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ORIGINAL PAPER

Localization of proteasomes and proteasomal proteolysis in the mammalian interphase cell nucleus by systematic application of immunocytochemistry

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Abstract Proteasomes are ATP-driven, multisubunit proteolytic machines that degrade endogenous proteins into peptides and play a crucial role in cellular events such as the cell cycle, signal transduction, maintenance of proper protein folding and gene expression. Recent evidence indicates that the ubiquitin-proteasome system is an active component of the cell nucleus. A characteristic feature of the nucleus is its organization into distinct domains that have a unique composition of macromolecules and dynamically form as a response to the requirements of nuclear function. Here, we show by systematic application of different immunocytochemical procedures and comparison with signature proteins of nuclear domains that during interphase endogenous proteasomes are localized diffusely throughout the nucleoplasm, in speckles, in nuclear bodies, and in nucleoplasmic foci. Proteasomes do not occur in the nuclear envelope region or the nucleolus, unless nucleoplasmic invaginations expand into this nuclear body. Confirmedly, proteasomal proteolysis is detected in nucleoplasmic foci, but is absent from the nuclear envelope or nucleolus. The results underpin the idea that the ubiquitin-proteasome system is not only located, but also proteolytically active in distinct nuclear domains and thus may be directly involved in gene expression, and nuclear quality control.

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Introduction

Proteasomes are self-compartmentalized molecular machines that degrade the bulk, approximately 80% (Lee and Goldberg 1998), of intracellular proteins. The housekeeping 26S proteasome consists of a cylindrical 20S core that is composed of four stacked rings with constitutive alpha and beta subunits structured αββα and flanked by two 19S regulatory complexes (Groll et al. 2005). The two inner β-rings form a central chamber that harbours the proteolytic centres with chymotryptic, tryptic and caspase-like activities. According to the current "two-substrate" model, ubiquitinated or denatured proteins are (1) recognized by the ATPase subunit S6′ (Rpt5) of the 19S regulatory complex (Lam et al. 2002), (2) unfolded, (3) channelled via a central passageway into the degradation chamber, (4) degraded into peptides, and (5) released through the entry channel (Hutschenreiter et al. 2004).

Immunolabelling, ultrastructural studies and observation of autofluorescent protein fusions in living cells revealed that proteasomes are located in the cytoplasm and the cell nucleus, as well. In the advent of the post-genomic era the role of proteasomes, ubiquitin, and ubiquitin-like proteins in regulation of gene expression has become a major topic in research on spatial organization of nuclear function (Muratani and Tansey 2003; von Mikecz 2006). Specialized nuclear domains and subnuclear "organelles" contribute to a highly organized structural platform for gene expression during

interphase (Handwerger and Gall 2006): The nuclear envelope (NE) is a double membrane structure composed of an outer membrane that faces the cytoplasm and is continuous with the rough endoplasmic reticulum (RER), and an inner membrane that contacts chromatin fibres through interaction of the lamin B receptor (LBR) and heterochromatin-protein 1 (HP1). The nucleoplasmic surface of the NE is lined by a fibrillar meshwork, the nuclear lamina. Signature proteins of the lamina such as lamins A/C interact with both, proteins from the inner nuclear membrane and chromatin fibres. Nuclear DNA is organized into differentially condensed chromatin where chromosomes are arranged in heterochromatic (inactive, silenced chromatin) and euchromatic (active chromatin) regions. The cell nucleus additionally features distinct nuclear domains referred to as nuclear bodies (NBs) such as nucleoli, Cajal bodies (CBs), promyelocytic leukaemia bodies (PML NBs), and speckles that occupy the interchromatin space and are characterized by dynamic clustering of specific signature proteins in response to gene expression. NBs seem to store and contribute proteins for the first steps of gene expression, since transcription sites that are visualized by incorporation of nucleotide analog in in situ run on assays distribute (1) throughout the nucleoplasm, including on the periphery of splicing speckles and PML NBs, and (2) are concentrated in several thousand foci.

By means of immunofluorescent labelling and electron microscopy Franke et al. identified nuclear proteasomes in the oocytes of *Xenopus laevis* and in the HeLa cells (Hugle et al. 1983; Kleinschmidt et al. 1983). In the following decades analyses of proteasome distribution generated a plethora of, at times contradictory, results. Proteasomes were localized at the NE, diffusely throughout the nucleoplasm, in nucleoli, juxtaposed to PML NBs, in speckles, and in nucleoplasmic foci using cells from different species (yeast, amphibians, mammals), different antibodies, and different immunolabelling techniques (Rivett et al. 1992; Peters et al. 1994; Reits et al. 1997; Enenkel et al. 1998; Mattsson et al. 2001; Chen et al. 2002; Rockel and von Mikecz 2002). We recently reported that active proteasomal proteolysis occurs in transient focal subdomains throughout the nucleoplasm that partially colocalize with SC35–speckles and PML NBs, but are not found at the nuclear periphery or within nucleoli (Rockel et al. 2005). Proteomic studies of the nucleolus confirmed that this nuclear body is devoid of proteasomes under basal conditions (Andersen et al. 2002). Furthermore, proteasomal activity could be detected in nucleoplasmic cell fractions, but not in biochemical preparations of NEs or nucleoli. The absence of proteasomal proteolysis in the respective subnuclear domains raises the question if they merely represent storage sites for components of the ubiquitin-proteasome system or if certain localization results concerning nuclear proteasomes are due to the experimental conditions used. For example, proteasomes have been localized within nucleoli under conditions such as inhibition of proteasomal activity and overexpression of c-Myc (Mattsson et al. 2001; Arabi et al. 2003). Arabi et al. showed that in formaldehyde-fixed COS-7 cells proteasomes recruit to nucleoli in the presence of proteasome inhibitors, a distribution inconsistent with subnuclear localization of 20S proteasomes in living cells (Reits et al. 1997) or different primary cells and cell lines that were fixed by methanol-based procedures (Amsterdam et al. 1993; Rockel et al. 2005). Here, a systematic confocal immunolabelling analysis was undertaken in HEp-2 cells, a human epithelial cell line that is very well established for detection of nuclear proteins, in order to address the subnuclear distribution of endogenous 20S proteasomes and proteasomal proteolysis in correlation with signature proteins of nuclear domains/structures.

Materials and methods

Cell culture and treatment

Human epithelial HEp-2 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). 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 coincubated with 1–5 μM lactacystin for 24 h to inhibit proteasomal activity.

Immunofluorescence

Cells were seeded on coverslips, grown to subconfluence, fixed and permeabilized using procedures I–VI:

I. Rinsed in PBS, fixed/permeabilized in methanol (20 min, −20°C)
II. Rinsed in PBS, fixed in methanol (5 min, −20°C), permeabilized in acetone (2 min, −20°C)
III. Rinsed in PBS, fixed in 4% freshly prepared paraformaldehyde (PFA), 250 mM HEPES (pH 7.6; 10 min, 4°C), refixed in 8% PFA, 250 mM HEPES (pH 7.6, 50 min, 4°C), followed by permeabilization in 0.5% Triton X−100 (30 min room temperature (RT), gentle rocking) [Guillot et al. 2004]
IV. Rinsed in PBS, fixed/permeabilized in 0.1% Triton X−100 in 4% PFA, 250 mM HEPES (pH 7.6;

10 min, 4°C), refixed in 8% PFA, 250 mM HEPES (pH 7.6, 50 min, 4°C), followed by permeabilization in 0.5% Triton X−100 (30 min RT, gentle rocking) [Guillot et al. 2004]

V. Rinsed in PBS, fixed in 4% formaldehyde (10% formaldehyde methanol free, Polysciences), PBS (10 min, RT), permeabilized in 0.25% Triton X−100 (3 min, RT).

VI. Rinsed in PBS, fixed in 0.4% formaldehyde (10% formaldehyde methanol free, Polysciences), PBS (10 min, RT), permeabilized in 0.25% Triton X−100 (3 min, RT).

The different fixation and permeabilization procedures cover methodology that was previously used for localization of proteasomes in cell nuclei, and reflect current protocols that are applied in the laboratory. After fixation and permeabilization cells were washed in PBS and subjected to indirect immunofluorescence (IF) as described previously (von Mikecz et al. 1997) with the following primary antibodies: rabbit polyclonal antibody (pAb) PW8155 to 20S proteasome alpha and beta subunits (Affiniti, Exeter, UK), rabbit pAb to core histone protein H2A (Serotec), mouse monoclonal antibody (mAb) PG-M3 to PML (Santa Cruz), mouse mAb 636 to lamin A/C (Santa Cruz), mouse mAb mara3 to RNA polymerase IIo (kindly provided by Bart Sefton, Salk Institute, La Jolla, CA, USA), mouse mAb 8WG16 to RNA polymerase IIa (Covance), mouse mAb SC35 to splicing factor SC35 (Sigma), human autoimmune sera to SmB/B′, topoisomerase I, centromeres or nucleoli (all human sera were provided by the W.M. Keck Autoimmune Disease Center, Scripps Research Institute, La Jolla, CA, USA). Secondary anti-mouse, -human and -rabbit antibodies conjugated with fluorescein isothiocyanate (FITC), rhodamine or Cy5 were purchased from Jackson Immuno Research Laboratories, PA. DNA was stained by incubation of fixed cells with TO-PRO-3 (Molecular Probes) in PBS for 15 min at RT. Living cells were incubated with DRAQ5 (Biostatus Limited) in medium for 15 min at RT.

Proteasomal protein degradation in living cells

HEp-2 cells were seeded on coverslips and grown to subconfluence. For in situ localization of proteasomal protein degradation in subnuclear compartments DQ-ovalbumin (DQ-OVA) was dissolved in PBS to a final concentration of 0.5 mg/ml and microinjected into HEp-2 cells (Rockel et al. 2005). 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. DQ-OVA fluorescence was localized in microinjected cell nuclei by fixation after 60 min post injection according to protocols (I)–(VI).

Microscopy

All 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 exited at 488 nm and emission was detected between 510 and 550 nm. Rhodamine was excited at 568 nm and emission was detected between 585 and 640 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 fluorochromes. No cross talk was observed. The DNA stains DRAQ5 and TO-PRO-3 were excited at 647 nm and emission was detected above 660 nm.

Quantification of fluorescent signals

For in situ accumulation studies confocal scans of lactacystin-treated and control cells were recorded with identical settings. Quantitative analysis of fluorescence intensity was performed using the Metamorph image analysis software package (Universal Imaging, West Chester, PA). In order to measure fluorescence intensity within the nucleus, regions of interest (ROIs) were positioned manually based on corresponding differential interference contrast (DIC) images. Images were background corrected by reference regions outside the cells, but within the field of view. For each experiment, the intensities of 100–200 nuclei were determined.

Results and discussion

20S proteasomes participate in distinct subnuclear domains

A representative antibody against conserved alpha- and beta subunits of the 20S core was used to immunolocalize endogenous proteasomes within the interphase cell nucleus and compare the staining pattern with signature proteins of defined nuclear structures. In order to assure preservation of the antigens in a fashion that reflects the in vivo situation with respect to their distribution

one unconventional (VI) and five commonly used methanol- (I, II) or formaldehyde-based (III–VI) fixation procedures were applied. Proteasomes distribute throughout the nucleoplasm in reticulated speckles (compare to Fig. 1, SmB/B′), distinct foci, and in a diffuse localization pattern, respectively (Fig. 1, 20S). Diffuse nucleoplasmic distribution of proteasomes is prominent using common formaldehyde-fixation (panels III–V), less revealed in methanol/acetone-based procedures and absent from cells that were fixed with a low concentration of formaldehyde (VI). The latter fixation results in localization of 20S proteasomes in multiple nucleoplasmic foci that resemble replication or transcription foci (Fig. 1, 20S, column VI). Application of this fixation procedure (VI) to well-defined nuclear proteins such as SmB/B′, H2A, and topoisomerase I rules out that the focal proteasome staining pattern represents random precipitation of proteasomes onto undefined nuclear structures (Fig. 1; Table 1, and data not shown), but rather suggests extraction of the homogeneous and speckled distribution which could mask proteasome localization within foci. Nucleoli are excluded from immunolabelling suggesting that 20S proteasomes are not located quantitatively within these NBs. In contradiction to results obtained from yeast (Enenkel et al. 1998; Takeda and Yanagida 2005), proteasomes do not accumulate at the nuclear periphery (lamin A/C), e.g. NE, of HEp-2 (Fig. 1, note corresponding DICs, first column) and other mammalian cells (data not shown). We conclude that nuclear distribution of proteasomes is species specific and differs in higher versus lower eukaryotes.

The nuclear localization pattern of 20S proteasomes obtained by different fixation procedures partly resembles nuclear distribution of spliceosomal component SmB/B′ (speckles) and histone protein H2A (homogeneous distribution), whereas no similarity is detectable with the staining patterns of nuclear signature proteins such as lamin A/C (Fig. 1, NE pattern), fibrillarin (data not shown, Table 1, nucleolar pattern), and centromeres (data not shown, Table 1, centromere pattern). These results are consistent with previous colocalization

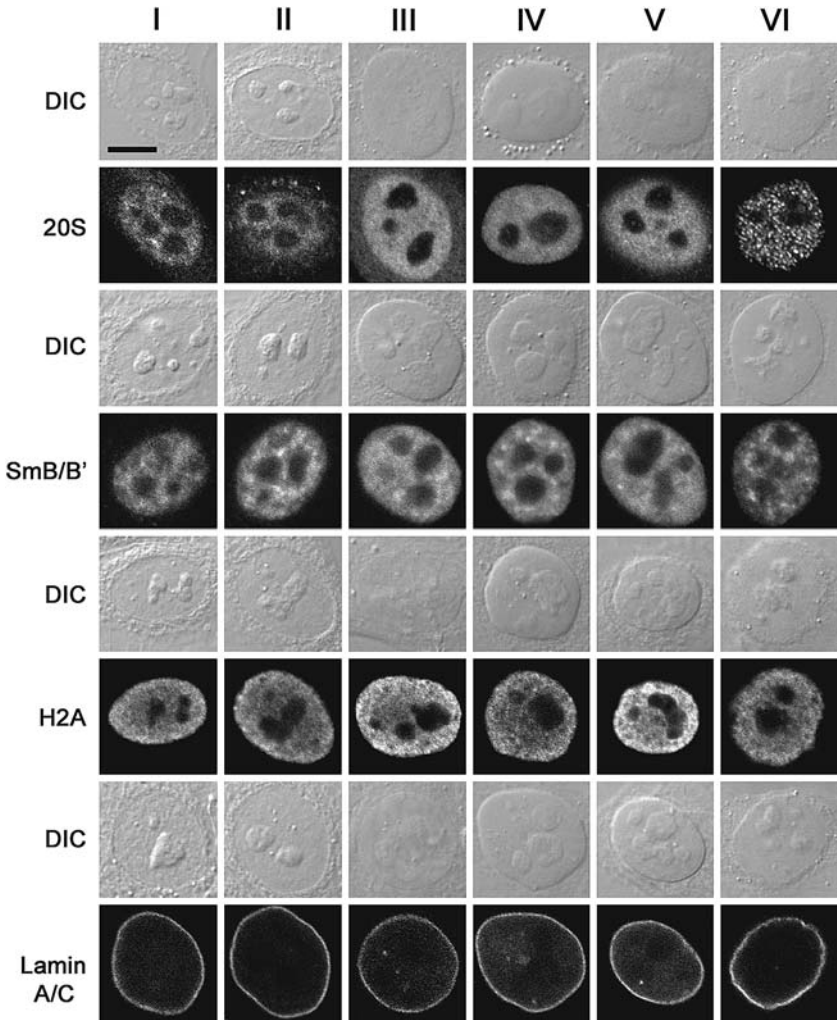

Fig. 1 Nuclear localization of 20S proteasomes, spliceosomal component SmB/B′, histone protein H2A, and lamin A/C. Subconfluent HEp-2 cells in interphase were fixed with methanol- and formaldehyde-based fixation procedures (I–VI) as detailed in Materials and methods and immunolabelled with rabbit polyclonal antibody to the 20S core of the proteasome, human autoimmune serum to SmB/B′, rabbit polyclonal antibody to H2A or mouse monoclonal antibody to lamin A/C. Micrographs of representative cells were detected by confocal microscopy. Corresponding cell morphology is obtained by differential interference contrast (*DIC*). *Bar* 5 μm

Table 1 Localization of nuclear proteasomes in comparison to signature proteins of the cell nucleus as detected by confocal immunofluorescence

Nuclear component	Nuclear distribution pattern in different fixation procedures					
	I	II	III	IV	V	VI
Group 1						
20S	npl,h,sp,f	npl,h,sp,f	npl,h	npl,h	npl,h	npl,f
Group 2						
DNA	npl,chr	npl,chr	npl,chr	npl,chr	npl,chr	npl,chr
H2A	npl,h	npl,h	npl,h	npl,h	npl,h	npl,h
PML	npl,dots	npl,dots	npl,dots	npl,dots	npl,dots	npl,dots
Lamin A/C	ne	ne	ne	ne	ne	ne
Nucleoli	nlr	nlr	nlr	nlr	nlr	nlr
SmB/B'	npl,h,sp	npl,h,sp	npl,h,sp	npl,h,sp	npl,h,sp	npl,h,sp
Centromeres	npl,dots	npl,dots	npl,dots	npl,dots	npl,dots	npl,dots
Group 3						
RNA pol IIo	npl,sp	npl,sp	weak f	npl,sp,f	npl,sp,f	npl,sp
RNA pol IIa	npl,h,sp,f	npl,h,sp,f	npl,h,f	npl,h,f	npl,h,f	npl,h,sp,f
Topo I	npl,h,nlr/r	npl,h,nlr/r	npl,h	npl,h	npl,h	npl,h,nlr/r

20S 20S proteasomes, *f* foci, *h* diffuse homogeneous, *H2A* histone protein 2A, *ne* nuclear envelope, *nlr* nucleolar, *nlr/r* nucleolar ring, *npl* nucleoplasmatic, *SmB/B′* spliceosomal component SmB/B′, *RNA pol IIo* RNA polymerase Iio, *RNA pol IIa* RNA polymerase Iia, *sp* speckles, *Topo I* topoisomerase I

studies of endogenous proteasomes and signature proteins of the cell nucleus that were obtained in our lab (Chen et al. 2002; Rockel and von Mikecz 2002; data not shown). SmB/B′ distributes homogeneously throughout the nucleoplasm, and concentrates in 20–40 interconnected or "reticulated" speckles that are prominent in methanol-based fixation procedures (I, II), and formaldehyde-based fixations (IV, VI), but masked by a diffuse nucleoplasmic staining in fixation procedures III and V. However, in contrast to a subfraction of 20S proteasomes, SmB/B′ does not localize to nucleoplasmic dots nor foci that represent a typical nuclear staining pattern of PML NBs, RNA polymerase I, and transcription factories (Table 1, data not shown). In order to characterize the spatial relationship between proteasomes, speckles and PML NBs in more detail, double labelling experiments were performed with respective signature proteins of these subnuclear structures, namely, the splicing factor SC35 (for speckles) and PML protein (for PML NBs). Figure 2 shows partial colocalization of SC35–speckles (red) with 20S proteasomes (green) in methanol/acetone- and formaldehyde-fixed cells (Fig. 2a, b, merge, arrows). Similarly, proteasomes (green) colocalize or juxtapose to a subpopulation of PML NBs (red) in both methanol/acetone- and formaldehyde-fixed cells (Fig. 2c, d, merge, arrows). The second column represents an inversed visualization of single IF-staining (black signal on a white background) that underpins the speckle or PML NB pattern as part of proteasomal distribution in the cell nucleus.

The application of a panel of commonly used fixation procedures results in different nuclear localization patterns of proteasomes, and gradually variable localization of spliceosomal component SmB/B′, whereas the distribution of nuclear proteins that participate in nucleosomes (histones), lamina, nucleolus, PML NBs, and centromeres remains unchanged (Fig. 1; Table 1). Formaldehyde is widely used and considered an excellent fixative for the localization of most nuclear proteins, while methanol-based fixation is generally applied to examine components of the cytoskeleton. Despite its "bad" reputation to precipitate proteins, methanol-based cell fixation did not induce alteration of the subnuclear distribution of most nuclear proteins we examined (Fig. 1; Table 1). Changes were observed concerning the subnuclear localization of 20S proteasomes, RNA polymerase II, and topoisomerase I (Table 1, group 1 and group 3, all enzymes). The fact that these alterations do not exactly correlate with application of formaldehyde- or methanol-based cell fixation procedures raises the following question: which proteasomal staining pattern represents the localization of endogenous proteasomes in living cell nuclei ?

Nuclear morphology corresponds in methanol-/acetone-fixed and living HEp-2 cells: implications for subnuclear localization of endogenous proteasomes

An ideal fixation procedure should make the antigen accessible, cause minimal denaturation of the antigen, and preserve cell morphology. In order to analyse the morphology of HEp-2 cells, DIC was compared after application of different fixation methods (Fig. 3a, DIC, I–VI). Nomarski-based DIC micrographs show a typical

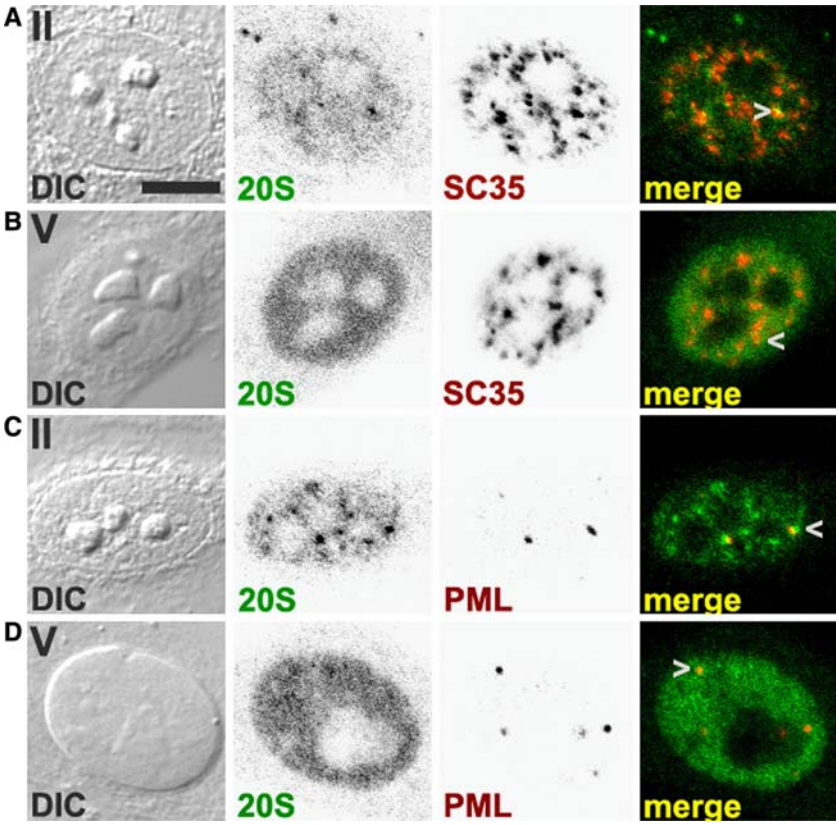

Fig. 2 Distribution of 20S proteasomes and nuclear bodies. Subconfluent HEp-2 cells in interphase were fixed with a methanol- (II) or formaldehyde-based fixation procedure (V) and co-immunolabelled with rabbit polyclonal antibody to the 20S core of the proteasome and one of the following: mouse monoclonal antibody against splicing factor SC35 (**a**, **b**) or mouse monoclonal antibody to PML (**c**, **d**). Merged images show colocalization and/or juxtaposition of 20S proteasomes with SC35-speckles or PML nuclear bodies (**a**–**d**, *last column*, *arrows*). Single immunofluorescence staining was transformed to greyscale and inverted using Adobe Photoshop in order to visualize nuclear bodies (**a**–**d**, *middle columns*). Micrographs of representative cells were detected by confocal microscopy. Corresponding cell morphology is obtained by differential interference contrast (**a**–**d**, *first column*, DIC). *Bar* 5 μm

bas-relief effect that reveals a highly structured cytoplasm in methanol-based fixation procedures and in living cells, whereas the cytoplasm appears less structured after methods that apply formaldehyde. Resemblance between living cells and methanol-fixed cells was also observed concerning the cell nucleus: sharp borders separate the nucleus from the cytoplasm in the NE region and nucleoli from the nucleoplasm (Fig. 3a, DIC, I–II, living cell). In contrast, formaldehyde-fixed nuclei display less distinct subnuclear structure and enlarged nucleoli (Fig. 3a, DIC, III–VI). Simultaneous labelling of DNA showed no apparent differences of localization of euchromatic and heterochromatic regions using different fixation methods; however, nucleolar DNA was exclusively stained in living cells (Fig. 3a, second column, DNA). The DIC analyses suggest that while all fixation methods used in this study preserve the general morphology of the cell nucleus, methanol-based procedures preserve nuclear morphology in a manner that resembles the one in living cells. Similarity of methanol-fixed cells and living cells was additionally observed concerning the morphology in *z*-axis, e.g. cell height (data not shown; Weidtkamp-Peters et al. 2006). This is consistent with the results showing that the majority of endogenous nuclear proteins do not alter their distribution due to application of methanol- or formaldehyde-based cell fixation (Table 1). The implication for the nuclear localization of endogenous proteasomes is that in HEp-2 cells they occur diffusely throughout the nucleoplasm, participate in speckles, nuclear dots and foci, but are absent from nucleoli and the NE. This subnuclear distribution pattern corresponds to the literature: (1) fusions of GFP and proteasomal subunits were localized diffusely throughout the nucleoplasm, but not within nucleoli nor the NE (Reits et al. 1997), (2) endogenous proteasomes partially colocalize with splicing factor SC35 and spliceosomal components (Chen et al. 2002; Rockel and von Mikecz 2002), (3) PML NBs contain components of the ubiquitin-proteasome system (UPS; Fabunmi et al.

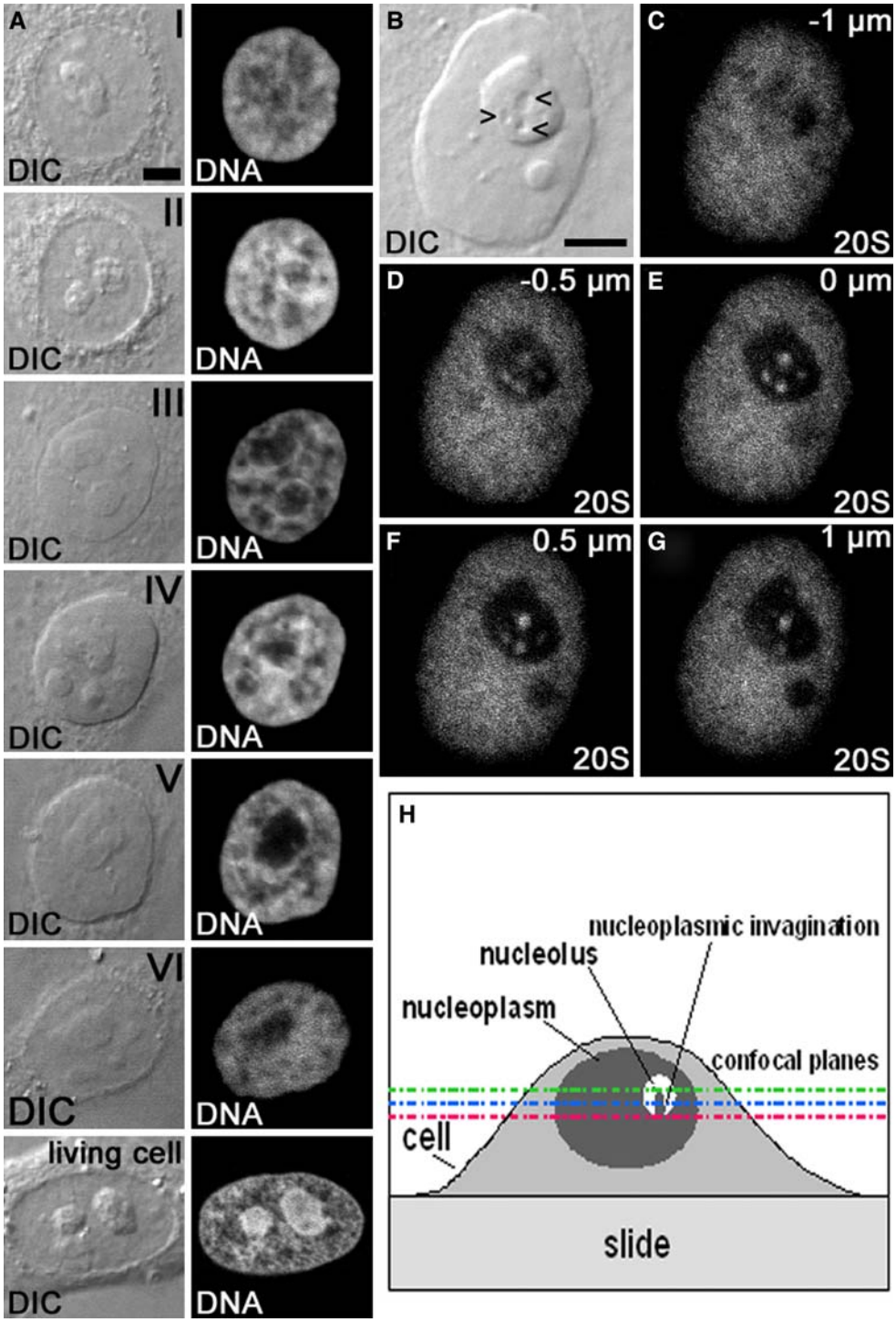

Fig. 3 Comparison of cell morphology and chromatin distribution in differently fixed cells and living cells. **a** DNA of interphasic HEp-2 cells was labelled with the DNA stain DRAQ5 (living cell, *bottom panel*) or cells were fixed with different procedures (I–VI) as detailed in Materials and methods and DNA was stained by TO-PRO-3 (*second column*). Micrographs of representative cells were detected by confocal microscopy and morphology was obtained by DIC (*first column*). **b** DIC of a HEp-2 cell (fixed and permeabilized according to procedure IV) with nucleoplasmic invaginations in the nucleolus seen as holes (*arrows*). **c**–**g** Confocal immunofluorescence sections of the cell depicted in **b** with a rabbit polyclonal antibody against the 20S proteasome shows nucleoplasmic invaginations into the nucleolus seen as rounded structures corresponding to the holes in the DIC. **h** Scheme of three confocal planes of one cell showing how nucleoplasmic invaginations cause false nucleolar localization. *Bar* 2.5 µm

2001; Rockel and von Mikecz 2002) and have been described as proteasomal degradation sites of a viral antigen (Anton et al. 1999), and (4) proteasome-dependent proteolysis occurs in transient nucleoplasmic foci (Rockel et al. 2005).

Our fixation results and the literature suggests that endogenous proteasomes participate in different populations in the interphase cell nucleus: (1) a soluble, highly mobile population that is diffusely localized throughout the nucleoplasm and extractable by methanol or a fixation procedure based on a low formaldehyde concentration (0.4%), (2) a population that is associated with subnuclear domains such as splicing speckles and PML NBs, and (3) a population that participates in protein degradation within transient proteolytic foci. The latter two proteasome populations may

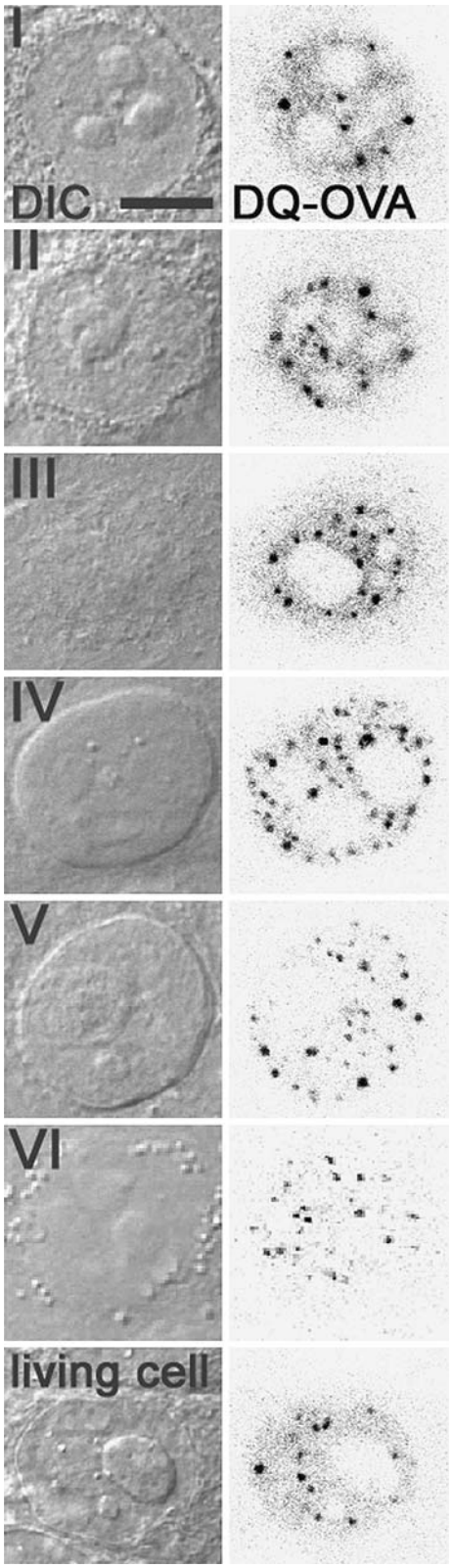

Fig. 4 Localization of proteasomal proteolytic foci in differently fixed cells versus living cells. Interphasic HEp-2 cells were microinjected with 0.5 mg/ml fluorogenic substrate protein DQ-OVA into the nucleus and living cells were observed (*bottom panel*) or fixed with different procedures (I–VI) as detailed in Materials and methods. Micrographs of representative cells were detected by confocal laser scanning microscopy (*second column*) and morphology is obtained by DIC (*first column*). Fluorescent signals were transformed to greyscale and inverted using Adobe Photoshop in order to visualize nucleoplasmic foci. *Bar* 5 μm

be masked by commonly used formaldehyde-based cell fixation and IF as was observed for the association of PCNA and replication sites (Bravo and MacDonald-Bravo 1987).

Controversial results have accumulated concerning the localization of proteasomes within nucleoli (reviewed in Wojcik and DeMartino 2003). We addressed this issue by observation of cells by means of DIC and show that depending on the cell fixation procedure nucleoplasmic invaginations expand into the nucleolus (Fig. 3b, arrows) that may induce false positive nucleolar IF signals of anti-proteasome antibodies in respective confocal planes (Fig. 3c–g; scheme in h). Nucleoplasmic invagination of nucleoli occurs with a low frequency (<5%) in methanol-based procedures, however, may reach a frequency as high as 30% in HEp-2 cells that were pre-extracted with TritonX−100 and subsequently fixed with formaldehyde. Spatially modulated illumination (SMI) microscopy confirmed that proteasomes are excluded from nucleoli in HEp-2 cells (U. Birk, C. Cremer, A. Scharf, A. von Mikecz, unpublished results).

Localization of proteasomal proteolysis in the interphase cell nucleus

Various approaches have been applied to visualize sites of nuclear functions such as replication and RNA synthesis. ^{3}H-labelled nucleosides, and the nucleotide analog dBrUTP or BrUTP, respectively, have enabled detailed analysis of replication and transcription in situ (Fakan and Puvion 1980; Jackson et al. 1993; Wansink et al. 1993). We showed recently by means of microinjection of an ectopic fluorogenic protein substrate that proteasomal protein degradation occurs in distinct nucleoplasmic foci which partially overlap with signature proteins of subnuclear domains such as splicing speckles, or PML NBs, ubiquitin, nucleoplasmic proteasomes, and RNA polymerase II (Rockel et al. 2005, Rockel and von Mikecz, 2002). These results established proteasomal proteolysis as an intrinsic function of the cell nucleus. In order to characterize the nuclear localization of proteasome-dependent proteolytic foci in more detail, HEp-2 cells were microinjected with the fluorogenic substrate DQ-ovalbumin (DQ-OVA) into the cell nucleus, subjected to living cell observation or different fixation procedures (I–VI) and analysed by

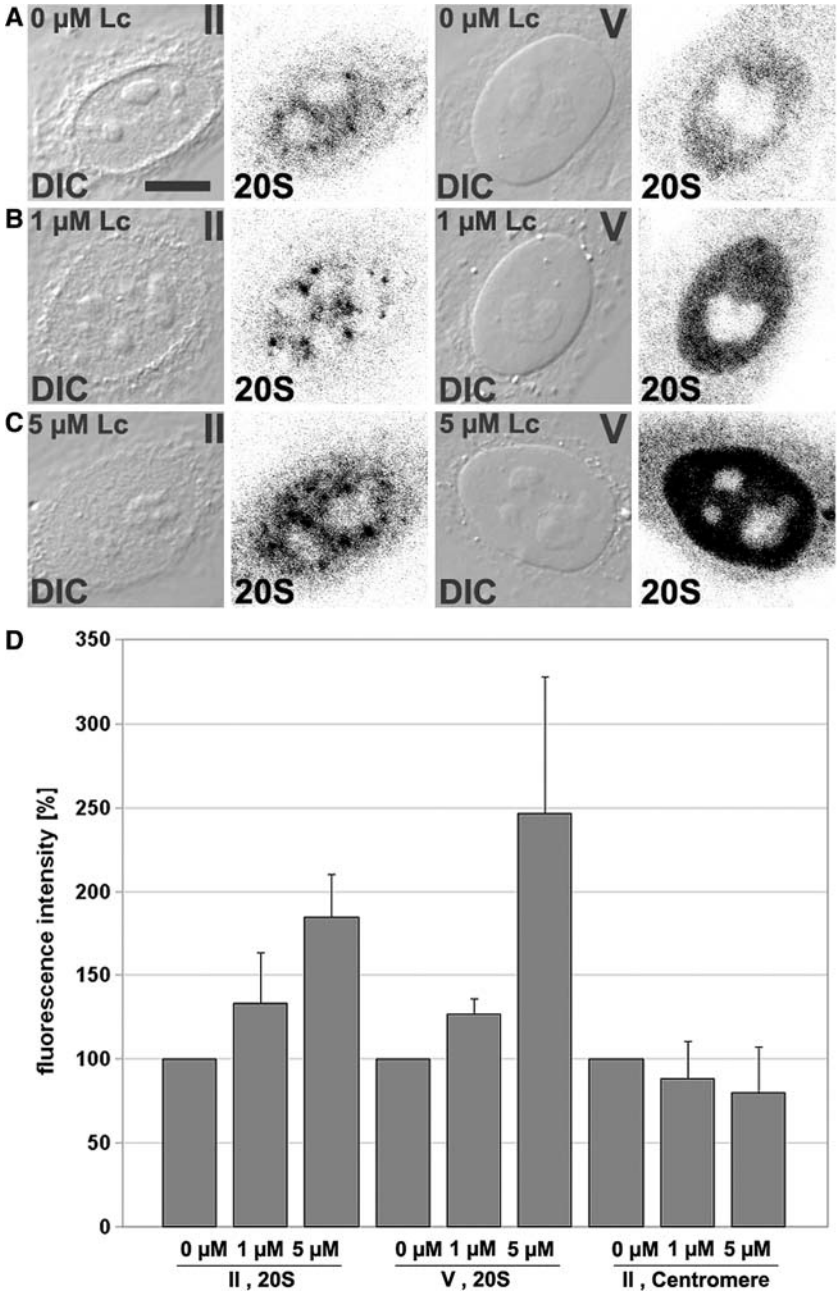

Fig. 5 Distribution of proteasomes during inhibition of proteasome-dependent proteolysis. Subconfluent HEp-2 cells in interphase were **a** left untreated or **b**, **c** treated with increasing concentrations of proteasome inhibitor lactacystin (Lc), fixed with a methanol- (II) or formaldehyde-based fixation procedure (V) and immunolabelled with rabbit polyclonal antibody to the 20S core of the proteasome. Micrographs of representative cells were detected by confocal microscopy. Immunofluorescence staining was transformed to greyscale and inverted using Adobe Photoshop in order to visualize nuclear bodies (20S). All images were acquired and processed under identical conditions. **d** Fluorescence intensity was quantified in 100–200 cell nuclei as detailed in Materials and methods. Values represent averages from three experiments ± SD. Corresponding cell morphology is obtained by differential interference contrast (DIC). II, fixation and permeabilization procedure II; V, fixation and permeabilization procedure V; 20S, 20S proteasomes. *Bar* 5 µm

confocal IF. DQ-OVA represents a fluorogenic substrate that is quenched by heavy labelling with BODIPY dyes. Upon hydrolysis of the DQ-OVA to single, dye-labelled peptides by proteases, this quenching is relieved, producing brightly fluorescent products.

Focal sites of DQ-OVA degradation occur in different sizes throughout the nucleoplasm excluding the nucleoli and NE region (Fig. 4, I–VI, living cell). Different sizes of DQ-OVA foci reflect their transient nature that was detected previously by time lapse experiments (Rockel et al. 2005), and may be due to the selection of the focal plane. In addition, a diffuse nucleoplasmic staining pattern was observed (Fig. 4, DQ-OVA, I–V, living cell) that seems to be extracted by the fixation procedure that includes low concentration formaldehyde fixation followed by permeabilization with TritonX-100 (Fig. 4, DQ-OVA, VI). The results suggest that focal sites of proteasomal degradation are localized throughout the nucleoplasm irrespective of the fixation procedure. Comparison with DQ-OVA foci in living cells corroborates the idea that proteasome-dependent proteolysis is an intrinsic function of the nucleoplasm. It is less clear if the diffuse staining pattern represents proteasomal proteolysis, since, in contrast to formation of proteolytic foci, diffuse nucleoplasmic DQ-OVA localization does not disappear as a consequence of inhibi-

tion of proteasomes (Rockel et al. 2005, Rockel and von Mikecz 2002). Again, there is no indication that nucleoli or the NE region contain any proteasomal activity, e.g. represent nuclear sites of proteasome-dependent protein degradation in basal cells. Lack of DQ-OVA penetration into the dense nucleolar body does not account for absence of proteasomal proteolysis foci within the nucleolus. We recently observed that ectopic D-peptides which are similarly sized in comparison to DQ-OVA are concentrated in nucleoli of HEp-2 cells (M. Chen, A. von Mikecz, Wiesehan, Willbold, unpublished observation).

In contrast to proteolytic foci, the subnuclear distribution of proteasomes is not altered when proteasome-dependent proteolysis is blocked. Lactacystin is a peptide-analogon that represents a specific inhibitor of the chymotryptic and tryptic proteasome activities. Treatment of cells with increasing concentration of lactacystin does not change the subnuclear distribution of 20S proteasomes (Fig. 5). In methanol/acetone-fixed cells (procedure II) proteasomes remain localized diffusely throughout the nucleoplasm and associated with speckles and foci (Fig. 5a–c, II, second column). A similar, although more diffuse, distribution pattern is observed in formaldehyde-fixed cells (Fig. 5a–c, V, last column). However, inhibitor treatment induces a significant increase of proteasome staining intensities (Fig. 5a–c micrographs, and d quantification) that cannot be attributed simply to experimental procedures, e.g. unspecific immunoaffinity, since IF of centromere, actin and nucleolar proteins does not accumulate in lactacystin-treated cells (Fig. 5d, centromeres; Rockel et al. 2002). It has to be further explored if the cells increase the expression of proteasomes in order to compensate for inhibition of proteolysis. Contradicting previous results (Arabi et al. 2003) proteasomes are not recruited to nucleoli after inhibition of proteasomal activity. We conclude that the experimental design accounts for these differences: Arabi et al. used (1) COS-7 cells that were transfected with GFP-myc and (2) proteasome inhibitor ALLN which is not as specific as lactacystin since it also inhibits calpains and other proteases. Overexpression of c-Myc and inhibition of other protein degradation pathways might not reflect the localization of proteasomes in cells with blocked proteasome-dependent proteolysis. Furthermore, nucleoplasmic invaginations into the nucleolus are more frequent in formaldehyde-based fixation methods and attribute to false positive nucleolar patterns (see above). Application of DIC represents a powerful tool to clarify these issues.

Systematic localization analyses show that endogenous proteasomes as well as proteasomal proteolysis distribute to distinct subnuclear domains (Fig. 6). Considering the central role of the ubiquitin-proteasome system in cellular processes, detailed knowledge of the time and place a substrate is ubiquitinated and degraded will prove to be essential to our understanding of the molecular mechanisms that regulate cell structure, function, and development. Elucidation of genetic codes from a variety of eukaryotes, including man, has provided us with the knowledge that thousands of genes

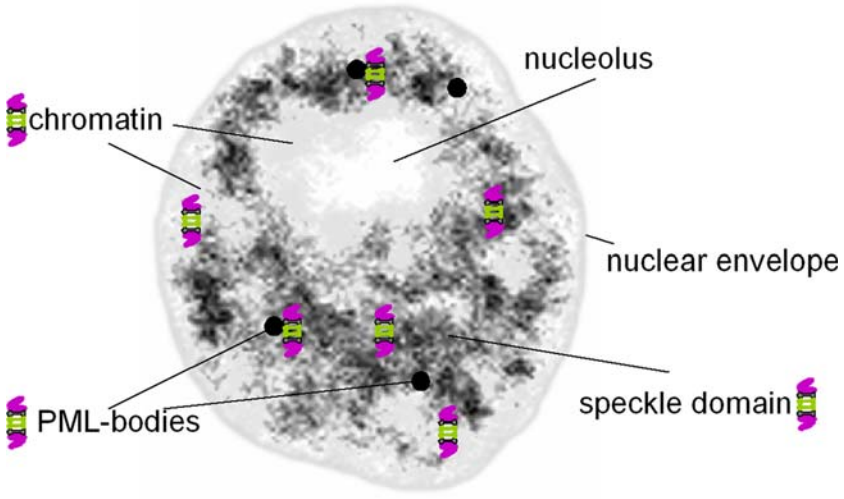

Fig. 6 The distribution of proteasomes in the mammalian interphase cell nucleus (working model). Proteasomes occur in three nuclear populations: (1) a soluble, highly mobile population that is diffusely localized throughout the nucleoplasm (Reits et al. 1997) and extractable by methanol or a fixation procedure based on a low formaldehyde concentration (0.4%), (2) a population that is associated with subnuclear domains such as PML NBs and speckles, and (3) a population that participates in protein degradation within transient proteolytic foci (von Mikecz 2006)

have to be organized and expressed in the cell nucleus. Hundreds of macromolecules including chromatin modulators, transcription factors and ribonucleoprotein particles (RNPs), cluster to form the molecular machineries for replication, DNA repair, transcription, RNA splicing, and ribosome biogenesis. It is largely unknown how the dynamics of nuclear structure and function are regulated. A major challenge of future research will be to further characterize nucleoplasmic protein clusters that contain proteasomes with respect to their (1) protein composition, (2) ubiquitination capacity, and (3) proteasomal activity in order to distinguish functional from pathological protein aggregates/clusters. The tight balance of ubiquitination and proteasomal proteolysis within or near such nucleoplasmic clusters may help to control and sustain nuclear function.

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