Biochemical and Biophysical Research Communications 433 (2013) 579–585

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

The metalloid arsenite induces nuclear export of Id3 possibly via binding to the N-terminal cysteine residues

Hisanori Kurooka [a,c,*], Manabu Sugai [b], Kentaro Mori [a], Yoshifumi Yokota [a,c,*]

[a] Division of Molecular Genetics, Department of Biochemistry and Bioinformative Sciences, School of Medicine, Faculty of Medical Sciences, University of Fukui, Fukui, Japan
[b] Department of Experimental Therapeutics, Translational Research Center, Kyoto University Hospital, Kyoto, Japan
[c] Research and Education Program for Life Science, University of Fukui, Fukui, Japan

ARTICLE INFO

Article history:
Received 8 March 2013
Available online 22 March 2013

Keywords:
Id3
Transcriptional repressor
NES
Arsenite
Cysteine

ABSTRACT

Ids are versatile transcriptional repressors that regulate cell proliferation and differentiation, and appropriate subcellular localization of the Id proteins is important for their functions. We previously identified distinct functional nuclear export signals (NESs) in Id1 and Id2, but no active NES has been reported in Id3. In this study, we found that treatment with the stress-inducing metalloid arsenite led to the accumulation of GFP-tagged Id3 in the cytoplasm. Cytoplasmic accumulation was impaired by a mutation in the Id3 NES-like sequence resembling the Id1 NES, located at the end of the HLH domain. It was also blocked by co-treatment with the CRM1-specific nuclear export inhibitor leptomycin B (LMB), but not with the inhibitors for mitogen-activated protein kinases (MAPKs). Importantly, we showed that the closely spaced N-terminal cysteine residues of Id3 interacted with the arsenic derivative phenylarsine oxide (PAO) and were essential for the arsenite-induced cytoplasmic accumulation, suggesting that arsenite induces the CRM1-dependent nuclear export of Id3 via binding to the N-terminal cysteines. Finally, we demonstrated that Id3 significantly repressed arsenite-stimulated transcription of the immediate-early gene *Egr-1* and that this repression activity was inversely correlated with the arsenite-induced nuclear export. Our results imply that Id3 may be involved in the biological action of arsenite.

1. Introduction

Id proteins function as negative regulators of basic helix-loop-helix (bHLH) transcription factors that control cell fate determination [1]. The gene products of the four Id family members (Id1–Id4) share a similar overall structure consisting of a highly conserved helix-loop-helix (HLH) domain and less conserved N- and C-terminal regions. Extensive analyses of knock-out mice have revealed that Ids play crucial roles in neurogenesis, angiogenesis, and immune cell development [2].

Id proteins primarily form inactive heterodimers with ubiquitously expressed bHLH E proteins such as E2A, E2-2, and HEB. On the other hand, Ids have been shown to interact with non-bHLH transcription factors and suppress their functions [1]. For example, Id1, Id2, and Id3 inhibit the ETS transcription factor Elk-1, which activates immediate-early gene expression in response to mitogenic stimulation [3]. They also antagonize the paired homeodomain transcription factor Pax-5 to ensure proper B-cell development [4,5] and prevent the runt-related transcription factor Runx2/Cbfa1 from promoting osteogenesis [6]. Thus, Ids are versatile transcriptional repressors that regulate cell proliferation and differentiation.

Altered subcellular distribution of Id proteins has been associated with differentiation in some cell lineages and a subset of human cancers [2,7,8]. Therefore, transport between the nucleus and the cytoplasm, which is referred to as nucleo-cytoplasmic shuttling, is likely to be involved in the regulation of Id protein functions. Nucleo-cytoplasmic shuttling is mediated through the nuclear pore complex, where transport receptors called importins and exportins facilitate the nuclear import and export of cargo proteins via recognition of nuclear localization signals (NLSs) and nuclear export signals (NESs), respectively [9]. Previously, we demonstrated that Id2 is a nucleo-cytoplasmic shuttling protein containing a strong NES that dominates over its weak NLS [10]. We also identified a distinct functional NES in Id1 [11], while the similar NES-like sequences of Id3 and Id4 are inactive at steady state [10,11].

Abbreviations: bHLH, basic helix-loop-helix; HLH, helix-loop-helix; NLS, nuclear localization signal; NES, nuclear export signal; LMB, leptomycin B; PAO, phenylarsine oxide; CA, constitutively active; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase.

* Corresponding authors. Address: Division of Molecular Genetics, Department of Biochemistry and Bioinformative Sciences, School of Medicine, Faculty of Medical Sciences, University of Fukui, 23-3 Matsuoka-Shimoaizuki, Eiheiji, 910-1193 Fukui, Japan. Fax: +81 776 61 8164.

E-mail addresses: hkurooka@u-fukui.ac.jp (H. Kurooka), yokota@u-fukui.ac.jp (Y. Yokota).

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.03.027

Here we describe the arsenite-induced nuclear export of Id3. We demonstrate that the N-terminal cysteine residues of Id3 interact with arsenite and are essential for the metalloid-induced nuclear export. We also show that the arsenite-induced nuclear export of Id3 counteracts its repression activity in Elk-1-mediated transcription. Our results provide novel insights into the regulation of Id3 protein localization and suggest its possible involvement in the arsenic response.

2. Materials and methods

2.1. Reagents

Sodium arsenite and anisomycin were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). SB203580 and PD98059 were obtained from Calbiochem (San Diego, CA). SP600125 was from Sigma–Aldrich (St. Louis, MO). Leptomycin B (LMB) was a generous gift from Dr. M. Yoshida (RIKEN Wako Institute). 4-amino-phenylarsine oxide (4-amino-PAO) was purchased from Toronto Research Chemicals, Inc. (Toronto, Ontario, Canada). Affi-Gel 10 gel was from Bio-Rad Laboratories (Hercules, CA).

2.2. Plasmids

The N-terminally GFP-tagged mouse Id3 plasmid has been described previously [10]. To generate Id3 point mutation and deletion constructs, appropriate fragments were PCR-amplified and inserted into pEGFP-C1 (Clontech, Mountain View, CA) and pFLAG-CMV-10 (Sigma). The expression plasmid encoding constitutively active MEK1 (pCMV-MEK1(CA)) was kindly provided by Dr. A. Sharrocks (The University of Manchester). To generate firefly luciferase reporter constructs containing the Elk-1-binding sites, the mouse *Egr-1* promoter region (from positions −425 to +22) was amplified by PCR using genomic DNA as a template and subsequently cloned into pGL3-Basic and pGL4.12 (Promega, Madison, WI).

2.3. Cell culture and DNA transfection

Mouse NIH3T3 fibroblasts and human embryonic kidney 293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. NIH3T3 cells were transfected with plasmids using FuGENE 6 reagent (Roche Diagnostics, Tokyo, Japan), according to the manufacturer's instructions. 293T cells were transfected using TransFectin lipid reagent (Bio-Rad).

2.4. Microscopic analysis

NIH3T3 plated on 2-well Lab-Tek chamber slides (Nalge Nunc International, Naperville, IL) were transfected with 1 μg of GFP fusion constructs. After 24 h, the cells were treated with the indicated agents and fixed with 3% formaldehyde in phosphate-buffered saline for 15 min at room temperature, and finally mounted with the Vectashield reagent containing 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI; Molecular Probes, Carlsbad, CA) to stain the nuclear DNA. Microscopic analysis was conducted as described previously [10].

2.5. PAO bead conjugation and pull-down assay

PAO-conjugated Affi-Gel 10 beads were prepared as described previously [12]. For the pull-down assay, transfected 293T cells were lysed on ice in pull-down buffer (25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol) for 30 min. Cell lysates were then cleared by centrifugation at 17,000×*g* for 20 min, and 200 μg of total proteins were incubated with 40 μl of PAO affinity beads (50% slurry) by rotating for 2 h at 4 °C. The beads were washed three times with pull-down buffer and twice with Tris–EDTA buffer, and bound proteins were eluted with Tris–EDTA buffer containing 100 mM DTT. The samples were separated by 14% SDS–PAGE and transferred onto a nitrocellulose filter. The filter was blotted with anti-FLAG M2 monoclonal antibody (Sigma) and immunoreactive bands were visualized by chemiluminescence using the ECL Plus kit (GE Healthcare, Buckinghamshire, UK).

2.6. Luciferase assay

NIH3T3 cells seeded in 24-well plates were transfected with 1 μg of total DNA. Forty-eight hour (for MEK1-stimulated transcription) or 28 h (for arsenite-induced transcription) after transfection, luciferase assays were performed using the Dual-Glo Luciferase Assay System (Promega), as previously described [10]. Relative luciferase activity was normalized to renilla luciferase activity conferred by the internal control plasmid, phRL-TK (Promega).

3. Results

3.1. Id3 is predominantly localized in the cytoplasm in cells treated with sodium arsenite

NES has been characterized by a short peptide sequence enriched in regularly spaced hydrophobic residues, defined as $\varphi X2\text{-}3\varphi X2\text{-}3\varphi X\varphi$, where φ is leucine, isoleucine, valine, phenylalanine, or methionine, and X is any amino acid [13]. All members of the Id protein family contain a conserved NES-like sequence that is located at the end of the HLH domain (Fig. 1A and B). Despite their high conservation, our previous study demonstrated that only the NES of Id1 is active in nuclear export [11]. We also showed that Id2 possesses a functional NES in the C-terminal region [10]. However, it seems that Id3 as well as Id4 has no additional NESs.

Although Id3 lacks an active NES, it is possible that its subcellular localization is regulated by unidentified mechanisms. To explore this possibility, we transfected NIH3T3 cells with an expression plasmid encoding Id3 fused to the C-terminus of GFP (Fig. 1B), and treated the cells with various stimuli that cause genotoxic and cellular stress. In accordance with our previous observation [10], fluorescence microscopic examination showed that GFP-Id3 was distributed throughout the untreated cells (Fig. 1C, left panels). Intriguingly, we found that it was predominantly localized in the cytoplasm when the cells were treated with sodium arsenite (100 μM) for 2 h (right panels). Cytoplasmic accumulation was detectable 1 h after the treatment and was observed in HeLa, U2OS, and HepG2 cells (data not shown). Importantly, a mutation in the last hydrophobic residue Val-82 to Ala (V82A) within the NES-like sequence (Fig. 1B) severely compromised the sodium arsenite-induced accumulation of GFP-Id3 in the cytoplasm (Fig. 1D). The localization of GFP-Id3 was not changed by the other stimuli tested, suggesting that the NES of Id3 is specifically activated by sodium arsenite.

3.2. Sodium arsenite induces nuclear export of Id3 in a CRM1-dependent and MAPK-independent manner

Sodium arsenite is a potent activator of p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) [14,15] and is known to stimulate extracellular signal-regulated kinase (ERK) activity in certain cell types [16]. Therefore, we

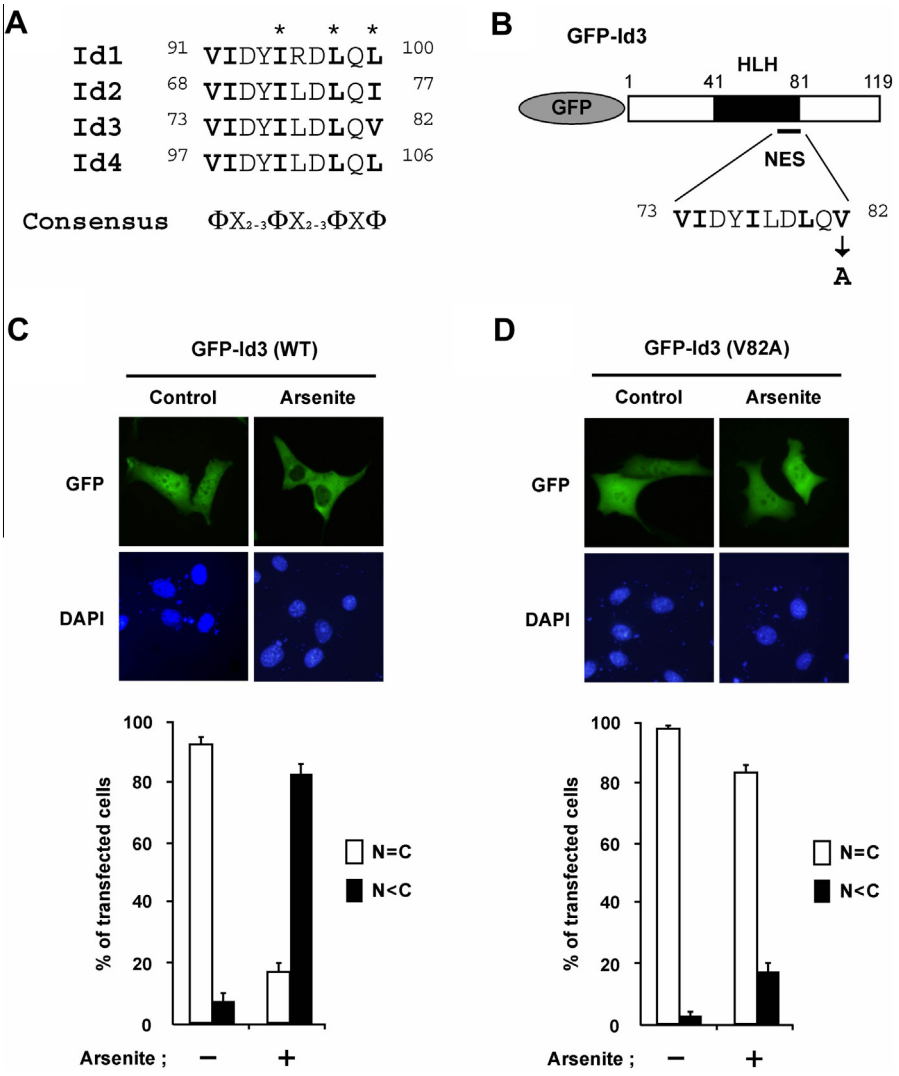

Fig. 1. Cytoplasmic accumulation of GFP-tagged Id3 in response to sodium arsenite. (A) Alignment of the conserved NES-like sequences of the mouse Id protein family. The NES consensus sequence was presented at the bottom. The positions of amino acid residues are indicated and conserved hydrophobic residues of the aligned sequences are highlighted in bold. Asterisks indicate critical residues whose mutation abrogated Id1 NES activity [11]. (B) The location of the NES-like sequence of Id3 is schematically shown in the GFP-Id3 fusion protein. The helix-loop-helix (HLH) domain is drawn as a black box. The numbers above boxes indicate the positions of amino acid residues of the Id3 protein. (C and D) NIH3T3 cells were transfected with the plasmid encoding wild-type GFP-Id3 (WT) (C) or the NES mutant (V82A) (D), and were either treated or not treated with 100 μM sodium arsenite for 2 h. Representative images are shown in the upper panels labeled by GFP and DAPI, which demonstrate the localization of the fusion proteins and the nucleus, respectively. The percentages of cells with nuclear and cytoplasmic (N = C) or predominantly cytoplasmic (N < C) localization were also depicted. At least 100 cells were scored for each sample. The values presented are the mean ± SD from three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

investigated whether the parallel MAPK pathways are involved in the nuclear exclusion of Id3. As shown in Fig. 2A, none of the three MAPK inhibitors (the p38 MAPK inhibitor SB203580, the JNK inhibitor SP600125, and the ERK inhibitor PD98059) affected the cytoplasmic accumulation of GFP-Id3 that was induced by sodium arsenite. In contrast, the accumulation was effectively blocked by co-treatment with LMB, a specific inhibitor of the nuclear export receptor CRM1 [17]. To confirm the result with kinase inhibitors, we examined the effect of the distinct stress-inducer anisomycin, which has been shown to activate all three classes of MAPKs [15,16]. The localization of GFP-Id3 was not altered even when cells were treated with a high concentration (10 μg/ml) of anisomycin (Fig. 2B). Taken together, these results suggest that the arsenite-induced cytoplasmic accumulation of Id3 is mediated by CRM1-dpendent nuclear export and does not depend on MAPK activation.

3.3. Arsenite binds to the N-terminal cysteines of Id3

Next, we generated N- and C-terminal deletion constructs of GFP-Id3 (Δ1–39 and Δ86–119) (Fig. 3A) and assessed their responsiveness to sodium arsenite. Both fusion proteins displayed nucleocytoplasmic distribution in control untreated cells (Fig. 3B), as did the full-length form. However, the distribution of N-terminally deleted GFP-Id3 (Δ1–39) was not influenced by treatment with sodium arsenite. On the other hand, about 60% of C-terminally deleted GFP-Id3 (Δ86–119) was predominantly localized in the cytoplasm of drug-treated cells. Thus, the N-terminal region of Id3 is indispensable for the arsenite-induced nuclear export.

Another feature of arsenite is its reactivity with protein thiol groups [14,18]. Arsenite has been shown to associate with many cellular proteins via closely spaced cysteine residues [18,19]. Since such cysteine residues (Cys-10, Cys-15, and Cys-16) are found in

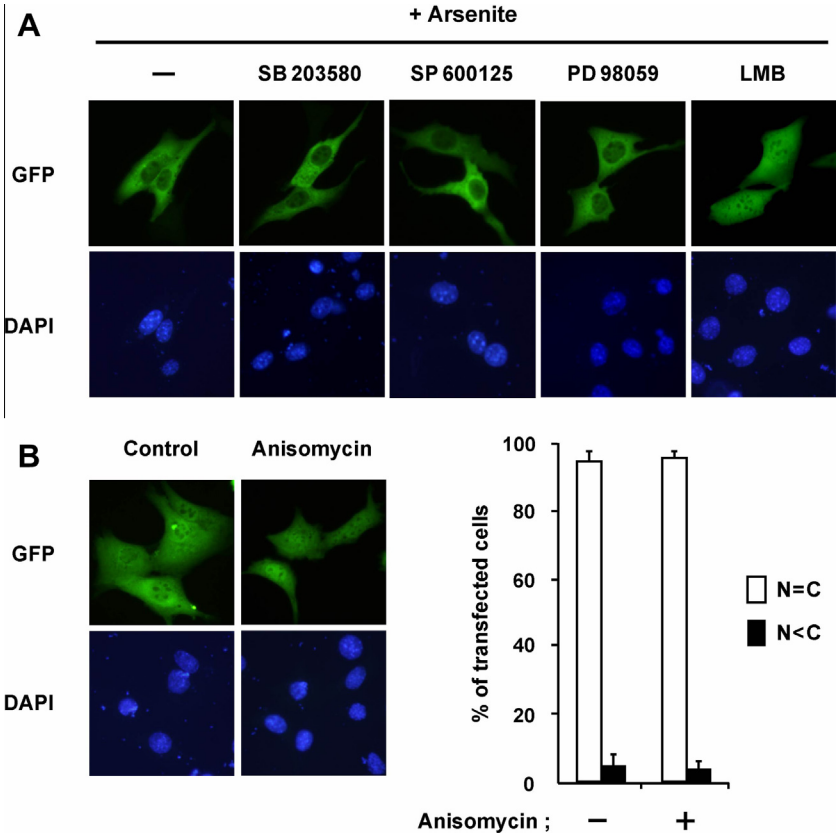

Fig. 2. The sodium arsenite-induced nuclear exclusion of Id3 is CRM1-dependent and MAPK-independent. (A and B) NIH3T3 cells were transfected with the GFP-Id3 (WT) plasmid, and its subcellular localization was examined as described in Section 2. Panels labeled by GFP and DAPI demonstrate the localization of the fusion protein and the nucleus, respectively. (A) Transfected cells were pre-treated for 30 min with 10 μM SB203580, 20 μM SP600125, 50 μM PD98059, or 10 ng/ml LMB. Cells were then exposed to 100 μM sodium arsenite for 2 h in the presence of each inhibitor. (B) Transfected cells were treated with 10 μg/ml anisomycin for 2 h. The percentages of cells with nuclear and cytoplasmic (N = C) or predominantly cytoplasmic (N < C) localization were depicted. At least 100 cells were scored for each sample. The values presented are the mean ± SD from three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the N-terminal region of Id3 (Fig. 3A), we asked whether arsenite binds to it. To this end, Id3 was expressed in 293T cells and then incubated with PAO (Fig. 3C), an organic arsenic compound that interacts preferentially with vicinal cysteine thiols of a protein [12]. A FLAG-tagged version of Id3 was used in this experiment, due to the absence of any cysteine residue in the epitope sequence. PAO-conjugated affinity beads, but not the control Affi-Gel 10 beads, efficiently pulled down FLAG-Id3 (Fig. 3D), indicating that arsenite binds to Id3. PAO binding was completely abolished by replacement of all five cysteine residues with serine (5 Cys), while it was sustained when the three N-terminal cysteines were unchanged and the others were substituted (C47/118S) (Fig. 3E). This data indicates that arsenite interacts with the N-terminal cysteine residues of Id3.

3.4. Arsenite-induced nuclear export of Id3 requires N-terminal cysteines and counteracts its transcriptional repression activity

To evaluate the relative importance of cysteine residues in the arsenite-induced nuclear export of Id3, we analyzed the subcellular distribution of three cysteine mutants of GFP-Id3 (C10/15/16S, C47A, and C118S). The Cys-47 to Ser (C47S) mutant was excluded from the analysis because it was cytoplasmic in untreated cells (data not shown). Similar to the wild-type protein, the accumulation of GFP-Id3 (C47A) and GFP-Id3 (C118S) was high in the cytoplasm when cells were exposed to sodium arsenite (Fig. 4A, middle and lower panels). In contrast, GFP-Id3 (C10/15/16S) exhibited diffuse distribution in drug-treated cells as well as control cells (upper panels). This result clearly shows that closely spaced N-terminal cysteines are essential for the arsenite-induced nuclear export of Id3.

Besides mitogenic stimuli, expression of the immediate-early genes such as *c-Fos* and *Egr-1* has been shown to be up-regulated in response to sodium arsenite [14–16]. Also in these cases, the ETS transcription factor Elk-1 is phosphorylated by MAPKs and thereafter binds to the promoter regions to activate transcription [15,16]. To address the functional significance of the arsenite-induced nuclear export of Id3, we measured the repression activity of wild-type GFP-Id3 (WT) and nuclear export-defective mutants (V82A and C10/15/16S) in Elk-1-mediated transcription. As shown in Fig. 4B, luciferase activity of the *Egr-1* promoter containing the Elk-1-binding sites was greatly enhanced by expression of the constitutively active (CA) form of MEK1, the upstream kinase for ERK [3]. This enhanced activity was pronouncedly reduced by co-expression of each fusion construct (approximately 90% reduction), ensuring the integrity of the expressed proteins. Meanwhile, the marked response of the reporter construct to sodium arsenite was hardly affected by wild-type GFP-Id3, whereas it was moderately, but significantly suppressed by the two nuclear export-defective mutants (55% and 65% suppression by the V82A and C10/15/16S mutants, respectively) (Fig. 4C). Since all fusion proteins repressed MEK1-stimulated transcription of the *Egr-1* gene, the differential effect on arsenite-stimulated transcription was attributed to their individual activities in arsenite-induced nuclear export. Accordingly, we conclude that the arsenite-induced nuclear export of Id3 counteracts its repression activity in Elk-1-mediated transcription.

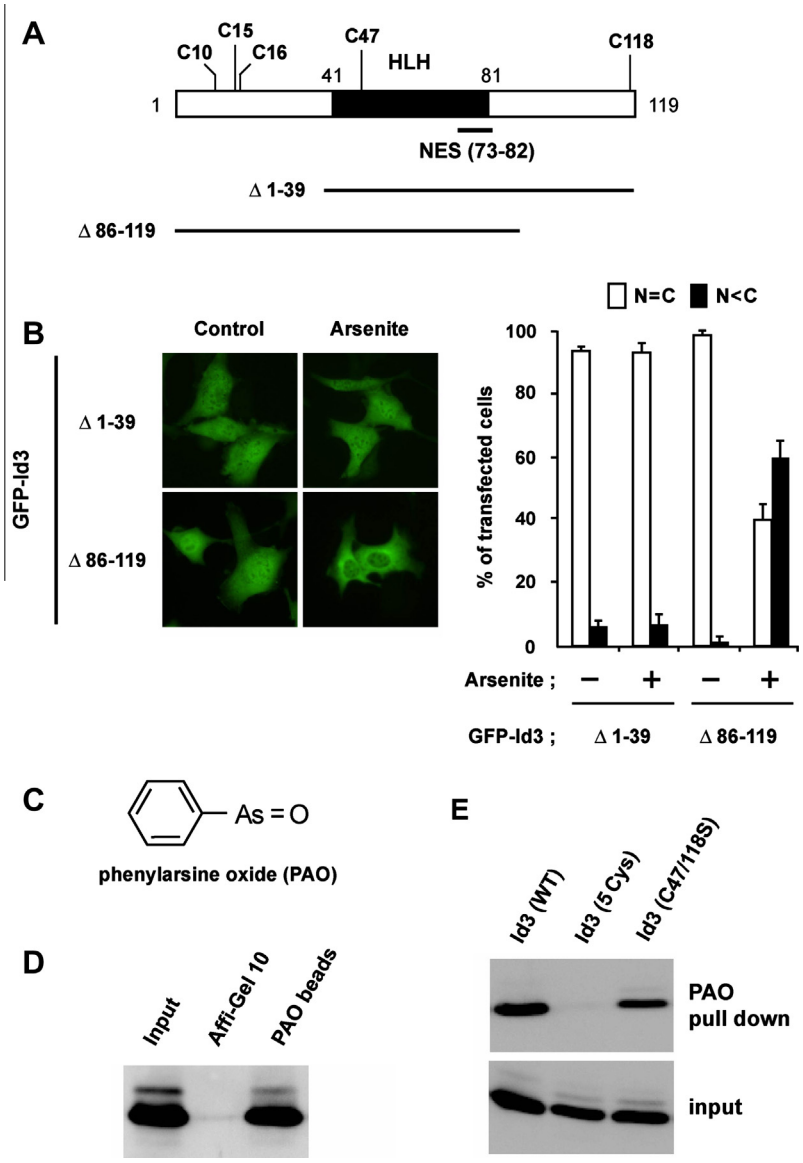

Fig. 3. Binding of PAO to the N-terminal cysteine residues of Id3. (A) Five cysteine residues of Id3 are schematically illustrated at the top. N- and C-terminal deletion constructs of GFP-Id3 are drawn below. (B) NIH3T3 cells were transfected with the deletion constructs, and were either treated or not treated with 100 μM sodium arsenite for 2 h. The left panels are representative images of green fluorescent cells. The percentages of cells with nuclear and cytoplasmic (N = C) or predominantly cytoplasmic (N < C) localization were also depicted. At least 100 cells were scored for each sample. The values presented are the mean ± SD from three independent experiments. (C) Structure of phenylarsine oxide (PAO). (D) Binding of PAO to Id3. 293T cells were transfected with the expression plasmid encoding wild-type 3×FLAG-Id3, and the cell lysates were prepared and incubated with PAO-conjugated affinity beads or control Affi-Gel 10 beads. The pull-down of Id3 was visualized by immunoblotting with anti-FLAG antibody. (E) Binding of PAO to Id3 cysteine mutants. The wild-type and 5 Cys and C47/118S mutants were expressed in 293T cells (lower panel) and pulled-down by PAO affinity beads (upper panel). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

In the present study, we investigated the regulation of subcellular localization of the transcriptional repressor Id3. We found that treatment with sodium arsenite resulted in the substantial accumulation of GFP-tagged Id3 in the cytoplasm. We consider that this accumulation may be directed by a CRM1-dpendent nuclear export mechanism because it was prevented by co-treatment with LMB (Fig. 2A). Cytoplasmic accumulation was also impaired by mutation of the Val-82 residue (Fig. 1D), which has been predicted to be the most critical for NES function [13]. These findings suggest that sodium arsenite activates the NES of Id3 to allow its CRM1-dpendent nuclear export.

Arsenite is a toxic metalloid that persists in the environment. It perturbs cell-cycle progression and differentiation through up- and down-regulation of numerous genes [18,20]. Although MAPK pathways are frequently engaged in the toxic effects of arsenite [21], their involvement in the arsenite-induced nuclear export of Id3 seems unlikely. Instead, our results suggest that the binding of arsenite to Id3 is important. We showed that the N-terminal cysteine residues of Id3 interacted with PAO, an organic derivative of arsenite (Fig. 3D), and that they are essential for the arsenite-induced nuclear export (Fig. 4A). In addition to thiol binding, arsenite has been shown to generate reactive oxygen species [22], which may lead to the oxidative modification of Id3 cysteine residues. However, the cytoplasmic accumulation of GFP-Id3 was not inhibited by the antioxidant N-acetyl cysteine and was not recapitulated by hydrogen peroxide (Kurooka et al., unpublished observations), contradicting the possibility that the arsenite-induced nuclear export of Id3 is caused by oxidative stress.

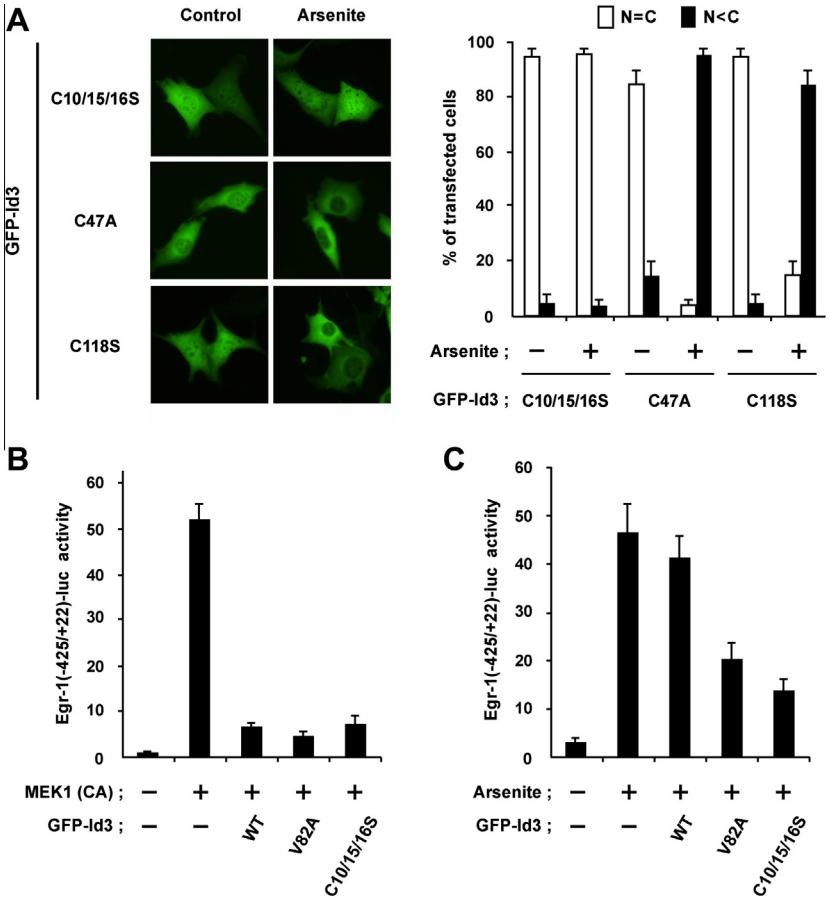

Fig. 4. The sodium arsenite-induced nuclear export of Id3 requires N-terminal cysteine residues and decreases its transcriptional repression activity. (A) NIH3T3 cells were transfected with the cysteine mutants of GFP-Id3 (C10/15/16S, C47A, and C118S), and were either treated or not treated with 100 μM sodium arsenite for 2 h. The left panels are representative images of green fluorescent cells. The percentages of cells with nuclear and cytoplasmic (N = C) or predominantly cytoplasmic (N < C) localization were also depicted. At least 100 cells were scored for each sample. The values presented are the mean ± SD from three independent experiments. (B and C) The repression activity of Id3 in Elk-1-mediated transcription was monitored by luciferase assay. (B) NIH3T3 cells were co-transfected with 100 ng of pGL3-Egr-1(−425/+22), 150 ng of pCMV-MEK1(CA), and 750 ng of each GFP-Id3 fusion construct. The error bars indicate the standard error of the mean. (C) NIH3T3 cells were co-transfected with 200 ng of pGL4.12-Egr-1(−425/+22) and 750 ng of each GFP-Id3 fusion construct. At 24 h post-transfection, the cells were treated with 100 μM sodium arsenite for 4 h. The error bars indicate the standard error of the mean. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The regulation of protein localization by thiol binding has been described previously. As opposed to Id3, the yeast AP-1 like transcription factors YAP1 and YAP8 were translocated into the nucleus upon stimulation with sodium arsenite [23,24]. The distribution of Yap2 was also shifted to the nucleus in response to cadmium, a toxic metal with similar thiol reactivity [25]. Conversely, the Nrf2-related transcriptional repressor Bach1 accumulated in the cytoplasm when cells were treated with heme, which has affinity for the cysteine–proline dipeptide sequence [26]. These effects were all ascribed to the interactions with cysteine residues of these proteins and the concomitant modulation of their NES function. Thiol binding appears to be one of the mechanisms regulating the nuclear export of eukaryotic transcription factors.

Our results strongly suggest that the binding of arsenite is responsible for the metalloid-induced nuclear export of Id3, but it remains unclear how arsenite binding leads to activation of the NES, which is apart from the N-terminal cysteine residues. One explanation would be intramolecular unmasking of NES. It is possible that the NES of Id3 is masked by conformational restraint at steady state and arsenite binding to the N-terminal cysteines may relieve it. An alternative possibility is intermolecular unmasking of NES. Upon arsenite binding, the NES of Id3 may be exposed by interaction with other molecules like the 14-3-3 proteins [27]. In that regard, it should be noted that the NES of Id1 is negatively regulated by N-terminal modification. Nishiyama et al. [28] reported that the nuclear export of Id1 was attenuated by protein kinase A-mediated phosphorylation at the Ser-5 residue in vascular endothelial cells. Thus, the N-terminal region of Ids is critical for regulation of the conserved NESs.

The above-mentioned YAP1 and YAP8 mediate arsenic tolerance by controlling the expression of detoxification genes [23,24]. Likewise, it is conceivable that Id3 plays a role in the biological action of arsenite. We have demonstrated that Id3 repressed arsenite-induced *Egr-1* expression and this repressive effect was inversely correlated with its nuclear export activity in response to sodium arsenite (Fig. 4C). This implies that sodium arsenite may induce nuclear export of Id3 to facilitate transcriptional activation of its responsive genes. It is necessary to comprehensively identify Id3-regulated genes in the arsenic response.

Acknowledgments

We are grateful to Y. Matsui for her technical assistance. We also thank M. Yoshida for LMB and A. Sharrocks for the constitutively active MEK1 plasmid. This work was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (H.K.: #20570128, Y.Y.: #24390077) and by Research Grants from the University of Fukui (H.K.).

References

[1] J.D. Norton, ID helix-loop-helix proteins in cell growth, differentiation and tumorigenesis, J. Cell Sci. 113 (2000) 3897–3905.
[2] J. Perk, A. Iavarone, R. Benezra, Id family of helix-loop-helix proteins in cancer, Nat. Rev. Cancer 5 (2005) 603–614.
[3] P.R. Yates, G.T. Atherton, R.W. Deed, J.D. Norton, A.D. Sharrocks, Id helix-loop-helix proteins inhibit nucleoprotein complex formation by the TCF ETS-domain transcriptional factors, EMBO J. 18 (1999) 968–976.
[4] E.C. Roberts, R.W. Deed, T. Inoue, J.D. Norton, A.D. Sharrocks, Id helix-loop-helix proteins antagonize Pax transcription factor activity by inhibiting DNA binding, Mol. Cell. Biol. 21 (2001) 524–533.
[5] H. Gonda, M. Sugai, Y. Nambu, T. Katakai, Y. Agata, K.J. Mori, Y. Yokota, A. Shimizu, The balance between Pax5 and Id2 activities is the key to AID gene expression, J. Exp. Med. 198 (2003) 1427–1437.
[6] Y. Luan, X.-P. Yu, N. Yang, S. Frenkel, L. Chen, C. Liu, P204 protein overcomes the inhibition of core binding factor α-1-mediated osteogenic differentiation by Id helix-loop-helix proteins, Mol. Biol. Cell 19 (2008) 2113–2126.
[7] S. Fong, R.J. Debs, P.-Y. Desprez, Id genes and proteins as promising targets in cancer therapy, Trends Mol. Med. 10 (2004) 387–392.
[8] A. Iavarone, A. Lasorella, ID proteins as targets in cancer and tools in neurobiology, Trends Mol. Med. 12 (2006) 588–594.
[9] I.G. Macara, Transport into and out of the nucleus, Microbiol. Mol. Biol. Rev. 65 (2001) 570–594.
[10] H. Kurooka, Y. Yokota, Nucleo–cytoplasmic shuttling of Id2, a negative regulator of basic helix-loop-helix transcription factors, J. Biol. Chem. 280 (2005) 4313–4320.
[11] J. Makita, H. Kurooka, K. Mori, Y. Akagi, Y. Yokota, Identification of the nuclear export signal in the helix-loop-helix inhibitor Id1, FEBS Lett. 580 (2006) 1812–1816.
[12] X. He, Q. Ma, Nrf2 cysteine residues are critical for oxidant/electrophile-sensing, Kelch-like ECH-associated protein-1-dependent ubiquitination-proteasomal degradation, and transcription activation, Mol. Pharmacol. 76 (2009) 1265–1278.
[13] T. la Cour, L. Kiemer, A. Mølgaard, R. Gupta, K. Skriver, S. Brunak, Analysis and prediction of leucine-rich nuclear export signals, Protein Eng. Des. Sel. 17 (2004) 527–536.
[14] M. Cavigelli, W.W. Li, A. Lin, B. Su, K. Yoshioka, M. Karin, The tumor promoter arsenite stimulates AP-1 activity by inhibiting a JNK phosphatase, EMBO J. 15 (1996) 6269–6279.
[15] C.P. Lim, N. Jain, X. Cao, Stress-induced immediate-early gene, egr-1, involves activation of p38/JNK1, Oncogene 16 (1998) 2915–2926.
[16] M. Bébien, S. Salinas, C. Becamel, V. Richard, L. Linares, R.A. Hipskind, Immediate-early gene induction by the stresses anisomycin and arsenite in human osteosarcoma cells involves MAPK cascade signaling to Elk-1, CREB and SRF, Oncogene 22 (2003) 1836–1847.
[17] N. Kudo, B. Wolff, T. Sekimoto, E.P. Schreiner, Y. Yoneda, M. Yanagida, S. Horinouchi, M. Yoshida, Leptomycin B inhibition of signal-mediated nuclear export by direct binding to CRM1, Exp. Cell Res. 242 (1998) 540–547.
[18] M.F. Hughes, B.D. Beck, Y. Chen, A.S. Lewis, D.J. Thomas, Arsenic exposure and toxicology: a historical perspective, Toxicol. Sci. 123 (2011) 305–332.
[19] Y.-Y. Chang, T.-C. Kuo, C.-H. Hsu, D.-R. Hou, Y.-H. Kao, R.-N. Huang, Charecterization of the role of protein-cysteine residues in the binding with sodium arsenite, Arch. Toxicol. 86 (2012) 911–922.
[20] L.C. Platanias, Biological responses to arsenic compounds, J. Biol. Chem. 284 (2009) 18583–18587.
[21] Z. Dong, The molecular mechanisms of arsenic-induced cell transformation and apoptosis, Environ. Health Perspect. 110 (2002) 757–759.
[22] S.J.S. Flora, Arsenic-induced oxidative stress and its reversibility, Free Radic. Biol. Med. 51 (2011) 257–281.
[23] R. Wysocki, P.-K. Fortier, E. Maciaszczyk, M. Thorsen, A. Leduc, Å. Odhagen, G. Owsianik, S. Ulaszewski, D. Ramotar, M.J. Tamás, Transcriptional activation of metalloid tolerance genes in *Saccharomyces cerevisiae* requires the AP-1-like proteins Yap1p and Yap8p, Mol. Biol. Cell 15 (2004) 2049–2060.
[24] R.A. Menezes, C. Amaral, A. Delaunay, M. Toledano, C. Rodrigues-Pousada, Yap8p activation in *Saccharomyces cerevisiae* under arsenic conditions, FEBS Lett. 566 (2004) 141–146.
[25] D. Azevedo, L. Nascimento, J. Labarre, M.B. Toledano, C. Rodrigues-Pousada, The *S. cerevisiae* Yap1 and Yap2 transcription factors share a common cadmium-sensing domain, FEBS Lett. 581 (2007) 187–195.
[26] H. Suzuki, S. Tashiro, S. Hira, J. Sun, C. Yamazaki, Y. Zenke, M. Ikeda-Saito, M. Yoshida, K. Igarashi, Heme regulates gene expression by triggering Crm1-dependent nuclear export of Bach1, EMBO J. 23 (2004) 2544–2553.
[27] A.J. Muslin, H. Xing, 14-3-3 proteins: regulation of subcellular localization by molecular interference, Cell. Signaling 12 (2000) 703–709.
[28] K. Nishiyama, K. Takaji, Y. Uchijima, Y. Kurihara, T. Asano, M. Yoshimura, H. Ogawa, H. Kurihara, Protein kinase A-regulated nucleo–cytoplasmic shuttling of Id1 during angiogenesis, J. Biol. Chem. 282 (2007) 17200–17209.