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Changes in intracellular localization of proteasomes in immortalized ovarian granulosa cells during mitosis associated with a role in cell cycle control

(cell division/spindle microtubules/cyclins/multicatalytic proteinase/immunofluorescence microscopy)

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ABSTRACT **We describe the isolation and characterization of proteasomes from recently established immortalized ovarian granulosa cell lines and their intracellular distribution during mitosis and during cAMP-induced differentiation, as revealed by immunofluorescence microscopy. In interphase, proteasomes were localized in small clusters throughout the cytoplasm and the nuclear matrix. In prophase, a substantial increase in proteasomal staining was observed in the perichromosomal area. A dramatic increase occurred in metaphase and in early anaphase; the chromosomes remained unstained. In late anaphase, intensive staining remained associated mainly with the spindle fibers. In telophase and early interphase of the daughter cells, intensive staining of proteasomes persisted in the nuclei. In contrast, in cells stimulated to differentiate by forskolin, which substantially elevates intracellular cAMP in these cell lines, only a weak staining of proteasomes was revealed in both the nucleus and the cytoplasm. Double staining of nondividing cells with antibodies to proteasomes and to tubulin did not show colocalization of proteasomes and microtubules. In contrast, dividing cells show a preferential concentration of proteasomes around spindle microtubules during metaphase and anaphase. The observed spatial and temporal distribution pattern of proteasomes during mitosis is highly reminiscent of the behavior of cyclins [Pines, J. & Hunter, T. (1991) *J. Cell Biol.* 115, 1–17]. Since proteasome accumulation appears to coincide with disappearance of cyclins A and B1 from the spindle apparatus, it is suggested that proteasomes may play a role in termination of mitosis by degrading the cyclins, which act as regulatory elements.**

The proteasome is a nonlysosomal proteinase complex (1, 2), which is present in all eukaryotic cells examined so far. The barrel-shaped complex has a molecular mass of 700 kDa and contains 15–20 different subunits, all encoded by members of one gene family (3, 4). Recently, proteasomes almost identical in size and shape but much simpler in subunit composition were discovered in the archaebacterium *Thermoplasma acidophilum* (5). In contrast to the wealth of experimental data on the proteolytic properties of proteasomes (6, 7) and growing insights into their structural organization (8–10), their physiological role has remained enigmatic.

From the localization of some genes encoding proteasomal subunits in the region of the major histocompatibility complex (MHC) class II (11–15) and from the transcriptional up-regulation of the genes encoding the MHC glycoprotein, the peptide transporter genes (16–18) and the proteasomal genes, it has been concluded that the proteasome is part of the antigen processing machinery (19). It has been proposed that proteasomes generate the peptide fragments from the intact antigen, which are then transported into the endoplasmic reticulum (19, 20). Proteasomes may well have acquired this function; however, their evolution must have preceded the evolution of the immune defense system.

A more general function of proteasomes has been suggested to be related to the ubiquitin proteolytic pathway (21, 22). Yeast mutants defective in the proteasome subunit carrying the chymotrypsin-like activity show reduced degradation of ubiquitinated proteins (23). Therefore, ubiquitinated proteins must be regarded as prime candidates for being the *in vivo* targets of the proteasome. It is still controversial, however, whether proteasomes are a component—possibly the catalytic core—of the 26S complex implicated as the effector of the ubiquitin proteolytic pathway (24–28). A recent review by Rechsteiner (22) lists the cellular substrates to which ubiquitin is conjugated. Since cyclins are among the proteins to which ubiquitin is conjugated (29), it is tempting to hypothesize that proteasomes have a role in cyclin degradation. To test this hypothesis, we have investigated the intracellular distribution of proteasomes during the somatic cell cycle and during differentiation. Previous experimental data on the intracellular distribution of proteasomes have been scarce and rather diverse. Proteasomes have been localized in the cytoplasm, in the nucleoplasm, and in both (30–34). Proteasomes were also reported to be associated with cytoskeletal elements (35). We have chosen the granulosa cell system for investigating their spatial and temporal distribution pattern, since stable lines derived from primary cells have recently been established (36, 37) that can be stimulated to differentiate by substances elevating their intracellular cAMP level (38, 39).

MATERIALS AND METHODS

Cell Cultures. Immortalized granulosa cell lines transfected by simian virus 40 (SV40) DNA (POGS5) and by SV40 DNA plus Ha-*RAS* oncogene (POGRS1) were cultured on 150-mm tissue culture plates (Falcon) in F-12/Dulbecco's modified Eagle's medium containing 5% fetal calf serum (FCS) as described (37). For immunostaining and ultrastructural observations, cells were grown for 48 h on 18-mm square glass coverslips, either in the presence of 5% FCS (proliferating cultures) or in the absence of FCS and in the presence of 100 μM forskolin, known to substantially inhibit growth and induce differentiation (37, 39).

Cell Harvesting and Isolation of Proteasomes. Proteasomes were isolated from both strains POGRS1 and POGS5. Cells were cultivated in 100 150-mm tissue culture dishes. The cells were harvested shortly before confluence. After a wash with 5 ml of phosphate-buffered saline (pH 7.5), cells were collected in 1 ml of 20 mM Tris·HCl/1 mM EDTA/1 mM NaN_3/1

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Abbreviation: SV40, simian virus 40.

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mM dithioerythritol/0.2 mM phenylmethylsulfonyl fluoride (PMSF) (TEADP buffer), pH 7.5, without trypsin treatment, by scraping with a rubber spatula. Harvesting yielded ≈12 ml of a loose pellet. The cells were broken by two cycles of freezing and thawing. The procedure was monitored by light microscopy. Particulate material, including intact nuclei, was sedimented with a Beckman microcentrifuge (4°C; 30 min; 13,500 × *g*). The supernatant was chromatographed on a DEAE-Sephacel ion-exchange column (Pharmacia). After washing the column with TEADP buffer, a linear salt gradient (0–500 mM NaCl) was applied (flow rate, 1 ml/min). As indicated by testing for chymotrypsin-like proteolytic activity with Suc-Ala-Ala-Phe-7-aminomethylcoumarin (40), the proteasomes eluted as a single peak. Fractions containing the major activity were pooled, concentrated with a Centricon-30 microconcentrator, and loaded on a Sephacryl HR S-400 gel-filtration column (Pharmacia). Fractions containing the main activity were subjected to another cycle of ion-exchange chromatography using Mono Q fast protein liquid chromatography (FPLC) (Pharmacia). After equilibration with TEAD buffer (TEADP buffer without PMSF), a salt gradient (0–500 mM NaCl; flow rate, 1 ml/min) was applied. All proteolytically active fractions were examined by SDS/PAGE. The fractions containing the proteasomes as the major protein were pooled, concentrated, and chromatographed on a Superose 6 gel-filtration column (Pharmacia).

Gel Electrophoresis and Western Blot. Electrophoresis in 15% polyacrylamide/Tricine/SDS-containing gels was performed as described (41) and proteins were transferred to a nitrocellulose membrane by wet blotting. Blots were treated with antibodies, and antigen–antibody complexes were visualized by using alkaline phosphatase-conjugated anti-rabbit IgG antibodies (42).

Electron Microscopy of Isolated Proteasomes. For electron microscopy, purified proteasomes were negatively stained with 2% (wt/vol) ammonium molybdate (pH 7.5). Conditions for recording electron micrographs and image analysis procedures have been described in detail (43).

Immunofluorescence Microscopy. Four different protocols were used for fixation of the cells on coverslips: (*i*) fixation at 24°C with 3% formaldehyde for 30 min, followed by 4-min exposure to 1% Triton X-100 as specified (38); (*ii*) fixation at 24°C for 15 min with methanol/acetone (35); (*iii*) fixation at −20°C with methanol for 15 min; (*iv*) fixation with 15% picric acid/2% paraformaldehyde solution for 30 min at 24°C followed by 70% methanol for 30 min (44). Cell morphology of fixed cells was examined by phase-contrast microscopy. After neutralizing free aldehyde groups by intensive washing with phosphate-buffered saline containing bovine serum albumin (10 mg/ml) and 10 mM glycine (pH 7.4), cells were incubated at 24°C for 1 h with a 1:50 dilution of two different antisera raised against rat muscle proteasomes and an antiserum against rat liver proteasomes (kindly supplied by B. Dahlmann, Düsseldorf University) followed by 1 h of incubation with goat anti-rabbit IgG coupled to rhodamine (1:40; Sigma). Nonspecific staining was examined on specimens incubated with nonimmune serum followed by goat anti-rabbit IgG conjugated to rhodamine. Specific intense staining of proteasomes was evident in the range of 1:50 to 1:200 dilution of the anti-proteasome antisera; nonimmune serum in a similar range of dilution gave a very weak nonspecific staining of the cells. For double-labeling of proteasomes and microtubules, cells were first labeled with antibodies to rat proteasomes and fluorescein goat anti-rabbit antibodies as described above. Next, the same cultures were incubated with mouse monoclonal antibodies to β-tubulin (Sigma) diluted 1:50–1:200 at 4°C for 12 h and subsequently with goat anti-mouse antibodies coupled to rhodamine at a 1:40 dilution for 1 h at 24°C. Cells mounted with Gelvanol (polyvinylalcohol/glycerol) medium were visualized by both phase-contrast and fluorescent microscopy using the Axophot microscope equipped with BP546/455 filters (Zeiss). Photographs were taken using immersion oil and an objective lens with a magnification of ×100.

RESULTS

Characterization of Proteasomes. Proteasomes from the two ovarian granulosa cell lines were purified from the cytoplasmic fractions as described. Electron micrographs of negatively stained proteasomes show the characteristic ring-shaped end-on views and rectangular side-on views with a striated pattern (Fig. 1). A yield of 500 μg of highly purified proteasomes was obtained from POGRS1 cells (100 culture dishes) compared to 200 μg from POGS5 cells. Since the POGS5 line has a much more developed actin cytoskeleton than the POGRS1 line (37, 45), one cannot exclude the possibility that a significant fraction of proteasomes was not released into the extraction medium in the POGS5 line because of their association with specific cytoskeletal proteins. The activity when the substrate Suc-Ala-Ala-Phe-7-aminomethylcoumarin was used was similar to that of proteasomes from rat skeletal muscle measured under the same conditions (40).

Upon SDS/PAGE (Fig. 2), proteasomes from the two cell lines showed a pattern of 8–10 subunits, all in the range of 25–31 kDa, characteristic of eukaryotic proteasomes. When purified, proteasomes and total cell lysates were subjected to SDS/PAGE, transblotted onto nitrocellulose, and reacted with three different polyclonal antibodies raised against proteasomes from rat skeletal muscle and rat liver. Only one subunit reacted strongly with one of the antisera raised against rat muscle proteasomes, while a second subunit showed a weak reaction. In the immunoblot of the cell lysates, no additional protein was detected. Another antiserum to muscle proteasomes showed a strong interaction with three different proteasomal subunits and a weak interaction with a fourth subunit, while antiserum to rat liver proteasomes reacted only with three different subunits. The same pattern of interaction on Western blots was revealed when

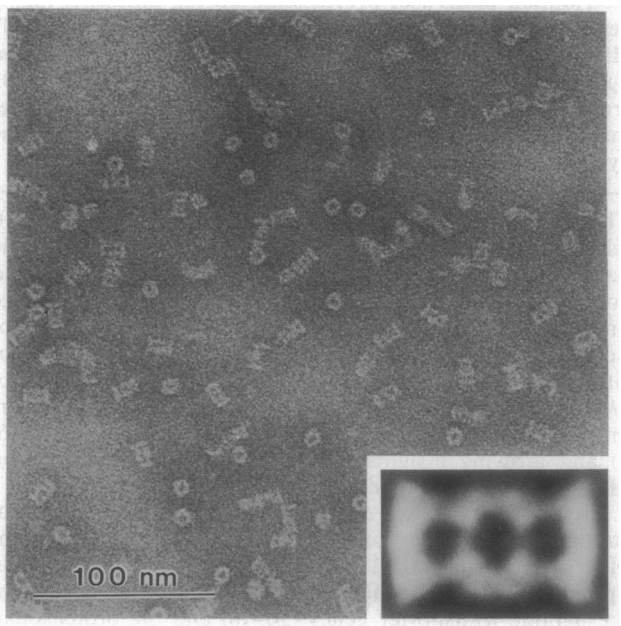

FIG. 1. Electron micrograph of purified proteasomes isolated from the POGS5 cell line. The preparation was negatively stained with ammonium molybdate (pH 7.3). The barrel-shaped protein complex is viewed end-on (ring-shaped particles) and side-on (rectangular particles). (*Inset*) After averaging, the side-on views show the characteristic tripartite structure reflecting the stacked ring structure of proteasomes.

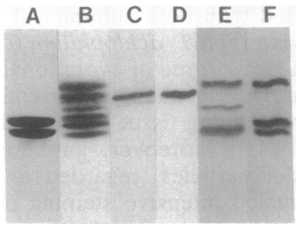

FIG. 2. SDS/PAGE of purified proteasomes and immunoblots of purified proteasomes compared with whole cell lysate. One microgram of purified *T. acidophilum* proteasomes (25 and 27 kDa) (lane A) and 1.5 μg of purified POGRS1 proteasomes (lane B) were subjected to SDS/PAGE. After electrophoresis (at 20 mA for 180 min) lanes A and B were directly stained with Coomassie blue. Lanes C, E, and F, POGRS1 highly purified proteasomes were subjected to PAGE and transferred to a nitrocellulose membrane by blotting at 60 mA for 16 h. The membrane was incubated with two different antisera against proteasomes of rat muscle (lanes C and E) and antiserum against rat liver proteasomes (lane F); antigen–antibody complexes were visualized by a second antibody labeled with alkaline phosphatase. Lane D, 20 μg of soluble protein fraction of cell lysate, excluding the nuclei, was separated on SDS/polyacrylamide gel. Antibody binding was performed as described above with the same antiserum as in lane C.

antibodies react with highly purified proteasome preparations or with homogenates (data not shown) of the oncogene-transformed granulosa cells. It was therefore concluded that the different antibodies react exclusively with proteasomes, although with different subunits.

Intracellular Localization of Proteasomes During the Cell Cycle. To follow the distribution of proteasomes in immortalized granulosa cells, cell cultures were fixed with formaldehyde, permeabilized briefly with Triton X-100, and stained with specific antibodies to rat muscle or rat liver proteasomes and subsequently with goat anti-rabbit IgG coupled to rhodamine. The fixation protocol described above (see *Materials and Methods*) resulted in a higher quality of structural preservation, although other methods of fixation gave essentially similar results.

In interphase, both nuclei and cytoplasm were stained weakly with specific antibodies to rat proteasomes. The staining occurred in numerous small clusters spread through the entire cytoplasm and the nuclear matrix; intracellular vesicular organelles and nucleoli were devoid of staining (Fig. 3). In cultures stimulated to differentiate by 48 h of incubation with 100 μM forskolin, staining was very low in both the POGRS1 and POGS5 lines. In proliferating cultures, all stages of the cell cycle could easily be monitored, especially in the POGS5 line in which the cells were well spread and attached firmly to the plastic dish. In early prophase, condensation of chromosomes was evident by their negative images, contrasting with the positive staining of the interchromosomal space by the antibodies, which was significantly higher than in interphase nuclei. In metaphase, a

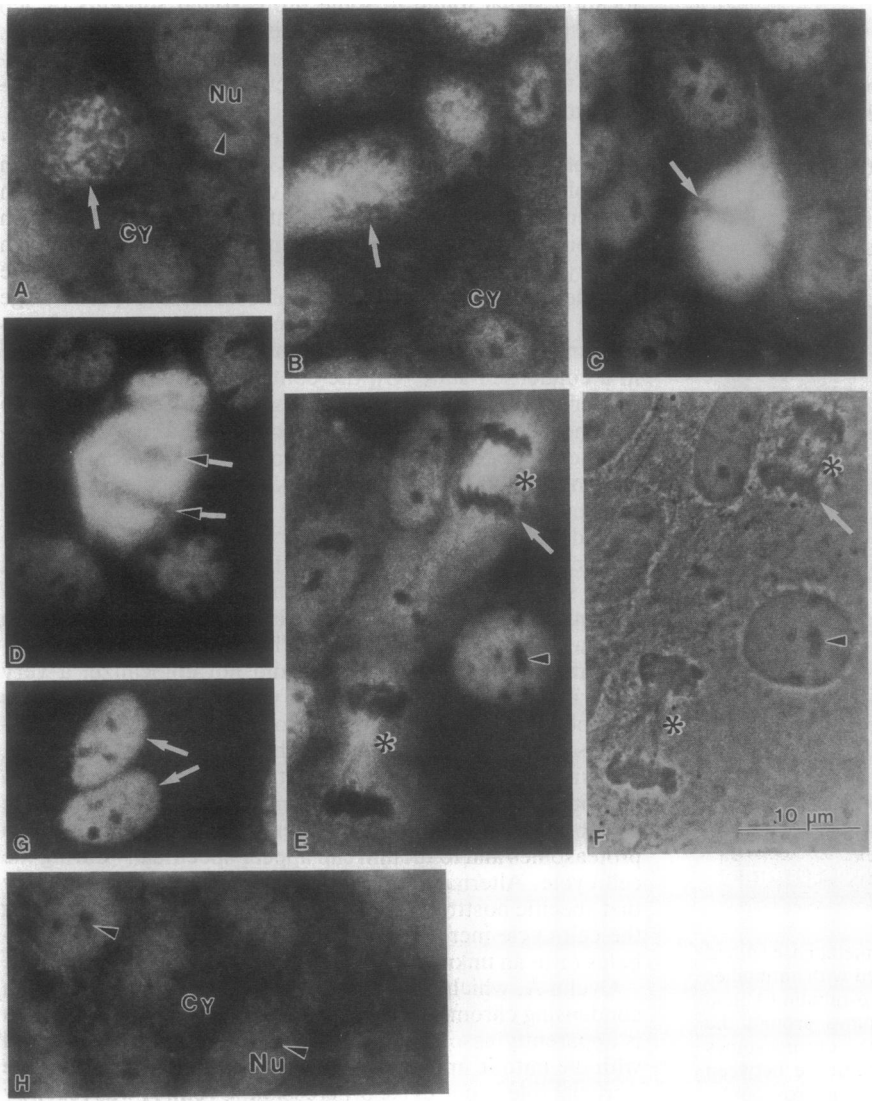

FIG. 3. Localization of proteasomes during the cell cycle. POGS5 cells were fixed with formaldehyde, permeabilized by Triton X-100, and stained sequentially with polyclonal antibodies to proteasomal protein followed by goat anti-rabbit IgG coupled to rhodamine. (*A*) Cells in interphase–early prophase. Chromosomal organization is evident due to surrounding positive staining for proteasomes (arrow). Nuclei (Nu) of interphase cells are less heavily stained, leaving the nucleoli unstained (arrowhead). (*B*) Late prophase. Chromosomes are well delineated by their negative staining with proteasome antibodies (arrow). Cytoplasm (Cy) shows particulate staining, suggesting clusters of proteasomes. Vesicular organelles in the cytoplasm are unstained. (*C*) Heavy labeling of a cell in metaphase. Shaded chromosome in the equatorial plane of the spindle appears unstained (arrow). (*D*) Early anaphase. Negatively stained chromosomes (arrows) start to move poleward. The rest of the cell is heavily stained. (*E*) Middle and late anaphase. Proteasomal staining is associated with the spindle fibers. Staining is less pronounced at late anaphase (asterisk at lower left). (*F*) Same cells as in *E* visualized by phase contrast. (*G*) Late telophase–early interphase. Daughter nuclei remain highly stained. (*H*) Cells in interphase after stimulation for differentiation by 48-h incubation with 100 μM forskolin. Particulate weak staining is seen throughout the nuclei and in the cytoplasm, leaving nucleoli (arrowheads) and some cytoplasmic organelles unstained.

dramatic increase in staining was observed; the chromosomes remained unstained. Since, at this stage, some rounding of the cells occurred, the negative image of the individual chromosomes was often less sharp. The intense staining of the cells was retained during early anaphase; as anaphase progressed, the cells were less intensively stained and proteasomes seemed to be associated mainly with the spindle fibers, leaving the daughter chromosomes clearly unstained. However, high-intensity proteasome staining was evident around the chromosomes, especially in early anaphase. In late telophase and early interphase, the daughter nuclei remained significantly stained; staining faded later in interphase. Identical spatial and temporal distribution of proteasomes was evident with all three antisera. We therefore conclude that this pattern is characteristic of the intact multisubunit proteasome molecule rather than of individual dissociated subunits. Antiserum raised recently against granulosa cell proteasomes gave an identical pattern of proteasomal localization.

To study possible interaction of proteasomes with microtubules, cells were double stained with fluorescein-labeled rabbit polyclonal antibodies to rat proteasomes and rhodamine-labeled mouse monoclonal antibodies to β-tubulin (Fig. 4). Colocalization of proteasomes and microtubules could not be detected in nondividing cells. In contrast, a preferential high concentration of proteasomes was clearly evident in the close vicinity of spindle microtubules in metaphase and anaphase, with a significantly lower concentration of proteasomes outside the spindle apparatus.

DISCUSSION

Proteasomes isolated from oncogene-transformed granulosa cell lines transfected either by SV40 DNA alone or by SV40 DNA and the Ha-*RAS* oncogene (37) showed a molecular architecture (Fig. 1) virtually indistinguishable from proteasomes isolated from other eukaryotic cells and cross-reacted with antibodies raised against proteasomes from rat muscle or rat liver. More strikingly, antibodies raised against proteasomes from the granulosa cell lines, at later stages of this work, cross-reacted with both subunits of the proteasome from the archaebacterium *T. acidophilum* (data not shown). This testifies to a high degree of evolutionary conservation.

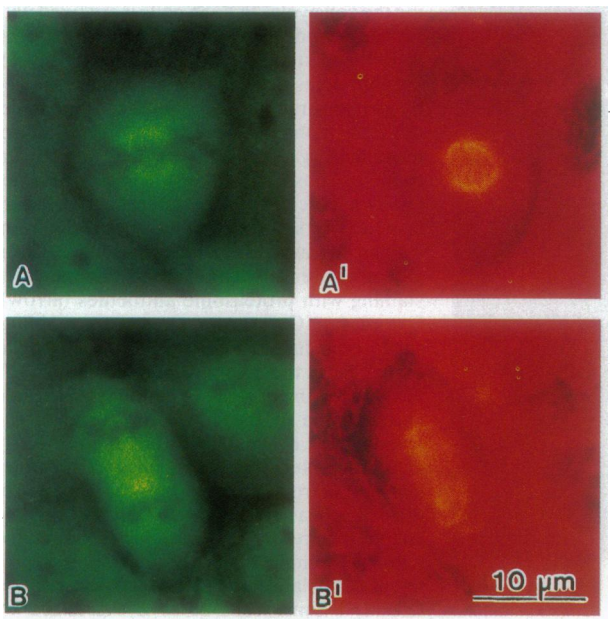

FIG. 4. POGS5 cells during mitotic division in metaphase (*A* and *A'*) and in early anaphase (*B* and *B'*) doubly stained with antibodies to proteasomes (*A* and *B*) and with antibodies to β-tubulin (*A'* and *B'*). Preferential high concentration of proteasomes around the microtubules of the spindle is evident, leaving the chromosomes unstained. In nondividing cells, there is no coincidence between microtubules and proteasome location (fluorescence microscopy).

Proteasomes are highly abundant in embryonic insects, particularly in proliferating tissues (46), and in transformed mammalian cells (47). Moreover, antibodies to *Xenopus laevis* 22S cylinder particles, regarded to be identical to proteasomes, revealed intensive staining of cells in metaphase (48). In the late blastula of *Pleurodeles waltli*, an association of proteasomes with the spindle fibers of a dividing cell was observed (34). However, there is no systematic study available to date on the distribution of proteasomes during different stages of the cell cycle and during differentiation.

Using specific antibodies to rat proteasomes, we have demonstrated that proteasomes are more abundant in proliferating cells than in cells stimulated to differentiate by elevating their intracellular cAMP by forskolin. This may be due to changes in expression and/or more rapid protein turnover of proliferating cells compared to the differentiated phenotype. Using immunofluorescence microscopy, we found significantly higher densities of proteasomes in prophase nuclei compared to interphase nuclei of forskolin-stimulated cells in which concentrations of proteasomes were relatively low, in both the nucleoplasm and the cytoplasm. The increase in nuclear proteasome concentration in prophase suggests that proteasomes can be rapidly transported from the cytoplasm to the nucleus. The nuclear location signal found in some proteasomal subunits (4, 49) could provide the molecular basis for such a mechanism. However, it is not yet clear whether nuclear envelope breakdown takes place prior to their accumulation in the perichromosomal region.

Our most striking finding is the extremely high concentration of proteasomes surrounding the mitotic apparatus in metaphase and in early anaphase; during late anaphase, high concentrations of proteasomes continue to be associated with the spindle fibers. This distribution is an indication of a high affinity between microtubules or other components of the spindle apparatus and the proteasomes. However, at no stage of the cell cycle was there evidence for interaction of proteasomes with the chromosomes, at least not while they were in a condensed state. On the contrary, chromosomes were rendered visible under the fluorescence microscope due to their negative staining with anti-proteasome antibodies. Nevertheless, since high concentrations of proteasomes were present around the chromosomes, some association of proteasomes with the surface of the chromosomes cannot be excluded.

Since our data on proteasome distribution during the cell cycle suggested a possible association of proteasomes with microtubules, we stained the cells with antibodies to both proteasomes and microtubules. While microtubules and proteasomes in nonstimulated cells did not colocalize, a very close association of proteasomes with spindle microtubules was observed during mitosis. However, since the proteasomes did not seem to decorate the microtubules precisely, and since there was no association of proteasomes with microtubules in nondividing cells, one could not exclude the possibility that a third component that can bind both to proteasomes and to tubulin can appear specifically during the cell cycle. Alternatively, we cannot exclude the possibility that specific posttranslational changes in proteasomes during the cell cycle increase their affinity to the spindle microtubules or to an unknown component of the spindle apparatus.

Cyclin A, which has been suggested to be associated with condensing chromosomes in prophase, and cyclin B1, which is apparently associated with condensed chromosomes and with the mitotic apparatus in prophase and metaphase, have a crucial role in cell cycle progression. Cyclin A was recently

shown to be destroyed during metaphase and cyclin B1 was destroyed at the metaphase–anaphase transition (50). There is also evidence that cyclins are degraded by ubiquitin-dependent proteolysis (29). The dramatic accumulation of proteasomes we observed coincides with the degradation of cyclins A and B1. Therefore, it is natural to suggest that a high proteasome concentration in the vicinity of the spindle apparatus is important to ensure the timely degradation of ubiquitinated cyclins, which is necessary for termination of mitosis, implying that proteasomes are an important element in the control of the cell cycle.

Maintenance of a high concentration of proteasomes in daughter nuclei after the nuclear membrane is reformed, and their gradual disappearance during differentiation, deserves further investigation. The dynamics of proteasome appearance and disappearance during the cell cycle and differentiation can be due either to shuttling of these complexes or to their turnover. Since data from other systems indicate a low rate of proteasome turnover (51), while there is rapid cell division in the transformed cells (37), it seems more probable that proteasome accumulation during mitosis is due to redistribution of the complexes within the cells, while the long-term effects in differentiation could be due to proteasome degradation and/or to down-regulation of their expression. Measuring the turnover rates of proteasomes and their possible association with ubiquitin at different stages of the cycle in synchronized cultures may give a more detailed insight into the contribution of shuttling versus turnover of proteasomes.

Note. A paper by Kawahara and Yokosawa on proteasome distribution in mitosis (52) was published after submission of this manuscript.

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