Amino-terminal domain of ATRIP contributes to intranuclear relocation of the ATR–ATRIP complex following DNA damage

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Abstract ATM and rad3-related protein kinase (ATR), a member of the phosphoinositide kinase-like protein kinase family, plays a critical role in cellular responses to DNA structural abnormalities in conjunction with its interacting protein, ATRIP. Here, we show that the amino-terminal portion of ATRIP is relocalized to DNA damage-induced nuclear foci in an RPA-dependent manner, despite its lack of ability to associate with ATR. In addition, ATR-free ATRIP protein can be recruited to the nuclear foci. Our results suggest that the N-terminal domain of the ATRIP protein contributes to the cell cycle checkpoint by regulating the intranuclear localization of ATR.

Keywords: ATR; ATRIP; Checkpoint; Genotoxic stress; Nuclear foci

1. Introduction

To maintain the integrity of genomic information in organisms, elaborate devices have been developed that ensure the ordered progression of cell cycle events. The DNA damage checkpoint system is one such device; it executes functions that protect cells from tumorigenesis and death. Recent studies revealed that this checkpoint is highly conserved from yeasts to mammals and requires the participation of a family of checkpoint kinases that share homology with phosphatidylinositol 3-kinase.

In mammals, two structurally related checkpoint kinases, ATM and ATR, are critical for cellular responses to altered DNA structures, such as breaks or modifications [1,2]. It is believed that ATM and ATR act in parallel pathways that respond to different DNA stresses, including double-strand breaks (DSBs) and replication blockage [3]. Both proteins show an affinity for DNA in vitro [4,5] and a significant fraction of each protein is recovered in the chromatin fraction [6–8]. ATM and ATR are members of a multi-protein complex that form large nuclear foci when cells are treated with reagents that interfere with DNA synthesis, and are thought to serve as sensors for DNA damage.

The yeast orthologs of ATR, Rad3 in *Schizosaccharomyces pombe* and Mec1 in *Saccharomyces cerevisiae*, are present as complexes with the partner proteins Rad26 and Ddc2/Lcd1/Pie1, respectively [9–12]. ATRIP was isolated as an ATR-associating protein [13] and is thought to be a mammalian counterpart of Rad26 and Ddc2. Based on observations that a mutation in a yeast ATR ortholog led to phenotypes identical to those produced by a loss of its associating protein, it is presumed that the two proteins constitute a functional complex [14]. Ddc2 displays DNA binding activity in vitro [15] and ATRIP is capable of binding to RPA-coated single-stranded DNA [16], suggesting that these partner proteins assist in recruiting the ATR kinase complex to damaged sites in yeasts. It has been shown that Rad26 and Ddc2 are required for kinase activity of the ATR orthologs, both in vivo and in vitro [14,17]. In addition, data have been accumulated that show a potential role of ATRIP in ATR-mediated phosphorylation of RPA and MCM [18,19].

In this work, we examine the domains of ATRIP that are implicated in the action of ATR upon genotoxic stresses. We show that the amino- and carboxyl-terminal regions contribute separately to intranuclear localization of the ATR–ATRIP complex. We discuss the roles of two functional domains of ATRIP involved independently in the ATR-mediated process.

2. Materials and methods

2.1. Antibodies

The following antibodies were used: N-19 (Santa Cruz Biotech) and ab-2 (Oncogene) for ATR, 9E10 (Babco) and #2272 (Cell Signaling Technology) for myc, NA13 (Oncogene) for RPA p70, 9H8 (Neo-Markers) for RPA p34, and ac-15 (Sigma) for β-actin.

To prepare a rabbit polyclonal antibody against the carboxyl terminus of ATRIP (ATRIP-C), the 0.5-kb *Pvu*II–*Xho*I fragment of ATRIP cDNA was inserted into the *Sma*I and *Xho*I sites of pGEX4T-2 to yield the pGST-ATRIP-C plasmid. The GST-ATRIP-C fusion protein, which was purified from *Escherichia coli* harboring pGST-ATRIP-C, was used for immunization. A rabbit polyclonal antibody against the amino-terminal domain of ATRIP (ATRIP-N) was raised using the synthetic peptide GTGHPPSKRARGFS, which corresponds to amino acid residues 25–38 of ATRIP. The antibodies were used after affinity purification.

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2.2. Construction of plasmids

A retroviral vector derived from the murine leukemia virus pMX-puro [20] was kindly provided by Prof. T. Kitamura (University of Tokyo). A *Bam*HI–*Xho*I fragment of hATRIP full-length cDNA was inserted into the *Bam*HI–*Xho*I sites of pMX-puro. To insert carboxyl-terminal tags into ATRIP, an *Xho*I site was created immediately before the termination codon, and a tag sequence from pEF4-mycHis (Invitrogen) or pEGFP-c1 (Clontech) was inserted. Retrovirus-based vectors expressing either the ATRIP N-terminal fragment (1–298 amino acid) containing the coiled-coil domain tagged with the myc epitope (N32-myc), or the C-terminal fragment without the coiled-coil domain (198–791 amino acid) tagged with the myc epitope (C64-myc) were constructed by creating restriction sites using PCR, followed by insertion of the fragment into the pMX-puro vector.

2.3. Western blotting and immunoprecipitation

For Western blot analysis, 1×10^6 cells were lysed in 100 μl of alkaline extraction buffer (0.1 N NaOH, 0.5% 2-mercaptoethanol) at 4 °C for 15 min and 1 ml of chilled acetone was added. After storage at −20 °C for 1 h, the cell lysates were centrifuged at $13\,000 \times g$ for 10 min, and the pellets were washed with 1 ml of chilled acetone and subjected to SDS–PAGE.

For immunoprecipitation, the cells were lysed in lysis buffer [50 mM Tris–HCl, 150 mM NaCl, 1% Tween 20, 0.3% NP-40, 1 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 0.5 mM cantharidin, and 1× complete mini (Roche)] at 4 °C for 30 min and the supernatant was recovered after centrifugation at $13\,000 \times g$ for 30 min. Lysates were incubated with each antibody at 4 °C for 1 h and for an additional 1.5 h with protein G-Sepharose (Amersham Biosciences). The immunoprecipitate was washed five times with wash buffer containing 50 mM Tris–HCl, 150 mM NaCl, and 1 mM NaF, and analyzed by SDS–PAGE.

2.4. Immunofluorescence

Immunofluorescence of cells grown on glass coverslips was performed as described previously [21]. Briefly, cells grown on glass coverslips were washed with PBS, fixed with 4% paraformaldehyde in PBS for 10 min, and permeabilized with 0.5% Triton X-100 in PBS for 5 min. The coverslips were blocked with 10% FCS in PBS, incubated with a primary antibody at RT for 1 h, washed with PBS three times, and incubated with a secondary antibody labeled with AlexaFluor 488 or AlexaFluor 594 (Molecular Probes Inc.). Slides were examined on a confocal microscope (BX-FLA, Olympus) equipped with epifluorescence.

2.5. Small interference RNA

Transfection of dsRNA into HeLa cells was carried out using Oligofectamine (Invitrogen) according to the manufacturer's instructions. Small interfering RNA (siRNA) duplexes contained 21 base pairs, including a 2-deoxynucleotide overhang. For ATRIP, the RNA coding sequence is 5′-GGUCCACAGAUUAUUAGAUdTdT-3′ [13], and for RPA70, it is 5′-CACUCUAUCCUCUUUCAUGdTdT-3′ [16]. Control double-stranded siRNA for GFP was purchased from Qiagen. The cells were analyzed 3–4 days after the first transfection.

2.6. Transfection and retroviral infection

Retroviral gene delivery was performed as described previously [21]. Pantropic retroviral packaging was carried out using a pVSV-G plasmid (Stratagene). Infection frequencies were typically 10–30% for HeLa cells and 70–90% for A549 cells.

3. Results

3.1. The carboxyl-terminal region of ATRIP contributes to the interaction with ATR

ATRIP consists of three regions, namely the amino-terminal region, the coiled-coil domain in the middle, and the carboxyl-terminal region. To determine which ATRIP domain interacts with ATR, we expressed a truncated version of the ATRIP protein using the retroviral promoter. The truncated products, tagged with the myc epitope, encompassed the amino terminus plus the coiled-coil domain (N32-myc) or the carboxyl

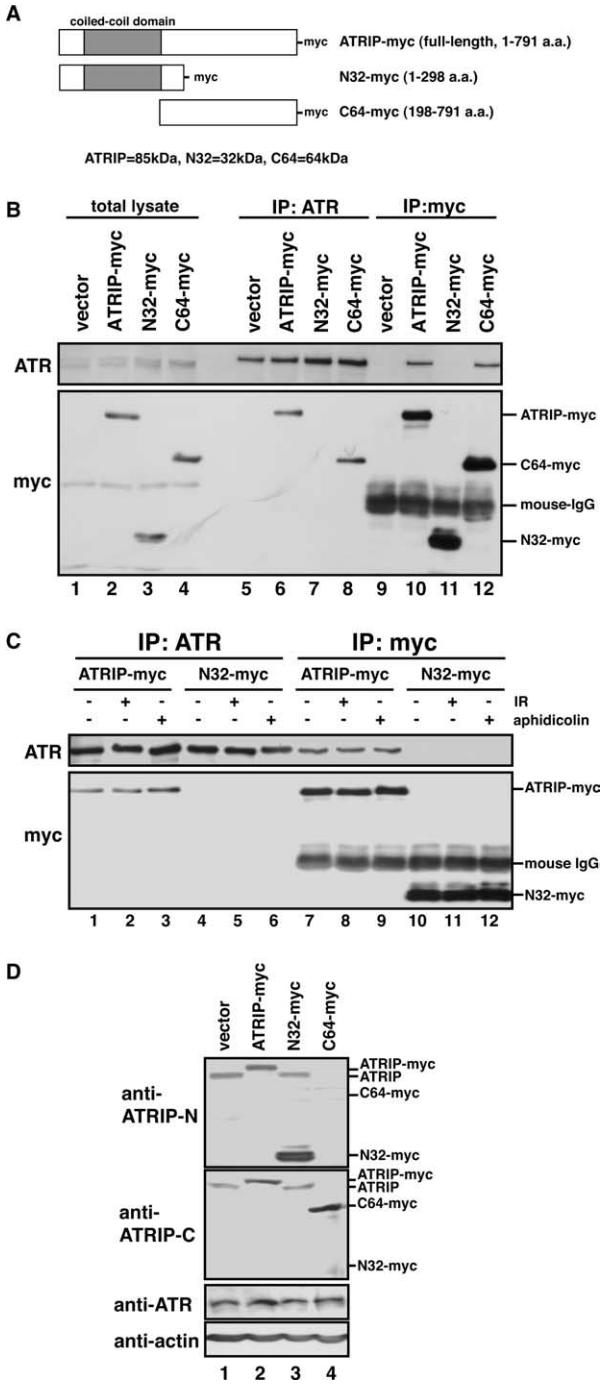

Fig. 1. ATRIP associates with ATR through its C-terminal region. (A) Truncated forms of ATRIP. (B) Co-immunoprecipitation of ATR with truncated ATRIP proteins. A549 cells infected with a control vector or a retrovirus harboring ATRIP-myc, N32-myc, or C64-myc were collected, and the lysates were analyzed by immunoprecipitation using anti-ATR or anti-myc antibody. The immunoprecipitates were subjected to Western blot analysis using anti-ATR or anti-myc antibody. (C) The ATRIP N-terminal fragment does not associate with ATR after DNA damage. Cells expressing full-length ATRIP-myc or N32-myc and a control vector were subjected to 10 Gy of X-irradiation or 10 μg/ml aphidicolin. Cell lysates were prepared 1 h after irradiation or 24 h after adding aphidicolin and then immunoprecipitated using anti-ATR or anti-myc antibody. (D) Effect of the truncated forms of ATRIP on the endogenous expression of ATRIP. Lysates of A549 cells expressing ATRIP-myc, N32-myc, or C64-myc were collected, and the cell lysates were analyzed with Western blotting using anti-ATRIP-N, anti-ATRIP-C, anti-ATR, or anti-β-actin antibody.

terminus (C64-myc; Fig. 1A). We found that ATR and ATRIP immunoprecipitated each other reciprocally (Fig. 1B), confirming previous observations that ATR and ATRIP form a complex [13]. Moreover, we found that C64-myc, but not N32-myc, retained the ability to interact with ATR (compare lanes 8 and 12 with lanes 7 and 11). We also performed a co-immunoprecipitation assay to examine whether the association between ATR and N32-myc changed under conditions in which ATR–ATRIP was relocated to nuclear foci. As shown in Fig. 1C, the association between ATR and ATRIP was unchanged after IR irradiation or aphidicolin treatment; moreover, N32-myc did not co-immunoprecipitate with ATR under any conditions tested.

A previous study showed that the amounts of ATR and ATRIP were regulated interdependently in vivo [13]. We found that expression of the full-length ATRIP-myc, as well as that

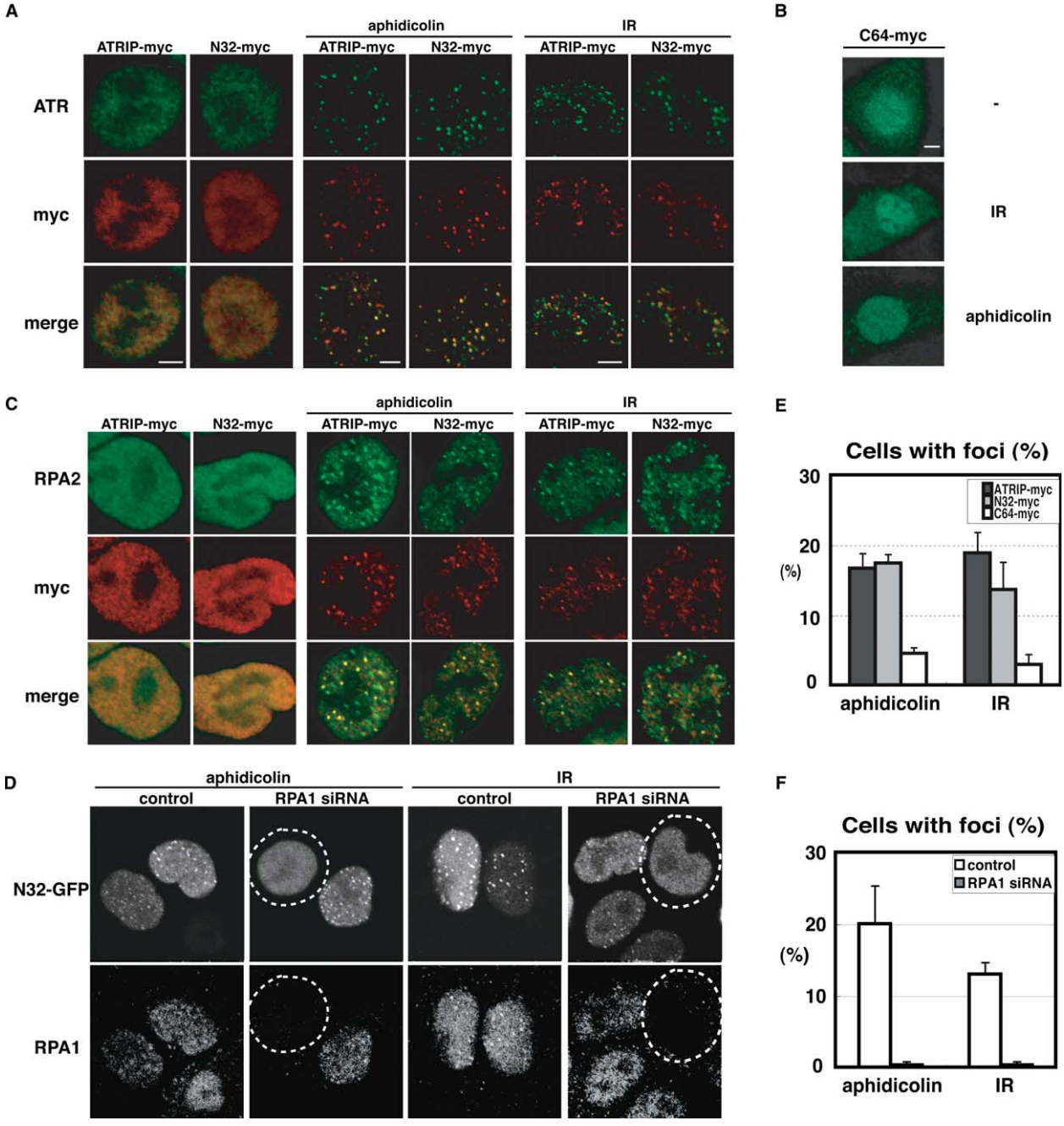

Fig. 2. RPA-dependent focus formation of the ATRIP N-terminus with DNA damage. (A–D) A549 cells expressing truncated versions of ATRIP were treated with 10 Gy of X-irradiation or with 10 μg/ml aphidicolin and then fixed in paraformaldehyde after 1 or 24 h, respectively. The cells were immunostained with anti-ATR, anti-myc, or anti-RPA p34 antibodies. In (C), the cells were transfected with siRNA against RPA p70 and after 3 days the cells were subjected to genotoxic stress. The cells used were (A and C) A549 with full-length ATRIP-myc or N32-myc, (B) A549 with C64-myc, and (D) A549 with N32-GFP. Dotted circles indicate cells with RPA levels that decreased with siRNA. Bar: 10 μm. (E and F) The proportion of 200 cells harboring anti-myc-stained foci in A and B, or that harboring GFP foci in D. With RPA siRNA columns, the proportion of focus-forming cells that lost the RPA signal was calculated. The data are shown as averages ± S.D. of three independent experiments.

of the C64-myc, downregulated endogenous ATRIP (Fig. 1D). The finding that the carboxyl-terminal region readily associates with ATR suggests that the fraction of ATRIP protein associated with ATR is a determinant of the level of endogenous ATRIP protein.

3.2. The amino terminus of ATRIP contributes to relocalization to nuclear foci after DNA damage

It has been reported that ATRIP formed nuclear foci together with ATR after DNA damage [13]. The truncated ATRIP protein N32-myc was defective in its association with ATR (Fig. 1B) and consequently it did not show apparent colocalization with ATR under normal conditions (Fig. 2A). Interestingly, we found that after DNA damage N32-myc formed foci efficiently, comparable to full-length ATRIP protein with the carboxyl-terminal myc tag. The foci co-stained for BRCA1 and endogenous ATR (Fig. 2A and E, and data not shown). By contrast, the carboxyl-terminal fragment C64-myc was mainly localized in the nucleus, but was defective in focus formation (Fig. 2B and E). Since N32-myc did not interact with ATR under any conditions tested (Fig. 1C), this suggests that the N-terminal region of ATRIP is sufficient for relocalization to nuclear foci and that the relocalization is not mediated by the direct association with ATR.

3.3. Relocalization of the ATRIP N-terminus to nuclear foci is RPA-dependent

ATRIP can bind the chromatin *via* RPA-dependent and -independent mechanisms [8,16,22]. As previously reported [16], we observed that RPA and the full-length ATRIP were recruited to the same sites of DNA damages. In addition, colocalization with RPA was apparent in case of N32-myc (Fig. 2C). As formation of the ATRIP foci depends on normal RPA function [16], we examined the possible contribution of RPA to DNA damage-induced relocation of the ATRIP N-terminal fragment using the RNA interference technique.

As shown in Fig. 2D and F, the formation of N32-GFP foci after treatment with aphidicolin or IR was completely abrogated in the RPA knockdown cells. This indicates that the ATRIP N-terminus requires the RPA function to relocalize to nuclear foci after DNA damage.

3.4. ATRIP can be relocated to nuclear foci in the absence of ATR

To further analyze ATRIP function independent of ATR, we used cells that expressed ATRIP from the viral promoter. The cellular amounts of ATR and ATRIP were co-regulated, and the siRNA-mediated knockdown of either ATR or ATRIP caused a reduction in the amount of the other protein [13]. By contrast, we found comparable amounts of the viral promoter-driven ATRIP, even when the expression of ATR was effectively repressed (Fig. 3A and B). Therefore, this system provides the advantage of stable ectopic ATRIP expression in the ATR-knockdown condition.

When HeLa cells expressing ATRIP-myc were transfected with ATR siRNA, immunofluorescent signals specific to ATR were lost in approximately 70% of the cells within 3 days. In the ATR knockdown cells, ATRIP-myc protein was expressed

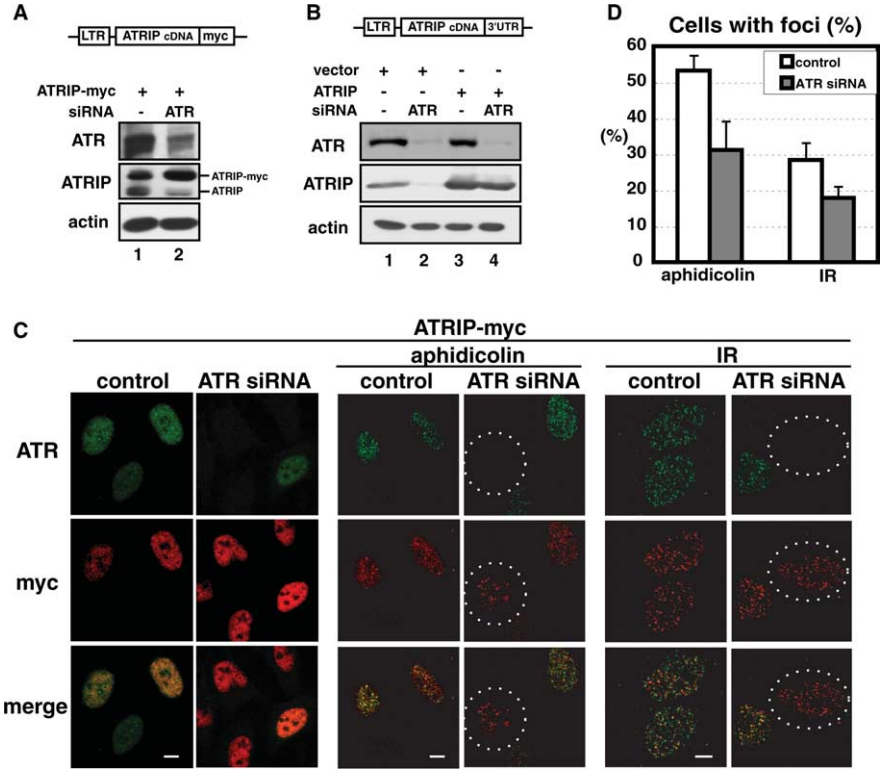

Fig. 3. ATR-independent focus formation of ATRIP. (A and B) Exogenously expressed ATRIP is not subject to quantitative regulation. ATRIP-myc or ATRIP without the myc tag was expressed with a retroviral promoter in HeLa cells. The cells were analyzed 3 days after ATR siRNA transfection. (C) ATRIP forms foci at sites of DNA damage under ATR-repressed conditions. HeLa cells expressing ATRIP-myc were transfected with ATR siRNA and after 3 days the cells were treated with 10 Gy of X-irradiation or with 10 μg/ml aphidicolin. The cells were fixed and stained with anti-ATR and anti-myc. Dotted circles indicate cells with ATR levels that decreased with siRNA in which exogenously expressed ATRIP-myc still formed foci. Bar, 10 μm. In (D), the proportion of cells harboring the myc foci was recorded as in Fig. 2.

in abundance compared with non-repressed cells (Fig. 3C). Furthermore, even in the absence of ATR, ATRIP formed foci when cells were treated with 10 μg/ml aphidicolin or 10 Gy IR (Fig. 3C). These results show that ATRIP alone has the potential to relocalize to nuclear foci after DNA damage. Nevertheless, siRNA-mediated knockdown of ATR reduced the frequency of ATRIP foci formation significantly both after aphidicolin treatment and after IR irradiation (Fig. 3D). Therefore, although ATR activity is not required absolutely for ATRIP focus formation, it may contribute to the stability of the foci after they are formed.

4. Discussion

In this study, we focused on the roles of the associating protein ATRIP in the stability and dynamic relocation of ATR. Our results highlight the physiological significance of two functional interfaces of ATRIP: the amino-terminal region for dynamic relocation of the ATR kinase complex after DNA damage and the carboxyl-terminal region for interaction with ATR.

We found that the endogenous expression of ATRIP was decreased by the ectopic expression of ATRIP. This repression appears to be achieved post-transcriptionally, since mRNA levels of endogenous ATRIP were comparable in the ATRIP-myc-expressing cells (data not shown). As reduction of endogenous ATR has already been shown to decrease endogenous ATRIP in a post-transcriptional mechanism [13], it seems that one of the most likely explanations of these results is that ATRIP must bind to ATR to be stable.

Using the RNA interference technique, we demonstrated that ATR-free ATRIP had the ability to be localized to damage-induced foci in vivo. A previous study showed that kinase-negative ATR failed to relocalize to damage-induced foci [23]. This observation does not necessarily contradict our results because our study suggests that ATR can contribute substantially to the efficiency of ATRIP focus formation. A significant portion of ATRIP exists free of ATR in cell lysates [8,18]. Moreover, ATR and ATRIP were easily separated at high ionic strength [5], and they were in equilibrium between association and dissociation at physiological ionic strength [18]. These biochemical characteristics are compatible with a model in which a fraction of ATRIP is first relocalized to foci to which ATR is subsequently targeted. Alternatively, ATR–ATRIP might be recruited to sites of damage as a complex and then partners are exchanged through dissociation-association events. In either case, the recruited ATR might modify the biochemical properties of ATRIP (or the ATR–ATRIP complex) or those of other proteins recruited to the sites of damage; this might in turn contribute to the stabilization or modulation of the protein complex. RPA is one candidate protein with a function that is modulated by ATR; RPA is involved in the efficient loading of the ATR–ATRIP complex to chromatin and is phosphorylated by ATR [16,23]. The histone H2A variant H2AX is another candidate, as it is phosphorylated by ATR and contributes to the accumulation of ATR at the lesion sites [24,25].

We showed that the amino-terminal portion of ATRIP (N32-myc) was relocalized to DNA damage-induced foci containing endogenous ATR–ATRIP complex. As no direct association of the N32-myc fragment with ATR was evident, we surmise that the amino terminus of ATRIP has the potential to relocate to the multiprotein complex formed at the site of DNA damage and the recruitment is independent of ATR. Although ATRIP interacts with RPA and MCM7 [18,19], we could not detect direct interaction between N32-myc and RPA or MCM7 through a co-immunoprecipitation experiment (data not shown). Further studies are required to elucidate the mechanism that directs the N-terminal domain-mediated recruitment of ATRIP to nuclear foci.

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