Proteolytic Processing and Assembly of the C5 Subunit into the Proteasome Complex

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Assembly of mammalian 20 S proteasomes from individual subunits is beginning to be investigated. Proteasomes are made of four heptamer rings in the configuration α7β7β7α7. By using anti-proteasome and anti-subunit-specific antibodies, we characterized the processing and assembly of the β subunit C5. The C5 precursor (25 kDa) remains as a free non-assembled polypeptide in the cell. The conversion of the C5 precursor to mature C5 (23 kDa) occurs concomitantly with its incorporation into 15 S proteasome intermediate and 20 S mature proteasome complexes. This processing is dependent on proteasome activity and takes place in the cytosol. These results are not fully compatible with the hypothesis that postulates that assembly of proteasomes takes place via a “half-proteasome” intermediate that contains one full α-ring and one full β-ring of unprocessed β subunit precursors.

The 20 S proteasome is the enzyme responsible for most non-lysosomal protein degradation in eukaryotes, and structural homologues are present in archeons and eubacteria (1, 2). The overall structure of the proteasome is a hollow cylinder composed of four heptamer rings in the configuration α7β7β7α7. The crystal structure of the Thermoplasma and yeast 20 S proteasomes (3, 4) implies the hydroxyl group of the NH2-terminal threonine residue of the 7 identical yeast 20 S proteasomes (3, 4) implies the hydroxyl group of the NH2-terminal proteolytic processing had taken place in the C5 subunit in mammalian systems by using C5 subunit-specific antibodies. These antibodies immunoprecipitate the C5 precursor (25 kDa) but not the native 20 S proteasome complex that contains the mature C5 (23 kDa). The C5 precursor re-
mains free in the cell, and its conversion to the 23-kDa polypeptide occurs concomitantly with its incorporation into 15 S proteasome intermediate and 20 S mature proteasomes.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Antisera—**NRK (rat), CHO (hamster), and HeLa (human) cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and grown to 60–80% confluency in 10-cm dishes. Rabbit anti-proteasome and anti-C5-specific antibodies, as well as other anti-subunit-specific antibodies (anti-C2, -C8, and -C9), have been described (22, 25, 26).

**Preparation of Subcellular Fractions—**NRK cells (70% confluent) were cooled on ice and washed 3 times with cold PBS. All subsequent steps were performed at 4 °C. Cells were scraped in PBS, centrifuged, and the pellet lysed by up and down pipetting in a buffer containing 10 mM Tris-Cl, pH 8.0, 7.5 mM (NH4)2SO4, 1 mM EDTA, 0.025% Nonidet P-40, and 1 mM dithiothreitol. After incubation on ice for 5 min, sucrose was added to the homogenate (0.3 M final concentration). Complete cell lysis was checked by phase contrast microscopy and trypan blue staining. Subcellular fractions were obtained by differential centrifugation of the cell homogenate as follows: nuclei, pellet of the cell homogenate after centrifugation at 10,000 x g for 10 min; mitochondria, pellet of the nuclear supernatant after centrifugation at 100,000 x g for 60 min; microsomes and cytosol (S100), pellet and supernatant of the postmitochondrial supernatant after centrifugation at 100,000 x g for 60 min, respectively. All pellet fractions were washed once with lysis buffer containing sucrose by gentle resuspension and recentrifugation as indicated above. Approximately 50 μg of protein of each of the subcellular fractions was used for immunoblot analysis with the indicated antibodies.

**Characterization of Anti-C5-, Anti-C8-, and Anti-C9-specific Antibodies and Protein Analysis—**Rat liver proteasome was purified as described (22, 27). The anti-subunit-specific antibodies were characterized by immunoprecipitation of the purified rat liver proteasomes under native or denaturing conditions. Native conditions for rat proteasomes in immunoprecipitation buffer are as follows: TBS (50 mM Tris-Cl, pH 7.5, 150 mM NaCl) containing 5% Nonidet P-40, 1 mM phenethylsulfonyl fluoride, and 10 μg/mL of leupeptin. Denaturing conditions for purified proteasomes were in the same buffer as above but containing 0.1% SDS and were boiled for 2 min before immunoprecipitation. For ELISA, 50 μl of a solution containing 50 μg/mL purified rat liver proteasome in TBS was used to coat 96-well plates (Nunc) by incubation overnight at 4 °C. Plates were then washed with TBS and blocked for 3 h at room temperature with 200 μl of blocking buffer (TBS with 3% bovine serum albumin) containing 0.1% Tween 20. Sera (50 μl) at different dilutions in blocking buffer with detergent were added to the wells and incubated for 3 h at room temperature or overnight at 4 °C. After washing with TBS, the secondary peroxidase-labeled antibody body at 1/1000 dilution in blocking buffer with detergent was added and incubated for 1 h at room temperature. After extensive washes, reaction was developed and quantitated in an EL340 reader at 490 nm. In each experiment, assays were run in triplicate, and control wells containing no primary antibody or preimmune sera were included to subtract background.

Immunoprecipitations were performed with the indicated antibodies previously coupled to protein A-Sepharose (Sigma) by incubation in TBS for 2 h at room temperature with rocking and washed 5 times with immunoprecipitation buffer (see above) by spinning in an Eppendorf microcentrifuge (10,000 rpm for 15 s). The samples to be immunoprecipitated were added to the beads containing the coupled antibodies and incubated for 4 h at 4 °C with rocking. The beads were washed three times with 1 ml of immunoprecipitation buffer (by spinning in an Eppendorf centrifuge, as above) and once with distilled water. The proteins were eluted with SDS-sample buffer and analyzed by SDS-PAGE.1 Proteins were analyzed on 10–20% gradient or 13% continuous gels, denaturing conditions, see above) or loaded onto 10–30% glycerol gradients, prepared as described (27). Proteasome and catalase activities were used as sedimentation markers, 20 S and 13 S sedimentation coefficients, respectively. Fractions from the glycerol gradients were analyzed by immunoprecipitation under native or denaturing conditions as described above.

**RESULTS**

**Characterization of Anti-C5-, Anti-C8-, and Anti-C9-specific Antibodies—**The anti-C5 antisera failed to immunoprecipitate the native proteasome complex (Fig. 1, A and B) which was readily immunoprecipitated by the anti-proteasome antisera (26). In contrast, denaturation of proteasomes by treatment with 0.1% SDS and boiling for 2 min allowed the anti-C5 antibodies to immunoprecipitate the 23-kDa C5 polypeptide in a dose-dependent manner (Fig. 1). Similar experiments to those shown in Fig. 1B blotted with other anti-subunit-specific antibodies (anti-C2, -C8, and -C9) failed to detect those subunits in the anti-C5 immunoprecipitates, but they were present, as expected, in the anti-proteasome immunoprecipitates (data not shown). Immunoblot analysis of subcellular fractions of NRK cells with the anti-C5 antibodies (Fig. 1C) showed that the 23-kDa C5 polypeptide is present in the nuclear, microsomal, and cytoplasmic (S100) fractions. Similar results were obtained with CHO and HeLa subcellular fractions (data not shown). Although the anti-C5-specific antibodies were unable to immunoprecipitate the native proteasome, they detected the purified proteasome complex when adsorbed to the wells of an ELISA plate (data not shown). Almost identical results to those shown in Fig. 1 were obtained with three different rabbit and four different mice anti-C5 antisera (data not shown).

The anti-C8 and anti-C9 antibodies were characterized in a similar way. Both antisera were unable to immunoprecipitate the native proteasome complex, whereas they immunoprecipitated the corresponding C8 and C9 subunits after denaturation of proteasomes (Fig. 2). The anti-C8 and anti-C9 antibodies gave similar results to those shown in Fig. 1C with immunoblots of NRK subcellular fractions (detecting the corresponding 29-kDa polypeptides) and were also able to detect the purified

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1 The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; MDP, multicatalytic proteinase, proteasome; ELISA, enzyme-linked immunosorbent assay; CHO, Chinese hamster ovary; NRK, normal rat kidney; PBS, phosphate-buffered saline.
proteasome complex when adsorbed to the wells of an ELISA plate (data not shown).

All these data permit us to draw the following conclusions with respect to the anti-C5, anti-C8, and anti-C9 antibodies obtained: 1) the epitopes recognized by these antibodies in their corresponding subunits are masked in the soluble native form of proteasomes; 2) those epitopes are clearly accessible after denaturation of the proteasome complex, as demonstrated by immunoprecipitation of the corresponding subunits after denaturation of proteasomes and by detection of these subunits in Western immunoblots; and 3) similarly, these epitopes are made accessible to recognition after adsorption of proteasomes to the wells of an ELISA plate.

Precursor Processing of the β-Subunit C5—To begin the study of the C5 subunit processing, we performed in vitro transcription/translation experiments of the full-length C5 cDNA. Fig. 3 shows the results of one of these experiments together with immunoprecipitation of the translated products with anti-C5 antibodies. The primary translation product of the in vitro transcribed rat C5 mRNA (similar results were obtained with mouse C5 mRNA, not shown) rendered a 25-kDa protein with a mobility in SDS-PAGE identical to the purified recombinant C5 protein (Fig. 3, compare lanes 2 and 5) and being immunoprecipitated by the anti-C5 antibodies (Fig. 3, lane 3). These in vitro experiments demonstrated that the precursor C5 protein (pro-C5, 25 kDa) is readily immunoprecipitated by our anti-C5 antisera.

To analyze the processing of the pro-C5 subunit, we used pulse-chase experiments and glycerol gradient sedimentation of cell-free extracts. The different samples were analyzed by immunoprecipitation with anti-proteasome, anti-C8-, or anti-C5-specific antibodies. As shown in Fig. 4A, the anti-C5 antibodies immunoprecipitated a labeled polypeptide of 25 kDa from total cell extracts, whose amount decreased during the chase period. Under the same conditions, the anti-proteasome antibodies immunoprecipitated a set of labeled polypeptides at the beginning of the chase experiment, and both the amount of total radioactivity and the number of immunoprecipitated polypeptides increased during the chase period (Fig. 4B). In contrast, anti-C8 antibodies (Fig. 4C) immunoprecipitated a set of polypeptides at the beginning of the chase experiment, and both the amount of total radioactivity and the number of immunoprecipitated polypeptides increased during the chase period (Fig. 4B). In contrast, anti-C8 antibodies (Fig. 4C) immunoprecipitated a set of polypeptides at the beginning of the chase experiment, and both the amount of total radioactivity and the number of immunoprecipitated polypeptides increased during the chase period (Fig. 4B). In contrast, anti-C8 antibodies (Fig. 4C) immunoprecipitated a set of polypeptides at the beginning of the chase experiment, and both the amount of total radioactivity and the number of immunoprecipitated polypeptides increased during the chase period (Fig. 4B). In contrast, anti-C8 antibodies (Fig. 4C) immunoprecipitated a set of polypeptides at the beginning of the chase experiment, and both the amount of total radioactivity and the number of immunoprecipitated polypeptides increased during the chase period (Fig. 4B). In contrast, anti-C8 antibodies (Fig. 4C) immunoprecipitated a set of polypeptides at the beginning of the chase experiment, and both the amount of total radioactivity and the number of immunoprecipitated polypeptides increased during the chase period (Fig. 4B). In contrast, anti-C8 antibodies (Fig. 4C) immunoprecipitated a set of polypeptides at the beginning of the chase experiment, and both the amount of total radioactivity and the number of immunoprecipitated polypeptides increased during the chase period (Fig. 4B). In contrast, anti-C8 antibodies (Fig. 4C) immunoprecipitated a set of polypeptides at the beginning of the chase experiment, and both the amount of total radioactivity and the number of immunoprecipitated polypeptides increased during the chase period (Fig. 4B). In contrast, anti-C8 antibodies (Fig. 4C) immunoprecipitated a set of polypeptides at the beginning of the chase experiment, and both the amount of total radioactivity and the number of immunoprecipitated polypeptides increased during the chase period (Fig. 4B). In contrast, anti-C8 antibodies (Fig. 4C) immunoprecipitated a set of polypeptides at the beginning of the chase experiment, and both the amount of total radioactivity and the number of immunoprecipitated polypeptides increased during the chase period (Fig. 4B). In contrast, anti-C8 antibodies (Fig. 4C) immunoprecipitated a set of polypeptides at the beginning of the chase experiment, and both the amount of total radioactivity and the number of immunoprecipitated polypeptides increased during the chase period (Fig. 4B). In contrast, anti-C8 antibodies (Fig. 4C) immunoprecipitated a set of polypeptides at the beginning of the chase experiment, and both the amount of total radioactivity and the number of immunoprecipitated polypeptides increased during the chase period (Fig. 4B).
In vitro translation of in vitro transcribed rat C5 mRNA. In vitro transcribed C5 mRNA was translated in a rabbit reticulocyte lysate and analyzed by 13% SDS-PAGE. Lanes 1–3 show the autoradiogram of the nitrocellulose filter. Lane 1, total translation reaction no mRNA added; lane 2, total translation reaction with in vitro transcribed rat C5 mRNA; lane 3, immunoprecipitation with anti-C5 antibodies of the translation products. Lanes 4 (proteasome) and 5 (recombinant rat C5 protein) show the immunoblot of the same nitrocellulose filter developed with the anti-C5 antibodies.

To examine the complexes that contained the C5 precursor and its processed form, we used glycerol gradient sedimentation of total radiolabeled cell extracts and immunoprecipitation. All fractions from glycerol gradients were denatured (SDS and boiling) before immunoprecipitation with the anti-C5 antibodies. Immediately after the pulse, the anti-C5 anti-serum immunoprecipitated the free non-assembled C5 (peak at fraction 20, top of the gradient, Fig. 5A). Similar results were obtained when glycerol gradient fractions were used directly for immunoprecipitation without prior denaturation. After an 8-h chase, the free C5 (Fig. 5C) was no longer detectable; and the processed C5 subunit (23 kDa) was found to sediment with 20 S sedimentation coefficient. Proteasome peptidase activity, detected by hydrolysis of N-succinyl-LLVY-methyleumarin, was observed peaking at fraction 6, confirming the position of the active mature proteasome at 20 S. After 3 h of chase an intermediate situation was obtained (Fig. 5B), free C5 precursor (25 kDa) at the top of the gradient and the processed C5 subunit (23 kDa) mainly sedimenting at 20 S, and some at ~15 S were detected.

These results were in apparent conflict with previous research (17, 29) that reported the presence of precursor C5 subunit in pre-proteasome complexes. To clarify this issue we performed pre-clearing experiments. After a 30-min pulse, radiolabeled cell extracts were pre-cleared with an excess of anti-proteasome, anti-C8, or anti-C9 antibodies. Afterward, the pre-cleared lysates were immunoprecipitated with the same antibodies or with anti-C5 antibodies. Pre-cleared with any of the three antibodies and re-immunoprecipitation with the same antibody failed to reveal any labeled polypeptides. Anti-C8 and anti-C9 antibodies failed to immunoprecipitate any labeled polypeptides from lysates pre-cleared with anti-proteasome antibodies. Anti-proteasome antibodies also failed to immunoprecipitate any complex from the anti-C8 and anti-C9 pre-cleared lysates, although some free C2 subunit was immunoprecipitated (data not shown). Immunoprecipitations of these pre-cleared lysates with anti-C5 antibodies demonstrate that the C5 precursor remained in the supernatants and was effectively immunoprecipitated by the anti-C5 antibodies. Similar results were obtained when we used pre-cleared extracts obtained after a 3-h chase period (data not shown). A short pulse (30 min) may not have allowed enough accumulation of labeled pro-C5, and as a consequence its incorporation into a complex could be undetectable. To deal with this possible criticism, we conducted pre-clearing experiments with extracts prepared from cells continuously labeled for 3 h and then chased for 24 h. Fig. 6A shows that anti-proteasome, anti-C8, and anti-C9 antibodies immunoprecipitate similarly labeled complexes. After a 24-h chase, only the anti-proteasome antibodies show the immunoprecipitation of an apparently mature proteasome complex. This complex is no longer recognized by the anti-C8 and anti-C9 antibodies, as expected, because these antibodies are unable to immunoprecipitate native mature proteasomes (Fig. 2). The anti-C5 antibodies immunoprecipitated the C5 precursor under native conditions, and after a 24-h chase no labeled C5 polypeptide was immunoprecipitated (Fig. 6A). These results were as predicted, all the C5 precursor is processed after 24 h of chase and incorporated into mature proteasomes (not immunoprecipitated by the anti-C5 antibodies, Fig. 1). Under denaturing conditions the anti-C5 antibodies immunoprecipitated both precursor and processed C5 subunit (Fig. 6B, pulse control (Con.) lane) from total 3-h pulse-labeled cell extracts. The pre-cleared lysates from the different immunoprecipitations shown in Fig. 6A were made to 0.1% SDS (final concentration), boiled for 2 min, and then immunoprecipitated with the anti-C5 antibodies (Fig. 6B). Immediately after the 3-h pulse, the anti-C5 antibodies readily immunoprecipitated the precursor C5 from extracts pre-cleared with the anti-proteasome, anti-C8, and anti-C9 antibodies, whereas only the processed C5 is present in lysates pre-cleared with anti-C5 antibodies (Fig. 6B). After a 24-h chase, no C5 precursor remains (Fig. 6A), and only extracts pre-cleared with anti-proteasome antibodies show complete removal of processed C5 subunit. In contrast, the processed C5 subunit remained present and was immunoprecipitated by the anti-C5 antibodies in those extracts that have been pre-cleared with anti-C5, anti-C8, and anti-C9 antibodies, respectively.

These results clearly demonstrated that most of the C5 precursor (25 kDa) is free and non-assembled in the cell, and only the processed C5 (23 kDa) is part of intermediate and mature proteasome complexes.

Processing of C5 Precursor Is Dependent on Proteasome Activity and Takes Place in the Cytosol—To investigate the possible dependence of C5 processing on proteasome activity, and to study where in the cell it takes place, we performed a series of experiments summarized by the data presented in Figs. 7 and 8. Radiolabeled total lysates prepared from NRK cells, treated or untreated with 5 μM lactacystin, were used directly or pre-cleared with an excess of anti-proteasome antibody before immunoprecipitation with the anti-C5 antibodies under denaturing conditions. Fig. 7A shows immunoprecipitation of total cell extracts with anti-C5 antibodies under denaturing conditions. The results show that treatment with lactacystin prevented the processing of subunit C5 (as expected), and the amount of total labeled C5 subunit is similar under all experimental conditions. Fig. 7B shows that anti-proteasome antibodies (under native conditions) immunoprecipitated an initial complex whose formation is not affected by treatment with lactacystin (pulse lanes, Fig. 7B). However, lactacystin treatment prevented the incorporation of labeled subunits and the disappearance of subunit pro-Z during the chase period (compare chase lanes, Fig. 7B). When cell lysates were pre-cleared with an excess of anti-proteasome antibody and the supernatants immunoprecipitated with anti-C5 antibodies under denaturing conditions, the C5 precursor was readily immunopre-
cipitated and diminished during the chase period in the absence of lactacystin (Fig. 7C) as expected (see Fig. 4A). In contrast, the C5 precursor remains unchased and not immunoprecipitated by the anti-proteasome antibodies when the cells are incubated in the presence of lactacystin (Fig. 7C). These data further reinforced the conclusion that the C5 precursor (25 kDa) is a free subunit, because its processing to the 23-kDa species is dependent on proteasome activity, and blocking its processing prevents its incorporation into a complex. Fig. 8 shows the results of immunoprecipitation with anti-C5 antibodies under denaturing conditions of nuclear and cytosolic fractions of NRK cells continuously labeled for 3 h. The precursor (25 kDa) and the processed C5 (23 kDa) subunit are clearly observed in the cytoplasmic fraction, whereas only the processed C5 subunit is present in the nuclear fraction. Similar results were obtained in pulse-labeled experiments of HeLa and CHO cells (data not shown). These results showed that the processing of the C5 subunit takes place in the cytosol, and as a consequence, only the processed C5 subunit is present in the cell nucleus.

### DISCUSSION

Mammalian proteasomes are composed of seven different α subunits and seven different β subunits that have to be assembled into an ordered structure with the configuration α7β7β7α7 (1, 2). Proteasome subunits are synthesized as independent polypeptides encoded by different mRNAs, and initially they should behave as free non-assembled subunits in the cell. α subunits of the mature proteasome complex seem to have the same amino acid sequence as their primary translation product, whereas most of the β subunits are synthesized as precursors (pro-β subunits) that subsequently undergo proteolytic processing in their NH2 terminus (5). The steady state levels of free α and β subunits in the cell seem too low to be detected by conventional analysis (gradient sedimentation or gel filtration followed by immunoblot of the corresponding fractions). Yang et al. (17) clearly demonstrated the presence of free non-assembled subunit C9 in RMA cells after a radioactive pulse for 30 min. We have data (not shown) that demonstrate the presence of free non-assembled subunit C2 and C9 after a 30-min pulse in NRK, CHO, and HeLa cells. With regard to the pro-β subunits, Yang et al. (17) mentioned the detection of free pro-LMP2, and Thomson and Rivett (13) showed the presence of free pro-N3. We have shown here the presence of free pro-C5. Therefore, from direct analysis of cells pulse-labeled for a short period with anti-α- and anti-β subunit-specific antibodies, we can draw a tentative conclusion that all newly synthesized α and pro-β subunits are initially free non-assembled subunits.

The half-lives of proteasomes are between 8 and 16 days in liver (30, 31), 2 days in H6 cells (29), and 5 days in HeLa cells (32). This long half-life of proteasomes (longer than the doubling time of cells in culture) implies that after a radioactive pulse, labeled subunits once incorporated into mature proteasomes would persist for long periods, just the opposite of what is usually obtained for most cell proteins in pulse-chase experiments. Therefore, a critical issue is whether synthesized subunits (labeled subunits after a short pulse) also have a long half-life like the mature proteasomes or shorter, because free subunits or proteasome intermediates are degraded. We have measured (densitometric scanning of autoradiograms) the radioactivity incorporated into single subunits and proteasome complexes during pulse-chase experiments in NRK and CHO cells. The amount of radioactivity incorporated into α subunits C2, C8, and C9 (data not shown) and into β subunit C5 (see Figs. 4 and 6) remained constant after the pulse and for the next 24 h of chase. To study the fate of proteasome intermediates, we used anti-proteasome antibodies that recognize both

![Figure 4](http://www.jbc.org/)

**FIG. 4.** Study of the synthesis and processing of subunit C5 by pulse-chase experiments in NRK cells. Sub-confluent NRK cells were labeled for 30 min with 0.25 mCi/ml [35S]methionine/cysteine and then chased with complete medium for the times indicated at the top of each lane. At the times indicated cells were processed for immunoprecipitation under native (A–C) or denaturing conditions (D). Anti-C5 antibodies (A and D), anti-proteasome (anti-MCP, B), and anti-C8 (C) antibodies. The figure shows the corresponding autoradiograms of 10–20% SDS-PAGE gels used to analyze the immunoprecipitates. Exposure time was 12 h for A, B, and D and 20 h for C (to make more visible the decay of the immunoprecipitated complex). Note that the proteasome polypeptide moves more clustered on this gradient SDS-PAGE compare with continuous SDS-PAGE (to).
intermediate and mature proteasomes and anti-C8 antibodies that only recognize proteasome intermediates. The total amount of radioactivity immunoprecipitated by the anti-proteasome antibodies increases during the chase, up to 5-fold with respect to the amount of radioactivity immunoprecipitated just after the pulse (Fig. 4B) and then remained constant up to 24 h of chase (Fig. 6). The anti-C8 antibodies immunoprecipitated a set of polypeptides just after the radioactive pulse, and the total amount of radioactivity immunoprecipitated increases at the beginning of the chase (up to 1.4-fold) and then starts to decrease, disappearing completely after 24 h of chase (Fig. 6). These results are in perfect agreement with a cell situation in which the rate of degradation of newly synthesized subunits and proteasome intermediates is not very extensive together with a very efficient incorporation of labeled subunits into mature long-lived proteasomes. This efficiency is specifically demonstrated by the data presented for subunit C5. The conversion of pro-C5 to the processed C5 is close to 100% (see Fig. 4, A and B), during the chase we observed a 5-fold decrease in pro-C5 and a 5-fold increase in the processed C5 subunit. Moreover, all the processed C5 subunit is finally incorporated into 20 S mature proteasomes (Fig. 5) with a long half-life (24 h, Fig. 6). A similar situation can be deduced from the data published with RMA cells by Yang et al. (17), using anti-C9 antibodies that recognize both proteasome intermediates and mature proteasomes, and by Frentzel et al. (12) with antibodies to mature proteasomes that also recognize proteasome intermediates. Our results also indicate that the level of pre-formed proteasome intermediates in the cell lines used in this study is low. This situation (highly efficient incorporation of subunits into mature long-lived proteasomes and low levels of proteasome intermediates) may not be applicable to all cell lines, as demonstrated for H6 cells (29) where proteasome intermediates are only 3–4-fold less abundant than mature proteasomes, and labeled subunits are not very efficiently incorporated into mature proteasomes.

Regarding the incorporation of C5 subunit into proteasome complexes (intermediate and mature proteasomes), the current model of proteasome assembly postulates the existence of a half-proteasome, an intermediate containing a ring of 7 a sub-
Fig. 7. Precursor C5 polypeptide processing depends on proteasome activity. NRK cells were labeled for 30 min with 0.25 mCi/ml [35S]methionine/cysteine and then chased with complete medium for 3 h. Total extracts of labeled NRK cells treated or untreated with 5 μl lactacystin were prepared and used for immunoprecipitation. A, anti-C5 antibodies under denaturing conditions. B, anti-proteasome antibodies (anti-MCP) under native conditions. C, immunoprecipitation with anti-C5 antibodies under denaturing conditions of radiolabeled extracts pre-cleared with an excess anti-proteasome antibodies (as in with anti-C5 antibodies under denaturing conditions.

Fig. 8. Precursor C5 polypeptide processing takes place in the cytoplasm. NRK cells were labeled for 3 h with 0.25 mCi/ml [35S]methionine/cysteine and fractionated into nuclear and total cytoplasmic fractions, and the fractions were subjected to immunoprecipitation with the anti-C5 antibody under denaturing conditions. Panel show the autoradiograms of the corresponding immunoprecipitates run on 13% SDS-PAGE.

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