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**Tissue- and developmental-specific changes in the subcellular localization of the 26S
proteasome in the ovary of *Drosophila melanogaster*.**

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Abstract

The intracellular localization of the 26S proteasome in the different ovarian cell types of *Drosophila melanogaster* have been studied by immunofluorescence staining and laser scanning microscopy, using monoclonal antibodies specific for regulatory complex subunits of the 26S proteasome. During the previtellogenic phase of oogenesis (stages 1-6) there is strong cytoplasmic staining in the nurse cells and follicular epithelial cells, but the proteasome is not detectable in the nuclei of these cell types. The subcellular distribution of the 26S proteasome was completely different in the oocyte. Besides a constant and very faint cytoplasmic staining there was a gradual nuclear accumulation of proteasomes during the previtellogenic phase of the oogenesis. Characteristic subcellular redistribution of the 26S proteasome has occurred in the ovarian cells during the vitellogenic phase of the oogenesis. There was a gradual decline in the concentration of the 26S proteasome in the nucleus of the oocyte, in stage 10 oocyte the proteasome is undetectable in the nucleus. This is accompanied by a massive nuclear accumulation of proteasomes in the follicular epithelial cells. Our results indicate that in higher eukaryotes the subcellular distribution of the 26S proteasome is strictly tissue- and developmental-specific.

Results and Discussion

The ubiquitin-proteasome system is responsible for the controlled intracellular proteolysis of short-lived regulatory proteins. A ubiquitinating enzyme cascade recognizes the degradation signals present in short-lived proteins, and marks them by the covalent attachment of a

multiubiquitin chain. A large proteolytic complex, the 26S proteasome, can selectively recognize, bind and degrade the multiubiquitinated proteins. In an ATP-dependent reaction, the 26S proteasome can reversibly dissociate into two multiprotein subcomplexes: the catalytic core and the regulatory complex. 20S proteasome, the catalytic core, is a non-specific, multicatalytic protease which can not discriminate between multiubiquitinated or non-ubiquitinated proteins. The selectivity of the 26S proteasome is ensured by the regulatory complex which can recognize and bind multiubiquitinated proteins, and feed them into the catalytic core for degradation.

The fate of short-lived regulatory proteins depends on the efficiency of the ubiquitinating enzyme cascade, the concentration and distribution of the deubiquitinating enzymes, as well as on the availability of the 26S proteasome in the different compartments of the cell. The intracellular distribution of the 26S proteasome, being an important regulatory factor, has been studied in different eukaryotic cells. In lower eukaryotes, immunolocalization studies and *in vivo* analysis of the distribution of GFP-tagged proteasomes revealed that the nuclear periphery and the endoplasmic reticulum network are the predominant localization sites of proteasomes (Eneken et al., 1998; Wilkinson et al., 1998). In addition, in the fission yeast *Saccharomyces pombe* it was shown that a dramatic redistribution of the proteasomes occurred during the meiotic divisions. A more dispersed nuclear distribution was observed during the first meiotic cleavage, while a sharp concentration of the proteasomes to the area between the separating DNAs occurred during the second meiotic cleavage (Wilkinson et al., 1998). In higher eukaryotes, localization studies of the proteasomes have led to more conflicting results. While several studies claimed a uniform distribution of the proteasomes both in the cytoplasm and the nucleus (Peters et al., 1994; Palmer et al., 1996), other studies have provided evidence for a preferential or almost exclusive nuclear localization (Grossi de Sa et al., 1988; Pal et al., 1988; Stauber et al., 1987). There are also reports claiming an exclusive cytoplasmic localization of the proteasome (Kloetzel et al., 1987;

Beyette and Mykles, 1992). The predominant localization of the proteasomes at the nuclear periphery, characteristic in lower eukaryotes, has not been observed in higher eukaryotic cells. Besides a uniform cytoplasmic localization, cell-cycle dependent changes in the distribution of nuclear proteasomes have been detected in the ascidian *Halocynthia roretzi* (Kawahara and Yokosawa, 1992) and in immortalized ovarian granulosa cell lines (Amsterdam et al., 1993), indicating the direct involvement of proteasomes in cell cycle events. The accumulation of nuclear proteasomes has been reported during the early embryonic development of *Drosophila melanogaster* (Klein et al., 1990).

The conflicting results in higher eukaryotes may be due to the artefactual interaction of the proteasomes with different cellular constituents during the preparation of the sample. Alternatively, the differential compartmentalization of the proteasomes may represent a novel regulatory mechanism of the controlled intracellular proteolysis. To discriminate between these alternatives, we have analyzed the subcellular distribution of the regulatory complex of the 26S proteasome in the ovary of *Drosophila melanogaster*.

The *Drosophila* egg chambers are buds of the germariums, which are at the anterior end of the ovarioles (Spradling A. 1993). Each egg chamber is composed of 16 stem cell-derived germ cells and is encapsulated by somatic follicular cells. During egg chamber formation one of the germ cells differentiate to become the oocyte (positioned posteriorly), while the others acquire the nurse cell fate. The egg chambers undergo 14 distinct developmental stages, which can be divided into two major phases: the previtellogenic (stages 1-6) and the vitellogenic (stages 7-14) phases of oogenesis. During the previtellogenic stages the size of the egg chambers increase but their shape remains spherical. There is no difference in the size of the oocyte and the nurse cells because they grow at the same rate. Vitellogenesis starts at stage 7 when the oocyte begins to endocytose the yolk proteins synthesized by the fat bodies and follicular cells.

The vitellogenesis has a dramatic effect on the morphology and physiology of the egg chambers: they grow more rapidly than before, the growing rate of the oocyte is higher than that of the nurse cells, so it occupies a constantly growing portion of the egg chamber. The cytoskeletal network is completely rearranged, e.g., the polarity of the microtubules in the oocyte is totally reversed (Theurkauf et al. 1992), and the morphogenetic molecules which will determine the anterior-posterior and dorso-ventral axes in the embryo are asymmetrically deposited (Lasko P.1999). The follicular epithelium is also rearranged during the vitellogenic stages. Most of the follicular cells migrate to the posterior half of the egg chamber retaining their columnar shape, while those follicular cells which remain over the nurse cells become flat (Spradling A. 1993). The egg chamber seems to be an ideal test object to study the subcellular distribution of the proteasomes in functionally different cell types, under strictly identical sample preparation conditions.

Egg chambers of wild type *Drosophila melanogaster* were stained with a monoclonal antibody developed against subunit p54 of the regulatory complex of the 26S proteasome. p54 is the multiubiquitin-binding subunit of the regulatory complex, the *Drosophila* homologue of the human S5a and the yeast Rpn10 subunits (Hölzl et al., 2000). The monoclonal antibody MAb 439 has strict specificity for p54: it recognizes a single polypeptide in a total protein extract prepared from *Drosophila* embryos or flies, and this polypeptide corresponds to subunit p54, as revealed by immunoblotting a 2D gel-separated purified 26S proteasome preparation (Kurucz et al., 2002). Immunostaining analysis revealed a dramatic subcellular redistribution of the proteasomes in the ovaries during the previtellogenic-vitellogenic transition. In the previtellogenic phase the proteasome is detectable from the earliest stages: a faint signal is seen in the cell cysts and the cystoblast, the staining level increases in the stage 2 egg chamber (Fig 1A). Between stages 2-6 intense staining is seen in the cytoplasm of the nurse cells and the follicular epithelium, but the nuclei of these cells are devoid of a detectable amount of proteasomes (Fig. 1A, C and D). This is

in sharp contrast with the nucleus of the oocyte, which contains detectable amounts of proteasomes from the earliest stage, and the nuclear concentration of proteasomes continuously increases till stage 6. Nucleoli of the oocytes are not stained. Constant and faint cytoplasmic staining is seen in the oocyte during this phase of development.

During the vitellogenesis there is an immense subcellular redistribution of the proteasomes. From stage 7 the concentration of proteasomes in the oocyte nucleus declines, and by stage 10 it is not detectable at all (Fig. 2). The subcellular distribution of the proteasomes does not change in the nurse cells during the whole maturation period. There are, however, characteristic changes in the compartmentalization of the proteasomes in the follicular epithelial cells. At stage 10 the nuclei of the columnar follicular cells are packed with proteasomes, and this nuclear staining is just as intense as that of the cytoplasm.

The tissue specific subcellular distribution of the 26S proteasome in the different ovarian cell types, and its developmental changes were indistinguishable from that shown in Figs. 1 and Fig. 2 using a monoclonal antibody (MAb12A1) specific for subunit p48A (data not shown). p48A is an ATPase subunit of the regulatory complex, the *Drosophila* homologue of the human S6 and the yeast Rpt 3 subunits (Hölzl et al., 2000; Kurucz et al., 2002).

The data presented in Figs. 1 and 2 indicate that the subcellular localization of the 26S proteasome in the ovary of *Drosophila melanogaster* exhibits strict tissue- and developmental specificity. Although the oocyte and the nurse cells are derived from the same germline stem cell, the differentiation of this stem cell induces a major subcellular redistribution of the 26S proteasome. In a later phase of the developmental program of the oogenesis a second wave of tissue-specific subcellular redistribution of the 26S proteasome occurs.

The tissue- and developmental stage-specific differences in the subcellular localization of the 26S proteasome in the various ovarian cell types strongly argues against the assumption that

artefactual effects of sample preparation would produce the different patterns. It is much more reasonable to suppose that the subcellular distribution reflects the functional needs for the 26S proteasome in the different cellular compartments, which can vary according to cell types or developmental programs. All the changes, described so far during the vitellogenic phase of oogenesis affects only cytoplasmic structures or functions in the different ovarian cell types (endocytosis, microtubular network, mRNA localization, etc). The characteristic changes in the nuclear localization of the 26S proteasome in the oocyte and the follicular cells, however, strongly argue for hitherto unknown nuclear event(s) during oogenesis, in which the 26S proteasome may have a pivotal role.

The unidirectional transport of the 26S proteasome from the cytoplasm to the nucleus have been demonstrated in human fibrosarcoma cells (Reits et al., 1997). The presence of nuclear localization signals (NLS) on several α -type subunits of the catalytic core may explain the transport of the 26S proteasomes into the nucleus. These NLSs have been found to direct reporter proteins into the nucleus in an *in vitro* nuclear transport assay (Nederlof et al., 1995; Knuehl et al., 1996). The *in vivo* functional relevance of these NLSs in the nuclear transport of the complete 26S proteasome particle, however, has not been demonstrated. Assuming the involvement of NLSs in the nuclear transport of the 26S proteasome, the mode of regulation of this transport is still unresolved. What prevents the nuclear transport of the 26S proteasome in the ovarian follicular epithelium and nurse cells during the previtellogenic phase of the oogenesis? What is the specific role of the 26S proteasomes in the nucleus in cells having a large nuclear pool? Elucidation of these questions will be required to understand the functions of the 26S proteasome.

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Materials and Methods

Monoclonal antibodies developed against the subunits of the regulatory complex have been characterized earlier (Kurucz et al., 2002). The ovaries were dissected in PBS, fixed in 4% formaldehyde for 30 min and washed three times in PBS containing 1% Triton X-100 (PBS-T). The ovaries were incubated in blocking solution (PBS-T containing 2% bovine serum albumin and 5% fetal calf serum). The monoclonal antibody MAb 439 was diluted 1: 200 in blocking solution and was applied overnight at room temperature. After washing three times for 1hr in PBS-T, the ovaries were incubated overnight at room temperature with Cy3-conjugated goat anti-mouse IgG, 1:100 dilution in blocking solution (Jackson ImmunoResearch Laboratories). This was followed by three 1hr washing steps in PBS-T. Phalloidin-FITC was used at 1:2000 dilution (Molecular Probes). Confocal images were taken using Leica TCS-SP1T confocal microscopes. Images were processed using Photoshop 5.0 (Adobe).

Figure legends

Fig.1. Subcellular distribution of the proteasomes in the previtellogenic egg chambers. Anti-p54 monoclonal antibody, MAb 439 was used to stain egg chambers (red), the actin cytoskeleton was revealed by FITC-phalloidin staining (green).

Panel A. Overlay of 20 optical sections. Mass accumulation of the proteasomes in the oocyte (oo) nuclei (arrow heads) is visible as early as stage 2 (st2) during egg chamber development. Proteasome is not detectable in the nuclei of the nurse cells (nc) and the follicular cells (fc, arrows).

Panel B. Actin staining of the same ovary.

Panel C. Proteasome staining in a single optical section.

Panel D. Overlay of B and C. Scale bar: 50µm

Fig.2. Subcellular distribution of the proteasomes in the vitellogenic egg chambers. Stage 10 egg chambers were stained as described in Fig.1.

Panel A. Overlay of 20 optical sections. Pronounced proteasomes accumulation in the nuclei of the posterior follicular cells (arrows), no staining in the nucleus of the oocyte (arrow head).

Proteasomes were not detectable in the nurse cell nuclei either.

Panel B. Actin staining of the same ovary.

Panel C. Proteasome staining in a single optical section.

Panel D. Overlay of B and C. Scale bar: 50µm

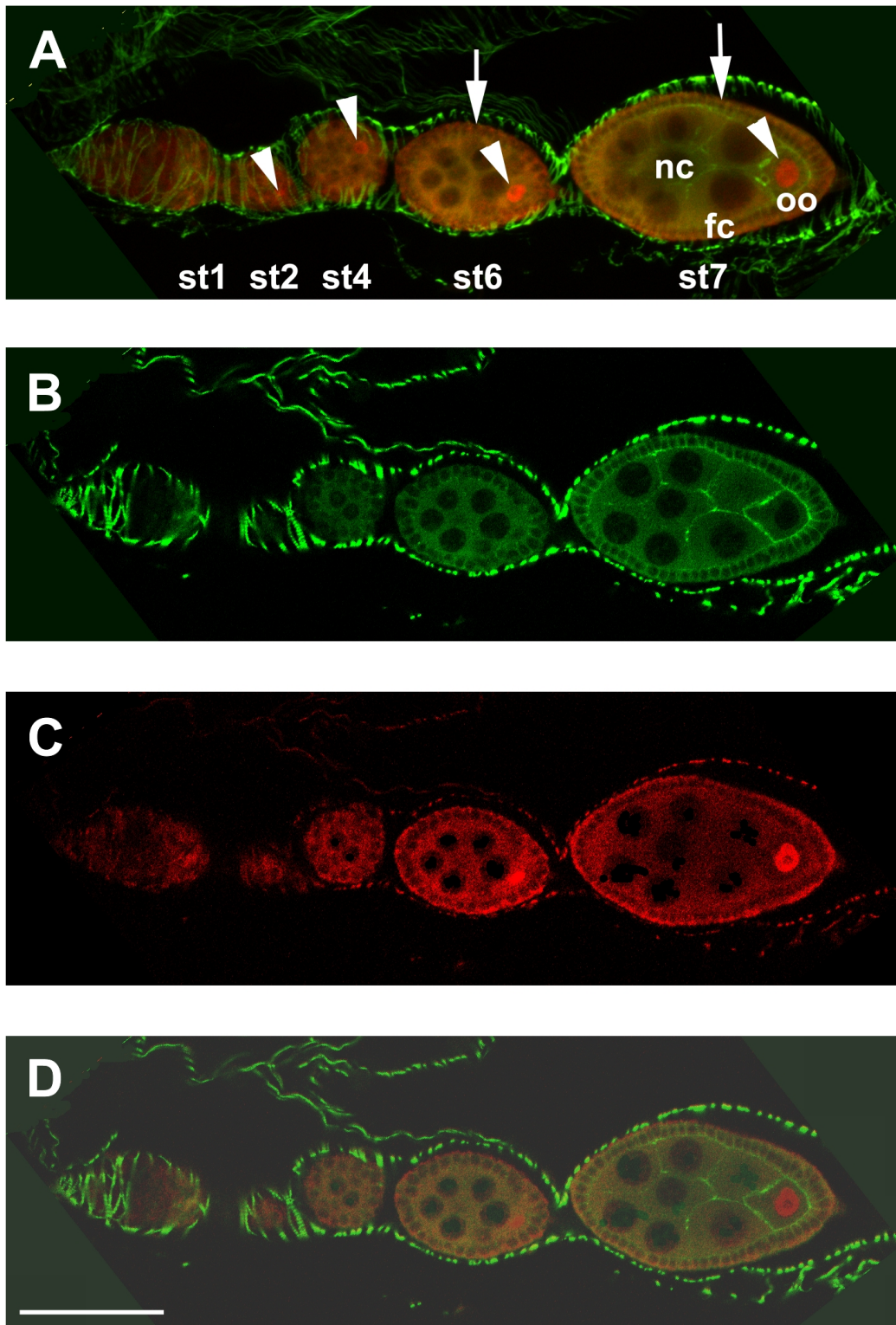


Figure 1

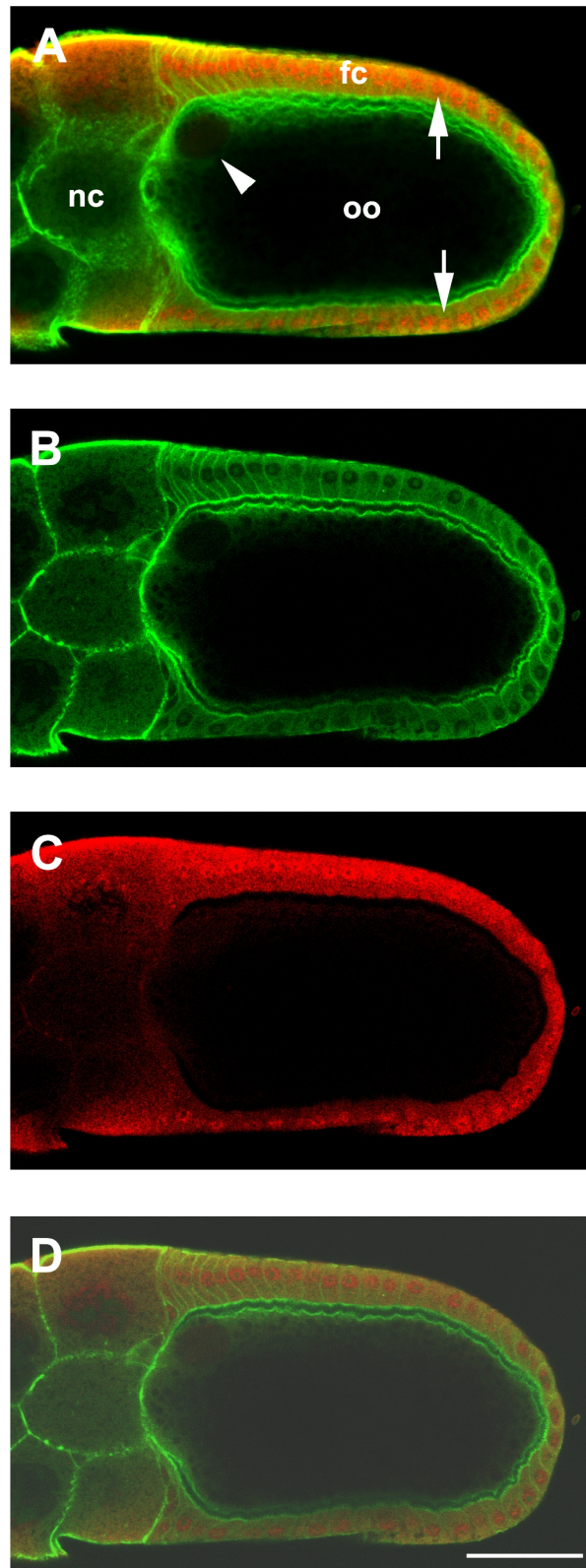


Figure 2