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Mechanism of Specific Nuclear Transport of Adriamycin: The Mode of Nuclear Translocation of Adriamycin-Proteasome Complex[1]

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ABSTRACT

Adriamycin (ADM), an anthracycline anticancer agent, is selectively stored in the nuclei of a variety of proliferating cells, but the precise mechanism of specific nuclear transport of ADM is not well known. Recently, we demonstrated that ADM shows high binding affinity to the cytoplasmic proteasomes of L1210 mouse leukemia cells and that taken up ADM by the cells selectively binds to proteasomes. Nuclear targeting of proteasome in proliferating cells may be mediated by the nuclear localization signals that are found in several of the α-type subunits of the 20S proteasome. To confirm nuclear transport of the ADM-proteasome complex, we synthesized a photoactive ADM analogue, *N*-(*p*-azidobenzoyl)-ADM, and generated a photoaffinity-labeled proteasome complex. The 26S proteasome purified from the cytosol of L1210 cells had a high affinity to *N*-(*p*-azidobenzoyl)-ADM. SDS-PAGE analysis of the photoaffinity-labeled proteasome showed that low molecular weight bands (~21–31 kDa) of 20S proteasome had the highest photoaffinity. The photoaffinity-labeled proteasome was distributed in the cytoplasm and nuclei of digitonin-permeabilized L1210 and B-16 mouse melanoma cells in the presence of the cytosolic fraction and ATP. The rate of nuclear translocation of the proteasome was low in the absence of ATP. These results suggest that the proteasome is a specific translocator of ADM from the cytoplasm to the nucleus and that 20S proteasome components are the dominant ADM-binding sites. The nuclear transport of ADM-proteasome complex is regulated by an ATP-dependent nuclear pore-mediated mechanism.

INTRODUCTION

The nucleus is the target organelle of the anthracycline anticancer agent ADM[3] (1–3). The affinity of ADM for nuclear DNA is thought to account for the nuclear events that are responsible for the cytotoxic activity of this agent. Although ADM enters cancer cells by a passive diffusion process (4), the precise mechanism of nuclear transport of ADM is not well understood. Recently, we demonstrated that ADM shows high binding affinity to the cytoplasmic proteasomes of L1210 mouse leukemia cells (5) and that taken up ADM by the cells selectively binds to the proteasomes (6).

Proteasomes are intracellular proteinase complexes involved in nonlysosomal mechanisms of protein degradation. The 26S proteasome is essential for the normal turnover of cytosolic and nuclear proteins and plays a role in processing and degradation of regulatory proteins that control cell growth and metabolism (7–9). The 26S proteasome consists of three large multisubunit complexes, *i.e.*, a 20S proteasome core particle and two 19S cap structures (10, 11). Immunocytochemical studies have shown that proteasomes are present both in the nucleus and in the cytoplasm of a variety of eukaryotic cells and tissues (12–14). Although the regulation of proteasome distribution between the nucleus and the cytoplasm is not well known in transformed cells and proliferating tissues, proteasomes have been found to occur predominantly in the nuclei (12, 15–18). Proteasomes isolated from the nuclei and cytoplasm of rat liver show the same basic properties (19). Active transport of proteasomes through the nuclear pores has been proposed to be mediated by nuclear localization signals, which have been found in several of the α-type subunits of the 20S proteasome (20–23).

In the present study, we demonstrated that the proteasome is a nuclear transporter of ADM and also clarified the mode of nuclear translocation of the ADM-proteasome complex. To visualize the ADM-proteasome complex and confirm its nuclear transport, we synthesized a photoactive ADM analogue and covalently coupled it to the proteasome by photoaffinity labeling. To monitor nuclear transport of the photoaffinity-labeled proteasome, digitonin-permeabilized cells were analyzed by confocal laser scanning microscopy. We showed that the proteasome had high affinity to the photoactive ADM analogue and that the photoaffinity-labeled proteasome was transported into the nuclei in the presence of ATP.

MATERIALS AND METHODS

Materials. ADM-HCl was purchased from Sigma Chemical Co. (St. Louis, MO), *N*-hydroxysuccinimidyl-4-azidobenzoate was from Pierce Chemical Co. (Rockford, IL), Suc-LLVY-MCA was from the Peptide Institute (Osaka, Japan), and digitonin was from Wako Pure Chemicals (Osaka, Japan). All other chemicals and reagents were of high commercial quality.

Cell Culture. L1210 mouse lymphocytic leukemia cells and B-16 mouse melanoma cells were cultured in 15 mM HEPES-buffered (pH 7.3) DMEM supplemented with 5% heat-inactivated FCS at 37°C under an atmosphere of 5% CO_2 in air. The cells were used for the following studies during log growth phase after 2–3 days in culture.

Synthesis of Photoactive ADM Analogue. The photoactive ADM analogue, NAB-ADM was synthesized by *N*-azidobenzoylation of ADM with *N*-hydroxysuccinimidyl-4-azidobenzoate according to the method of Felsted *et al.* (24). A yield of ~70% was obtained. The final product gave a symmetrical single peak on reverse phase (C_{18}) high performance liquid chromatography [4.6 mm × 250 mm; solvent system, 70% acetonitrile, 0.1% acetic acid, and water, at a flow rate of 1 ml/min; retention time, 4.1 min (NAB-ADM), 2.2 min (ADM)] and no other UV (270 nm)-visible (488 nm) absorbing peaks were seen on the chromatography. The UV-visible absorption spectrum of NAB-ADM was a composite of the spectra of its component chromophores, *i.e.*, the *p*-azidobenzoyl group (λ_{max} 270 nm) and the adriamycinone ring (λ_{max} 488 nm). The fluorescence emission spectrum of NAB-ADM was identical with that of ADM (data not shown).

Purification of the Proteasome. The 26S proteasome was purified from the cytosol of L1210 cells by ultracentrifugation, followed by hydroxyapatite, gel permeation, and DEAE ion exchange chromatography. At the final step, the 20S proteasome and 26S proteasome were separated by passage through a DEAE-Toyopearl column (1.3 × 3.8 cm; Tosho Co., Tokyo, Japan) equilibrated with buffer A (50 mM Tris-HCl, pH 7.5/2 mM ATP/1 mM DTT, 5 mM $MgCl_2$/20% glycerol). Protease-active proteins remaining in the column were eluted with two bed volumes of buffer A containing 100 mM NaCl and 200 mM NaCl, respectively. The chymotrypsin-like protease activity (19) of the proteasome was measured using the fluorogenic substrate Suc-LLVY-MCA (5).

Photoaffinity Labeling of Proteasomes with NAB-ADM. The purified 26S proteasome (570 μg/ml) was incubated with 10 μM NAB-ADM in buffer A. After incubation at 4°C for 3 h, unbound NAB-ADM was removed by

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[3] The abbreviations used are: ADM, Adriamycin; NAB-ADM, *N*-(*p*-azidobenzoyl)-adriamycin; Suc-LLVY-MCA, succinylleucylleucylvalyltyrosine 4-methylcoumaryl-7-amide.

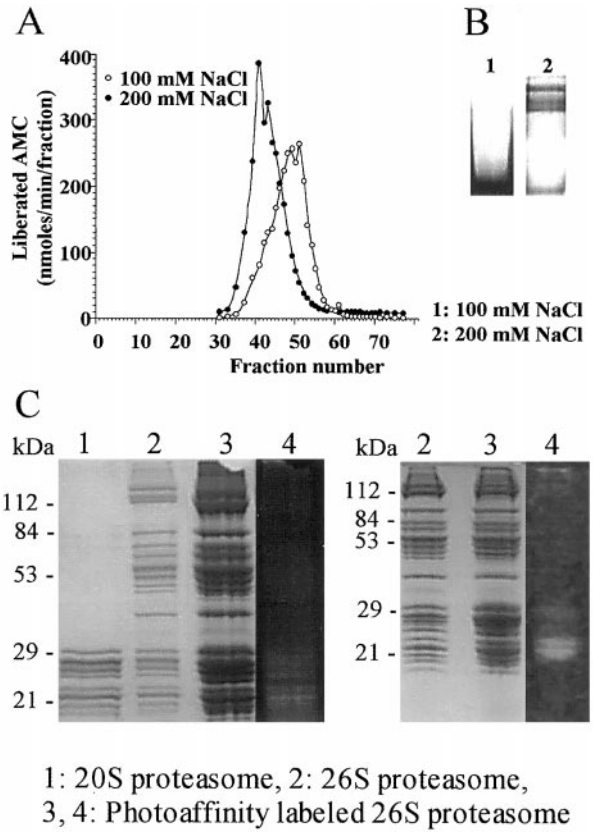

Fig. 1. Bio-Gel A-5 m molecular-sieve chromatography and nondenaturing PAGE of the two-protease active fractions obtained by DEAE-Toyopearl chromatography. In *A*, the 100 or 200 mM NaCl fraction obtained by chromatography was applied separately to a Bio-Gel A-5 m column (1 × 97 cm). Fractions of 1 ml were collected to measure Suc-LLVY-MCA-hydrolyzing activity. The activity is shown as liberated 7-amino-4-methylcoumarin (*AMC*). In *B*, samples (10 μg) obtained by DEAE-Toyopearl chromatography were separated by native 4.5% PAGE. Protein staining was performed with Coomassie Brilliant Blue R-250. *C*, SDS-PAGE of photoaffinity-labeled 26S proteasome. The photoaffinity-labeled samples (50 μg) were separated by SDS-10% PAGE. Protein staining was performed with Coomassie Brilliant Blue R-250 *(Lanes 1–3)*. The fluorescence of the photocovalent adduct derived from NAB-ADM was detected on a UV fluorescent table equipped with two 10-W self-filtering 312 nm lamps *(Lane 4)*.

passage through a Sephadex G-100 (Pharmacia, Tokyo, Japan) column equilibrated with buffer A. The eluent was immediately UV irradiated at 4°C for 5 min with two 10-W self-filtering 312 nm lamps at a distance of 2 cm. Following irradiation, the sample was applied to a Sephadex G-25 (Pharmacia) column equilibrated with nuclear import assay buffer (20 mM HEPES-KOH, pH 7.3/110 mM potassium acetate/2 mM magnesium acetate/1 mM EGTA/2 mM DTT/2 mM ATP), and the void volume fraction was collected. The fraction was immediately used for nuclear import assay.

Nuclear Import Assay. We used the digitonin-permeabilized cell method to study nuclear import of the photoaffinity-labeled proteasome by a slight modification of the method reported by Moore and Biobel (25) as follows.

L1210 cells or B-16 cells were grown in the chambers of slide glasses to which was attached an O-ring (inside diameter, 9 mm) with silicone grease. For permeabilization, the slide glass was placed on ice, and the chamber was washed once with 0.1 ml of ice-cold nuclear import assay buffer. The cells were treated with 0.1 ml of the same buffer containing 35 μg/ml digitonin on ice for 5 min. Then, the chamber was washed twice with 0.1 ml of ice-cold nuclear import assay buffer. The permeabilized cells were incubated with 50 μl of the photoaffinity-labeled proteasome (NAB-ADM at 2.4 μM, protein at 380 μg/ml), and the cytosolic fraction (15 μg protein) was prepared from L1210 cells at 25°C for 30 min in the presence or absence of ATP-regenerating system (2 mM ATP/5 mM phosphocreatine/20 units/ml creatine phosphokinase). After incubation, the cells were washed twice with 0.1 ml of ice-cold nuclear import assay buffer and fixed in 0.1 ml of ice-cold 3% paraformaldehyde in nuclear import assay buffer without DTT on ice for 15 min.

Confocal Laser Scanning Microscopy. The fixed cells were mounted in a drop of 10% PBS and 90% glycerol. The edges of the coverslip were sealed with nail polish. The sample was viewed under a confocal laser scanning microscope (Leica, Heidelberg, Germany), with the 488 nm line of the argon laser used for excitation.

Quantification of NAB-ADM. For quantification of photocovalent adduct formation, the photoaffinity-labeled proteasome was treated with ice-cold 7% trichloroacetic acid at 4°C for 2 h and centrifuged at 5000 × *g* for 5 min. The protein pellet was washed three times with ice-cold ethanol:ether (1:1) to remove residual trichloroacetic acid. The dried sample was dissolved in 1 ml of 0.3 N HCl, 50% ethanol, and 1% SDS, and ADM was measured fluorometrically (excitation, 480 nm; emission, 588 nm) after hydrolysis at 90°C for 15 min.

Gel Electrophoresis. SDS/PAGE was performed as described by Laemmli (26) in 10% polyacrylamide gels. Nondenaturing gels consisted of 3% polyacrylamide stacking and 4.5% polyacrylamide resolving gels cast in 90 mM Tris/80 mM boric acid, pH 8.3/0.1 mM EDTA buffer containing 2.5% sucrose. The gels were run at 50 V for 400 V-h at 4°C in 90 mM Tris/80 mM boric acid, pH 8.3/0.1 mM EDTA buffer. Protein staining was performed with Coomassie Brilliant Blue R-250. The photocovalent adducts were detected by visualizing the fluorescence of the NAB-ADM on a UV-fluorescent table equipped with two 10-W self-filtering 312 nm lamps.

RESULTS

Purity of Two Proteasome Fractions. The purity of the proteasome preparations was examined by gel permeation chromatography, nondenaturing PAGE, and SDS-PAGE. An aliquot of the each fraction eluted from the DEAE-Toyopearl column was applied to a Bio-Gel A-5m (Bio-Rad, Richmond, CA) column (1 × 97 cm) equilibrated with buffer A. The protease-active protein in the 100 mM NaCl fraction was eluted at a median molecular mass of ~700 kDa and that of the 200 mM NaCl fraction was eluted at a median molecular mass of ~1500 kDa (Fig. 1*A*). As shown in Fig. 1*B*, nondenaturing PAGE revealed two major protein complexes in the large protease-active fraction from the DEAE-Toyopearl column, whereas only a single major band was present in the smaller protease-active fraction obtained by chromatography. The preparation showed the same SDS-PAGE pattern characteristic of the 20S and 26S proteasomes, respectively (Fig. 1*C*, *Lanes 1* and *2*). The 700-kDa fraction eluted by DEAE-Toyoperl column chromatography contained a closely related 20S proteasome, whereas a 26S proteasome was found in the 200 mM NaCl fraction (1500 kDa). Approximately 1.4 mg of the purified protein were obtained from 533 mg of the cytosol of L1210 cells with a yield of 15% of total proteolytic activity. The protease activity was purified by 70-fold by this procedure.

Photoaffinity Labeling of the Proteasome. *In vitro* photoaffinity labeling of the purified 26S proteasome with NAB-ADM showed high binding affinity of NAB-ADM to the proteasome. The yield of trichloroacetic acid-precipitable photocovalent adducts from the proteasome was 36%, and the specific binding activity was 6.3 nmol/mg protein (Table 1). The photoaffinity-labeled proteasome showed low protease activity, and ~70% inhibition of the activity was observed (Table 1). The NAB-ADM photoaffinity labeling pattern of the 26S

Table 1 *Photoaffinity labeling of 26S proteasome with NAB-ADM*

The purified 26S proteasome (570 μg/ml) was incubated with 10 μM NAB-ADM at 4°C for 3 h. After incubation, unbound NAB-ADM was removed by gel chromatography and UV irradiated (312 nm) at 4°C for 5 min. Suc-LLVY-MCA-hydrolyzing activity and content of the photocovalent adduct were measured as described in the text. Control protease activity was measured in the absence of NAB-ADM.

Photoaffinity labeling	Protease activity (control %)	NAB-ADM	
		μM	nmol/mg protein
−	97.7	10.0 (added)	17.5 (added)
+	29.7	3.6	6.3

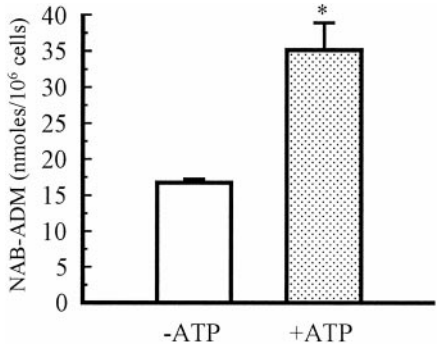

Fig. 2. Cellular uptake of photoaffinity-labeled 26S proteasome by L1210 cells. Digitonin-permeabilized cells were incubated in the photoaffinity-labeled proteasome and the cytosolic fraction of L1210 cells for 30 min at 25°C in the presence or absence of an ATP-generating system. After incubation, the cells were washed three times with ice-cold PBS and treated with ice-cold 7% trichloroacetic acid. The cellular content of the photocovalent adduct was determined using the trichloroacetic acid method as described in the text. Each column represents the mean ± SD of three cultures; *, $P < 0.01$ relative to −ATP (Dunnett's multiple comparison test).

proteasome preparation was determined by SDS-PAGE. The pattern of the fluorogenic labeling derived from NAB-ADM of most major and minor photolabeled components (Fig. 1*C, Lane 4*) was dependent on the Coomassie Brilliant Blue-stained polypeptide patterns of 26S proteasome (Fig. 1*C, Lane 3*). However, the 20S proteasome polypeptide was the most prominently photolabeled component of the 26S proteasome.

Cellular Uptake and Nuclear Import of the Photoaffinity-labeled Proteasome. Cellular uptake of the photoaffinity-labeled 26S proteasome by digitonin-permeabilized L1210 cells was ATP dependent (Fig. 2). The contents of photocovalent adducts were far greater in the nuclei than in the postnuclear fraction of the cells (data not shown).

The photoaffinity-labeled 26S proteasome was distributed in the cytoplasm and nuclei in digitonin-permeabilized L1210 cells (Fig. 3*A, left*) and B-16 cells (Fig. 3, *B* and *C, left*) in the presence of ATP. The predominantly nuclear distribution of photoaffinity-labeled proteasomes was observed in both cell lines. When digitonin-permeabilized cells of both lines were viewed by inverted phase contrast fluorescent microscopy (Olympus, Tokyo, Japan; excitation filter, 490 nm; barrier filter, 530 nm) during incubation for nuclear import, the orange-red fluorescence of photoaffinity-labeled proteasomes was detected in the nuclei of both cell lines even in the presence of photoaffinity-labeled proteasomes in the extracellular medium (data not shown). This nuclear condensation of the photoaffinity-labeled proteasome suggested active transport from the cytoplasm to the nucleus. The rate of nuclear translocation of photoaffinity-labeled proteasomes in both cell lines was remarkably low in the absence of ATP (Fig. 3, *right*). The slight amount of proteasome levels were detected in the cytoplasm.

DISCUSSION

ADM is selectively stored in the nuclei of a variety of tissues and cells (1–3), but the precise mechanism of specific nuclear transport is not well understood. ADM specifically induces cytotoxicity at lower concentrations in proliferating than in nonproliferating cells, and higher cellular uptake of ADM was reported in proliferating than in nonproliferating cells (27–29). Recently, we demonstrated that ADM shows high binding affinity to the cytoplasmic proteasomes of L1210 cells (5) and that taken up ADM by the cells selectively binds to the proteasomes by using *in situ* photoaffinity labeling with NAB-ADM (6). Proteasomes are multicatalytic proteinase complexes that possess both a cytosolic and a nuclear proteinase function and have been isolated from a wide variety of eukaryotic cells and tissues (30–32). Immunohistochemical studies showed proteasomes to be located in the cytoplasm and nuclei of these cells and tissues (12–14). Interestingly, in neoplastic cells as well as in undifferentiated normal cells undergoing proliferation, proteasomes have been found to occur predominantly in the nuclei (12, 15, 18), and proteasome gene expression has been shown to be more pronounced than in nonproliferative, differentiated normal cells and tissues (15–17). The high levels of proteasome gene expression and nuclear localization are down-regulated in these cells by induction of differentiation (17, 18). Thus, the high cellular uptake and cytotoxicity of ADM in proliferating cells appears to be due to the marked nuclear localization and high-level gene expression of proteasomes in the cells.

To visualize and analyze the cellular distribution and nuclear transport of the ADM-proteasome complex, photoactive NAB-ADM was synthesized, and 26S proteasome purified from the cytosol of L1210 cells was cross-linked with NAB-ADM by UV irradiation. The formation of ADM-proteasome complex by photoaffinity labeling is based on the assumption that a reversible complex is formed between the photoactive NAB-ADM and the proteasomes acceptor sites, which specifically recognize structural characteristics of the drug. Then, irradiation converts NAB-ADM into a reactive nitrene intermediate, which will covalently attach at or near the acceptor-binding sites (33, 34). Because the nitrene intermediate has a short half-life, photoactivated NAB-ADM should efficiently label only those acceptors to which it is closely associated. NAB-ADM showed high affinity to the 26S proteasome, and the photoaffinity-labeled proteasome showed

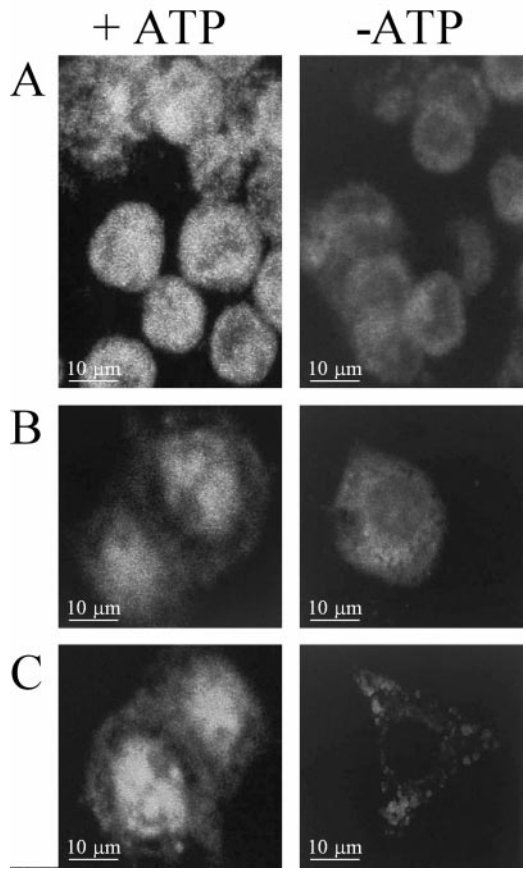

Fig. 3. Nuclear import of photoaffinity-labeled 26S proteasome in L1210 cells (*A*) and B-16 cells (*B* and *C*). Digitonin-permeabilized cells were incubated with the photoaffinity-labeled proteasome and the cytosolic fraction of L1210 cells at 25°C for 30 min in the presence (*left*) or absence (*right*) of an ATP-generating system. Fluorescence was analyzed by confocal laser scanning microscopy.

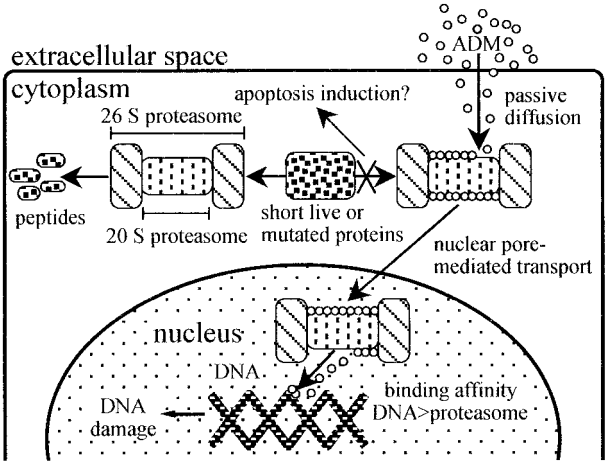

Fig. 4. Schematic representation of the hypothetical mechanism of nuclear translocation of ADM-proteasome complex. See text for details.

low chymotrypsin-like protease activity. The β-type subunit and the NH_2-terminal threonine of the 20S proteasome contribute to the catalytic active sites (9, 35, 36). Figueiredo-Pereira *et al.* (37) showed that aclacinomycin A, an anthracycline anticancer agent, is a reversible noncompetitive inhibitor of the chymotrypsin-like protease activity of the 20S proteasome. We have found that ADM inhibited the protease activity in the same manner.[4] Although the precise binding sites of ADM in the 20S proteasomal subunit are not yet known, the photoaffinity-labeled components of the 20S proteasome with NAB-ADM may be necessary for the chymotrypsin-like protease activity of 20S proteasome. ADM may bind to an allosteric site, causing distortion of the catalytic site and obstructing its access to the scissile bond.

Nuclear import experiments using digitonin-permeabilized cells showed that the photoaffinity-labeled proteasome is distributed in the cytoplasm and the nuclei in the presence of ATP, although nuclear localization is predominant. In contrast, the rate of nuclear translocation of the photoaffinity-labeled proteasome was remarkably low in the absence of ATP. Macromolecular transport into the nuclei is mediated by the nuclear pore complex, which is dependent on ATP hydrolysis (38). The nuclear targeting of proteasomes may be directed by some of the α-type subunits containing nuclear localization signal sequences (20–23). The ATP dependency of nuclear condensation of the photoaffinity-labeled proteasome may reflect nuclear pore-mediated macromolecular transport into the nucleus, which is mediated by nuclear localization signals in the proteasomal α-type subunits.

On the basis of these observations, we propose the following mechanism for the nuclear translocation of ADM-proteasome complexes as shown in Fig. 4: (*a*) ADM enters cancer cells by simple diffusion (4), and ADM-proteasome complex is formed in the cytoplasm due to the high affinity of ADM to the proteasome; (*b*) ADM-proteasome complex is translocated from the cytoplasm into the nucleus of the cell. The complex may be translocated into the nucleus via nuclear pores because the proteasome possesses nuclear localization signals (20–23); (*c*) In the nucleus, DNA-binding affinity of ADM is higher than that of the proteasome (5). Thus, ADM dissociates from the proteasome and intercalates into the DNA, thereby inhibiting DNA synthesis; (*d*) On the other hand, ADM bound to the proteasome shows low protease activity. Inhibition of proteasome activity induces inhibition of processing and degradation of regulatory proteins that control cell growth and metabolism (7–9), thereby inducing apoptosis of cancer cells (39, 40). Thus, agents that target the proteasome may be useful for the treatment of cancer. This hypothetical mechanism based on the results of the present study suggests that the proteasome is a specific translocator of ADM from the cytoplasm to the nucleus, and this agent might therefore be useful as a new candidate for cancer chemotherapy.

[4] K-I. Kiyomiya, H. Nakagawa, S. Matsuo, and M. Kurebe, unpublished observations.

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