunit rather than a dimer or a complex of the subunit with other proteins.

DISCUSSION

2D PAGE of human placental proteasomes show 19 spots besides lmp2 and lmp7. In most tissues the lmp subunits are found only in a minor population of proteasomes, and deletion mutants are fully viable (Spies et al., 1990). We shall therefore concentrate on the major population of non-lmp proteasomes.

Analysis of the 19 non-lmp subunits with a panel of monoclonal antibodies showed that three of them are present in isoforms that differ in pI. For subunits 10 and 12 this conclusion was corroborated by peptide mapping and by the presence of only one isoform of each subunit in cell cultures.

Subunit 7 has been identified as subunit HC2 (Tamura et al., 1991) by partial sequencing (K. Hendil, P. Kristensen and W. Uerkvitz, unpublished work) and the two isoforms of subunit 7 may result from alternative mRNA splicing (Pereira et al., 1992). A similar explanation may also account for the presence of three isoforms of each of the subunits 10 and 12. However, a stepwise post-translational modification of these subunits may be more likely. The nature of the modification has not yet been determined. Deamidation or phosphorylation of the most basic isoforms, present in cell cultures, would produce derivatives with decreasing pI, like those found in the tissues. Phosphorylation of some subunits has indeed been demonstrated (Haass and Kloetzel, 1989; Pereira and Wilk, 1990; Arrigo and Mehlen, 1993; Ludemann et al., 1993).

The detection of isoforms of three of the subunits brings the number of different non-lmp subunits down to 14 (spots 1–14 in

Figure 1). The most alkaline of these subunits (12 and particularly 14) are sometimes lost during pH-gradient electrophoresis. This may account for the lower number of subunits detected by some investigators. The presence of 14 different core subunits fits well with the model built by analogy with the simpler archaebacterial proteasome that contains only two different subunits. It consists of four stacked rings, each made up of seven subunits (Pühler et al., 1992). The α subunits make up the ends of the cylinder, with the β subunits forming the two middle rings (Grziwa et al., 1991). In addition, electron microscopy of complexes between proteasomes and some of the antibodies (Kopp et al., 1993) has shown that the human proteasome is also a complex dimer. Most likely, the outer rings of human proteasomes consist of seven different α-type subunits and the inner rings of seven different β-type subunits, making up the observed total of 14 different subunits. Six of the presumably 7 α-type subunits are detected with antibody MCP231 in Figure 2(p).

Besides the fraction of proteasomes containing lmp2 and lmp7, subpopulations of non-lmp proteasomes have been claimed to be present in some cells (Kloetzel et al., 1987; Grossi de Sa et al., 1988; Falkenburg and Kloetzel, 1989; Haass and Kloetzel, 1989; Kloetzel et al., 1991; Ahn et al., 1991; Strack et al., 1992; Yang et al., 1992; Früh et al., 1992). We could only find 14 non-lmp subunits in a variety of cells and tissues, even when the proteasomes were precipitated with antibodies to four different subunits. Proteasome subpopulations are therefore likely to consist of the same 14 subunits. However, some of them are apparently post-translationally modified. These modifications may account for the reported heterogeneity of proteasomes. The specific positions of at least four different subunits in proteasomes, as revealed by immunoelectron microscopy (Kopp et al., 1993; F. Kopp, P. Kristensen, K. Hendil, A. Johnsen, A. Sobek and B. Dahlmann, unpublished work) also argue that the proteasome is a highly ordered structure.

The induction of lmp2 and lmp7 by interferon-γ gives rise to subsets of proteasomes (Früh et al., 1992; Yang et al., 1992; Brown et al., 1993; Patel et al., 1994) where subunits 2 (delta) and 14 (MB1) have been replaced by lmp2 and lmp7 respectively (Aki et al.,1994; Akiyama et al., 1994; Belich et al.,1994). The quaternary structure of the proteasome is therefore probably maintained.

Like others (Hoffman et al., 1992; Aki et al., 1993; Ugai et al., 1993), we found that cell extracts contain two electrophoretic variants of the 26 S proteinase and two variants of proteasomes. The 26 S proteinases may differ in the ratio of non-proteasome components bound to the proteasome (Sawada et al., 1993). The two forms of proteasomes in cell extracts both migrate more slowly than purified proteasomes, perhaps because the proteasomes gain higher electrophoretic mobility during purification, either by acquiring a higher net negative charge or by decreasing their hydrodynamic diameter. This interpretation cannot be ruled out, but seems unlikely, since our purification procedure is fast and the putative conversion is quantitative (Figure 7). More likely the proteasomes in cell extracts have formed complexes with one or more of the several known activators or inhibitors (Murakami and Etlinger, 1986; Li et al., 1991; Hoffman et al., 1992; Dubiel et al., 1992; Ma et al., 1992a,b, 1994; Driscoll et al., 1992; Li and Etlinger, 1992). Both the two 26 S proteinases and the proteasome variants contain all the 11 subunits that could be detected with the monoclonal antibodies.

The presence in cell extracts of free subunit 1 may mean that the subunit is loosely bound in proteasomes, so that it dissociates during preparation and analysis of the extracts. However, proteasomes are very stable and need high concentrations of denaturants to be dissociated. Also, the absence of streaking in the immunoblots (e.g. Figure 7) suggests that proteasomes are stable during electrophoresis. Finally, as judged from Coomassie Blue-stained gels, purified proteasomes seem to contain approximately the same amount of protein in subunit 1 as in the other subunits (Figure 1). It appears likely, then, that subunit 1 (zeta) is also found in a free state in intact cells.

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