Overexpression of a Dominant-Negative Mutant Ubc9 Is Associated with Increased Sensitivity to Anticancer Drugs

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ABSTRACT

Ubc9 is an E2-conjugating enzyme required for sumoylation and has been implicated in regulating several critical cellular pathways. We have shown previously that Ubc9 is important for sumoylation and nuclear delocalization of topoisoformase (topo) I in response to topo I inhibitors such as topotecan. However, the role for Ubc9 in tumor drug responsiveness is not clear. In this study, we found that although MCF7 cells expressing a Ubc9 dominant-negative mutant (Ubc9-DN) display decreased activity of topo I, these cells are more sensitive to the topo I inhibitor topotecan and other anticancer agents such as VM-26 and cisplatin. In addition, we found that alteration of Ubc9 expression correlates with drug responsiveness in tumor cell lines. To understand possible mechanisms of Ubc9-associated drug responsiveness, we examined several proteins that have been shown to interact with Ubc9 and that may be involved in drug responsiveness. One such protein is Daxx, which is a Fas-associated protein that plays a role in Fas-mediated apoptosis by participating in a caspase-independent pathway through activation of apoptosis signal-regulating kinase 1 and c-Jun NH2-terminal kinase. We found that cells expressing Ubc9-DN accumulate more cytoplasmic Daxx than the control cells. Because cytoplasmic Daxx is believed to participate in cellular apoptosis, we suggest that the interaction of Ubc9 with Daxx and subsequent alteration in the subcellular localization of Daxx may contribute to the increased sensitivity to anticancer drugs in the cells expressing Ubc9-DN. Finally, we found that overexpression of Daxx sensitizes cells to anticancer drugs possibly in part through alterations of the ratio of cytoplasmic and nuclear Daxx. Together, our results suggest a role for Ubc9 in tumor drug responsiveness.

INTRODUCTION

Ubc9 is an essential E2 enzyme required for small ubiquitin-related modifier conjugation or sumoylation (1, 2). Small ubiquitin-related modifier-1 is a 10 kDa protein that forms a covalent bond at a lysine residue of target proteins in a manner similar to ubiquitination (3). However, unlike ubiquitination, which leads to degradation of conjugated proteins, sumoylation seems to stabilize the targeted proteins (4). In addition, sumoylation is implicated in the regulation of transcriptional activity and protein subcellular localization (5, 6).

Many proteins involved in critical cellular pathways are sumoylated (5, 6). Among them is DNA topoisomerase I (topo I; 7), which plays a critical role in DNA metabolism and transcription. Topo I is a target for anticancer agents such as camptothecin and its clinically important derivatives, topotecan (TPT) and irinotecan (8, 9).

We have demonstrated recently that sumoylation of topo I is associated with its nuclear delocalization in response to treatment of topo I inhibitors (10), suggesting that sumoylation serves as an addressing tag for this protein. Furthermore, when the potential lysine sumoylation sites (K103/K117/K153) of topo I are mutated to arginines, the enzyme shows little sumoylation and is trapped in the nucleolus in the presence of camptothecin (11). Thus, sumoylation seems to prevent topo I binding to sites in nucleoli. More recently, it was shown that arginine substitutions of two sumoylated sites (R117 and R153) reduced camptothecin-induced cleavable complexes without influencing its in vitro catalytic activity; the camptothecin-induced cleavable complexes of wild-type topo I increased in a sumoylation-dependent manner (12). This finding implies that sumoylation of topo I enhances the cytotoxicity of topo I inhibitors.

Ubc9 is the sole E2-conjugating enzyme required for protein sumoylation (2). Ubc9 is therefore believed to play a central role in the above-mentioned biological processes through sumoylation. Ubc9 physically interacts with topo I (7), but whether this interaction has any effect on topo I activity is not known. Importantly, Ubc9 is implicated in apoptosis and DNA repair, because it interacts with such proteins as p53 and Rad51 (13–16). Moreover, Ubc9 is also required for normal mitosis and recovery from DNA damage or S-phase arrest in fission yeast (17, 18). Yeast expressing temperature-sensitive mutant Ubc9 are more sensitive to topo I inhibitors (7). Ubc9 was shown recently to interact with proliferating cell nuclear antigen, thereby implicating it in the Rad6-mediated DNA repair pathway (19). However, it is not clear whether Ubc9 plays any role in tumor drug responsiveness.

In the present study, we found that overexpression of a dominant-negative Ubc9 (Ubc9-DN) sensitized tumor cells to anticancer drugs such as inhibitors of topo I (TPT) and topo II (VM-26). Of interest, we observed that compared with the vector control, the Ubc9-DN-expressing cells accumulated more cytoplasmic Daxx, a Fas-associated protein implicated in Fas-induced apoptosis (20, 21), than the vector control. Furthermore, we found that overexpression of Daxx increased the sensitivity of cells to anticancer drugs such as TPT, suggesting a role for Ubc9 in tumor drug responsiveness in part through regulation of subcellular distribution of Daxx.

MATERIALS AND METHODS

Cell Culture. HeLa cells (obtained from American Type Cell Collection, Manassas, VA) were grown in DMEM (BioWhitaker, Walkersville, MD). MCF7 cells (American Type Cell Collection) and mouse mammary cancer cells CRL2116 (American Type Cell Collection) were grown in RPMI (Bio-Whitaker). All media were supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units of penicillin/ml and 100 μg of streptomycin/ml. Cells were incubated at 37°C in a humidified chamber supplemented with 5% CO2.

Plasmids. Human Ubc9 wild type and a DN mutant that carries the C93S mutation were cloned into the pCMV-Myc vector (Clontech, Palo Alto, CA) as described previously (10). A plasmid carrying glutathione S-transferase (GST)-Ubc9 (pGEX-Ubc9) was constructed by PCR amplification of the full-length gene and cloned into pCR2.1. After verification of the sequence by DNA sequencing, the fragment was cloned into pGEX-2T (Amersham, Piscataway, NJ) at BamHI and EcoRI sites. The PCR primers were Ubc9-5′.5′. GGATCCCTCGGGGATCGCCCTCAGCA (sense) and Ubc9 –3.1, CTTATGAGGGCCTTCGGGATCGCCCTCAGCA (antisense). To construct enhanced green fluorescent protein (EGFP)-Daxx fusions, the Daxx coding region (minus start codon ATG) was inserted into pGEX-Ubc9. The resulting fusion gene and cloned into pCR2.1. After verification of the sequence by DNA sequencing, the fragment was cloned into pGEX-2T (Amersham, Piscataway, NJ) at BamHI and EcoRI sites. The PCR primers were Ubc9-5′.5′. GGATCCCTCGGGGATCGCCCTCAGCA (sense) and Ubc9 –3.1, CTTATGAGGGCCTTCGGGATCGCCCTCAGCA (antisense). To construct enhanced green fluorescent protein (EGFP)-Daxx fusions, the Daxx coding region (minus start codon ATG)
was amplified by reverse transcription-PCR from HeLa cells and cloned into pEIGFP-C3 by standard procedures.

**Transfection.** Transfection of MCF7 cells was performed by electroporation using the electroporator II with extender II (Bio-Rad, Hercules, CA) following a published protocol (22) with a slight modification. In brief, exponentially growing MCF7 cells (~6 million) were harvested and resuspended in 0.4 ml of electroporation buffer (22). After mixing with plasmid DNA, the cells were incubated for 10 min at room temperature in the hood. The cells were then subjected to a pulse at 220 V and 950 μF of capacity. To establish stably transfected cell lines, Ubc9-DN was introduced into MCF7 cells along with pTK-Hg (Clontech) as above, and was selected in the presence of 200 μg of hygromycin/ml (Invitrogen, Carlsbad, CA). The expression of the exogenous Ubc9-DN gene was determined by Western blot using antibodies specific to either Ubc9 or Myc-tag. The vector control was a pool of over 20 individual clones. To generate stable transfectants of EGFP-Daxx, MCF7 cells expressing EGFP-Daxx were selected in the presence of G418 (1 mg/ml). Positive clones were confirmed by fluorescence microscopy and Western blot analysis. It should be noted that the vector control was also a pool of over 20 individual clones.

**GST Pull-Down Assays.** Isolation and purification of the GST fusion protein (GST or GST-Ubc9) were performed as described previously (23). For pull-down assays, total protein was isolated from different cell lines with the pull-down buffer. After removal of cell debris by centrifugation, 500 μg of protein was mixed with beads carrying GST or GST-Ubc9 and incubated for 2 h at 4°C. After three washes with PBS, proteins were solubilized in 1 × SDS protein sample buffer and separated in 8% SDS polyacrylamide gels.

**Isolation of Cytoplasmic and Nuclear Proteins.** Cells were harvested and resuspended in cellular lysis buffer [0.1% NP40, 10 mM Tris (pH 7.9), 10 mM MgCl₂, and 15 mM NaCl] on ice for 10 min. After brief centrifugation, the supernatant was saved as the cytoplasmic fraction, and the nuclear pellet was lysed in nuclear lysis buffer [0.5 M NaCl, 20 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) (pH 7.9), 20% glycerol] on ice for 15 min. Protein concentration was determined by Bio-Rad protein assay kit (Bio-Rad) per the manufacturer’s instruction.

**Western Blot.** Western blot analysis was carried out as described previously (24). Topo I-specific antibody TH-1 (25) was used to detect topo I. Anti-Myc antibody (Zymed, South San Francisco, CA) was used to detect overexpression of Ubc9-DN protein, and the same anti-Ubc9 antibody was used to detect both endogenous and Ubc9-DN.

**Fluorescence Microscopy.** To detect the nuclear localization of EGFP-Daxx, MCF7 cells were transfected with plasmid DNA and then examined in a fluorescence microscope (Carl Zeiss, Thornwood, NY) equipped with a filter for EGFP. To determine the transfection efficiency in transient transfection experiments, we used pEIGFP-C3 as a reporter, and a total of 200 cells were counted for each transfection.

**Cytotoxicity Assays.** 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assays were used to determine the response of tumor cells to anticancer drugs according to standard methods. Briefly, cells were seeded in 96-well plates and treated with drugs at concentrations indicated and incubated at 37°C for 4 days.

**RESULTS**

**Overexpression of Ubc9-DN Decreases Sumoylation and Catalytic Activity of Topo I.** We have shown previously that overexpression of Ubc9-DN reduces sumoylation of topo I in response to TPT in HeLa cells (10). Consistent with these results, we have found reduction of topo I sumoylation by Ubc9-DN in MCF7 cells (Fig. 1A). Accordingly, we asked whether overexpression of Ubc9-DN has any effect on topo I activity. We initially used cells transiently transfected with Ubc9-DN and found that Ubc9-DN decreased topo I activity by about 2-fold when compared with the vector control (not shown). We then generated several stable Ubc9-DN transfectants in MCF7 cells and selected two (Ubc9-DN-7 and Ubc9-DN-8) of over a dozen clones. Expression of the exogenous gene in these two clones was detected by Western blot (Fig. 2A). As with the transient transfectants, relaxation assays with the stable transfectants revealed a lower activity of topo I in both clones compared with the vector control (Fig. 1B). Because overexpression of Ubc9-DN decreases sumoylation of topo I, the results suggest that Ubc9-DN-associated decrease in topo I activity is possibly attributable to decreased sumoylation of topo I.

**MCF7 Cells Expressing Ubc9-DN Display Increased Drug Sensitivity.** Because topo I activity is directly correlated with the sensitivity to topo I inhibitors, the decreased topo I activity in MCF7 cells expressing Ubc9-DN is expected to make the cells more resistant to topo I inhibitors. To test the effect of Ubc9-DN on drug responsiveness, we tested these two Ubc9-DN clones (Ubc9-DN-7 and Ubc9-DN-8) for their response to TPT. Unexpectedly, MTT assays revealed that these Ubc9-DN cells are in fact more sensitive to the drug (Fig. 2B), suggesting that mechanisms other than reduction of topo I activity are likely to be responsible for the increased sensitivity to TPT. Indeed, these Ubc9-DN-expressing cells are also more sensitive to VM-26 (Fig. 2C), a topo II inhibitor, as well as cisplatin, a DNA alkylation agent (not shown). In fact, these data are consistent with the observation that yeast temperature-sensitive Ubc9 mutants were more sensitive to DNA-damaging agents (7), supporting the notion that drug responsiveness is multifactorial. Hence, the results suggest that Ubc9/small ubiquitin-related modifier interact with many other pro-
teins in addition to topo I and affect their activities or their subcellular localization, which ultimately contribute to drug responsiveness.

Altered Expression of Ubc9 in Tumor Cells Correlates with Their Sensitivity to Anticancer Agents. In light of the above findings, we tested some ovarian tumor cell lines with different levels of intrinsic sensitivity to cisplatin for Ubc9 expression. For instance, OVCAR3 cells were 3- and 5-fold (at the IC50) more sensitive to cisplatin than OVCAR5 and OVCAR8 cells, respectively. As shown in Fig. 3A, the level of Ubc9 in OVCAR3 cells was about 3-fold less than that of OVCAR5 or OVCAR8 cells. This correlation was also supported by semi-quantitative reverse transcription-PCR data (not shown), suggesting that Ubc9 could be a contributing factor to drug responsiveness. In support of this notion, we found a similar relationship between Ubc9 and resistance to VM-26 in CEM and its sublines, CEM/V1-5 (to VM-26) cells expressed the highest level of Ubc9 (Fig. 3B), which was twice as much as in CEM cells.

Detection of More Cytoplasmic Daxx in MCF7 Cells Expressing Ubc9-DN by Cellular Fractionations. To determine possible mechanisms underlying the increased drug sensitivity of MCF7 cells expressing Ubc9-DN, we examined proteins that have potential roles in drug responsiveness, including those associated with apoptosis and DNA repair. One such protein is Daxx, a Fas death domain-associated protein implicated in Fas-induced apoptosis (20, 21). Daxx has been shown to interact with Ubc9 (28) and is a substrate for sumoylation (29). Because Ubc9 is implicated in regulating the subcellular localization of proteins, we first examined the interaction of Ubc9 with Daxx by glutathione S-transferase (GST) pull-down assays. Total protein was isolated from MCF7, CRL-2116, and HeLa cells, respectively, and incubated with GST-Ubc9 as described in “Materials and Methods.” The membrane was probed with anti-Daxx antibody. Amount of protein extract for the input was about half of that used in the pull-down assays. B-C, subcellular distribution of Daxx in parental MCF7 cells (B) and MCF7 cells expressing vector alone or Ubc9-DN (C). Cytoplasmic and nuclear protein was isolated from MCF7 cells as described in “Materials and Methods.” About 50 μg of protein were loaded per lane. Topoisomerase I (topo I) serves as a nuclear marker, in addition to serving as a loading control; α-tubulin serves as a cytoplasmic marker as well as a loading control. Shown here (C) is a representative of more than three separate experiments. Note about a 2-fold increase in the cytoplasmic Daxx in Ubc9-DN-expressing MCF7 cells. D, subnuclear distribution of enhanced green fluorescent protein (EGFP)-Daxx as detected by a fluorescence microscope.
As well as its subcellular distribution. Although there was no obvious difference in the Daxx protein from total extracts among different cell lines (not shown), we detected more cytoplasmic Daxx in both the Ubc9-DN-7 and Ubc9-DN-8 cells than in the vector control cells (Fig. 4C). Similarly, no difference was seen for nuclear Daxx between controls and Ubc9-DN cells presumably because only a small portion of nuclear Daxx moves out in these Ubc9-DN cells. Cytoplasmic Daxx is believed to participate in the caspase-independent apoptosis pathway (30). Hence, an increase in the cytoplasmic Daxx suggests that these Ubc9-DN-expressing cells are more prone to apoptosis, and thus, would be more sensitive to TPT and other anticancer drugs.

Because previous studies suggested that the nuclear Daxx can also be recruited to promyelocytic leukemia oncogenic domain, which requires sumoylation of promyelocytic leukemia (31, 32), we also examined subnuclear distribution of Daxx in these cells by using EGFP-Daxx. However, we did not detect any alteration of subnuclear distribution of Daxx between Ubc9-DN cells and the vector control cells (Fig. 4D). Although we cannot exclude the possibility that suppression of the endogenous Ubc9 by Ubc9-DN is insufficient to detect any difference in the subnuclear distribution of Daxx, it appears that Ubc9 has no detectable effect here. Finally, we used the same pEGFP-Daxx construct and monitored the subcellular localization of the fusion protein. However, we did not detect differences in the subcellular localization between the vector control and Ubc9-DN cells.

Overexpression of Daxx Increases Drug Sensitivity. To evaluate the role of Daxx in drug-induced cell death, we overexpressed pEGFP-Daxx in MCF7 cells and generated several stable cell lines expressing EGFP-Daxx. Expression of the fusion protein for one such clone (EGFP-Daxx-1) was detected by Western blot, as shown in Fig. 5A. Although microscopic examination of these cells revealed that the fusion protein was predominantly nuclear (Fig. 5B), cellular fractionation indicated a higher ratio of cytoplasmic versus nuclear EGFP-Daxx than that of the endogenous Daxx (Fig. 5C). For instance, whereas we detected about 5% of endogenous Daxx in the cytoplasm, we detected about 35% (as measured by densitometry) of EGFP-Daxx in the cytoplasm (Fig. 5C).

MTT assays indicated that the EGFP-Daxx-expressing MCF7 cells are more sensitive to TPT (−7-fold at the IC_{50}; Fig. 5D) and to VM-26 (−5-fold at the IC_{50}; Fig. 5E) than the vector alone. Similar responses were seen for other EGFP-Daxx clones (not shown). Because this increase in drug sensitivity corresponded with a higher ratio of cytoplasmic versus nuclear EGFP-Daxx than that of the endogenous Daxx (Fig. 5C), we suggest that more cytoplasmic EGFP-Daxx may contribute to the increased drug sensitivity of these cells.

DISCUSSION

Protein sumoylation has been implicated in the regulation of protein stability, subcellular localization, and the activity of transcription factors (5, 6). Because Ubc9 is the sole E2-conjugating enzyme required for sumoylation (2), it is believed to play a central role in these processes. Although some evidence suggests that Ubc9 may be involved in a DNA repair pathway in yeast (17, 19), little is known about whether Ubc9 plays any role in tumor drug responsiveness. In the present study, we found that overexpression of Ubc9-DN decreases topo I sumoylation, sensitizes tumor cells to a variety of anticancer agents including TPT, VM-26, and others, suggesting that Ubc9-dependent sumoylation plays a role in drug responsiveness. Moreover, we found a good correlation between Ubc9 levels and drug resistance in ovarian cancer and acute lymphoblastic leukemia cell lines, further supporting this notion.

Although the MCF7 cells expressing Ubc9-DN display a reduced topo I activity, they are paradoxically more sensitive to antitumor agents. To determine molecular mechanisms underlying the alteration of drug response in these Ubc9-DN-expressing MCF7 cells, we examined the Fas-associated protein, Daxx, which is implicated in Fas-mediated apoptosis. Intriguingly, we found that more cytoplasmic Daxx is accumulated in MCF7 cells expressing Ubc9-DN, implying that Ubc9 plays a role in regulating the subcellular localization of Daxx. Although fluorescence microscopy did not indicate any change of subcellular localization of Daxx by Ubc9-DN, cellular fractionation combined with Western blot revealed such changes. Despite several possibilities for this discrepancy, we postulate that the cytoplasmic Daxx may associate only loosely with nuclear membrane. When the cells are intact, the cytoplasmic Daxx surrounds the nucleus and thus appears to be in the nucleus; however, once cells are lysed, Daxx dissociates from the nuclear membrane and is released into the cytoplasmic fraction. Although it is possible that the cytoplasmic fraction prepared in this study may be contaminated with the nuclear Daxx,
because we did not detect topo I, an abundant nuclear protein in the cytoplasmic fraction, this is not likely to be the case. Daxx has been shown to be sumoylated (29), so overexpression of Ubc9-DN should affect sumoylation of Daxx. However, we detected no difference in Daxx sumoylation between controls and transfectants. This is presumably because sumoylated Daxx is only detected when Daxx, small ubiquitin-related modifier-1, and Ubc9 are all overexpressed, and the ratio of sumoylated versus nonsumoylated Daxx is only ∼1–2% (29).

Because Daxx is a component of the Fas-induced apoptosis pathway (20), the increase of the cytoplasmic Daxx may explain in part why these Ubc9-DN-expressing cells are more sensitive to drugs. Given that Ubc9 plays a role in regulating the subcellular localization of interacting proteins (33, 34), we suggest that Daxx is another member of such Ubc9-mediated proteins.

As shown in Fig. 6, Fas/Fas ligand is one of the key elements of a well-defined apoptotic pathway (35, 36). Interaction of Fas/Fas ligand leads to the recruitment of death-associated proteins to the cytoplasmic domain of Fas. Depending on which adaptor protein associates with Fas, two distinct Fas-mediated pathways have been identified (i.e., caspase dependent and caspase independent). Fas-associated death domain mediates a caspase-dependent pathway through recruitment of procaspase-8 (34, 35), whereas Daxx mediates a caspase-independent pathway by activating a proapoptotic kinase, apoptosis signal-regulating kinase 1 (Ask1; 37). Although the Fas-associated death domain pathway plays a major role at an early stage of apoptosis, the Daxx pathway is believed to play a role in a late stage of apoptosis (20).

Daxx resides in both the cytoplasm and nucleus and has a dual function (Fig. 6). Although the cytoplasmic Daxx participates in Fas-induced apoptosis, the nuclear Daxx acts as a negative transcriptional regulator (38). In the cytoplasm, Daxx interacts with the death domain of Fas and Ask1, and activation of Ask1 by binding to Daxx leads to activation of c-Jun NH2-terminal kinase (36). By contrast, the nuclear Daxx controls expression of several proteins such as PAX3 (38), a transcription factor that is involved in development. Sequestration of Daxx from the nucleoplasm and into promyelocytic leukemia oncogenic domain leads to relief of the repressive activity of Daxx (32).

Regulation of Daxx in enhancing Fas-mediated apoptosis is complex, and several factors have been shown to be involved in this process (39–41). For instance, although Fas activation leads to the accumulation of cytoplasmic Daxx, HSP27 is able to block Fas-induced translocation of Daxx from the nucleus to the cytoplasm and formation of Fas/Daxx/Ask1 complexes, leading to cell death through Daxx-dependent apoptosis (39, 40). Because overexpression of Ubc9-DN causes accumulation of cytoplasmic Daxx, this suggests that Ubc9 could be another factor regulating the subcellular localization of Daxx.

Nevertheless, alteration of the subcellular localization of Daxx may not be the only explanation for the observed increased sensitivity to a variety of anticancer drugs in these Ubc9-DN cells. It is well known that drug responsiveness is a consequence of multiple cellular factors. For instance, alterations in DNA repair pathways can also contribute to tumor drug responsiveness. Anticancer drugs such as TPT and VM-26 cause DNA damage by stabilizing DNA-protein complexes (8, 9). Ubc9 has been shown to be important for DNA repair (19), and it has been speculated that sumoylation of topo I could be a DNA damage-rescuing mechanism (7). We have found previously that nucleolar delocalized topo I formed nuclear body-like structures (10), which could be nuclear foci where DNA repair takes place (Fig. 6). Furthermore, Ubc9 was originally identified through its involvement in the degradation of cyclins in yeast (42), implicating its role in cell cycle control, although there is no biochemical evidence to support this notion. If this occurs in mammalian cells, Ubc9 could also contribute to the responsiveness of cells to DNA-damaging agents such as TPT through regulation of the cell cycle. Nevertheless, the present study suggests that Ubc9 could impact the response of the
cells to anticancer drugs through alteration of the subcellular localization of Daxx.

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