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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 h post infection. ATM substrates, Mcm3 and the γH2AX 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 pertubation, resulting from cessation of host protein synthesis, as an early event in Sindbis vector infection.

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 and 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).

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 h 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 eIF2α (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, 2008). Inhibition 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, 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., 2002, 2004a,b, 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

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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, 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, 100 μg/ml penicillin–streptomycin and 0.5 μg/ml amphotericin B (Mediatech Inc., Manassas, VA).

2.2. 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 αMEM supplemented with 10% FCS for 12 h. After 12 h, the media was replaced with OPTI-MEM (Gibco) supplemented with 100 mg/l $CaCl_2$ and cells were incubated at 37 °C for 24 h, at which time the supernatant was collected, spun at 1500 g, 4 °C to remove debris, and frozen at −80 °C. Vectors were titered as previously described (Tseng et al., 2002). Repication 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 (MOI) 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 1 h prior to removal of virus or media, washed 1× 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.

2.3. Western blotting

Cell lysates were prepared using Whole Cell Lysis Buffer (25 mM HEPES pH 7.4, 300 mM NaCl, 1.5 mM $MgCl_2$, 1 mM 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 30 min before spinning at 12,500 × *g* at 4 °C for 15 min 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) (1 μg/ml) (Millipore), anti-Mcm3 (G-19) (0.2 μg/ml), anti-Chk1 (G-4) (0.4 μg/ml) and β-actin (C-4) (0.2 ng/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 (40 ng/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.

2.4. Immunoprecipitation

Dynal beads (50 μl slurry) (Invitrogen) were incubated with 10 μg anti-ATM phospho-Ser1981 or anti-Mcm3 for 1 h at RT with rotation followed by two washes with PBS, 0.05% Tween 20 (PBST). Beads were then rotated with 150 μg lysate overnight at 4 °C. Samples were washed 4× with PBST. Protein was eluted from beads with 20 μl SDS-PAGE sample buffer. For mass spectrometry analysis, 550 μg whole cell lysate was first pre-cleared with mouse IgG1-bound beads before overnight incubation with anti-phospho-ATM. Beads were washed 3× with PBST, 0.3 M KCl, then 3× with PBST.

2.5. Mass spectrometry

Cell lysate (550 μg) prepared after 24 h 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 were 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 Fig. 1A, a high molecular weight band is observed at 2–3 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 competent Sindbis virus (RCS). Fig. 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