Phosphorylation of ATPase subunits of the 26S proteasome

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Received 18 May 1998

Abstract The 26S proteasome complex plays a major role in the non-lysosomal degradation of intracellular proteins. Purified 26S proteasomes give a pattern of more than 40 spots on 2D-PAGE gels. The positions of subunits have been identified by mass spectrometry of tryptic peptides and by immunoblotting with subunit-specific antipeptide antibodies. Two-dimensional polyacrylamide gel electrophoresis of proteasomes immunoprecipitated from [^{32}P]phosphate-labelled human embryo lung L-132 cells revealed the presence of at least three major phosphorylated polypeptides among the regulatory subunits as well as the C8 and C9 components of the core 20S proteasome. Comparison with the positions of the regulatory polypeptides revealed a minor phosphorylated form to be S7 (MSS1). Antibodies against S4, S6 (TBP7) and S12 (MOV34) all cross-reacted at the position of major phosphorylated polypeptides suggesting that several of the ATPase subunits may be phosphorylated. The phosphorylation of S4 was confirmed by double immunoprecipitation experiments in which 26S proteasomes were immunoprecipitated as above and dissociated and then S4 was immunoprecipitated with subunit-specific antibodies. Antibodies against the non-ATPase subunit S10, which has been suggested by others to be phosphorylated, did not coincide with the position of a phosphorylated polypeptide. Some differences were observed in the 2D-PAGE pattern of proteasomes immunoprecipitated from cultured cells compared to purified rat liver 26S proteasomes suggesting possible differences in subunit compositions of 26S proteasomes.

Key words: Proteasome; Phosphorylation; Mass spectrometry; ATPase

1. Introduction

The 26S proteasome is a large (approximately 2 MDa), ATP-dependent proteolytic complex which catalyzes the majority of non-lysosomal protein degradation within the cell in either a ubiquitin-dependent or ubiquitin-independent manner (reviewed in [1,2]). The 26S proteasome is composed of a central catalytic core comprising the smaller (approximately 700 kDa) 20S proteasome to the ends of which are associated regulatory complexes. In eukaryotes, the catalytic core is composed of 14–17 different subunits which are divided into two groups, α and β, depending on their sequence similarity to the α and β subunits of the proteasome from *Thermoplasma acidophilum* [3]. At least five distinct peptidase activities have been found to be associated with this complex [4,5].

The regulatory complex (also called PA700) consists of approximately 21 subunits with masses ranging from 25–110 kDa [2]. These subunits may be divided into two groups, ATPases and non-ATPases. Six ATPase subunits have been shown to be associated with the human 26S proteasome and homologues for all of them have been cloned from yeast and shown to be essential gene products. Analysis of the entire yeast genome has shown that no more members of this ATPase family are present [2]. The ATPases are presumably required to provide energy for the unfolding of protein substrates of the complex. The ATPase subunit sequences show greatest similarity in a central 200 amino acid region which contains the putative ATP-binding site [6]. The ATPase subunits are part of a large protein family called the AAA proteins all of which contain a conserved domain containing an ATP-binding domain [7] but which have very diverse functions within the cell.

Thirteen non-ATPase subunits of the human 26S regulatory complex have recently been cloned and sequenced. Whilst the function of some of these subunits is unknown, studies with yeast homologues have indicated possible functions for some including S1 which is a homologue of yeast SEN3, a factor affecting t-RNA splicing [8], and S14 which is a homologue of NIN1, a yeast cell cycle gene whose product is required for G1/S and G2/M transitions [9]. S5a and its homologue the multi-ubiquitin-binding protein-1 from *Arabadopsis thaliana* have been demonstrated to specifically recognize poly-ubiquitin chains [10,11], one signal known to mark proteins for destruction by the 26S proteasome. The fact that several of the human non-ATPase subunits do not have homologues in the yeast genome suggests species-specific functions for some of them [2].

The 26S proteasome is found in both the cytoplasm and nucleus of the cell and is responsible for the degradation of many short-lived proteins which play important regulatory roles within the cell. Such proteins include the transcription factors c-jun [12], c-fos [13], NFκ-B [14] and its regulator Iκ-B [15], the tumor suppressor protein p53 [16], a number of cyclins [17,18]. Thus, the proteasome itself plays an important role in cellular homeostasis, proliferation and differentiation and this is reflected both in its ubiquitous presence throughout evolution and the fact that the majority of its subunits are essential gene products [2].

Here we report on the analysis of components of the 26S proteasome isolated from rat liver and from cultured cells, and the identification of phosphorylated regulatory subunits.

2. Materials and methods

2.1. Purification of rat liver proteasomes and preparation of subunit-specific antibodies

20S and 26S proteasomes were purified from rat liver as previously reported [19–21]. Monoclonal antibodies immunospecific for proteasome subunit C2 (MCP20) were from Dr. K.B. Hendil (August Krogh Institute, Copenhagen, Denmark) [22]. Monoclonal antibodies specific for proteasome regulatory complex subunit S3 were from Dr. J. Dys-

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PII: S0014-5793(98)00676-0

on (MRC Clinical Sciences Centre, London). Polyclonal antibodies immunospecific for proteasome subunits S4, S10 and S12 were prepared by immunizing New Zealand White rabbits with the subunit-specific peptides MGQSQSGGHGPGGGC, TNRPDSKNWQYQC and AVPFDEDDKDDSC, respectively. The peptide sequences used (human S4, residues 1–14; human S10, residues 356–367; mouse S12, residues 55–66) were chosen using the Peptidestructure command of the Mol GCG program (Genetics Computer Group, 1991). Rabbits were immunized with BCG vaccine one month prior to immunization with peptide conjugated to tuberculin purified protein derivative [23]. The primary peptide injection (30 μg) was mixed with Freund's complete adjuvant, and Freund's incomplete adjuvant was used for subsequent injections (30 μg each).

2.2. Electrophoresis

SDS-PAGE and two-dimensional PAGE, using 12.5% separating gels, electrophoretic blotting and autoradiography were carried out as described previously [24].

2.3. Mass spectrometric identification of subunits

Proteasome subunits separated by two-dimensional PAGE and blotted onto polyvinylidene difluoride membrane (PVDF) were stained with Ponceau S, excised, and subjected to tryptic digestion. They were identified by matrix-assisted laser-desorption ionization (MALDI) mass spectrometry and screening of peptide databases [25] using the MOWSE search program at SEQNET facility, Daresbury, UK. Esterification of the samples with thionyl chloride (Aldrich) was carried out to increase the search specificity and subunits were identified by obtaining peptides covering the full length of the polypeptide.

2.4. Cell culture

L-132 human embryonic lung cells [26] were routinely grown in Dulbecco's modified essential medium (DMEM) (GIBCO BRL) supplemented with 10% (v/v) new-born bovine serum and penicillin/streptomycin (50 iu/ml/50 μg/ml, respectively) in a humidified atmosphere of 5% (v/v) CO_2/air.

2.5. Metabolic labelling of cellular proteins

Cellular proteins were labelled with ^{32}Pi and [^{35}S]methionine (Amersham) as described [24] with the exception that for labelling with ^{32}Pi, cells were incubated in media containing radiolabel for 4 h, and calyculin A (0.1 μM) and okadaic acid (1 μM) (Calbiochem) were added for the last 30 min.

2.6. Immunoprecipitation of 26S proteasomes

In the case of [^{35}S]methionine-labelling, the medium was removed from the cells which were rinsed with 137 mM NaCl, 1.5 mM KH_2PO_4, 20 mM $Na_2HPO_4.2H_2O$, 2.7 mM KCl, pH 7.2 (PBS) before being released from the flask with trypsin/versene (1:10) (GIBCO BRL). Cells were resuspended in PBS and sedimented at 1000×*g* for 5 min. In the case of ^{32}Pi-labelling, the addition of calyculin A and okadaic acid caused the cells to detach from the flasks. Thus, cells were collected as a suspension in labelling medium, sedimented at 1000×*g* for 5 min, washed with PBS, and resedimented. Lysis buffer (20 mM Tris pH 7.5, 20% (v/v) glycerol, 5 mM ATP, 5 mM sodium pyrophosphate, 50 mM sodium fluoride, 0.1 mM sodium orthovanadate, 0.1 μM okadaic acid and 10 mM β-glycerophosphate, 0.2% (v/v) Nonidet P-40) was added to the cell pellets and the cells were lysed by passing them through a 25 gauge needle. The lysates were removed to Eppendorf tubes and centrifuged for 15 min to sediment any insoluble material and the resulting supernatants were immediately diluted 10-fold with lysis buffer without Nonidet P-40. Protein A agarose-MCP20 (100 μl of a 50% suspension in PBS) were added to cell lysates and the mixtures tumbled end-over-end for 16 h at 4°C. The agarose was then sedimented by centrifugation and the supernatants removed. The gel pellets were washed three times for 10 min each with lysis buffer containing 0.02% (v/v) Nonidet-P40.

2.7. Double immunoprecipitation of ATPase subunits

26S proteasomes from ^{32}Pi-labelled cells were immunoprecipitated as above. Following the final washes, 50 μl of 10% SDS were added (for 15 min) to the protein A agarose pellets to dissociate bound proteins. The agarose was sedimented and the supernatants removed to clean tubes and diluted to 5 ml with lysis buffer containing 0.02% (v/v) Nonidet-P40. Protein A agarose-coupled antibodies specific for individual ATPase subunits were added and immunoprecipitation carried out as above.

Table 1
Identification of rat 26S proteasome subunits in Fig. 1 by mass spectrometry and immunoblotting

Spot number (see Fig. 2)	Subunit name (and homologues)
1	S3, p58, p91A
2, **3**, 13	*S4, MTS2
4	*TBP1
5, 8	*S6, TBP7
6, **7**	*S7, MSS1, CIM5
9, 10, 11, 12	*S8, p45, SUG1, CIM3, TRIP1
14, 15	S12, MOV34, p40
16, 17	POH1, PAD1
18, 19	C2
20, 21	C9
22, 23, 24, 25, **26**	C8
27	C6
28	iota
29	zeta
30, 31, **32**	C3
33	delta
34	C7

[a]Antibodies were used to identify spots in the case of S3, S4, S10 and S12 and with the exception of S3 the spots were also identified by mass spectrometry. Where subunits gave more than one spot, the spot number indicated in bold was the major form.
*ATPase subunits.

3. Results

3.1. Identification of 26S proteasome subunits following two-dimensional PAGE

In order to identify the subunits of the 26S proteasome, the complex was first purified from rat liver and subjected to two-dimensional PAGE analysis. Fig. 1 shows the characteristic pattern of subunits obtained following Coomassie blue staining of the gels. The subunits of the core 20S proteasome lie between approximately 21 and 34 kDa, and the subunits of the regulatory complex range from 25 to 110 kDa [2]. 26S

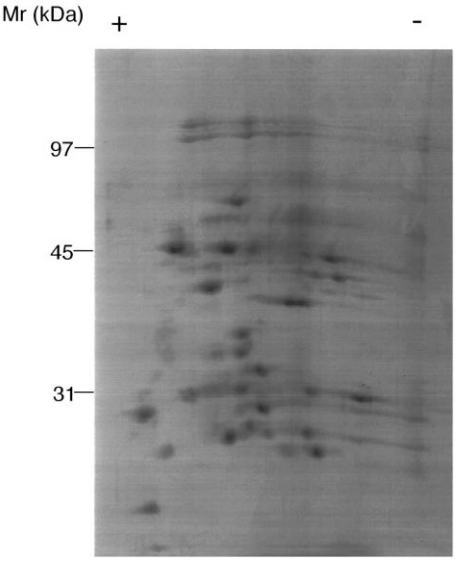

Fig. 1. Two-dimensional PAGE gel of 26S proteasomes purified from rat liver stained with Coomassie blue.

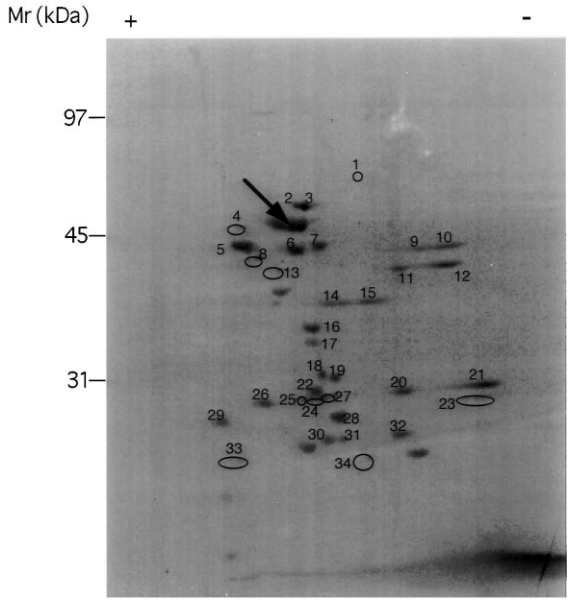

Fig. 2. Identification of 26S proteasome subunits by mass spectrometry following separation by two-dimensional PAGE. 26S proteasomes purified from rat liver were separated by two-dimensional PAGE and transferred to PVDF. Spots visualized with Ponceau S were excised, digested with trypsin and identified by mass spectrometry as described in Section 2. Only those spots for which a positive identification could be made are numbered. See Table 1 for subunit assignments. The arrow highlights a contaminant (the dihydrolipoamide-succinyltransferase component of the pyruvate dehydrogenase multi-enzyme complex) found in early preparations.

proteasomes contain all the core proteasome subunits including the non-essential MHC-encoded subunits, LMP2 and LMP7 (Murray, R.Z. and Rivett, A.J., unpublished observations). The position of the majority of the subunits on two-dimensional PAGE was determined by mass spectrometry of tryptic peptides and immunoblot analysis. More than 30 spots were assigned to subunits (Fig. 2 and Table 1). Many of the spots were found to be related to one another suggesting that post-translational modification of subunits frequently occurs. The positions of S3, S4, S10 and S12 were confirmed by immunoblotting with subunit-specific antipeptide antibodies (data not shown). The large spot indicated by the large arrow in Fig. 2 is a contaminant that was present in some of our earlier proteasome preparations and has been identified by mass spectrometry as the dihydrolipoamide-succinyltransferase component of pyruvate dehydrogenase. This contaminant has now been removed from our preparations (Fig. 1).

3.2. Specificity of immunoprecipitation of 26S proteasomes

In order to check the specificity of the immunoprecipitation conditions, MCP20 immunoprecipitations from [^{35}S]methionine-labelled human embryonic lung L-132 cells were subjected to two-dimensional PAGE. A comparison of the pattern of radiolabelled material (Fig. 3) with that of proteasomes purified from rat liver (Fig. 1) shows that the monoclonal antibody used specifically immunoprecipitates 26S proteasomes from L-132 cell lysates under the conditions used, and also that a similar pattern of subunits is present in human and rat proteasomes from the different cell types. There are, however, some differences with respect to a number of individual subunits as highlighted by the arrows in Fig. 3. The open arrows indicate the weak labelling by [^{35}S]methionine in L-132 cells of spots clearly visible by Coomassie blue staining from rat liver (see Fig. 1). The closed arrows indicate some examples where darker spots appear to be present in [^{35}S]methionine-labelled immunoprecipitates. Assuming that there are no gross differences in the numbers of methionines in these proteins these results suggest some differences in the ratio of individual subunits present in 26S proteasomes from different cell types.

3.3. Phosphorylation of 26S proteasome subunits

L-132 cells were grown in the presence of ^{32}Pi to determine whether any of the 26S proteasome regulatory subunits are phosphorylated. The phosphatase inhibitors okadaic acid and calyculin A were added to the cell media for the final 30 min of incubation with ^{32}Pi and the cells then lysed in a non-denaturing buffer containing phosphatase inhibitors. The lysates were subjected to immunoprecipitation and two-dimensional PAGE as above, the separated polypeptides were transferred to nitrocellulose and then autoradiographed (Fig. 4). The autoradiographs revealed the presence of three consistently labelled major spots (labelled I, II and III in Fig. 4) amongst the regulatory subunits with molecular masses of 45–50 kDa. In addition, a number of minor phosphorylated spots were observed (such as IV in Fig. 4). However, these spots, because of their relatively low level of labelling, were not seen in every experiment. Two subunits of the core complex, C8 and C9, were also seen to be labelled as previously reported [24]. Experiments were also carried out in which calyculin A and okadaic acid were not added to the culture medium during labelling. In this case, reduced phosphorylation of spots

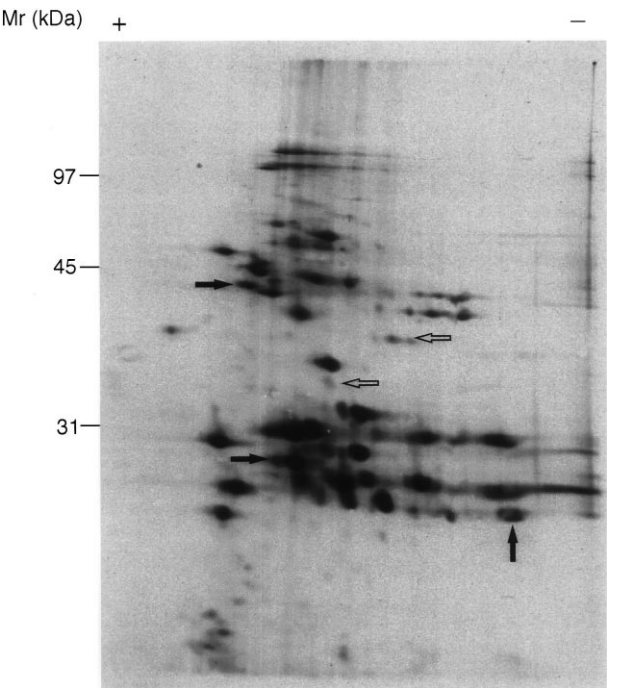

Fig. 3. Two-dimensional PAGE gel of 26S proteasomes immunoprecipitated from L-132 cells. Proteasomes immunoprecipitated from [^{35}S]methionine-labelled human L-132 cells separated by two-dimensional PAGE, transferred to nitrocellulose and autoradiographed. A comparison with the Coomassie blue stained pattern of rat liver proteasome subunits (Fig. 1) shows that some subunits are less well represented in the immunoprecipitates (open arrows) whilst others are more marked (closed arrows).

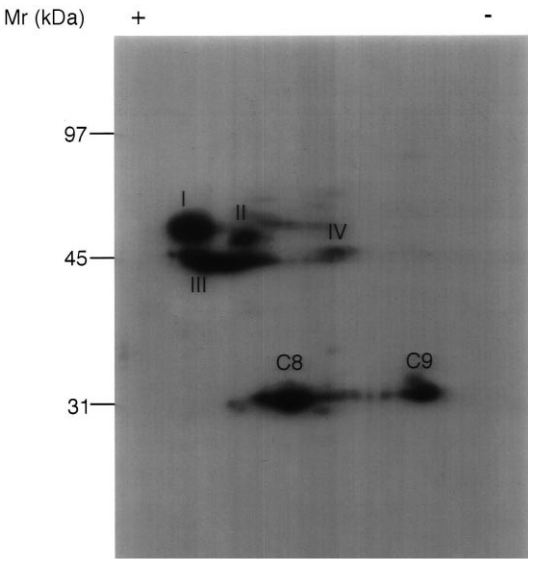

Fig. 4. Phosphorylation of 26S proteasomes. Proteasomes immunoprecipitated from ^{32}Pi-labelled human L-132 cells separated by two-dimensional PAGE, transferred to nitrocellulose and autoradiographed. Potential phosphorylated regulatory subunits are labelled I–IV.

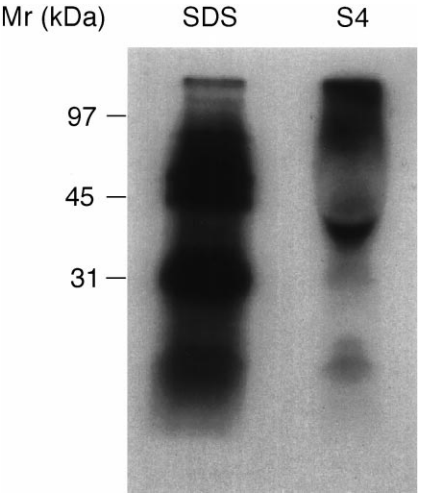

Fig. 5. Phosphorylation of S4. Proteasomes immunoprecipitated from ^{32}Pi-labelled human L-132 cells using MCP20 were dissociated in 10% SDS (lane 1) and immunoprecipitated with anti-S4 subunit-specific antibodies (lane 2) before separation by SDS-PAGE. Separated polypeptides were transferred to nitrocellulose and autoradiographed.

I–IV was observed. There was little change in the phosphorylation of C8 under these conditions and a decrease in the phosphorylation of C9 (data not shown).

3.4. *Identification of phosphorylated regulatory subunits by immunoblotting*

The major phosphorylated subunits did not coincide exactly with stained polypeptides which we had identified above (Fig. 2). However, one minor phosphorylated protein, IV in Fig. 4, coincided with a stained spot. From its relative position on the blot we identified this subunit as S7 (MSS1), an ATPase subunit. In order to identify the other phosphorylated spots, which had probably moved position due to their phosphorylation, we probed the nitrocellulose membranes with antibodies we had raised against peptides specific to individual regulatory subunits S4, S6 (TBP7), S10 and S12 (MOV34).

Immunoblotting with these antibodies resulted in, with the exception of S10, all of them reacting with material in approximately the area around the phosphorylated spot labelled II in Fig. 4, in addition to areas coinciding with their positions as determined by mass spectrometry. The antibody against the S10 subunit failed to detect any material coinciding with phosphorylated spots, suggesting that S10 which has previously been suggested to be phosphorylated [27] is not phosphorylated under the conditions used at least in the L-132 cells tested.

3.5. *Identification of phosphorylated regulatory subunits by double immunoprecipitation*

In order to verify whether the S4, S6 (TBP7) and S12 were phosphorylated, 26S proteasomes were immunoprecipitated from ^{32}Pi-labelled cell lysates with MCP20. The 26S proteasomes were then dissociated with 10% SDS, the SDS diluted out and individual subunits immunoprecipitated from the mixture using the subunit-specific antibodies. These second immunoprecipitates were then analyzed by SDS-PAGE, blotted onto nitrocellulose and autoradiographed. A number of phosphorylated bands were present in an aliquot removed from the SDS-treated sample (Fig. 5, lane 1). These represent all the phosphorylated subunits present in the complex. The second immunoprecipitation with anti-S4 antibodies resulted in a single phosphorylated band of the slightly lower molecular mass than expected (Fig. 5, lane 2). This slightly anomalous migration is believed to be due to the proximity of excess immunoglobulin heavy chains of the polyclonal antibodies which migrate just above S4, resulting in the rounded shape of the band. Second immunoprecipitations with the antibodies against S6 (TBP7) and S12 (MOV34) were not successful. These antibodies either failed to immunoprecipitate any material under conditions where the 26S complex dissociated, or when less stringent conditions were used, immunoprecipitated the whole 26S complex (data not shown). Similar results were obtained when 9 M urea was used to dissociate the complex. Although the latter observations do not independently confirm the identity of the phosphorylated subunits, they do provide evidence that phosphorylated proteins are not simply substrates of 26S proteasomes and/or artifactually bound cellular proteins.

4. Discussion

We have analyzed the subunits of 26S proteasomes following their separation by two-dimensional PAGE. The 30 spots identified were found to be associated with only 15 distinct subunits suggesting that post-translational modifications of subunits is not a rare event. Indeed, as many as four forms of some subunits were observed. These results are in accordance with previous studies which have shown a greater number of spots arising from two-dimensional PAGE separation of the core 20S complex than is accounted for by the 17 possible constituent subunits [19]. Whilst some of these modifications are due to phosphorylation (see below), not all are. What the other modifications are remains to be determined.

The monoclonal antibody MCP20 immunoprecipitates both

20S and 26S proteasomes. Following phosphorylation of proteasome complexes in the presence of phosphatase inhibitors calyculin A and okadaic acid, strong phosphorylation of what appeared to be three subunits of the 26S proteasome regulatory complex was observed as well as the relatively low phosphorylation of some other subunits in the regulatory complex. Phosphorylation of 20S proteasome subunits C8 and C9 was observed as reported previously [24,28]. The absence of these phosphatase inhibitors in the labelling media caused an increase in the labelling of C9 whilst not effecting that of C8 and decreasing that of the regulatory subunits. These data support the view that the phosphorylations of C8 and C9 are carried out by different pathways and that casein kinase II which has been demonstrated to phosphorylate C8 at two serines close to the C-terminus [28] is not responsible for the phosphorylation of C9. Indeed, we have postulated earlier that C9 might be phosphorylated by a cGMP-dependent kinase [24].

In addition to our preliminary report [29], two other recent studies have reported the observation of phosphorylated regulatory subunits of 26S proteasomes [27,30]. However, different subunits are reported in each case and both of these studies have identified phosphorylated subunits on the basis of molecular mass or sequencing of bands cut from SDS-PAGE gels. These methods do not allow for any increase in apparent mass often seen in cases of multiple phosphorylation and for the fact that minor amounts of a phosphorylated subunit may migrate to the same position as a larger non-phosphorylated subunit. The results presented here show the position of phosphorylated subunits on 2D-PAGE gels.

From its relative position on blots we were able to identify the minor phosphorylated spot IV as S7 (MSS1), an ATPase subunit. The highly phosphorylated spots I–III could not be unequivocally identified. However, our data demonstrate that subunit S4 is definitely phosphorylated and also suggest that S6 (TBP7) and S12 (MOV34) are phosphorylated and are responsible, at least in part, for spots II and III. The role of these phosphorylations is unknown but they might be expected to regulate the activity of the ATPase subunits and therefore influence protein degradation. The ATPase subunits have been suggested to be responsible for the unfolding of protein substrates [31], prior to their entering the central cavity of the complex where the catalytic sites lie.

Antibodies against the non-ATPase subunit S10 failed to cross-react with any phosphorylated polypeptides indicating that S10, under these conditions, is not phosphorylated. This is in contrast to the suggestions of Yang et al. [27] who reported the phosphorylation of two regulatory subunits of 26S proteasomes from murine lymphoma RMA cells and identified them as S6 and S10 based only on their relative mobilities on SDS-PAGE gels. However, our findings suggest that this method of identification is inadequate since the phosphorylated subunits have an altered electrophoretic mobility. It was also reported [27] that the addition of calyculin A and okadaic acid to the culture media during phosphorylation increased the phosphorylation of C9 and decreased that of the regulatory subunits in direct contrast to the results presented here. A possible explanation for this discrepancy is that there are differences in the levels of the kinases and phosphatases in the differing cell types as we have previously observed with respect to C8 and C9 phosphorylation in L-132 and Rat-1 fibroblast cells [24]. It is also possible that the phosphatase inhibitors have varying effects depending on the proliferative state of the cell.

The phosphorylation of the ATPase subunit S8 (TRIP1) has been reported previously based on separation by SDS-PAGE and sequencing of the phosphorylated band [30]. We observed no phosphorylation in the position expected for S8 but cannot rule out that any phosphorylated form would not move to a more acidic position where the phosphorylated spots are. Certainly, the phosphorylation of S8 would be consistent with the possibility that all the ATPase subunits can be phosphorylated.

A comparison of the two-dimensional PAGE pattern of 26S proteasome subunits purified from rat liver (Fig. 1) and immunoprecipitated from [^{35}S]methionine-labelled human L-132 cells (Fig. 3) reveals that whilst the subunit patterns are quite similar, there are some differences in the relative amounts of some subunits. Taking into account the fact that many subunits give rise to more than one spot, the number of spots representing regulatory subunits (Figs. 1–3) does not appear to account for the 20 or more regulatory subunits already cloned [2,32]. This suggests that there may be heterogeneity in the subunit composition of 26S complexes in different cell types or under varying physiological conditions [33]. Also some of the subunits of the 26S proteasome appear to be present in complexes distinct from the proteasome and may even have unrelated functions. For example S8 (TRIP1) has recently been shown to be part of a complex other than the proteasome and to have DNA helicase activity dependent on its ability to bind ATP [34]. However, the presence of these subunits in the 26S proteasome may reflect an ability to recognize substrates of a particular type related to their other functions. This may explain why six different ATPases are present. The [^{35}S]methionine-labelling pattern in Fig. 3 shows the presence of a number of minor spots which might be minor forms of modified subunits or low amounts of coprecipitating proteins. It seems very likely that some of these spots may be substrate proteins. For example, c-fos has been shown to associate with the complex [35].

Acknowledgements: The authors wish to thank Dr. K.B. Hendil (August Krogh Institute, Copenhagen, Denmark) for the MCP20 hybridoma and Dr T. Harrison (University of Leicester) for the culturing of MCP20 hybridomas and harvesting of culture supernatants. We thank Dr. K.B. Hendil and Professor K. Tanaka for monoclonal antibodies to some of the ATPase subunits and Dr J. Dyson for p91A antibodies. This work was supported by the Cancer Research Campaign (G.G.F.M.), the Medical Research Council (R.Z.M.), and by the Lister Institute of Preventive Medicine and the Wellcome Trust (A.J.R.).

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