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Identification of p32 as a novel substrate for ATM in heart

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Abstract

Chemotherapeutic agents to induce DNA damage have been limited to use due to severe side effects of cardiotoxicity. ATM (Ataxia–telangiectasia mutated) is an essential protein kinase in triggering DNA damage responses. However, it is unclear how the ATM-mediated DNA damage responses are involved in the cardiac cell damage. To elucidate these functions in heart, we searched for specific substrates of ATM from mouse heart homogenate. Combining an in vitro phosphorylation following anion-exchange chromatography with purification by reverse-phase high-performance liquid chromatography (HPLC), we successfully identified p32, an ASF/SF2-associated protein, as a novel substrate for ATM. An in vitro kinase assay using recombinant p32 revealed that ATM directly phosphorylated p32. Furthermore, we determined Ser 148 of p32 as an ATM phosphorylation site. Since p32 is known to regulate mRNA splicing and transcription, p32 phosphorylation by ATM might be a new transcriptional regulatory pathway for specific DNA damage responses in heart.

Keywords: Protein kinase; ATM; High-performance liquid chromatography; p32; In vitro kinase assay

DNA double-strand breaks (DSBs) that arise from ionizing radiation or chemotherapeutic agents are the most severe threat to the genomic integrity in eukaryotic cells. One of the principal molecules concerned with DSBs is the ATM gene, which is mutated in individuals of Ataxia–telangiectasia, and it encodes a serine/threonine protein kinase that belongs to phosphatidylinositol-3-phosphate kinase-related kinase (PIKK) family [1]. ATM protein exists as an inactive dimer (or higher-order oligomer) in unstressed cells and DNA damage causes dissociation of this dimer. Dissociation requires both kinase activity and intermolecular autophosphorylation of both ATM proteins on Ser 1981 [2]. ATM acts as a central transducer of DSB signals via phosphorylating key effector substrates including the tumor suppressor p53, breast cancer-associated gene-1 (BRCA1), and Checkpoint kinase 2 (Chk2) to initiate the cell cycle arrest or apoptosis [3]. The molecular function of ATM is not thought to be restricted to these responses. Other ATM substrates were identified with a role for chromatin remodeling [4], or gene transcription [5,6], but their function has not been fully elucidated. Many responses that appear to be dependent on ATM are likely to involve in unknown substrates of ATM. Therefore, it is necessary to identify the specific substrates of ATM directly linked to these functions.

Adriamycin is highly effective chemotherapeutic agent used to treat a wide variety of tumors, but its usage is limited due to its dose-related cardiotoxicity [7]. The major tumor suppressing effects arise from the induction of DSBs in cancer cells; however, these effects have not been studied well in cardiomyocytes. Adriamycin-induced cardiac cell

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death was previously reported to be induced by p53 activation [8] and/or the generation of reactive oxygen species [9,10]. Although it has been demonstrated that adriamycin induces phosphorylation of p53 at Ser 15, which is targeted by ATM [11–13], it is unclear whether the cardiac cell death is mediated by ATM-induced phosphorylation of p53 and/or of other molecules yet to be identified.

In the present study, we screened de novo substrates for ATM in mouse heart in combination with an in vitro kinase assay and the unique purification techniques using two-step column chromatography. In consequence, we identified p32, which was initially identified as an ASF/SF2 splicing factor-associated protein, as a novel ATM substrate.

Materials and methods

Cell culture and transfection. 293T cells were obtained from American Type Cell Culture and maintained with DMEM supplemented with 10% fetal bovine serum (FBS). Lipofectamine 2000 or Optifect (Invitrogen) was used to all transfection experiments according to manufacturer's instructions.

Plasmids. pcDNA3/Flag-ATM wild-type (WT) and kinase-dead (KD) plasmids were kindly gift from M.B. Kastan [12]. The coding sequence of mouse p32 was amplified by PCR from mouse heart cDNA library and subcloned into pENTR/D-TOPO vector (Invitrogen), named pENTR/p32. Glutathione-*S*-transferase (GST)-fusion p32 constructs was generated as follows: mouse mature p32 corresponding to aa 72–279 from pENTR/p32 was amplified by PCR and subcloned into the bacterial expression vector pGEX-5X-2 (GE Healthcare). For expression in mammalian cells, pENTR/p32 plasmid was recombined into pcDNA3.1/Flag vector. The constructs that replaced various serine residues to alanine in this study were generated by PCR-based mutagenesis using pENTR/p32 plasmid as a template. GST-p53 expression construct was generated as described previously [12]. All constructs were verified by sequencing.

Recombinant proteins. Recombinant Flag-ATM and Flag-p32 was purified as follows: 293T cells transfected Flag-ATM WT or KD, or Flag-p32 plasmids were lysed with Buffer A consisting of 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Tween-20, 0.3% NP-40, and protease inhibitor cocktail (Nacalai) and immunopreciptated with anti-FLAG M2 agarose (Sigma) at 4 °C for 1 h. The beads were washed twice with Buffer A and three times with Buffer A containing 0.5 M LiCl, and then Flag-ATM was eluted with Buffer B (25 mM Tris–HCl, pH 8.0/100 mM NaCl/10% glycerol/1% Tween-20/2 mM DTT) containing 0.25 mg/ml Flag peptide at 4 °C for 1 h. After centrifugation, the supernatant was used as recombinant ATM protein. The purity of recombinant ATM was analyzed by SDS–PAGE and Coomassie staining (Bio-Rad). For preparation of GST-fusion protein, the expression plasmid pGEX-5X-p32,was transformed into DH5α cells and the protein expression was induced by incubating the bacteria with 1 mM isopropyl-β-thiogalactopyranoside for 2 h at 37 °C. The cells were collected by centrifugation and lysed by sonication in phosphate-buffered saline (PBS) containing 1 mM EDTA, 1% Triton-X 100, and protease inhibitor cocktail. The cell lysates were pulled down with glutathione sepharose 4 Fast Flow (GE Healthcare) at 4 °C for 1 h. After extensive washing, proteins were eluted with 10 mM reduced glutathione, and dialyzed against kinase buffer (as described below).

Screening of ATM substrates. One C57BL/6J mouse was killed by cervical dislocation, and its heart was perfused with ice-cold PBS, removed and washed with ice-cold PBS twice. The tissue was homogenated using Polytron homogenizer in homogenize buffer consisting of 30 mM Mops (pH 7.5), 10% glycerol, 2 mM EDTA, 1 mM DTT, 50 mM β-glycerophosphate, 10 mM sodium fluoride, 0.02% Tween-20 and protease inhibitor cocktail (Nacalai), and then NP-40 and Tween-20 were added to the homogenates at a final concentration of 0.3% and 1%, respectively, and incubated at 4 °C with gentle rocking for 1 h. The homogenates were centrifuged at 16,000*g* for 30 min. The supernatant was filtered and loaded onto Super Q-5PW (7.5 × 75 mm, TOSOH) anion-exchange column pre-equilibrated with Column Buffer consisting of 30 mM Mops (pH 7.5), 10% glycerol and 0.02% Tween-20. After washing with Column Buffer, proteins were eluted with a linear gradient of NaCl (0–1 M) at a flow rate of 0.5 ml/min. Fractions (0.5 ml each) were collected, and aliquot of each fraction (50 μl) was analyzed with an in vitro kinase assay as described below.

Purification by reverse-phase HPLC. The active fractions containing the 30 kDa of phosphorylated band after an in vitro kinase assay was concentrated with acetone precipitation and reconstituted with 0.3% trifluoroacetic acid (TFA) and 5% acetonitrile. The samples were then applied to a phenyl reverse-phase HPLC column (4.6 × 50 mm, Nacalai) pre-equilibrated with 0.1% TFA. Fractions were eluted with a linear gradient of 5–80% acetonitrile at a flow rate of 0.5 ml/min. Each fraction was lyophilized and separated by SDS–PAGE, and the gels were stained with silver and analyzed with autoradiography. For identification of the amino acid sequence, TFA and acetonitrile were added to the active fractions from Super Q-5PW column at a final concentration of 0.3% and 5%, respectively. These samples were then applied to the reverse-phase HPLC column. Fractions were eluted and analyzed as described above. The band corresponding to the phosphorylated protein was excised, digested with trypsin, and analyzed by matrix-assisted laser desorption/ionization and time-of-flight mass spectrometry (MALDI-TOF/MS) (Hitachi High-Tech Manufacturing and Service Co., Ltd.).

In vitro kinase assay. GST-p32 purified from bacteria or Flag-p32 purified from 293T cells was incubated with 10 μCi [γ-^{32}P]ATP and purified Flag-ATM in kinase buffer containing 30 mM Mops (pH 7.5), 50 mM NaCl, 10% glycerol, 0.02% Tween-20, 1 mM DTT, 10 mM $MgCl_2$, 10 mM $MnCl_2$, and 5 μM ATP at 30 °C for 30 min, and then analyzed by SDS–PAGE, stained with Coomassie, and autoradiography.

Phosphoamino acid analysis. Phosphoamino acid analysis was performed as described previously [14]. Briefly, GST-p32 was incubated with Flag-ATM in the presence of [γ-^{32}P]ATP as described above. The reaction mixture was then separated by SDS–PAGE, stained with Coomassie, and visualized by autoradiography. The radiolabeled band was excised from the gel and digested with trypsin. The digested sample was partially hydrolyzed by boiling in 6 M HCl for 60 min at 110 °C. The hydrolysate was lyophilized and resuspended in 5% TFA, 50% acetonitrile containing phosphoamino acid standards, and spotted onto thin-layer cellulose plates. Electrophoresis was performed using pH 1.9 buffer for the first dimension and pH 3.5 buffer (5% acetic acid, 0.5% pyridine) for the second dimension. The standards were then stained with ninhydrin, and the plates were analyzed by autoradiography.

Results

Screening of novel substrates for ATM

To search for novel substrates of ATM, mouse heart homogenate was initially fractionated with a Super Q-5PW anion-exchange chromatography (Fig. 1). Each fraction was incubated with [γ-^{32}P]ATP in the presence of purified Flag-tagged ATM wild-type (WT) or kinase-dead (KD). Among several ^{32}P-incorporated bands detected in Flag-ATM WT, but not in KD, we focused on approximately 30 kDa phosphorylated band (designated as AP30, ATM-phosphorylated 30 kDa protein) eluted at high salt concentration (~0.7 M) (Fig. 1B, fraction 44–50) because other bands was thought to be matched to the molecular weight of proteins that were previously identified as ATM substrates.

Purification and identification of AP30

Next, we further purified these ^{32}P-incorporated fractions using a reverse-phase high-performance liquid chro-

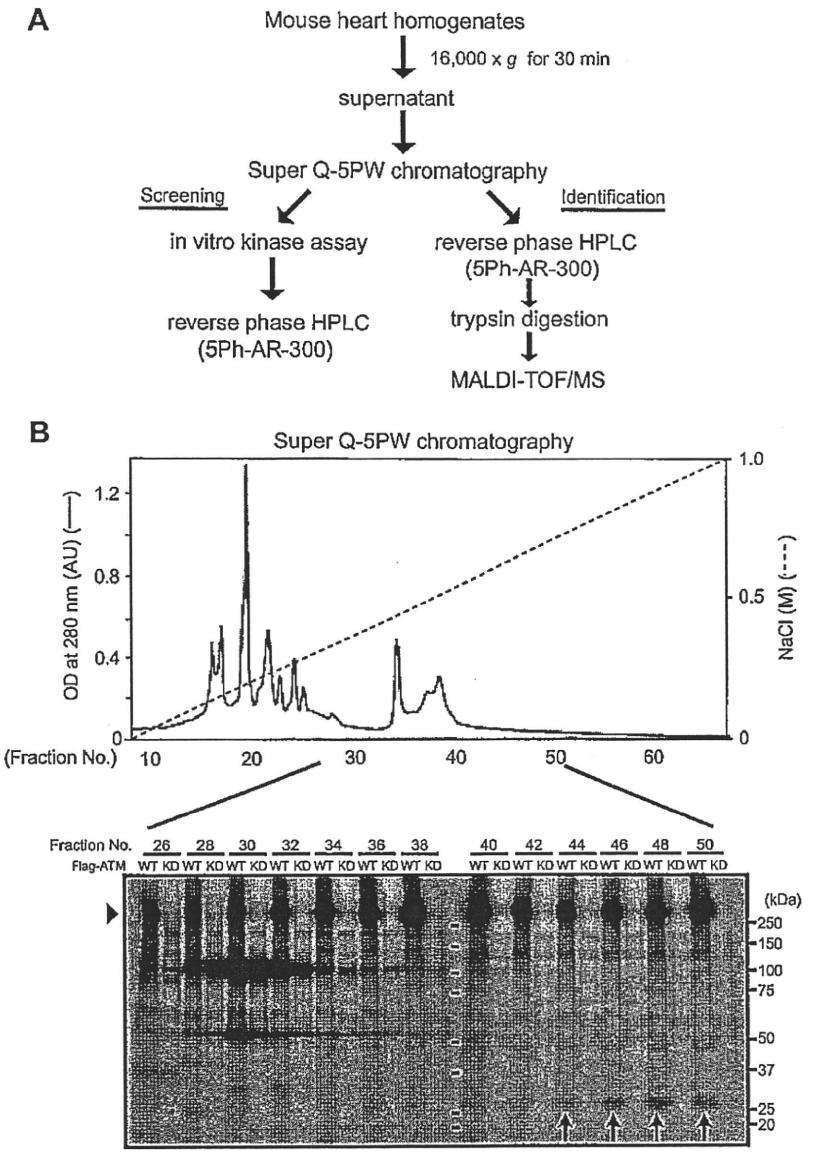

Fig. 1. Screening of novel substrates for ATM from mouse heart. (A) The strategy for identification of the novel substrates for ATM is shown. (B) Mouse heart homogenate was loaded onto a Super Q-5PW column. The protein was eluted with a linear gradient of 0–1 M NaCl (upper panel). Each fraction was incubated with recombinant Flag-ATM WT or KD in the presence of [γ-^{32}P]ATP, and then ^{32}P-incorporated fraction was separated by SDS–PAGE and visualized by autoradiography (lower panel). Arrowhead indicates the autophosphorylation of ATM. Arrows indicate AP30 (see the text).

matography (HPLC) (Fig. 1A). These fractions were precipitated with acetone, resuspended in 0.3% TFA and 5% acetonitrile, and applied to a reverse-phase phenyl HPLC column (Fig. 2A). After eluting with a linear gradient of acetonitrile, each fraction was analyzed by SDS–PAGE, silver staining, and autoradiography. ^{32}P-incorporated AP30 protein bands were only detected in fraction 49 and 50 of HPLC, which were matched with those of silver stained-gel (Fig. 2B). To characterize AP30, the AP30-containing fractions from Super Q-5PW column were directly applied to the reverse-phase phenyl HPLC column without an in vitro phosphorylation. The protein band corresponding to AP30 could be detected in the same fraction when in vitro phosphorylation was performed in the presence of [γ-^{32}P]ATP (Fig. 2C). The band was excised, subjected to in-gel digestion with trypsin and analyzed by MALDI-TOF/MS. Peptide mass fingerprinting of the AP30 determined five peptide sequences: AFVEFLTDEIKEEK, MSGDWELEVNGTE AK, ITVTFNINNSIPPTFDGEEEPSQGQK, AEEQEPE LTSTPNFVVEVTK, and EVSFQATGDSEWR. These sequences were identical to those of mouse p32/gC1QBP/HABP1, which was originally identified as an ASF/SF2 splicing factor (Fig. 2D) [15].

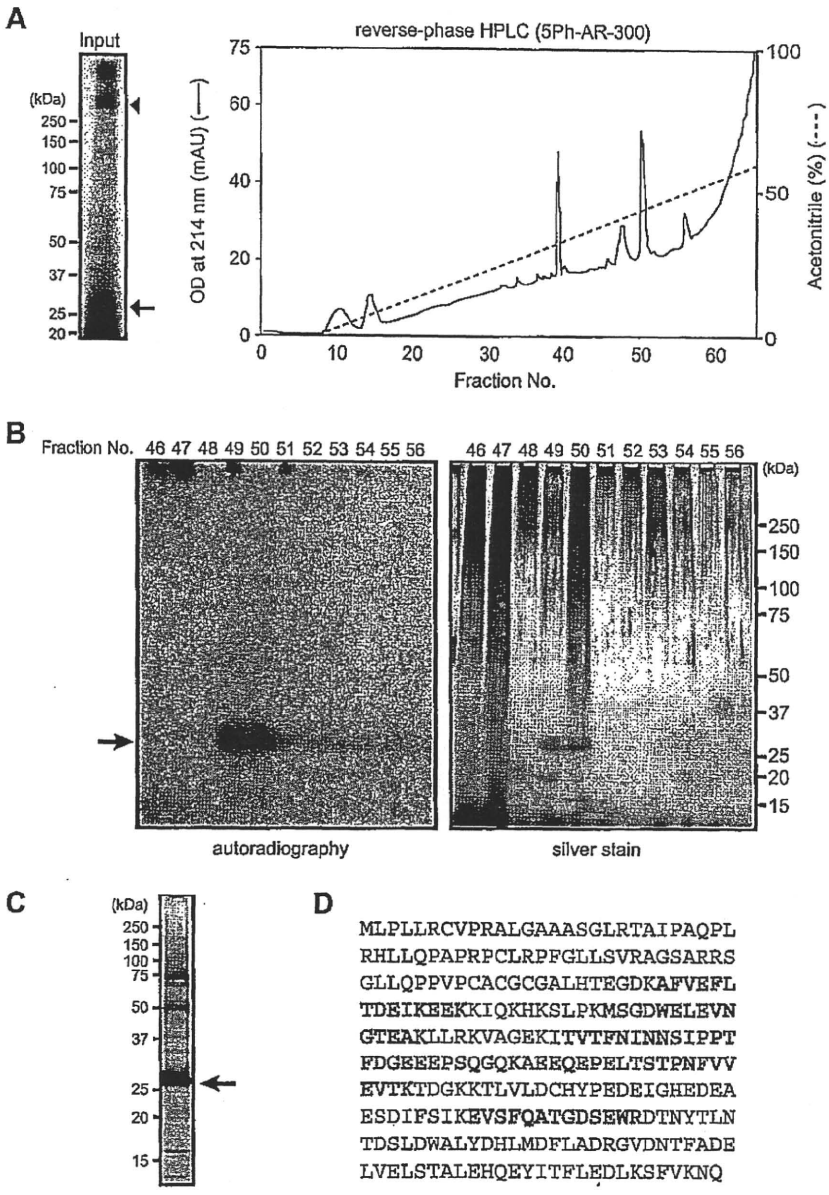

Fig. 2. Purification of AP30 using a reverse-phase HPLC. (A) The mixture of ^{32}P-labeled fractions 44–50 from a Super Q-5PW column was precipitated with acetone and reconstituted with 0.3% TFA/5% acetonitrile. The sample was applied to a reverse-phase HPLC column. After equilibration, the proteins were eluted with a linear gradient of 5–80% acetonitrile. The input before apply to the column is shown in the left panel (arrowhead; p-ATM, arrow; AP30). (B) The eluates were lyophilized, resolved in sample buffer, separated by SDS–PAGE, and stained with silver. Arrow indicates AP30. (C) The mixture of fractions 44–50 from a Super Q-5PW column was applied to reverse-phase HPLC column in the absence of [γ-^{32}P]ATP. The fraction 50 was separated by SDS–PAGE and stained with silver. Arrow indicates AP30. (D) The amino acid sequence of mouse p32 is indicated. Matched peptides by mass spectrometric analysis are shown in red. (For interpretation of the references in color in this figure legend, the reader is referred to the web version of this article.)

ATM phosphorylates p32 in vitro

To confirm that ATM indeed phosphorylates p32 protein in vitro, we initially generated recombinant GST-fusion p32 protein from bacteria, and performed an in vitro kinase assay. GST-p53 was used as a positive control, and efficiently phosphorylated by recombinant ATM WT. As shown in Fig. 3A, ATM WT, but not KD, strongly phosphorylated GST-p32. The extent of p32 phosphorylation by the equal amounts of ATM is almost comparable with that of p53, suggesting that p32 is also endogenous substrate for ATM. Because the recombinant protein purified from bacteria is often phosphorylated due to inappropriate conformation, we also used Flag-p32 generated from mammalian cells. We separately purified Flag-ATM and Flag-p32, and then we performed an in vitro kinase assay

using them. In the case of Flag-p32 protein from mammalian cells, ATM efficiently phosphorylated recombinant p32 in a dose-dependent manner (Fig. 3B). These data suggest that ATM directly phosphorylates p32 in vitro.

Identification of Ser 148 as a p32 phosphorylation site by ATM

To determine the phosphorylation sites of p32 by ATM, phosphorylated p32 was subjected to tryptic digestion followed by the phosphoamino acid analysis. The analysis revealed that the phosphorylation of p32 by ATM occurred in serine, but neither threonine nor tyrosine residues (Fig. 4A). Next, we attempted to identify the phosphorylated residues in p32 by site-directed mutagenesis. It is well known that ATM phosphorylates a consensus motif, serine or threonine followed by glutamine residue [16]. p32 carries only one such motif ($S^{148}Q$) and it is highly conserved among human, rat, and mouse (Fig. 4B). We replaced this serine to alanine (S148A) and performed an in vitro kinase assay using this mutant. Mutation of S148 in GST-p32 completely abolished its phosphorylation by ATM (Fig. 4C). Although we performed in vitro kinase assay using various serine mutant in p32, no other sites were affected by the mutation (data not shown). These data suggest that ATM phosphorylates p32 at S148 in vitro.

Discussion

In the current study, we attempted to identify the novel substrates for ATM from adult mouse heart. We screened substrates for the kinase using the methods that

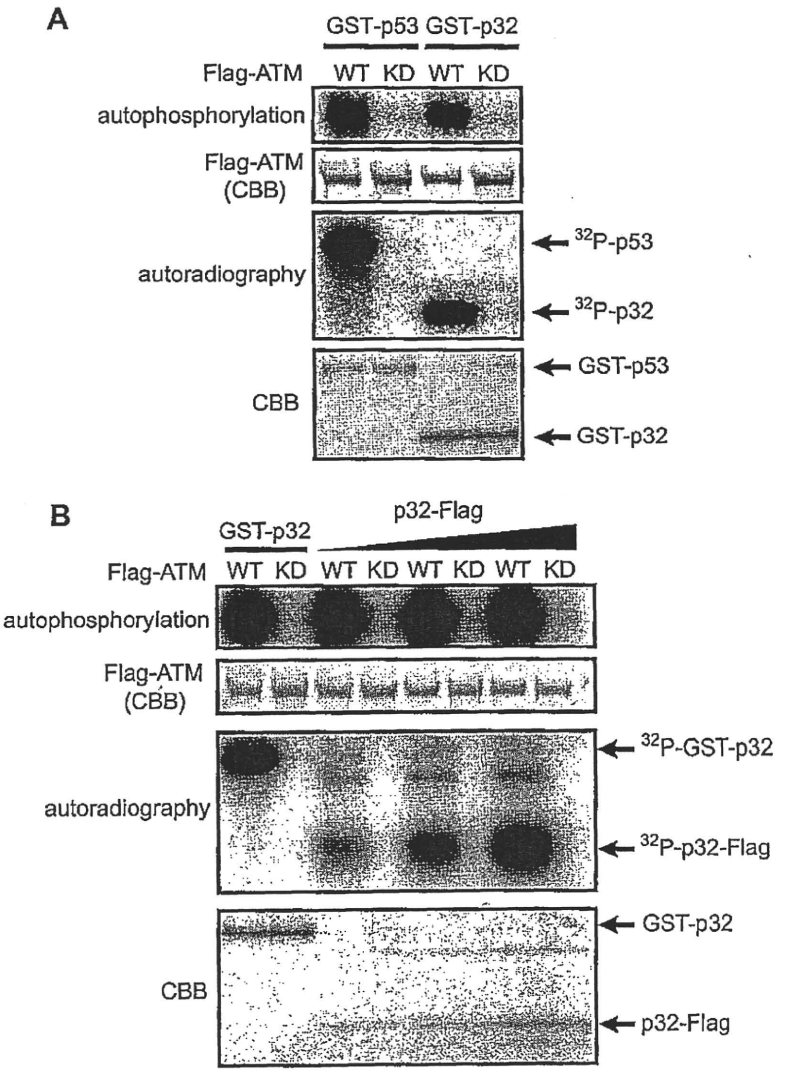

Fig. 3. ATM directly phosphorylates in vitro. (A) GST-p53 or GST-p32 purified from bacteria was incubated with recombinant Flag-ATM WT or KD in the presence of [γ-^{32}P]ATP at 30 °C for 30 min, and then the reaction mixture was analyzed by SDS–PAGE, stained with Coomassie, and visualized by autoradiography. (B) Flag-p32 purified from transiently transfected 293T cells was used for the in vitro kinase assay as described in (A).

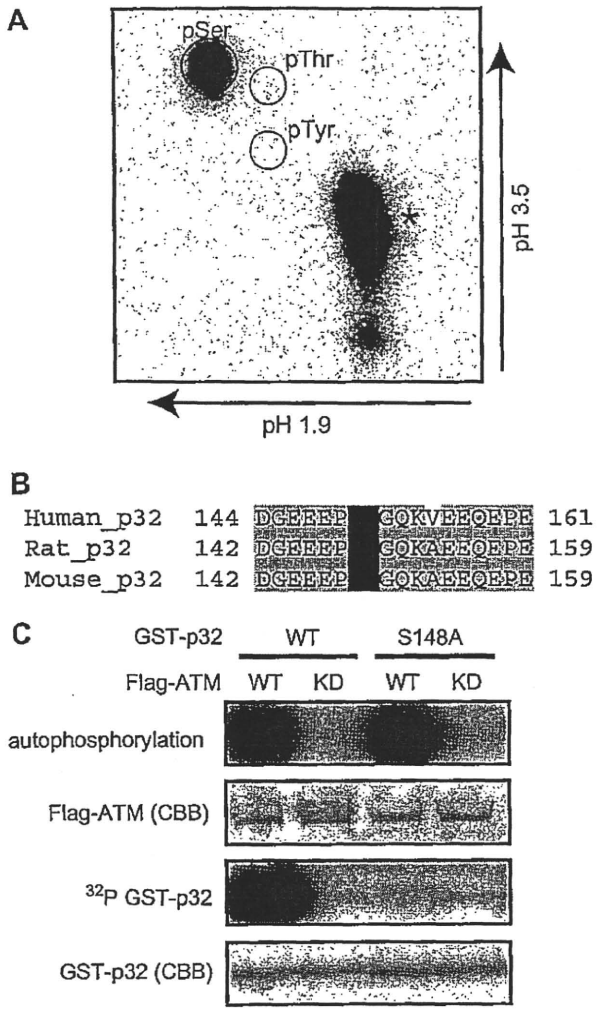

Fig. 4. Identfication of Ser 148 as a phosphorylation site in p32. (A) Phosphoamino acid analysis of p32. Phosphorylated p32 in vitro was excised from the gel and hydrolyzed in hydrochloric acid, and the resulting phosphoamino acids were separated with thin-layer electrophoresis using pH 1.9 for the first dimension and pH 3.5 for the second dimension. Autoradiography shows radiolabeled material comigrating with the phosphoserine (pSer) standard, but not with phosphothreonine (pThr) or phosphotyrosine (pTyr). Asterisk shows incomplete hydrolysis. The origin is shown as plus. (B) One Ser-Gln motif, which is targeted by PIKK, is present in mouse p32 and conserved among several species. Yellow backgrounds indicate identical amino acids among species. The consensus SQ motif is shown in red box. (C) An in vitro kinase assay was performed using wild-type or S148A mutant of GST-p32 as shown in Fig. 3. (For interpretation of the references in color in this figure legend, the reader is referred to the web version of this article.)

combined an in vitro kinase assay with ion-exchange chromatography followed by reverse-phase HPLC. We purified and identified p32 as a novel substrate for ATM. ATM efficiently phosphorylated recombinant p32 in vitro. Furthermore, we determined Ser 148 as a phosphorylation site of p32. Thus, we concluded p32 is a novel substrate of ATM in vitro.

In proliferating cells, when DNA damage has occurred, ATM phosphorylates diverse effector molecules, leading to cell cycle arrest or apoptosis. ATM deficiency in mice or humans, affects to the diverse cellular functions including hypersensitivity to ionizing radiation, premature aging, abnormal behavior, and cancer predisposition [1]. However, in terminally differentiated cells such as neuron or cardiomyocytes, DNA replication no longer occurs. In these cells physiological functions of ATM might be different from those in proliferating cells. Indeed, anti-cancer medicines like adriamycin, which activate ATM signaling pathways, are known to damage non-proliferating cardiomyocytes as well as rapidly proliferating cancer cells. These clinical data also suggest that a specific ATM-mediated signaling pathway exists in non-proliferating cardiomyocytes. Therefore we started the de novo screening of new substrate for ATM in heart.

We recently developed the unique methods for the protein purification using ion-exchange or affinity chromatography followed by reverse-phase HPLC, and these methods was useful for detecting new protein-binding partners [17]. Taking advantage of this strategy for high-resolution purification, we combined the detection of kinase activity with the protein fractionation, and could succeed in identifying novel substrates for several kinases [18,19]. In the present study, we further applied this method to purify the substrates of ATM, and identified p32.

p32 was originally isolated from HeLa cell nuclear extracts as a component of ASF/SF2, known as one of SR protein family [15]. Although the biological functions of p32 have not yet been entirely elucidated, several studies have shown that p32 interacts with various nuclear proteins including cellular and viral proteins [20]. It has been demonstrated that p32 binds ASF/SF2, which results in inhibition of splicing activity of ASF/SF2 due to the attenuation of its phosphorylation [21]. Studies about cardiomyocyte-specific ASF/SF2-deficient mice showed that depletion of ASF/SF2 in cardiomyocytes caused aberrant splicing pattern of calmodulin-dependent protein kinase IIδ, led to hypercontraction phenotype of these mice [22]. These data implied strong involvement of ASF/SF2 and its regulation by p32 phosphorylation in cardiac homeostasis. Identification of ATM as a p32 kinase in this study might shed light on the new molecular interaction between splicing machinery and DNA damage responses in heart.

In summary, we identified p32 as a novel substrate for ATM using unique purification techniques, and ATM phosphorylates p32 at Ser 148 in vitro. Future investigation will be needed to clarify the physiological importance of p32 phosphorylation by ATM in vivo, and how ATM regulates the mRNA processing through the phosphorylation of p32 in DNA damage responses in heart.

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