Nuclear Localization of Proteasomes Participates in Stress-inducible Resistance of Solid Tumor Cells to Topoisomerase II-directed Drugs

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ABSTRACT

Physiological cell conditions of solid tumors, such as glucose starvation and hypoxia, induce cellular resistance to topoisomerase II-directed drugs. Here, we show that the induction of drug resistance is mediated by nuclear accumulation of proteasomes, large multicatalytic protease complexes. We found that the nuclear proteasome accumulation during glucose starvation was attenuated by stable expression of a mutant type of proteasome subunit, XAPC7, that lacked the nuclear localization signal (NLS). It is important that the expression of NLS-defective XAPC7 also diminished the induction of resistance to etoposide and doxorubicin, typical topoisomerase II-directed drugs. Under normal conditions, however, the NLS-defective XAPC7 had little effect on either nuclear proteasome distribution or etoposide sensitivity. Our findings demonstrate that stress-induced nuclear proteasome accumulation occurs through up-regulation of the NLS-dependent transport. Inhibition of the nuclear proteasome accumulation can be a novel approach to circumventing resistance to topoisomerase II-directed drugs.

INTRODUCTION

Cancer cells present in solid tumors are often surrounded by stressful microenvironmental conditions, such as glucose starvation, hypoxia, low pH, and other nutritional deprivation (1, 2). These physiological stress conditions are sources of cellular drug resistance. Indeed, cancer cells under glucose starvation and hypoxia become resistant to multiple drugs, including the topoisomerase II-directed drugs etoposide and doxorubicin (2, 3). In a previous study, we found that the stress-induced resistance to topoisomerase II-directed drugs was effectively prevented by luteinizing and other proteasome inhibitors (3). Furthermore, luteinizing significantly enhanced the antitumor activity of etoposide in a solid tumor model (4). Thus, proteasomes can be involved in the stress-induced resistance to topoisomerase II-directed drugs in vitro and in vivo.

Proteasomes are large, multicatalytic protease complexes implicated in the degradation of various cellular proteins (4, 5). The 20S proteasome, the catalytic core of proteasomes, has a cylindrical structure made up of 14 different subunits, 7 α-type and 7 β-type (6). Proteasomes are localized in both the nucleus and the cytoplasm (7, 8), and the intracellular distribution of proteasomes changes during various events or stress responses (8–11). Although the regulation mechanisms are not understood fully, two pathways have been described for the distribution between the cytoplasm and the nucleus (12). Proteasomes can be taken up into the nucleus on nuclear membrane reassembly during cell division (12). They can also be transported unidirectionally over the nuclear membrane (12), and nuclear targeting of proteasomes may be directed by some of the α-subunits that contain NLS sequences (13–16). In fact, these NLSs can function to direct the reporter molecules to the nucleus (14), although the significance of the NLSs in nuclear proteasome transport has not been verified in living cells.

Previously, we observed that proteasomes accumulated in the nucleus under stress conditions (10, 11). However, little is known about the mechanisms of nuclear proteasome accumulation or its role in stress-induced drug resistance. In this study, we investigated these points by establishing stable transfectants that express a NLS-truncated α-subunit of the 20S proteasome.

MATERIALS AND METHODS

Cell Culture and Treatments. The human colon adenocarcinoma HT-29 cells were maintained in RPMI 1640 (Nissui, Tokyo, Japan) supplemented with 5% heat-inactivated fetal bovine serum and 100 μg/ml of kanamycin, and they were cultured at 37°C in a humidified atmosphere containing 5% CO2. Glucose deprivation was achieved by culturing cells in the glucose-free RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 5% heat-inactivated fetal bovine serum. For the colony formation assay, cells were cultured for 4 h with etoposide (Bristol Myers Squibb Co., Ltd., Tokyo, Japan), doxorubicin (Kyowa Hakko Co., Ltd., Tokyo, Japan), or vincristine (Eli Lilly Japan Co., Ltd., Kobe, Japan) and seeded at appropriate dilutions in fresh medium. After 7–8 days, colonies were fixed with 10% formaldehyde, stained with 0.01% crystal violet, and counted. For the growth-inhibition assay, cells were seeded in 96-well plates at a density of 1000 cells/well. After a 24-h culture, drug solutions were directly added to the culture medium, and the cells were further cultured for 72 h. Relative cell growth was determined by a methylthiazolotetrazolium assay (17). The cell cycle distributions were determined by using a Becton Dickinson fluorescence-activated cell analyzer, and the data were interpreted using the R-FIT model program provided by the manufacturer.

Plasmids and Transfection. Full-length human XAPC7 cDNA was generated by PCR from a cDNA library of HT-29 cells. The PCR fragment was cloned into pcDNA3.1/V5-His vector using a TA-TOPO cloning kit (Invitrogen, San Diego, CA) so that XAPC7 was tagged with a V5-His epitope sequence consisting of 45 amino acid residues in total. Similarly, a mutant type of XAPC7 that lacked the NLS sequence (a.a. 241–248) at the COOH terminus (Fig. 1A) was amplified by PCR with the above construct as a template, and it was cloned into the pcDNA3.1/V5-His vector. The constructs were confirmed by sequencing. After linearization of the plasmids by digesting at the Pvu I site, transfection was performed by electroporation (960 μF, 270 mV; Gene Pulser; Bio-Rad, Richmond, CA). After 2 days of drug-free incubation, the cells were cultured for 2 weeks in the culture medium containing 400 μg/ml of G418 and G418-resistant cells were subsequently cloned.

Western Blot Analysis. Total cell lysates were prepared by solubilizing cells in × SDS sample buffer [10% glycerol, 5% 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl (pH 6.8)], as described previously (3, 10, 11). To analyze nuclear proteasome expression levels, we isolated the nuclei and solubilized them in × SDS sample buffer, as previously described (3, 11). Equal amounts of proteins were subject to SDS-PAGE and electroblotted onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). Membranes were probed with mouse monoclonal antibodies against XAPC7, HCA9, HN3 (subunits of 20S proteasome; AFFINITI Research Product, Ltd., United Kingdom), topo IIα (clone KF4; Cambridge Research Biochemicals, Wimington, DE), and topo IIβ (clone 8P8; PharMingen, San Diego, CA). To detect exogenously expressed XAPC7, we used an anti-V5 antibody-conjugated horseradish peroxidase (Invitrogen). The specific signals were detected using...
Using anti-V5 antibody.

anti-V5 antibody and the immunoprecipitates were subjected to Western blot analysis. HXAdNLS-1 (HXAdNLS-1) were immunoprecipitated by agarose-immobilized anti-HC3 proteasome complexes. Cell lysates of HT-29 (HXAWT), and HXAdNLS-1 (HXAdNLS-1) cells were prepared and subjected to Western blot analysis using anti-V5 (top panel), anti-XAPC7 (middle panel), and anti-HC9 antibodies (bottom panel).exo, exogenous XAPC7/WT or XAPC7/dNLS; endo, endogenous XAPC7. C, incorporation of exogenously expressed XAPC7s into proteasome complexes. Cell lysates of HT-29 (Parent), HXAWT-1 (HXAWT), and HXAdNLS-1 (HXAdNLS) were immunoprecipitated by aga-rate-immobilized anti-HC3 antibody MCP21, and the immunoprecipitates were subjected to Western blot analysis using anti-V5 antibody. XAPC7 (exo), XAPC7/WT or XAPC7/dNLS.

an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Tokyo, Japan).

**Immunoprecipitation.** Cells were suspended in a lysis buffer [1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium orthovanadate, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5% NP40] and passed several times through a 26-gauge needle to disperse any large aggregates. Insoluble materials were removed by centrifugation. Equal amounts of proteins were precleared and immunoprecipitated using aga-rate-immobilized anti-HC3 antibody MCP21 (AFFINITI). The immunoprecipitates were washed three times with 1 ml of the lysis buffer and then resuspended in 2X SDS sample buffer. After boiling for 5 min, the complexes were evaluated using Western blot analysis.

**Measurement of Nuclear Proteasome Activity.** Preparation of nuclear extracts and measurement of proteasome activities were carried out as described previously (10, 11). In brief, nuclear extract was obtained by rocking the nuclei in extraction buffer containing 20 mM HEPES-KOH (pH 7.5), 0.1% (w/v) 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS), 2 mM EDTA, 5 mM DTT, 400 mM KCl, 10 μg/ml of leupeptin, 20 μg/ml of pepstatin, 10 μg/ml of aprotinin, and 1 mM benzamidine and, subsequently, by dialyzing against the assay buffer containing 20 mM HEPES-KOH (pH 7.5), 2 mM DTT, and 10% (v/v) glycerol. After removal of insoluble materials by centrifugation, the nuclear extracts were used for a peptide cleavage assay. Equal amounts of proteins were incubated with 20 μM fluorogenic proteasome substrate, Suc-LLVY-MCA (Peptide Institute Inc., Osaka, Japan). After 1 h at 37°C, the reaction was stopped with 2% SDS, and proteasome activity was assessed using a spectrofluorometer (excitation at 380 nm and emission at 460 nm).

**RESULTS**

**Establishment of Stable Transfectants Expressing NLS-deleted XAPC7.** XAPC7, a 20S proteasome subunit, has a NLS sequence (a.a. 241–248) in its COOH-terminal region (13, 14). We constructed expression vectors of wild-type (XAPC7/WT) and NLS-truncated mutant of XAPC7 (XAPC7/dNLS) with a V5-His epitope tag (Fig. 1A). After transfection of the constructs into HT-29 cells, two independent clones from each transfection were obtained and designated HXAWT-1, HXAWT-2, HXAdNLS-1, and HXAdNLS-2, respectively.

These clones expressed similar levels of exogenous XAPC7/WT or XAPC7/dNLS, as shown by Western blot analysis using an anti-V5 antibody (Fig. 1B and data not shown). XAPC7/WT and XAPC7/dNLS showed similar apparent molecular weights, but they were distinguished from endogenous XAPC7 by Western blot analysis using an anti-XAPC7 antibody (Fig. 1B). The expression levels of exogenous XAPC7/WT and XAPC7/dNLS were approximately twice those of endogenous XAPC7 in the stable transfectants. The endogenous levels of XAPC7, as well as another proteasome subunit, HC9, were nearly equal to those in nontransfected parental HT-29 cells (Fig. 1B).

XAPC7/WT and XAPC7/dNLS were immunoprecipitated with proteasome complexes, as shown by using the antibody MCP21 that recognizes another proteasome subunit, HC3, and precipitates proteasomes as intact complexes (Ref. 12; Fig. 1C). Thus, the exogenously expressed XAPC7/WT and XAPC7/dNLS can be incorporated into proteasome assemblies.

**Attenuation of Stress-induced Nuclear Proteasome Accumulation by Expression of XAPC7/dNLS.** As we have previously reported (10, 11), nuclear distribution of proteasomes is enhanced under glucose-starvation conditions. Indeed, the nuclear expression levels of XAPC7 increased without changes in total expression levels (Fig. 2A). Similarly, the exogenously expressed XAPC7/WT accumulated in the nucleus under glucose starvation (Fig. 2C). However, the stress-induced nuclear accumulation of XAPC7/dNLS was significantly attenuated, although the nuclear level of XAPC7/dNLS under normal conditions was comparable with that of XAPC7/WT. Total expression levels of XAPC7/WT and XAPC7/dNLS, as well as endogenous XAPC7, were relatively constant under normal or glucose-starvation conditions (Fig. 2B).

In HXAWT-1 cells, endogenous XAPC7 and other proteasome subunits HC9 and HN3 accumulated in the nucleus, similar to exogenous XAPC7/WT (Fig. 2C). The nuclear proteasome activities were also enhanced 3.8 and 3.6 times by glucose starvation in HXAWT-1 and HXAWT-2 cells, respectively (Fig. 2D). The increase in nuclear proteasome activities was comparable with that in the glucose-starved parental HT-29 cells (Fig. 2D). When compared with these cells, the stress-induced nuclear accumulation of endogenous XAPC7, HC9, and HN3 was diminished in the XAPC7/dNLS-transfected cells (Fig. 2C). Furthermore, the increase in nuclear proteasome activities was confined by 2.4 and 2.2 times in HXAdNLS-1 and HXAdNLS-2 cells, respectively (Fig. 2D). These results indicated that the nuclear accumulation of proteasome complexes was attenuated in the XAPC7/dNLS transfectants under glucose-starvation conditions.

Using the total cell lysates and the nuclear extracts of parental HT-29 and the transfected cells, we also examined the expression levels of topo IIα and β. As previously reported (3), the expression levels of topo IIα were decreased under glucose-starvation conditions in HT-29 cells (Fig. 2A). Similarly, the decreased topo IIα expression under glucose-starvation conditions also occurred both in HXAWT-1 and in HXAdNLS-1 cells (Fig. 2, B and C). However, the XAPC7/dNLS-transfected cells showed slightly higher, but reproducibly higher, expression levels of topo IIα under stress conditions than XAPC7/WT-transfected cells. Under normal conditions, the expression levels of topo IIα were essentially the same in both transfectants. In contrast to topo IIα, the expression levels of topo IIβ were constant regardless of cellular conditions.
A starvation conditions (Fig. 3 and -2 cells, a strong etoposide resistance was induced under glucose-starvation conditions (Fig. 3). The cellular sensitivities were determined by the colony-formation assay after 4-h drug treatments. In HXAWT-1 and HXAdNLS, typical topo II-directed drugs, under normal or glucose-starvation conditions (Fig. 3). We compared the cellular sensitivities of parental HT-29 and XAPC7/WT- and XAPC7/dNLS-transfected cells to vincristine, an antitumor microtubule inhibitor. As reported previously (3), induction of vincristine sensitivities were somewhat varied under normal conditions.

The cell growth rates and the cell cycle profiles of XAPC7/WT and XAPC7/dNLS transfectants were similar to those of parental HT-29 cells under normal conditions (Fig. 4A; Table 1). In addition, cellular proliferation of these cell lines was similarly inhibited by 72-h exposures to etoposide, doxorubicin, and vincristine (Fig. 4B), supporting the observation that the transfectants had essentially the same cell growth properties under normal conditions. Furthermore, the transfectants, like parental HT-29 cells (3, 11), showed G1 phase arrest or delay when starved of glucose (Table 1). Therefore, we concluded that the attenuation of stress-inducible etoposide resistance in XAPC7/dNLS transfectants cannot be explained by alteration in cell proliferation or by cell cycle arrest.

**DISCUSSION**

Proteasomes are found both in the cytoplasm and in the nucleus (7, 12), and nuclear distribution of proteasomes is enhanced under glucose-starvation conditions in cancer cells (10, 11). Stress conditions also lead to the induction of topo II-directed drug resistance (2, 3). In this study, we showed that both the nuclear proteasome accumulation...
and the inducible drug resistance were attenuated in the stable transfectants of the NLS-truncated mutant XAPC7/dNLS. The expression of this mutant, however, did not affect nuclear proteasome distribution or etoposide sensitivity under normal conditions. These results demonstrate that nuclear proteasome localization is involved in the development of drug resistance under glucose-starvation conditions.

With respect to the inhibitory effect of XAPC7/dNLS on the nuclear proteasome accumulation, we showed that the exogenously expressed XAPC7/dNLS was incorporated into proteasome complexes (Fig. 1C). In the XAPC7/dNLS-transfected cells, the attenuated nuclear accumulation under stress conditions was observed for not only the exogenous XAPC7/dNLS but also the endogenous XAPC7. In addition, other proteasome components, HC9 and HN3, were not accumulated normally (Fig. 2C), and increase in the nuclear proteasome activities under stress conditions were attenuated in the XAPC7/dNLS-transfected cells (Fig. 2D). These findings collectively suggested that XAPC7/dNLS inhibited the nuclear accumulation of all (or the majority of) components consisting of 20S proteasome complexes under stress conditions, possibly through being incorporated in the complexes. This notion would also be supported by the fact that, at least in yeast, proteasome assembly to form 13–16S precursor complexes occurs in the cytoplasm before import of proteasomes into the nucleus (18–20).

The exact mechanisms of the XAPC7/dNLS-mediated inhibition remain to be determined. However, several lines of evidence have demonstrated that NLS-mediated transport through the nuclear pores plays a role in the nuclear distribution of proteasomes. The functionality of NLS sequences contained in four α-subunits, including XAPC7, has been shown in a permeable cell system using reporter molecules fused with the NLSs (14–16). In addition, nuclear pore complexes appear able to translocate large protein complexes like proteasomes (12). Indeed, purified 20S proteasomes can accumulate in the nuclei of digitonin-permeabilized cells, and this translocation is inhibited by blocking the nuclear pores with wheat germ agglutinin (16). Therefore, the NLSs of proteasome subunits could function in cells to translocate proteasomes into the nucleus as intact complexes. Together with these previous studies, our findings suggested that XAPC7/dNLS may inhibit the NLS-mediated nuclear proteasome transport after being incorporated into mature or precursor complexes of proteasome. Interestingly, however, the XAPC7/dNLS had little effect on nuclear proteasome distributions under normal conditions. Therefore, the regulation of nuclear proteasome transport under normal conditions is different from the regulation of nuclear proteasome transport under stress conditions, and a NLS-dependent pathway may be up-regulated in the stressed cells.

It should be noted that the nuclear level of XAPC7/dNLS itself was enhanced substantially, but not fully, under glucose-starvation conditions (Fig. 2B). This observation indicates that the NLS of XAPC7 is not the sole determinant for the stress-induced nuclear accumulation of this subunit. It is possible that alternative, functional NLSs in α-subunits other than XAPC7 also operate in the stress-induced nuclear transport of proteasomes. Thus, XAPC7/dNLS, incorporated into proteasome complexes, may be transported to the nucleus in a manner that depends on other NLSs. However, at present, we cannot rule out the possibility of the involvement of a NLS-independent pathway. In fact, proteasomes can also diffuse into the nucleus on nuclear membrane reassembly during cell division (12). Such a NLS-independent pathway (or pathways) might also provide an explanation for the marginal effect of XAPC7/dNLS expression seen on nuclear proteasome distribution under normal conditions.

The mechanisms of drug resistance mediated by nuclear proteasome accumulation remain to be determined. In the present study, we demonstrated that XAPC7/dNLS-transfected cells showed slightly higher expression levels of topo IIα under glucose-starvation conditions than did XAPC7/WT-transfected cells (Fig. 2, B and C). Because the expression of topo IIα is a determinant of the cellular sensitivity to topo II-directed drugs, the difference in topo IIα levels could contribute, at least in part, to the attenuation of inducible resistance in XAPC7/dNLS-transfected cells. Furthermore, this observation raises the possibility that the degradation of nuclear proteins, including topo IIα, is facilitated by the nuclear proteasome accumulation under stress conditions. Actually, it is understood that changes in the subcellular localization of proteasomes play an important role in the efficiency of proteasome-mediated protein degradation (4). In addition, proteasomes diffuse rapidly within the nucleus as well as within the cytosol.
plasm; therefore, the protein breakdown by proteasomes may be mediated by a simple collision between them and the target proteins (12). Thus, the increased numbers of proteasomes in the nucleus may facilitate degradation of nuclear proteins, and the efficient degradation may be associated with the stress-induced drug resistance.

Proteasomes are ubiquitously expressed in normal tissues and play essential roles in various cellular functions, such as proliferation, differentiation, and antigen presentation (4, 5). Therefore, total inhibition of proteasome activities may have adverse effects. In this context, inhibiting nuclear distribution of proteasomes can be a selective approach to preventing stress-inducible drug resistance of solid tumors. Indeed, microenvironmental stresses, such as glucose starvation and hypoxia, are not observed in normal tissues, and the NLS-dependent pathway appears important particularly for stress-induced nuclear transport of proteasomes in cancer cells. Thus, additional investigations into the mechanisms of nuclear proteasome transport would provide tumor-selective strategies to circumvent the inducible resistance to topo II-directed drugs.

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