Cdc34-mediated degradation of ATF5 is blocked by cisplatin

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Running Title: Degradation of ATF5 during Apoptosis

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ATF5, a member of ATF/CREB family of b-ZIP transcription factors, contributes to neural cell differentiation and is involved in cell apoptosis in response to cisplatin and a number of environment factors. However, the mechanisms governing the regulation of ATF5 protein during apoptosis are largely unknown. In this study, we reported that ATF5 protein was a substrate of the ubiquitin-proteasome pathway. Interestingly, the ubiquitin-dependent degradation of exogenous ATF5 protein was independent of lysine residues. Instead, addition of a large N-terminal EGFP tag increased the stability of ATF5 protein and the free amino acid group of the N-terminal methionine of ATF5 protein was a site for ubiquitinylation, indicating that exogenous ATF5 was degraded via the ubiquitin-proteasome system through N-terminal ubiquitinylation. Furthermore, cisplatin increased ATF5 protein expression via preventing its ubiquitin-dependent degradation, which might be associated with its promoting the nucleus-to-cytoplasm translocation of E2 ubiquitin-conjugating enzyme Cdc34 and reducing the interaction between ATF5 and Cdc34. In summary, a down-regulation of proteasome-mediated degradation of ATF5 might contribute to cisplatin-induced apoptosis, providing a new mechanism of cisplatin-induced apoptosis.

ATF5 transcription factor, a member of ATF/CREB family of b-ZIP transcription factors (1), plays a critical role in regulating cAMP response element (CRE)-dependent genes and functions as an essential role in neural cell differentiation (2-4).

Accumulated data from our laboratory and other investigators indicated that ATF5 functions as an apoptosis-related protein in response to a number of environment factors and DNA damage (5,6). Interference with the function or expression of ATF5 in glioma cells leaded to their death in vitro and in vivo (7), indicating that ATF5 might be an attractive target for therapeutic intervention in
glioblastoma. Consistent with this, ATF5 was widely expressed in carcinomas and interference with its function caused apoptotic cell death of neoplastic breast cell lines (8). Furthermore, ATF5 functioned as an anti-apoptotic role in an interleukin 3 (IL-3)-dependent cell line (9). And, we have showed that ATF5 was up-regulated by cisplatin and its over-expression increased cisplatin-induced apoptosis in HeLa cells (5), indicating the pro-apoptotic role of ATF5 in DNA damage-induced apoptosis. Previously, Debanda et al. showed that ATF5 interacted with E2 ubiquitin-conjugating enzyme Cdc34 (10). Cdc34 and RAD6 had been reported to be important components of the ubiquitin-proteasome system in the nucleus which was responsible for the degradation of some transcriptional factors (11). Although the progresses in ATF5 interacting partners, function and its relation to some diseases have been made in recent years, the precise molecular mechanisms of ATF5 protein regulation during apoptosis are largely unknown.

Cisplatin is a well-known DNA-damaging agent and is widely used in the treatment of solid tumors, including testicular, ovarian, bladder, cerebral, head and neck, and small-cell and non-small-cell lung cancers (12-14). Recent studies provided evidence that the stability of many proteins including Bax, Bak and p53 contributed to cisplatin cytotoxicity in cancer cells (15-17), indicating the contribution of proteolysis in cisplatin-induced apoptosis. Consistent with this, the proteasome inhibitor PS-341, the representative of a new class of chemotherapeutic drugs, was capable of inducing apoptosis in cisplatin-resistant squamous cell carcinoma cell (18). In addition, cisplatin-resistant ovarian cancer cells were defective in 26S proteasome (19). These data indicated that cisplatin might induce cell apoptosis by regulating the ubiquitin-dependent degradation of protein.

In this study, we provided evidence that ATF5 was degraded via the ubiquitin-proteasome pathway and exogenous ATF5 was degraded via N-terminal ubiquitinylation. And, cisplatin increased ATF5 protein expression by preventing its proteasome-mediated degradation. Furthermore, we reported for the first time that cisplatin-reduced ubiquitin-dependent degradation of ATF5 might be associated with its promoting the nucleus-to-cytoplasm translocation of Cdc34 and inhibiting the interaction between ATF5 and Cdc34. These data indicated that a down-regulation of proteasome-mediated degradation of ATF5 ubiquitinylation might contribute to cisplatin-induced apoptosis, providing a new mechanism of cisplatin-induced apoptosis.

**EXPERIMENTAL PROCEDURES**

*Reagents—* Cisplatin, cycloheximide (CHX), ubiquitin protein, Flag-ubiquitin, ubiquitin-Ald, Cdc34 protein, phosphocreatine, phosphocreatine kinase, hexokinase, 2-deoxyglucose, anti-β-Tubulin antibody and anti-Flag antibody were purchased from Sigma. MG132 was purchased from Calbiochem. Rabbit anti-ATF5 polyclonal antibody was purchased from Aviva System Biology Ltd. Goat Anti-ATF5 polyclonal antibody was
purchased from Abcam Ltd. Mouse anti-EGFP antibody was purchased from Roche. Rabbit Anti-PARP antibody was purchased from Cell Signal Technology. Mouse anti-myc and mouse anti-HA antibodies were purchased from Invitrogen Company. Mouse anti-Ubiquitin, mouse anti-Histone and mouse anti-GAPDH antibodies were purchased from Santa Cruz Biotechnology Inc. Mouse anti-Cdc34 antibody was purchased from BD Pharmingen Ltd.

Cell culture and cell transfection—HeLa, HEK293 and MDA231 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 50 μg/ml streptomycin at 37 °C in a humidified CO₂ incubator (5% CO₂, 95% air). Cell transfection was performed using Lipofectamine2000 (Invitrogen) according to the manufacturer’s instructions.

Plasmid construction and mutagenesis—Expression constructs for HA-tagged Ub and C-terminally EGFP-tagged pEGFPN3-ATF5 have been described previously (20,21). Myc-tagged ATF5 plasmid was constructed by inserting ATF5 coding sequence into the EcoR I/BamH I sites of the pcDNA3.1/myc(-) vector. The deletion mutants of ATF5 were designated as ΔC1 (aa 1–40), ΔC2 (aa 1-204), ΔN1 (aa 41-282), ΔN2 (aa 121-282) and ΔN3 (aa 205-282), and were constructed by PCR amplification using pcDNA3.0-ATF5 as a template and the listed primers: ΔC1 sense, 5'-ATAGAATTCCATGTCACTCCTGGCGACCCCTG-3', and ΔC2 anti-sense, 5'-AATGGATCCCGAGGATGGGTAGGG-3'; ΔN1 sense, 5'-ATAGAATTTCATGCCGCTACGGTGCTCTGTCGAGGAGGACCGAGTACG-3'; ΔN2 sense, 5'-ATAGAATTTCATGCCGCTACGGTGCTCTGTCGAGGAGGACCGAGTACG-3'; ΔN3 sense, 5'-AATGGATCCGCAGCTACGGTGCTCTGTCGAGGAGGACCGAGTACG-3'. To construct N-terminally EGFp-tagged pcDNA3.0-EGFP-ATF5 plasmid, the ATF5 coding sequence was inserted into the EcoR I/Not I sites of the pcDNA3.0-EGFP vector which has previously described (22). The point-mutants of ATF5 were derived from pEGFPN3-ATF5 plasmid by PCR amplification using Takara MutanBEST mutagenesis kit. Mutated constructs were sequenced and correct ones were selected for further experiments. The prokaryotic expression plasmid pET23b-HA-ATF5 was constructed by inserting the coding sequence of HA-tagged ATF5 into the Nde I/Xho I sites of the pET23b vector. The recombinant plasmid pET23b-HA-Xa-ATF5 which contained an HA tag followed by a factor Xa protease cleavage site (Ile-Glu-Gly-Arg) after the first methionine was constructed using Takara MutanBEST mutagenesis kit. PcDNA3.0-Cdc34 plasmid was constructed by PCR amplification using pCS2+cdc34 plasmid (a kind gift from Carlos R. Herrera) as a template.

In vivo ubiquitination assay—Cells treated with
25 μM MG132 for 12 h were re-suspended in modified RIPA buffer (150 mM NaCl, 0.5% NP-40, 0.1% (w/v) deoxycholate, 0.1% SDS, 10 mM Tris-HCl (pH 7.5), 1 mM PMSF, 10 mg/ml aprotinin and 5 mg/ml leupeptin) with 1% SDS to disrupt protein-protein interaction, boiled for 10 min, then diluted in 10 volumes of RIPA buffer. The lysates were sonicated at 4 °C and subsequently pre-cleared with protein G agarose for 1 h at 4 °C. The lysates were subsequently divided for individual immunoprecipitation with the appropriate antibodies. Immunoprecipitated proteins were analyzed using western blot assay.

**In vitro ubiquitination assay**—His-tagged ATF5 protein was purified from bacteria BL21. 50 μg cell extracts was mixed with 20 μl ATF5 protein and nickel agarose in 120 μl reaction buffer (20 mM Tris (pH 7.6), 50 mM NaCl, 10 mM MgCl2, 1 mM DDT, 1 μM Ubiquitin-Ald, 1 mg/ml Ubiquitin or Flag-Ubiquitin, 25 μM MG132) at 30 °C for 2 h with constant rotation. Reactions were carried out in the presence of either 0.5 mM ATP and an ATP regeneration system (10 mM phosphocreatine, 5 U/ml phosphocreatine kinase) or 50 U/ml hexokinase and 10 mM 2-deoxyglucose to deplete endogenous ATP. Then the nickel agarose was washed, and the bound proteins were eluted in protein sample buffer and subjected to western blot analysis. For Factor Xa cleavage, the protein was pre-treated with Factor Xa for 16 h at 25 °C before SDS-PAGE analysis.

**Half-life assay**—HeLa cells were transiently transfected with pEGFPN3-ATF5 construct or its mutants. 40 h after transfection, cells were treated with 100 μg/ml CHX for 0, 0.5, 1.0, 1.5 and 2 h. The cells were harvested and 50 μg of cell lysates were analyzed by immunoblotting with anti-EGFP and anti-GAPDH antibodies. The protein levels were calculated using densitometry analyzing software and the values of ATF5 were plotted against the values of GAPDH. The ratio of ATF5/GAPDH at 0 h was taken as 100%. The assay was repeated independently at least three times.

**Determining the expression efficiency of protein by fluorescent microscopy and flow cytometry**—Cells were washed twice with 1 x PBS and re-suspended in 0.5 ml of 4% paraformaldehyde. EGFP expression was analyzed by flow cytometry with excitation and emission settings of 488 nm and 530 nm respectively. The assay was repeated independently at least three times. Immunofluorescence and confocal assay were performed as previously described (22).

**Reverse transcription (RT)-PCR and co-immunoprecipitations**—RT-PCR was performed as described previously (5). Primers used for PCR were as follows: ATF5 sense, 5’-AAGTCGGCGGCTCTGAGGTA-3’, and ATF5 anti-sense, 5’-GGACTCTGCCCCGTTCCTTCA-3’; P21 sense, 5’-GACACCCACTGGAGGGTACT-3’ and P21 anti-sense, 5’-GGCGTTTGGAGTGTTAGAAA-3’. The co-immunoprecipitation and immunoblotting assays were performed as previously described. Nuclear and cytoplasmic protein was isolated according to the method of Schreiber et al (23).
RESULTS

Cisplatin increases ATF5 protein expression via inhibiting its ubiquitin-mediated degradation—
Our previous study showed that ATF5 protein was up-regulated during cisplatin-induced apoptosis in HeLa cells (5). To investigate the mechanism of cisplatin-induced ATF5 expression, ATF5 mRNA expression was examined using RT-PCR in HeLa cells treated with cisplatin at different concentration. The level of ATF5 mRNA expression did not significantly changed in response to cisplatin, and the level of P21 mRNA expression was significantly increased in response to cisplatin which was consistent with previous report (24) (Fig. 1A). These data indicated that cisplatin might regulate ATF5 expression at protein level. To verify if cisplatin caused accumulation of ATF5 by preventing its proteasome-mediated degradation, the level of ATF5 protein expression in HeLa cells pretreated with DMSO or proteasome inhibitor MG132 for 1 h followed by cisplatin treatment for 12 h was analyzed using western blot assay. As expected, the ATF5 protein expression was increased by cisplatin in a dose-dependent manner in the absence of MG132 (Fig. 1B, lanes 1-3). The positive effect of cisplatin on ATF5 protein expression was eliminated in the presence of MG132, although the total amount of ATF5 protein was increased after MG132 treatment (Fig. 1B, lanes 4-6). The same experiments were performed in HEK293 (Fig. 1C) and MDA231 (Fig. 1D) cells and similar results were obtained, indicating that cisplatin might regulate ATF5 expression via proteasome-dependent degradation.

Efficient degradation of proteins by the proteasome generally requires the conjugation of multiple molecules of ubiquitin to the substrate (25). To determine whether ATF5 was ubiquitinylated in vivo, myc-tagged ATF5 plasmid was transiently co-transfected into HeLa cells with HA-tagged ubiquitin. Followed by MG132 treatment, the myc-tagged ATF5 was immunoprecipitated using anti-myc antibody and analyzed by immunoblotting using anti-HA antibody. We detected a high-molecular-weight of HA-marked molecules whose intensity was increased by MG132 treatment (Fig. 1E, lane 3 versus lane 4). Furthermore, when the same sample was immunoblotted with anti-ATF5 antibody, the film demonstrated a similar ladder of ATF5 species (Fig. 1E, lanes 7 and 8). These results strongly suggested that ATF5 was poly-ubiquitinylated in vivo.

Next, we investigated the contribution of cisplatin in ubiquitin-dependent degradation of ATF5. Upon MG132 treatment, endogenous ATF5 formed efficient conjugation of ubiquitin-ATF5 which was eliminated markedly by cisplatin (Fig. 1F, lane 3 versus lane 4). Furthermore, we obtained the similar result using anti-ATF5 antibody to detect the same sample (Fig. 1F, lane 7 versus lane 8). These results suggested that cisplatin increased ATF5 protein level, at least partly through inhibition of the ubiquitin-mediated of ATF5.

Cisplatin promoted the nucleus export of Cdc34 and reduced the interaction between ATF5 and Cdc34—Previously, ubiquitin-conjugating enzyme
E2 Cdc34 has been reported to target ATF5 degradation (10). Consistent with this, the forced expression of Cdc34 decreased the protein level of endogenous ATF5 significantly (Fig. 2A). To investigate the mechanism of cisplatin-reduced ATF5 ubiquitination and degradation, we first examined the effect of cisplatin on the expression of Cdc34. Upon cisplatin treatment, the total level of Cdc34 protein expression in HeLa cells was not significantly changed (data not show). Interestingly, we observed that Cdc34 translocated from the nuclear to the cytoplasm in response to cisplatin treatment. Cdc34 was randomly localized in both nucleus and cytoplasm and co-localized with ATF5 in nucleus in HeLa cells, and Cdc34 was expressed predominantly in the cytoplasm and did not co-localize with ATF5 after a 12 h exposure to cisplatin (Fig. 2B). These changes were confirmed using western blot analysis. ATF5 co-immunoprecipitated with Cdc34 was decreased significantly by cisplatin treatment (Fig 2C). Furthermore, the level of Cdc34 in cytoplasm was increased but the level of Cdc34 in nuclear extraction was decreased after cisplatin treatment in a dose-dependent manner (Fig. 2D). However, ATF5 expression was limited to the nucleus of HeLa cells and was increased by cisplatin in a dose-dependent manner (Fig. 2D). And, the half-life of endogenous ATF5 in the nucleus of HeLa cells was prolonged from approximately 45 min to almost 80 min after cisplatin treatment (Fig. 2E). To confirm these results, we examined the ability of nuclear extracts prepared from HeLa cells treated with or without cisplatin to ubiquitinylate ATF5 in vitro. Whereas nuclear extracts from control HeLa cells produced efficient ubiquitin conjugation of purified ATF5 protein in an ATP-dependent manner, extracts from cisplatin-treated HeLa cells had hardly any activity above background controls (Fig. 2F). Next, we investigate the contribution of Cdc34 in the degradation of ATF5 in the presence or absence of cisplatin. The ability of nuclear extracts prepared from HeLa cells to ubiquitinylate ATF5 was enhanced by purified Cdc34 protein, and the negative effect of cisplatin on the ubiquitination of ATF5 was inhibited in the presence of purified Cdc34 protein in vitro (Fig. 2G). These results suggested that cisplatin-reduced ubiquitin-dependent degradation of ATF5 in the nucleus might be associated with the translocation of Cdc34 from the nucleus to the cytoplasm.

The N-terminal 204 amino acids of exogenous ATF5 contain the major domain for its degradation— ATF5 protein contains a transcriptional activation domain (AD domain; aa 1-204) including a proline-rich region and a DNA-binding domain (DBD domain; aa 204-282) which has the high homology comparing with ATF4 (6). To map the region responsible for the degradation of ATF5, various deletion mutants of ATF5 were constructed (Fig. 3A), and were transiently co-transfected into HeLa cells with 1 μg pEGFPN3 empty vector which was used as a control of transfection efficiency. 24 h after transfection, cells were treated with DMSO or MG132. We observed that MG132 significantly increased the expression of ATF5 FL, ΔC1, ΔC2,
ΔN1 and ΔN2 without changing the expression of ΔN3 (Fig. 3B), indicating that the N-terminal 204 amino acids of ATF5 was essential for its degradation. To further verify the domain of ATF5 contributing to cisplatin-reduced ATF5 degradation, we investigated the effect of cisplatin on the expression of ATF5 full length (FL), the AD domain (ΔC2) and the DBD domain (ΔN3) in HeLa cells pretreated with DMSO or MG132. The expression of ATF5 full length (FL) and the AD domain (ΔC2) were increased by cisplatin treatment in the absence of MG132 (Fig. 3C, (a) and (b)). However, neither cisplatin nor MG132 had effect on the expression of the DBD domain (Fig. 3C, (c)). These results suggested that the essential region for degradation of exogenous ATF5 by proteasome pathway was the N-terminal 204 amino acids (the AD domain).

In vivo degradation of ATF5 by the proteasome is independent of lysine residues— The conjugation of ubiquitin to an internal lysine was the initial step in the degradation of the majority of the substrates of the ubiquitin system (25). To investigate the contribution of 3 lysine residues (K29, K106 and K107) in ATF5 AD domain in its ubiquitination, three lysine mutants (K29R, K106107R or K29R+K106107R) were constructed into pEGFPN3 vector by replacing lysine with arginine at one, two or three positions (Fig. 4A, left panel), and were transiently transfected into HeLa cells. We found that all these mutants were expressed at low levels similar to that of the wild-type protein by fluorescence microscopy and flow cytometry (supplementary S1). Consistent with this, immunoblotting analysis showed that expression levels of all these mutants were increased by MG132 significantly (Fig. 4A), suggesting that none of the lysine was essential for ATF5 degradation in vivo. Next, we measured the half-lives of these mutants by CHX chase experiments. Both wild-type ATF5 and its lysine mutants were rapidly degraded, with half-lives of approximately 45 min (Fig. 4B, lanes 1-5). And, their half-lives were prolonged to approximately 90 min by MG132 treatment (Fig. 4B, lanes 6-10). Although the DBD domain of ATF5 was not required for ATF5 degradation, it may provide lysine residues that could serve as attachment sites for ubiquitylation of ATF5. To address the contribution of DBD domain in the ubiquitylation of ATF5, ATF5 and its three lysine mutants deleting DBD domain were constructed (Fig. 4C, upper panel). These plasmids were transiently transfected into HeLa cells and western blot analysis showed that their expression were increased by MG132 significantly (Fig. 4C, lower panel). We also investigated the ubiquitination of wide type ATF5 and its lysine mutants containing or deleting DBD domain in vivo. pEGFPN3-ATF5, Mut3, ΔC2, Mut6 or control vector were co-transfected with HA-Ub into HeLa cells. Followed by MG132 treatment, the EGFP-tagged ATF5 was immunoprecipitated using anti-EGFP antibody from cellular extracts and analyzed by immunoblotting using anti-HA antibody. As expected, we detected a high-molecular-weight of HA-marked ubiquitin conjugated ATF5 and its mutants (Fig. 4D). On the basis of these results, we
concluded that the ubiquitin-dependent degradation of exogenous ATF5 was independent of lysine residues in vivo.

Addition of a large N-terminal tag stabilizes exogenous ATF5 but does not block its ubiquitinylation— Many authors reported that addition of a large tag such as a 6 x Myc or an EGFP at the N-terminal of the proteins could stabilize those N-terminal ubiquitinated proteins (26,27). Since ATF5 ubiquitinylation did not require the presence of lysine residues, we sought to examine the possibility that it might be ubiquitinylated at N-terminus. To address this point, we constructed an N-terminally EGFP-tagged ATF5 plasmid (Fig. 5A, (a)). We found that the N-terminal EGFP-tag dramatically increased ATF5 expression compared to the C-terminal EGFP-tag by fluorescence microscopy observation (Fig. 5A, (b)), flow cytometry analysis (Fig. 5A, (c)) and western blot assay (Fig. 5A, (d)). CHX chase experiments clearly showed that N-terminal EGFP-tag dramatically increased the stability of ATF5 protein compared to the C-terminal EGFP-tag (Fig. 5B).

Since a large N-terminal EGFP tag could stabilize ATF5 protein, we investigated whether the tag blocked its ubiquitinylation. ATF5-EGFP or EGFP-ATF5 was co-transfected with HA-Ub into HeLa cells followed by MG132 treatment or not and immunoprecipitated using anti-EGFP antibody. As expected, immunoblotting using anti-HA antibody detected the ubiquitin-ATF5 conjugates whose intensity was increased after MG132 treatment whatever the EGFP tag was at the C-terminus or the N-terminus (Fig. 5C), indicating that the N-terminal large tag could not block its ubiquitinylation in vivo. We confirmed this result by in vitro ubiquitinylation assay using N-terminally GST-tagged ATF5 protein purified from bacteria (supplementary S2). These results suggested that the N-terminal large tag did not block the recognition site for ubiquitin, although it could increase the stability of exogenous ATF5.

Exogenous ATF5 is ubiquitinylated at the N-terminus— For several proteins, it was reported that the first ubiquitin is conjugated to the N-terminal residue rather than to an internal lysine (27). In the case of MyoD, it was clearly shown that chemical blocking of the α-NH2 group completely stabilized the protein by in vitro an in vivo degradation assay (28). To address whether the conjugation site of ATF5 to ubiquitin was the free N-terminal NH2, we generated constructs of ATF5 that after the first methionine contained an HA tag (which did not contain any lysine: Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala) followed by a factor Xa protease cleavage site (Ile-Glu-Gly-Arg) or not. The purified HA-ATF5 and HA-Xa-ATF5 protein were used as substrates and incubated with whole HeLa cell extracts in in vitro ubiquitination assay. After reaction, the protein was treated with or without factor Xa protease. If ATF5 was ubiquitinylated at the N-terminus, ubiquitin-conjugated HA-Xa-ATF5 treated with factor Xa protease could not be detected by immunoblotting using anti-ubiquitin antibody for the ubiquitin-HA has been cut away. Compared to control (Fig. 6, upper panel, lane 1),
we did observe the ubiquitinylated HA-ATF5 and HA-Xa-ATF5 without treatment of factor Xa protease (Fig. 6, upper panel, lanes 3 and 5). After factor Xa proteasome treatment, the ubiquitinylated HA-Xa-ATF5 was eliminated completely but the ubiquitinylated HA-ATF5 was not diminished (Fig. 6, upper panel, lanes 4 and 6). When the same sample was blotted with antibody to ATF5 itself, the film demonstrated a similar ladder of ATF5 species and the HA-Xa-ATF5 had its N-terminal HA tag removed in the presence of factor Xa as shown by the increase in gel migration (Fig. 6, lower panel), indicating that exogenous ATF5 was ubiquitinylated at the N-terminal methionine.

**DISCUSSION**

In this study, we revealed that cisplatin increased the expression of ATF5 protein by preventing its ubiquitin-dependent degradation. This conclusion was based on the following evidence. First, cisplatin increased ATF5 protein expression without changing its mRNA expression. Second, the amount of ATF5 protein was increased by cisplatin in the absence of MG132, not in the presence of MG132. Interestingly, cisplatin decreased the level of ATF5 protein expression in the presence of MG132 in HeLa cells, not in the other examined cells, indicating the specificity of cisplatin-reduced ATF5 expression in the presence of MG132 in HeLa cell. Although protein ubiquitination has been best characterized as a mechanism for targeting proteins to the proteasome for degradation (29), recently it has become clear that this modification could also serve many other functions, largely by serving as a protein–protein interaction motif (30,31). Ubiquitination could also modulate the activity of transcription factors, by recruiting coactivators or corepressors or by serving as a trigger for nuclear export (32). In contrast, there are currently only a couple of examples of ubiquitin serving as a nuclear import signal. The nuclear translocation of the transcription factor NF-κB was indirectly controlled by ubiquitination (33). So we conjectured that the expression and translocation of certain unknown protein regulated by cisplatin in the presence of MG132 might negative regulate the expression of ATF5, which needed further investigation. Third, we identified ATF5 protein as a substrate of the ubiquitin-proteasome pathway in vivo and in vitro. Furthermore, cisplatin reduced poly-ubiquitin conjugates of ATF5 in vivo and in vitro. Taken together, these results suggested that cisplatin-induced ATF5 accumulation was associated with a down-regulation of ubiquitin-proteasome-mediated degradation.

The ubiquitin-proteasome system plays an important role in controlling the levels of various cellular proteins and therefore regulates basic cellular processes such as cell cycle progression, signal transduction, cell transformation and cell apoptosis (25,29,34). Accumulated evidence indicated that cisplatin might induce cell apoptosis by regulating the ubiquitin-dependent degradation of proteins (16,18,19). However, the mechanisms by which cisplatin regulates ubiquitination and degradation of protein are largely unknown. Here, we reported for the first time that cisplatin might...
accommodate the ubiquitin-proteasome system through regulating the nucleus-to-cytoplasm translocation of E2 ubiquitin-conjugating enzyme Cdc34. Cdc34 and RAD6 had been reported to be important components of the ubiquitin-proteasome system in the nucleus which was responsible for the degradation of some transcriptional factors (11). Functionally, Cdc34 in association with different ubiquitin protein ligases has been shown to target many different substrates for ubiquitination and degradation during cell division, signal transduction and development (10,35-39). Previously, Debanda et al. showed that ATF5 interacted with E2 ubiquitin-conjugating enzyme Cdc34 and Cdc34 overexpression reduced the expression of ATF5 in JEG3 cells (10). Consistent with this, co-transfection of a Myc-tagged ATF5 with Cdc34 resulted in a significant loss of ATF5 protein in HeLa cells, which could be reversed by incubation of MG132 (data not shown). Furthermore, we found that cisplatin promoted the translocation of Cdc34 from the nucleus to the cytoplasm and reduced the interaction between Cdc34 and ATF5, thus inhibiting the degradation of ATF5. Since Cdc34 was an essential gene for the transition into S phase of the cell division cycle (10, 35-39), it might reveal a new cross-talking between cell cycle and DNA damage and a new mechanism of cisplatin-induced apoptosis.

Another interesting finding was that exogenous ATF5 degradation was independent of lysine residues. MyoD, p21, p16, ERK3, the Epstein-Barr virus LMP1 and HPV-16 E7 are degraded following attachment of the first ubiquitin moiety to their N-terminal moiety (26-28,40-42). Addition of a large tag at the N-terminus of these proteins increases the stabilization of the proteins by largely unknown mechanisms (27). Here, we identified ATF5 as a new protein degraded in this fashion. Addition of a large N-terminal tag blocked the degradation of exogenous ATF5 and increased its stability significantly. Furthermore, we provided direct proof that exogenous ATF5 was ubiquitinylated at the N-terminus in vitro. Interestingly, the large N-terminal EGFP tag of exogenous ATF5 could not block the recognition site for ubiquitin, in agreement with the previously findings about p21 reported by Bloom and coworkers (43). The EGFP tag contained lysine and it could at least theoretically be ubiquitinylated at the N-terminus. Some authors considered that a large tag caused a steric hindrance that interferes with unfolding by and/or translocation into the proteasome independent of the presence of the ubiquitin chain (26). On the basis of these results, we concluded that exogenous ATF5 was degraded via N-terminal ubiquitinylation. However, it was observed that there was difference between the degradation of exogenous ATF5 and endogenous ATF5 in the presence of cisplatin, suggesting that the endogenous ATF5 might have varied method of degradation. For several substrates such MyoD, P21, which has been reported that the first ubiquitin is conjugated to the N-terminal, the internal lysines also play a role in modulating their stability (26,28). Several substrates are degraded in a manner that is ubiquitination-independent yet proteasome-dependent. Thus, the modulatory
pathway of endogenous ATF5 might be complex and multipath need more research.

In summary, the present studies demonstrated that ATF5 was degraded by proteasome pathway. Cisplatin prevented the degradation of ATF5, at least partly through promoting the translocation of Cdc34 from the nucleus to the cytoplasm. The precise mechanisms of N-terminal ubiquitination of exogenous ATF5 protein and cisplatin-mediated Cdc34 translocation need further investigations.

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Abbreviations—ATF5, activating transcription factor 5; MG132, z-Leu-Leu-Leu-CHO; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PARP, poly ADP-ribose polymerase; Ub, ubiquitin; CHX, cycloheximide; EGFP, enhanced green fluorescence protein.

FIGURE LEGENDS

Fig. 1. Cisplatin increases ATF5 protein expression via inhibiting its ubiquitin-mediated degradation. A. The effect of cisplatin on ATF5 mRNA expression. RT-PCR analysis of ATF5 and P21 mRNA expression in HeLa cells treated with different concentration of cisplatin (+ 10 μM; ++ 20 μM) for 12 h. The expression of β-Actin mRNA was used as an internal control. B. Cisplatin-induced ATF5 protein expression was correlated with proteasome pathway. Apoptosis was induced by different concentration of cisplatin (+ 10 μM; ++ 20 μM, 12 h) in HeLa cells pretreated without (lanes 1-3) or with (lanes 4-6) MG132. Western blot analysis was performed using antibodies against ATF5 and PARP. GAPDH expression was served as a loading control. The same experiments were performed in HEK293 cells (C) and MDA231 cells (D). E. HeLa cells were co-transfected with control vector (lanes 1, 2, 5 and 6) or pcDNA3.1-ATF5-myc (lanes 3, 4, 7 and 8) and HA-Ub. 36 h after transfection, the cells were treated with (lanes 2 and 4) or without (lanes 1 and 3) MG132 for 12 h. Cell extracts were prepared as described in ‘EXPERIMENTAL PROCEDURES’ and myc-tagged ATF5 was immunoprecipitated using anti-myc antibody. The immune complexes were analyzed by immunoblotting using anti-HA antibody (left panel). The same sample was analyzed by immunoblotting using anti-ATF5 antibody (right panel). The ubiquitin-conjugated form of ATF5 was indicated. F. Cisplatin inhibited the ubiquitination of ATF5 in vivo. Ubiquitination assay of endogenous ATF5 was performed in HeLa cells treated (lanes 2 and 4) or
untreated (lanes 1 and 3) with cisplatin in the absence (lanes 1 and 2) or in the presence (lanes 3 and 4) of MG132 (25 μM). Endogenous ATF5 was immunoprecipitated using anti-ATF5 rabbit polyclonal antibody and the ubiquitin-conjugated form of ATF5 was detected by immunoblotting using anti-Ub antibody (left panel) or anti-ATF5 goat polyclonal antibody (right panel).

**Fig. 2.** The effects of cisplatin on the translocation of Cdc34 and the interaction between ATF5 and Cdc34. A. The effect of Cdc34 overexpression on endogenous ATF5 expression. HeLa cells were transiently transfected with control or pcDNA3.0-Cdc34 plasmids (+ 1 μg; ++ 2 μg). 48 h after transfection, endogenous ATF5 expression was analyzed by immunoblotting using anti-ATF5 antibody. GAPDH expression served as a loading control. B. Intracellular localization of Cdc34 and ATF5 in response to cisplatin treatment. HeLa cells treated with or without cisplatin (20 μM, 12 h) were stained using anti-Cdc34 antibody and anti-ATF5 antibody and co-staining of nucleus was performed using Hochest33258. The images were captured with a confocal microscope and software provided by Carl Zeiss. C. Cisplatin reduced the interaction between Cdc34 and ATF5 in vivo. The whole cell lysates from HeLa cells treated without (lanes 1 and 2) or with (lanes 3 and 4) cisplatin (20 μM 12 h) were immunoprecipitated with anti-Cdc34 antibody (lanes 2 and 4) or control normal IgG (lanes 1 and 3). Immunoprecipitated complex were analyzed by immunoblotting using anti-ATF5 antibody or anti-Cdc34 antibody (upper panel). 5% whole cell lysates (WCL) were used as input (lower panel). D. The cytoplasm (Cyto) and nuclear (Nu) proteins were isolated from HeLa cells treated with different concentration of cisplatin (+ 10 μM; ++ 20 μM, 12 h). The expression of endogenous Cdc34 and ATF5 in the cytoplasm (lanes 1-3) and the nucleus (lanes 4-6) were analyzed by western blot. β-Tubulin and Histone were used for the internal control of the cytoplasmic and nuclear fractions as well as to determine possible cross-contamination between the fractions. E. The half-life of endogenous ATF5 in the nucleus was prolonged by cisplatin. The cytoplasm and the nucleus protein were isolated from HeLa cells treated without or with cisplatin. The half-life of endogenous ATF5 was determined as described in ‘EXPERIMENTAL PROCEDURES’ (upper panel). The half-life of endogenous ATF5 in the nucleus was prolonged from approximately 45 min to 80 min by cisplatin treatment (lower panel). The graph represented ATF5/Histone ratio as measured by densitometry. The value of ATF5/Histone at 0 h was taken as 100%. F. Cisplatin inhibited the ubiquitination of ATF5 in vitro. ATF5 protein purified from bacteria BL21 using nickel agarose was used as a substrate in an in vitro ubiquitination assay in the presence of Flag-ubiquitin and 50 μg of nuclear fractions from HeLa cells which was pretreated without (lanes 1, 3, 5 and 7) or with (lanes 2, 4, 6 and 8) cisplatin (10 μM, 12 h). The reactions were carried out with an ATP-regenerating system (lanes 5-8) or an ATP-depleting condition (lanes 1-4) as described in ‘EXPERIMENTAL PROCEDURES’. The ubiquitin-conjugated form of ATF5 was detected using
anti-Flag antibody (upper panel) or anti-ATF5 antibody (lower panel). The contribution of Cdc34 in cisplatin-inhibited ATF5 ubiquitinylation. The ubiquitination assay in vitro was performed as described in Fig. in the absence (lanes 1, 3) or in the presence (lanes 2, 4) of purified Cdc34 protein (0.2 μM). The ubiquitin-conjugated form of ATF5 was detected using anti-Flag antibody (upper panel) or anti-ATF5 antibody (lower panel).

**Fig. 3.** The N-terminal 204 amino acids of ATF5 contain the major domain for its degradation. A. Schematic representation of ATF5 and its deletion mutants used in the following studies. B. Effect of MG132 on expression of ATF5 full length and its deletion mutants. pcDNA3.1-ATF5-myc or ATF5 deletion mutants were transiently transfected into HeLa cells. 36 h after transfection, the cells were treated for 12 h with (lanes 2, 4, 6, 8, 10 and 12) or without (lanes 1, 3, 5, 7, 9 and 11) MG132 (25 μM). The cell lysates were analyzed by immunoblotting using anti-myc antibody. C. AD domain was the major region for ATF5 degradation. pcDNA3.1-ATF5-myc (a), ATF5 ΔC2 (AD domain) (b) or ATF5 ΔN3 (DBD domain) (c) was transiently transfected into HeLa cells. 24 h after transfection, apoptosis was induced by cisplatin pretreated with (lanes 3 and 4) or without MG132 (lanes 1 and 2) for 1 h. The expression of ATF5 and its mutants were analyzed by immunoblotting using anti-myc antibody. In B and C, 1 μg pEGFPN3 empty vector was co-transfected into HeLa cells which was used as a control of transfection efficiency. GAPDH was served as a loading control.

**Fig. 4.** In vivo degradation of ATF5 by the proteasome is independent of lysine residues. A. Schematic representation of ATF5 and its lysine mutants used in the following studies (left panel). pEGFPN3-ATF5 or its lysine mutants (Mut1, Mut2 or Mut3) were transiently transfected into HeLa cells. 36 h after transfection, the cells were treated for 12 h with (lanes 2, 4, 6 and 8) or without (lanes 1, 3, 5 and 7) MG132. The cell lysates were analyzed by immunoblotting with anti-EGFP antibody (right panel). B. The half-lives of ATF5 and its lysine mutants (Mut1, Mut2 and Mut3) were determined as described in ‘EXPERIMENTAL PROCEDURES’ (left panel). The half-lives of ATF5 and its lysine mutants were approximately 45 min which could be prolong to 90 min by MG132. The graph represented ATF5/GAPDH ratio level as measured by densitometry (right panel). The value of ATF5/GAPDH at 0 h was taken as 100%. C. The structural schematics of pEGFPN3-ATF5 ΔC2 and its lysine mutants (Mut4, Mut5 and Mut6) (upper panel). pEGFPN3-ATF5 ΔC2 or its lysine mutants were transiently transfected into HeLa cells. 36 h after transfection, the cells were treated for 12 h with (lanes 2, 4, 6 and 8) or without (lanes 1, 3, 5 and 7) MG132. The cell lysates were analyzed by immunoblotting using anti-EGFP antibody (lower panel). D. pEGFPN3-ATF5 WT, Mut3, ΔC2, Mut6 or control vector were co-transfected into HeLa cells with HA-Ub. 36 h after transfection, cells were treated with MG132 for 12 h, and the cell
extracts were prepared as described in ‘EXPERIMENTAL PROCEDURES’ and EGFP-tagged ATF5 was immunoprecipitated using anti-EGFP antibody. The immune complexes were analyzed by immunoblotting using anti-HA antibody (upper panel). The cell lysates were analyzed by immunoblotting using anti-EGFP antibody (lower panel).

**Fig. 5.** Addition of a large N-terminal tag stabilizes ATF5 but does not block its ubiquitinylation. A. Addition of a large N-terminal tag increased ATF5 protein expression. (a) Schematic representation of the constructs used in these experiments. C-terminally or N-terminally EGFP-tagged ATF5 was designated as ATF5-EGFP (pEGFPN3-ATF5) or EGFP-ATF5 (pcDNA3.0-EGFP-ATF5). (b) The same amount plasmids of empty vector, ATF5-EGFP or EGFP-ATF5 were transiently transfected into HeLa cells. 48 h after transfection, cells were observed by fluorescence microscopy. (c) The expression efficiency of EGFP was determined by flow cytometry. The EGFP expression efficiency of empty vector, ATF-EGFP and EGFP-ATF5 was found to be 92%, 17% and 58% respectively. (d) The expression of ATF5-EGFP (lane 2) or EGFP-ATF5 (lane 3) was detected using anti-EGFP antibody. B. The half-life of ATF5-EGFP or EGFP-ATF5 was determined as described in ‘EXPERIMENTAL PROCEDURES’. The half-life of ATF5-EGFP was approximately 45 min. The half-life of EGFP-ATF5 was approximately 120 min. The graph represented ATF5/GAPDH ratio level as measured by densitometry. The value of ATF5/GAPDH at 0 h was taken as 100%. C. N-terminal EGFP tag did not block the ubiquitinylation of ATF5. HA-Ub was transiently co-transfected into HeLa cells with and ATF5-EGFP (lanes 1 and 2) or EGFP-ATF5 (lanes 3 and 4). 36 h after transfection, the cells were treated with (lanes 2 and 4) or without (lanes 1 and 3) MG132 for 12 h. Cell extracts were prepared as described in ‘EXPERIMENTAL PROCEDURES’ and EGFP-tagged ATF5 was immunoprecipitated using anti-EGFP antibody. The immune complexes were analyzed by immunoblotting using anti-HA antibody (upper panel). The cell lysates were analyzed by immunoblotting using anti-EGFP antibody (lower panel). The ubiquitin-conjugated form of ATF5 was indicated.

**Fig. 6.** ATF5 is ubiquitinylated at the N-terminus. HA-ATF5 and HA-Xa-ATF5 with an N-terminal HA-tag followed by a Factor Xa cleavage site were purified from bacteria BL21 using nickel agarose. The HA-ATF5 or HA-Xa-ATF5 protein was used as a substrate in ubiquitination assay in the presence of 50 μg of total lysate from HeLa cells in vitro. After the reaction, the products were incubated with (lanes 2, 4 and 6) or without (lanes 1, 3 and 5) Factor Xa for 16 h at 25 °C. The ubiquitin-conjugated form of ATF5 was detected by immunoblotting using anti-Ub antibody (upper panel) and anti-ATF5 antibody (lower panel).
Figure 5

A

(a) ATF5-EGFP

(b) Vector ATF5-EGFP EGFP-ATF5

(d) ATF5-EGFP  - + -

EGFP-ATF5  - - +

ATF5-EGFP  

EGFP-ATF5  

GAPDH

(c) Expression efficiency (%)

Vector ATF5-EGFP EGFP-ATF5

B

CHX(h) 0 0.5 1 1.5 2

ATF5-EGFP

GAPDH

EGFP-ATF5

GAPDH

C

ATF5-EGFP + + - -

EGFP-ATF5 - - + +

HA-Ub + + + +

MG132 - + - +

conj.

ATF5

Lysates

Lane: 1 2 3 4

IP:α-EGFP

IB:α-HA

IB:α-EGFP
Figure 6

|        | Lane: 1 | 2 | 3 | 4 | 5 | 6 |
|--------|---------|---|---|---|---|---|
| HA-ATF5-his | − | − | + | + | − | − |
| HA-Xa-ATF5-his | − | − | − | − | + | + |
| Factor Xa | − | + | − | + | − | + |

**IB:** α-Ub

**IB:** α-ATF5

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