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ARD1 binding to RIP1 mediates doxorubicin-induced NF-κB activation

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ABSTRACT

NF-κB is activated by several cellular stresses. Of these, the TNFα-induced activation pathway has been examined in detail. It was recently reported that receptor-interacting protein 1 (RIP1) is involved in DNA damage-induced NF-κB activation by forming a complex with the p53 interacting death domain protein (PIDD) and NF-κB essential modulator (NEMO) in the nucleus, although the underlying mechanism of this interaction has yet to be clarified. This study shows that siRNA knock-down of arrest-defective 1 protein (ARD1) abrogated doxorubicin- but not TNFα-induced activation. Conversely, the over-expression of ARD1 greatly enhanced NF-κB activation induced by doxorubicin. Immunoprecipitation experiments revealed that ARD1 interacted with RIP1 via the acetyltransferase domain. Furthermore, the over-expression of several domain-deleted ARD1 constructs demonstrated that the N-terminal and acetyltransferase domains of ARD1 were required for doxorubicin-induced NF-κB activation. Treatment of deacetylase inhibitor, trichostatin A, significantly increased doxorubicin-induced NF-κB activation in the presence of ARD1 but not acetyltransferase-defective ARD1 mutant. Moreover, N-terminal domain-deleted ARD1 could not be localized in the nucleus in response to doxorubicin treatment. These data indicate that the interaction between ARD1 and RIP1 plays an important role in the DNA damage-induced NF-κB activation, and that the acetyltransferase activity of ARD1 and its localization in to the nucleus are involved in such stress response.

1. Introduction

Chemotherapeutic compounds are well known to induce DNA damage in targeted tumor cells, leading to cell death. In parallel, DNA damage induces the activation of nuclear factor κB (NF-κB) followed by the transcription of cell survival genes, which results in enhanced cell proliferation and escape from cell death [1]. Prior to activation, NF-κB is sequestered in the cytoplasm by the inhibitory protein IκB, which masks the nuclear localization signal of NF-κB. Once activated, IκB is phosphorylated through IκB kinase complex consisting of IKKα, IKKβ, and NEMO (NF-κB essential modulator, also known as IKKγ), followed by ubiquitination and subsequent proteasomal degradation. NF-κB liberated from IκB enters the nucleus and induces transcription by binding to the consensus regions of various genes [2].

Receptor-interacting protein 1 (RIP1) is typically involved in the two pathways, the tumor necrosis factor (TNF) α-induced and DNA damage-induced NF-κB activation pathways [3,4]. In the TNFα-induced NF-κB pathway, RIP1 is recruited to the TNFα receptor and forms a complex with the TNFR-associated death domain protein (TRADD), TNF receptor-associated factor 2 (TRAF2), and cellular inhibitor of apoptosis proteins (cIAP)-1/2, and is modified with polyubiquitin chains by TRAF2 and cIAP. Ubiquitinated RIP1 recruits TGFβ activated kinase 1 (TAK1) and TAK1-binding proteins (TAB) 1/2/3, which leads to the activation of the IKK complex [5,6]. Also, RIP1 is indispensable for DNA damage-induced NF-κB activation, as was demonstrated in RIP1-deficient mouse embryonic fibroblast (MEF) [4]. In this pathway, RIP1 forms a distinct complex with p53-induced protein with a death domain (PIDD) and NEMO in the nucleus [7]. NEMO is sequentially modified with a small ubiquitin-like modifier (SUMO) by protein inhibitor of activated STATy (PIAsy) and followed by Ataxia telangiectasia mutated protein (ATM)-dependent phosphorylation and cIAP-1-dependent monoubiquitination, resulting in the nuclear export of NEMO [8–10]. Cytoplasmic NEMO forms an IKK complex in the same way as the TNFα-induced pathway. RIP1 is involved in both TNFα-induced and DNA damage-induced NF-κB pathways, and functions in the cytoplasm and nucleus, respectively. In DNA damage-induced NF-κB pathway, RIP1 translocates into the nucleus and forms a complex with PIDD and NEMO. However, it is unclear how RIP1 senses DNA damage and is activated in the nucleus.

Abbreviations: ARD1, arrest-defective 1 protein; RIP1, receptor-interacting protein 1; HA, hemagglutinin; Myc, c-Myc; TNF, tumor necrosis factor; HEK, human embryonic kidney; MEF, mouse embryonic fibroblast; NLS, nuclear localization signal.

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Arrest-defective 1 protein (ARD1) was initially identified as an N-terminal α-acetyltransferase (NAT) in yeast [11]. The enzymes that mediate protein acetylation are classified in two categories: one targets the α-amino group of N-terminal amino acids and the other targets the ε-amino group of lysines [12]. At present, mammalian ARD1 appears to be a unique acetyltransferase with both NAT and lysine acetyltransferase activities [13–15]. In mammalian cells, ARD1 has been reported to be necessary for DNA damage-induced apoptotic cell death by doxorubicin [16]. Doxorubicin, a cancer chemotherapeutic compound, is a topoisomerase II inhibitor that induces DNA damage and concomitantly induces NF-κB activation [17]. Considering that NF-κB activation induces resistance against cancer chemotherapy, dissecting the regulatory mechanism of the NF-κB activation is crucial.

In the current study, we present the novel finding that ARD1 is required for doxorubicin-induced NF-κB activation. Moreover, we show that ARD1 interacts with RIP1, and that an ARD1 mutant that lacks the N-terminal or acetyltransferase domain could not induce doxorubicin-induced NF-κB activation. Furthermore, deacetylase inhibitor further enhanced doxorubicin-induced activation in the presence of ARD1 but not mutated ARD1. These results indicate that the binding of ARD1 to RIP1 and acetyltransferase activity of ARD1 is necessary for DNA damage-induced NF-κB activation.

2. Materials and methods

2.1. Antibodies and reagents

Mouse anti-HA, anti-Myc, anti-GAPDH, anti-β-actin, anti-Myc and anti-ubiquitin antibodies and rabbit anti-ARD1 and anti-NEMO antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-Myc antibody was from Bethyl Laboratories (Montgomery, TX). TNFα was from Sigma (St. Luis, MO). Doxorubicin was purchased from Enzo Life Sciences (Farmingdale, NY). Trichostatin A was purchased from Biomol (Plymouth Meeting, PA).

2.2. Cell culture

HEK293 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA). The cells were cultivated in Dulbecco's modified Eagle's medium (Sigma–Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA). Cultures were kept at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

2.3. Construction of plasmids

The cDNAs encoding human ARD1 and RIP1 were amplified by reverse transcription-polymerase chain reaction (RT-PCR) using RNA from HEK293 cells as a template. RNA was isolated from cells using Trizol reagent (Invitrogen). RT-PCR was performed with Superscript reverse transcriptase, oligo $(dT)_{12-18}$ primer (Invitrogen), and synthesized first-stranded cDNA as a template. PCR primers were synthesized based on the GenBank ID: NM_003491 and NM_003804, respectively. The obtained ARD1 cDNA was subcloned into the pEF vector with an additional sequence encoding an HA epitope at the 5′-end (pEF-HA-ARD1), and RIP1 cDNA was ligated into a pcDNA6 vector (Invitrogen) with a C-terminal Myc-His tag sequence (pcDNA6-RIP1-Myc-His). For the construction of deletion mutants of ARD1, PCR was employed using wild type cDNAs as a template and then subcloned into pEF-HA. A plasmid encoding mutated ARD1(R82A/G85A) was generated by using KOD-plus mutagenesis kit (Toyobo, Osaka, Japan) and then subcloned into pEF-HA. The nucleotide sequence of each construct was confirmed by DNA sequencing (Greiner Bio-One, Tokyo, Japan) and protein expression was confirmed by Western blotting with anti-HA or anti-Myc antibodies.

2.4. Transfection, immunoprecipitation, and Western blotting

Transfection of plasmids into HEK293 cells was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Cells were treated with 10 mM z-VAD-fmk (R&D Systems, Minneapolis, MN) to inhibit apoptotic cell death by over-expression of RIP1. After 48 h of transfection, the cells were washed with cold phosphate buffered saline and lysed in ice-cold lysis buffer (20 mM Tris–HCl, pH 7.5, containing 150 mM NaCl, 0.5% NP-40, 0.5 mM PMSF, 10 μg/ml leupeptin, 1 mM DTT, 25 mM β-glycerophosphate, and 1 mM sodium orthovanadate). The cell lysates were cleared by centrifugation at 16,000*g* for 5 min at 4 °C and the nuclear fraction was spun down. For immunoprecipitation of the cytoplasmic fraction, supernatants were incubated with the appropriate antibodies for 2 h at 4 °C and then mixed with protein A-agarose beads (Roche Diagnostics, Mannheim, Germany) that had been equilibrated in lysis buffer. After the mixtures were incubated overnight at 4 °C, the beads were recovered by centrifugation and washed five times with cold lysis buffer. The beads were then resuspended in gel loading buffer (50 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 0.25 mg/ml bromophenol blue, 5% β-mercaptoethanol) and boiled for 5 min. For Western blot analysis, samples were separated on a polyacrylamide gel and electrotransferred to polyvinylidene fluoride membranes (PVDF) (PALL, Pensacola, FL). Membranes were blocked with 5% nonfat milk in wash buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20) and incubated with primary antibodies overnight at 4 °C. After three washes with wash buffer, the membranes were incubated with alkaline phosphatase-conjugated second antibodies (Promega, Madison, WI) for 1 h at room temperature. Membranes were washed three times with wash buffer and once with AP buffer (100 mM Tris–HCl, pH 9.6, 150 mM NaCl, 1 mM $MgCl_2$), and the immunoblotted proteins were detected by a chromogenic method using BCIP/NBT color development substrate (Promega).

2.5. Reporter assay

To measure NF-κB activation, HEK293 cells were co-transfected with a luciferase reporter plasmid driven by three tandem repeats of the immunoglobulin κ light chain NF-κB enhancer region and pCMV-LacZ, which constitutively expresses β-galactosidase as an internal control of transfection efficiency. Luciferase activity was measured with a Lumat LB9507 (Berthold, Melbourne, Australia) using the Picagene kit (Toyo Ink, Tokyo, Japan) as a substrate. β-Galactosidase activity was measured with a Bio-Rad Model 550 microplate reader (Bio-Rad, Hercules, CA) at a wavelength of 450 nm using *o*-nitrophenyl-β-galactopiranoside as a substrate. Statistical analysis of the results was performed using Microsoft Excel. The *p*-values were calculated using a paired Student's *t*-test and statistical significance was determined when a *p*-value was less than 0.05.

2.6. RNA interference

To knock-down the expression of ARD1, the following RNA oligonucleotides were designed:

siARD1–1 sense, 5′-CCAGAUGAAAUACUACUUCTT-3′;
siARD1–1 antisense, 5′-GAAGUAGUAUUUCAUCUGGTT-3′;
siARD1–2 sense, 5′-CUCACCCCACGAGCUUUCACA-3′;
siARD1–2 antisense, 5′-UGAAAGCUCGUGGGGUGAGGA-3′;

siARD1–3 sense, 5′-CAUCGAGAACAAGGUGGAGAGCAAA-3′;
siARD1–3 antisense, 5′-UUUGCUCUCCACCUUGUUCUCGAUG-3′;
control siRNA sense, 5′-GUACCGCACGUCAUUCGUAUC-3′; and
control siRNA antisense, 5′-UACGAAUGACGUGCGGUACGU-3′.

The annealed siRNAs were introduced into HEK293 cells by reverse transfection using Lipofectamine 2000. The cells were cultured for 72 h and then used for each assay.

3. Results

3.1. ARD1 is required for doxorubicin-induced NF-κB activation

ARD1 was reported to be necessary for DNA damage-induced apoptotic cell death by doxorubicin in a genome-wide RNAi screening study [16]. To examine the role of human ARD1 in NF-κB activation, we examined the effect of ARD1 knock-down on DNA damage- and TNFα-induced NF-κB activation. For DNA damage-induced activation of NF-κB, doxorubicin was used since it breaks double strand DNA in cells and concomitantly induces NF-κB activation [17]. To measure NF-κB activation, HEK293 cells that had been treated with siRNAs were transiently transfected with a reporter plasmid encoding luciferase under the control of NF-κB activation. Three kinds of siRNAs were used for the knock-down of ARD1, and Western blotting data showed that all of these effectively reduced the expression of ARD1 (Fig. 1A). Cells stimulated with doxorubicin increased NF-κB activity by approximately 8-fold compared with non-stimulated cells; however, NF-κB activity in ARD1 knock-down cells was significantly attenuated ($p < 0.001$). By contrast, in TNFα-induced NF-κB activation, there was no significant difference between the cells transfected with control siRNA and those with ARD1-targeting siRNAs ($p > 0.05$; Fig. 1B). Next, we examined the effect of over-expressing ARD1 on doxorubicin-induced activation under the same conditions. When ARD1 was over-expressed in the cells, the activation of NF-κB by doxorubicin was increased by up to 1.9-fold compared to untreated cells ($p < 0.05$; Fig. 1C). These findings clearly indicated that ARD1 was required for doxorubicin-induced NF-κB activation but not for TNFα-induced activation.

3.2. ARD1 promotes RIP1-mediated NF-κB activation

RIP1 is known as a mediator of NF-κB signaling induced by both TNFα [18] and DNA-damaging drugs [4,7]. Therefore, we examined the effect of ARD1 on RIP1-mediated NF-κB activation. HEK293 cells were transiently transfected with RIP1 cDNA and NF-κB activation was measured in the absence or presence of ARD1. Over-expression of RIP1 greatly induced NF-κB activation, as reported previously [19], and this activation was further increased by up to 1.9-fold by the over-expression of ARD1 in a dose-dependent manner ($p < 0.05$; Fig. 1D). To examine the cooperation of ARD1 and RIP1 on NF-κB activation in the cell, we next performed immunoprecipitation using HA-tagged ARD1 and Myc-tagged RIP1.

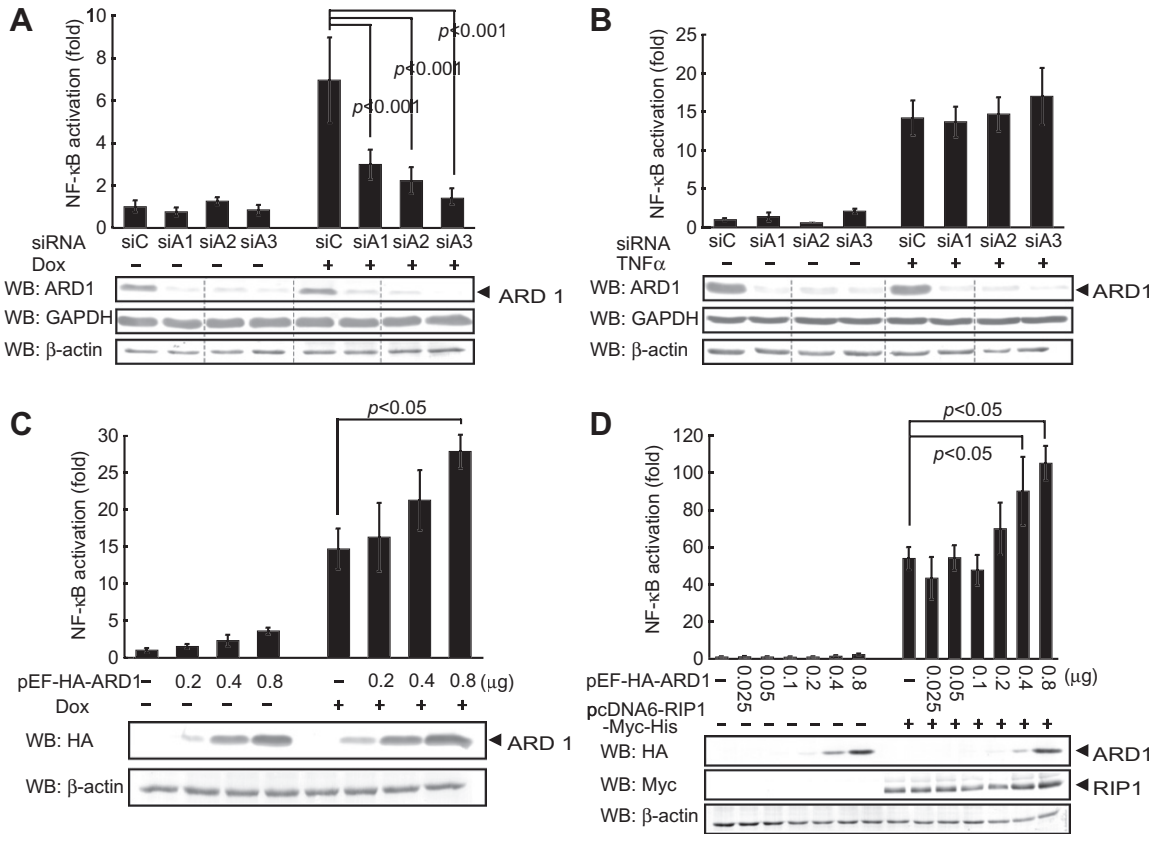

Fig. 1. Effect of ARD1 on NF-κB activation induced by doxorubicin and TNFα. Each ARD1-specific siRNA (5 nM) was introduced into HEK293 cells. After 24 h, NF-κB-dependent luciferase (p[kB3]-tk-Luc) and β-galactosidase reporters (pCMV-LacZ) were co-transfected into cells. The cells were treated with 0.5 μM of doxorubicin (Dox) for 12 h (A) or TNFα (20 ng/ml) for 18 h (B). (C) After the introduction of pEF-HA-ARD1 with p[kB3]-tk-Luc and pCMV-LacZ plasmids into HEK293 cells, cells were stimulated with or without 0.5 μM doxorubicin for 12 h. (D) The effect of ARD1 on RIP1-induced NF-κB activation was investigated. pEF-HA-ARD1 and reporter plasmids with or without pcDNA6-RIP1-Myc-His were introduced into HEK293 cells. Luciferase assays were performed as described in Section 2 and normalized to β-galactosidase activity. Western blotting was carried out at 48 h post-transfection. Results shown represent an average of triplicate experiments and error bars indicate SD.

3.3. ARD1 interacts with RIP1 via the acetyltransferase domain

To examine the association between ARD1 and RIP1, we performed immunoprecipitation using the cytosolic fraction of lysates from HEK293 cells expressing HA-tagged ARD1 and Myc-tagged RIP1. Expression of HA-tagged ARD1 and Myc-tagged RIP1 were confirmed by Western blotting with anti-HA and anti-Myc antibodies, respectively (Fig. 2A). When HA-tagged ARD1 was precipitated with anti-HA antibody, a band corresponding to Myc-tagged RIP1 was detected by anti-Myc antibody only in the lysate of cells expressing both ARD1 and RIP1. Conversely, HA-tagged ARD1 was detected in samples precipitated with anti-Myc antibody when both ARD1 and RIP1 were expressed together. These results suggested that ARD1 interacted with RIP1 directly or indirectly in the cells. To determine the domain of ARD1 that interacted with RIP1 in more detail, we constructed deletion mutants of ARD1 (Fig. 2B). Since ARD1 consists of an N-terminal domain (1–44 amino acids), an acetyltransferase domain (45–130 amino acids), and a C-terminal domain (179–235 amino acids), each domain-deleted ARD1 (ARD1ΔN, ARD1ΔAT, and ARD1ΔC) was expressed with Myc-tagged RIP1 and immunoprecipitation was performed using anti-Myc antibody. Each domain-deleted ARD1 was equally expressed in the cells (Fig. 2C). ARD1ΔN and ARD1ΔC maintained the interaction with Myc-tagged RIP1. By contrast, ARD1ΔAT was not precipitated with RIP1, indicating that the acetyltransferase domain of ARD1 was necessary for interaction with RIP1.

3.4. Both the N-terminal and acetyltransferase domains of ARD1 are required for doxorubicin-induced NF-κB activation

Next, we examined the effect of ARD1 deletion mutants to determine whether the acetyltransferase domain of ARD1 is important for doxorubicin-induced NF-κB activation. Plasmids expressing ARD1 mutants were transfected into HEK293 cells along with an NF-κB luciferase reporter gene. Over-expression of wild type ARD1 activated NF-κB, whereas ARD1 mutants with a deletion in the N-terminal domain or acetyltransferase domain had no effect on NF-κB activation (Fig. 3A).

In DNA damage-induced NF-κB activation pathway, RIP1 forms a distinct complex with PIDD and NEMO in the nucleus [7] and subsequently NEMO is modified with ubiquitin [8,9]. Next we examined the effect of ARD1 on NEMO ubiquitination, which is upstream of NF-κB activation induced by doxorubicin. As expected, the ubiquitination of NEMO was accelerated by over-expression of ARD1 2 and 4 h after doxorubicin treatment of HEK293 cell, and ubiquitinated NEMO was then degraded or deubiquitinated 6 h after the doxorubicin treatment (Fig. 3B). Then, we tried to monitor the effect of ARD1 over-expression on doxorubicin-induced NF-κB activation at early stage. Next we performed NF-κB reporter assay using HEK293 cells over-expressing ARD1 or acetyltransferase-defective ARD1 mutant in the presence of deacetylase inhibitor, trichostatin A, to determine whether the acetyltransferase activity of ARD1 is necessary for doxorubicin-induced NF-κB activation. After 6 h-treatment of doxorubicin in the presence of trichostatin A, NF-κB activation of the HEK293 cell was increased approximately 5-fold and the activation was further enhanced up to 12-fold by over-expression of HA-tagged ARD1 (Fig. 3C, upper panel). ARD1 has an acetyl-CoA contact site from residues 82 to 87 (RRLGLA) and the acetyltransferase activity of ARD1 was abrogated by introducing alanine substitutions at amino acid residues Arg82 and Gly85 [14]. Then the cell was over-expressed with ARD1(R82A/G85A) mutant instead of wild type in the presence of trichostatin A and doxorubicin-induced NF-κB activation was measured. Though the expressed amount of mutated ARD1 was same as that of wild type, level of NF-κB activation of mutated ARD1-expressing cell was same as that of untransfected cell (Fig. 3C). Combined with the data showing ARD1(R82A/G85A) was coprecipitated with RIP1 (data not shown), these data strongly suggest that acetyltransferase activity of ARD1 was required for doxorubicin-induced NF-κB activation of the cell.

As shown in Figs. 2C and 3A, ARD1ΔN did not enhance doxorubicin-mediated NF-κB activation although the mutant bound to RIP1. To clarify the mechanism of ARD1ΔN, we examined subcellular localization of wild type ARD1, ARD1ΔN, and ARD1(R82A/G85A). Both HA-tagged wild type ARD1 and ARD1(R82A/G85A) were localized in the nucleus 3 h after doxorubicin treatment, whereas ARD1ΔN could not be detected in the nuclear fraction, though it was expressed in the cytosol of cells (Fig. 4). The data suggested that nuclear localization signal (NLS) of ARD1 may be present in N-terminal region and that translocation of ARD1 to the nucleus may be required for enhancement of doxorubicin-mediated NF-κB activation.

4. Discussion

It is well known that NF-κB is activated by a variety of agents via distinct signaling pathways. Using a luciferase reporter assay, we demonstrated that the knock-down of ARD1 expression led to the abrogation of NF-κB activation induced by doxorubicin but not TNFα (Fig. 1A and B), and that the over-expression of ARD1 enhanced doxorubicin-induced activation of NF-κB in a dose-dependent manner (Fig. 1C). These results suggested that ARD1 is a positive mediator of DNA damage- but not TNFα-induced NF-κB activation. In DNA damage-induced NF-κB signaling, it has been already reported that RIP1 plays an important role as an adaptor molecule that relays the PIDD signal to NEMO in the nucleus [4,7,20]. Interestingly, we showed that the over-expression of ARD1 enhanced NF-κB activation induced by RIP1 in a dose-dependent manner (Fig. 1D), and further demonstrated that ARD1 interacts with RIP1 via its acetyltransferase domain (Fig. 2C). Such interaction between ARD1 and RIP1 may explain how ARD1 is specifically involved in DNA damage-induced NF-κB signaling.

Overexpression of ARD1ΔAT did not enhance doxorubicin-induced NF-κB activation as expected since ARD1ΔAT mutant could not associate with RIP1. Similarly, ARD1ΔN could not enhance NF-κB activation even though the mutant could bind to RIP1 (Fig. 3A). However, intracellular localization of ARD1ΔN was different from that of wild type ARD1, and wild type but not ARD1ΔN was localized in the nucleus 3 h after doxorubicin treatment (Fig. 4). The data suggested that NLS of ARD1 was present in N-terminal domain and that localization of ARD1 in the nucleus may be required for enhancement of doxorubicin-mediated NF-κB activation.

ARD1 is an acetyltransferase and we hypothesized that the acetyltransferase activity of ARD1 might be required for the activation of NF-κB by doxorubicin. To investigate this possibility, we examined the effect of an ARD1 mutant lacking acetyltransferase activity. We found that ubiquitination of NEMO, which is upstream of DNA damage-induced NF-κB activation, occurred within 4 h (Fig. 3B), which was in good agreement with previous report showing that sumoylation and ubiquitination of NEMO were detected in HEK293T cell at 4 h after doxorubicin treatment [7] and that the peak of these modifications of NEMO was from 45 to 90 min [8]. Since acetyltransferase activity of ARD1 seems to be involved in early step(s) of NF-κB signaling pathway, we examined the effect of acetyltransferase activity of ARD1 on NF-κB activation in the presence of deacetylase inhibitor, trichostatin A, at 6 h after doxorubicin treatment. The cell over-expressing ARD1 but not acetyltransferase-defective ARD1(R82A/G85A) enhanced doxorubicin-induced NF-κB activation compared with untransfected cell under the same condition (Fig. 3C). These findings suggest that

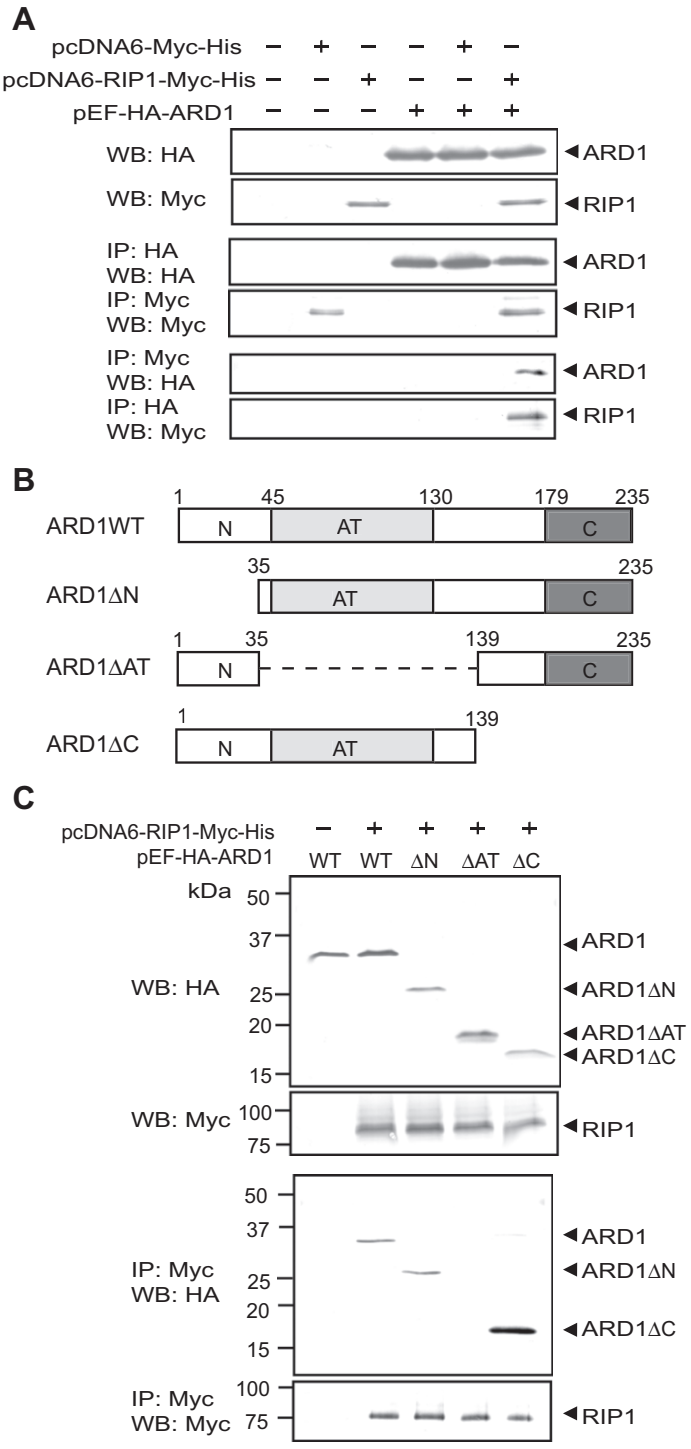

Fig. 2. ARD1 co-precipitated with RIP1. (A) HEK293 cells were transfected with pEF-HA-ARD1 and either pcDNA6-RIP1-Myc-His or a mock vector. Expression of ARD1 and RIP1 was confirmed with anti-HA and anti-Myc antibodies, respectively (upper two panels). Immunoprecipitated ARD1 and RIP1 were confirmed with anti-HA (third panel) and anti-Myc antibodies (fourth panel), respectively. Co-precipitated ARD1 or RIP1 was monitored by Western blotting using anti-Myc or anti-HA antibodies, respectively (lower two panels). (B) Schematic diagram of the human ARD1 domain structure and its deletion mutants. In the acetyltransferase domain, the acetyl-CoA binding site (RRLGLA) is present from amino acid residues 82 to 87 and from 78 to 83, respectively. ARD1WT, ΔN, ΔAT, and ΔC indicate expressed proteins of wild type, N-terminal-deleted, acetyltransferase domain-deleted, and C-terminal-deleted ARD1. (C) Co-immunoprecipitation assays using RIP1 and deletion mutants of ARD1 were performed. Expression vectors encoding HA-tagged ARD1, ARD1ΔN, ARD1ΔAT, or ARD1ΔC and Myc-tagged RIP1 were transfected into HEK293 cells. Expression of ARD1 mutants and RIP1 was confirmed with anti-HA and anti-Myc antibodies, respectively (upper two panels). Co-precipitated ARD1 with Myc-tagged RIP1 was monitored by Western blotting using anti-HA (third panel). Immunoprecipitated RIP1 was confirmed with anti-Myc (fourth panel).

acetyltransferase activity of ARD1 is involved in doxorubicin-induced NF-κB activation.

Trichostatin A is a well-known histone deacetylase inhibitor. This inhibitor was also used to enhance ARD1-mediated acetylation of HIF-1α [13]. Though target molecule of ARD1-mediated acetylation in doxorubicin-induced NF-κB activation has not been determined yet, trichostatin A seemed to function as a deacetylation inhibitor against substrates of ARD1. RIP1 is thought to be a

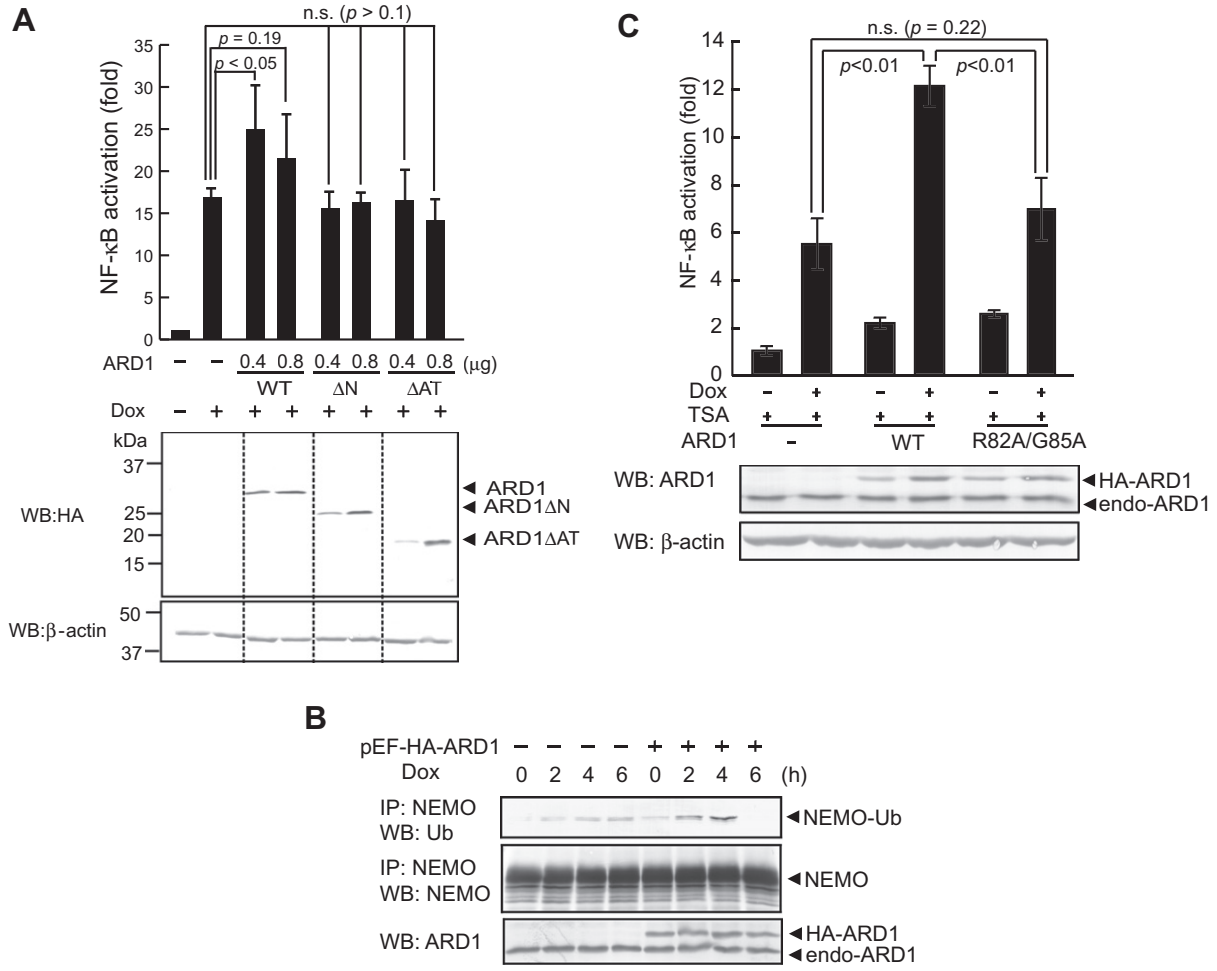

Fig. 3. The acetyltransferase activity of ARD1 is required for doxorubicin-induced NF-κB activation. (A) Each expression vector encoding either HA-tagged ARD1, ARD1ΔN or ARD1ΔAT was transfected into HEK293 cells along with p[kB3]-tk-Luc and pCMV-LacZ plasmids. Twenty-four hours after transfection, cells were treated with 0.5 μM of doxorubicin (Dox) for 12 h and NF-κB activation was measured (upper panel). Expression of each ARD1 mutant was confirmed by Western blotting using anti-HA antibody (lower). The measurement of luciferase activity and Western blotting are described in the legend of Fig. 1. Results shown represent the means of triplicate experiments and error bars indicate SD. (B) HEK293 cells were transfected with pEF-HA-ARD1 or a mock vector. Forty-eight hours after the transfection, cells were stimulated with 3.2 μM doxorubicin for 0, 2, 4 or 6 h, respectively. Expression of HA-tagged and endogenous ARD1 were monitored by Western blotting using anti-ARD1 antibody (lower panel). Immunoprecipitated NEMO was confirmed with anti-NEMO antibody (middle panel). Ubiquitinated NEMO (NEMO-Ub) was monitored by using anti-ubiquitin antibody (upper panel). (C) Forty-eight hours after transfection of pEF-HA-ARD1 (WT), pEF-HA-ARD1(R82A/G85A), or mock (−) plasmids along with reporter plasmid into HEK293 cells, cells were treated with 0.5 μM doxorubicin and 1 μM trichostatin A (TSA) for 6 h and NF-κB activation was measured by luciferase assay. Results shown represent the means of triplicate experiments and error bars indicate SD.

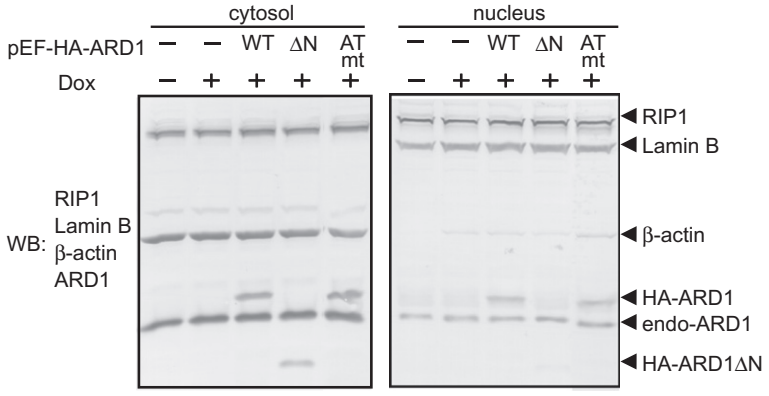

Fig. 4. Subcellular localization of ARD1 and its mutants after doxorubicin treatment. HEK293 cells were transfected with expression vector encoding either HA-tagged ARD1 (WT), ARD1ΔN (ΔN), or ARD1(R82A/G85A) (ATmt), respectively. Forty-eight hours after the transfection, cells were treated with 3.2 μM doxorubicin for 3 h. The cells were fractionated into cytosol and nuclear fractions and analyzed by Western blotting using anti-RIP1, anti-Lamin B, anti-β-actin, and anti-ARD1 antibodies. β-Actin and Lamin B are markers for cytosolic and nuclear proteins, respectively.

convincing candidate for ARD1-mediated acetylation because we demonstrated that RIP1 associated with ARD1 in this study (Fig. 2A). Identification of a target protein of ARD1-mediated acetylation and mutual interaction between ARD1, RIP1, PIDD and NEMO should be investigated in detail in the near future.

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