Dynamics of proteasome distribution in living cells

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Proteasomes are proteolytic complexes involved in non-lysosomal degradation which are localized in both the cytoplasm and the nucleus. The dynamics of proteasomes in living cells is unclear, as is their targeting to proteins destined for degradation. To investigate the intracellular distribution and mobility of proteasomes *in vivo*, we generated a fusion protein of the proteasome subunit LMP2 and the green fluorescent protein (GFP). The LMP2–GFP chimera was quantitatively incorporated into catalytically active proteasomes. The GFP-tagged proteasomes were located within both the cytoplasm and the nucleus. Within these two compartments, proteasomes diffused rapidly, and bleaching experiments demonstrated that proteasomes were transported slowly and unidirectionally from the cytoplasm into the nucleus. During mitosis, when the nuclear envelope has disintegrated, proteasomes diffused rapidly throughout the dividing cell without encountering a selective barrier. Immediately after cell division, the restored nuclear envelope formed a new barrier for the diffusing proteasomes. Thus, proteasomes can be transported unidirectionally over the nuclear membrane, but can also enter the nucleus upon reassembly during cell division. Since proteasomes diffuse rapidly in the cytoplasm and nucleus, they may perform quality control by continuous collision with intracellular proteins, and degrading those proteins that are properly tagged or misfolded.

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Introduction

The 20S proteasome is an intracellular proteinase complex involved in non-lysosomal mechanisms of protein degradation. Its substrates include transcription factors, oncoproteins and cyclins, and it is responsible for the generation of peptides for presentation by MHC class I molecules to the immune system (Goldberg, 1995; Jentsch and Schlenker, 1995; Cerundolo *et al.*, 1997). The 20S proteasome forms the core of a larger 26S protease complex, which catalyses the ATP-dependent degradation of ubiquitinated proteins (Hochstrasser, 1995). Alternatively,

the 20S proteasome can associate with the PA28 activator which is involved in antigen presentation (Groettrup *et al.*, 1996). The barrel-shaped structure of the proteasome is composed of 28 subunits arranged in four seven-membered rings. The two outer rings are formed by α subunits and the two inner rings by β subunits, with eukaryotic proteasomes consisting of multiple different α - and β -related subunits (Löwe *et al.*, 1995; Groll *et al.*, 1997). The combined β subunits exhibit proteolytic activity, and are synthesized as inactive precursors to avoid unrestrained degradation. The β subunit prosequence is removed either just before or during the dimerization of the two $\alpha\beta$ ring structural complexes (Yang *et al.*, 1995; Schmidtke *et al.*, 1996). Three β subunits, delta (β 1), MB1 (β 5) and Z (β 2), are down-regulated in proteasomes after induction with interferon γ (IFN γ) (a potent stimulator of MHC class I presentation) and replaced by the IFN γ -inducible subunits LMP2 (β 1i), LMP7 (β 5i) and MECL-1 (β 2i) (Belich *et al.*, 1994; Nandi *et al.*, 1996). The mammalian proteasome exhibits up to five different peptidase activities, including those that preferentially cleave peptides at the carboxy side of hydrophobic, basic and acidic residues, commonly referred to as chymotrypsin-like, trypsin-like and peptidyl-glutamyl-peptide hydrolysing activities. The activities of proteasomes can be assayed with a variety of different substrates, including fluorogenic peptides (Driscoll *et al.*, 1993; Gaczynska *et al.*, 1993). Incorporation of LMP2 and LMP7 into the proteasome results in an increased chymotryptic and tryptic peptidase activity *in vitro*, modulating the cleavage site preferences of the proteasome (Kuckelkorn *et al.*, 1995), although larger peptide substrates can give different results (Boes *et al.*, 1994).

Proteasomes are localized both in the nucleus and in the cytoplasm of eukaryotic cells (Palmer *et al.*, 1994, 1996). They may be free in the cytoplasm or associated with cytoskeletal elements. In addition, some proteasomes may be bound to the endoplasmic reticulum (ER) (Yang *et al.*, 1995) and may deliver degraded antigenic peptides directly to TAP (transporter associated with antigen processing). It is unknown whether proteins marked for degradation require specific targeting or reach the proteasome by diffusion. The regulation of proteasome distribution between the cytoplasm and the nucleus is also unknown. This may either occur only during mitosis, when the nuclear envelope has disintegrated, or through active nuclear import. Nuclear targeting of the proteasome may be directed by some of the α subunits containing sequences for nuclear localization signals (NLSs) (reviewed by Tanaka *et al.*, 1990). These NLSs are able to direct reporter molecules to the nucleus *in vitro* and could, therefore, be functional *in vivo* to translocate proteasomes into the nucleus as intact complexes, although this has not been verified experimentally (Nederlof *et al.*, 1995).

In order to visualize the proteasome and to investigate

its intracellular distribution and mobility *in vivo*, we coupled the green fluorescent protein (GFP) to the C-terminus of the proteasome subunit LMP2. To follow proteasomal mobility, living LMP2–GFP-transfected cells were analysed by confocal microscopy. We show that proteasomes are divided over the nuclear and the cytoplasmic compartments, within which they rapidly diffuse. Fully assembled proteasomes can enter the intact nucleus either through the nuclear pore or during reassembly of the nuclear envelope after cell division.

Results

Quantitative incorporation of GFP-tagged LMP2 into proteasomes

HT1080 fibrosarcoma cells were stably transfected with a chimera of the proteasome subunit LMP2 with GFP attached to the C-terminus (LMP2–GFP). Since antisera raised against the C-terminus of LMP2 can detect intact proteasomes (Belich, 1996), we anticipated that the C-terminus of LMP2 would be exposed at the surface of the proteasome. To measure the relative amount of incorporation of LMP2–GFP into the proteasome, LMP2–GFP was detected in cell lysates prior to and after removal of proteasomes by three consecutive rounds of immunoprecipitation with the monoclonal antibody (mAb) MCP21 (recognizing the proteasome α subunit HC3/ α 2) (Hendil *et al.*, 1995). Western blotting with anti-GFP antisera showed that the complete LMP2–GFP fusion protein was quantitatively removed from the lysate by MCP21 (the dilution factor of the lysates due to immunoprecipitations was $<10\%$). Since LMP2–GFP was not detectable in the lysate after MCP21 precipitation, LMP2–GFP was incorporated quantitatively into proteasomes (Figure 1A). In addition, no higher molecular weight band that might correspond to unprocessed LMP2–GFP was observed.

To show that the GFP modification of LMP2 did not affect the generation of fully assembled proteasomes, proteasomes were immunoprecipitated with anti-GFP antibodies from lysates of the transfectant prior to, or after, 72 h of IFN γ treatment. IFN γ up-regulates the expression of LMP2 and LMP7, which replace the delta and MB1 subunits, respectively (Belich *et al.*, 1994). The immunoprecipitates were separated by SDS–PAGE, transferred to nitrocellulose membranes and the delta and LMP7 subunits were detected with specific antibodies as indicated (Figure 1B). As expected, LMP7 was incorporated after IFN γ treatment into LMP2–GFP-containing proteasomes. Moreover, the delta subunit was recovered from the GFP-containing proteasomes, indicating that these proteasomes can contain two β rings, one of which has incorporated subunit delta and the other containing LMP2–GFP. As anticipated, IFN γ treatment reduced the level of delta, due to the incorporation of endogenous LMP2.

Thus, LMP2–GFP appears to be incorporated quantitatively into fully assembled proteasome structures. This was confirmed further by separating cytosol from both the LMP2–GFP transfectant and a control GFP-only transfectant over a sucrose density gradient. LMP2–GFP migrated at a high density together with other proteasome subunits incorporated into proteasomes. No LMP2–GFP was detected at the density corresponding to low molecular weight proteins such as free GFP (not shown).

Fig. 1. Quantitative incorporation of GFP-tagged LMP2 into proteasomes. The human fibrosarcoma cell line HT1080 was stably transfected with a construct containing GFP coupled to the C-terminus of the proteasome subunit LMP2. (A) HT1080 cells and transfectants were lysed followed by three subsequent immunoprecipitations with MCP21, as indicated above the figure. Immunoprecipitates and total lysate before (lane 1) and after immunoprecipitation (lane 5) were analysed by 10% SDS–PAGE followed by Western blot analysis with anti-GFP antisera. (B) HT1080 cells (lanes C) and LMP2–GFP transfectants (lanes GFP) were cultured with or without 300 U/ml human IFN γ for 72 h and lysed. Immunoprecipitation with anti-GFP antisera was followed by Western blotting using delta and LMP7 antisera. (C) LMP2–GFP-containing proteasomes are active. Proteasomes were immunoprecipitated from HT1080 or HT1080–LMP2–GFP using either MCP21 (solid and dotted bars) or anti-GFP antisera (dashed and open bars) coupled to protein A–Sepharose beads. Equal amounts of proteasomes were assayed against 100 μ M of each of the peptide substrates z-Gly-Gly-Leu-AMC (chymotryptic activity) (solid and dashed bars) and z-Ala-Ala-Arg-AMC (tryptic activity) (dotted and open bars) and released fluorescence was determined. The experiments were performed in duplicate. Error bars are indicated.

GFP-tagged proteasomes are functional

To determine any effect of incorporation of LMP2–GFP on the catalytic activity of the proteasome, the peptidase activity of proteasomes isolated with either the mAb

Fig. 2. Distribution of GFP-tagged proteasomes in living cells. LMP2–GFP-transfected HT1080 cells were cultured and analysed by confocal microscopy in a tissue culture chamber at 37°C. Bar, 10 μ m.

MCP21 (against the α subunit HC-3/ α 2) or anti-GFP antisera was assayed. The chymotrypsin-like (Leu) and the trypsin-like (Arg) activities were determined using fluorogenic peptide substrates (Figure 1C). Similar levels of activity were observed from proteasomes isolated with either the anti-proteasome antibody (MCP21) or the anti-GFP antibody in the LMP2–GFP-expressing cell line. The parental HT1080 cell line, lacking LMP2–GFP, did not show any proteolytic activity after precipitating with anti-GFP antibody. The assay was performed on similar amounts of proteasomes, as determined by Western blot analysis (not shown). Incorporation of GFP-tagged LMP2 into proteasomes had little effect on the chymotryptic and tryptic activities. Similar assays with non-specific immunoprecipitates and GFP immunoprecipitates from the GFP-only-expressing cell line showed no peptidase activity (not shown).

Distribution of GFP-tagged proteasomes in living cells

Since LMP2–GFP was incorporated quantitatively into active proteasomes, we conclude that the fluorescence of LMP2–GFP-transfected cells corresponds to proteasomes and not to single LMP2–GFP subunits. The intracellular distribution of GFP-tagged proteasomes was determined by confocal scanning laser microscope (CSLM) on living cells cultured at 37°C (Figure 2). Green fluorescence was observed in both the cytoplasm and the nucleus of the transfectants. However, proteasomes were excluded from nucleoli, and less fluorescence was observed in a ring around the nucleus (the nuclear envelope) and within a perinuclear region. This perinuclear region corresponded to the ER/Golgi, as shown by fixing cells and staining with antibodies against various markers (TAP, calreticulin, PDI) for the ER (not shown). This membrane-rich region exhibits reduced fluorescence because the proteasome is not present within the lumen of the ER/nuclear envelope.

Mobility of proteasomes in the cytoplasm and nucleus

It is unclear whether proteasomes are sequestered as relatively immobile complexes or whether they move

freely through the cell. Furthermore, it is unknown whether proteins destined for degradation are transported to proteasomes or whether protease and substrate meet by continuous transport or diffusion. In order to address these questions, the mobility rate of GFP-tagged proteasomes in living cells was determined. A small region of the nucleus (indicated by a box in Figure 3A) was bleached by scanning this region for various periods, as indicated. After bleaching of the discrete boxed region for 10 or 30 s, the entire nucleus lost fluorescence, whereas the cytosol remained bright (Figure 3A). To verify that there was no leakage to non-bleached areas of laser light during bleaching, cells were fixed in methanol prior to applying the bleaching protocol (Figure 3C). Again the boxes denote the bleached areas. A well-defined region in both the nucleus and the cytoplasm (upper and lower cell respectively) was permanently bleached. Note that the pattern of fluorescence is markedly different from that of living cells because the fixation resulted in the precipitation of proteasomes. However, in living cells, the entire nucleus lost fluorescence within 30 s of bleaching of a small fraction of the nucleus, implying that most proteasomes moved through the bleached region within this period.

To study whether the proteasomes moved by active transport or by diffusion, the cells were depleted of ATP by pre-treatment with sodium azide (NaAz) and 2-deoxyglucose prior to the bleaching experiments. This is sufficient to arrest active transport processes, as shown by examining fluid phase endocytosis by the LMP2–GFP transfectant of the red fluorophore sulforhodamine 101 (SR101) upon pre-treatment (Figure 3D). Negligible amounts of SR101 were endocytosed after pre-treatment with NaAz and 2-deoxyglucose. Bleaching of a box in the nucleus of cells depleted of ATP resulted in loss of fluorescence in the entire nucleus (Figure 3A; bottom panel). Most proteasomes moved through the bleached box in the nucleus within this period in an ATP-independent fashion, indicating that proteasomes diffused rapidly throughout the nucleus.

The mobility of proteasomes in the cytosol was studied in a similar way. Bleaching of the boxed area in the cytosol for 30 or 90 s progressively reduced the fluorescence in the cytosol, but not in the nucleus (Figure 3B). Longer bleaching periods were required for the cytoplasm than for the nucleus, since the cytoplasm is considerably larger. ATP depletion, followed by bleaching of a part of the cytoplasm for 90 s (Figure 3B; bottom panel), also showed uniform loss of cytoplasmic fluorescence, indicating that proteasomes rapidly diffuse within the cytoplasm. The nuclear envelope apparently imposes a barrier, since rapid diffusion between cytoplasm and nucleus was not observed.

It is noteworthy that no cytosolic or nuclear areas outside the bleached region retained fluorescence upon bleaching, indicating that the majority of the proteasomes were freely mobile. To quantitate this, fluorescence recovery after photobleaching (FRAP) experiments were performed. The immobile fraction of proteasomes in the nucleus was calculated to be $3 \pm 2\%$ ($n = 10$). The same protocol was applied to the cytoplasm. Here $8 \pm 3\%$ ($n = 10$) of the fluorescent proteasomes were immobile. The higher immobile proteasome fraction within the cytosolic compartment might be due to a small quantity

Fig. 3. Mobility of proteasomes in the cytoplasm and nucleus. Living transfectants were analysed by confocal microscopy at 37°C. (A) A small region of the nucleus (indicated by the box) was bleached by slow scan for either 10 or 30 s as indicated. To deplete ATP, cells were incubated for 30 min with a mixture of NaAz and 2-deoxyglucose prior to the bleaching protocol (panel NaAz). (B) A small region of the cytoplasm (indicated by the box) was bleached by slow scan for either 30 or 90 s as indicated. Bottom panel: a mixture of NaAz and 2-deoxyglucose was used to deplete ATP prior to initiating the bleaching experiment. (C) To validate the bleaching procedure, cells were fixed with methanol prior to applying the bleaching protocol. The boxes denote the bleached cytoplasmic and nuclear areas. Bleaching of fixed cells results in a stable well-defined box not affected by further incubation for 40 min (right panel). (D) To control for depletion of ATP, living cells were treated for 30 min with a mixture of NaAz and 2-deoxyglucose and incubated with the fluid phase marker SR101 to demonstrate inhibition of endocytosis. Bar, 10 µm.

of proteasomes associated with ER membranes (Yang *et al.*, 1995).

Transport of proteasomes between cytoplasm and nucleus

Proteasome subunits are assembled in the cytoplasm, and most subunits, including LMP2, do not contain an NLS. It is thus unclear how proteasomes enter the nucleus. They may be transported through the nuclear pore or, alternatively, they may enter before assembly of the nuclear envelope during cell division. To study transport of GFP-tagged proteasomes over the nuclear membrane, the nucleus of the transfected cells was bleached (Figure 4A, middle panel) followed by culturing the cells at 37°C. Fluorescence slowly reappeared in the nucleus after long periods of culture (Figure 4A).

It is possible that the newly synthesized pool of (partially assembled) proteasomes is imported into the nucleus, rather than the pre-existing pool. To analyse this, the experiment was repeated in the presence of the translation inhibitor cycloheximide (Figure 4B). As before, some fluorescence in the nucleus reappeared, indicating that pre-formed proteasomes were imported slowly into the

nucleus. This was not due to restoration of the bleached GFP fluorophore because bleaching of the entire cell followed by culture for 4 h in the presence of cycloheximide, to prevent new synthesis of LMP2–GFP, did not show any return of fluorescence (data not shown). To analyse whether this transport is uni- or bidirectional, the cytoplasm was bleached and cells were cultured in the presence of cycloheximide (Figure 4C). After 3 h, no increase in fluorescence in the cytoplasm and no decrease in fluorescence in the nucleus was observed, consistent with unidirectional proteasome transport from the cytoplasm to the nucleus. Quantitation of fluorescence confirmed both the lack of recovery of fluorescence in the cytoplasm and the slow decay of nuclear fluorescence at the same rate as the surrounding cells (not shown).

Free GFP redistribution is bidirectional

To demonstrate that slow unidirectional transport from cytoplasm to nucleus is not a general phenomenon for intracellular proteins, we analysed the transport of GFP expressed in the HT1080 cell line. GFP is a small ~27 kDa protein which is below the exclusion size for passive transport through the nuclear pore complex, in contrast

Fig. 4. Proteasome transport between the cytoplasm and the nucleus. Living transfectants were bleached in the indicated region and cultured for 3 h in a tissue culture chamber at 37°C. Images were collected during this period by confocal microscopy. **(A)** The nuclei of two transfectants (indicated by arrows) were bleached by slow scan for 30 s (as indicated by the box). Timepoint $t = 0$ min is upon bleaching of the second nucleus (middle panel). The bottom panel is after 180 min. **(B)** Transfectant cells were incubated with cycloheximide before bleaching to inhibit synthesis of new proteasome subunits. The nuclei of two transfectant cells (indicated by arrows) were bleached by slow scan for 30 s (as indicated by the box). Timepoint $t = 0$ min is just after bleaching of the second nucleus (middle panel). The bottom panel is after 180 min. **(C)** Transfected cells were incubated with cycloheximide followed by bleaching of the cytoplasm for 90 s (as indicated by the box) to analyse for transport of proteasomes from the nucleus to the cytoplasm. Timepoint $t = 0$ min is just after bleaching of the cytoplasm (middle panel). The bottom panel is after 180 min of culture. Bar, 10 μ m.

to proteasomes (reviewed by Fabre and Hurt, 1994). Expression of untagged GFP in HT1080 cells gave a fluorescence distribution very similar to that of GFP-tagged proteasomes. Fluorescence was distributed equally, with the exception of the nucleoli and the nuclear envelope/ER region where the fluorescence was reduced (Figure 5). Bleaching of the nucleus or the cytoplasm rapidly resulted in loss of fluorescence outside the bleached region (Figure 5; middle panel). Bleaching of the nucleus was followed by redistribution of fluorescence within 4 min (Figure 5A), and bleaching of the cytosol resulted in a similar redistribution pattern (Figure 5B). The redistribution of fluorescence after bleaching was also observed after depletion of ATP with NaAz and 2-deoxyglucose (not shown). Thus GFP diffused rapidly both within and between the nuclear and cytosolic compartments, unlike proteasomes.

Proteasome mobility during cell division

Slow transport of proteasomes from the cytoplasm to the nucleus is not necessarily the only mechanism for nuclear import. Proteasomes may also enter before assembly of the nuclear membrane, after cell division. LMP2-GFP transfectants were analysed at different stages throughout cell division (Figure 6). Cells in the process of division have a markedly different appearance from cells in G_1 because they round up (compare with Figure 2). In addition, the nuclear envelope, ER and nucleoli cannot be distinguished, but the chromosomes can still be seen as areas excluding GFP-tagged proteasomes. A cell in metaphase shows alignment of the chromosomes on the

mitotic spindle (Figure 6A). When the indicated region was bleached for 90 s, loss of fluorescence was observed throughout the cells, demonstrating the absence of diffusion barriers (such as the nuclear membrane) during metaphase. Figure 6B shows two cells after completion of cell division. They still contain decondensed chromosomes visible as a negative image that excludes proteasomes (also observed with visible light; not shown). Bleaching within the outlined box for 90 s resulted in a loss of fluorescence in the entire cell, but not the adjacent daughter cell. Later, during completion of the cell cycle (as shown in Figure 6C), the nuclear envelope has assembled, although the nucleoli have not been fully formed. Bleaching of the box in the cytoplasm or the nucleus for 90 or 30 s respectively only decreased the fluorescence in these compartments, indicating that the sealed nuclear envelope prevented rapid exchange of proteasomes between the nucleus and the cytoplasm. Apparently GFP-tagged proteasomes move by diffusion through the entire metaphase cell until the nuclear envelope has been reconstituted.

Discussion

Proteasomes are large, abundant proteinase complexes involved in non-lysosomal degradation of proteins. Proteasomes are found within both the cytoplasm and the nucleus, but it was unclear whether they are free or associated with other proteins, cytoskeletal components or ER membranes. Association of proteasomes with a cellular scaffold would imply that proteins destined for degradation are targeted to these complexes. Alternatively,

Fig. 5. Mobility of GFP in the cytoplasm and nucleus. Living HT1080 cells transfected with GFP only were analysed by confocal microscopy at 37°C. (A) A small region of the nucleus (indicated by the box) was bleached by slow scan for 10 s (middle panel). The bottom panel is the bleached cell after 4 min of culture. (B) A small region of the cytoplasm (indicated by the box) was bleached by slow scan for 30 s (middle panel). The bottom panel is the bleached cell after 4 min of culture. Bleaching of either the nucleus or the cytoplasm shows rapid redistribution of fluorescence within 4 min. Bar, 10 μ m.

if proteasomes existed freely within the cell, their substrates could interact by collision with no need for a targeting mechanism. In addition, it is unclear how proteasomes enter the nucleus, as only a few α subunits contain a putative NLS. They may be transported through the nuclear pore or they may enter the nucleus during reassembly after cell division.

To visualize and follow the mobility of the proteasome in living cells, GFP was linked to the C-terminus of LMP2 (LMP2-GFP). The fusion of GFP with LMP2 did not prevent its incorporation into the complex, as LMP2-GFP is quantitatively associated with functional proteasomes. Further analysis of the GFP-tagged proteasomes showed that the delta subunit could also be recovered through precipitation of LMP2-GFP. Since delta is exchanged for LMP2 upon IFN γ stimulation, at least a fraction of the isolated 20S GFP-tagged proteasomes contain >14 different subunits, implying that the two seven-membered β rings of one proteasome can contain different subunits without perfect 2-fold symmetry.

Previous immunohistochemical studies using fixed cells showed proteasomes to be located in the cytoplasm and parts of the nucleus, occasionally associated with cytoskeletal components (Palmer *et al.*, 1994). This contrasts with our experiments using living cells, which show proteasomes equally distributed in the nucleus, although excluded from the nucleoli, in the cytoplasm, and at a reduced level in the ER/Golgi region and nuclear envelope (since proteasomes are excluded from the ER lumen). The differences between these observations can be explained by effects of fixation, as proteasomes may precipitate on cytoskeletal components. Furthermore, we observed no change in distribution of proteasomes upon treatment with

Fig. 6. Proteasome mobility during cell division. Living transfectants were observed by confocal microscopy at 37°C. (A) A cell in metaphase shows alignment of the chromosomes on the mitotic spindle. The indicated region was bleached for 90 s resulting in a general loss of fluorescence (right panel). (B) Two cells after completion of cell division, still containing decondensed chromosomes. The indicated region was bleached for 90 s, resulting in a general loss of fluorescence of the bleached cell (right panel). (C) Cells during completion of the cell cycle, when the nuclear envelope has assembled. The box in the cytoplasm or the nucleus was bleached for 90 or 30 s respectively. Only the bleached compartments lost fluorescence. Bar, 10 μ m.

IFN γ , suggesting that incorporation of different subunits into the proteasome does not influence cellular localization of proteasomes.

Bleaching experiments using transfectants showed that the GFP-labelled proteasomes diffused rapidly within the nucleus and the cytosol, although they diffuse ~ 7 times more slowly than free GFP (not shown). FRAP experiments to quantitate the mobile fraction indicated that virtually all nuclear proteasomes were mobile ($97 \pm 2\%$), whereas the mobile fraction in the cytoplasm was slightly less ($92 \pm 3\%$). This small difference may be due to the small amount of proteasomes associated with ER membranes (Rivett *et al.*, 1992; Yang *et al.*, 1995). Proteasomes are involved directly in generating peptides for antigen presentation (Rock *et al.*, 1994), but it is unclear whether peptides are generated at specific locations and freely diffuse to TAP for entry in the ER, or are transported to TAP by specialized chaperones like hsp70 (Srivastava *et al.*, 1994). Alternatively, proteasomes associated with TAP may be responsible for the generation of antigenic peptides. The observed diffusion of proteasomes in the cytoplasm suggests that antigens can be degraded everywhere within the cell. The resulting peptides probably diffuse at similar rates and will contact the high affinity peptide-binding site of TAP (Endert *et al.*, 1995) in the ER membrane, which is present throughout the cytoplasm. Since nuclear antigens are presented efficiently by MHC class I molecules, they may be degraded by diffusing nuclear proteasomes, with the resulting peptides passing through the nuclear pore into the cytoplasm (as shown for free GFP) before binding to TAP. It is unclear

whether chaperones like hsp70 or other proteins are required for transfer of these peptides to TAP.

During metaphase, and just after cell division, the nuclear envelope disperses, removing the diffusion barrier between cytoplasm and nucleus and enabling proteasomes to migrate throughout the entire cell. Upon nuclear envelope formation, which seems to occur at a later stage than formation of the plasma membrane between the two daughter cells, proteasomes are again sequestered within either the cytoplasm or the nucleus. The nuclear envelope forms a diffusion barrier for GFP-tagged proteasomes but not for small proteins like GFP (or free proteasomal subunits), which can diffuse rapidly from the cytoplasm to the nucleus and vice versa, albeit at a slower rate than within the respective compartments. The intact nuclear envelope only allows very slow, unidirectional import of GFP-tagged proteasomes. Thus, proteasomes enter the nucleus both by rapid exchange during cell division and by slow import after assembly of the nuclear envelope.

Apparently, the nuclear pore is able to translocate large protein complexes like the proteasome, possibly mediated by NLS signals in α subunits or through signals contained within associating complexes such as the 19S activator. The 20S (yeast) proteasome is $113 \text{ \AA} \times 148 \text{ \AA}$ and the channel in the nuclear pore has been reported to be $260 \text{ \AA}$ in diameter (Feldherr *et al.*, 1984; Gerace, 1992). This allows transit of the proteasome without affecting its structure. Since the turnover of proteasomes is reported to be very slow (Tanaka and Ichihara, 1989), nuclear import should also be slow to enable balanced replacement of older proteasomes. However, during cell division, the cytoplasmic pool of proteasomes rapidly mixes with the nuclear pool until the nuclear envelope has reassembled. Although transport between the cytosolic and nuclear compartment is slow, proteasomes diffuse rapidly within the two compartments. This suggests that proteins marked for degradation by the proteasome do not require specific targeting but may encounter the proteasome by simple collision. Diffusing proteasomes will interact continuously with cytosolic or nuclear proteins, and perform quality control to degrade the proteins that are either tagged by ubiquitination, or otherwise already in an appropriate conformation for degradation. Our data suggest that substrate-specific recognition is sufficient for the proteasome to degrade its targets without the need for directed transport.

Materials and methods

Antibodies

Polyclonal rabbit anti-GFP serum was raised against a fusion protein of GST-GFP (kindly provided by Dr K.Sawin, ICRF, London, UK). Polyclonal rabbit anti-LMP2, anti-LMP7 and anti-delta sera were raised against carboxy-terminal peptides from LMP2, LMP7 and delta, respectively (kindly provided by Dr M.Belich, ICRF). Proteasomes were precipitated using the mAb MCP21 (kindly provided by Dr K.Hendil, August Krogh Institute, Copenhagen, Denmark) which recognizes the α subunit HS3.

Construction of the LMP2-GFP fusion protein and transfection

The human LMP2 cDNA was derived from an 800 bp full-length LMP2 cDNA insert (Kelly *et al.*, 1991). Fluorescence activated cell sorting (FACS)-optimized mutant GFP cDNA was kindly provided by Dr B.Cormack (Department of Microbiology and Immunology, Stanford

University School of Medicine, Stanford, CA). An LMP2 fragment without the final stop codon was generated by PCR, with a *Bam*HI site at the 5' end (using oligo 5'CGCGGATCCGCGATGCTGCGCGCGGG-AGAAGTC), ending with the first codons of GFP (using oligo 5'TCCTT-TACTCATCTCATCATAGAATTTTGGCAG). A GFP fragment was created by PCR with the end of LMP2 (without the stop codon) at the 5' end (using oligo 5'TTCTATGATGAGATGAGTAAAGGAGAAGAA-CTT) and an *Eco*RI site at the 3' end (using oligo 5'CGGAATTCGGTTTGTATAGTTCATCCATGCC). The amplified fragments served as primers for the complementary strands, creating an LMP2-GFP fusion protein which was cloned into pcDNA3 (Invitrogen, San Diego, CA) by *Bam*HI-*Eco*RI digest and checked by sequence analysis.

HT1080 (human fibrosarcoma) cells were transfected using the Lipofectamine procedure (Gibco Life Technologies, Paisley, UK). Positive clones were selected for fluorescent appearance and maintained in Iscove's medium supplemented with 7.5% fetal calf serum (FCS) and G418 (0.4 mg/l).

Immunoprecipitation and Western blotting

Cells were washed twice with phosphate-buffered saline (PBS) at a concentration of 3×10^7 /ml, and lysed for 30 min at 4°C in 0.5% NP-40-containing lysis buffer (0.01 M Tris-HCl pH 8.0, 0.14 M NaCl, 0.025% NaAz, 5 mM iodoacetamide, 2 mM phenylmethylsulfonyl fluoride). Lysates (100 μ l/sample) were cleared by centrifugation for 30 min at 10 000 g and pre-cleared twice for 1 h with glycine-Sepharose CL-4B beads (Pharmacia, Uppsala, Sweden). Immunoprecipitations were performed using either 2.5 μ l of the mAb MCP21 or GFP antisera and washed twice in lysis buffer with NP-40, once with TSA (0.01 M Tris-HCl pH 8.0, 0.14 M NaCl, 0.025% NaAz) and once with 0.05 M Tris-HCl pH 7.5. In this protocol, the original lysate was diluted <10%. Pellets were dissolved in SDS sample buffer, separated by SDS-PAGE and transferred to PVDF membranes. Blots were blocked for 1 h in PBS, 0.2% Tween-20 (PBS/T) and 5% non-fat milk, and incubated with specific antibodies for 1 h at room temperature. Blots were washed four times with PBS/T for 10 min, and incubated with peroxidase-conjugated second antibodies for 45 min. Non-bound antibodies were removed by washing, and visualization was performed by enhanced chemiluminescence (Amersham, UK).

Proteasome activity assay

Approximately 2×10^7 cells were washed three times with PBS and pellets were lysed in a hypotonic buffer (10 mM triethanolamine, 10 mM acetic acid, 1 mM EDTA, 0.25 M sucrose pH 7.4), disrupted using an EMBL cell cracker and subsequently kept at 4°C. Equal amounts of supernatant (corresponding to 300 μ g of protein) were used per immunoprecipitation. Overnight pre-clearance with normal mouse serum and protein A beads was followed by another round of protein A for 2 h. Immunoprecipitations were done for 1.5 h using either MCP21, GFP antisera or Tap 1 antisera as a negative control. After washing five times, the pellets were split into equal portions and assayed against 100 μ M of the peptide substrates z-Ala-Ala-Arg-AMC and z-Gly-Gly-Leu-AMC [both in dimethylsulfoxide (DMSO) carrier] (Novabiochem and Bachem, Switzerland) in a total volume of 200 μ l of hypotonic buffer at 37°C. Aliquots of 10 μ l in duplicate were quenched into 1 ml of ethanol after 8 h of incubation, and the fluorescence of the free AMC ($\lambda_{\text{excitation}} = 370 \text{ nm}$, $\lambda_{\text{emission}} = 460 \text{ nm}$) measured on a spectrofluorimeter (Perker Elmer, The Netherlands). Incubation buffer with peptides and incubation buffer with control immunoprecipitates gave negligible fluorescence.

Confocal analysis and bleaching

Living cells were analysed in a tissue culture device at 37°C using a 600MRC confocal microscope equipped with an argon-krypton laser (BioRad Labs, Hercules, CA). Green fluorescence was detected at $\lambda > 515 \text{ nm}$ after excitation at $\lambda = 488 \text{ nm}$. Cells were incubated for 1 min before and throughout the rest of the analysis period with cycloheximide (200 μ M) or for 30 min before analysis with a mixture of NaAz (0.05%) and 2-deoxyglucose (50 μ M). Endosomes were labelled by internalization of SR101 (25 μ g/ml) (Wubbolts *et al.*, 1996). To bleach GFP-tagged proteasomes in living cells, a small region of the nucleus or the cytoplasm was scanned for the times indicated. Cells were fixed for 5 min with methanol at -20°C, followed by washing with PBS before analysis by confocal microscopy.

Fluorescence recovery after photobleaching

To quantitate the mobile fraction of GFP-tagged proteasomes, FRAP experiments were performed (Edidin *et al.*, 1976; Cole *et al.*, 1996). A

box in the nucleus was bleached and the effect on the fluorescence in this box versus a number of boxed regions outside the bleach box was quantitated. The latter boxes were averaged to compensate for local variations. From these data, the immobile fraction can be determined using the formula $\% \text{immobile} = (F_{\text{Bafter}}/F_{\text{Bbefore}}) - (F_{\text{Aafter}}/F_{\text{Abefore}})$. F_{A} is the fluorescence in the bleach box and F_{B} the fluorescence in the box outside this region; the ratio of F_{A} and F_{B} corrects for differences in the thickness of the boxes (the z -direction). The fluorescence is determined before and after bleaching, and whereas both F_{A} and F_{B} will decrease due to equal bleaching of the mobile fraction, only F_{A} will lose the immobile fraction.

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