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ATM Kinase is Activated by Sindbis Viral Vector Infection

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Abstract

Sindbis virus is a prototypic member of the *Alphavirus* genus, *Togaviridae* family. Sindbis replication results in cellular cytotoxicity, a feature that has been exploited by our laboratory for treatment of *in vivo* tumors. Understanding the interactions between Sindbis vectors and the host cell can lead to better virus production and increased efficacy of gene therapy vectors. Here we present studies investigating a possible cellular response to genotoxic effects of Sindbis vector infection. The Ataxia Telangiectasia Mutated (ATM) kinase, a sentinel against genomic and cellular stress, was activated by Sindbis vector infection at 3 hours post infection. ATM substrates, Mcm3 and the γ H2AX histone, were subsequently phosphorylated, however, substrates involved with checkpoint arrest of DNA replication, p53, Chk1 and Chk2, were not differentially phosphorylated compared with uninfected cells. The ATM response suggests nuclear perturbation, resulting from cessation of host protein synthesis, as an early event in Sindbis vector infection.

Keywords

Sindbis; alphavirus; ATM; gene therapy vector

1. Introduction

Sindbis virus (SINV), an enveloped, single-stranded, positive-sense RNA virus is a prototypic member of the *Alphavirus* genus (Frolov et al., 1996; Strauss and Strauss, 1994). Replication of SINV has been extensively studied in vertebrate cells (reviewed in Strauss & Strauss, 1994, Frolov *et al.*, 1996). The SINV genome resembles cellular mRNA, having a capped 5' and polyadenylated 3' end. Following viral particle entry and uncoating, the RNA genome is translated, producing non-structural proteins that form a replication complex. A full-length negative strand is first synthesized to serve as a template for amplified genome copies. A partial genomic transcript, encoding the structural proteins, is also initiated from a subgenomic promoter. Synthesis of negative strand, positive strand and subgenomic RNA is temporally regulated by proteolysis of the non-structural proteins to modulate the replication complex (Gorchakov et al., 2008; Lemm et al., 1994).

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Interplay between virus and host cell factors determines the outcome of viral infections. SINV infection markedly alters cellular physiology. Within a few hours post infection, cellular transcription and translation are down-regulated; by 8 hours post infection (hpi) 80%–90% of cellular protein and RNA synthesis is inhibited (Gorchakov et al., 2005). Early after infection, activation of the double stranded-RNA protein kinase (PKR), presumably sensing the SINV replicative intermediates that exist in double-stranded form, leads to translational inhibition by phosphorylation of initiation factor eIF2 α (Venticinque and Meruelo, 2010; Ventoso et al., 2006). Cellular stress pathways are also initiated including the formation of stress granules, which sequester cellular translation factors and mRNA thereby augmenting the inhibition of protein synthesis (McInerney et al., 2005; Sanz et al., 2009; Venticinque and Meruelo, 2010; Ventoso et al., 2006). PKR has also been linked to apoptosis through activation of the JNK stress kinase (Venticinque and Meruelo, 2010). Cytopathic effects are observed 12–16 hpi and cell death occurs 24–48 hpi (Frolov and Schlesinger, 1994).

Selection of non-cytopathic SINV mutants points to the role of non-structural protein, nsP2, as a major factor influencing viral-host cell interactions (Frolova et al., 2002). NsP2 cytotoxicity correlates with its ability to inhibit host cell transcription (Frolov et al., 2009; Garmashova et al., 2006; Gorchakov et al., 2005; Gorchakov et al., 2008). Elimination of host transcription counters the cells anti-viral response by preventing the synthesis of proteins such as α/β IFNs (Garmashova et al., 2006; Gorchakov et al., 2005; Gorchakov et al., 2008).

Our laboratory has exploited the cytopathic properties of SINV for treatment of *in vivo* tumors (Hurtado et al., 2006; Meruelo, 2004; Tseng et al., 2004a; Tseng et al., 2002; Tseng et al., 2004b; Tseng et al., 2006). SINV can bind to the cell surface via the high affinity laminin receptor (Wang et al., 1992), a molecule that, opportunely, is upregulated on the surface of many tumor cell types (Menard et al., 1998) hence providing a virtual tumor-specific target for Sindbis (Tseng et al., 2004b). Construction of Sindbis vectors was patterned on SINV replicons, virus particles that contain genomic RNA but, which lack, all or some, structural gene sequences (Bredenbeek et al., 1993; Frolov et al., 1996; Xiong et al., 1989). The particles can infect cells and generate replicative forms that cannot, however, be transmitted to other cells (Frolov and Schlesinger, 1994) a factor that is advantageous for the safety of viral gene therapy. Substitution of the structural genes with genes encoding potentially therapeutic proteins, such as interleukin 12 (Tseng et al., 2004a) or HSV-1 thymidine kinase (Tseng et al., 2006) can increase vector efficacy..

Understanding the interactions between Sindbis vectors and the host cell can lead to better virus production and increased efficacy of gene therapy vectors. Our recent studies systematically examined the cellular pathways culminating in apoptosis of Sindbis vector-infected transformed and fibroblast cell lines. The role of JNK and Mcl-1 proteins, linking translational arrest, cellular stress and apoptosis, was elucidated (Venticinque and Meruelo, 2010). Considering the observed transcriptional inhibition in host cells (Frolov et al., 2009; Garmashova et al., 2006; Gorchakov et al., 2005; Gorchakov et al., 2008), we present studies investigating possible genotoxic effects of the Sindbis virus vector. The Ataxia Telangiectasia Mutated (ATM) kinase, a sentinel against genomic and cellular stress, was found to respond to SINV infection.

2. Materials and Methods

2.1. Cell Lines

Murine NIH3T3 cells were obtained from the American Type Culture Collection. Cells were maintained in Dulbecco's Modified Eagles Media supplemented with 10% Fetal Bovine

Sera, 100ug/ml penicillin-streptomycin and 0.5ug/ml amphotericin B (Mediatech Inc, Manassas, VA).

2.1 Sindbis vector, replication competent virus and Infection

Sindbis vector (SV-EGFP) was produced as previously described (Tseng et al., 2002). Briefly, plasmids expressing the replicon SinRep-EGFP or DHBB helper RNAs were linearized with *PacI* or *XhoI* (New England Biolabs) respectively. *In vitro* transcription was performed using the mMessage mMachine RNA transcription kit (Ambion). Helper and replicon RNAs were then electroporated into BHK cells and incubated in α MEM supplemented with 10% FCS for 12 hours. After 12 hours, the media was replaced with OPTI-MEM (Gibco) supplemented with 100 mg/l CaCl_2 and cells were incubated at 37°C for 24 hours, at which time the supernatant was collected, spun at 1500g, 4°C to remove debris, and frozen at -80°C. Vectors were titered as previously described (Tseng et al., 2002). Replication competent virus, carrying the luciferase gene, was also produced from DNA plasmids (Tseng et al., 2009).

Cells were infected with SV-EGFP in OPTI-MEM + CaCl_2 at a multiplicity of infection of 100, to achieve greater than 85% infectivity as assessed by fluorescent microscopy. Mock infected cells were incubated in OPTI-MEM + CaCl_2 . Cultures were gently rocked at 4°C for one hour prior to removal of virus or media, washed 1X with PBS and then incubated in complete media at 37°C for indicated times; time post infection was calculated from the time at 37°C incubation.

2.2 Western Blotting

Cell lysates were prepared using Whole Cell Lysis Buffer (25mM Hepes pH 7.4, 300mM NaCl, 1.5mM MgCl_2 , 1mM EDTA, 0.5% NP-40) supplemented with protease inhibitor cocktail (Roche) and phosphatase inhibitor (Pierce). Cells were harvested, washed once in PBS, then rotated at 4°C for 30min before spinning at 12,500g at 4°C for 15min to remove debris. Protein concentrations were measured using BioRad Dc Protein Reagent. Protein samples were run on 4–15% gradient SDS-polyacrylamide gels (BioRad) under reducing conditions. Protein was transferred to polyvinylidene fluoride membrane (Millipore) in Tris-glycine buffer pH 7.5 containing 10% methanol. Antibodies utilized: anti ATM phospho-Ser1981 (mab 10H11.E12) (1ug/ml) (Millipore), anti Mcm3 (G-19) (0.2ug/ml), anti Chk1 (G-4) (0.4ug/ml) and β -actin (C-4) (0.2ng/ml) (Santa Cruz), anti p53 phospho-Ser15 (1:1000 dil), anti phospho-H2A.X Ser139 (1:1000 dil) and anti Chk2 (1:500 dil) (Cell Signaling Technologies) antibodies. Horseradish peroxidase conjugated secondary antibodies (40ng/ml) were used (Santa Cruz) and filters developed with SuperSignal West Pico Chemiluminescence substrate (Pierce) and exposed to autoradiography film (Hyblot CL). Densitometry of scanned autoradiographs was performed using NIH Image J1.44f software.

2.2 Immunoprecipitation

Dynal beads (50ul slurry) (Invitrogen) were incubated with 10ug anti ATM phospho-Ser1981 or anti Mcm3 for one hour at RT with rotation followed by two washes with PBS, 0.05% Tween 20 (PBST). Beads were then rotated with 150ug lysate overnight at 4°C. Samples were washed four times with PBST. Protein was eluted from beads with 20ul SDS-PAGE sample buffer. For mass spectrometry analysis, 550ug whole cell lysate was first pre-cleared with mouse IgG1-bound beads before overnight incubation with anti phospho-ATM. Beads were washed 3x with PBST, 0.3M KCl, then 3x with PBST.

2.3 Mass Spectrometry

Cell lysate (550ug) prepared after 24 hrs SV-EGFP infection was immunoprecipitated and run on a 12.5% SDS-PAGE gel. The gel was visualized with Coomassie Blue stain. Protein isolation, digestion and analysis by MALDI-TOF was performed by the Rockefeller University Proteomics Facility (proteomics.rockefeller.edu). MS/MS data was analyzed using Mascot (Matrix Science) to search the non-redundant *Mus Musculus* database.

3. Results

3.1. Sindbis vector infection activates ATM

Analysis of a potential cellular genotoxic stress response to SV-EGFP infection was initiated with the examination of ATM protein activation. The ATM protein is a major sensor of many types of cellular stress. At various times after SV-EGFP infection of murine NIH3T3 fibroblast cells, cell lysates were prepared and examined by western blot analysis using an antibody recognizing the autophosphorylated activation site encompassing ATM Ser1981 (Bakkenist and Kastan, 2003). In Figure 1A, a high molecular weight band is observed at 2 to 3 hpi corresponding to the ~370 kDa phosphorylated ATM protein. Another strong band of approximately 100 kDa appeared 24 hpi. The earliest appearance of the 100 kDa band was 8 hpi (Fig.1B). In addition, presence of the specific ATM inhibitor, KU-55933 (Hickson et al., 2004), during infection diminished the level of phosphorylated ATM along with the 100 kDa band indicating ATM activation and phosphorylation of an apparent ATM substrate.

The ATM response was also observed after infection of cells with replicative Sindbis virus. Figure 1D. shows comparable levels of ATM phosphorylation and induction of the 100 kDa band. Effects on cell viability for Sindbis vector (MOI,100) and replicative Sindbis (MOI, 2) were similar while replicative virus at MOI 20 had a faster cytopathic effect (Fig. 1C.). These results are in agreement with Sindbis vectors or SINV replicons showing similar patterns of RNA synthesis compared with infectious virus (Sawicki et al., 2006). Absence of structural genes does not avert the cytotoxicity of SINV; Sindbis vectors suppress host RNA and protein synthesis and induce apoptotic cell death (Bredenbeek et al., 1993; Frolov and Schlesinger, 1994).

Treatment of cells with the ATM kinase inhibitor, KU-55933, diminished ATM autophosphorylation and the appearance of the 100kDa band (Fig. 1D.) but did not alter the cytotoxicity of either virus or vector infection (Fig. 1C).

3.2 Identification of the Sindbis vector-induced ATM substrate as Mcm3

The ATM antibody, recognizing the phospho-Ser1981 site, was used to immunoprecipitate the 100 kDa protein from SV-EGFP infected cells (Fig. 2). The corresponding protein band, isolated from a Coomassie stained PAGE gel, was analyzed by mass spectrometry. Four proteins within the expected MW range had acceptable MS/MS Mascot scores (Table I). A literature search revealed that the minichromosome maintenance protein, Mcm3, had been previously identified as an ATM target in response to genotoxic stress (Shi et al., 2007). Shi *et al* also found that antibodies generated against peptides containing the DpSQ ATM phosphorylation site are polyreactive, increasing the probability that at least one of the three potential ATM phosphorylation sites, within the carboxy-terminus of the murine Mcm3 protein, accounts for the presence of the 100 kDa band (Figure 3A). The identity of the 100 kDa band was verified when immunoprecipitates, from SV-EGFP infected cell lysates, using either phospho-ATM or Mcm3 antibodies, were shown to be cross-reactive (Fig. 3B).

3.3 ATM response pathways and Sindbis Vector infection

To understand the ATM response to SV-EGFP infection, phosphorylation of the DpSQ site was examined for various targets of the ATM pathway and compared with different modes of ATM activation. NIH3T3 cells were treated using predetermined optimal conditions for ATM activation: SV-EGFP infection for 24 hrs, 250uM H₂O₂ for 30 min, or 10ug per ml bleomycin for 3 hrs. Cell lysates were analyzed for the presence of phosphorylated ATM and subsequent substrates, Mcm3, p53 (Ser15), H2AX (Ser139), Chk1(Ser317) and Chk2(Thr68) kinases (Fig.4).

Induction of oxidative stress with H₂O₂ and DNA double-strand breaks (DSB) with bleomycin (Guo et al., 2010) elicited strong phospho-ATM Ser(1981) signals and correspondingly high phosphorylation signals from Mcm3. Densitometry measurements indicate that phospho-ATM levels and phospho-Mcm3 levels were stimulated 15.4 ± 8.06 fold and 26.86 ± 8.38 fold, respectively, by H₂O₂ and 11.96 and 12.13 fold, respectively, by bleomycin treatment. SV-EGFP infection induced an 11.84 ± 4.31 fold phospho-ATM and a 16.01 ± 3.41 fold induction of phospho-Mcm3. In all cases, similar levels of non-phosphorylated proteins were observed

SV-EGFP infection differed in the induction of p53 (Ser15) phosphorylation compared with both H₂O₂ and bleomycin. The absence of p53 phosphorylation is not due to diminished expression as comparable protein levels were observed on Western blots. ATM phosphorylation is thought to enhance the function of p53 as a transcriptional activator (Kastan and Lim, 2000). SINV, which inhibits cellular transcription, may preclude this ATM response.

Like bleomycin, SV-EGFP stimulated the phosphorylation of H2AX, a variant of histone H2A that, when phosphorylated on Ser139, is associated with DSB and loss of chromatin integrity (Li et al., 2005). ATM activation by H₂O₂ oxidative stress did not result in H2AX phosphorylation, consistent with previous observations (Guo et al., 2010).

ATM phosphorylation of Check kinases 1 and 2 leads to arrest of cell-cycle progression through DNA replication and mitosis, preventing aberrant chromosome formation due to DNA damage (Kastan and Lim, 2000; Shiloh, 2006). Two forms of Chk1 are seen after H₂O₂ and bleomycin treatment indicating phosphorylated (top arrow) and unphosphorylated (bottom arrow) protein, whereas, SV-EGFP treatment shows no difference between Chk1 in infected and non-infected cells. Examining Chk2, H₂O₂ and bleomycin treatment resulted in the complete shift of bands to the upper position while in SV-EGFP infected cells, the bottom band remained prominent. Although it is not, in fact, clear that it is the ATM Chk1(Ser317) and Chk2(Thr68) sites that were phosphorylated (as repeated attempts with various antibodies for these epitopes did not result in successful western blots), the observed differences between patterns of Chk1 and Chk2 bands following SV-EGFP infection compared with both H₂O₂ and bleomycin treatment suggest an alternative ATM response.

3.4 Sindbis shut-down of host protein synthesis could be detected by ATM

As inhibition of host protein synthesis is a major outcome of Sindbis infection, the ability of other translation inhibitors to stimulate ATM phosphorylation was examined. Treatment of cells with cycloheximide (Bandhakavi et al., 2010), puromycin (Nathans, 1964), or thapsigargin (Paschen et al., 1996) all resulted in the stimulation of ATM (Figure 5). It appears, however, that Sindbis infection results in higher levels of phospho-Mcm3.

4. Discussion

The ATM kinase is known to be a major regulator of cellular defense against a variety of stimuli including, DNA damage (Ismail et al., 2005), oxidants (Cosentino et al., 2011; Guo et al., 2010), hypotonic stress (Bakkenist *et al.*, 2003, (Bakkenist and Kastan, 2003; Schou et al., 2008; Soutoglou and Misteli, 2008), hypoxia (Bencokova et al., 2009) and perturbation of chromatin structure (Schou et al., 2008; Soutoglou and Misteli, 2008). Upon infection, several viral processes can provoke a cellular stress response: binding, entry, uncoating, replication complex formation, biosynthesis of intermediate and mature viral products. Some DNA viruses have been shown to elicit and exploit the ATM mediated DNA damage response (DDR) to facilitate their replication while others have evolved mechanisms to avoid or subvert this pathway (reviewed in (Weitzman et al., 2010). Both the Hepatitis C Virus, like SINV, a single-strand (+) sense RNA virus (Ariumi et al., 2008), and the non-enveloped, segmented double-stranded RNA Avian Reovirus (Chulu et al., 2010) require ATM/DDR for replication.

In our studies, ATM is activated relatively early after SV-EGFP infection at a point when viral (+) strand RNA synthesis occurs, PKR activity is stimulated and host protein synthesis starts to decrease. ATM Ser1981 phosphorylation has been previously associated with decreased protein synthesis resulting from cycloheximide treatment or amino acid starvation (Bandhakavi et al., 2010). We have also observed (Figure 5) ATM activation in cells treated with the translation inhibitors, cyclohexamide, puromycin (Nathans, 1964) or thapsigargin (Paschen et al., 1996). Shutdown of host protein translation by Sindbis virus could, therefore, be sensed in the nucleus through ATM. Although the exact nature of this activation is unknown, Bandhakavi *et al.* (2010) speculate that cessation of protein synthesis may activate ATM/DDR by affecting short-lived proteins required for genomic integrity.

Two downstream substrates of ATM, Mcm3 and H2AX, were phosphorylated following SV-EGFP infection. In contrast, ATM substrates involved with checkpoint arrest of DNA replication, p53, Chk1 and Chk2, were not differentially phosphorylated compared with uninfected cells. Mcm3 is a subunit of the hexameric protein complex, consisting of Mcm2-7, that is a key component of the genomic DNA pre-replication complex (Forsburg, 2004). Mcm proteins also form subcomplexes, however, that could have non-replicative functions in the cell, such as Mcm3 and Mcm5, which bind with Stat-1 α for transcription of IFN γ response genes (DaFonseca et al., 2001).

We observe Mcm3 phosphorylation 8 hours after Sindbis infection. At this time, translation of subgenomic transcripts is prominent and the Sindbis full length genome is no longer amplified. Based upon studies indicating the requirement of the Mcm complex for *in vitro* synthesis of the Influenza single-stranded (–) sense RNA virus, (Kawaguchi and Nagata, 2007), we examined whether Mcm3 plays a role in Sindbis-EGFP vector replication and, if so, whether phosphorylation would then alter this function. Using siRNA, we knocked down Mcm3 and measured (–) and (+) strand Sindbis RNA synthesis by quantitative RT PCR and EGFP synthesis by FACS analysis at 1, 2, 3, 4, and 6 hours after infection. The presence or absence of Mcm3 protein, determined by western blot, was found to have no effect on the replication of SV-EGFP (Hurtado and Pampeno, unpublished results).

Although serine residues within the C-terminus of Mcm3 were identified as targets of ATM in response to genotoxic stimuli, the functional significance of their modification is not understood as the phosphorylated proteins are still able to form Mcm complexes and bind to chromatin (Shi et al., 2007). Preferential localization of phosphorylated Mcm3 in nucleoplasmic *versus* chromatin fractions has been reported (Shi et al., 2007). The appearance of phospho-Mcm3 at 8 hours, with a more intense signal at 24 hours after SV-

EGFP infection, may reflect the progressive dissociation of the chromatin structure with the onset and continuation of the apoptotic process; nucleoplasmic Mcm3 providing a better or more accessible target for ATM. Strong phosphorylation of Mcm3 by ATM was also observed after cytotoxic treatment of cells with H₂O₂ and bleomycin, further suggesting an apoptotic effect.

Phosphorylation of the H2AX histone protein on Ser139 (γ H2AX) mobilizes γ H2AX to sites of DSBs or complexes of DDR proteins that have been observed in the absence of DNA damage (Soutoglou and Misteli, 2008). SV-EGFP induced phosphorylation of ATM and downstream Mcm3 and H2AX substrates, but not cell cycle checkpoint substrates, p53, Chk1 and Chk2, may indicate a response to chromatin perturbation. Although the Sindbis non-structural protein, nsP2, has been observed in the cell nucleus (Peränen et al., 1990; Rikkinen, 1996), the association between nuclear localization and cytotoxicity is not clear (Frolov et al., 2009; Tamm et al., 2008). In this regard, we have expressed exogenous Sindbis nsP2 protein but did not observe an ATM response (Pampeno, unpublished results).

The present data indicates that ATM can sense the shutdown of host cell protein synthesis by Sindbis vector infection. This response is in addition to stimulation of the mitochondrial apoptotic pathway and the JNK stress kinase (Venticinque and Meruelo, 2010). Inhibition of ATM activation does not prevent or delay the cytopathic effect of Sindbis. However, it remains interesting that checkpoint kinases and p53 are not mobilized by ATM. Whether this occurs through viral activity or if crosstalk among cellular reactions result in the predominance of the apoptotic pathway remains unresolved.

Analysis of ATM substrates using proteomic techniques has revealed a vast network of interactions related to the genotoxic stress response (Bensimon et al., 2010; Matsuoka et al., 2007; Shi et al., 2007). Further investigations are needed to fully comprehend the role of ATM activation at early stages of replication. It is possible that other ATM substrates are called into play prior to the phosphorylation of Mcm3. In an *in vivo* setting, it is also possible that ATM substrates could mobilize innate immune responses for organism survival. Study of Sindbis interaction with the ATM pathway may provide new tools to create better gene therapy vectors and add to the growing knowledge of the DDR that protects genome integrity.

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Figure 1. SV-EGFP infection induces ATM phosphorylation

(A) NIH3T3 lysates were prepared at the times indicated after SV-EGFP infection. (B) Specific ATM inhibitor (KU) was added to cells (+) 1 hour before and during infection. (-) cells were treated with DMSO vehicle. (C&D) Cells were infected with SV or RCS (MOI 2 or 20) in the presence or absence of KU. At 6 and 24 hours cells were trypsinized and assayed for viability with trypan blue prior to preparation of lysates. Infections were performed in triplicate. (A,B,D) Protein samples (50ug) were applied to 4–15% SDS-PAGE gels, which were analyzed by western blotting with ATM phospho-Ser1981 antibody. Markers on the right are in kDa.

Figure 2. The phospho-ATM antibody specifically precipitates the 100kDa protein

Lysate from NIH3T3 cells prepared 24 hpi with SV-EGFP, was immunoprecipitated with ATM phospho-Ser1981 antibody bound to protein G beads. Flow through (FT), unbound proteins; Eluate (E), proteins eluted into SDS PAGE sample buffer; (0), beads only; (IgG), non-specific mouse IgG attached to beads; (CL), total cell lysate. Equivalent volumes of each sample were applied to the gel, which was analyzed by western blotting with ATM phospho-Ser1981 antibody. Bands of approximately 50 and 25 kDa, are heavy and light chain immunoglobulin respectively. Markers are in kDa. Results are representative of at least three independent experiments.

A.

P-ATM epitope	SLAEEEGSQSTTIS
mMcm3 aa 674-688	LEDEEEKSQEDTEQK
718-732	HTPKTDDSQEKTDDS
735-749	SQEKTDDSQETQDSQ

B.

Figure 3. The ATM phospho-Ser1981 antibody recognizes Mcm3

(A) Shown is the phospho-ATM epitope and alignment of three potential ATM kinase substrate sites in murine Mcm3. (B) Lysate from NIH3T3 cells, prepared 24 hpi with SIN-EGFP (+) or mock infected (-), was immunoprecipitated with murine IgG, ATM phospho-Ser1981, or Mcm3. (-) samples are total cell lysates. Western blotting was with Mcm3 antibody for phospho-ATM IP and ATM phospho-Ser1981 antibody for Mcm3 IP. Markers are in kDa.

Figure 4. Comparison of ATM activation by SV-EGFP (SV), oxidation (H₂O₂) or double-strand DNA breaks (DSB)

Cell lysates were prepared from NIH3T3 cells, mock treated (–) or treated (+) with SV-EGFP, H₂O₂, or bleomycin (DSB). Protein (50ug) was analyzed by western blotting using antibodies designated on the right. Arrows on the left point to phosphorylated (upper) and unphosphorylated (lower) forms of Chk kinases. Results are representative of several independent experiments.

Figure 5. Activation of ATM by protein translation inhibitors

NIH3T3 cells were treated with thapsigargin (1uM, 2uM, 4uM), cycloheximide (20uM, 40uM, 80uM) or puromycin (20nM, 40nM, 80nM) for 2 hours or infected with SV (3, 6, 24 hours). Inhibition of protein synthesis was monitored by ^{35}S Met incorporation in parallel cultures (data not shown). Protein samples (50ug) were applied to 4–15% SDS-PAGE gels, which were analyzed by western blotting with ATM phospho-Ser1981 antibody. First lane of gels are lysates of mock treated cells. Markers on the right are in kDa. Results are representative of several independent experiments.

Table 1

MS/MS Mascot candidates for the identity of the 100 kDa band recognized by the ATM phosphor-Ser1981 antibody.

Protein	Gene ID	Mr	MS Peptides	Sequence Coverage	Mascot Score
Ac1262	32264629	117,756	7	12%	174
Hsp60-like (rat)	109506917	94,066	8	19%	60
Mcm3 (mouse)	33859484	92,230	16	33%	51
Mcm3 (rat)	109486863	92,229	15	31%	51