

## $\gamma$ -Interferon decreases the level of 26 S proteasomes and changes the pattern of phosphorylation

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In mammalian cells proteasomes can be activated by two different types of regulatory complexes which bind to the ends of the proteasome cylinder. Addition of two 19 S (PA700; ATPase) complexes forms the 26 S proteasome, which is responsible for ATP-dependent non-lysosomal degradation of intracellular proteins, whereas 11 S complexes (PA28; REG) have been implicated in antigen processing. The PA28 complex is upregulated in response to  $\gamma$ -interferon ( $\gamma$ -IFN) as are three non-essential subunits of the 20 S proteasome. In the present study we have investigated the effects of  $\gamma$ -IFN on the level of different proteasome complexes and on the phosphorylation of proteasome subunits. After treatment of cells with  $\gamma$ -IFN, the level of 26 S proteasomes decreased and there was a concomitant increase in PA28–proteasome complexes. However, no free 19 S

regulatory complexes were detected. The majority of the  $\gamma$ -IFN-inducible proteasome subunits LMP2 and LMP7 were present in PA28–proteasome complexes, but these subunits were also found in 26 S proteasomes. The level of phosphorylation of both 20 S and 26 S proteasome subunits was found to decrease after  $\gamma$ -IFN treatment of cells. The C8 alpha subunit showed more than a 50% decrease in phosphorylation, and the phosphorylation of C9 was only barely detectable after  $\gamma$ -IFN treatment. These results suggest that association of regulatory components to 20 S proteasomes is regulated, and that phosphorylation of proteasome alpha subunits may be one mode of regulation.

**Key words:** 19 S regulatory complex, antigen processing, ATPase complex, PA28, ubiquitin.

### INTRODUCTION

Proteasomes (20 S proteasomes) have a cylindrical structure composed of four heptameric rings (reviewed in [1]). Yeast proteasomes have 14 different subunits arranged as a stack of four rings with seven different alpha-type subunits in each of the outer rings and seven different beta-type subunits in each of the inner rings. The proteasome is a threonine protease, where the N-terminal threonine of beta subunits acts as the catalytic nucleophile and the catalytic sites are located inside the cylindrical structure. Only three of the yeast beta subunits have a catalytic N-terminal threonine residue. In animal cells there are an additional three catalytic beta subunits, LMP2, LMP7 and MECL1. These three non-essential subunits are inducible by  $\gamma$ -interferon ( $\gamma$ -IFN) and become incorporated into proteasomes in place of the constitutive catalytic beta subunits, delta, MB1 and Z, to form immunoproteasomes which have been implicated in antigen processing (reviewed in [2–4]). The results of several studies have demonstrated that incorporation of the  $\gamma$ -IFN-inducible subunits into proteasomes alters their catalytic activities [5–8], and that decreased levels of LMP2 and LMP7 lead to defects in antigen processing and presentation [9,10].

Two different types of proteasome regulatory complexes can bind to the ends of the 20 S proteasome cylinder (reviewed in [11]). An 11 S regulator (PA28; REG) which is also inducible by  $\gamma$ -IFN has been implicated in antigen processing [12] and recently in the assembly of immunoproteasomes [13]. The PA28 is composed of two types of subunits, alpha and beta, which form a 200 kDa complex [14]. The addition of a 19 S regulatory complex to each end of the 20 S proteasome results in the formation of the 26 S proteasome. Binding of the 19 S regulatory

complexes is essential for ATP-dependent proteolysis by proteasomes. These complexes consist of six ATPase subunits and approx. 15 other components, some, but not all, of which are encoded by essential genes in yeast. ATP is required for the formation of 26 S proteasomes and also for protein degradation, where it may play a role either in unfolding protein substrates or in translocating them into the central cavity where the catalytic sites are located. It is the 26 S form of proteasomes which is responsible for the degradation of the majority of short-lived cellular proteins, including ubiquitin-dependent proteolysis [15,16].

We and others have reported previously [17,18] that the C8 and C9 components in the core 20 S proteasome are phosphorylated *in vivo*. At least four different components of the 19 S regulatory complexes of 26 S proteasomes, including some, but not all, of the ATPase subunits, are also phosphorylated [19]. In the present study we have analysed the effect of  $\gamma$ -IFN on the relative levels of different proteasome complexes and on the phosphorylation of proteasome subunits. The results show a substantial decrease in the level of 26 S proteasomes after treatment with  $\gamma$ -IFN and an increase in PA28–proteasome complexes.  $\gamma$ -IFN also changes the level of phosphorylation of proteasome subunits.

### MATERIALS AND METHODS

#### Cell culture

Human embryonic lung L-132 cells were grown in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with either

Abbreviation used:  $\gamma$ -IFN,  $\gamma$ -interferon.

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10% (v/v) newborn bovine serum and penicillin/streptomycin (50 units/ml per 50 µg/ml) in a humidified atmosphere of air/CO<sub>2</sub> (19:1). Cells were grown in the absence and presence of 250 units/ml human γ-IFN for 2 days.

### Antibodies and immunoblotting

A mouse monoclonal antibody, MCP72 [20], which recognizes the C8 alpha-type proteasome subunit, was used to investigate the total proteasome population. Mouse monoclonal antibody MCP20, which recognises the C2 alpha-type subunit, was used for immunoprecipitation of proteasomes [19,20]. Mouse monoclonal p45-110, which recognises the p45 ATPase subunit of the 19 S regulatory complex, was generously given by Dr K. B. Hendil (University of Copenhagen, Denmark) and Dr K. Tanaka (The Tokyo Metropolitan Institute of Medical Science, Japan). MCP72 was kindly provided by Dr K. B. Hendil and rabbit polyclonal antiserum against PA28 (REGβ) was generously given by Dr M. Rechsteiner (University of Utah, UT, U.S.A.). The antibodies, Lmp2-13 and Lmp7-1 [21], were used for detection of the γ-IFN-inducible proteasome subunits LMP2 and LMP7 respectively. Immunoblotting was carried out as described [21] using chemiluminescence reagent plus (NEN Life Science Products).

### Preparation of cell extract and separation of proteasome complexes by gel filtration

Cells were lysed in 20 mM Tris/HCl buffer, pH 7.5, containing 20% glycerol, 5 mM ATP and 0.2% Nonidet P40 (26 S buffer conditions) or 20 mM Tris/HCl buffer, pH 7.5, containing 20% glycerol and 0.2% Nonidet P40 (PA28 buffer conditions). Extracts were centrifuged at 11 500 g for 10 min at 4 °C. Gel filtration was carried out using a Pharmacia Superose 6 FPLC column equilibrated in 20 mM Tris/HCl buffer, pH 7.5, containing 10% glycerol, 5 mM ATP and 100 mM NaCl (26 S buffer conditions) or 20 mM Tris/HCl buffer, pH 7.5, containing 10% glycerol (PA28 buffer conditions). For each fractionation, 0.5 ml fractions were collected and samples analysed by SDS/PAGE and immunoblot analysis. The peak fractions containing 26 S and 20 S proteasomes were determined by activity assays (see below) and also from the elution profile of purified rat liver 20 S and 26 S proteasomes [22,23].

### Proteasome activity assays

Chymotrypsin-like activity of 20 S and 26 S proteasomes was assayed in fractionated cell extracts, as described previously [22], using 40 µM succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (Peptide Research Institute, Osaka, Japan) as substrate. All assays were performed using a Perkin-Elmer LS-3B Fluorescence Spectrometer (excitation 370 nm, emission 430 nm). 20 S proteasome activity was measured in the presence of 0.02% SDS which activates 20 S proteasomes and partially inhibits 26 S proteasomes. Assays of 26 S proteasome and PA28-proteasome complexes were carried out in the presence of 5 mM ATP, which stimulates 26 S proteasome activity but which has no effect on the activity of the PA28-proteasome complexes. The presence of unbound PA28 in fractionated cell extracts was assayed by comparing the activity in the presence and absence of purified rat liver 20 S proteasomes.

### Metabolic labelling of cellular proteins

Cells were grown in the presence or absence of 250 units/ml IFN for 2 days. For *in vivo* phosphorylation experiments, cells were

then labelled with [<sup>32</sup>P]P<sub>i</sub> for 4 h, as described [19], with the exception that phosphatase inhibitors, calyculin A (0.1 µM) and okadaic acid (1 µM) (Calbiochem), were added for the last 30 min. Phosphorylation experiments were also carried out with [<sup>32</sup>P]P<sub>i</sub> added at the same time as the γ-IFN. Labelling with <sup>35</sup>S was carried out as described [19]. For labelling in the presence of γ-IFN, cells were grown in the presence of γ-IFN for 36 h and then labelled with [<sup>35</sup>S]methionine in the presence of γ-IFN for a further 16 h.

### Immunoprecipitation of proteasomes

In the case of [<sup>35</sup>S]methionine-labelling, the medium was removed from the cells, which were rinsed with PBS before being released from the flask with trypsin/versene (1.25:10; Gibco BRL). Cells were resuspended in PBS and centrifuged at 1000 g for 5 min. In the case of <sup>32</sup>P-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, centrifuged at 1000 g for 5 min, washed with PBS, and re-sedimented. 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 P40] 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 at 11 500 g to sediment any insoluble material, and the resulting supernatants were immediately diluted 10-fold with lysis buffer without Nonidet P40. Protein-A-agarose-MCP20 (100 µl of a 50% suspension in PBS) was 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 prior to analysis by two-dimensional PAGE [19], with purified rat liver 26 S proteasomes [23] added to aid recovery of labelled polypeptides.

## RESULTS

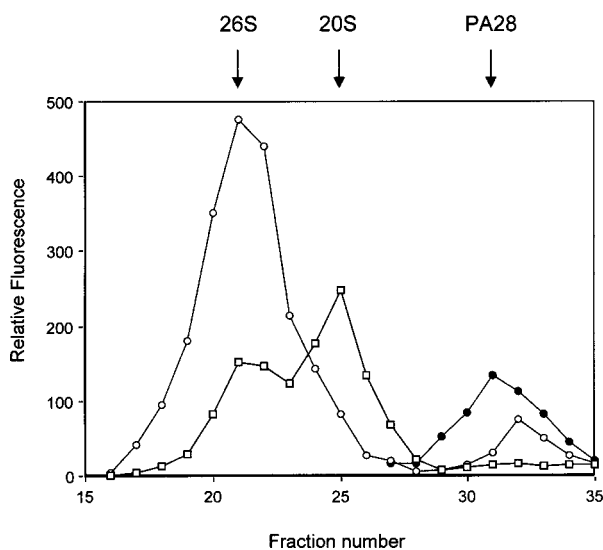
### IFN causes a decrease in 26 S proteasome levels

Immunoblot analysis of extracts prepared from L132 cells (Table 1) showed that treatment with γ-IFN caused a decrease (approx. 20%) in 26 S proteasome levels, while having little effect on total 20 S proteasomes. This effect was confirmed by carrying out similar experiments with COS-7 cells where the results were similar (Table 1). Proteasome complexes from L132 cells were also analysed by chromatography of cell lysates on a Superose 6 column. Peak activities of 26 S and 20 S proteasomes eluted in fractions 21 and 25 respectively, as indicated by the arrows in Figure 1. The low peak of protease activity in fraction 32 was unrelated to proteasomes. Immunoblots carried out with anti-C8 antibody to measure total proteasomes showed a roughly even distribution between 26 S and 20 S proteasomes in untreated L132 cells (Figure 2). After γ-IFN treatment there was a marked shift in distribution between the 20 S and 26 S proteasome fractions. Following γ-IFN treatment the proportion of C8 in the 26 S proteasome fractions was significantly lower than that in 20 S proteasome-containing fractions. Immunoblots with the anti-p45 antibody confirmed the decrease in 26 S proteasome levels following γ-IFN treatment and also showed that the decrease in 26 S proteasome was not accompanied by an increase in free 19 S complexes. An increase in 20 S proteasome activity was also observed after γ-IFN treatment (results not shown). The decrease in 26 S proteasome levels caused by γ-IFN was not

**Table 1** Quantification of the levels of 26 S and total proteasomes in  $\gamma$ -interferon-treated and untreated cells

Proteasome levels in both L132 and COS-7 cells were calculated by immunoblot analysis using anti-p45 antibodies for 26 S proteasomes and anti-C8 antibodies for total 20 S proteasomes, with purified rat liver proteasomes as standards. Treatment with  $\gamma$ -IFN was for 2 days. The mean of six (L132) and five (COS-7) separate experiments ( $\pm$ S.D.) is shown. The values obtained for the level of 26 S proteasome in treated cells were significantly different from that of untreated cells at  $P < 0.05$  (\*), while that of total 20 S proteasomes in treated and untreated cells was not significantly different. For analysis of the phosphorylation of C8 in L132 cells, the ratio of the intensity of C8 labelling for  $\gamma$ -IFN-treated cells to untreated cells was determined from the same autoradiograph in each of seven separate experiments; results are expressed as means  $\pm$  S.D. The level of phosphorylation of C8 in treated cells is significantly different from that in untreated cells at  $P < 0.0005$  (\*\*). Statistical analyses were carried out using the Student's *t* test.

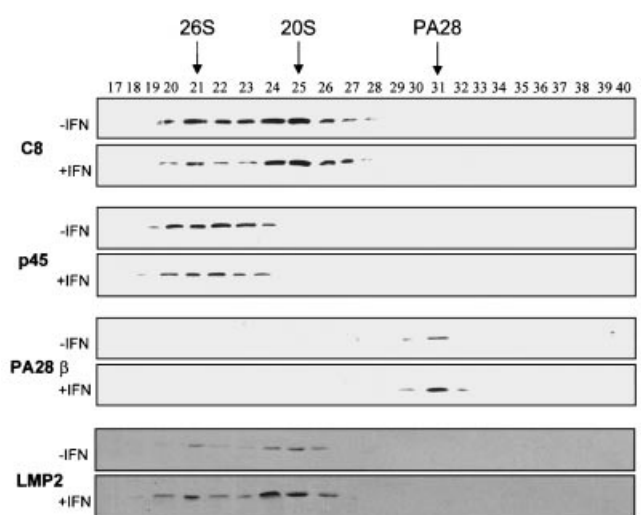
	Control	$\gamma$ -IFN treated	Ratio $\gamma$ -IFN treated/control
L132 cells			
26 S proteasome ( $\mu$ g/mg cell lysate)	9.4 $\pm$ 1.02	7.6 $\pm$ 1.26	0.81*
Total 20 S proteasome ( $\mu$ g/mg cell lysate)	26.34 $\pm$ 2.33	29.06 $\pm$ 9.39	1.10
Phosphorylation of C8	—	—	0.23 $\pm$ 0.091**
COS-7 cells			
26 S proteasome ( $\mu$ g/mg cell lysate)	7.40 $\pm$ 0.88	5.90 $\pm$ 1.20	0.80*
Total 20 S proteasome ( $\mu$ g/mg cell lysate)	19.08 $\pm$ 7.45	16.83 $\pm$ 4.78	0.90

**Figure 1** Separation of 26 S and 20 S proteasomes

Cell extracts were chromatographed on a Superose 6 column equilibrated in buffer containing 0.1 M NaCl as described in the Materials and methods section. Assays for 26 S proteasomes (○) contained 5 mM ATP, and assays for 20 S proteasomes (□) contained 0.02% SDS. PA28 (●) was assayed by addition of purified rat liver 20 S proteasomes [21] as described in the Materials and methods section. The elution position of 26 S proteasomes, 20 S proteasomes and PA28 complexes are indicated by the arrows.

easily detectable by activity measurements, probably due to changes in activity with the incorporation of  $\gamma$ -IFN-inducible subunits [5–8].

Fractions from the chromatographed lysates were immunoblotted with anti-LMP2 antibody (Figure 2) and anti-LMP7 antibody (results not shown) to investigate whether these  $\gamma$ -IFN-inducible subunits are incorporated into different proteasome complexes. Although the majority of LMP2 subunits were present in 20 S proteasomes (peak fraction 25) some of these subunits were also found in 26 S proteasomes (peak fraction 21), especially after treatment with  $\gamma$ -IFN when their total levels were increased (Figure 2). Similar observations were made with the anti-LMP7 antibody. These results confirm that all the newly synthesized LMP2 and LMP7 subunits induced by  $\gamma$ -IFN treatment are rapidly incorporated into mature proteasomes in L132 cells. Low levels of precursor complexes containing unprocessed LMP2 and

**Figure 2** Immunoblot analysis of 26 S and 20 S proteasomes

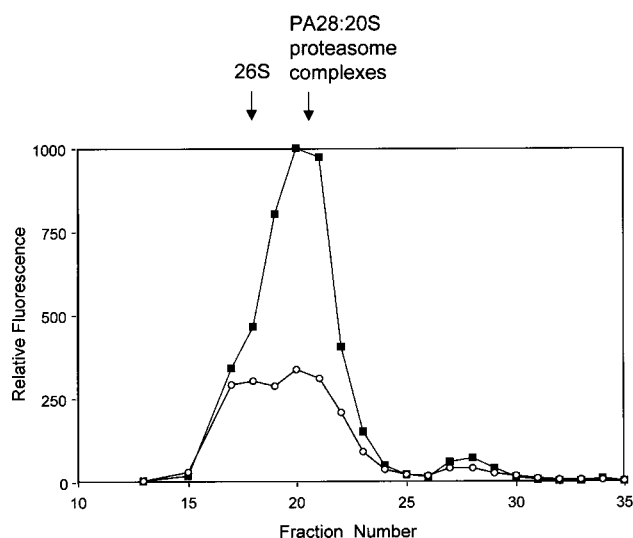
Fractions from Figure 1 were subjected to SDS/PAGE and immunoblotted with antibodies as indicated.

LMP7 could be detected after shorter (24 h) of  $\gamma$ -IFN treatment (results not shown).

To investigate whether  $\gamma$ -IFN-induced decreases in 26 S proteasomes occurred in other cell types, similar experiments were performed using COS-7 cells. After  $\gamma$ -IFN treatment the increase in levels of LMP2 and LMP7 were similar to those observed in L132 cells, and there was a dramatic decrease in the level of 26 S proteasomes (results not shown). Similarly, no free 19 S regulatory complexes were detected.

#### Analysis of PA28 and PA28–proteasome complexes

The levels of the PA28 regulator and its association with 20 S proteasomes were also investigated. Fractions were immunoblotted with anti-PA28 $\beta$  antibody (Figure 2). PA28 complexes elute in fractions 30–32 (peak fraction 31) and this was confirmed using antibodies to PA28 $\alpha$ . Thus under the high salt conditions employed for the gel filtration, PA28 eluted as the free regulator complex, the levels of which increase after  $\gamma$ -IFN treatment. To confirm this, fractions containing free PA28 were preincubated



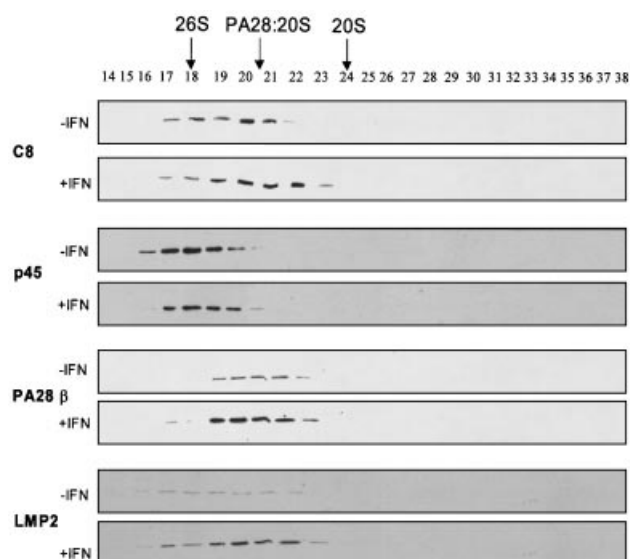
**Figure 3** Gel filtration of PA28–proteasome complexes

Cell extracts prepared before and after  $\gamma$ -IFN treatment were chromatographed on a Superose 6 column equilibrated in the low salt buffer. Assays of 26 S proteasomes and PA28–proteasome complexes from extracts before (○) and after (■)  $\gamma$ -IFN treatment contained ATP, and were carried out as described in the Materials and methods section. The elution position of 26 S proteasomes and PA28–20 S proteasome complexes are indicated by the arrows.

with exogenous 20 S proteasome, and these samples were assayed as described. The results showed activation of 20 S proteasomes by PA28-containing fractions and an approx. 3-fold increase in PA28 levels after  $\gamma$ -IFN treatment, consistent with data from immunoblots (Figure 2).

The data presented in Figure 2 raise the question of whether the decrease in 26 S proteasome levels, and the shift to 20 S proteasomes observed after  $\gamma$ -IFN treatment, may be due to an increase in PA28–proteasome complex formation. In order to investigate this possibility, gel filtration of extracts was carried out under low salt conditions which favour PA28–20 S complexes. Purified rat liver 20 S proteasomes eluted with a peak in fraction 24. Equivalent fractions from the gel filtration of cell extracts could not be activated by SDS, suggesting that there is very little, if any, free 20 S proteasome under these conditions (results not shown). Activities measured in the presence of ATP (Figure 3) showed the presence of two peak fractions, 18 and 20. Fraction 18 represents the peak 26 S proteasome activity under the conditions of this column run. The peak activity measured in fraction 20 was due to PA28–proteasome complexes. Addition of exogenous 20 S proteasomes did not result in activation by later fractions, indicating that no free PA28 was present.

After  $\gamma$ -IFN treatment of cells, there was an increase in the levels of PA28–proteasome complexes which account for the majority of the activity assayed with succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (Figure 3). In the absence of  $\gamma$ -IFN, total proteasomes were present in fractions 17–22, with peak fractions at 18 and 20 (Figure 4), consistent with the activity data in Figure 3. Immunoblotting with anti PA28 $\beta$  antibody (Figure 4) revealed that PA28 complexes were present in fractions 19–22, with the peak at fraction 20. These fractions coincide with the anti-C8 immunoblot, implying that the PA28 complexes are associated with 20 S proteasomes. Neither free PA28 (fractions 29–32) nor any free 20 S proteasomes (fractions 23–25) were observed. Immunoblotting with anti-LMP2 antibody (Figure 4) showed that the majority of immunoproteasomes were present in



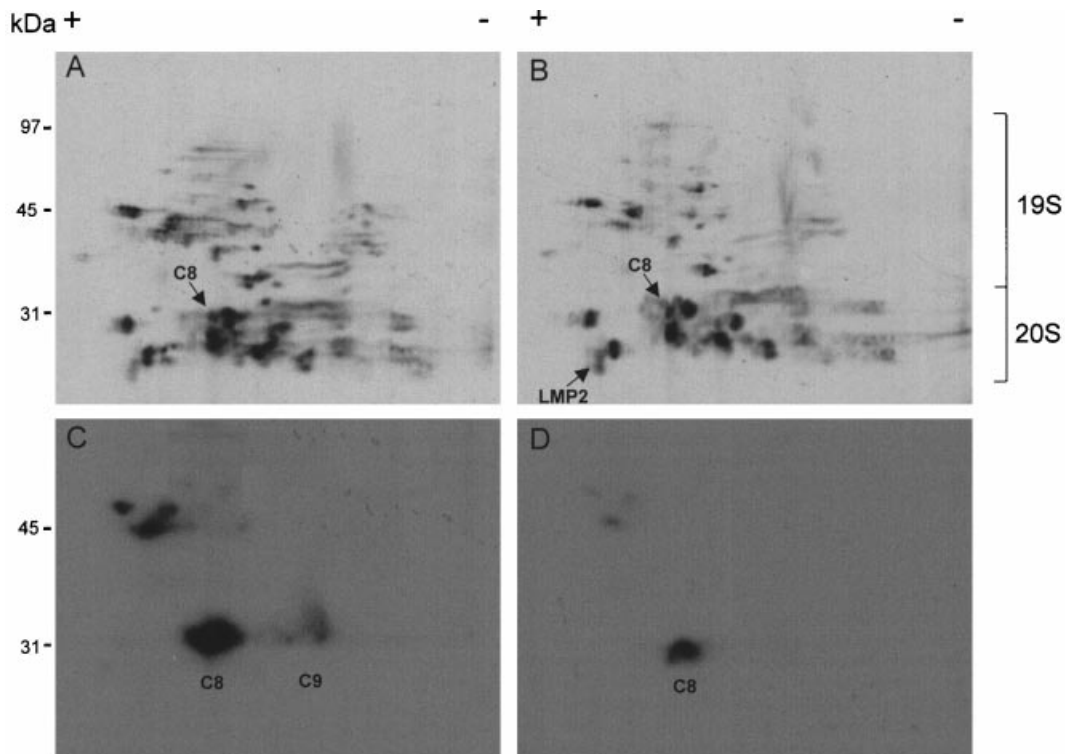
**Figure 4** Immunoblot analysis of PA28–proteasome complexes

Fractions from Figure 3 were subjected to SDS/PAGE and immunoblotted with antibodies as indicated.

PA28–20 S complexes, although some are present in 26 S proteasomes. Similar results were obtained with the anti-LMP2 antibody.

#### $\gamma$ -IFN decreases the phosphorylation of proteasome subunits

Previously, we have shown that two alpha subunits of the core 20 S proteasome and several subunits of the 26 S proteasome are phosphorylated *in vivo* [19]. In the present study, we examined the effect of  $\gamma$ -IFN on the phosphorylation of proteasome subunits to determine whether the changes in the different proteasome complexes are accompanied by changes in phosphorylation. Cells were treated with  $\gamma$ -IFN and labelled with [ $^{32}$ P]P<sub>i</sub> and  $^{35}$ S as described. Proteasomes were immunoprecipitated from cell lysates and analysed by two-dimensional PAGE followed by autoradiography. Figures 5(A) and 5(B) represent the pattern of protein subunits for 26 S proteasomes. Figure 5(C) shows the subunits that are phosphorylated in 20 S and 26 S proteasomes. In addition to the two alpha subunits of the core 20 S proteasome, C8 and C9, several subunits of the 19 S regulatory complex were phosphorylated in the absence of  $\gamma$ -IFN, in accordance with our published data [19]. After  $\gamma$ -IFN treatment, the level of 26 S proteasomes, as judged by  $^{35}$ S labelling intensity, was decreased by 15–20% (compare Figures 5A and 5B), in agreement with the data in Table 1. Interestingly, the C8 subunit remained phosphorylated after  $\gamma$ -IFN treatment (Figure 5D). The decrease in C8 phosphorylation observed after  $\gamma$ -IFN treatment was greater than the decrease in 26 S proteasome levels (Table 1). Phosphorylation of the C9 subunit was obvious in the absence of  $\gamma$ -IFN, but was barely detectable when cells were treated with  $\gamma$ -IFN for 2 days (compare Figures 5C and 5D). These changes occurred within the first few hours of  $\gamma$ -IFN treatment since there was no difference in the phosphorylation pattern obtained when the labelled phosphate was added at the same time as the  $\gamma$ -IFN (results not shown).



**Figure 5** Effect of  $\gamma$ -IFN on phosphorylation of proteasomes

Proteasomes immunoprecipitated from [ $^{35}\text{S}$ ]methionine-labelled cells were separated by two-dimensional PAGE, transferred to nitrocellulose and autoradiographed. (A) Cells untreated with  $\gamma$ -IFN, (B) cells after  $\gamma$ -IFN treatment. The ratio of total 19 S to total 20 S subunits decreased by 18% following  $\gamma$ -IFN treatment. The position of the phosphorylated C8 is shown by the arrow. (C) and (D) show the phosphorylation pattern obtained with proteasomes immunoprecipitated from [ $^{32}\text{P}$ ]P $_i$ -labelled cells which were untreated or treated with  $\gamma$ -IFN respectively. In this experiment [ $^{32}\text{P}$ ]P $_i$  was added 2 days after the start of  $\gamma$ -interferon treatment, although a similar decrease in phosphorylation was observed when  $\gamma$ -interferon and [ $^{32}\text{P}$ ]P $_i$  were added at the same time.

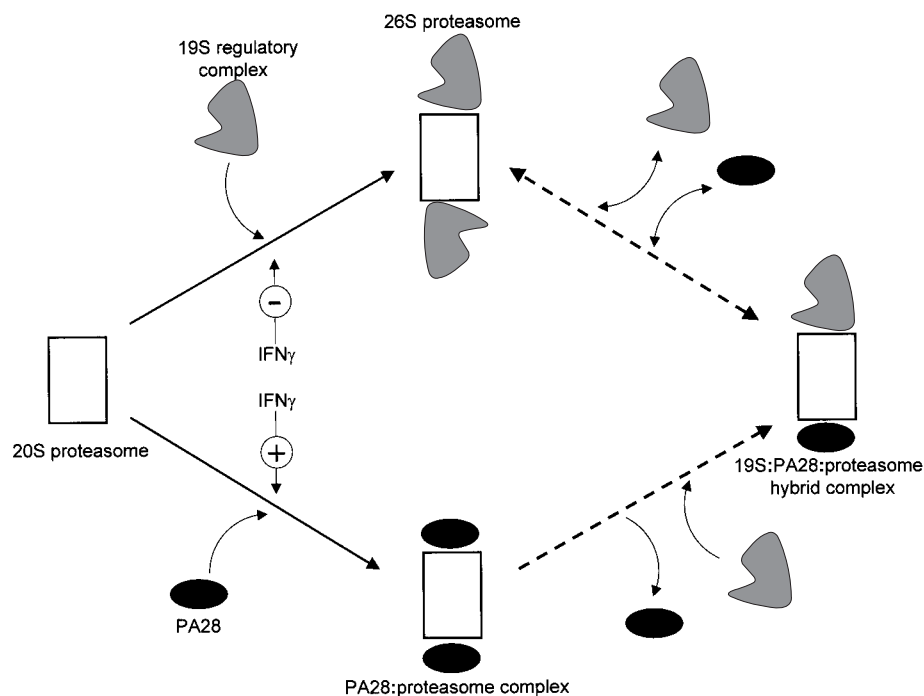
## DISCUSSION

These studies help to elucidate mechanisms by which  $\gamma$ -IFN can affect proteasome complexes in order to promote antigen processing in response to viral infection. The results presented are summarized in Scheme 1. It is well known that  $\gamma$ -IFN induces three non-essential catalytic subunits of proteasomes and the 11 S regulator proteins PA28  $\alpha$  and  $\beta$  [2–4,14]. This is the first report showing that  $\gamma$ -IFN causes a decrease in 26 S proteasome levels concomitant with a rise in the level of PA28–proteasome complexes. Since incorporation of  $\gamma$ -IFN-inducible subunits alters the peptidase activities of proteasomes [5,8], the changes in levels of the different complexes were best assessed either by immunoblot analysis of extracts prepared before and after  $\gamma$ -IFN treatment, or by comparison of immunoprecipitates from [ $^{35}\text{S}$ ] methionine-labelled cells. Both methods gave the same decrease of 15–20%. It is not clear whether the decrease in 26 S proteasomes is due to a decrease in the rate of synthesis or an increase in the turnover, or to a combination of both of these. The time course of the change over 2–3 days (results not shown) is consistent with either explanation, and experiments designed to address the rate of turnover did not give clear answers. It is interesting to note that no free 19 S regulatory complex was detected, even after  $\gamma$ -IFN treatment, suggesting that 19 S complexes do not exist in significant amounts on their own.

Along with the decrease in 26 S proteasome levels, we observed increased levels of PA28–20 S proteasome complexes. The conditions chosen for gel filtration are critical for detection of PA28–proteasome complexes. These complexes dissociate in the

presence of salt [24,25], whereas the presence of salt did not seem to affect the 26 S proteasome complex. Under low salt conditions, all 20 S proteasomes appeared to be associated with regulators with or without  $\gamma$ -IFN treatment. Interestingly, the two regulatory complexes have different subcellular distributions. The 11 S regulator is predominantly cytoplasmic, whereas 19 S complexes are present in the nucleus as well as the cytoplasm [21].  $\gamma$ -IFN treatment alters the ratio of different proteasome complexes and demonstrates the importance of PA28–proteasome complexes over 26 S proteasome complexes during the immune response. However, it is critical that 26 S proteasomes are not completely eliminated after  $\gamma$ -IFN treatment because they have crucial functions in the turnover of cell cycle regulators and other short-lived proteins [15,16].

The occurrence of LMP2 and LMP7 in 26 S as well as 20 S proteasomes is consistent with earlier observations [21,26,27] and with evidence that ubiquitin-dependent proteolysis can contribute to antigen processing [4]. The presence of hybrid 19 S complex–PA28 proteasome complexes cannot be eliminated, even though our experiments did not provide any direct evidence for their existence. Such complexes have been immunoprecipitated from HeLa cells [25]. Recently, it has been reported that there is impaired immunoproteasome assembly in PA28  $-/-$  mice, suggesting that PA28 is required for immunoproteasome assembly [13]. Thus it seems possible that these hybrid complexes are intermediates in the formation of 26 S proteasomes containing the  $\gamma$ -IFN-inducible subunits (Scheme 1). Preckel et al. [13] also found PA28 to be associated with the 15 S complexes containing precursor immunosubunits,



**Scheme 1** Regulation of proteasome complexes by  $\gamma$ -IFN and phosphorylation

$\gamma$ -IFN induces three catalytic subunits (LMP2, LMP7 and MECL1) which become incorporated into newly formed immunoproteasomes. The ratio of immunoproteasomes to constitutive proteasomes before  $\gamma$ -IFN exposure depends on the cell type. These 20 S proteasomes bind two types of regulatory complexes. The addition of 19 S regulatory complexes gives rise to 26 S proteasomes, which are critical for ATP-dependent protein degradation including degradation by the ubiquitin–proteasome pathway. PA28–proteasome complexes, which increase after  $\gamma$ -IFN treatment of cells, can be isolated after gel filtration in low salt buffer.  $\gamma$ -IFN causes a decrease in 26 S proteasome levels most likely due to a decrease in the formation of new complexes in favour of PA28–proteasome complexes. PA28 also appears to be involved in the assembly of immunoproteasomes [13]. Hybrid 19 S complex–PA28–proteasome complexes may be formed as intermediates in the formation of 26 S proteasomes, especially those containing  $\gamma$ -IFN-inducible subunits, or from 26 S proteasomes. Hybrid proteasomes have been reported to increase after  $\gamma$ -IFN treatment [28], but their assembly pathway has not been elucidated. The strong but reduced phosphorylation of 20 S proteasome subunit C8 after  $\gamma$ -IFN treatment suggests that C8 is phosphorylated in 26 S proteasomes, while C9 phosphorylation was barely detectable after  $\gamma$ -IFN treatment.

suggesting that perhaps PA28 promotes assembly of immunoproteasomes through conformational changes in the  $\alpha$  ring of the core proteasome. However, we found no evidence for the association of PA28 with proteasome precursor complexes. It is also possible that hybrids are formed from 26 S proteasomes after  $\gamma$ -IFN treatment, when their level increases [28].

Modifications in alpha subunits of the 20 S proteasome could dictate which regulatory complexes bind, and phosphorylation may be one such change [18,26,29]. Treatment of cells with  $\gamma$ -IFN resulted in substantially decreased phosphorylation of 20 S and 26 S proteasome subunits, with the exception of the C8 subunit, which was still phosphorylated but at a reduced level. Total cellular protein phosphorylation was also found to decrease in the presence of  $\gamma$ -IFN (results not shown), and the phosphorylation of the alpha subunit C9 was barely detectable following  $\gamma$ -IFN treatment. Since C8 remains phosphorylated, but at a lower level in 26 S proteasome complexes after  $\gamma$ -IFN treatment, these results are consistent with the suggestion that C8 phosphorylation may be involved in regulating 26 S proteasome formation [18,26]. Casein kinase II, which is the kinase responsible for phosphorylation of C8 [26], has been reported to co-purify with 20 S proteasomes [30].  $\gamma$ -IFN reduces the level of phosphorylation and changes the ratio of proteasome complexes, preferentially allowing binding of PA28 complexes to 20 S proteasomes.

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