Arsenite-induced Cdc25C degradation is through the KEN-box and ubiquitin–proteasome pathway

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Arsenite is a known human carcinogen that induces tumorigenesis through either a genotoxic or an epigenetic mechanism. In this study, the effect of arsenite on cell cycle regulation and the mechanisms that contribute to this effect were investigated. Treatment of the cells with arsenite suppressed cell proliferation and reduced cell viability in a dose- or time-dependent manner. Analysis of cell cycle profile and cell cycle regulatory proteins indicated that arsenite arrested the cell cycle at G_2/M phase, partially through induction of cell division cycle 25 (Cdc25) isoform C (Cdc25C) degradation via ubiquitin–proteasome pathways. Mutation of the putative KEN box within the region 151 to 157 of human Cdc25C or treatment of the cells with a peptide competitor encompassing the KEN box partially inhibited arsenite-induced ubiquitination of Cdc25C. Thus, these results indicate that the regulated ubiquitination of Cdc25C may be involved in the arsenite-induced proteolytic down-regulation of Cdc25C activity in the G_2/M phase of the cell cycle and suggest a link between cell cycle and the carcinogenic effects of arsenite.

Environmental and occupational exposure to high levels of inorganic arsenic, mainly trivalent arsenic [arsenite, As(III)], has been linked to a number of human diseases, including dermatosis, diabetes mellitus, and cardiovascular disorders, as well as cancers of the lung, bladder, liver, kidney, and skin (1). Experimental data indicate that arsenite is only weakly active or, more frequently, inactive in bacterial and mammalian cell mutation assays (2). Therefore, the carcinogenic effect of arsenic may be largely based on its epigenetic mechanisms by targeting several cellular proteins, principally through sulfhydyl interactions and oxidative stress. The cellular proteins possibly targeted by arsenite include signaling molecules, transcription factors, the cytoskeletal system, and the pathways of cell cycle regulation.

The regulation of cell cycle is frequently altered in human cancer cells (3). Central players of cell cycle regulation are the cyclin-dependent kinases (Cdks) and their upstream cell division cycle 25 (Cdc25) dual phosphatases that dephosphorylate and activate the Cdks (4). In mammalian cells, three Cdc25 isoforms, named Cdc25A, B, and C, have been cloned and functionally identified. Cdc25A has been considered to be a critical regulator of the G_1/S phase transition, whereas Cdc25B and C are predominantly expressed in G_2 and M phases to regulate entry of cells into M phase by dephosphorylating and activating the Cdc2(Cdk1)/cyclin B complex (5). A variety of protein kinases phosphorylate Cdc25C at S216 in response to DNA damage and replication block. Impairment of this checkpoint mechanism will cause rearrangement, amplification, and loss of chromosomes, events that are causally associated with cancer.

In the last few years, inducible degradation of cell cycle-regulatory proteins, especially the mitotic B type cyclins, by the ubiquitin–proteasome system has emerged as a process that is as critical as reversible phosphorylation in the control of cell cycle progression (6). Attachment of ubiquitin to a lysine residue on a substrate protein involves a cascade of ubiquitin transfer reactions and requires ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme E2, and ubiquitin-ligase E3 (7). Ubiquitin-tagged proteins are recognized and degraded by the 26S proteasome, a multisubunit protease complex specific for multiubiquitinated substrates (7). At present, at least six types of ubiquitin ligase complexes have been identified. These ligase complexes include the anaphase promoting complex (APC), the Skp1-cullin-F-box complex, the Von Hippel–Lindau Syndrome gene protein-elongin B-elongin complex, homologous to E6-AP C terminus (HECT) domain proteins, ring-finger E3s, and smurfs, among which the APC appears to be the most important ubiquitin ligase to mediate the inducible degradation of cell cycle proteins (6). By destroying anaphase inhibitory proteins and mitotic cyclins, the APC triggers the separation of sister chromatids and attenuates Cdk kinase activity, respectively, which are necessary for cytokinesis and for the next round of genome replication. The ubiquitination and degradation of cyclin B1 and other APC targets depend on either a destruction box (D box) composed of the sequence R-X-X-L-X-X-X-X-N found in the N terminus of the proteins (8) or a KEN box composed of the sequence K-E-N-X-X-X-N/D located in the N or C terminus of the proteins (9).

Previous studies by our group indicated that arsenite was a potent inducer of G_2/M phase cell cycle arrest, partially through the p53-independent but c-Jun-N-terminal kinase-dependent induction of GADD45α, an important inhibitor for Cdc2/cyclin B complex (10, 11). In this report, we extend this observation and demonstrate an additional mechanism that meditates arsenite-induced G_2/M phase cell cycle arrest: an inducible degradation of Cdc25C. We provide evidence that the arsenite-induced Cdc25C degradation is through the ubiquitin–proteasome pathway. We also demonstrate that a KEN box within the Cdc25C protein is accountable for the ubiquitination and degradation of this protein in the cells in response to arsenite.

Materials and Methods

Cell Culture. The human bronchial epithelial cell line, BEAS-2B, from American Type Culture Collection, was cultured as previously described (10). To determine the rate of cell proliferation, cells were seeded in six-well tissue culture plates at $\approx 3 \times 10^4$ cells per well with or without arsenite. At each time point, wells in triplicate were rinsed with PBS twice to remove dead cells and debris. Live cells on the plates were trypsinized and counted by using a hemocytometer. For the trypan blue dye exclusion assay, all cells were collected separately from each well at the time indicated. The cells were stained with trypan blue (Sigma) for 10 min. The stained (dead) and unstained (live) cells were counted. For flow cytometry, cells were labeled with propidium iodide and subjected to cell cycle profile analysis, as described previously (10).

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Abbreviations: Cdk, cyclin-dependent kinases; Cdc25, cell division cycle 25; APC, anaphase promoting complex; D box, destruction box; Chk, checkpoint kinase.

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Reagents. Arsenite was purchased from Aldrich. Antibodies against human Cdc25A, Cdc25B, Cdc25C, p55Cdc20, and ubiquitin were from Santa Cruz Biotechnology or Upstate Biotechnology (Lake Placid, NY). Enhanced chemiluminescence Western blotting detection reagents were from Amersham Pharmacia Life Science.

Tat-Cdc25C Peptides. A modified 11-mer Tat transduction domain (single-letter code, YGRK/AK/ARRQRRR) was used as a delivery sequence for wild-type Cdc25C peptide 141–160 encompassing a KEN box or a mutated Cdc25C peptide in which the KEN box was replaced with an AAA sequence. Because of a concern that the presence of lysine residues in the delivery peptide derived from HIV Tat protein would interfere with ubiquitination of intracellular proteins, two lysine residues (underlined in the indicated Tat-transduction domain) were replaced with alanines in the delivery peptide. The Tat-Cdc25C-KEN peptide contains a sequence of YGRAARRQRRR-G-RDAMCSSSAN**KEN**DNG**N**LVD. The Tat-Cdc25C-AAA peptide contains a sequence of YGRAARRQRRR-G-RDAMCSSAN**AAA**DNG***N***LVD. FITC-labeled Tat peptide was generated by fluorescein labeling. All peptides were purified by gel filtration through fast protein liquid chromatography. The purity of peptides was around 95%.

Immunoprecipitation and Western Blot. Cells were trypsinized, washed twice with ice-cold PBS, and harvested in cell lysis buffer containing the protease inhibitors antipain (10 μg/ml), pepstatin A (10 μg/ml), and leupeptin (10 μg/ml). Cells were lysed by sonication, and the protein supernatant was clarified by centrifugation at 25,000 $\times$ *g* for 5 min at 4°C. Protein concentration was determined by the Bradford protein assay. Immunoprecipitation was performed by adding anti-Cdc25C, antiubiquitin, or anti-Myc-tag antibody. Western blot analysis was performed by using the following primary antibodies: anti-checkpoint kinase 1 (Chk1) polyclonal antibody, anti-Cdc25A polyclonal antibody, anti-Cdc25B polyclonal antibody, anti-Cdc25C polyclonal antibody, anti-p55Cdc20, and antiubiquitin polyclonal antibody.

Site-Directed Mutation. Point mutation was introduced into plasmid pCMV-Myc-Cdc25C by using the QuickChange Site-Directed Mutagenesis kit (Stratagene). To generate a K to A mutation for the first lysine (K) residue in the KEN box of Cdc25C, the coding sequence AAA for lysine was changed to GCA by using a primer set encompassing the coding sequence of KEN box.

Results

Arsenite Treatment Causes Proliferative Arrest in BEAS-2B Cells. As described earlier (12, 13), arsenite is a potent inducer of stress responses of the cell, which possibly affect cell proliferation or differentiation. To determine the further effect of arsenite on cell growth, the cells were visually inspected daily after 5 or 10 μM arsenite treatment. Control cells showed no decrease in cell density over a 5-day period. In contrast, cells treated with arsenite showed a progressive decrease of cell density because of the detachment of dead cells. Treatment of cells with arsenite suppressed the proliferation of BEAS-2B cells that had been proliferating asynchronously, as indicated by cell numbers (Fig. 1*A*), whereas mock treatment had no effect (Fig. 1*A*). The reduced growth of the cells treated with arsenite appeared to be due to an increased rate of cell death. Trypan blue dye exclusion analysis indicated a pronounced decrease in cell viability beginning at day 2, which became prominent by day 5 (Fig. 1*B*). Treatment of the cells with arsenite for 2 days promoted the detachment of the cells. More than 90% of detached cells were stained by trypan blue.

Arsenite Causes Cell-Cycle Arrest in G_2/M Phase. To explore further the inhibitory effect of arsenite in human bronchial epithelial cell proliferation, we analyzed the cell cycle profile in the absence or presence of arsenite for 48 h by measuring the DNA content by flow cytometry analysis of propidium-iodide-stained cells (Fig. 2*A*). Because previous studies showed that arsenite was able to increase the number of asynchronized cells in both S phase and G_2/M phase, we speculated that the arsenite effect might be more pronounced if cells were synchronized in G_0 phase through prestarvation in serum-free medium for 36 h. The cells were cultured in the absence or presence of arsenite in serum-free medium for an additional 48 h after prestarvation. Note that serum starvation for an additional 48 h resulted in more than 60% of cells accumulating in G_0/G_1 phase. In contrast, over 50% of the cells were in G_2/M phase after 48 h of sustained treatment of the cells with 5–10 μM arsenite. An average cell cycle distribution of three independent experiments was presented in Fig. 2*B*, which indicated that the majority of the cells were in G_0/G_1 in the absence of arsenite. Arsenite treatment resulted in a dose-dependent decrease of G_0/G_1 cells concomitant with an increase of G_2/M cells. In addition, a small (statistically insignificant) increase of S phase cells in response to arsenite was observed. This result suggests that the S phase entry of the cells was normal in response to arsenite, but the exit from S phase might be partially impaired.

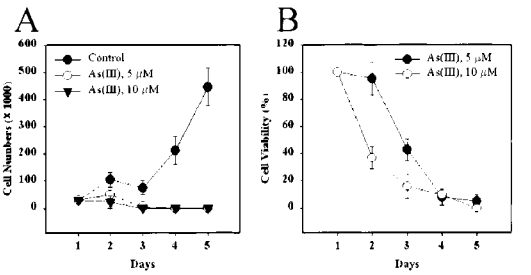

Fig. 1. Arsenite inhibits cell proliferation. (*A*) Aliquots containing 3×10^4 cells were seeded into six-well tissue culture plates in triplicate and cultured in the absence or presence of 5 or 10 μM arsenite in 5% FBS for the indicated number of days. Cells were counted at each time point. The results represent the average ± SD of triplicate analyses. (*B*) Time course analysis of cell viability. Cells seeded in six-well tissue culture plates were incubated in the absence or presence of arsenite for a varying number of days, and the viable cell number was counted by using the trypan blue exclusion method. Results are shown as means ± SD obtained from three independent experiments.

Arsenite-Induced G_2/M Arrest Involves Cdc25C Degradation. We presumed that arsenite-induced G_2/M arrest could be attributed to the activation of a checkpoint control. Gross overproduction of checkpoint proteins is rarely seen in normal cells because of the powerful growth-suppressing capabilities of these proteins. However, increased checkpoint protein expression or activity can often be observed after the treatment of the cells with DNA-damaging agents or other stress inducers. A number of checkpoint proteins, including GADD45α, p53, Chk1, and Chk2, can play a role in

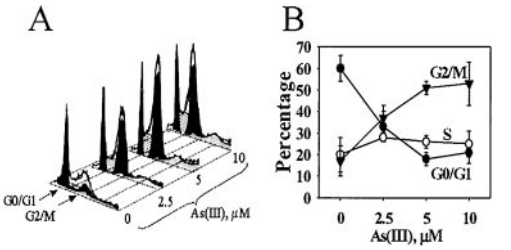

Fig. 2. Arsenite arrests cells at the G_2/M phase. (*A*) The cells were synchronized by serum deprivation. These cells were then left untreated or treated with various doses of arsenite in serum-free medium as indicated for 48 h. Cell cycle profile was determined by flow cytometry. (*B*) The average of cell cycle distributions was determined by three independent analyses.

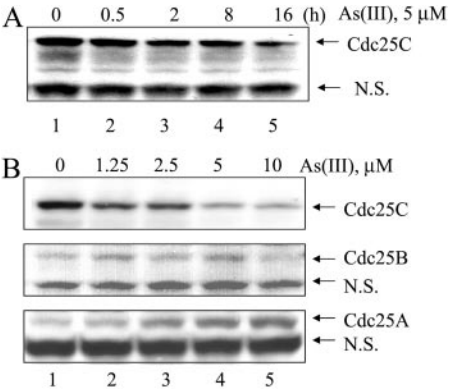

Fig. 3. The cell cycle-arresting effect of arsenite involves Cdc25C degradation. (*A*) Cells were synchronized at G_0/G_1 phase by serum deprivation and treated with 5 μM arsenite for the indicated times before extract preparation. Extracts were subjected to Western blot by using the anti-Cdc25C polyclonal antibody. (*B*) Cells were treated with indicated doses of arsenite for 12 h and subjected to Western blot by using antibodies against Cdc25C (*Top*), Cdc25B (*Middle*), and Cdc25A (*Bottom*). N.S., nonspecific bands that indicate equal loading of proteins.

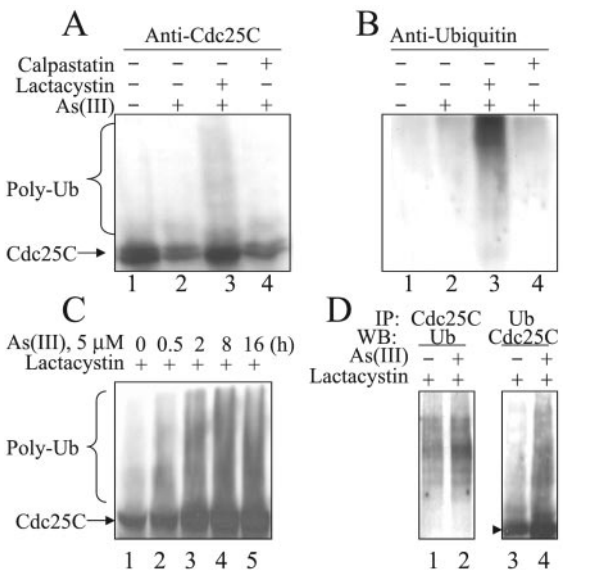

Fig. 4. Arsenite-induced Cdc25C degradation is mediated by a ubiquitin–proteasome pathway. (*A*) Cells were pretreated with vehicle solution DMSO (lanes 1 and 2), 20 μM lactacystin (lane 3), or 100 μM calpastatin peptide (lane 4) for 2 h. Then the cells were either left untreated (lane 1) or treated with 5 μM arsenite (lanes 2–4) for an additional 12 h. Cell lysates were prepared and subjected to Western blot for Cdc25C protein. PolyUb, polyubiquitin chains. (*B*) The Western blot membrane in *A* was stripped and reprobed with anti-ubiquitin antibody. (*C*) Time series of Cdc25C ubiquitination induced by arsenite. (*D*) Cells were pretreated with lactacystin for 2 h and then either left untreated or treated with 5 μM arsenite for 12 h. Normalized amounts of lysate were processed for immunoprecipitation (IP) by using either 20 μg of anti-Cdc25C antibody followed by Western blot with the antiubiquitin antibody (lanes 1 and 2) or vice versa (lanes 3 and 4).

regulating the G_2/M phase checkpoint. Because the function of p53 is deficient in BEAS-2B cells as previously reported (14), other checkpoint proteins that could account for arsenite-induced G_2/M phase arrest were investigated.

Our previous work demonstrated that arsenite was able to induce GADD45α expression, especially in cells where NF-κB signaling was inhibited (10, 11). In cells where NF-κB signaling was normal, the induction of GADD45α by arsenite was less potent, whereas the G_2/M arresting effect of arsenite was still substantial, indicating the involvement of other checkpoint protein(s). We assumed that Chk1 and Chk2 might account for this G_2/M arrest in addition to GADD45α. In response to DNA damage or other stress signals, activated Chk1 and Chk2 phosphorylate S216 of Cdc25C, causing it to bind to 14–3-3 proteins, which retain Cdc25C in the cytoplasm where it cannot dephosphorylate and activate Cdc2. As a consequence, cells will be arrested in G_2/M phase. Functional induction of Chk1 and Chk2 can be assessed by examining the protein levels and mobility of Cdc25C. When cells were cultured in the absence of arsenite, a steady level of Cdc25C was observed both in the continually serum-starved condition and in the presence of serum (5%) (data not shown). A rapid degradation of Cdc25C was observed after incubation of cells with 5 μM arsenite for different time periods (Fig. 3*A*). The earliest degradation of Cdc25C occurs at 30 min of arsenite stimulation. The arsenite-induced degradation of Cdc25C occurs in a dose-dependent manner (Fig. 3*B Top*). Because nonspecific protein degradation occurs when cells undergo cytotoxic responses, we determined whether the degradation of Cdc25C induced by arsenite represented a cytotoxic reaction. Thus, the protein levels of two other Cdc25 family proteins, Cdc25A and Cdc25B, were examined. Whereas a marginal degradation pattern of Cdc25B was observed in the cells treated with arsenite (Fig. 3*B Middle*), a dose-dependent increase of Cdc25A was noted (Fig. 3*B Bottom*). In contrast to the regulation of G_2/M by Cdc25B and Cdc25C, the major function of Cdc25A is the regulation of the G_1/S phase transition. The increased Cdc25A protein not only explains the observed small increase in the number of cells in S phase (Fig. 2 *A* and *B*) but also excludes the possibility that nonspecific cytotoxicity caused the observed degradation of Cdc25C in response to arsenite. Indeed, cytotoxicity did not occur until 24 h after the cells were treated with arsenite (Fig. 1).

Arsenite-Induced Cdc25C Degradation Depends on Ubiquitin–Proteasome. Emerging evidence indicates that ubiquitin–proteasome participates in the degradation of several cell cycle regulatory proteins, such as cyclin A, cyclin B, Cdc25A, Cdc25B, Cdc6, geminin, and securin/pituitary tumor-transforming gene (PTTG) (6). To test whether the degradation of Cdc25C induced by arsenite is mediated through ubiquitin–proteasome or other systems, cells were preincubated with a specific proteasome inhibitor, lactacystin, or a calpain inhibitor, calpastatin. Analysis of Cdc25C by immunoblotting of lysates from cells pretreated with lactacystin revealed that the degradation of Cdc25C protein induced by arsenite was inhibited in the presence of lactacystin (Fig. 4*A*; compare lane 2 with lane 3). In contrast, calpastatin peptide, a specific cell-permeable inhibitor for calpains, had no effect on arsenite-induced Cdc25C degradation (Fig. 4*A*, lane 4). Fig. 4*A* also shows that multiple conjugates with higher molecular weights than Cdc25C itself can be recognized by the anti-Cdc25C antibody. To test whether some of these conjugates could form from polyubiquitin modification on Cdc25C, we monitored the possible presence of ubiquitin conjugation by reprobing the stripped membrane with antiubiquitin antibody. Results depicted in Fig. 4*B* indicate that high molecular-weight conjugates can be recognized by the antibody against ubiquitin. The ubiquitination of Cdc25C induced by arsenite appears to be very rapid, because a time-course study indicates that the ubiquitination of Cdc25C occurred after as little as 30 min of arsenite stimulation in the presence of lactacystin (Fig. 4*C*). Thus, these results suggest that arsenite-induced degradation of Cdc25C is through the ubiquitin–proteasome system.

To confirm further the ubiquitination of Cdc25C in response to arsenite, we immunoprecipitated Cdc25C or ubiquitin from the lysates of cells treated with arsenite in the presence of lactacystin and analyzed the samples by immunoblotting. Ubiquitin conjugation with Cdc25C was confirmed by a reciprocal experiment in which proteins were first immunoprecipitated with Cdc25C anti-

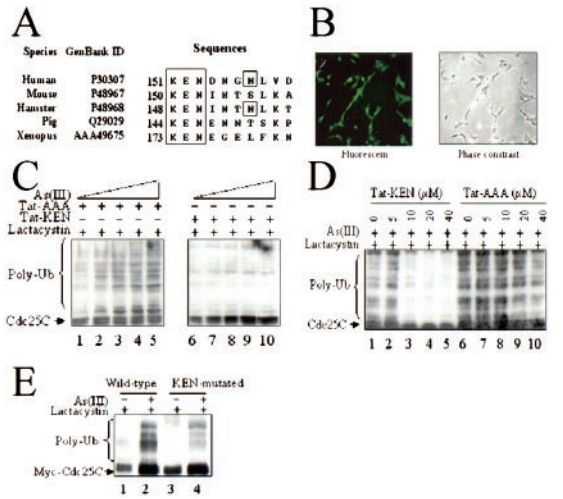

Fig. 5. Tat-Cdc25C-KEN peptide inhibits Cdc25C ubiquitination induced by arsenite. (*A*) Alignment of KEN box of several vertebrate Cdc25C proteins. (*B*) BEAS-2B cells were treated with 20 μM Tat-FITC. After 2 h, cells were fixed and observed by using fluorescein microscopy (*Left*) or phase-contrast microscopy (*Right*). (*C*) Cells were pretreated with 20 μM lactacystin and 20 μM Tat-AAA peptide (lanes 1–5) or 20 μM Tat-KEN peptide (lanes 6–10) for 2 h. The cells were then treated with increasing concentrations of arsenite (0, 1.25, 2.5, 5, and 10 μM) for 12 h. Cell lysates were subjected to immunoprecipitation by using anti-Cdc25C antibody. (*D*) Cells were pretreated with 20 μM lactacystin along with various concentrations of Tat-KEN-box peptide (lanes 1–5) or control peptide (lanes 6–10) for 2 h. Cdc25C ubiquitination was determined by immunoprecipitation after treatment of these cells with 10 μM arsenite for an additional 12 h. (*E*) BEAS-2B cells were transfected with either pCMV-Myc-tagged-Cdc25C (wild type; lanes 1 and 2) or pCMV-Myc-Cdc25C-KEN mutant (KEN mutated; lanes 3 and 4). The transfected cells were either untreated or treated with 5 μM arsenite for an additional 12 h. Normalized amounts of lysate were subjected to Western blot by using anti-Myc tag antibody.

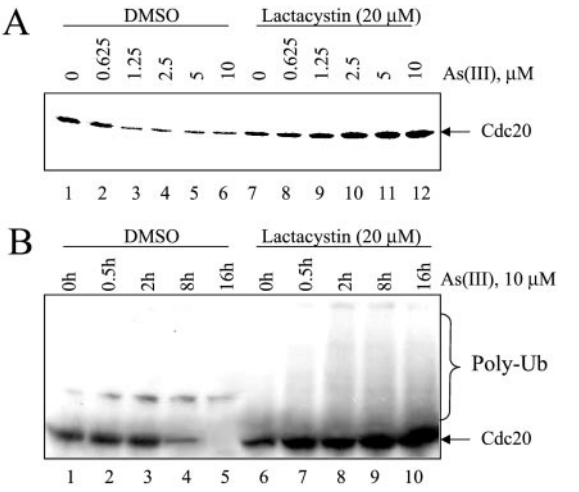

Fig. 6. Arsenite induces ubiquitination and degradation of Cdc20. (*A*) Cells were pretreated with 20 μM lactacystin (lanes 7–12) or vehicle solution (DMSO, lanes 1–6). After 2 h, the cells were further treated with the indicated concentrations of arsenite for an additional 12 h. The protein level of Cdc20 was determined by Western blot by using anti-p55Cdc20 antibody. (*B*) The pretreatment of the cells is the same as in *A*. The time-course study of Cdc20 ubiquitination was performed after the treatment of the cells with 10 μM arsenite for the indicated times. To visualize the ubiquitination of Cdc20, the exposure of the x-ray film was prolonged.

body and analyzed by Western blot with the ubiquitin antibody (Fig. 4*D*, lanes 1 and 2) or vice versa (Fig. 4*D*, lanes 3 and 4).

Arsenite-Induced Cdc25C Degradation Requires KEN Box. The ubiquitin–proteasome-mediated degradation of many cell cycle regulatory proteins requires either a D box composed of the sequence R-X-X-L-X-X-X-X-N or a KEN box with amino acid sequence K-E-N-X-X-X-N/D (9, 15, 16). Analysis of the amino acid composition of the human Cdc25C revealed the absence of a typical D box in its sequence. However, there is a KEN-like structure with a sequence K-E-N-D-N-G-N in the region of 151 to 157 of Cdc25C. Sequence alignment among several vertebrate Cdc25Cs shows that all Cdc25Cs analyzed contain a roughly conserved KEN box (Fig. 5*A*). To determine whether the KEN box within the region of 151 to 157 is required for the ubiquitination of Cdc25C, we synthesized a Tat-fusion peptide containing an 11-mer Tat transduction domain fused to a 20-aa peptide encompassing the KEN box of human Cdc25C or the control sequence in which the KEN residues were replaced with AAA. We used the Tat-Cdc25C-KEN box peptide as a competitor to inhibit the ubiquitination of Cdc25C protein in response to arsenite. To monitor the efficiency of Tat fusion peptides to transduce into cells, a Tat peptide conjugated to FITC was added to the cell culture medium in a concentration of 20 μM for 2 h. Consistent with previous reports indicating the efficient cellular entry of Tat-fusion peptides (17), fluorescein microscopic analysis demonstrated that more than 95% of cells were transduced with Tat-FITC peptide (Fig. 5*B*). Microscopy at higher magnification indicates that most of Tat-FITC peptides were accumulated in the nuclei (data not shown). To determine the effect of Tat-Cdc25C-KEN box on ubiquitination, cells were pretreated with lactacystin and 20 μM Tat-Cdc25C-KEN box or control peptide. After 2 h, cells were further treated with increasing concentrations of arsenite for 12 h. Cell lysates were immunoprecipitated and analyzed for ubiquitination of Cdc25C. The results showed that the Tat-Cdc25C-KEN-box peptide but not the control peptide, KEN-box-mutated Tat-Cdc25C-AAA peptide, was capable of inhibiting the ubiquitination of Cdc25C in the cellular response to increasing concentrations of arsenite (Fig. 5*C*; compare lanes 6–10 with lanes 1–5). Alternatively, we pretreated cells with lactacystin along with increasing concentrations of Tat-KEN-box peptide or control peptide for 2 h. A decreased arsenite-induced Cdc25C ubiquitination was observed in the cells in response to the increasing concentrations of Tat-KEN-box peptide (Fig. 5*D*, lanes 1–5) but not the increasing concentrations of control peptide (Fig. 5*D*, lanes 6–10).

To ascertain directly the requirement of KEN box for the ubiquitination of Cdc25C induced by arsenite, we generated Myc-tagged Cdc25C expression vector with a mutation of KEN box (K151A). Transfection of this mutated form of Cdc25C revealed that the mutant exhibited the same mobility and expression level as the wild-type form of Cdc25C (Fig. 5*E*). However, the ubiquitination of this mutated Cdc25C in response to arsenite was reduced appreciably in the immunoprecipitation by using anti-Myc-tag antibody. The remaining ubiquitin conjugation on the K151A mutated form of Cdc25C indicates that other regions in Cdc25C protein might also contribute, although less effectively, to the arsenite-induced ubiquitination of Cdc25C.

Arsenite Induces Degradation of Cdc20. The requirement of KEN box for arsenite-induced Cdc25C degradation suggests that arsenite may target other KEN-box-containing proteins for proteasomal degradation. The degradation of Cdc20, an activator of mitotic form APC, had been previously shown to be KEN box-dependent (9). To determine whether arsenite is also capable of inducing the degradation of Cdc20, we next assayed the ubiquitination and protein levels of Cdc20 in cells treated with arsenite. As depicted in Fig. 6, arsenite induced degradation of Cdc20 in both a dose- and time-dependent manner. However, in comparison with Cdc25C (Figs. 3 and 4), both ubiquitination and degradation of Cdc20 are relatively weak in response to arsenite.

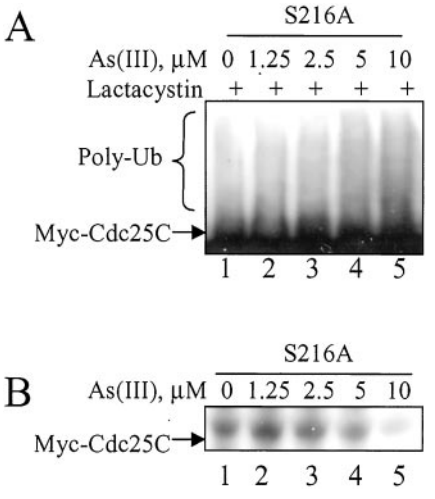

Fig. 7. Arsenite-induced Cdc25C ubiquitination and degradation is S216 phosphorylation independent. (*A*) Cells transfected with mutated Cdc25C (S216A) were treated with increasing concentrations of arsenite in the presence of 20 μM lactacystin for 12 h. Cell lysates were prepared for Western blot by using anti-Myc-tag antibody. (*B*) Transfected cells treated with arsenite in the absence of lactacystin and subjected to Western blot by using anti-Myc-tag antibody.

S216 Phosphorylation of Cdc25C Is Not Involved in Arsenite-Induced Cdc25C Degradation. It has been well documented that stress signals activate a subset of cellular kinases, such as mitogen-activated protein kinase p38 and Chk1, that phosphorylate S216 of Cdc25C (4, 18). Modification of Cdc25C by S216 phosphorylation regulates the binding of 14–3-3 proteins that sequester Cdc25C in the cytoplasm. It has been shown that phosphorylation of Cdc25A by Chk1 is an essential step for the ubiquitin–proteasome-mediated degradation of Cdc25A in the cellular response to DNA-damaging agents (19). To determine whether phosphorylation of S216 affects the ubiquitination and degradation of Cdc25C induced by arsenite, we transfected a Myc-tagged Cdc25C with S216A mutation. As indicated in Fig. 7 *A* and *B*, the mutation of S216 had no appreciable effect on the ubiquitination or degradation of Cdc25C protein in the cellular response to arsenite. Our previous studies showed a substantial activation of mitogen-activated protein kinases (MAPK), i.e., Erk, c-Jun N-terminal kinase (JNK), and p38, by arsenite (10, 11). A recent study by Bulavin *et al.* (18) suggested that genotoxic activation of p38 was responsible for the S216 phosphorylation of Cdc25C and subsequent G_2/M cell cycle arrest. To determine whether activation of MAPK contributes to arsenite-induced Cdc25C degradation, cells were preincubated with an Erk kinase inhibitor (PD98059), a p38 inhibitor (SB203580), or transfected with a kinase mutated SEK1 to block JNK activation. Analysis of the protein levels of Cdc25C in the cells treated with arsenite indicates that none of these inhibitors could restore the steady level of Cdc25C (data not shown), despite their effective inhibition on arsenite-induced activation of Erk, JNK, and p38, as previously described (10, 11).

Discussion

Although arsenite is a known human carcinogen, little attention has been given to the hypothesis that arsenite induces diseases through the impairment of normal cell cycle regulation. Previous work by our group and others indicated this possibility by demonstrating that arsenite was able to arrest the cell cycle at the G_2/M phase via its effects on some cell cycle regulatory molecules (10, 11). In the current study, we provide evidence that inducible degradation of Cdc25C by arsenite is also responsible for the perturbation of cell cycle transition. Moreover, our data suggest that the degradation of Cdc25C induced by arsenite is mediated by the ubiquitin–proteasome system. In addition, we identified a KEN box in the region of 151 to 157 of Cdc25C that is involved in arsenite-induced degradation of Cdc25C by the ubiquitin–proteasome system.

Cdc25C, a dual-specificity protein phosphatase that dephosphorylates and activates the critical cyclin-regulated protein kinase Cdc2 and thereby promotes passage of the cell into mitosis, has been considered as a stable protein during the whole cell cycle (20, 21). The mechanism for mitotic activation of Cdc25C is poorly understood. The majority of functional studies of Cdc25C thus far have been focused on the phosphorylation and consequent subcellular localization of Cdc25C protein (22). During interphase, the major phosphorylation site in human Cdc25C is S216 in response to the activation of Chk1 or C-TAK1, resulting in the binding of 14–3-3 proteins that retain Cdc25C in the cytoplasm (4, 23). The activity of Cdc25C can also be regulated by the binding of peptidyl-prolyl isomerase (Pin1) that regulates Cdc25C through conformational modification (24, 25). Unlike its other family members, Cdc25A and Cdc25B, that undergo rapid degradation by the ubiquitin–proteasome system either in response to certain stress signals or during cell cycle progression (19, 26), there is no established evidence for Cdc25C degradation by ubiquitin–proteasome, either under the conditions of stress or during cell cycle transition.

Regulated ubiquitin–proteasome-mediated protein turnover is one of the primary mechanisms governing passage through the cell cycle (6). The central part of this process is the APC, a ubiquitin ligase complex that covalently attaches the polypeptide ubiquitin to a number of cell cycle regulatory proteins (6). From a regulatory perspective, the orderly cell cycle transition can be considered as a sequence of timed association of substrate-specific APC activators that determine the destruction of certain cell cycle regulatory proteins at specific times during cell cycle progression. Two related Trp-Asp (WD) repeat-containing proteins, Cdc20 and Cdh1, have been identified as substrate-specific activators that may recruit substrates to the APC in a manner analogous to the substrate recognition subunits of Skp1-cullin-F-box-β-TrCP (27). Indeed, a recent study by Pfleger *et al.* (28) demonstrated that both Cdc20 and Cdh1 could directly bind to substrates via their N termini. Cdc20 recognizes substrates in a D box-specific manner, whereas Cdh1 recognizes substrates with either a KEN box, such as Cdc20, Nek2, and B99, or a D box, such as cyclin A (9, 15, 16, 29, 30). In addition to these cell cycle regulatory proteins, Cdh1 may also target proteins, such as SnoN, a transforming growth factor type-β signaling molecule, that are involved in other cellular function for APC-dependent ubiquitination and degradation (29, 31).

The mammalian Cdc25C proteins do not contain a typical D box but have a conserved KEN box (Fig. 5*A*). Site-directed mutation of this KEN box or the use of a KEN-box peptide as a competitor partially suppressed the ubiquitination of Cdc25C in response to arsenite, indicating the possible involvement of APC-Cdh1 in this process (Fig. 5 *D* and *E*). In addition, the evidence that arsenite also induces the ubiquitination and degradation of another KEN-box-containing protein, Cdc20 (Fig. 6), further supported this notion. The different potency of arsenite on the turnover of Cdc25C and Cdc20 (compare Fig. 6 with Fig. 5) may result from the involvement of different ubiquitination systems. Recent studies in chicken embryo indicate that Cdh1 family members have varied substrate specificities (32). Whereas chicken Cdh1C has a broad spectrum form targeting the APC substrates, including cyclin B, securin, Plk, Nek2, Ark2, Cdc20, and B99, chicken Cdh1A recognizes only Plk and chicken Cdh1B recognizes only Nek2.

The precise mechanism by which arsenite activates the ubiquitination process of Cdc25C, possibly through the activation of APC-Cdh1, remains to be investigated. In somatic cells, Cdh1

binding to the APC is cell cycle-dependent, resulting in a peak of APC-Cdh1 activity in G_1 phase (33, 34). In the S, G_2, and earlier M phases, Cdk-dependent phosphorylation of Cdh1 causes it to dissociate from the APC. In addition, the activity of APC-Cdh1 can also be negatively regulated by the spindle assembly checkpoint protein, MAD2L2 (35). In later mitosis and G_1 phase, dephosphorylation of Cdh1 by Cdc14 phosphatase liberated from nucleolus converts it to an active form (36). Consistent with this notion, several Cdh1 substrates accumulate in S and G_2 phases but disappear in G_1 phase (37). It is not clear whether arsenite is capable of modulating the Cdc14 phosphatase activity that dephosphorylates and activates Cdh1, leading to the KEN-box-dependent degradation of Cdc25C. Arsenite has been recently demonstrated as a potent inducer of the gene encoding the mitogen-activated protein kinase phosphatase 1 (MKP-1) through the phosphorylation and acetylation of histone H3 associated with the MKP-1 gene (38). It appears plausible that a similar mechanism might be involved in the effects of arsenite on Cdc14 phosphatase.

Arsenite is a known carcinogen that is associated with a number of human cancers (1). Despite the abundance of experimental and epidemiological data, how arsenite or arsenic compounds cause carcinogenic transformation is still a debatable issue. A number of studies observed aneuploidy, alteration of sister chromatid exchanges, and retarded cell proliferation in arsenite-exposed cells (39–41). The observed inducible degradation of Cdc25C in the present report, therefore, not only may indicate a self-protective mechanism of cells but may also suggest that certain important mitotic events are possibly perturbed or impaired by arsenite. G_2 and M phases are undoubtedly the most important parts of the cell cycle. Errors in the choreography of these two phases can cause aneuploidy or genetic instability, a hallmark of most human cancers. In addition, it is intriguing to note that whereas arsenite induces degradation of Cdc25C and possibly Cdc25B, arsenite also causes elevation of protein levels of Cdc25A (Fig. 3*C*), a protein phosphatase that regulates the G_1/S phase transition by dephosphorylating and activating the Cdk2/cyclin E complex. Cdc25A has also been postulated to be an oncogene because of its ability to cooperate with c-Myc and Ras to promote carcinogenic transformation (42, 43). Thus, the carcinogenic effect of arsenite may be through two mutually synergic mechanisms: facilitation of G_1/S transition by the induction of Cdc25A and enhancement of genome instability by the impairment of the G_2/M phase of cell cycle. A better understanding of the mechanisms of arsenite-induced cell cycle disregulation will aid not only in the development of treatment modalities but also in the promotion of preventive strategies.

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