Phosphorylation of 20S proteasome alpha subunit C8 (α 7) stabilizes the 26S proteasome and plays a role in the regulation of proteasome complexes by γ -interferon

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In animal cells there are several regulatory complexes which interact with 20S proteasomes and give rise to functionally distinct proteasome complexes. γ -Interferon upregulates three immunoproteasome beta catalytic subunits of the 20S proteasome and the PA28 regulator, and decreases the level of 26S proteasomes. It also decreases the level of phosphorylation of two proteasome alpha subunits, C8 (α 7) and C9 (α 3). In the present study we have investigated the role of phosphorylation of C8 by protein kinase CK2 in the formation and stability of 26S proteasomes. An epitope-tagged C8 subunit expressed in mammalian cells was efficiently incorporated into both 20S proteasomes and 26S proteasomes. Investigation of mutants of C8 at the two known CK2 phosphorylation sites demonstrated that these are the two

phosphorylation sites of C8 in animal cells. Although phosphorylation of C8 was not absolutely essential for the formation of 26S proteasomes, it did have a substantial effect on their stability. Also, when cells were treated with γ -interferon, there was a marked decrease in phosphorylation of C8, a decrease in the level of 26S proteasomes, and an increase in immunoproteasomes and PA28 complexes. These results suggest that the down-regulation of 26S proteasomes after γ -interferon treatment results from the destabilization that occurs after dephosphorylation of the C8 subunit.

Key words: γ -interferon, phosphorylation, proteasome, protein complexes, protein degradation, protein–protein interactions.

INTRODUCTION

Proteasomes are multimeric proteinase complexes which play a central role in protein degradation in animal cells. They are involved in many basic cellular processes including the cell cycle, apoptosis, the stress response, and the regulation of immune and inflammatory responses [1–5]. The proteolytic core 20S proteasome is composed of four stacked rings of seven subunits each. These are encoded by members of the same gene family most of which are essential for cell viability. In eukaryotic proteasomes the outer rings are each composed of seven different alpha subunits and the inner rings of seven different beta subunits. Three of the beta subunits contain a catalytic site. In animal cells three additional non-essential beta subunits are inducible by γ -interferon and replace the three catalytic subunits in the standard 20S proteasome in newly synthesized immunoproteasomes. Immunoproteasomes play a role in antigen processing for presentation by the MHC class I pathway (reviewed in [6–8]). The crystal structure of the related archaeobacterial and yeast proteasomes [9,10] have shown that proteins must be translocated into the central cavity to be degraded, because the catalytic sites are positioned on the inside of the cylindrical structure.

The catalytic activities have been shown to be activated by the association of regulatory complexes to the ends of the core 20S proteasome [11]. One such regulator is a multisubunit complex known as the 19S regulatory complex, ATPase complex or PA700 [11,12]. The association of 19S regulatory complexes to each end of the 20S proteasome results in the formation of the 26S proteasome that is active in the ubiquitin and ATP-dependent degradation of many substrates, including NF κ B, p53 and cyclin B [4,5]. The regulatory components of the 26S proteasome, which include a multi-ubiquitin-chain-binding subunit and 6 ATPases,

are thought to be involved in substrate recognition and unfolding. The 26S proteasome is the predominant form of the proteasome in lower eukaryotes and plays a major role in protein degradation by both ubiquitin-dependent and ubiquitin-independent mechanisms [4,13]. A second activator complex, which is known as PA28 or REG, contains alpha and beta subunits that are present only in organisms with an adaptive immune system, and are induced by γ -interferon, as are the three immunoproteasome-specific catalytic subunits [6–8]. The PA28–proteasome complex has, therefore, like the immunoproteasome, been implicated in antigen processing [reviewed in 14,15]. Hybrid 19S–20S–PA28 proteasomes have also been identified [16,17] but their role is not yet clear.

Phosphorylation of substrate proteins has been implicated in the regulation of protein degradation by the ubiquitin–proteasome pathway [4,5]. Phosphorylation of proteins plays an important role in the regulation of many protein–protein interactions and it may also regulate the formation of proteasome complexes. Two alpha subunits of the core 20S proteasome, C8 (α 7) and C9 (α 3) [18,19], and several subunits of the 19S regulator [20–22] are known to be phosphorylated in animal cells. Protein kinase CK2 (formerly casein kinase II), which is known to copurify with 20S proteasome preparations [23], appears to be responsible for the phosphorylation of C8. The CK2 phosphorylation sites have been mapped to residues Ser-243 and Ser-250 [19], which lie close to the C-terminus of the subunit. We have reported previously [24] that both the level of phosphorylation of C8 and the level of 26S proteasomes decrease following treatment of cells with γ -interferon, when the level of PA28-containing proteasome complexes increases. These observations suggest that phosphorylation may play an important role in regulating the formation of proteasome complexes in animal cells. In order to

Abbreviations used: CK2, protein kinase CK2 (formerly casein kinase II); DMEM, Dulbecco's modified Eagle's medium; p-DMEM, phosphate-free DMEM; LLVY-AMC, succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin; Ser243Ala etc., mutation of Ser-243 into alanine etc.

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investigate the possible functional role of phosphorylation of C8 in the assembly of 26S proteasomes, we have expressed an epitope-tagged C8 in COS-7 cells, and investigated the effect of mutation at the two CK2 sites.

The results presented here confirm that phosphorylation of C8 in animal cells does occur at these two CK2 sites and demonstrate that phosphorylation plays an important role in stabilizing the association of 19S regulatory complexes with 20S proteasomes to form the 26S proteasome. Moreover, they suggest that the decrease in phosphorylation of C8 in response to γ -interferon treatment results in unstable 26S proteasome complexes, thus facilitating the increase in PA28-containing complexes that occurs under these conditions.

EXPERIMENTAL PROCEDURES

Subcloning of rat C8 subunit into a mammalian expression vector

The rat C8 cDNA in pBluescript KS⁺ [25] was kindly provided by Dr Keiji Tanaka (The Tokyo Metropolitan Institute, Japan). A myc-tag was cloned into the C-terminus of the C8 sequence by PCR using the 5' primer 5'-TTTTTTTTTCTCGAGCGTTC-TGTGGCCTGCCG-3' and the 3' primer 5'-TTTTTTTTTTCGCGCCGCTACTTCAGGTCCTCCTCCGAGATC AGCT T-CTGCTCCATCATATTGTCGTCATC-3'. The primers carry *Xho*I and *Not*I sites respectively (underlined) inframe with the corresponding *Xho*I and *Not*I sites of the pCI-neo vector, a mammalian expression vector (Promega, Chilworth, Southampton, U.K.). The corresponding C8 myc-tag PCR product was ligated into the pCI-neo vector at the same sites in the multiple cloning region of the vector.

Site-directed mutagenesis

Mutagenesis of the CK2 C8 phosphorylation sites [19] was performed using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, Cambridge, U.K.). Site-directed mutagenesis of the myc-tagged C8 in pCI-neo was performed according to the manufacturer's guidelines using the following oligonucleotides: Ser243Ala forward primer 5'-GGAAGCAGAGAAATATGCC-AAGGAAGCATTGAAGGAAGAAGATG-3' and the reverse primer 5'-CATCTTCTTCCTTCAATGCTTCCTTGGCATAATTTCTCTGCTTCC-3', Ser250Ala forward primer 5'-GAAGGAAGAAGATGAAGCAGATGACGACAATATGATGGAGC-3' and the reverse primer 5'-GCTCCATCATATTGTCGTCATCTGCTTCATCTTCTTCCTTC-3', Ser243Asp forward primer 5'-GAGGAAGCAGAGAAATATGCCAAGGAAGACTTGAAGGAAGAAGATG-3' and the reverse primer 5'-CATCTTCTTCCTTCA-CGTCTTCCTTGGCATAATTTCTCTGCTTCCTC-3', Ser250Asp forward primer 5'-GAAGGAAGAAGATGAAGACGATGACGACAATATGATGGAGCAG-3' and the reverse primer 5'-CTGCTCCATCATATTGTCGTCATCGTCTTCATCTTCTTCCTTC-3'. The mutational changes are indicated by underlining. For double alanine mutants, the primers for Ser250Ala were used with the Ser243Ala DNA. Similarly, for double aspartic acid mutants, the primers for Ser250Asp were used with the Ser243Asp DNA. For some PCR reactions, 5 % DMSO was added to increase the yield of PCR products produced. Automated sequencing was used to confirm mutational changes (Sequencing Facilities, Department of Biochemistry, University of Bristol, Bristol, U.K.).

Cell culture and transfection of COS-7 cells

COS-7 cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10 % foetal bovine

serum containing penicillin (50 units/ml) and streptomycin (50 μ g/ml) in a humidified atmosphere of 5 % (by vol.) CO₂/air. Cells were transfected with the pCI-neo containing the myc-tagged C8 sequence (wild-type and mutants) or the empty vector using the FuGENE 6 transfection reagent (Roche, Lewes, East Sussex, U.K.). Transfections were carried out according to the manufacturers guidelines. Optimal transient transfection was obtained using 2–5 μ g DNA, 15 μ l FuGENE and 48 h incubation at 37 °C. Similar conditions were used for the mutants of C8 phosphorylation sites. Treatment with human γ -interferon was carried out as described previously [24].

Harvesting of cells

Original conditions

COS-7 cells were displaced from their flasks with trypsin/EDTA and washed with PBS (Sigma). Cells were resuspended in 300 μ l of lysis buffer [25 mM Tris/HCl, pH 7.5, 100 mM NaCl, 5 mM ATP, 0.2 % (v/v) Nonidet P-40 and 20 % glycerol] and lysed using a 21-gauge needle. The cell debris was sedimented at 11 600 g in a microcentrifuge at 4 °C for 10 min.

Mild low salt conditions

Cells were displaced from their flasks by gentle scraping into PBS. Cells were resuspended in 300 μ l of low-salt lysis buffer [25 mM Tris/HCl, pH 7.5, 5 mM ATP, 0.2 % (v/v) NP40 and 20 % glycerol] and incubated on ice for 30 min and then cell debris was sedimented as above. The supernatant was used for analysis either by SDS/PAGE or by gel filtration (see below).

Electrophoresis and immunoblotting

SDS/PAGE was performed using a 15 % polyacrylamide separating gel. Proteins separated by SDS/PAGE were electrophoretically blotted to nitrocellulose sheets using a BioRad Trans-BlotTM apparatus. Non-denaturing PAGE was performed using a 2.5 % stacking gel and 4.5 % separating gel cast in Tris/borate buffer [26]. Immunoblotting was performed as described previously [24] using anti-myc (9E10) antibody kindly prepared by Dr T. M. Harrison (University of Leicester, UK), and anti-proteasome monoclonal antibodies [27], anti-p45-110, anti-C2 and anti-C8 (mcp72), kindly provided by Dr K. B. Hendil (University of Copenhagen, Denmark). Rabbit polyclonal PA28 antibodies against the alpha subunit were purchased from Affiniti.

Separation of proteasome complexes

Separation of 26S and 20S proteasomes

COS-7 cell extracts (prepared under original conditions as described above) were fractionated by FPLC on a Superose 6 column (Pharmacia) equilibrated in 20 mM Tris/HCl, pH 7.5, 5 mM ATP, 10 % glycerol, 100 mM NaCl in order to separate 26S and 20S proteasomes, which were assayed as described below. Either 1 ml (Figure 2) or 0.5 ml (Figure 5) fractions were collected.

Gel filtration under low salt conditions to avoid dissociation of PA28–proteasome complexes

In other experiments fractionations were carried out using the same elution buffer except that it lacked the NaCl and ATP. In each

case the elution position of the different proteasome complexes was determined by proteasome assays as described below and by immunoblot analysis using anti-p45 antibodies for 19S regulatory complex/26S proteasome, anti-PA28, and anti-C8 or anti-C2 for all proteasomes. The elution positions of purified 20S proteasomes [28], 26S proteasomes [29] and PA28–20S complexes [30] were also determined.

Proteasome activity assays

Chymotrypsin-like activity of 20S and 26S proteasomes was assayed in fractionated cell extracts as described previously [28] using 40 μ M succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (LLVY-AMC; Peptide Institute, Scientific Marketing Associates, Barnet, Herts, U.K.) as substrate. All assays were performed using a PerkinElmer LS-3B Fluorescence Spectrometer (Excitation 370 nm, Emission 430 nm). Assays of 26S proteasome complexes were carried out in the presence of 5 mM ATP which stimulates 26S proteasome activity. 20S proteasome activity was measured in the presence of 0.02 % SDS which activates 20S proteasomes and partially inhibits 26S proteasomes. Martin Rechsteiner (University of Utah, Utah, U.S.A.) kindly provided purified human recombinant PA28.

Phosphorylation of cellular proteins and immunoprecipitation of C8-myc

COS-7 cells were labelled with 32 P_i as described previously [18]. Essentially, the media was removed from subconfluent flasks and the cells were rinsed with phosphate-free DMEM (P-DMEM). Cells were then grown in P-DMEM supplemented with 10 % dialysed fetal calf serum for 40 min in a humidified atmosphere of 5 % (by vol.) CO₂/air. 32 P_i (0.5 mCi) was then added to each flask. Cells were cultured for a further 4 h. The medium was carefully removed from the culture flasks, which were then rinsed three times with PBS. Ice-cold RIPA buffer (1 ml) containing phosphatase inhibitors [50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1 % (v/v) Nonidet P-40, 0.1 % SDS, 0.5 % sodium deoxycholate, 5 mM sodium pyrophosphate, 50 mM sodium fluoride, 0.1 mM sodium orthovanadate, 0.1 μ M okadaic acid and 10 mM glycerol-2-phosphate] was added directly to the culture flask. The flasks were placed on ice for 30 min with occasional gentle agitation to ensure complete cell lysis. The lysates were placed in Eppendorf tubes and centrifuged in a microfuge at maximum speed (11 600 g) for 10 min to sediment any insoluble material, and the resulting supernatants were placed in clean tubes. Anti-myc antibody [50 % (v/v) suspension of anti-myc Protein-A–agarose] was added to each lysate and the tubes were tumbled for 16 h at 4 °C. The agarose was sedimented by 1000 g and the supernatants removed. The gel pellets were then washed three times for 5 min each with NET buffer [50 mM Tris/HCl, pH 7.0, 150 mM NaCl, 5 mM EDTA, 0.1 % SDS, 0.1 % sodium deoxycholate, 0.5 % (v/v) Nonidet P-40, 5 mM sodium pyrophosphate, 50 mM sodium fluoride, 0.1 mM sodium orthovanadate, 0.1 μ M okadaic acid and 10 mM glycerol-2-phosphate].

In vitro phosphorylation of 20S proteasome or 26S proteasome (2.5 pmoles) was carried out with 180 ng of purified human recombinant CK2 (Roche) in 20 mM Tris/HCl buffer, pH 7.0, containing 5 mM MgCl₂, 0.05 mM ATP (specific activity 500–2000 c.p.m./pmol), 0.5 mM EDTA, 5 % glycerol (v/v) and 0.1 % 2-mercaptoethanol. Assays were incubated at 37 °C for 30–60 min and stopped by heating in SDS/PAGE sample buffer. Proteins were separated by SDS/PAGE and analysed by autoradiography.

Figure 1 Expression and phosphorylation of epitope-tagged C8

(A) Extracts from control COS-7 cells (C) and cells transfected with C8-myc (T) were separated by SDS/PAGE and immunoblotted with anti-C8 antibody (left-hand panel) or anti-myc antibody (right-hand panel). Endogenous C8 and expressed C8-myc are indicated with arrows. (B) Control COS-7 cells and transfected cells expressing C8-myc were labelled with 32 P_i. Proteasomes were immunoprecipitated from cell extracts with anti-myc antibodies as described in Experimental procedures and run on SDS/PAGE gels. An autoradiogram from control cell extracts (C) and extracts of transfected C8-myc expressing cells (T) is shown (left-hand panel) as well as an immunoblot of samples from control (C) and transfected (T) cells with anti-myc antibodies (right-hand panel).

RESULTS

Expression and phosphorylation of epitope-tagged C8

When C8-myc was expressed in COS-7 cells the epitope-tagged protein was detected at a similar level to that of the endogenous C8, as judged by immunoblot analysis with anti-C8 antibody (Figure 1A). We next investigated whether the myc-tagged C8 subunit was phosphorylated in the same way as the endogenous C8 protein, by labelling control and transfected cells with 32 P_i and then immunoprecipitating from cell extracts using anti-myc antibodies. Analysis of samples by SDS/PAGE followed by autoradiography and immunoblot with anti-myc antibodies clearly showed phosphorylation of the C8-myc (Figure 1B).

To investigate whether the myc-tagged C8 was incorporated into proteasome complexes, cell extracts were fractionated by gel filtration. The conditions used in these experiments, which included salt and ATP in the buffer, were chosen to dissociate PA28–proteasome complexes and provide a good separation of 26S and 20S proteasomes [24]. COS-7 cells were transiently transfected with either the empty vector (control) or vector containing the C8-myc insert. The separation of proteasome complexes determined by immunoblotting with anti-p45 for 26S proteasomes and anti-C8 for 20S and 26S proteasomes were consistent with the elution profiles of purified 26S and 20S proteasomes (peak fractions 10, 11 and 13, respectively). Samples of eluted fractions were run on SDS/PAGE gels for immunoblotting with anti-C8 antibody to detect both endogenous C8 and the slower migrating C8-myc bands. The majority (> 90 %) of the expressed C8-myc was found to be distributed in 26S and 20S proteasomes in the same way as endogenous C8 (Figure 2C). A small proportion of the total expressed C8-myc was seen in high molecular mass aggregates. The trace amount of C8 in fraction 18 (Figures 2C and D) could be due to the formation of rings of C8 subunits such as those observed following expression of mammalian C8 in bacteria [31]. There was no cross-reacting material in the later

Figure 2 Incorporation of Myc-tagged C8 into 20S and 26S proteasomes

COS-7 cell extracts were fractionated on a Superose 6 column for the identification of fractions containing 26S and 20S proteasomes. Fractions from control cells (panels **A**, **B**) and cells expressing C8-myc (panels **C**, **D**) were run on SDS/PAGE gels and immunoblotted with anti-p45, anti-C8 and anti-myc antibodies as indicated. No cross-reacting material was found in later column fractions (19–25). The peak fractions for elution of purified 26S proteasomes (fractions 10–11) and 20S proteasomes (fraction 13) are shown by the arrows and are in agreement with peak fractions determined by proteasome assays of the fractionated cell extracts.

fractions [19–25] and therefore no evidence for the presence of any uncomplexed endogenous C8 or C8-myc subunits.

The high level expression and incorporation of myc-tagged C8 into 26S proteasomes and 20S proteasomes confirmed that the COS-7 cell-expression system was suitable for functional studies. The myc tag had no effect on the incorporation of C8 into 20S or 26S proteasomes and no effect on phosphorylation at the CK2 sites. Interestingly, the observation that immunoprecipitation with anti-myc antibodies pulls down only phosphorylated C8-myc and not phosphorylated endogenous C8 (Figure 1B) indicates that endogenous C8 is not present at significant levels in proteasomes containing C8-myc. It therefore follows that in cells expressing C8-myc, the amount of endogenous C8 incorporated into proteasomes is very small and that the endogenous C8 detected in extracts (Figure 1A) is predominantly derived from untransfected cells. This suggests a transfection efficiency of around 50%.

Phosphorylation of C8 ($\alpha 7$) by protein kinase CK2 *in vitro* and in COS-7 cells

We next investigated the phosphorylation of purified 26S and 20S proteasomes by protein kinase CK2. CK2 phosphorylated a single protein band in purified 20S proteasomes (Figure 3), consistent with the report of copurification of the kinase with proteasomes [23], and with the known phosphorylation of C8 *in vitro* [19]. Although C9 is a similar size to C8, and the recombinant rat C9 subunit and has been reported to show a low level of phosphorylation by CK2 *in vitro* [19], the known CK2 site Ser-248 of the yeast Y13/ $\alpha 3$ /C9 subunit [32] is not conserved in mammalian proteasomes. The band at approx. 28 kDa is due to autophosphorylation of CK2. The action of CK2 on purified 26S proteasomes gave rise to a band of the same size, but much less intense, than with an equivalent amount of 20S proteasome (Figure 3). No other phosphorylated bands were detected. Since it was necessary to remove the ATP that was present in the buffer in order to carry out the labelling experiment, we

Figure 3 Phosphorylation of purified rat liver 20S and 26S proteasome preparations by purified CK2

Purified CK2 was incubated with radiolabelled ATP in assays containing purified 20S proteasome (20S), purified 26S proteasome (26S) or no proteasome (–). Assays without CK2 were included as a control. Samples were analysed by autoradiography of SDS/PAGE gels (**A**) and immunoblot with anti-C8 antibody (**B**). Molecular mass markers (kDa) are indicated on the left.

cannot rule out the possibility that 26S proteasomes were partially dissociated. The results are consistent with the idea that C8 is present predominantly in the phosphorylated form in 26S proteasomes, which may become partially dephosphorylated during purification. Although the lower phosphorylation of 26S proteasomes than 20S proteasomes could also be explained by steric hindrance and lower accessibility of CK2 sites in the 26S proteasome, we already know, from earlier investigations using $^{32}\text{P}_i$, that C8 is phosphorylated in the 26S proteasome in cultured cells [18]. Therefore the *in vitro* phosphorylation by CK2 was not characterized further. However, it is interesting to note from the control samples in Figure 3(A) that neither 20S nor 26S proteasomes are phosphorylated in the absence of added kinase, suggesting that our purified preparations were free from significant amounts of any copurifying proteasome kinase activities [23,33]. No other proteasome subunits were found to be phosphorylated by CK2.

Sequence alignments show that the CK2 sites in C8, Ser-243 and Ser-250, are conserved in animals (Figure 4A), but not in lower eukaryotes. In order to confirm that the two CK2 sites in C8 are phosphorylated in cells, and to investigate the role of C8 phosphorylation in proteasome complex formation in animal cells, site-directed mutagenesis was performed. Single-site alanine (Ser243Ala or Ser250Ala) mutants were generated by site-directed-mutagenesis as indicated in Figure 4(B). Serine to aspartate mutants (Ser243Asp or Ser250Asp) were generated to mimic the negatively charged phosphoserine residues. The double alanine mutant and the double aspartic acid mutants were also prepared. Expression conditions for the mutants were optimized as they were for the expression of wild-type myc-tagged C8. The levels of expressed mutant proteins (Figure 4C) were comparable with those of endogenous protein for all of the single-site mutants and the double alanine mutant. However, the levels of the double aspartic acid mutant were always very low compared with the others and this mutant was not characterized extensively because the level of expression was so low.

Figure 4 CK2 phosphorylation sites of C8

(A) Sequence alignments of the C-terminal sequences of mouse, human and rat proteasome alpha-type subunit C8 ($\alpha 7$). The two CK2 sites [Ser-243 and Ser-250 (14)] are indicated in bold. (B) A schematic diagram of the epitope-tagged rat C8 subunit showing the sites of mutation. (C) Expression of the mutant C8-myc proteins in COS-7 cells. COS-7 cells were untransfected (lane 1), transfected with wild-type C8-myc (lane 2), or transfected with mutant C8-myc: Ser243Asp (lane 3), Ser243Ala (lane 4), Ser250Ala (lane 5), Ser250Asp (lane 6), Ser243Ala and Ser250Ala double mutant (double alanine; lane 7) and double aspartic acid (lane 8). Samples of cell extracts were run on SDS/PAGE gels and immunoblotted with anti-C8 antibody. The top band is C8-myc and the lower band the endogenous C8. (D) Phosphorylation of expressed C8-myc protein. COS-7 cells were transfected with empty vector (lanes 1 and 4), wild-type C8-myc (lanes 2 and 5), or double alanine mutant of C8 (lanes 3 and 6). Cells were labelled with ^{32}P , and then myc-tagged proteasomes were immunoprecipitated with anti-myc antibody for analysis by autoradiography (lanes 1–3) and by immunoblotting with anti-myc antibody (lanes 4–6). Molecular mass markers (kDa) are indicated on the left.

In order to confirm that the two CK2 sites in C8 are the ones that are phosphorylated in cells, phosphorylation of the double alanine mutant was compared with that of the wild-type C8-myc (Figure 4D). As predicted, the double alanine mutant was not phosphorylated, confirming that C8 Ser-243 and Ser-250 are its usual phosphorylation sites, and this accounts for the stoichiometry of labelling of 1.5–2 moles/mole C8 observed in animal cells [18].

We investigated the subcellular distribution of expressed wild-type C8-myc and double alanine C8-myc to determine whether the phosphorylation status of C8 had any effects on the subcellular localization of proteasomes. In each case immunofluorescent labelling with anti-myc antibodies was observed in the nucleus and in the cytoplasm (results not shown), as expected from the known distribution of proteasomes in COS-7 cells [34]. A proportion of the myc label appeared to be associated with what looked like nuclear bodies, where proteasomes have been found previously [35]. Since there was no difference between the location of wild-type C8 myc and the double alanine mutant, this was not investigated further. The phosphorylation of C8 appeared to have little, if any, effect on proteasome localization.

Figure 5 Incorporation of myc-tagged C8 proteins into 20S and 26S proteasomes

(A) COS-7 cell lysates were fractionated on a Superose 6 column to separate 26S and 20S proteasomes. Assays for 26S proteasomes (closed symbols) contained 5 mM ATP, and assays for 20S proteasomes (open symbols) contained 0.02% SDS as described in Experimental procedures. Fractions equal to half the size (0.5 ml) of those in Figure 2 were collected. Therefore the fraction numbers indicated (19–28) correspond to fractions 10–14, inclusive, in Figure 2. Fractions collected from column runs with control cell extracts were analysed by SDS/PAGE gels and immunoblotting with anti-C2 antibody to detect the elution positions of both 26S and 20S proteasomes and anti-p45 antibodies to detect 26S proteasomes (inset). (B) Fractionation was carried out with extracts of COS-7 cells transfected with wild-type C8-myc (WT), and the following mutants of C8-myc: Ser243Ala (S243A), Ser250Ala (S250A), Ser243Asp (S243D), Ser250Asp (S250D), Ser243Ala (S243A) and Ser250Ala double mutant (Double Ala) as indicated. Samples of fractions were run on SDS/PAGE gels and immunoblotted with anti-C8 antibody. Assays of 26S and 20S proteasomes were carried out to confirm that the elution positions were the same for all samples and for control cells as shown in (A).

Effect of phosphorylation of C8 on its assembly into proteasome complexes

The incorporation of C8 and wild-type C8-myc into 20S and 26S proteasomes was compared with that of the various mutants (Figure 5). The elution profiles of 26S and 20S proteasomes from gel filtration were confirmed by enzyme assays and by immunoblotting with anti-p45 and anti-C2 antibodies (Figure 5A). The fraction numbers are different in this figure compared with those shown in Figure 2 because half-size fractions were collected to get better separation of the different proteasome complexes. As stated above, C8-myc was found in both 20S and 26S proteasomes to the same level as endogenous C8 (Figure 5B). In contrast, single-site alanine mutants of the CK2 phosphorylation sites of C8 resulted in reduced incorporation of the myc-tagged subunit into 26S proteasomes while their incorporation into 20S proteasomes was unaffected. Single-site aspartate mutants of the same sites allowed normal levels of incorporation of the subunit into 26S proteasomes. These results suggest that the negative charge in these positions mimicking the phosphorylation is sufficient to partially stabilize the 26S proteasome. The myc-tagged C8 double

Figure 6 Activity in extracts of cells expressing wild-type C8 and double alanine mutant

Cells expressing wild-type C8-myc (WT) or double alanine mutant (Double Ala) were lysed using our original lysis conditions and also under the mild low salt conditions as detailed in Experimental procedures. **(A)** Extracts were assayed for proteasome activity (mean $\pm$ S.E.M., $n=3$) and immunoblotted with anti-C8 antibody. **(B)** Cell extracts were fractionated by gel filtration as in Figure 5, (left-hand panel) and peak 26S proteasome activity fractions for cells expressing WT and double alanine C8-myc were run on SDS/PAGE gels and immunoblotted with anti-C8 antibodies (right-hand panel).

alanine mutant was present in 20S proteasomes, but none of this subunit was detected in peak 26S proteasome fractions 20–21 (Figure 5B), even after a long exposure of the blots. The barely detectable C8-myc band in fraction 22 in Figure 5(B) (double alanine) was likely due to 20S proteasome and the slight C8-myc band detected in fraction 19 can be explained by the low level of high molecular weight aggregates observed with all of the C8-myc proteins. There was no significant difference in activity in peak 26S proteasome fractions in alanine or aspartic acid mutants (results not shown).

If phosphorylation was essential for assembly of 26S proteasomes we would expect the double alanine mutant C8 to act in a dominant negative way preventing 26S complex formation. Initially it looked as though this was indeed the case and that expression of the double alanine mutant reduced proteasome activities in cell extracts (Figure 6). However, the results of further experiments carried out with cells lysed under very mild conditions showed that proteasome activity was not significantly inhibited in cells transfected with double alanine C8-myc. Over-expression of wild-type C8-myc or double alanine C8 myc had no effect on proteasome level or activity when cells were lysed under these mild low-salt conditions. However, when cells were lysed with a needle under the extraction conditions used in the earlier experiments, a substantial decrease in activity of the double alanine mutant was observed (Figure 6). This decrease in activity was comparable with the extent of transfection supporting the conclusion that 26S proteasomes containing double alanine C8-myc were highly unstable in these lysates. Thus the results suggest that the stability of the 26S proteasome containing double alanine C8 was reduced, making it sensitive to the disruptive forces of the original lysis procedure. Further analysis of extracts by gel filtration showed that when cells were lysed under mild conditions double alanine C8-myc was found in 26S proteasomes at a similar level to wild-type C8-myc (Figure 6C). Taken

Figure 7 Effect of γ -interferon on phosphorylation of C8-myc

COS-7 cells were transfected with C8-myc and either not treated (– IFN) or treated (+ IFN) with γ -interferon. The cells were then labelled for 4 h with 32 P. Cell extracts were prepared and immunoprecipitations carried out with anti-myc antibodies as described in Experimental procedures. **(A)** Samples were analysed by SDS/PAGE and autoradiography (left-hand panel) and by immunoblot with anti-C8 and anti-myc antibodies (right-hand panel) as indicated in the figure. **(B)** Immunoblot analysis of control cells and cells treated with γ -interferon. The γ -interferon-inducible increase in immunoproteasomes is illustrated with LMP7 (left-hand panel) and the decrease in relative levels of 26S to 20S proteasomes is shown with anti-p45 (26S proteasomes only) and anti-C2 (26S and 20S proteasomes) antibodies (centre panel). The relative density of p45/C2 bands before and after treatment with γ -interferon was 0.775 and 0.655, respectively. The γ -interferon-inducible increase in PA28, measured by blotting with anti PA28 α antibodies, is shown in the right-hand panel. **(C)** Separation of proteasome complexes by non-denaturing PAGE. Immunoblots were carried out with anti-C8 (for all proteasomes), anti-S5a (for 19S regulators and 26S proteasomes) and anti-PA28 antibodies, as indicated.

together these observations demonstrate that phosphorylation is not absolutely essential for the assembly of 26S proteasomes but that it does have a significant influence on their stability.

Changes induced by treatment with γ -interferon

Following treatment of COS-7 cells with human γ -interferon the level of phosphorylation of expressed C8-myc was reduced dramatically while the level of C8 protein showed no change (Figure 7A). These results are consistent with our earlier studies of proteasome phosphorylation in human embryo lung cells after γ -interferon treatment where 2D-PAGE analysis showed a decrease in phosphorylation of both C8 and C9 [24]. Proteasome activity measured in COS-7 cell extracts with LLVY-AMC as substrate was increased 1.3–2-fold following γ -interferon treatment. This was due to a change in the content of different proteasome complexes that was confirmed by immunoblot analysis. There was a 3-fold increase in PA28 levels after γ -interferon treatment (Figure 7B) and this, together with the induction of immunosubunits, accounts for the observed increase in proteasome activity. 26S proteasome levels decreased by around 20% (Figure 7B). All of our results are consistent with the γ -interferon causing a decrease in phosphorylation of C8 that would decrease the stability of association of 19S complexes and thereby favour formation of PA28–proteasome complexes.

Analysis of cell extracts by non-denaturing PAGE confirmed these results. Some dissociation of proteasome complexes was apparent under the electrophoresis conditions used (Figure 7C). 26S proteasomes, PA28–20S-proteasome complexes and 20S proteasomes were present at significant levels. There was little if any of the hybrid complex in these COS-7 cell extracts either before or after treatment with γ -interferon. The total C8 (all proteasomes) level was the same before and after γ -interferon treatment, but the distribution between the different complexes changed with more being present in free 20S proteasomes and PA28–20S complexes and less in 26S proteasomes following γ -interferon treatment. The results are consistent with earlier analysis and quantification of complexes by immunoblot analysis of SDS/PAGE gels [24].

DISCUSSION

Our results provide direct evidence that proteasome alpha subunit C8 ($\alpha 7$) is phosphorylated by protein kinase CK2 in animal cells and they are in full agreement with the phosphorylation sites Ser-243 and Ser-250 identified previously [19]. The phosphorylation of C8 appears to be constitutive. C8 is phosphorylated in both 26S and 20S proteasomes in a variety of different cell types [18–20,24] and (S. Bose, G. G. F. Mason and A. J. Rivett, unpublished results). We have not observed significant changes in the level of phosphorylation under a variety of conditions that are known to affect signalling pathways (S. Bose, G. G. F. Mason and A. J. Rivett, unpublished results), with the exception of treatment with γ -interferon [24]. CK2 is often responsible for the constitutive phosphorylation of proteins and plays an important role in maintaining cell viability [36,37].

The phosphorylation of purified 20S proteasomes by purified CK2, the reported co-purification of CK2 with 20S proteasomes [23], and the dephosphorylation of proteasomes after γ -interferon treatment [24] all suggest that CK2-dependent phosphorylation of C8 may be involved in the regulation of proteasome complexes. All of our results are consistent with the phosphorylation of C8 at the two CK2 sites taking place when the subunit is already assembled into the 20S core particle. In this regard it is of interest that the double aspartic acid mutant C8 (negatively charged residues mimicking phosphorylation at both CK2 sites) was expressed only at very low levels compared with the double alanine mutant or wild-type C8. It is possible that the double aspartic acid mutant subunit is unstable. Destabilization by CK2-dependent phosphorylation of the C-terminus of a protein that leads to proteasome-mediated degradation has been observed for the tumour suppressor protein PTEN [38].

The fact that we observed no difference in activity in cell extracts expressing the wild-type and the double alanine mutant C8-myc when extracts were made by lysing cells under very mild conditions, suggests that phosphorylation of C8 has little if any effect on proteasome peptidase activities. These observations are in full agreement with data from the *in vitro* phosphorylation of 20S proteasomes by CK2 [19]. Although binding to C8 has recently been identified as an important factor in the degradation of p21^{WAF1/CIP1} [39], there is no evidence to suggest that the constitutive phosphorylation of C8 is a mechanism of regulating protein degradation. From our present results, it is clear that phosphorylation of C8 by CK2 does not play a significant role in determining the intracellular localization of proteasomes, although this also is an area where there are differences between yeast and mammalian cells [40–42].

The position of Ser-243 and Ser-250 on the outside of the 20S proteasome cylindrical structure led us to suggest a role in determining which regulatory complexes are bound, rather than

having a direct effect on proteolytic activity [18,24]. All of the data presented here support this model. Phosphorylation of the C8 alpha subunit of 20S proteasomes could affect the strength of binding of 19S regulatory complexes to 20S proteasomes through changes in charge at the C-terminus of C8, or through local conformational changes. The CK2 sites, conserved in mammals, are found within a highly acidic region at the C-terminus. The location of this site is accessible on the outside of the 20S proteasome and occurs in the region that is not well defined in the yeast structure. There is a structural requirement of the ATPase subunits of the 19S regulatory complex for ATP [43], and phosphorylation of subunits of the 19S complex, such as the ATPase subunit p45, may also be important for 26S proteasome stability [22]. The CK2-dependent constitutive phosphorylation of C8 clearly has a substantial effect on the stability of 26S proteasomes to mechanical forces and high-salt conditions. Indeed, partial dephosphorylation of 26S proteasomes during purification may contribute to the instability of the purified complex and losses of 26S proteasomes during the purification procedure.

In cells, the destabilization of 26S proteasomes following dephosphorylation of C8 probably contributes to the observed changes in the levels of the different proteasome complexes after treatment with γ -interferon. There is a decrease in 26S proteasome levels following γ -interferon treatment of cells [24] and a marked increase in levels of PA28-containing proteasome complexes [15,16,24]. Decreased phosphorylation of C8 could lead to unstable 26S proteasome complexes and allow the replacement of 19S regulators with PA28. This model is consistent with our earlier observations that 26S proteasomes contain a higher level of the phosphorylated form of C8 than 20S proteasomes [18].

Such a function for the phosphorylation of C8 may also explain why the CK2 sites are not conserved in yeast, where there is no PA28 regulator, and the 20S proteasome is almost exclusively present as 26S proteasome. Yeast C8 [$\alpha 7$] can be phosphorylated but only in a polylysine-dependent fashion [44]. Interestingly additional proteasome subunits can be phosphorylated in yeast proteasomes. In *Candida albicans*, $\alpha 3$ (C9/Y13), $\alpha 5$ (Pup2/zeta) and $\alpha 6$ (Pre5/C2) are phosphorylated [32]. The phosphorylation of the yeast $\alpha 3$ subunit is catalysed by CK2 [32], but the Ser-248 phosphorylation site of the yeast subunit is not conserved in mammalian proteasomes. A low level of phosphorylation of the recombinant C9 subunit by CK2 *in vitro* has been reported [19], but polo-like kinase has been implicated in the phosphorylation of C9 in mammalian cells [45]. The proteasome C2 ($\alpha 6$) subunit is also phosphorylated in rice [46] but there is no report of phosphorylation of this subunit in animal cells. Further analysis will be required to establish the functional significance of the phosphorylation of other proteasome subunits.

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