# Proteasomes degrade proteins in focal subdomains of the human cell nucleus

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## Summary

**The ubiquitin proteasome system plays a fundamental role in the regulation of cellular processes by degradation of endogenous proteins. Proteasomes are localized in both, the cytoplasm and the cell nucleus, however, little is known about nuclear proteolysis. Here, fluorogenic precursor substrates enabled detection of proteasomal activity in nucleoplasmic cell fractions (turnover 0.0541 μM/minute) and nuclei of living cells (turnover 0.0472 μM/minute). By contrast, cell fractions of nucleoli or nuclear envelopes did not contain proteasomal activity. Microinjection of ectopic fluorogenic protein DQ-ovalbumin revealed that proteasomal protein degradation occurs in distinct nucleoplasmic foci, which partially overlap with signature proteins of subnuclear domains, such as splicing speckles or promyelocytic leukemia bodies, ubiquitin, nucleoplasmic proteasomes and RNA polymerase II. Our results establish proteasomal proteolysis as an intrinsic function of the cell nucleus.**

Key words: Cell nucleus, Nuclear bodies, Nucleolus, Proteasomes, Proteolysis, Ubiquitin-proteasome system

## Introduction

Intracellular proteolysis is executed by complex biochemical machineries that play a crucial role in cellular events, such as the cell cycle, signal transduction, gene expression, development, maintenance of proper protein folding and antigen processing (Kirschner, 1999). Eukaryotic cells contain two major pathways of protein degradation. Exogenous proteins that are taken up by endocytosis, and membrane proteins are degraded via the lysosomal pathway into peptides. The majority of intracellular proteins, however, are proteolysed by the ubiquitin-proteasome pathway. In most cultured mammalian cells 80-90% of the protein breakdown occurs by the ubiquitin-proteasome pathway (Lee and Goldberg, 1998), whereas inhibitors of lysosomal proteolysis block only 10-20% of total protein degradation (Gronostajski et al., 1985). The ubiquitin-proteasome pathway provides for cellular quality control since it prevents accumulation of misfolded proteins and protein aggregation diseases (Goldberg, 2003). Hydrolysis of proteins requires a high-molecular-mass proteolytic complex, called proteasome, which utilizes ATP (Hershko et al., 1984). Proteasomes were identified as large 2500 kDa structures (26S), containing a 700 kDa cylindrical core particle (20S), that harbours six active sites in its cavity and is flanked by two 900 kDa regulatory domains (19S). This 19S-20S-19S architecture sequesters the catalytic sites from potential substrates, defining the proteasome as a self-compartmentalizing protease (Baumeister et al., 1998). According to the current 'two substrate'-model ubiquitinated or denatured proteins are: (1) recognized by the 19S regulatory subunits; (2) unfolded; (3) channeled via a central passageway into the degradation chamber; (4) degraded into peptides; and (5) released through the entry channel (Finley, 2002; Hutschenreiter et al., 2004). The mammalian proteasome provides up to five different peptidase activities, including those peptide-hydrolysing activities commonly referred to as chymotrypsin-, trypsin- and caspase-like. Proteasome activity can be analysed with a variety of different substrates, including fluorogenic peptides (Driscoll et al., 1993; Gaczynska et al., 1993).

Proteasomes are abundant multisubunit proteases (approximately 0.6% of total cell protein in HeLa cells) that are found in the cytoplasm, both free and attached to the endoplasmatic reticulum (ER), and in the nucleus of eukaryotic cells (Wojcik and DeMartino, 2003). Nuclear localization signals (NLSs) in human α-type subunits of the 20S core particle, and tyrosine phosphorylation of the same subunits direct translocation of proteasomes into the cell nucleus (Nederlof et al., 1995). In the cell nucleus proteasomes are present throughout the nucleoplasm, concentrating in subnuclear structures such as splicing speckles (Chen et al., 2002), PML nuclear bodies (Anton et al., 1999; Lallemand-Breitenbach et al., 2001; Rockel and von Mikecz, 2002) and clastosomes (Lafarga et al., 2002), while nucleoli are devoid of proteasomes (Reits et al., 1997; Wojcik and DeMartino, 2003). In particular conditions, such as elevated Myc expression (Arabi et al., 2003; Welcker et al., 2004), and altered localization of nuclear protein PML (Mattson et al., 2001), proteasomes also accumulate in nucleoli. However, a pilot proteomic analysis has corroborated that nucleoli do not contain proteasomes in basal cells (Andersen et al., 2002). Besides proteasomes, the cell nucleus also contains all components of the ubiquitin-proteasome system, such as ubiquitin (Anton et al., 1999), the 19S (Peters et al., 1994) and

11S regulator subunits (Lallemand-Breitenbach et al., 2001) and the ubiquitin-attachment machinery, e.g. HDM2 (Lain et al., 1999). The ubiquitin-conjugating enzyme UbcM2 is imported into nuclei by the transport receptor importin-11 (Plafker et al., 2004).

While it is undisputed that proteasome-dependent degradation occurs in the cytoplasm, the function of nuclear proteasomes has only recently come into the focus of scientific interest. Consistent with the idea of nuclear proteolysis it has been shown that mutated forms of influenza virus nucleoprotein (NP) misfold and rapidly cluster with proteasomes in subnuclear structures known as PML bodies (Anton et al., 1999). PML bodies represent putative proteolytic centres in the nucleus since interferon γ and virus infection induce recruitment of proteasomes, regulator 11S (Fabunmi et al., 2001), and misfolded proteins to this location (Wojcik and DeMartino, 2003). Substrates that were reported to be degraded within the cell nucleus include: (1) transcriptional repressor Matα2 whose rapid degradation is observed only when the protein is efficiently imported into the nucleus (Lenk and Sommer, 2000); (2) skeletal muscle transcription factor MyoD, a physiological substrate of the ubiquitin proteasome pathway (Floyd et al., 2001); and (3) p53, which is degraded by nuclear proteasomes in stress conditions, such as DNA damage (Shirangi et al., 2002).

Proteasomal proteolysis within the cell nucleus may enable a tight regulation of nuclear function. This concept is substantiated by recent reports that suggest control of transcription by the ubiquitin-proteasome system (Muratani and Tansey, 2003). Ubiquitin positively regulates mammalian coactivator CIITA by enhancing its assembly at the MHC class II promoter (Greer et al., 2003). Specific proteasome inhibitors strongly inhibit oestrogen receptor-mediated transcription and block cycling of the oestrogen receptor, and other transcription factors, on and off oestrogen-responsive promoters (Reid et al., 2003). Co-immunoprecipitation experiments reveal a physical association between RNA polymerase II and the proteasome at sites of stalled transcription (Gillette et al., 2004). Thus, Gillette et al. propose the hypothesis that 26S proteasomes associate with transcriptionally active genes at the 3′ ends of those genes and sites of DNA damage.

Despite the mounting evidence for mechanistic connections between nuclear function and the ubiquitin-proteasome system, the localization of proteasomal proteolysis in the cell nucleus is unknown. In general it is not clear if nuclear substrates are hydrolysed in distinct subnuclear domains or transported to the cytoplasm for degradation. A unique feature of the cell nucleus is its composition of visually and functionally defined subnuclear compartments, such as the nuclear envelope (Wilson, 2000), nucleoli (Scheer and Hock, 1999), splicing speckles (Spector, 1993), PML bodies (Zhong et al., 2000) and Cajal bodies (Gall, 2000). We have shown previously that proteasomal degradation of nuclear proteins is correlated with their localization in the nucleus, and that nuclear substrate proteins accumulate in their 'resident' subnuclear compartments upon inhibition of the ubiquitin-proteasome pathway (Chen et al., 2002; Rockel and von Mikecz, 2002). Here we report confinement of proteasomal activity to specific nuclear compartments, and introduce an in situ proteolysis assay, which demonstrates that proteasomes degrade proteins in distinct subnuclear foci.

## Materials and Methods

### Cell culture and treatment

Human epithelial HEp-2 cells, epidermoid KB cells and mouse L929 fibroblast were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Primary dermal fibroblasts F38 were isolated from foreskin as described before (Berneburg et al., 1999). Human keratinocytes (HaCaT) were obtained from Petra Boukamp (Boukamp et al., 1988). All cells were grown as recommended to 70-80% confluence and detached by trypsinization. Cell viability was assessed by trypan blue exclusion. Where indicated, cells were co-incubated with 1-10 μM lactacystin (Alexis Biochemicals, CA), or 5-20 μM MG132 (Merck Bioscience, Darmstadt, Germany) for 4 hours to inhibit proteasomal activity.

### Immunofluorescence

Cells were seeded on coverslips, grown to subconfluence, fixed with methanol/acetone or 3.7% formaldehyde/0.25% Triton X-100, and subjected to indirect immunofluorescence (IIF) as described previously (Chen et al., 2002) with the following primary antibodies: mouse monoclonal antibody (mAb) PW8195 to 20S proteasome α subunits and rabbit polyclonal antibody (pAb) PW8155 to 20S proteasome α and β subunits (Affiniti, Exeter, UK), mAb Tub 2.1 to β-tubulin (Sigma, St Louis, MO), mAb αSC35 to SC35 (BD Bioscience, San Jose, CA), mAb 72B9 to fibrillarin (Reimer et al., 1985), mAb PG-M3 to PML, mAb 636 to Lamin A/C and mAb P4D1 to ubiquitin (Santa Cruz Biotechnology, CA), and mAb mara3 to RNA Polymerase II (Patturajan et al., 1998) (kindly donated by Bart Sefton, Salk Institute, La Jolla, CA). Secondary anti-mouse and anti-rabbit antibodies conjugated with fluorescein isothiocyanate (FITC) or CY5 were purchased from Jackson Immuno Research Laboratories, PA.

### Cell fractionation

Cytoplasmic, nuclear and nucleolar fractions were prepared from HEp-2 cells, using a method described by Harris Busch and colleagues (Rothblum et al., 1977) with the following modifications. Aliquots (1 ml) containing ~$1\times10^7$ HEp-2 cells were washed in PBS, resuspended in 4 ml RSB-8 buffer (0.01 M Tris-HCl, 0.01 M NaCl, 8 mM MgAc, pH 7.4) and incubated for 30 minutes at 4°C. The cells were centrifuged at 1000 ***g*** for 8 minutes at 4°C, resuspended in 4 ml RSB-NP40 (0.01 M Tris-HCl, 0.01 M NaCl, 1.5 mM MgAc, pH 7.2, 0.5% NP40) and dounce homogenized using a tight pestle. Dounced cells were centrifuged at 800 ***g*** for 8 minutes at 4°C. The supernatant was collected as cytoplasmic fraction and the nuclear pellet was resuspended in 1 ml 0.88 M sucrose, 5 mM MgAc and centrifuged at 2500 ***g*** for 20 minutes at 4°C. Nuclei were checked by phase contrast microscopy to exclude residual intact cells, resuspended in 1 ml 0.34 M sucrose containing 0.5 mM MgAc and sonicated for 10×0.5 seconds using a microtip probe sonicator (Labsonic U, B. Braun Medical, Bethlehem, PA) at 'low' power setting. The sonicated sample was then layered over 3 ml 0.88 M sucrose and centrifuged at 3000 ***g*** for 20 minutes at 4°C. The pellet contained the nucleoli, while the supernatant consisted of the nucleoplasmic fraction. Purity of cell fractions was determined by immunoblotting of signature proteins for subcellular compartments. Equal loading of protein samples was controlled by Coomassie blue (gel) and Ponceau S (nitrocellulose) staining. Concentration of total protein in cell fractions was determined by Bradford assays.

### Nuclear envelope preparation

Nuclear envelope fractions were prepared from HEp-2 cells, using a method based on that first decribed by Gerace and Blobel (Gerace and Blobel, 1982). Briefly, nuclei from ~$1\times10^7$ cells were collected and resuspended in 10 mM triethanolamine-HCl, 292 mM sucrose, 0.1 mM $MgCl_2$, pH 8.5 including 1 μg/ml DNase I. The suspension was

incubated for 15 minutes at 22°C, layered over 10 mM ethanolamine-HCl, 876 mM sucrose, 0.1 mM $MgCl_2$, pH 7.5, and centrifuged at 13,000 ***g*** for 10 minutes at 4°C. The pellet was resuspended in 10 mM triethanolamine, 292 mM sucrose, 0.1 mM $MgCl_2$, pH 7.5. DNase I (5 μg/ml) was added for 15 minutes at 22°C. The digested nuclei were recovered by centrifugation at 13,000 ***g*** for 10 minutes at 4°C. The pellet was resuspended in 10 mM triethanolamine, 292 mM sucrose, 0.1 mM $MgCl_2$, pH 7.5, extracted with Triton-X100 [final concentration of 2% (w/v)], incubated for 10 minutes at 4°C and centrifuged at 10,000 ***g*** for 10 minutes at 4°C. The pellet was resuspended in 2 M NaCl, 100 mM triethanolamine-HCl, pH 7.5 and collected as nuclear envelope fraction. Purity of nuclear envelope fractions was tested by immunoblotting with signature proteins lamin A/C.

### Immunoblotting

Protein amount of cell fractions was determined according to Bradford: cytoplasmic fraction 275.3 μg/μl, nucleoplasmic fraction 289.7 μg/μl, nucleolar fraction 215.7 μg/μl, and nuclear envelope fraction 197.5 μg/μl. Equal volumes of cell fractions were separated by SDS-PAGE, transferred to Hybond N (Amersham, Arlington Heights, IL), and reacted with either of the following: mAb PW8195 to 20S proteasome α-subunits (Affiniti, Exeter, UK), mAb Tub 2.1 to β-tubulin (Sigma, St Louis, MO), mAb 636 to Lamin A/C (Santa Cruz Biotechnology, CA), mAb αSC35 to SC35 (BD Bioscience, San Jose, CA), rabbit pAb to fibrillarin (Chen et al., 2002), mAb PC-M3 to PML (Santa Cruz) in PBS containing 0.5% Tween 20 with 5% nonfat dried milk. Protein transfer and sample loading was controlled by Ponceau S staining. Specific proteins were visualized using enhanced chemoluminescence according to the manufacturer's instructions (Amersham).

### Proteasome activity assays

Proteasomal acitivity in cell fractions was determined by cleavage of the fluorogenic precursor substrate N-Succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (Suc-LLVY-AMC; Affiniti, Exeter, UK). 10 μM substrate was added to cellular fractions (~$1\times10^6$ cells), and incubated in a reaction buffer (500 mM Hepes, 10 mM EDTA, pH 7.6). Fluorescent increase resulting from degradation of Suc-LLVY-AMC at 37°C was monitored over time by means of a fluorometer (Fluoroscan Ascent, Thermo Labsystems, Santa Fe, NM) at 340 nm excitation and at 460 nm emission, using free AMC as a standard. Resulting product curves were followed for up to 24 hours. Each value of fluorescence intensity represents a mean value obtained from three independent experiments. After 2 hours of activity measurement, 10 μM proteasome inhibitor lactacystin (Alexis Biochemicals, San Diego, CA) was applied to the substrate/lysate reaction where indicated. Measurement of proteasomal activity in living cells was performed by microinjection of Suc-LLVY-AMC into the cell nucleus.

### Turnover number of nuclear proteasomes

To determine the enzyme activity subcellular fractions were incubated with an excess of Suc-LLVY-AMC substrate (10 mM) (Dahlmann et al., 1993) at 37°C for 115 minutes and fluorescence signals were calibrated against a free AMC standard. The enzyme activity (EA) was calculated by the gradient of linearization of the activation curve. To determine the turnover number (TN) of proteasomal hydrolysis, the protein content of cellular fractions was analysed by Bradford protein assay. We defined the absolute amount of 26S proteasomes (P) based on their molecular weight (2.1 MDa) (Tanaka and Tsurumi, 1997), and the condition that 0.6% of the whole protein amount in HeLa cells represents 26S proteasomes (Hendil, 1988). Proteasomal (TN) was calculated by the following equation:

$$\text{EA}\ (\mu\text{mol}\times\text{min}^{-1})\ /\ \text{P}\ (\mu\text{mol}) = \text{TN}\ (\mu\text{mol}\times\text{min}^{-1}\ \mu\text{mol}^{-1})\ .$$

To estimate in vivo proteasomal enzyme activity fluorescence data obtained from the living cell substrate accumulation experiments were normalized and compared with fluorescence data acquired in the in vitro experiments.

### Proteasomal activity, protein degradation in living cells

HEp-2 cells were seeded onto coverslips and grown to subconfluence. For proteasomal activity assays the proteasome substrate Suc-LLVY-AMC (Affiniti, Exeter, UK) was microinjected into the cell nucleus by micromanipulation (InjectMan NI2, Eppendorf, Hamburg, Germany). Caspase-3 specific substrate Ac-DEVD-AMC (BD Bioscience, San Jose, CA) or proteasome-predigested Suc-LLVY-AMC were microinjected as specificity and negative controls, respectively. All substrates were dissolved in DMSO and diluted to a final concentration of 10 μM in PBS. Approximately 5% of the cell volume was injected. Each experiment was done in triplicate. Fluorescent signals were detected by epifluorescent light microscopy. For in situ localization of proteasomal protein degradation in subnuclear compartments DQ ovalbumin (DQ-OVA) or predigested DQ-OVA was dissolved in PBS to a final concentration of 0.5 mg/ml and microinjected into HEp-2 cells. DQ-OVA is a fluorogenic substrate for proteases. A strong fluorescence quenching effect is observed when proteins are heavily labelled with BODIPY dyes. Upon hydrolysis of the DQ ovalbumin to single, dye-labelled peptides by proteases, this quenching is relieved, producing brightly fluorescent products. Accumulation of DQ-OVA fluorescence was measured in microinjected cell nuclei of untreated and lactacystin-treated cells after 5-15 minutes incubation and fixation with methanol/acetone. DQ-OVA peptides (predigested DQ-OVA) were produced by preincubation of DQ-OVA for 1 hour at 37°C with 0.05 mg/ml Trypsin-EDTA (Invitrogen, Karlsruhe, Germany). The resulting DQ-OVA peptides were heat activated for 20 minutes at 70°C, cooled down to RT and injected into the cells. Time lapse analysis of fluorescent signals in living cells were acquired with low laser intensity (5%) to minimize phototoxic effects. In situ localization of proteasomal degradation was performed by double labelling of DQ-OVA with antibodies against signature proteins of subnuclear domains, ubiquitin and proteasomes. Cells were fixed 10 minutes post microinjection. For endocytic uptake of DQ-OVA HEp-2 cells were grown in RPMI medium containing 200 μg/ml DQ-OVA for 30 minutes. The cells were washed with PBS and analysed in RPMI/10% FCS.

### Microscopy

Images showing the Suc-LLVY-AMC accumulation in living cells were acquired with a Zeiss Axiovert 100 TV fluorescence microscope using a 60× oil objective (Plan-Neofluar, Zeiss, Jena, Germany) with a charge coupled device (CCD) camera (ORCA II, Hamamatsu, Bridgewater, NJ). AMC and DAPI were excited at 330-385 nm and emission detected at 420-460 nm. All other images were obtained with a confocal laser scanning microscope from Olympus (Fluoview 2.0, IX70 inverted microscope; Lake Success, NY) using a 60× oil objective (UPlanFl, Olympus). FITC and DQ-OVA were excited at 488 nm and emission was detected between 510 and 550 nm. Cy5 was excited at 647 nm and emission was detected above 660 nm. Controls established the specificity of fluorochrome-conjugated antibodies for their respective Igs, and that signals in green, red and far-red channels were derived from the respective fluorochrome. No cross talk was observed in double-staining experiments.

### In situ quantification of fluorescence signals

Quantitative analysis of fluorescence intensity in subcellular domains was determined using the Metamorph image analysis software

package (Universal Imaging Corp., West Chester, PA) as described recently (Chen et al., 2002). Briefly, regions of interest (ROIs) were positioned manually according to corresponding differential interference contrast (DIC) images. Images were background-corrected by reference regions outside the cells, but within the field of view, which matched with identical-sized ROIs. In double labelling experiments signals were defined as 'yellow' and co-localizing in the range of red 108-255, green 108-255, blue 0-255 (RGB colour model). For each experiment, the fluorescence intensities of 100-200 microinjected cells were determined. Cells that were not injected or microinjected exclusively into the cytoplasm were excluded from quantification.

### Statistical analysis

For statistical analysis of fluorescence data the software GraphPad Prism (GraphPad Software, San Diego, CA) was used. Differences in mean measurements were compared by unpaired *t*-test. The two-tailed *P*-value given shows the probability of the observed discrepancy being due to chance, assuming a Gaussian distribution.

## Results

### Subcellular localization of proteasomes

HEp-2 cells and a variety of cell lines and primary cells were subjected to confocal immunofluorescence with mouse monoclonal antibodies against 20S proteasomes to determine the localization of endogenous proteasomes. In all mammalian cells analysed so far endogenous proteasomes distribute throughout the cytoplasm and nucleoplasm, however, they are neither detectable in the nuclear envelope nor in nucleoli (Fig. 1B,D). In addition to a diffuse nucleoplasmic localization pattern proteasomes concentrate in speckles and punctate dots. These results were confirmed by immunoblotting of subcellular fractions (see Materials and Methods). Cytoplasmic and nucleoplasmic fractions contain multiple proteins sized between 20 and 30 kDa that correspond to α- and β-subunits of the 20S proteasome core particle (Fig. 1C, lanes cy and nu). By contrast, the nucleolar and nuclear envelope protein fractions do not contain subunits of 20S proteasomes (Fig. 1C, lanes no and ne). Subnuclear localization of proteasomes was not affected by treatment with proteasomal inhibitors, such as MG132 and lactacystin (Fig. 1E). Proteasome inhibitors were used at concentrations that block proteasome-dependent protein degradation, but do not induce changes of cell morphology nor kill the cells as controlled by differential interference contrast (DIC, data not shown). The results on localization of endogenous proteasomes in distinct subcellular compartments could be confirmed (1) in different cell lines, and primary cells, (2) using a variety of monoclonal or polyclonal antibodies against 20S and 26S

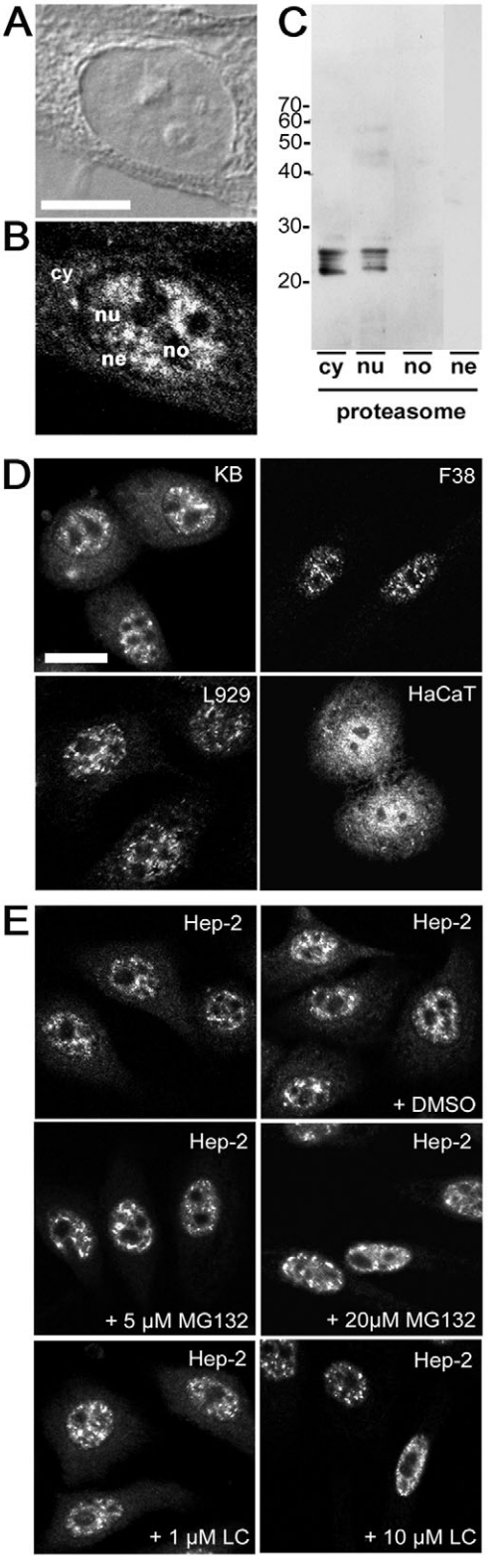


**Fig. 1.** Subcellular localization of proteasomes. (A) Differential interference contrast (DIC) of a representative HEp-2 cell during interphase. (B) Confocal immunofluorescence micrograph of the cell seen in (A) with mouse monoclonal antibody PW8195 against 20S proteasomes shows cytoplasmic localization (cy), and a speckled staining pattern in the nucleoplasm (nu). Large unlabelled areas correspond to nucleoli (no) and the nuclear envelope (ne). (C) Immunoblot of biochemically fractionated HEp-2 cells with antibody PW8155 confirms that proteasomes occur in the cytoplasm (cy) and in the nucleoplasm (nu), but neither in the nucleolar (no), nor the nuclear envelope (ne) fraction. 20, 30, 40, 50, 60, 70 molecular mass in kDa. NB: the same subcellular fractions were used in Fig. 1C, Fig. 2A-D. (D) Subcellular distribution of proteasomes was analysed by confocal immunofluorescence with antibody PW8155 in a variety of mammalian cell types, such as human epidermoid cells (KB), primary fibroblasts (F38), murine fibroblasts (L929), human keratinocytes (HaCaT) and in HEp-2 cells treated with proteasome-inhibitors (E). LC, lactacystin. Scale bars, 5 μm.

proteasomes, and (3) by application of different staining procedures (data not shown) (Chen et al., 2002; Rockel and von Mikecz, 2002; Guillot et al., 2004).

### Subcellular localization of proteasomal activity

To localize proteasomal activity in subcellular compartments HEp-2 cells were grown to subconfluence, lysed and fractionated into cytoplasmic, nucleoplasmic, nucleolar and nuclear envelope proteins, as detailed in Materials and Methods. The purity of subcellular protein fractions was confirmed by immunoblotting with signature proteins of cellular compartments such as β-tubulin (cytoplasm), SC35 (splicing speckles, nucleoplasm), fibrillarin (dense fibrillar component, nucleolus) and lamin A/C (nuclear lamina) (Fig. 2A-D, left panel). HEp-2 fractions were co-incubated in vitro with fluorogenic precursor Suc-LLVY-AMC, which is a substrate for proteasome-dependent degradation that fluoresces when cleaved. Suc-LLVY-AMC is specifically cleaved by the rate-limiting chymotrypsin activity of the 20S proteasome (Rock et al., 1994) with preferential cleavage after large hydrophobic residues. The increase of fluorescence, which can be measured with a spectrofluorometer, is proportional to enzymatic activity. Fluorometric detection showed a linear increase of fluorescence intensity over time due to Suc-LLVY-AMC cleavage in cytoplasmic and nucleoplasmic protein fractions (Fig. 2A,B, right panel, closed circles). This activity could be blocked completely by addition of proteasome-specific inhibitor lactacystin (Fig. 2, open circles). By contrast, nucleolar and nuclear envelope fractions did not contain proteasomal activity as shown by flat product curves (Fig. 2C,D, right panels). The results suggest that subcellular compartments differ concerning their proteasome-dependend activity, and that this activity is correlated to localization of proteasomes. Cytoplasmic HEp-2 cell fractions are characterized by high proteasomal activity with a turnover number (TN) of 73.97 (substrate/minute/enzyme). The nucleoplasm contains proteasomes, and a significant proteasomal activity [(TN) 40.68], whereas nucleolus and nuclear envelope cell fractions are void of proteasomes and

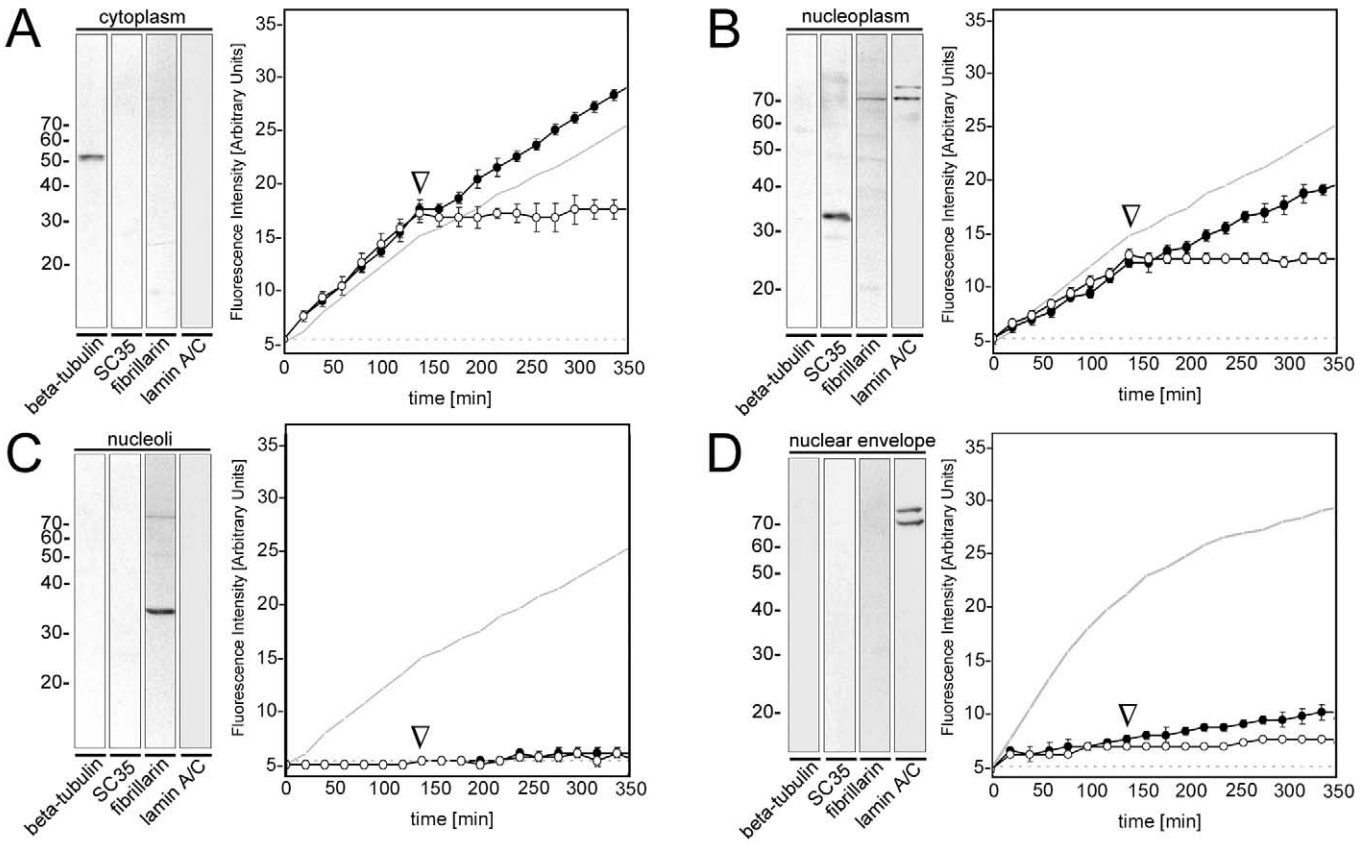


**Fig. 2.** Proteasomal activity in subcellular fractions. HEp-2 cells were grown to subconfluence, followed by biochemical purification of cytoplasmic (A), nucleoplasmic (B), nucleolar (C) and nuclear envelope (D) cell fractions as detailed in Materials and Methods. The purity of cell fractions was monitored by immunoblotting. Proteins from preparations of cellular fractions were separated by SDS-PAGE electrophoresis, transferred to nitrocellulose and immunolabelled with signature proteins of subcellular compartments, such as β-tubulin (cytoplasm), SC35 (splicing speckles, nucleoplasm), fibrillarin (nucleolus) and lamin A/C (nuclear envelope). Proteasomal activity of subcellular fractions was determined by their incubation with fluorogenic precursor Suc-LLVY-AMC at 37°C and fluorometric detection of cleaved substrates. The resulting product curves (filled circles) were observed over time. Fluorescence intensity values represent mean values of three independent experiments with standard errors between 0.00 and 1.25 (fluorescence intensity, arbitrary units). To control substrate specificity proteasome-inhibitor lactacystin (10 μM) was added after 2 hours (open arrowheads) to double determination assays (open circles). Biochemically purified 20S proteasomes were included as positive controls (grey curves). 20, 30, 40, 50, 60, 70 molecular mass in kDa.

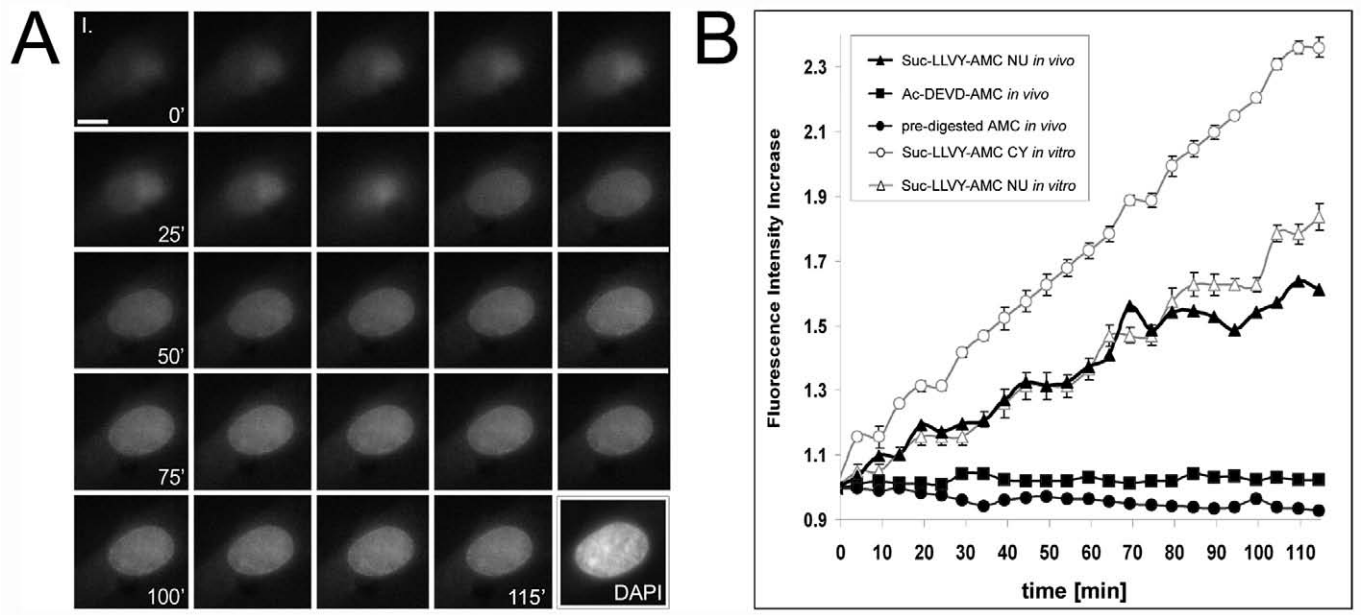


**Fig. 3.** Proteasomal activity in the nucleus of living cells. (A) Fluorogenic precursor substrate Suc-LLVY-AMC was microinjected into the nucleus of a HEp-2 cell and proteasomal cleavage measured by detection of fluorescence intensity increase over time. Images were acquired every 5 minutes by means of epifluorescent light microscopy. After 115 minutes the same cell was microinjected with DAPI to detect DNA and to confirm localization of the cell nucleus. (B) Fluorometric quantification of time-lapse experiments. Comparison between proteasomal activity of cell nucleus-microinjected Suc-LLVY-AMC (filled triangles), caspase-specific substrate Ac-DEVD-AMC (filled squares), and predigested Suc-LLVY-AMC (filled circles), with Suc-LLVY-AMC that was digested in vitro by cytoplasmic (CY, open circles), or nucleoplasmic (NU, open triangles) cell fractions. Note the similar proteasomal activity in the nucleus of the living cell and in nucleoplasmic cell fractions. Scale bar, 5 μm.

proteasomal activity, as well [(TN) 2.97 and 2.89, respectively]. Proteasomal activity was calculated as detailed in the Materials and Methods section.

## Proteasomal activity in nuclei of living cells

To investigate proteolytic activity of proteasomes in the cell nucleus of living cells we bypassed endosomal uptake and cytoplasmic protein degradation by microinjection of fluorogenic precursor substrate Suc-LLVY-AMC into nuclei of HEp-2 cells growing on coverslips. Nuclei were imaged by epifluorescence microscopy 5-115 minutes after microinjection at 5-minute intervals (Fig. 3A). Cells that were exclusively microinjected into the cell nucleus show a linear increase of nuclear fluorescence intensity over time indicating nuclear activity of proteasomes, while cytoplasmic fluorescence remains unchanged (Fig. 3A,B). Microinjection of Suc-LLVY-AMC into the cytoplasm resulted in weak cytoplasmic fluorescence, whereas accumulation of fluorescence in the cell nucleus was not observed (data not shown). To exclude autofluorescence of the dye AMC in living cells and confirm that the cleavage of Suc-LLVY-AMC is proteasome-specific pre-digested Suc-LLVY-AMC or caspase-specific substrate Ac-DEVD-AMC were microinjected into nuclei as controls. The flat product curves indicate that both controls did not induce an increase of fluorescence intensity within the cell nucleus (Fig. 3B). The position of cell nuclei was determined by phase-contrast microscopy (data not shown) and microinjection of DAPI (Fig. 3A). A comparison of fluorescence intensity increase induced by in vitro co-incubation of cell fractions with Suc-LLVY-AMC (compare with Fig. 2), and in living cells suggest similar proteasomal activities in the nucleoplasmic cell fraction (open triangles) and in nuclei of living cells (closed triangles, note similar product curves, Fig. 3B). Turnover of Suc-LLVY-AMC is 0.0969 (μM/minute) in cytoplasmic fractions, 0.0472 (μM/minute) in nucleoplasmic fractions, and 0.0541 (μM/minute) in cell nuclei of living cells. The identification of nuclear cleavage of fluorogenic precursor substrate Suc-LLVY-AMC is a strong indication for proteasome-dependent activity that enables monitoring of proteasomal proteolysis in the cell nucleus. Taken together the results obtained with Suc-LLVY-AMC, cell fractions and microinjection suggest that the cell nucleus contains active proteasomes.

## Localization of proteasomal protein degradation in nucleoplasmic foci

Our experiments with the peptide substrate Suc-LLVY-AMC detected proteasomal activity in the cell nucleus. Next, we investigated if nucleoplasmic proteasomes degrade proteins. To this end HEp-2 cells were microinjected with the ectopic protein DQ-ovalbumin (DQ-OVA). OVA is a well characterized substrate for proteases that is generally used as a probe for antigen processing and presentation (Rock et al., 1994). A strong fluorescence quenching effect is observed when OVA is heavily conjugated with BODIPY dyes, exhibiting less than 3% of the fluorescence of the corresponding free dyes. Upon hydrolysis of the DQ-OVA to single, dye-labelled peptides by proteases, this quenching is

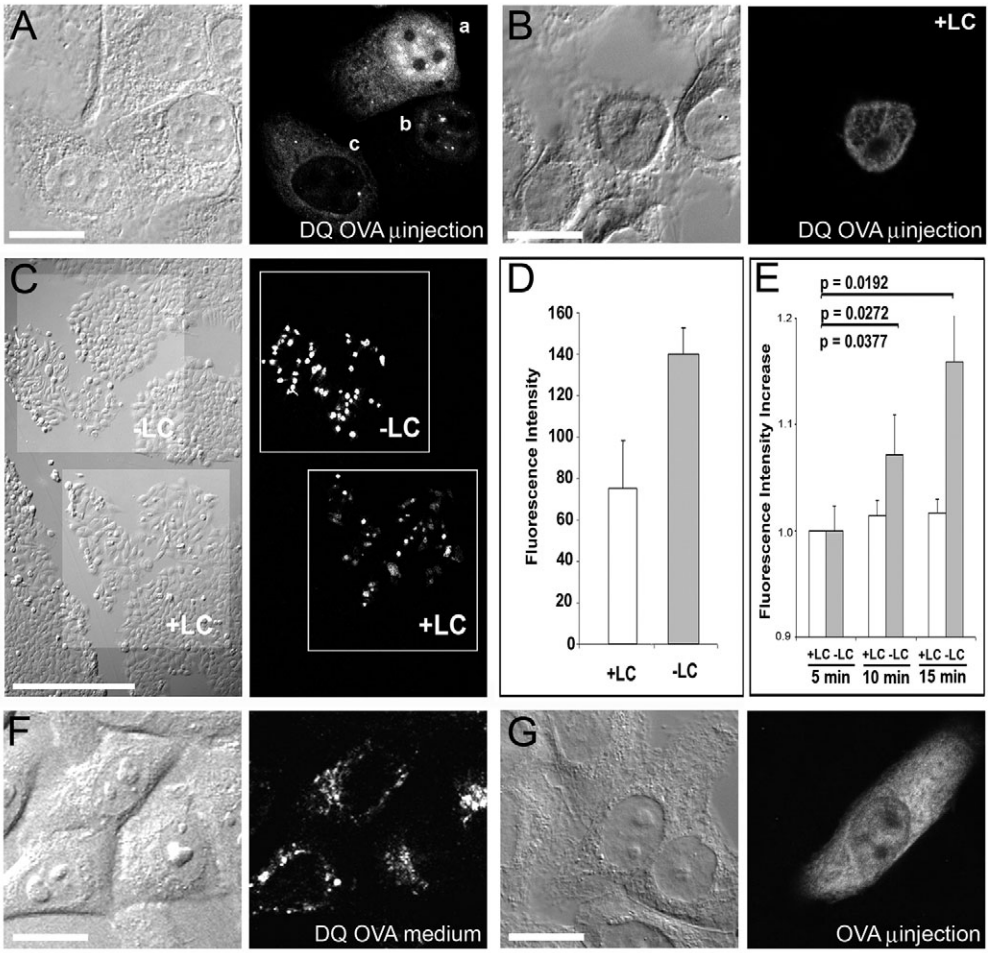


**Fig. 4.** In situ localization of proteasome-dependent protein degradation. Panels (A-C) and (F,G) show confocal fluorescence micrographs of HEp-2 cells with the corresponding differential interference contrast (DIC) image on the left. (A) HEp-2 cells were microinjected with 0.5 mg/ml fluorogenic substrate protein DQ-OVA into the cytoplasm and cell nucleus (cell a) or into the cell nucleus (cell b) or into the cytoplasm (cell c). Microinjected DQ-OVA shows a granular cytoplasmic localization. In the cell nucleus DQ-OVA localizes to distinct nucleoplasmic foci. Unlabelled areas correspond to nucleoli. Note, DQ-OVA is excluded from the nucleus in cells that were exclusively microinjected into the cytoplasm. (B) A representative HEp-2 cell that was microinjected with DQ-OVA and 10 μM proteasome inhibitor lactacystin shows a diffuse localization of DQ-OVA in the nucleus without formation of nucleoplasmic foci. (C) Equal numbers of HEp-2 cells in highlighted areas were microinjected with DQ-OVA (–LC) or DQ-OVA and 10 μM lactacystin (+LC). Corresponding fluorescence micrographs show decreased DQ-OVA fluorescence in proteasome inhibitor-treated (+LC) versus untreated (–LC) cells. (D) Fluorescence-quantification of (C). (E) HEp-2 cells were treated as in (C), and fluorescence of DQ-OVA quantified after 5, 10 and 15 minutes (min). (F) HEp-2 cells were co-incubated for 30 minutes with 200 μg/ml DQ-OVA, which localizes to endosomal vesicles in the cytoplasm, whereas the nucleus is unlabelled. (G) Confocal immunofluorescence of a representative HEp-2 cell that was microinjected with 200 μg/ml plain ovalbumin, fixed and immunolabelled with anti-ovalbumin antibodies. The ectopic protein ovalbumin distributes diffusely throughout the cytoplasm, and cell nucleus. Scale bars, 10 μm (A,B,F,G); 150 μm (C).

relieved, producing brightly fluorescent products. Fluorometric in vitro experiments confirmed that DQ-OVA fluorescence is quenched in distilled water, PBS, and cell culture medium (RPMI/FCS), but released by addition of 0.05 mg/ml trypsin (data not shown). HEp-2 cells that were grown in medium containing DQ-OVA, showed bright staining of endosomal vesicles in the cytoplasm (Fig. 4F, micrograph). We observed rapid uptake of DQ-OVA into the endosomal system within minutes that immediately resulted in bright fluorescence (data not shown). Large unlabelled areas correspond to cell nuclei (even after incubation periods of 1-16 hours) suggesting that DQ-OVA does not translocate to the cell nucleus. To detour endosomal protein degradation, and detect proteasome-dependent proteolysis HEp-2 cells were microinjected into the cytoplasm and nucleus (Fig. 4A, cell a), the nucleus (cell b) or the cytoplasm (cell c). In contrast to co-cultivated DQ-OVA (compare Fig. 4F), microinjected DQ-OVA localizes in a granular pattern throughout the cytoplasm and nucleoplasm, and concentrates in bright nucleoplasmic foci. Unlabelled areas correspond to nucleoli (Fig. 4A, micrograph and corresponding DIC). Since exclusive microinjection of DQ-OVA into the cytoplasm or nucleoplasm results in exclusive fluorescent staining of the respective cell compartment (Fig. 4A, cells b and c), we conclude that little or no nucleocytoplasmic transport of microinjected DQ-OVA occurs. To determine if the subnuclear localization of DQ-OVA is dependent on proteasomal activity cells were microinjected simultaneously with DQ-OVA and 10 μM proteasome-specific inhibitor lactacystin (Fig. 4B). We observed a reduced, and diffuse DQ-OVA staining in nuclei of proteasome-inhibited cells. Thus, we conclude that the focal localization of microinjected DQ-OVA in cell nuclei of untreated cells is due to proteasomal protein degradation in nucleoplasmic foci. Next we quantified proteasome-dependend protein degradation in DQ-OVA microinjected cells. HEp-2 cells in selected areas of subconfluent cell monolayers were microinjected with DQ-OVA or DQ-OVA and 10 μM lactacystin (Fig. 4C). Fluorescent intensities vary significantly in untreated versus lactacystin-treated cells. About 50% decrease of DQ-OVA fluorescence was observed in lactacystin-treated HEp-2 cells (Fig. 4C, micrograph, frame +LC; quantification in Fig. 4D), which represents a remarkable efficiency of proteasomal inhibition

since lactacystin does not block all of the six proteolytic centres of the 20S core particle. Time kinetic experiments showed rapid proteolysis of DQ-OVA that occurred immediately after microinjection (Fig. 4E). Fluorescence intensity was visualized and quantified in 5 minute intervals 5 to 15 minutes after microinjection. DQ-OVA fluorescence remained unchanged in cells that were pretreated with lactacystin, whereas a significant linear increase occurred in microinjected cells that were not treated with proteasome inhibitors (Fig. 4E). Microinjection experiments with DQ-OVA show that this technique allows localization of proteasomal protein degradation in distinct foci of the cell nucleus since (1) DQ-OVA is not subjected to nucleocytoplasmic translocation, (2) formation of proteolysis foci is specifically inhibited by lactacystin, (3) about 50% of DQ-OVA fluorescence results from proteasome-dependent protein hydrolysis in whole cells, and (4) time-dependent fluorescence increase of DQ-OVA exclusively represents proteasomal proteolysis as no increase could be detected in lactacystin-treated cells. To control that the distinct distribution of microinjected DQ-OVA in nucleoplasmic foci is correlated with proteolysis, and does not result from the localization of ovalbumin in the nucleus, plain ovalbumin was microinjected into HEp-2 cells. Confocal immunofluorescence with antibodies against ovalbumin detected a diffuse localization of the ectopic protein throughout the cytoplasm and nucleoplasm (Fig. 4G, micrograph), however, a focal distribution of microinjected ovalbumin did not occur.

To exclude the possibility that nucleoplasmic DQ-OVA foci represent subnuclear domains of unspecific DQ-OVA peptide, e.g. cleaved substrate, diffusion instead of proteolytic centres HEp-2 cells were microinjected either with DQ-OVA or predigested DQ-OVA on the same coverslip, and observed over time (Fig. 5). DQ-OVA peptides emit bright fluorescence and localize diffusely throughout the nucleoplasm and cytoplasm (Fig. 5A, cell a). After 30 minutes the fluorescent signal of predigested DQ-OVA disappeared completely. Accumulation of predigested DQ-OVA (DQ-OVA peptides) in nucleoplasmic foci was not observed (Fig. 5A, cell a, and C). By contrast, microinjection of DQ-OVA induced fluorescence signals that occurred in distinct focal domains of the nucleoplasm that form and fade over time (Fig. 5A, cell b, inset, and B). Taken together the microinjection experiments suggest that DQ-OVA foci represent proteolytic centres with proteasomal activity. Such proteasomal protein degradation foci were detected in different cell types such as primary fibroblasts, and using different substrates, e.g. DQ-BSA (bovine serum albumin, data not shown).

## Co-localization of proteasomal protein degradation foci with subnuclear domains

Since the interphase cell nucleus is characterized by a compartmentalized architecture double labelling localization studies were performed with DQ-OVA and signature proteins of subnuclear domains to detect proteasomal protein degradation in situ and/or in nucleo (Fig. 6). Subnuclear localization of DQ-OVA was analysed in cells that were fixed 10 minutes after microinjection and subjected to confocal immunofluorescence. In cells that were microinjected into cell nuclei DQ-OVA appears in bright foci throughout the nucleoplasm in addition to a granular distribution. β-tubulin localizes in a granular pattern throughout the cytoplasm that does neither co-localize with cytoplasmic, nor nucleoplasmic

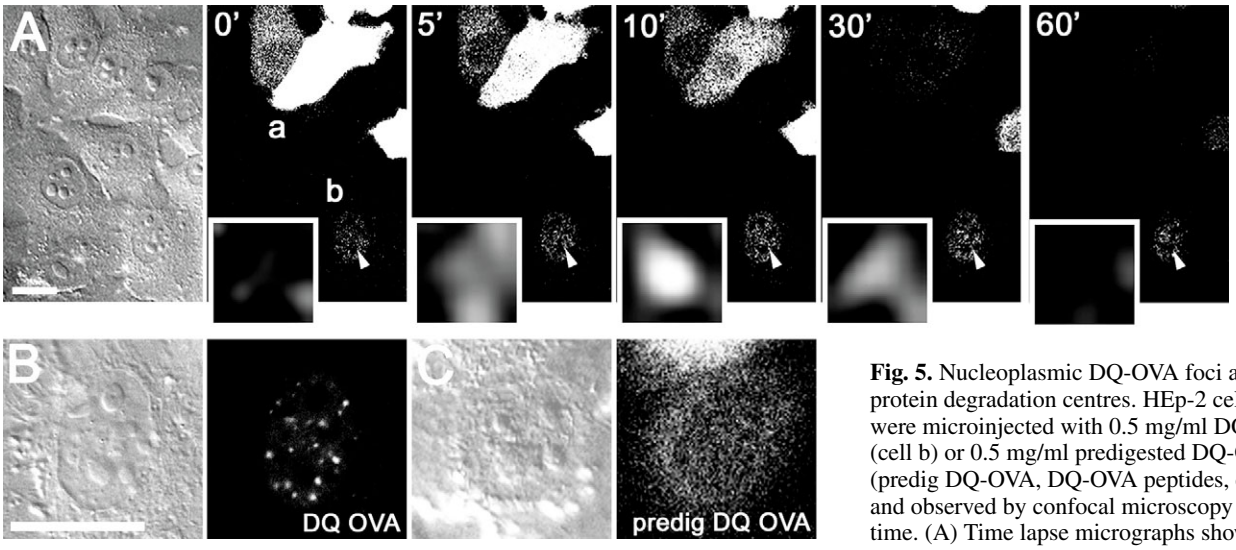


**Fig. 5.** Nucleoplasmic DQ-OVA foci are protein degradation centres. HEp-2 cells were microinjected with 0.5 mg/ml DQ-OVA (cell b) or 0.5 mg/ml predigested DQ-OVA (predig DQ-OVA, DQ-OVA peptides, cell a), and observed by confocal microscopy over time. (A) Time lapse micrographs show strong fluorescence, and diffuse distribution of microinjected DQ-OVA peptides throughout the nucleoplasm and cytoplasm that decreases rapidly, and disappears within 30 minutes (cell a). Microinjected DQ-OVA shows weaker fluorescence in the cell nucleus that concentrates in distinct foci, and persists over 60 minutes (cell b). The insets show blow ups of a representative DQ-OVA degradation centre (arrows) that forms within 10 minutes, persists for about 20 minutes, and has disappeared after 60 minutes (insets). Note, for time-lapse observation of living cells fluorescent signals were acquired with low laser intensity (5%) and high confocal aperture to minimize cellular stress and photobleaching effects, and to enable simultaneous observation of fluorescent signals in different focal planes. (B) After the time-lapse observation cell b was scanned with routine confocal settings (20% laser intensity, low confocal aperture). (C) Confocal microscopy (settings as in B) of a HEp-2 cell microinjected with predigested DQ-OVA shows that DQ-OVA peptides distribute diffusely throughout the nucleoplasm. Bars, 10 μm.

DQ-OVA (Fig. 6A). Lamins A/C are constituents of the nuclear lamina forming a ring-like structure around the nucleoplasm. No overlap was detected between lamin A/C and DQ-OVA (Fig. 6B). The clumpy nucleolar localization of fibrillarin, a component of the dense fibrillar compartment (DFC) in the nucleolus, did also not co-localize with microinjected DQ-OVA (Fig. 6C). By contrast, significant co-localization was observed between splicing factor SC35, a signature protein of splicing speckles, and DQ-OVA in bright nucleoplasmic foci (Fig. 6D, yellow). A subpopulation of PML nuclear bodies, visualized by antibodies to PML, did also contain DQ-OVA (Fig. 6E). Further analysis revealed significant co-localization of DQ-OVA with RNA-polymerase II (pol II), ubiquitin and proteasomes in nucleoplasmic foci (Fig. 6F-H, respectively). The co-localization between subnuclear structures and DQ-OVA is partial, which corroborates the idea that sites of concentrated DQ-OVA fluorescence in the nucleoplasm represent distinct foci of protein degradation instead of fortuitous DQ-OVA accumulation due to diffusion of cleaved substrate. Proteolysis-dependent localization of DQ-OVA is further confirmed by a study on the fate and dynamics of intracellular peptides in living cells. It was shown that fluorescently labelled peptides distribute diffusely within the cell nucleus, and do not concentrate in nucleoplasmic foci (Reits et al., 2003). We suggest that proteasome-dependent protein degradation occurs in nucleoplasmic foci that contain proteasomes and DQ-OVA (Fig. 6H). These proteasomal degradation foci partially overlap or juxtapose with subnuclear domains, such as splicing speckles and PML bodies. Partial co-localization between DQ-OVA degradation foci and 20S proteasomes (15%, Fig. 6I) is consistent with previous observations in living cells that proteasomes are highly mobile

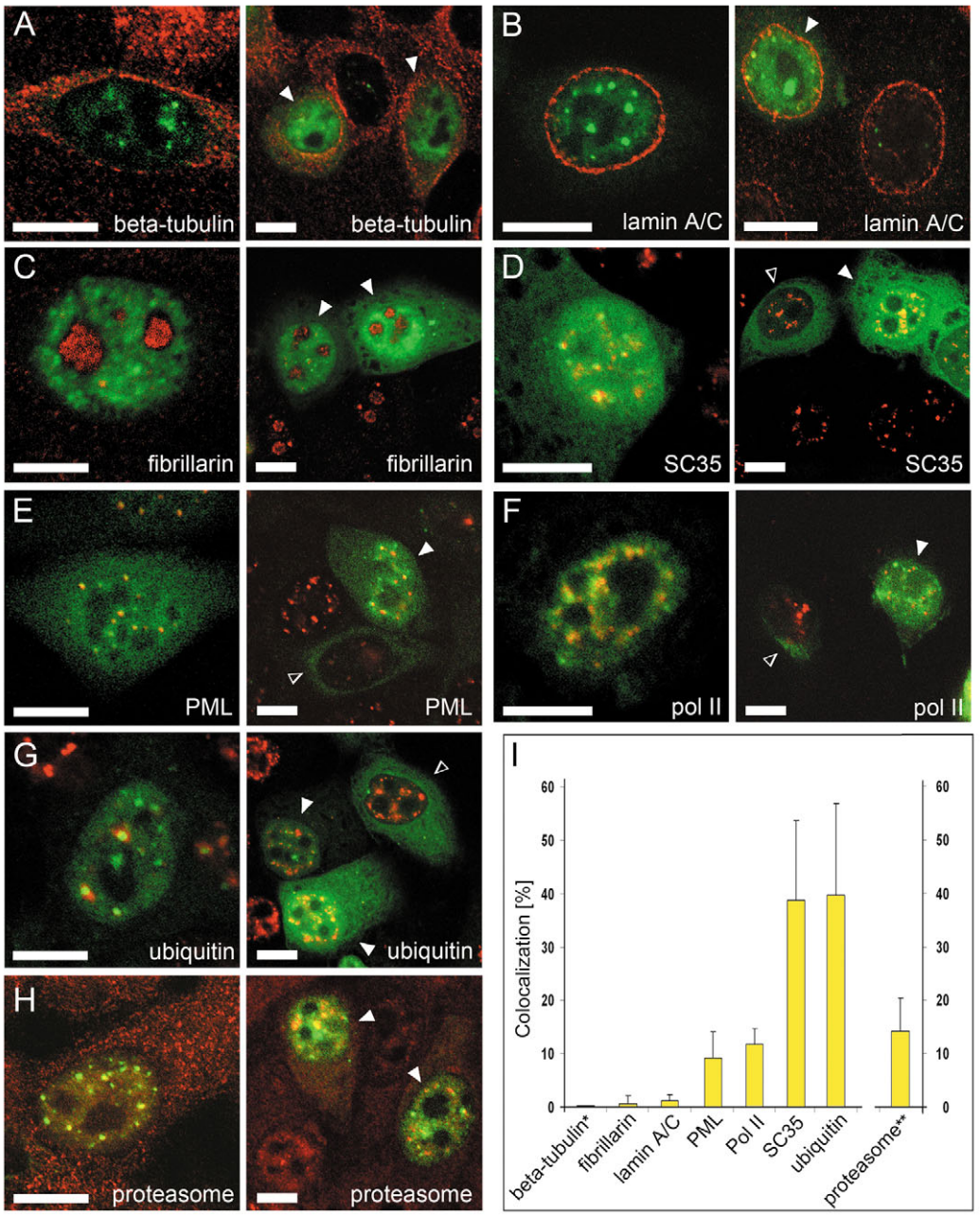


**Fig. 6.** Proteasome-dependent degradation of DQ-OVA occurs in distinct subnuclear domains. Panels (A-H) show a confocal fluorescence micrograph of a representative HEp-2 cell microinjected with DQ-OVA (green), and a corresponding overview of untreated and microinjected cells (arrowheads) on the left. HEp-2 cells were double labelled (red) with β-tubulin (A), lamin A/C (B), fibrillarin (C), SC35 (D), PML (E), RNA polymerase II (pol II, F), ubiquitin (G), and proteasomes (H). Microinjected DQ-OVA co-localizes partially with SC35, PML, pol II, ubiquitin and proteasomes in nucleoplasmic foci (yellow). (I) Quantification of co-localization between DQ-OVA and β-tubulin or nuclear proteins in microinjected cells. Co-localization of DQ-OVA with β-tubulin was quantified by defining whole cells as regions of interest (asterisk). Co-localization with nuclear proteins and nucleoplasmic proteasomes (two asterisks) was determined using cell nuclei as regions of interest. Note, exclusion of DQ-OVA from the nucleus in cells that were exclusively microinjected into the cytoplasm (open arrowheads). Scale bars, 5 μm.

in the cell nucleus (Reits et al., 1997), and the idea that these molecular machines perform quality control by continuous collision with potential substrates degrading those proteins that are properly tagged or misfolded.

## Discussion

In this study, we used cell fractionation and microinjection of fluorogenic substrates Suc-LLVY-AMC, and DQ-OVA to localize proteasomal activity and proteasome-dependent protein degradation within the cell during interphase. Proteasomal activity was detected in the cytoplasm and nucleoplasm, but not in nucleolar or nuclear envelope fractions. In the cell nucleus proteasome-dependent protein degradation of the model substrate DQ-OVA occurred in distinct nucleoplasmic foci that partially overlap with splicing speckles and PML bodies, respectively. In a previous study we showed that proteasomal degradation of nuclear proteins is correlated with their subnuclear localization (Rockel and von Mikecz, 2002). Upon specific inhibition of proteasome-dependent proteolysis by lactacystin spliceosomal, and PML body components accumulated in splicing speckles or PML bodies, respectively. Since nucleolar proteins, such as fibrillarin and UBF (upstream binding factor), did not accumulate we concluded that they do not represent proteasomal substrates. Here, we offer a possible explanation for the selective degradation of nuclear proteins: splicing factors and PML are degraded by proteasomes because splicing speckles and PML bodies partially overlap with focal sites of proteasomal protein degradation (Fig. 6). By contrast, nucleolar proteins generally do not co-localize with protein degradation foci in interphase nuclei, and nucleolar protein fractions do not contain proteasomal activity (Fig. 2C).

These results are consistent with the literature on the localization of proteasomes and components of the ubiquitin-proteasome system (UPS). By electron microscopy, immunofluorescence, and analyses of GFP-variants in living cells it was shown that subunits of the 20S proteasome core, proteasomal regulators, ubiquitin and ubiquitin ligases distribute specifically in the cytoplasm and the nucleoplasm, but not within the nucleolus (Chen et al., 2002) (for reviews, see Rivett, 1998; Wojcik and DeMartino, 2003). Localization of proteasomes in the nucleolus was observed under unique conditions such as inhibition of proteasomal protein degradation by MG132 (Mattsson et al., 2001), and overexpression of Myc (Arabi et al., 2003). However, we could neither confirm nucleolar localization of proteasomes in HEp-2 cells and a variety of primary cells and cell lines that were treated with proteasome inhibitors MG132 and lactacystin (Fig. 1; data not shown), nor could we detect any proteasomal activity in nucleolar fractions of such cells (data not shown). The lack of proteasomal activity defines the nucleolus as an unlikely localization for proteasomal protein degradation, which does not exclude that nucleolar proteins are degraded elsewhere in the nucleus. Consistent with this idea we recently described degradation of nucleolar protein fibrillarin in nucleoplasmic foci under conditions that specifically inhibit nucleolar transcription (Chen et al., 2002).

Although it has been shown about two decades ago that proteasomes localize to the cell nucleus (Hügle et al., 1983), research has been focussing on cytoplasmic protein breakdown and antigen processing. Thus, little is known on the functional organization and role of nuclear proteasomes. The present knowledge about nuclear proteolysis in general is limited. To investigate mechanisms of protein degradation in the cell nucleus *Escherichia coli* β-galactosidase (β-gal) variants were expressed in COS cells and used as model proteins (Tsuneoka and Mekada, 1992). Half-life of β-gal strongly correlated with the presence or absence of nuclear localization signals (NLSs). However, the authors could not determine whether nuclear β-gal was degraded in the cell nucleus or transported to the cytoplasm and hydrolysed by cytoplasmic proteases. The introduction of leptomycin B, an inhibitor of nuclear export, enabled two recent studies that described degradation of myoD (Floyd et al., 2001) and endogenous p53 (Shirangi et al., 2002) in the cell nucleus. Protein degradation in these studies is attributed to the ubiquitin-proteasome system. Few reports identify other proteases than proteasomes in the cell nucleus. Human SUMO-1 protease SENP2 binds to Nup 153, a nucleoporin that is localized to the nucleoplasmic face of the nuclear pore complex (Hang and Dasso, 2002). Lysosomal protease cathepsin L exists in an isoform capable of trafficking to the nucleus and activating the CDP/Cux transcription factor (Goulet et al., 2004). However, since both papers describe localization of ectopic proteins, proteasomes remain the only endogenous proteases identified in the cell nucleus so far. Recently, a novel protein quality control system based on the ubiquitin-protein ligase San1p has been identified in *Saccharomyces cerevisiae* and it is proposed that analogous systems exist in other eukaryotes (Gardner et al., 2005).

In the present study we confirm the idea that the ubiquitin-proteasome system is not only abundant in the cell nucleus, but may play a major role in regulation of nuclear structure and function by protein degradation in distinct subnuclear compartments. We detect significant proteasomal activity in nucleoplasmic cell fractions [(TN)=69.01], whereas such protease activity is not detectable in nucleoli [(TN)=6.26] or nuclear envelope protein fractions [(TN)=4.82]. Due to considerable flaws of biochemical cell fractionation techniques we additionally used microinjection of fluorogenic precursor substrate Suc-LLVY-AMC to monitor proteasomal activity in situ. The comparison between the cell fractionation and microinjection experiments (Fig. 3B) reveals similar cleavage rates of Suc-LLVY-AMC in vitro, in nucleoplasmic fractions (turnover=0.0541 μM/minute), and in the cell nucleus of a living cell (turnover=0.0472 μM/minute). Thus, we suggest that proteasomal proteolysis should be added to the list of nuclear functions, such as replication, transcription and splicing. The advantage of proteolysis within the cell nucleus is tight regulation of processes involved in gene expression, since active proteasomes may directly interact with molecular machines such as ribonucleoprotein complexes. Recently, a model was introduced that describes recruitment of 26S proteasomes to activated genes (Muratani and Tansey, 2003). Recruitment of proteasomes to the basal transcription machinery would convert RNA polymerase from an initiation- to elongation-competent form. A report by Gillette et al. shows transcription-dependent association of 26S proteasomes with *GAL1, GAL10* and *HSP82* genes (Gillette et al., 2004). These authors suggest that proteasomes are generally recruited to DNA at sites of stalled RNA polymerase and may serve in transcriptional termination, as well as clearance of dead

complexes blocked by DNA damage. Consistent with these findings we provide evidence here that nucleoplasmic foci of protein degradation, visualized by cleavage of fluorogenic substrate DQ-OVA, significantly overlap with sites of active RNA polymerase II (pol II), e.g. pol II that is phosphorylated in its CTD (C-terminal domain; Fig. 6F,I). This result should fuel further investigations on the role of nuclear proteasomes in the regulation of transcriptional protein networks. The distribution of proteasomal protein degradation in nucleoplasmic foci that overlap with splicing speckles and PML bodies may likewise reflect involvement of the ubiquitin-proteasome system in gene expression. The present study establishes techniques to vizualize proteasomal activity in situ, especially in nucleo, which may aid to put the nuclear function of proteasomes into focus.

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