Phosphorylation and membrane association of the Rubella virus capsid protein is important for its anti-apoptotic function

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Summary

Rubella virus (RV), a member of Togaviridae, is an important human pathogen that can cause severe defects in the developing fetus. Compared to other togaviruses, RV replicates very slowly suggesting that it must employ effective mechanisms to delay the innate immune response. A recent study by our laboratory revealed that the capsid protein of RV is a potent inhibitor of apoptosis. A primary mechanism by which RV capsid interferes with programmed cell death appears to be through interaction with the pro-apoptotic Bcl-2 family member Bax. In the present study, we report that the capsid protein also blocks IRF3-dependent apoptosis induced by the double-strand RNA mimic polyinosinic-polycytidylic acid. In addition, analyses of cis-acting elements revealed that phosphorylation and membrane association are important for its anti-apoptotic function. Finally, the observation that hypo-phosphorylated capsid binds Bax just as well as wild-type capsid protein suggests that interaction with this pro-apoptotic host protein in and of itself is not sufficient to block programmed cell death. This provides additional evidence that this viral protein inhibits apoptosis through multiple mechanisms.

Introduction

Rubella virus (RV) is a member of Togaviridae, a family of enveloped positive strand RNA viruses. It is a human pathogen normally associated with the relatively mild, self-limiting disease Rubella, or German measles. Common complications include arthralgia, arthritis and thrombocytopenia; however, severe neurological disorders such as progressive panencephalitis may also occur. Significantly, RV can cause severe birth defects when contracted during the first trimester of pregnancy (reviewed in Hobman, 2013). While little is known about the pathogenic mechanisms of RV infection, it has been suggested that viral persistence is important. For example, long-term shedding of virus has been observed in infants with congenital rubella syndrome (Phillips et al., 1965) and in adults patients suffering from RV-associated arthritis (Chantler et al., 1982; 1985).

An important aspect of viral persistence is escape from apoptosis, a process that can be induced by the immune system or through cellular stress caused by viral replication (reviewed in Griffin and Hardwick, 1997). Unlike alphaviruses, which comprise the majority of togaviruses, RV is a slowly replicating virus that has an unusually long eclipse period. Infection of mammalian cells by RV has been reported to cause apoptosis (Pugachev and Frey, 1998; Hofmann et al., 1999), but generally this does not occur until long after the peak virus production periods. We recently demonstrated that RV-infected cells are in fact resistant to multiple apoptotic stimuli (Ilkow et al., 2011), a property that would be expected to provide a window of opportunity for replication. The resistance of RV-infected cells to apoptosis is thought to be dependent wholly or in part, on the capsid protein, which binds to and inhibits the pro-apoptotic host protein Bax. In the present study we demonstrate that binding to Bax is not sufficient for the apoptotic function of capsid; however, loss of phosphorylation and membrane association leads to decreased anti-apoptotic activity. These data suggest that
specific localization and or the ability to interact with additional factors are critical for its function in blocking apoptosis.

Results

Recombinant capsid protein affects the integrity of isolated mitochondria

Previous studies from this laboratory established that expression of the RV capsid protein has multiple effects on mitochondria (Beatch and Hobman, 2000; Beatch et al., 2005; Ilkow et al., 2010; 2011). In order to further understand how this viral protein affects mitochondrial physiology, we examined the effects of adding recombinant capsid protein to freshly isolated mitochondria. Because capsid protein blocks apoptosis in part through its interaction with Bax, we anticipated that treatment of isolated mitochondria with purified capsid protein would protect from pro-apoptotic factors such as tBid (Li et al., 1998). Surprisingly, addition of capsid to mitochondria had a similar effect as tBid in vitro (Fig. 1A). Specifically, mitochondria treated with recombinant RV capsid or tBid exhibited a dramatic loss of membrane potential compared to buffer alone, an event that often precedes release of cytochrome c during the early stages of apoptosis (Liu et al., 1996). Consistent with these results, exposure of mitochondria to recombinant capsid protein resulted in efflux of cytochrome c (Fig. 1B). Addition of a control protein, Sec17, did not induce release of cytochrome c from the mitochondria. Similar to RV capsid protein Sec17 is a peripheral membrane protein with a molecular mass of 34 kDa (Griff et al., 1992). The effect of RV capsid on cytochrome c efflux was not linear but rather appeared to have a threshold limit.

Hypo-phosphorylated capsid protein is less effective in blocking apoptosis

The data from the in vitro experiments were at first unexpected; however, it was important to consider that the recombinant capsid protein used in these experiments is different from the bona fide capsid that is produced in mammalian cells. First, the latter is phosphorylated on multiple serine and threonine residues, a process that is dependent upon initial phosphorylation of serine 46 (Law et al., 2003; 2006). Therefore, we assayed apoptotic resistance of cells expressing wild-type capsid and point mutants in which serine 46 was changed to alanine (S46A) or a phosphomimetic amino acid aspartic acid (S46D). Cells were transduced with lentiviruses encoding wild-type and mutant capsid proteins and after 2 days, were challenged with apoptotic stimuli that activate death receptor pathways (anti-Fas) or through intracellular pathways that detect viral RNA (poly I:C). Apoptosis in the transduced cells was determined by indirect immunofluorescence with antibodies to activated caspase 3. We have previously shown that RV capsid blocks Fas-dependent apoptosis in multiple cell types (Ilkow et al., 2011) however, the effect of poly I:C on capsid-expressing cells has not been investigated previously.

Data in Fig. 2A show that compared to wild-type capsid protein, both S46A and S46D were significantly less effective in protecting cells from Fas-dependent apoptosis. Comparable results were obtained when capsid-expressing cells were challenged with polyI:C (Fig. 2B), a dsRNA mimic that activates apoptosis through
a pathogen-associated molecular pattern receptor-based pathway (Peng et al., 2009; Kubler et al., 2010).

We next investigated whether phosphorylation of capsid was important for blocking apoptosis in the context of infection. Cells were infected with viruses encoding wild-type, S46A or S46D capsids followed by treatment with anti-Fas. In Fig. 3, it can be seen that compared to the mock population treated with anti-Fas, the proportion of apoptotic cells infected with wild-type RV was approximately half. Conversely, infection with S46A virus or S46D virus conferred virtually no protection compared to mock-infected cells. Together, these data are consistent with a scenario where phosphorylation of the RV capsid protein, particularly at Serine 46, is important for its role in blocking apoptosis during virus replication. Interestingly, earlier data from our laboratory showed that infectious titres of recombinant RV strains harbouring S46A mutations are only 10-fold lower than wild-type RV (Law et al., 2003). Similarly, an S46D mutant RV strain did not exhibit a dramatic replication defect in vitro (data not shown). However, it should be noted that other recombinant RV strains with multiple mutations that impair the anti-apoptotic ability of capsid (Ilkow et al., 2011) are extremely unstable and revert to wild-type in less than two passages. As such, it is possible that any significant replication defects associated with the S46A and D mutants are masked by rapid reversion.

Interaction with Bax is not sufficient for RV capsid to inhibit apoptosis

We observed that when higher lentiviral transduction levels were employed, cells expressing S46A and S46D capsids were protected from anti-Fas to a significant degree (Fig. 4A). This was not due to differences in stabilities of the capsid mutants as all of the constructs were expressed at comparable levels to wild-type capsid (data not shown). This suggests that even hypo-phosphorylated capsid protein harbours residual anti-apoptotic activity. One mechanism by which RV capsid may block programmed cell death is through sequestration of the pro-apoptotic host protein, Bax (Ilkow et al., 2011). Accordingly, we questioned whether the mutant capsids, particularly S46A, had a lower affinity for Bax. Surpris-
ingly, co-immunoprecipitation with anti-Bax, consistently resulted in recovery of more S46A capsid compared to wild-type or S46D capsid (Fig. 4B). These data suggest that sequestration of Bax may not be sufficient for capsid to block apoptosis.

Membrane-association is important for the anti-apoptotic function of RV capsid

Another major difference between recombinant capsid protein from *Escherichia coli* (Fig. 1) and *bona fide* capsid produced in infected (or transfected cells), is that a significant proportion of the latter is membrane-associated via a hydrophobic peptide that serves as the signal peptide (SP) for the E2 glycoprotein (Suomalainen et al., 1990; Law et al., 2001). Therefore, we analysed the potential importance of the SP domain in the anti-apoptotic role of capsid protein. Cells that were transduced with lentivirus encoding wild-type, S46A or S46D capsids were challenged with anti-Fas and activation of caspase 3 was determined using indirect immunofluorescence (Fig. 5). The levels of apoptosis among transduced cells expressing capsid proteins that lack the SP domain were significantly higher with S46AΔSP being the least protective.

One potential explanation for the reduced anti-apoptotic activity of some capsid mutants is that they are localized away from mitochondria and/or ER membranes. For example, most of the ΔSP mutants localized to cytoplasmic granules, nuclei and/or nucleoli (Fig. 6). It is interesting to note that the least protective capsid mutant S46AΔSP was localized predominantly in the nucleolus.
whereas a larger proportion of \(\Delta SP\) and S46D\(\Delta SP\) was detected in the cytoplasm. The punctate cytoplasmic structures to which \(\Delta SP\) mutants localized resembled processing bodies (P-bodies), which are RNA-rich granules that function in mRNA metabolism (Anderson and Kedersha, 2009). Based on colocalization of the anchorless capsid mutants with the P-body marker DCP1 (Fig. 7, arrowheads), it is tempting to speculate that membrane-association and possibly phosphorylation, are important to prevent capsid protein from accumulating in these structures, which similar to the nucleolus, have high concentrations of RNA. One major consequence of capsid accumulating in these RNA-rich structures is its loss from the cytoplasm and consequently, ability to interact with host proteins or membranes that function in apoptosis. Together, these data suggest that localization of capsid to cytoplasmic membranes or the E2 SP itself is important for the anti-apoptotic role of RV capsid.

**Discussion**

The teratogenic effects of RV on the fetus likely require the virus to persist in utero, a process that in turn may involve interfering with the innate immune response. To this end, we showed that the capsid protein affects several mitochondria-dependent pathways including apoptosis (Ilkow et al., 2008; 2010; 2011). The ability of the RV capsid protein to interact with multiple host cell proteins may be related to the fact that its structure is flexible (Mangala Prasad et al., 2013). Data from the present study indicate that the ability of RV capsid to inhibit apoptosis is a complex process that does not simply involve sequestering the host protein Bax. Indeed, some capsid constructs that do not bind Bax can still protect cells from apoptotic stimuli (Ilkow et al., 2011) suggesting that this viral protein interferes with programmed cell death pathways through more than one mechanism. Certainly, some large DNA viruses employ a multi-pronged strategy to limit apoptosis. Human cytomegalovirus for example, utilizes two different proteins vMIA and vIBO to inhibit Bax and Bak respectively (Goldmacher et al., 1999; Cam et al., 2010). While the RV capsid does not protect cells from Bak overexpression (Ilkow et al., 2011), it cannot be ruled out at this point that it affects steps that lead to activation of Bak or Bax for that matter. Recent findings indicate that these proteins can be activated through multiple mechanisms, some of which are rather unconventional. For instance, in addition to promoting Bax and Bak activity through transcription-dependent mechanisms following detection of viral dsRNA, the transcription factor IRF3 can also activate Bax through direct binding (Chattopadhyay et al., 2010).

The unexpected findings from in vitro assays in which recombinant capsid was found to have pro-apoptotic effects on isolated mitochondria led us to examine cis-acting elements that are important for the anti-apoptotic function of capsid. Our findings indicate that phosphorylation and the membrane-binding region of capsid, the E2 SP, are both important for its ability to inhibit

*Fig. 6. Subcellular localization of capsid mutants. Cells were transfected with indicated constructs, incubated for 2 days before being labelled with Mitotracker and processed for indirect immunofluorescence. Capsid proteins were detected using a mouse monoclonal antibody and rabbit anti-calnexin was used to detect the endoplasmic reticulum.*
programmed cell death. Membrane-association per se may not be critical for blocking apoptosis, but rather, localization of capsid to specific cytoplasmic compartments such as the surface of the ER and/or mitochondria. The RV capsid does not possess catalytic activity and therefore changes in its phosphorylation state likely affects intermolecular interactions. For example, the S46A mutant and other hypo-phosphorylated mutants bind RNA with higher affinity than wild-type capsid (Law et al., 2003; 2006). Given that the S46D phosphomimic mutant is significantly less protective than wild-type capsid, we are left to conclude that serine 46 per se, or the phosphorylated version of this amino acid, plays a critical role in its anti-apoptotic function. While substitution of an aspartate at this position is sufficient to induce downstream phosphorylation of other residues in capsid, it may not mimic phosphoserine well enough to facilitate interaction with host cell proteins involved in apoptosis. Indeed, a hypo-phosphorylated capsid mutant (P3D), in which three phospho-serine/threonine residues in the RNA binding region were changed to aspartates, behaved similarly to S46A in an in vitro RNA-binding assay. Specifically, the aspartates were expected to decrease RNA-binding of capsid but surprisingly, the P3D mutant exhibited significantly higher affinity for viral genomic RNA than wild-type capsid protein (Law et al., 2006).

None of the capsid mutants when expressed in mammalian cells, including S46AΔSP, recapitulated the 'pro-apoptotic' effects of capsid protein in vitro. One potential explanation for these seemingly discordant data is that unlike recombinant capsid protein, which is completely devoid of phosphorylation, S46A mutants are not completely devoid of phosphorylation but instead are hypo-phosphorylated (Law et al., 2003). High concentrations of non-phosphorylated capsid protein may be toxic for mitochondria and certainly, the ratio of capsid to mitochondria is higher in the in vitro system than in transfected or transduced cells. Indeed, there appears to be a threshold after which recombinant capsid protein behaves in this manner. Specifically, whereas addition of 0.8 μg of protein to mitochondria had no effect, addition of 2 (or more) μg resulted in near complete efflux of cytochrome c. Finally, it cannot be ruled out at this point that the 'pro-apoptotic' activity of recombinant capsid is simply an artefact of the in vitro system. Nevertheless, data from the in vitro experiments prompted further investigation, which showed that

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both phosphorylation and membrane association are important for the anti-apoptotic function of RV capsid in situ.

We hypothesize that phosphorylation and membrane-association prevents the RV capsid from becoming sequestered in other areas of the cell with high RNA content; a situation that would allow capsid to remain concentrated at sites where it fulfills both structural (nucleocapsid assembly) and non-structural functions (blocking apoptosis). The fact that S46AΔSP, which has the lowest anti-apoptotic activity, localizes to organelles with high RNA content (P-bodies and the nucleus/nucleolus) is consistent with this idea. Indeed, even a pool of anchor-less but still phosphorylated capsid (ΔSP) localized to these RNA-rich structures. Whether this has any bearing on the inherent differences between RV capsid and the closely related alphanvirus capsid proteins is debatable. However, given that cells infected with alphaviruses generally produce much higher levels of antigen than RV-infected cells, it is possible that alphaviruses can better tolerate the mis-localization of antigen than RV-infected cells, it is possible that alphaviruses can better tolerate the mis-localization of antigen than RV-infected cells.

Experimental procedures

Reagents and cells

A549, Vero, BHK-21 and HEK 293T cells were obtained from the American Type Culture Collection (Manassas, VA). Protein A and G Sepharose beads were obtained from GE Healthcare Bio-Sciences Corp (Princeton, NJ). Media and fetal bovine serum (FBS) were obtained from Life Technologies-Invitrogen (Carlsbad, CA).

Plasmid construction and virus production

Several plasmids used in this study including pBRM33, pBRM33-S46A, pCMV5-Capsid, pCMV5-S46A, pCMV5-S46D and pCMV5-CapsidΔSP have been previously described (Yao and Gillam, 1998; Law et al., 2001; 2003; 2006). S46AΔSP and S46DΔSP cDNAs were constructed by PCR amplification of the template plasmid pCMV5-Capsid using a forward primer that begins at an internal Sal site (5′-GC GC CAT A5T CGA GCC GGT AGA AGA CC-3′) and a reverse primer that introduces a stop codon and BamHI site just before the E2 signal peptide (5′-TTG GAT CCT TAG CGG ATG CGC CAA GGA TG-3′). These fragments were then used to replace the 400 bp BamHI–SalI fragments in pCMV5-S46A or pCMV5-S46D respectively. The plasmid pBRM33-S46D was constructed by replacing the NotI–BsrGI fragment in pBRM33 with the analogous fragment from pCMV5-S46D followed by reinsertion of the NotI–NotI fragment. Platinum Taq® DNA Polymerase High Fidelity (Life Technologies) was used for all PCR amplification reactions.

For production of recombinant lentiviruses encoding capsid proteins, the vector pTRIP-AcGFP (Urbanowski and Hobman, 2013) was employed. To introduce capsid cDNAs into this plasmid, pCMV5-Capsid, pCMV5-Capsid S46A, pCMV5-S46D, pCMV5-CapsidΔSP, pCMV5 S46A ΔSP and pCMV5 S46D ΔSP were digested with EcoRI and BamHI. The capsid cDNAs were then purified and ligated together with EcoRI–BamHI, EcoRI–SpeI and SpeI–SpeI adaptors (5′-CTA GTG AGC TCA GCG-3′ and 5′-AA TAC TCG CTG AGC TCA-3′) into pTRIP-AcGFP that had been digested with BsrGI and SpeI.

To produce lentiviral stocks, HEK293T cells (2.5×10⁶ per 100 mm dish) seeded the previous day, were co-transfected with pTRIP AcGFP constructs and HIV gag-pol and vesicular stomatitis virus envelope protein plasmids in a ratio of 7:7:2 (Urbanowski and Hobman, 2013) using TransIT-LT1 (Mirus, Madison, WI) transfection reagent. Lentivirus-containing media were collected 3 days post transfection and then passed through 0.44 μm filters before aliquoting and storage at –80°C until use.

RV infectious RNA was transcribed from plasmids pBRM33, pBRM33-S46A and pBRM33-S46D using mMESSAGE...
mMACHINE® SP6 Kit (Ambion) as described (Yao and Gillam, 1999; Law et al., 2003). Virus was reconstituted by electroporating BHK-21 cells with infectious RNA and collecting the media 3 days post infection. Media were cleared by centrifugation at 900 g for 5 min before aliquoting.

Cell culture and virus infection
A549 and HEK293T cells were cultured in Dulbecco’s minimal essential medium (high glucose) containing 10% FBS, 2 mM glutamine, 1 mM Hepes, penicillin and streptomycin in a humidified incubator at 37°C with 5% CO. Where indicated, cells were infected with RV by adding appropriate amounts of viral stock on top of cells in half the normal volume of media (1 ml/35 mm dish). Cells were incubated for 6 h with virus inoculum after which media were replaced. RV-infected cells were maintained at 35°C in a humidified atmosphere containing 5% CO.

Cells were transduced with lentivirus by diluting an appropriate amount of viral stock in 4 ml/35 mm well of DMEM containing 3% FBS, 20 mM Hepes and 4 μg ml⁻¹ polybrene. Cells were then spinoculated by centrifuging at 1200 g for 45 min at 37°C. After spinoculation, lentivirus-containing media were removed and replaced with normal growth media containing 10% FBS.

In vitro mitochondria assays
Mitochondria from rat liver were prepared as described (Ilkow et al., 2010). For cytochrome c release assays, freshly isolated mitochondria were adjusted to 1 mg ml⁻¹ of total protein by diluting with 250 mM sucrose, 10 mM Hepes-KOH, 1 mM ATP, 5 mM Na succinate, 0.08 mM ADP, 1 mM DTT, 2 mM K₂HPO₄ (pH 7.5) and were then incubated with the indicated amounts of recombinant capsid or control protein (Sec17) for 30 min at 30°C. Mitochondria were then pelleted by centrifugation for 10 min at 8000 g and then pellets and supernatant were analysed by immunoblotting with a monoclonal rabbit antibody to cytochrome c (from Dr L. Berthiaume, University of Alberta, Canada).

To determine effect of RV capsid protein on mitochondrial membrane potential, crude mitochondria were prepared from A549 cells. Cells were homogenized in ice-cold mitochondria isolation buffer [0.2% (w/v) BSA, 200 mM mannitol, 70 mM sucrose, 1 mM EGTA, 10 mM Hepes-KOH, pH 7.5] supplemented with Complete™ EDTA-free protease inhibitors using a dounce homogenizer with a loose fitting pestle. Unbroken cells and nuclei were pelleted by centrifugation at 500 g for 10 min at 4°C. The supernatants were then centrifuged at 10 000 g for 20 min at 4°C to obtain crude mitochondrial pellets. Mitochondrial pellets were resuspended in mitochondria resuspension buffer (250 mM sucrose, 10 mM Hepes-KOH, 1 mM ATP, 5 mM Na succinate, 0.08 mM ADP, 1 mM DTT, 2 mM K₂HPO₄, pH 7.5). Mitochondria were incubated with recombinant proteins for 15 min at 30°C, then with 150 nM of tetramethylrhodamine, ethyl ester (TMRE) for 30 min at 37°C. Mitochondria were then washed twice with 0.25 M sucrose 2 mM Hepes buffer and analysed by fluorometry.

Apoptosis assays
Caspase 3 activation in capsid-encoding lentivirus-transduced or RV-infected cells was detected by indirect immunofluorescence as described (Ilkow et al., 2011). Unless otherwise indicated, A549 cells were transduced with lentiviruses at a multiplicity of transduction of two and then apoptosis was induced 2 days later by adding αFAS (0.25 μg ml⁻¹) and cycloheximide (1 μg ml⁻¹) for 24 h. Alternatively, apoptosis was induced in transduced cells by transfection with polynosinic-polycytidylic acid sodium salt poly(I:C) (Sigma-Aldrich, St. Louis, MO). Two days post transduction, cells were transfected with poly(I:C) (1 μg ml⁻¹) for 20 h prior to apoptosis assays. Transfection with an equal concentration of plasmid DNA (pCMV5) was used as a negative control. Finally, cells were also infected with wild-type (M33) and capsid mutant strains of RV at an moi of 0.5. Two days post infection, apoptosis was induced with αFAS (1 μg ml⁻¹) and cycloheximide (1 μg ml⁻¹) for 8 h before caspase 3 activation assays were performed. In the mock-infected cells, all caspase 3-positive cells were counted. However, in the experimental samples, only infected/transduced cells (which were positive for RV antigen or AcGFP) were counted in order to quantify caspase 3 activation levels.

All P-values were calculated using paired Student’s t-tests. Asterisks indicate P-values of < 0.05 compared to the wild-type capsid, except where otherwise noted.

Capsid and Bax co-immunoprecipitation
A549 cells were seeded at 1 × 10⁶ cells in 100 mm dishes and transfected 1 day later with pCMV5-Capsid, pCMV5-S46A or pCMV5-S46D using Lipofectamine 2000 (Invitrogen). Two days post transfection, cells were lysed in 350 μl of lysis buffer [1% Igepal, 50 mM Tris pH 7.4, 150 mM NaCl, 2 mM EDTA, 1× HalT™ Phosphatase Inhibitor (Thermo Scientific) and 1× Complete™ Protease Inhibitor Cocktail (Roche)]. Insoluble debris was removed by centrifugation at 10 000 g for 10 min. Lysates were pre-cleared by incubating with 33% protein A-Sepharose slurry (15 μl) for an hour at 4°C after which 4 μg of antibody Rabbit anti-Bax (Abcam) pre-bound to 15 μl of protein A sepharose slurry in 1% casein was added for 4 h at 4°C. Immune-complexes on the beads were washed three times with lysis buffer before elution by boiling in gel loading buffer. Immunoprecipitates together with lysate (9%) control were separated on 12% acrylamide gels before immunoblotting with a mouse monoclonal antibody (Wolinsky et al., 1991) to capsid (C-1) from Dr Jerry Wolinsky (University of Texas, Houston) and rabbit anti-Bax.

Indirect immunofluorescence
A549 cells were seeded on coverslips and then transfected the next day with Transit-LT1 (Mirus). Two days after transfection, cells were fixed and indirect immunofluorescence was conducted as previously described (Ilkow et al., 2011). Capsid proteins were detected using a mouse monoclonal (C-1). Rabbit antibodies to DCP1 (Abcam, Toronto, ON) and calnexin (Dr David Williams, University of Toronto) were used to detect P-bodies and endoplasmic reticulum respectively. To label mitochondria, cells were incubated with Mitotracker Red CMXRos (Molecular Probes®, Eugene, OR) 30 min before fixation. Images were acquired using a Quorum WaveFX-X1 Spinning Disc Confocal System with Volocity acquisition software.
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