ROLE OF MITOCHONDRIA DURING BOVINE ADENOVIRUS 3 INFECTION

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ABSTRACT

Bovine adenovirus (BAdV) -3 is a non-enveloped, icosahedral virus with a double-stranded DNA genome, and is being developed as a vector for vaccination of animals and humans. Mitochondria are multifunctional organelles, which are involved in various functions of the cell including but not limited to energy production, aging, regulation of cell cycle, anti viral responses. Thus, this makes them strategic targets for many pathogens. Although a number of viruses affect the structure and function of mitochondria, the effect of BAdV-3 infection on these organelles has not been well characterized. The aim of the present study was to ascertain the pathological effects of BAdV-3 infection on host mitochondria and the role of BAdV-3 encoded proteins in modulating mitochondrial functions.

Electron microscopy analysis revealed extensive damage to the inner mitochondrial membrane characterized by dissolution of cristae and amorphous appearance of mitochondrial matrix with little or no damage to the outer mitochondrial membrane. There were fewer cristae with altered morphology. Patches of protein synthesis machinary around mitochondria were observed at 12 hrs post infection. At 24 hrs post-infection, extensive damage to mitochondria was evident throughout the infected cell. ATP production, mitochondrial Ca^{2+} and mitochondrial membrane potential (MMP) peaked at 18 hrs post-infection but decreased significantly at 24 hrs post-infection. This decrease coincided with increased production of superoxide (SO) and reactive oxygen species (ROS), at 24 hrs post-infection indicating acute oxidative stress in the cells and suggesting a complete failure of the cellular homeostatic machinary.

Sequence analysis of BAdV-3 proteins revealed the presence of potential mitochodria localization signals (MLS) in 52K, VII, 33/22K and IVa2. Western blot analysis of isolated mitochondrial fractions suggested that all these proteins are localized in the mitochondria. However, a more stringent proteinase K assay confirmed the presence of 52K and pVII in the mitochondria suggesting that the other observed proteins were loosely attached to the surface of the mitochondria or may simple co-purify with the mitochondrial fraction. The presence of potential MLS in 52K and pVII was confirmed by localization of EYFP (Enhanced Yellow Fluorescent Protein; a predominantly cytoplasmic protein), when fused to MLS of pVII or 52K, to mitochondria of transfected cells.

Expression of pVII in transfected cells showed an increase in MMP and ATP production, and increased sequestration / retention of mitochondrial Ca^{2+} in the cells. However, there was no increase in reactive oxygen species (ROS) / superoxide (SO) production in pVII transfected cells indicating that pVII acts as an antiapototic protein. In contrast, expression of 52K in transfected cells significantly increased ROS/SO production with no significant change in ATP production, mitochondrial Ca^{2+} or MMP indicating that 52K alone causes an oxidative stress in cells following infection and causes apoptosis.

In conclusion, these results reveal an intricate relationship between Ca^{2+} homeostasis, the ATP generation ability of cells, SO and ROS production and regulation of MMP following infection by BAdV-3 or transfection of the cells with plasmid DNAs expressing pVII & 52K. While pVII appears to contribute to the survival of the cells during virus replication, 52K is involved in the death of the infected cells and thus may help in release of progeny virus.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS USED

ADP	Adenovirus Death Protein
ANT	Adenine nucleotide translocase
AP	Alkaline Phosphatase
BAdV	Bovine Adenovirus
CAR	Coxsackie and adenovirus receptor
DMEM	Dulbeco's minimum essential medium
EGTA	Ethylene glycol tetraacetic acid
EYFP	Enhanced Yellow Fluorescent Protein
FBS	Fetal Bovine Serum
HAdV	Human adenovirus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HSV	Herpes simplex virus
ITR	Inverted terminal repeat
kDa	Kilo Dalton
KRH	Krebs–Ringer-Hepes
MDBK	Madin-Darby bovine kidney
MLS	Mitochondria Localization Signal
MOI	Multiplicity of infection
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
ROS	Reactive Oxygen Species
SMP	Skimmed Milk Powder
SO	Superoxide
TMRM	Tetra methyl rhodamine methylester
VDAC	Voltage dependent anionic channels
VSV	Vesicular stomatitis virus
βME	Beta-mercaptoethanol

1.0 LITERATURE REVIEW

1.1 Adenoviruses

Adenoviruses were first isolated from adenoid tissues by two independent groups attempting to identify the causative agents of acute respiratory infections (Rowe et al., 1953; Hilleman and Werner, 1954). They have been isolated from mammals, birds, reptiles and other species (Enders et al., 1956; Benko et al., 2005; Berk, 2007). Their replication is mostly limited to single host species but can be present asymptomatically in species other than the natural host (Shenk, 2001).

1.1.1 Adenovirus classification

Adenoviruses belong to family *Adenoviridae*, which is divided into five genera (Davison et al., 2003; Benko et al., 2005) Members of genus *Mastadenovirus* exclusively infect mammalian species and are serologically distinct from members of other genera. Members of this genus include all identified human adenovirus (HAdV) serotypes 1 to 55 (Berk, 2007; Smith et al., 2010; Walsh et al., 2010). Their genome size ranges from 30.2 kb (canine adenovirus type 1) to 36.5 kb (simian adenovirus type 25) (Davison et al., 2003) with a GC content of 40.8 to 63.8%. Members of this genus have a considerably long inverted terminal repeat (ITR) (93-371 bp) compared to members of other genera. Proteins V and IX are unique to this genus and not found in members of other genera. Members of HAdV subgroup C are the most extensively studied and are type species for this genus.

Members of genus *Aviadenovirus* infect a variety of birds causing varying degree of pathogenicity. The members of this genus are serologically distinct from members of other genera and contain a genome of 45.4 kb in case of turkey adenovirus (TAdV) (Kajan et al., 2010) or 45kb in case of fowl adenovirus (FAdV) - 9 (Ojkic and Nagy, 2003). *Aviadenovirus* genome lacks the genes for protein V, IX and homologues for E1, E3, and E4 regions of *Mastadenoviruses* (Chiocca et al., 1996; Ojkic and Nagy, 2003). Members of this genus have two fibers per vertex of capsid. FAdV-1 (CELO) is type species for this genus.

Members of *Atadenovirus*, have been isolated from a variety of hosts including reptiles, birds and mammals excluding humans (Benko and Harrach, 1998; Both, 2002; Benko et al., 2005), and are serologically distinct from members of other genera. The members of this genus have high AT content in their genomes that formed the bases on which the genus was named (Benko and Harrach, 1998; Benko et al., 2005). *Atadenovirus* genome lack homologue of structural proteins V and IX and, the proteins coded by E1A and E3 regions (Benko et al., 2002; Both, 2004; Benko et al., 2005). All members of this genus encode a novel structural protein named p32K (Benko et al., 2005). Ovine adenovirus (OAdV)-287 is the type species for this genus.

The members of the *Siadenovirus* genus are serologically distinct from members of other genera. Members of this genus TAdV-3 and frog adenovirus type (FrAdV)-1 have shortest of the known adenoviral genomes 26.2 kb and 26.1 kb long, respectively (Pitcovski et al., 1998; Benko et al., 2005). The genome of other member raptor adenovirus (RAdV)-1 has only been partially characterized (Kovacs and Benko, 2009). Members of this genus lack proteins V and IX as well as homologue of regions E1, E2 and E3 (Benko et al., 2005; Kovacs and Benko, 2009) and contain a gene that codes for a protein related to bacterial sialidases (Davison et al., 2000; Benko et al., 2002). TAdV-3 was classified in this genus due to the absence of any complement fixing antigen common with other adenoviruses of birds classified into genera *At* or *Aviadenovirus*. FAdV-1 is non pathogenic and is the type species of this genus (Benko et al., 2005).

The only member of genus *Ichtadenovirus* was isolated from white sturgeon (Hedrick et al., 1985;) and named white sturgeon adenovirus (WSAdV)-1 (Kovacs et al., 2003). WSAdV-1 is non pathogenic and is the type species for the genus. The availability of partial genome sequence suggests that the genome contains a central conserved region (CCR) with genes organized on both sides of it. An ORF towards the left of the CCR encodes for the proteins that are significantly similar to the bacterial and phage proteins of unknown functions. The arrangement of genes to the right of CCR is also different from that of any of the other known adenoviruses. The genes for V, IX, VIII, μ and fiber and as are homologues encoded by E1, E2 and E3 regions of members of *Mastadenovirus*. The genome contains at least eight ORFs that have no known homologues (Davison et al., 2003; Benko et al., 2005). A phylogenetic analysis indicates

that WSAdV-1 represents a separate lineage as members of four genera and the fifth clade appear at considerable distance from each other, thus justifying the establishment of a fifth genus.

1.1.2 Human adenovirus

To date, over 55 serotypes of HAdV have been identified (Berk, 2007; Smith et al., 2010; Walsh et al., 2010). HAdVs are divided into seven species, A to G (Berk, 2007; Jones et al., 2007) based on their ability to agglutinate erythrocytes, oncogenicity in animals, and genetic homology. Since most of our understanding in adenoviral biology has been come from the work with HAdV-5, so information presented in the following sections relates to HAdV-5 unless stated otherwise.

1.1.2.1 Virus structure and genome organization

Human adenovirus consists of a non-enveloped icosahedral capsid of 75-90 nm in diameter, encapsidating a DNA-containing core (Niiyama et al., 1975; Berk, 2007) with fiber projecting outward from the icosahedron's vertices. The capsid is composed of major (hexon, penton and fiber) and minor (IIIa, VI, VIII and IX) capsid proteins (Vellinga et al., 2005). In addition to capsid proteins, core proteins (V, VII, mu, protease and terminal proteins [TP]) are present inside the capsid and bind directly to viral DNA. Non-structural proteins (100K, 33/22K and 52K) are not present in the mature virion, but are involved in the processing of pre-proteins and assembly of mature virus particles (Russell, 2009).

The virus core consists of the double-stranded DNA genome and the viral core proteins. HAdV genomes are 26 to 35 kb in length with GC content varying from 33 to 63% (Russell, 2009). Ends of the genome have inverted terminal repeats (ITRs) of variable length depending upon the species. The most predominant core protein is pVII. It has over 800 copies per virion (Berk, 2007) and forms nucleosome-like cores over which the viral DNA is condensed (Vayda and Flint, 1987). Binding of protein V to protein VI provides a link between the viral core and the capsid (Sundquist et al., 1973; Everitt et al., 1975; Berk, 2007). The terminal protein is also present in the core and is covalently attached to the 5' ends of the viral DNA (Rekosh et al., 1977). Viral protease,

which is involved in virus maturation, entry and un-coating (Greber, 2002) is also attached to terminal protein.

1.1.2.2 Virus entry

The virus attaches the cells primarily via the coxsackie and adenovirus (CAR) receptor (Bergelson et al., 1997), which is a member of the immunoglobulin super family and is involved in the adhesion between epithelial cells (Arnberg, 2009; Sharma et al., 2009). *In vitro,* CAR acts as a primary receptor (Arnberg et al., 2000a; Arnberg et al., 2000b; Russell, 2000) for the members of subgroups A, C, E and F, and few of subgroup D, but this may not be the case *in vivo* (Arnberg, 2009). Except for serotypes 3 and 7, HAdV's species B can use CD80, CD86, or CD46 as a receptor (Marttila et al., 2005; Short et al., 2006). Sialic acid has been shown to be used as a receptor by some members of subgroup D (Arnberg et al., 2000a). The interaction of adenovirus fibre with CAR or other receptors allows attachment of the virus to the host cell. Internalization step requires the interaction of RGD motif of penton base with the integrins and, a_vb_3 and a_vb_5 on the cell surface (Bai et al., 1993; Wickham et al., 1993; Nemerow and Stewart, 1999). All sequenced HAdVs pentons have RGD motif with the exception of HAdV-40 and -41, which have a gastrointestinal tropism and show delayed internalization (Zhang and Bergelson, 2005). Following this interaction, the virus internalizes by receptor mediated endocytosis in clathrin coated pits (Wang et al., 1998; Patel et al., 2009) creating early endosomes (Gastaldelli et al., 2008). The endosomal compartment has an acidic pH, which induces conformational changes in the viral capsid. After these conformational changes, the capsid starts to dismantle, starting with the dissociation of IIIa, penton, and VIII (Greber et al., 1993; Wiethoff et al., 2005). Consequently, an amphipathic helix in protein VI is exposed that disrupts the endosomal membrane and allows the virus to escape into the cytosol (Wiethoff et al., 2005). Once in cytosol, hexons bind directly to the microtubule motor protein dynein (Bremner et al., 2009), which directs the partially disassembled capsid towards the nucleus along the microtubules (Leopold et al., 2000).

Several protein-protein interactions including the binding of histone H1 to hexon promote further disassembly of the viral capsid (Kelkar et al., 2004; Kelkar et al., 2006). The capsid disassembly and the nuclear import of genome also depends upon the ability

of L3 protease to degrade the shuttle protein VI, which acts as a cement to hold the capsid and the core together (Honkavuori et al., 2004). Capsid disassembly and nuclear import of the viral DNA is also facilitated by hsc70 and CRM1 (Saphire et al., 2000; Strunze et al., 2005). Capsid then loses protein VIII, which also is a cementing protein (Matthews and Russell, 1998). Following disassembly of capsid, viral DNA along with proteins V and VII interact with a nuclear import receptor transportin, which takes DNA/protein V/protein VII complex into the nucleus (Hindley et al., 2007) where genes from early regions (E1, E2, E3 and E4) are transcribed.

1.1.2.3 Early gene expression

E1A is the first region of the viral genome to be transcribed (Nevins et al., 1979) and transcripts appear within 1 hr of virus infection. Five mRNA transcripts coding for 289R, 243R, 217R, 171R and 55R proteins are generated from E1A by differential splicing (Perricaudet et al., 1979; Russell, 2000). The two major E1A proteins, 289R and 243R regulate the viral and the cellular gene transcription in the infected cells. Five conserved regions (CRs) viz., the N-terminus, CR1, CR2, CR3, and CR4 (Avvakumov et al., 2004) have been identified. The 243R lacks a 46-amino acid region near the center of the protein, a gap that corresponds to CR3 (Avvakumov et al., 2004) but shares N- and C-termini with the 289R protein (Perricaudet et al., 1979). E1A induces the host cell to enter S phase to create a permissive environment for viral replication, and stimulates the expression of other early viral genes (Russell, 2000; Berk, 2007; Russell, 2009). E1A is a potent transactivator capable of stimulating transcription from a variety of promoters. It associates with the E1A DNA-binding domains of transcription factors bound to promoters and thus, activates transcription from a number of viral and cellular promoters (Liu and Green, 1994).

The E1A, on its own, has been shown to induce apoptosis. It can increase p53 levels and promotes p53-dependent apoptosis in mouse embryonic fibroblasts (MEFs) (Lowe and Ruley, 1993; Samuelson and Lowe, 1997) and tumor suppression in murine melanoma cells (Deng et al., 1998). A deletion in CR1 and the amino terminus of E1A abolishes apoptosis indicating its role in the apoptotic complex formation / induction of apoptosis (Querido et al., 1997). A tumor suppressor gene p19ARF is required for E1A

signaling to p53 through a pathway involving retinoblastoma (RB) gene inactivation (de Stanchina et al., 1998). It has also been observed that E1A is sufficient by itself to induce substantial apoptosis independent of p53 and other adenoviral genes. E1A acts by processing of caspase-3 and cleavage of poly (ADP-ribose)-polymerase (PARP). E1A also de-regulates cell cycle checkpoints by forcing the cell into S phase, and thus inducing apoptosis (White. 1995; White. 2001). E1A modulates the inducible nitric oxide synthase gene repressing nitric oxide (NO) production. Since NO is an antiviral effector of innate immune system, by repressing NO production, E1A increases the chances of survival in the cell (Higashimoto et al., 2006).

The E1B region of HAdV-5 encodes two proteins, E1B-55K and E1B-19K. These proteins prevent virus-induced apoptosis by countering the actions of the E1A proteins (Berk, 2007). Protein E1B-55K binds to p53 (a pro-apoptotic gene) and suppresses the transcription of p53-activated genes thereby preventing the induction of apoptosis by virus infection or by E1A transformation. E1B-55K also forms a ubiquitin ligase complex, after interaction with viral E4orf6 and cellular proteins that targets p53 for degradation. E1B-19K protein is expressed early and, localizes to the nuclear membrane (Rao et al., 1997) and mitochondria suppressing the adenovirus induced apoptosis by p53 dependent or independent pathways (Lomonosova et al., 2005; Berk, 2007). E1B-19K is a Bcl-2 homologue, which binds to pro-apoptotic proteins BAK and BAX, prevents them from forming pores in mitochondria thus preventing the release of Cyto C and thereby preventing apoptosis (Berk, 2007).

The E2 encodes proteins that are involved in viral DNA replication (Berk, 2007). The E2A region encodes a 72kDa viral DNA-binding protein (DBP). It is highly phosphorylated at N-terminus, which is essential for DNA replication. The C-terminus of DBP is highly conserved among DBPs of *Mastadenoviruses* and is involved in DNA binding, initiation and maintenance of DNA replication, and transcriptional control of the major late promoter (Linne and Philipson, 1980). The E2B region encodes viral DNA polymerase (Pol) and the pre-terminal protein (pTP)(Berk, 2007). pTP forms a heterodimer with DNA polymerase (pol), which is transported to the nucleus of infected cell (due to the presence of a strong nuclear localization signal in pTP) where it initiates the viral DNA replication (Fredman and Engler, 1993).

The E3 region encodes non-essential genes, which are mainly involved in suppressing host immune responses (Weeks and Jones, 1985; Wold et al., 1994; Wold et al., 1995; Horwitz, 2004). HAdV-5 E3 region transcribes nine mRNAs generated by alternate splicing of the common transcript initiating from E3 promoter (Horwitz, 2004). These transcripts encode gp19, 14.7K, 12.5K, 10.4K, 11.6K and 6.7K proteins (Wold et al., 1995; Tollefson et al.,1996), which help virus in evading innate and adaptive immune responses (Wold et al., 1994; Toth et al., 2003).

The E4 region, located on the right end of the genome, produces 18 distinct mRNAs by alternate splicing (Tigges and Raskas, 1984). These transcripts encode seven proteins (Orf1 to Orf6 and Orf6/7) that appear to have a wide variety of functions including but not limited to viral DNA synthesis, protein phosphorylation, RNA processing, nucleo-cytoplasmic transport of the late viral mRNA and the shut-off of host protein synthesis during virus infection (Weitzman and Ornelles, 2005).

1.1.2.4 Viral DNA replication

The DNA replication of HAdV-5 occurs between 5-8 hrs post-infection of HeLa cells at a multiplicity of infection (MOI) of 10 (Berk, 2007). The cis-acting DNA sequence (origin of DNA replication or Ori) is located within ITR. Three viral proteins encoded by E2 region namely viral DNA polymerase (Pol), the pre-terminal protein (pTP), and the DNA-binding protein (DBP) are required for DNA replication. A pre-initiation complex (PIC) consisting of the viral and the cellular proteins forms at Ori (Temperley and Hay, 1992). Pol and pTP interact to form a heterodimer that binds to the origin of DNA replication (Temperley and Hay, 1992). Initiation of replication occurs by a protein priming mechanism at PIC (Ikeda et al., 1982; de Jong et al., 2003). After formation of PIC, the chain elongation starts by strand displacement mechanism using residues 4-6 of the 3'-GTAGTA-5' sequence at the terminus of the ITR as a template. Elongation of the strand requires DBP and the polymerase proteins. Displaced strand can duplicate by formation of panhandle structure (Wang and Pearson, 1985) by a mechanism called type 1 DNA replication. Various cellular factors like NF1 and NFIII/OctI help in the formation of a stabilized structure at Ori, which results in enhancing the reaction rate of replication (Zijderveld et al., 1993; Zijderveld and van der

Vliet, 1994; Zijderveld et al., 1994). In addition, virus genome replication needs cellular topoisomerase I (Nagata et al., 1983) to relax torsional stress placed on the viral DNA by the replication forks (Schaack et al., 1990).

1.1.2.5 Intermediate and late gene expression

Intermediate genes are synthesized 5-8 hrs post-infection, which coincide with DNA replication. Viral proteins IVa2 and IX are classified as intermediate or delayed-early, which have been shown to activate the major late promoter (MLP) (Lutz and Kedinger, 1996). They are structural proteins whose expression is dependent on the viral DNA replication but their transcripts appear earlier than those of the other late proteins (Binger and Flint, 1984). pIX is a 140 amino acid protein (Rosa-Calatrava et al., 2001). Each virion contains 240 copies of pIX (Parks et al., 1999), which confers the heat stability to the capsid (Vellinga et al., 2005). The C-terminal domain of pIX is involved in the formation of trimers, which is not required for its incorporation in adenovirus capsid (Berk, 2007; Russell, 2009). Since pIX is exposed to the outer surface of capsid, it is one of the preferred site for the insertion of targeting ligands (Curiel, 1999). Protein IVa2 is 450 amino acid and localizes in the nucleolus of the infected cells (Lutz and Kedinger, 1996; Lutz et al., 1996). pIVa2 acts as a transcriptional repressor of MLP (Tribouley et al., 1994; Lutz and Kedinger, 1996) and also plays a role in DNA packaging. Cellular transcription repressors repress the expression of both of these intermediate proteins during viral replication. A cellular protein CBF-1 has been identified as a repressor for pIX (Chen et al., 1994; Dou et al., 1994).

Viral DNA replication sets up the stage for the transcription of the late genes. Their expression correlates with a reduction in early gene expression, decrease in host cell transcription and the start of virus assembly. The major late promoter activates the expression of the major late transcription unit (Shaw and Ziff, 1980), which generates at least 20 mRNAs, which are further subdivided into five families (L1-L5) based on usage of polyA sites (Shaw and Ziff, 1980). The late genes encode structural proteins (hexon, penton, fiber, IIIa, V, VI, VII, VIII, IX, and μ), non-structural proteins (52K, 100K, 33K, 22K) and the core viral protease.

The L1 region encodes two proteins viz., 55K/52K and IIIa (Lucher et al., 1986). Protein 55/52K regulates the transcription from MLP (Gustin et al., 1996; Gustin and Imperiale, 1998) and plays an important role in virus esncapsidation and assembly (Perez-Romero et al., 2005; Perez-Romero et al., 2006; Wohl and Hearing, 2008). This protein is also required for stable association between viral DNA and empty capsid (Gustin et al., 1996; Gustin and Imperiale, 1998). Protein IIIa is located under the vertex of icosahedrons and is involved in virus assembly (San Martin et al., 2008; Ma and Hearing, 2011). This protein can enhance its own synthesis by an auto stimulatory mechanism (Tormanen et al., 2006; San Martin et al., 2008).

The L2 region encodes three proteins viz., pIII, pV and mu. Protein III is a penton base protein and contains an RGD (Arg-Gly-Asp) motif, which plays an important role during virus entry into the cell (Shayakhmetov et al., 2004; Vellinga et al., 2005). Mutation or deletion of this motif affects the viral entry and also escape of virus from the endosomes. Protein V is located inside the capsid in association with viral DNA and helps in the delivery of DNA to the nucleus in association with protein VII (Mathews, 2006; Berk, 2007; Russell, 2009). Upon infection, protein V is localized in the nucleolus and mitochondria (Matthews and Russell, 1998) after interaction with the cellular protein p32. Protein pVII localizes to the nucleus (Wodrich et al., 2003; Wodrich et al., 2006) of the infected cells, associates with viral DNA and mediates transcription repression (Johnson et al., 2004) in association with E1A. Protein mu is an 11 kDa protein which in association with pVII, plays an important role in DNA condensation (Anderson et al., 1989).

The L3 region encodes three proteins viz., protease, hexon and VI. Protease is a 23 kDa protein required during virus maturation. This protein interferes with various cellular transcription and translation processes to the advantage of the virus. It also has de-ubiquitinating activity (Balakirev et al., 2002). Hexon is the most abundant structural protein in the virus. It is a trimeric protein and comprises of two loops and seven hyper variable regions (HVRs). Loop 1 contains HVR1-6 and loop 2 contains HVR7, which are sites of antigenic variability and contain type specific epitopes (Roberts et al., 2006; Kalyuzhniy et al., 2008; Lehmkuhl and Hobbs, 2008). Hexon contains strong nuclear localization signals and has shown to exert an adjuvant effect (Molinier-Frenkel et al.,

2002) for activation of immune system. Protein VI, an internal capsid protein exhibits pH-independent membrane lytic activity upon exposure of N-terminal amphipathic α-helix and helps in escape of adenovirus from the endosomes after entry (Wiethoff et al., 2005). In a mature virion, this N-terminal helix is buried within a hexon-protein VI interface. This protein contains strong nuclear localization and export signals, which helps virus to reach nucleus in very short time upon entry. This protein also helps in localization of the hexon capsomers to the nucleus (Vellinga et al., 2005).

The L4 region encodes a structural protein pVIII and two non structural proteins viz., 100K and 33K/22K. Protein VIII is associated with the hexon and is thought to provide structural stability during capsid formation (Vellinga et al., 2005; Berk, 2007). Protein 33K predominantly localizes in the nucleus of the infected cells (Russell, 2009), where it acts as a virus encoded RNA splicing factor (Ali et al., 2007) and plays an important role in the assembly of the virions (Fessler and Young, 1999; Finnen et al., 2001; Ali et al., 2007). Its expression is optimal at the beginning of the late phase of infection (Ali et al., 2007) and is essential for the late phase of transcription. Protein 22K shares the N-terminal 105 amino acids with 33K and plays an important role in viral DNA encapsidation (Ostapchuk and Hearing, 2005; Ostapchuk et al., 2005; Ostapchuk et al., 2006).

Protein 100K, a non-structural protein has been shown to transport hexon monomers from the cytoplasm to the nucleus and is involved in their trimerization in the nucleus (Hong et al., 2005, Koyuncu and Dobner, 2009). This is a scaffolding protein required for the viral capsid assembly (Berk, 2007). 100K is also involved in the translation of adenoviral mRNA by ribosome shunting and eliminates the cap-dependent translation pathway thus, preventing the cellular mRNA translation (Dolph et al., 1988; Cuesta et al., 2004). Protein 100K has been shown to be a substrate for Granazyme B (GrB) that prevents the granule induced apoptosis in the infected target cells. (Andrade et al., 2001; Andrade et al., 2003; Cuesta et al., 2004; Ursu et al., 2004; Andrade et al., 2007). 100K undergo tyrosine phosphorylation, which is essential for efficient ribosome shunting and late protein synthesis (Xi et al., 2005). 100K also undergo methylation at RGG motifs, which catalyzes the binding of 100K to the hexon, promotes capsid

assembly and modulates the tripartite leader-mRNA interaction (Koyuncu and Dobner, 2009).

The L5 region encodes an important structural protein named fiber (Ruigrok et al., 1994; Chroboczek et al., 1995). There are 12 fibers present on each of the 12 vertices of an adenovirus capsid. It has N- terminal tail attached to a penton base, a middle region named shaft and a C-terminal region named knob. The knob region helps in attachment of the virus to a cellular receptor. Variability in the knob sequence is responsible for viral antigenic diversity (Nanda et al., 2005; Schoggins et al., 2005). Fiber is also one of the targets considered for modification in the tailoring of viral tropism towards specific organs or cells for the purpose of gene delivery (Glasgow et al., 2004; Nanda et al., 2005). It consists of a proline –glycine rich sequence, which is important in the folding of the shaft. The length of the shaft plays an important role in virus tropism and providing stability to the virus capsid (Ambriovic-Ristov et al., 2003).

1.1.2.6 Virus release from the cell

Assembly of virus occurs in the nucleus following the transport of viral structural proteins form the cytoplasm to the nucleus. The first step in virus assembly is formation of capsomeres. The pVI protein, which contains both a nuclear localization and a nuclear export signal, attaches to hexon monomers and transports them to the nucleus (Wodrich et al., 2003). The cleavage of the pVI NLS stops its shuttling activity and the transport of hexon into the nucleus, and initiates the virus assembly instead (Kauffman and Ginsberg, 1976; Wodrich et al., 2003; Wiethoff et al., 2005). Using 100K (Hong et al., 2005) and IVa2 (Zhang and Imperiale, 2003) proteins as a scaffold, hexon then trimerizes in the nucleus of the cell. The penton base interacts with the fiber trimmers and assembles with the hexon capsomers to form intact capsids (Horwitz et al., 1969). During assembly, light and heavy assembly intermediates are observed. Once formed, a cis-acting DNA sequence located in the left end of the viral DNA mediates the DNA capsid interactions (Russell, 2009). The encapsidation initiates from the left end of the DNA and immature virions are formed. It is still unclear how viral DNA is inserted into the capsid. The viral protease then cleaves the precursor proteins IIIa, TP, VI, VII and VII to generate mature proteins (Russell, 2009), which stabilizes the viral structure rendering it infectious.

Mature virions remain in the infected cells and are released upon cell lysis. Escape of mature virus is facilitated by several mechanisms. The 11.6 kDa protein encoded by E3 region and known as adenovirus death protein (ADP), is expressed during early stages of infection and results in lysis of the infected cell. Expression of this protein is associated with increased oxidative stress and mitochondrial activity indicating the involvement of mitochondria in the process as a source of energy to effect cell lysis (Wold et al., 1995; Tollefson et al., 1996). Excess production of fiber during later stages of infection has also been suggested as an escape mechanism for progeny adenovirus (Walters et al., 2002). The fiber binds to CAR resulting in easier access to the apical surface and loosening of the tight junctions (Walters et al., 2002). Exact mechanisms behind both these processes are still elusive.

1.1.3 Bovine adenovirus

Bovine adenovirus (BAdV) was first isolated in the 1960s (Darbyshire et al., 1965). So far, eleven serotypes (Harrach and Benko, 2007; Lehmkuhl and Hobbs, 2008) have been identified from both healthy animals (Darbyshire et al., 1965) and animals with respiratory or enteric diseases (Lehmkuhl et al., 1975; Smyth et al., 1996).

1.1.3.1 Classification

Based on the phylogenetic analysis, the identified serotypes of bovine adenoviruses are classified as members of *Mastadenovirus* genus (BAdV-1, BAdV-2, BAdV-3, BAdV-9 and BAdV-10) or *Atadenovirus* genus (BAdV-4, BAdV-5, BAdV-8, BAdV-6, BAdV-7 and Rus) (Benko et al., 2005; Harrach and Benko, 2007; Lehmkuhl and Hobbs, 2008). The BAdV serotypes of *Mastadenovirus* genus are more closely related to ovine adenoviruses than they are to each other. In contrast, BAdV serotypes of *Atadenovirus* genus are more closely related to each other.

1.1.3.2 Bovine adenovirus type 3

The virus was isolated from the eye of an apparently healthy cow and later from African buffalo, sheep and deer (Darbyshire et al., 1965). However, experimental infections of cattle with BAdV-3 can produce sub-clinical infections or mild respiratory

disease with the production of neutralizing antibodies (Lehmkuhl et al., 1975; Mittal et al., 1999). BAdV-3 is a non-enveloped icosahedral particle of 75 nm in diameter and contains dsDNA genome of 34,446 base pairs with 54% G/C content (Reddy et al., 1998). The BAdV-3 genome has 33 predicted open reading frames (Reddy et al., 1998; Reddy et al., 1999a; Reddy et al., 1999b; Kulshreshtha et al., 2004). BAdV-3 genome is flanked on either end by the ITRs of 195 bases, which have an unusually high G/C content (Reddy et al., 1998). BAdV-3 is unique in that part of the E1A open reading frame (ORF) appear to be required for DNA packaging (Xing et al., 2003; Xing and Tikoo, 2006; Xing and Tikoo, 2007). BAdV-3 also lacks TATA or CAAT boxes between the left ITR and upstream of the E1A start codon suggesting that E1A promoter is located within the left ITR (Xing and Tikoo, 2006). The genome is organized into early (E) region comprising of four transcriptional units (E1 – E4), intermediate and late (L) regions comprising of seven regions (L1-L7) (Reddy et al., 1998).

1.1.3.2.1 Early gene expression

The E1 region of BAdV-3 encodes potential ORFs of 211 (E1A), 157 ($E1B_{small}$), and 420 ($E1B_{large}$) amino acids (Reddy et al., 1999a). The E1 region produces nine mRNA transcripts all sharing 5' and 3' termini (Reddy et al., 1999a). E1A region produces six transcripts, which translate into three proteins of 211, 115, and 100 amino acids that share a common N-terminus (Reddy et al., 1999a). E1A proteins have no predicted nuclear localization signal(s) suggesting that their interaction with cellular proteins may help in the nuclear localization of E1A proteins (Reddy et al., 1999a). Bovine E1A proteins are involved in transactivation of viral genes and are essential for viral replication (Reddy et al., 1999b; Zhou et al., 2001). Transcripts from E1B region encode two proteins of 420 ($E1B_{large}$) and 157 ($E1B_{small}$) amino acids (Reddy et al., 1999a) and are homologue of HAdV-5 proteins E1B-55K and E1B-19K proteins, respectively (Reddy et al., 1998; Zheng et al., 1994). Of these early proteins, E1A and $E1B^{large}$ are essential for BAdV-3 replication (Zakhartchouk et al., 2004). Unlike HAdV-5, BAdV-3 E1A and E1B transcripts are 3' co-terminal (Reddy et al., 1999a)

The E2 region is divided into E2A and E2B transcriptional units, and encode proteins involved in DNA replication (Reddy et al., 1998). The E2A codes for a 432

amino acid long DNA binding protein (DBP), which shows 38 to 47% similarity with DBPs of other members of the *Mastadenovirus* genus. The DBP is expressed as a 48 kDa protein in BAdV-3 infected cells (Zhou et al., 2001). The DBP expression is detected 6-12 hrs post-infection and continues throughout the infection cycle of the BAdV-3, which has helped in developing a titration method for BAdV-3 (Zhou et al., 2001). The E2B region codes for the viral DNA polymerase (Pol) and the pre-terminal protein (pTP), which share 59-62% and 58-60% similarity, respectively, with their homologue in other members of the *Mastadenovirus* genus (Reddy et al., 1998). In BAdV-3, the Pol protein is 1,023 amino acids (Reddy et al., 1998) and the pTP protein is 649 amino acids long (Baxi et al., 1998). pTP protein contains a YSRLVYR motif, which has been shown to be essential for protein priming of the DNA replication initiation. The transcripts of DNA pol and pTP are 3' co-terminal (Reddy et al., 1998).

The E3 region of BAdV-3 is located between the coding sequences for pVIII and the fiber protein (Reddy et al., 1998; Idamakanti et al., 1999). It is 1.5 kb long and is transcribed left to right on "r" strand (Idamakanti et al., 1999). The E3 region produces five transcripts originating from the E3 promoter and the MLP that have the potential to encode four proteins of 284, 121, 86, or 82 amino acids (Idamakanti et al., 1999). In infected cells, 284R appears as proteins of 48, 67, and 125 and 140 kDa. Further analysis confirmed that 48 kDa and 67 kDa represent forms containing high mannose and N-linked complex oligosaccharides. The 125 kDa and 140 kDA bands appeared to be homo and heterodimers of different forms (Idamakanti et al., 1999). Protein 121R is expressed as 14.5 kDa protein in BAV-3-infected cells and does not appear to undergo any post-translational modification (Idamakanti et al., 1999; Zakhartchouk et al., 2001). Since the E3 region is not essential for BAdV-3 replication *in vitro* (Zakhartchouk et al., 1998) or *in vivo* (Zakhartchouk et al., 1999), it has been used as site for the insertion and expression of vaccine antigen genes (Zakhartchouk et al., 1998; Baxi et al., 2000).

As in HAdV-5, E4 region of BAdV-3 is located near the right end of the genome (Reddy et al., 1998). It is transcribed right to left on "l" strand. The E4 region produces seven transcripts (Baxi et al., 1999), which have the potential to code at least five proteins viz., 143R (ORF1), 69R (ORF2), 286R (ORF3), 143R2 (ORF4) and 219R

(ORF5), which appear unique to BAdV-3. None of the individual E4 proteins are essential for BAdV-3 replication (Baxi et al., 1999; Baxi et al., 2001).

1.1.3.2.2 Intermediate gene expression

Like HAdV-5, BAdV-3 also has positional homolog of two identified intermediate proteins, pIX and IVa2 (Reddy et al., 1998). The BAdV-3 pIX protein is 125 amino acids long and shows 16-28% homology with pIX proteins from other members of the *Mastadenovirus* genus. The pIX protein is expressed as a 14 kDa protein (Reddy et al., 1999a) in BAdV-3 infected cells. Protein pIX is a component of viral capsid and its C-terminus is exposed on the surface of the viral capsid (Zakhartchouk et al., 2004); ligands have been fused to pIX which change the host-tropism of BAdV-3 (Zakhartchouk et al., 2004). The IVa2 protein of BAdV-3 is 376 amino acids long and shows 64-70% homology with the IVa2 proteins from other members of the *Mastadenovirus* genus. The IVa2 transcript has a polyadenylation signal unique from that of pTP and DNA Pol (Baxi et al., 1998; Zheng et al., 1999).

1.1.3.2.3 Late gene expression

The major late promoter (MLP) of BAdV-3 contains a TATA box, an inverted CAAT box, a binding site for USF, and an initiator element, which is similar to that in HAdV-3 (Reddy et al., 1998). The tripartite leader is 205 nucleotides in length (Reddy et al., 1998) and is attached to all the late gene transcripts. The first of the three parts is 40 nucleotides long and is located near the MLP. Second part is located within the DNA Pol gene on the non-coding strand and is 78 nucleotides long. The third part is located in the pTP gene on the non-coding strand and is 87 nucleotides in length. The late region of BAdV-3 produces several mRNAs, which are divided into seven families (L1 to L7) based on usage of 3' polyadenylation sites (Reddy et al., 1998).

The L1 region of BAdV-3 encodes four proteins viz., 52K, IIIa, penton, and pVII, which share a common, polyA signal. The BAdV-3 52K protein is predicted to encode a protein of 370 amino acids long (Reddy et al., 1998; Paterson, 2010) and predominantly localizes into the nucleus of the infected cells using active import pathways using the classical importin α /β pathway for nuclear import (Paterson, 2010). It can also transport

to nucleus in detergent permeabilized cells indicating its affinity to bind with nuclear components. The 52K protein specifically interacts with the nucleolar protein NFκB-binding protein (NFBP) and relocates it from the nucleolus to the nucleus of the infected cells (Paterson, 2010). It also interacts *in v*itro and *in vivo* with pVII (Paterson, 2010). The pIIIa protein is 568 amino acids long (Reddy et al., 1998) and contain a putative viral protease cleavage site located 19 residues from the C-terminus. The penton protein of BAdV-3 is 482 amino acids long (Reddy et al., 1998) and lacks the conserved RGD motif required for integrin binding. It also lacks a leucine-aspartic acid-valine motif but contains a methionine-aspartic acid-valine (MDV) motif. The core protein pVII of BAdV-3 is 171 amino acids long (Reddy et al., 1998), is highly basic (25% basic residues) and has a cleavage site for the adenovirus protease (MYGG↓A) located 19 nucleotides from the N-terminus of the protein (Reddy et al., 1998).

The L2 region of BAdV-3 codes for a single transcript encoding the core protein pV (Reddy et al., 1998). The pV protein is 410 amino acids long and contains a bipartite nuclear localization signal.

The L3 region of BAdV-3 encodes the μ protein, (also known as pX) (Reddy et al., 1998), which is 80 amino acids long and shares 53-64% homology with the μ proteins of other members of the *Mastadenovirus* genus. The protein contains two predicted protease cleavage sites and a nuclear localization signal (Reddy et al., 1998).

The L4 region of BAdV-3 encodes 263 amino acids long protein IV, which shares 32-39% homology with the pVI proteins of other members of the *Mastadenovirus* genus. It contains two consensus cleavage sites for the viral protease located after residue 33 (in N-terminus) and after residue 252 (in C-terminus) (Reddy et al., 1998).

The L5 region of BAdV-3 encodes two proteins viz., hexon and viral protease (Reddy et al., 1998). BAdV-3 hexon is 910 amino acids long and shows significant (66-71%) homology with the hexon proteins of other members of the *Mastadenovirus* genus. Hexon is expressed as 98 kDa protein in infected cells (Kulshrestha et al., 2004). The protease encoded by BAdV-3 is 204 amino acids long and shows homology with the proteases of other members of the *Mastadenovirus* genus (Reddy et al., 1998).

The L6 region of BAdV-3 encodes three non structural proteins viz., 100K, 33K, 22K, and a structural protein pVIII (Reddy et al., 1998; Kulshreshtha, 2009). The 100K

protein of BAdV-3 is largest among all the known members of *Mastadenovirus* genus. It is 850 amino acids long and share 50-52% homology with the 100K protein of other members of the *Mastadenovirus* genus. In BAdV-3, 22K and 33K proteins are 274 amino acids long and 279 amino acids long, respectively (Reddy et al., 1998; Kulshreshtha, 2009). The proteins localize to the nucleus of the infected cells and appear to be involved in the capsid assembly particularly in the encapsidation of DNA (Kulshreshtha et al., 2004). The 33K protein of BAdV-3 interacts with the 100K and pV proteins of BAdV-3 (Kulshreshtha and Tikoo, 2008), and can transactivate the MLP (Kulshreshtha, 2009). During the course of infection, 33K protein interacts and co-localizes with bovine presenilin-1-associated protein / mitochondrial carrier homolog 1 (BoPSAP / BoMtch1) in the mitochondria and can modulate the pro apoptotic effect of Stauroporine (Kulshreshtha, 2009). In BAdV-3, minor capsid protein pVIII is 216 amino acids long (Reddy et al., 1998) and shares 51-56% homology with pVIII proteins in other members of the *Mastadenovirus* genus. It contains two protease cleavage sites between amino acids 111 -112 and 146-147 (^{108}IAGG↓G and ^{143}LGGG↓S).

The L7 region of BAdV-3 encodes fiber protein. The fibre protein is 976 amino acids long and has relatively low homology (22-27%) with the fiber proteins of other members of the *Mastadenovirus* genus. A N-terminus hydrophobic sequence motif , [FNPVYPY (D/E)] (Caillet-Boudin, 1989) and C-terminal domain TLWT motif (Chroboczek et al., 1995) of HAdV-5 fiber are strongly conserved in BAdV-3 fiber (Reddy et al., 1998). Fiber comprises of tail, shaft and knob regions. Compared to HAdV-5 fiber, BAdV-3 fiber shaft is quite long and has structure with several bends (Ruigrok et al., 1994). BAdV-3 fiber is expressed as a 102 kDa glycoprotein (Wu et al., 2004), which is localized in the nucleus of the infected cells. The N- terminus of BAdV-3 fiber contains a NLS , which helps the fiber to localize to the nucleus of the transfected or infected cells (Wu et al., 2004). Mutant BAdV-3 expressing NLS deleted fiber shows significantly impaired growth (Wu et al., 2004). Fiber knob appears to determine virus tropism as replacing the knob of BAdV-3 fiber with that of HAdV-5 resulted in virus, which specifically infected human and not bovine cells (Wu and Tikoo, 2004).

1.2 Mitochondria

1.2.1 Introduction

Mitochondria are cellular organelles found in the cytoplasm of almost all eukaryotic cells. One of their important functions is to produce and provide energy to the cell in the form of ATP, which help in proper maintenance of the cellular processes thus making them indispensable for the cell. Besides acting as a powerhouse of the cell, they act as a common platform for the execution of a variety of cellular functions in normal or microorganism infected cells. Mitochondria have been implicated in aging (Wallace, 2005; Chan, 2006), apoptosis (Antignani and Youle, 2006; Chan, 2006; Gradzka, 2006; McBride et al., 2006; Kroemer et al., 2007), the regulation of cell metabolism (Chen and Chan, 2005; Mannella, 2006), the cell-cycle control (Hardie et al., 2003; Jones et al., 2005; Mandal et al., 2005), the development of the cell (Bakeeva et al., 1978; Bakeeva et al., 1983; Honda and Hirose, 2003), antiviral responses (Seth et al., 2005), signal transduction (Bossy-Wetzel et al., 2003) and diseases (Olanow and Tatton, 1999; Van Den Eeden et al., 2003; Martin, 2006; McFarland et al., 2007).

Although all mitochondria have the same architecture, they vary greatly in shape and size. The mitochondria are composed of the outer mitochondrial membrane, the inner mitochondrial membrane, the intermembrane space (space between outer and inner membrane) and the matrix (space enclosed by the inner mitochondrial membrane). The outer membrane is a smooth phospholipid bilayer, imbedded with different types of proteins (Rapaport, 2003). Most important of them are the porins, which freely allow the transport (export and import) of the molecules (proteins, ions, nutrients and ATP) less than 10 kDa across the membranes. The outer membrane surrounds the inner membrane creating an inter-membrane space that contain molecules such as cytochrome C (Cyt-C), second mitochondria-derived activator of caspases (SMAC)/ Diablo and endonuclease G. It also acts as a buffer zone between the outer membrane and the inner membrane of the mitochondria. The inner membrane is highly convoluted into structures called cristae, which increase the surface area of the membrane and are the seats of the respiratory complexes. The inner membrane of the mitochondria allows free transport of water, oxygen and carbon dioxide. The matrix contains enzymes for the aerobic respiration,

dissolved oxygen, water, carbon dioxide, and recyclable intermediates that serve as energy shuttles and perform other functions.

Mitochondria contain a single 16 kb circular DNA genome, which codes for 13 proteins (mostly subunits of respiratory chains I, II, IV and V), 22 mitochondrial tRNAs and 2 rRNAs (Shadel and Clayton, 1997; Shoubridge, 2002). The mitochondrial genome is not enveloped (like nuclear envelop), contains few introns and does not follow universal genetic code (Burger et al., 2003). Although the majority of the mitochondrial proteins are encoded by the nuclear DNA and imported into the mitochondria [reviewed by (Rapaport, 2003; Neupert and Herrmann, 2007; Chacinska et al., 2009; Schmidt et al., 2010; van der Laan et al., 2010)], mitochondria synthesize a few proteins that are essential for their respiratory function (Burger et al., 2003; Wallace, 2005).

Proteins destined to the mitochondria have amino terminal pre-sequences known as mitochondria/ matrix localization signals (MLS), which can be 10-80 amino acid long with predominantly positively charged amino acids (Rapaport, 2003). The combination of these pre-sequences with adjacent regions determines the localization of a protein in the respective mitochondrial compartments. The outer mitochondrial membrane contains two major translocators viz., a) the translocase of the outer membrane (TOM) 40, which functions as an entry gate for most mitochondrial proteins with MLS and b) sorting and assembly machinery (SAM) or the translocase of β-barrel (TOB) protein, which is a specialized insertion machinery for beta-barrel membrane proteins (Habib et al., 2005). Once proteins pass through the outer membrane, they are recruited by the pre-sequence translocase-associated motor (PAM) to the translocase of the inner mitochondrial membrane (TIM) 23 complexes, which mediates the import of proteins to the matrix. Finally, the pre-sequences are cleaved in the matrix, the proteins are modified to their tertiary structure and rendered functional (Schmidt et al., 2010).

Viruses are acellular obligate intracellular microorganisms that infect the living cells/organisms and are the only exception to the cell theory proposed by Schleiden and Schwann in 1838/1839 (Schwann, 1847). Viruses have an outer protein capsid and a nucleic acid core. Usually, the viral nucleic acids can be either DNA (double or single stranded) or RNA (+ or – sense single stranded or double stranded RNA). Some of the viruses are covered with an envelope embedded with glycoproteins. The viruses have

long been associated with the living organisms and it was in the later part of the 20th century that their relationship with various cellular organelles was studied in detail. To survive and replicate in the cell, viruses need to control various cellular organelles involved in defense and immune processes. They also require energy to replicate and escape from the cell. Once inside the host cell, they modulate various cellular signal pathways and organelles including mitochondria, and use them for their own survival and replication. This review summarizes the functions of mitochondria and how viruses modulate them (Figure 1).

1.2.2 Viruses regulate Ca^{2+} homeostasis in host cells

Ca^{2+} is one of the most abundant and versatile elements in the cell and acts as a second messenger to regulate many cellular processes (Berridge et al., 1998). Earlier, the outer membrane of mitochondria was thought to be permeable to Ca^{2+}, but recent studies suggest that the outer membrane contains a voltage-dependent anion channel (VDAC) that possess Ca^{2+} binding domains, which regulate entry of Ca^{2+} into the mitochondrial inter membrane space (Green, 1998; Chorna et al., 2010; Liu et al., 2011). The influx of Ca^{2+} through the inner membrane is regulated by the mitochondrial Ca^{2+} uniporter (MCU), which is a highly selective Ca^{2+} channel that regulates the Ca^{2+} uptake based on the mitochondrial membrane potential (MMP). The net movement of charge due to Ca^{2+} uptake is directly proportional to the decrease of MMP (Kirichok et al., 2004). A second mechanism that helps in Ca^{2+} movement across the mitochondria membrane is called "rapid mode" uptake mechanism (RaM) (Gunter and Gunter, 2001). In this process, Ca^{2+} transports across the mitochondrial membrane by exchange with Na^{+}, which in turn depends upon its exchange with H^{+} ion and thus MMP. This ion exchange across the mitochondrial membrane decreases the MMP and is dependent on electron transport chain (ETC) for its maintenance.

The Ca^{2+} efflux mechanism is regulated by the permeability transition pore (PTP). The PTP is assembled in the mitochondrial inner and the outer membranes (Halestrap, 2009; Halestrap, 2010) with Ca^{2+} binding sites on the matrix side of the inner membrane. The PTP regulates the mitochondrial Ca^{2+} release by a highly regulated "Flickering" mechanism that controls the opening and the closing of the pore (Huttemann et al.,

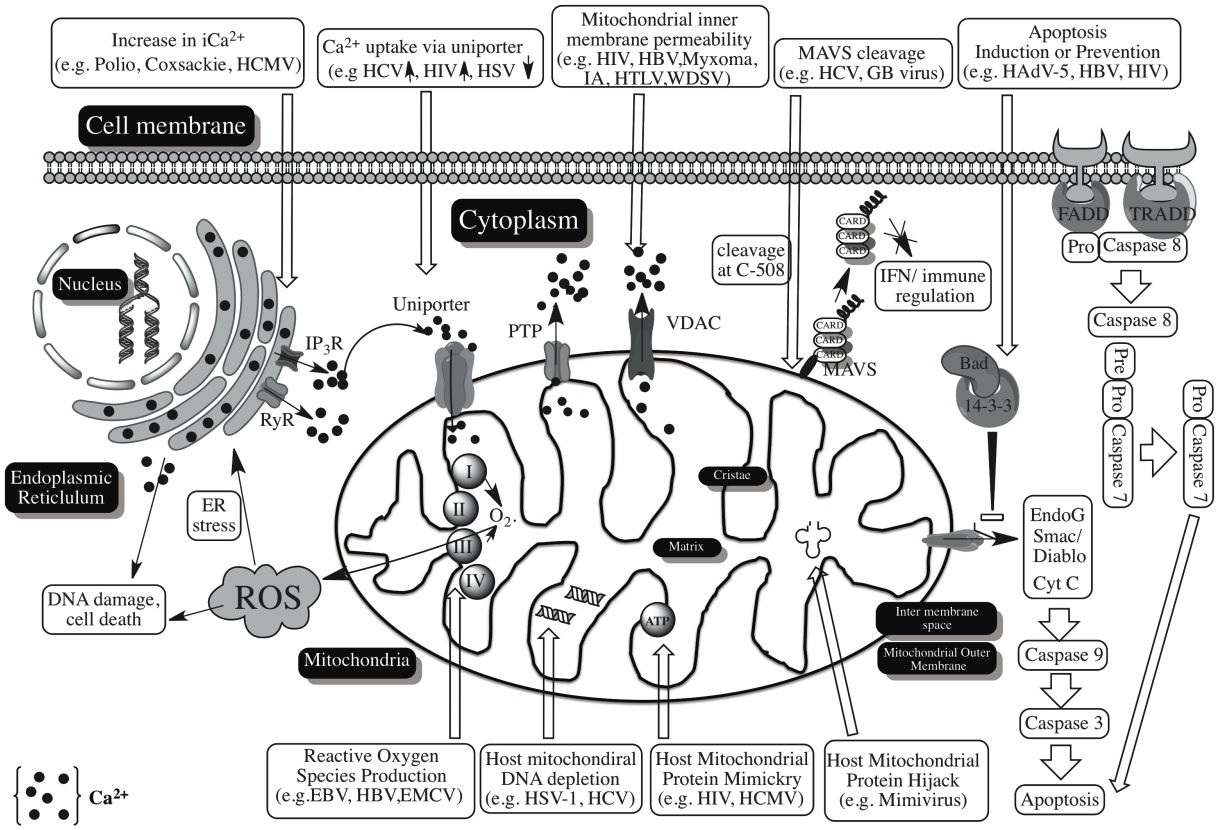


Figure 1.1 Schematic diagram of the cell showing mitochondria, nucleus, endoplasmic reticulum (ER) and cell membrane. iCa^{2+} (Intracellular calcium), FADD (Fas-Associated protein with Death Domain), TRADD (Tumor necrosis factor receptor type 1-associated Death domain protein), PTP (Permeability transition pore), VDAC (Voltage-dependent anion channel), IP_3R (Inositol 1,4,5-trisphosphate receptor), RyR (Ryanodine receptor), MAVS (Mitochondrial anti-viral signaling), I, II, III and IV are complex I to IV of electron transport chain, O_2· (superoxide radical), Bad (Bcl-2-associated death promoter), ROS (Reactive oxygen species), IFN (Interferon), HCMV (Human cytomegalovirus), HIV (Human immunodeficiency virus), HSV (Herpes simplex virus), HBV (Hepatitis B virus), HTLV (Human T-lymphotropic virus), IA (Influenza A virus), WDSV (Waleye dermal sarcoma virus), HCV (Hepatitis C virus), HAdV-5 (Human adenovirus-5), EBV (Epstein–Barr virus), EMCV (Encephalomyo-carditis virus).

2000). RaM works together with ryanodine receptor (RyR) isoform 1, which is another very important calcium release channel (Petronilli et al., 1991). Both, RyR and RaM regulate the phenomenon of excitation-metabolism coupling in which cytosolic Ca^{2+} induced contraction is matched by mitochondrial Ca^{2+} stimulation of ox-phos (Xia et al., 2006). However, mitochondrial Ca^{2+} overload can result in prolonged opening of the pore leading to pathology (Koopman et al., 2010).

Although Ca^{2+} is involved in the activation of many cellular processes including stimulation of the ATP synthase (Susin et al., 1999, Balaban, 2009), allosteric activation of Kreb's cycle enzymes (Wernette et al., 1981; McCormack and Denton, 1993) and the adenine nucleotide translocase (ANT) (Mildaziene et al., 1995), the primary role of mitochondrial Ca^{2+} is in the stimulation of ox-phos (Haworth et al., 1981; Copello et al., 2002; Nasr et al., 2003). Thus, the elevated mitochondrial Ca^{2+} results in upregulation of the entire ox-phos machinery, which then results in faster respiratory chain activity and higher ATP output, which can then meet the cellular ATP demand. Ca^{2+} also upregulates other mitochondrial functions including activation of N-acetylglutamine synthetase to generate N-acetylglutamine (Johnston and Brand, 1990), potent allosteric activation of carbamoyl-phosphate synthetase, and the urea cycle (McGivan et al., 1976). Thus, any perturbation in mitochondrial or cytosolic Ca^{2+} homeostasis has profound implications for the cell function. Moreover, mitochondrial Ca^{2+} particularly at high concentrations experienced in pathology, appears to have several negative effects on the mitochondrial functions (Peng and Jou, 2010).

A number of viruses alter the Ca^{2+} regulatory activity of the cell for their survival. Herpes simplex type (HSV) -1 virus causes a gradual decline (65%) in the mitochondrial Ca^{2+} uptake at 12 hrs post-infection (Lund and Ziola, 1985), which helps in virus replication. Although mitochondrial Ca^{2+} uptake fluctuates throughout the course of measles virus infection of the cells, the total amount of cellular Ca^{2+} remains the same (Lund and Ziola, 1985) indicating the tight control virus exerts over the cellular processes during its life cycle.

The core protein of hepatitis C virus (HCV) targets mitochondria and increases Ca^{2+} (Li et al., 2007; Campbell et al., 2009). The NS5A protein of HCV causes alterations in Ca^{2+} homeostasis (Gong et al., 2001; Kalamvoki and Mavromara, 2004;

Dionisio et al., 2009). Both of these proteins may be responsible for the pathogenesis of liver disorders associated with HCV infection. Even in the cells co-infected with HCV and human immunodeficiency virus (HIV), these viruses enhance the MCU activity causing cellular stress and apoptosis (Li et al., 2007; Baum et al., 2011). The p7 protein of HCV form porin like structures (Cook and Opella, 2010) and cause Ca^{2+} influx to cytoplasm from storage organelles (Griffin et al., 2004). These HCV proteins disturb the Ca^{2+} homeostasis at different stages of the infection and thus help to enhance the survival of the cell. Interestingly, interaction of protein X of hepatitis B virus (HBV) with VDAC causes the release of Ca^{2+} from the storage organelles (mitochondria/endoplasmic reticulum (ER)/ Golgi) into the cytoplasmic compartment, which appears to help virus replication (Bouchard et al., 2001; Choi et al., 2005).

The *Nef* protein of HIV interacts with IP_3R (Foti et al., 1999) and induces an increase in cytosolic Ca^{2+} through the promotion on T cell receptor-independent activation of the NFAT pathway (Manninen and Saksela, 2002). Activated NFAT, in turn, causes the low-amplitude intracellular Ca^{2+} oscillation, promoting the viral gene transcription and replication (Kinoshita et al., 1997).

Ca^{2+} is an important factor for different stages of rotavirus lifecycle, and for the stability to rotavirus virions (Ruiz et al., 2000). The NSP4 protein of rotavirus increases the cytosolic Ca^{2+} concentration by activation of phospholipase C (PLC) and the resultant ER Ca^{2+} depletion through IP_3R (Tian et al., 1995; Diaz et al., 2008). This alteration in Ca^{2+} homeostasis has been attributed to increased cell membrane permeability (Zambrano et al., 2008). A decrease in the cellular Ca^{2+} concentrations toward the end of the life cycle has been reported to enable rotavirus release from the cell (Ruiz et al., 2007).

The 2BC protein of poliovirus increases the intracellular Ca^{2+} concentrations in the cells 4 hrs post infection, which is necessary for the viral gene expression (Irurzun et al., 1995; Aldabe et al., 1997). Toward the end of the virus life cycle, the release of Ca^{2+} from the lumen of ER through IP_3R and RyR channels cause accumulation of Ca^{2+} in the mitochondria through uniporter and VDAC resulting in mitochondrial dysfunction and apoptosis (Brisac et al., 2010). In contrast, the 2B protein of Coxsackie virus decreases the membrane permeability by decreasing Ca^{2+} concentrations in the infected cells

(Nieva et al., 2003; van Kuppeveld et al., 2005) due to its porin like activity that results in Ca^{2+} efflux from the organelles. Reduced protein trafficking and low Ca^{2+} concentration in the Golgi and the ER favors the formation of the viral replication complexes, down-regulates host anti-viral immune response and inhibits apoptosis (de Jong et al., 2006; de Jong et al., 2008).

Enteroviruses orchestrate the apoptotic process during their life cycle to enhance their entry, survival and release from the infected cell. The perturbation in the cytoplasmic Ca^{2+} homeostasis at 2–4 hrs post infection coincides with the inhibition of the apoptotic response that can be attributed to decreases in the cytotoxic levels of Ca^{2+} in the cell and the mitochondria. This also provides the virus with optimum conditions for the replication and protein synthesis. Finally, a decrease in the mitochondrial and other storage organelles (ER and Golgi) Ca^{2+} levels causes an increase in the cytosolic Ca^{2+} concentration, leading to the formation of vesicles and cell death, thus assisting in the virus release (van Kuppeveld et al., 1997, van Kuppeveld et al., 2005; Campanella et al., 2004).

The pUL37 × 1 protein of human cytomegalovirus (HCMV) localize to the mitochondria (Bozidis et al., 2010) and causes the trafficking of Ca^{2+} from the ER to the mitochondria at 4-6 hrs post-infection (Sharon-Friling et al., 2006). Active Ca^{2+} uptake by mitochondria induces the production of ATP and other Ca^{2+} dependent enzymes accelerating virus replication, and a decrease in Ca^{2+} levels in the ER thus producing anti-apoptotic effects (Pinton et al., 2001).

The 6.7K protein encoded by E3 region of HAdV-2 localizes to the ER and helps to maintain the ER Ca^{2+} homeostasis in transfected cells, thus inhibiting apoptosis (Moise et al., 2002)

1.2.3 Viruses cause oxidative stress in host cells

The mitochondrial respiratory chain is the most significant source of reactive oxygen species (RO) in the cell. Superoxide ($O_{2-}\cdot$) is the primary ROS produced by the mitochondria. In the normal state, there is little or no leakage of electrons between the complexes of the electron transport chain (ETC). However, during stress conditions, a small fraction of the electrons leave complex III and reach complex IV (Niizuma et al.,

2009). This premature electron leakage to oxygen results in the formation of two types of superoxides viz., O_2-, in its anionic form and HO_2- in its protonated form.

Leakage of electrons takes place mainly from Q_o sites of complex III, which are situated immediately next to the inter-membrane space resulting in the release of superoxides in either the matrix or the inner-membrane space of the mitochondria (Muller et al., 2002; Muller et al., 2003; Muller et al., 2004; Skulachev, 2006). About 25-75% of the total electron leak through Complex III could account for the net extra-mitochondrial superoxide release (St-Pierre et al., 2002; Han et al., 2003; Miwa et al., 2003). Thus, the main source of $O_2^{-\cdot}$ in the mitochondria is the ubisemiquinone radical intermediate ($QH^{\cdot}$) formed during the Q cycle at the Q_O site of complex III (Tsutsui et al., 2006; Stowe and Camara, 2009; Tsutsui et al., 2009). Complex I is also a source of ROS, but the mechanism of ROS generation is less clear. Recent reports suggest that glutathionylation (Taylor et al., 2006) or PKA mediated phosphorylation (Ott et al., 2002; Raha et al., 2002; Taylor et al., 2006) of complex I can elevate ROS generation. Backward flow of electron from complex I to complex II can also result in the production of ROS (Stowe and Camara, 2009).

A variety of cellular defense mechanisms maintain the steady state concentration of these oxidants at non- toxic levels. This delicate balance between the ROS generation and the metabolism may be disrupted by various xenobiotics including viral proteins. The main reason for the generation of ROS in virus infected cells is to limit the virus multiplication. However, ROS also acts as a signal for various cellular pathways and viruses utilize the chaos generated inside the cell for their replication. A number of viruses cause oxidative stress to the host cells, which directly or indirectly helps them to survive. The core protein of HCV causes oxidative stress in the cell and alters apoptotic pathways (Nishina et al., 2008; Baum et al., 2011; de Mochel et al., 2010; Hsieh et al., 2010; Ming-Ju et al., 2011). The E1, E2, NS3 and core protein of HCV are potent ROS inducers, and can cause host DNA damage, independently (Hsieh et al., 2010; Machida et al., 2010; Ming-Ju et al., 2011) or mediated by nitric oxide (NO) thus aiding in virus replication.

The ROS are generated during HIV infection (Kruman et al., 1998; Baugh, 2000; Baum et al., 2011; Gil et al., 2010). H_2O_2, a ROS generated during HIV infection

strongly induces HIV long terminal repeat (LTR) via NF-kappa B activation. Impaired LTR activity ablates the LTR activation in response to the ROS thus aiding in virus replication (Pyo et al., 2008). HIV also causes extensive cellular damage due to increased ROS production and decreased cytosolic anti-oxidant production (Lin et al., 2011). Co-infection of HIV and HCV causes the hepatic fibrosis, the progression of which is regulated through the generation of ROS in an NF-κB dependent manner (Lin et al., 2011).

Epstein-Barr virus (EBV) causes increased oxidative stress in the host cells within 48 hrs during the lytic cycle indicating the role of ROS in the virus release (Lassoued et al., 2010). Oxidative stress activates the EBV early gene BZLF-1, which causes the reactivation of EBV lytic cycle (Lassoued et al., 2010). This has been proposed to play an important role in the pathogenesis of EBV-associated diseases including malignant transformations (Lassoued et al., 2008; Gargouri et al., 2009).

Interestingly, HBV causes both an increase and decrease in oxidative stress to enhance its survival in the host cells (Kim et al., 2010, Hu et al., 2011). HBV induces strong activation of Nrf2/ARE-regulated genes *in vitro* and *in vivo* through the activation of c-Raf and MEK by HBV protein X thus protecting the cells from HBV induced oxidative stress and promoting establishment of the infection (Schaedler et al., 2010). The protein X of HBV also induces the ROS mediated upregulation of Forkhead box class O4 (Foxo4), enhancing the resistance to the oxidative stress-induced cell death (Srisuttee et al., 2011). However, reports also suggest that upon exposure to oxidative stress, HBV protein X accelerates the loss of Mcl-1 protein via caspase-3 cascade thus inducing proapototic effects (Hu et al., 2011). Co-infection of HCV also causes the genotoxic effects in the peripheral blood lymphocytes due to increased oxidative damage and decreased MMP (Bhargava et al., 2010). It is possible that contradictory functions of protein X of HBV cold occur at different stages of virus replication.

Encephalomyocarditis virus (EMCV) causes oxidative stress in the cells damaging the neurons, which is an important process in the pathogenesis of EMCV infection (Ano et al., 2010).

1.2.4 Viruses regulate mitochondrial membrane potential in host cells

Membrane potential (MP) is the difference in voltage or electrical potential between the interior and the exterior of a membrane. The membrane potential is generated either by an electrical force (mutual attraction or repulsion between both positive or negative) and / or by diffusion of particles from high to low concentrations. The mitochondrial membrane potential (MMP) is a MP (≅ 180) across the inner membrane of mitochondria, which provides energy for the synthesis of ATP. Movement of protons from complex I to V of electron transport chain (ETC) located in the inner mitochondrial membrane create an electric potential across the inner membrane**,** which is important for the proper maintenance of ETC and ATP production. Reported MMP values for mitochondria *(in vivo)* differ from species to species and from one organ to another depending upon the mitochondria function, protein composition and the amount of oxidative phosphorylation activity required in that part of the body (Huttemann et al., 2008).

The voltage dependent anionic channels (VDACs) also known as mitochondrial porins form channels in the outer mitochondrial membranes and act as the primary pathway for the movement of metabolites across the outer membrane (Colombini et al., 1996; Forte et al., 1996; Han et al., 2003; Liu et al., 2011;Villinger et al., 2010). In addition, a number of factors including oxidative stress, calcium overload and ATP depletion induce the formation of non-specific mitochondrial permeability transition pores (MPTP) in the inner mitochondrial membrane, which is also responsible for the maintenance of MMP (Pebay-Peyroula et al., 2003; Chorna et al., 2010; Liu et al., 2011). The outer membrane VDACs, the inner membrane adenine nucleotide translocase (ANT) (Hunter and Haworth, 1979) and the cyclophilin D (CyP-D) in the matrix are the structural elements of the mitochondrial permeability transition pore (MPTP).

When open, MPTP increases the permeability of the inner mitochondrial membrane to ions and solutes up to 1.5 kDa, which causes dissipation of the MMP and diffusion of the solutes down their concentration gradients, by a process known as the permeability transition (Garlid et al., 1995; Bernardi, 1999). The MPTP opening is followed by the osmotic water flux, passive swelling, outer membrane rupture, and the release of pro-apoptotic factors leading to the cell death (Halestrap, 2006; Halestrap,

2010). Because of the consequent depletion of ATP and Ca^{2+} deregulation, opening of the MPTP had been proposed to be a key element in determining the fate of the cell before a role for the mitochondria in apoptosis was proposed (Bernardi, 1999).

The MMP can be altered by a variety of stimuli including sudden burst of ROS (Huttemann et al., 2008; Ming-Ju et al., 2011), Ca^{2+} overload in the mitochondria or the cell (Balaban, 2009; Peng and Jou, 2010; Szydlowska and Tymianski, 2010) and/or by proteins of invading viruses (Kruman et al., 1998; Piccoli et al., 2007; Gac et al., 2010). In general, an increase or decrease in MMP is related to the induction or the prevention of apoptosis, respectively. Prevention of apoptosis during early stages of the virus infection is a usual strategy employed by viruses to prevent host immune response, and promote their replication. On the contrary, induction of apoptosis during later stages of virus infection is a strategy used by viruses to release the progeny virions for dissemination to the surrounding cells.

Many viral proteins alter mitochondrial ion permeability and/or membrane potential for their survival in the cell. The p7, a hydrophobic integral membrane (Carrere-Kremer et al., 2002) viroprotein (Gonzalez and Carrasco, 2003) of HCV localizes to the mitochondria (Griffin et al., 2004) and controls the membrane permeability to cations (Pavlovic et al., 2003; Griffin et al., 2004) promoting the cell survival for virus replication (Gonzalez and Carrasco, 2003).

The R (Vpr) protein of HIV, a small accessory protein localizes to the mitochondria, interacts with ANT, modulates MPTP, and induces the loss of MMP promoting the release of Cyt-C (Azuma et al., 2006) leading to the cell death (Jacotot et al., 2000; Deniaud et al., 2004). The Tat protein of HIV also modulates MPTP leading to the accumulation of Tat in the mitochondria and induction of loss of MMP resulting in the caspase dependent apoptosis (Macho et al., 1999).

The M11L protein of myxoma poxvirus localizes to the mitochondria, interacts with the mitochondrial peripheral benzodiazepine receptor (PBR) and regulates MPTP (Everett et al., 2002) inhibiting MMP loss (Everett et al., 2000), and thus inhibiting the induction of apoptosis during viral infection (Macen et al., 1996). The FIL protein of vaccinia virus down regulates the pro-apoptotic Bcl-2 family protein Bak and, inhibits the loss of the MMP and the release of Cyt-C (Wasilenko et al., 2003; Wasilenko et al.,

2005). The crmA/Spi-2 protein of vaccinia virus, a caspase 8 inhibitor, modulates MPTP thus preventing apoptosis (Wasilenko et al., 2001).

The PB1-F2 protein of influenza A viruses localize to the mitochondria (Bruns et al., 2007;Chen et al., 2001; Gibbs et al., 2003; Henkel et al., 2010), interacts with VDAC1 and ANT3 (Danishuddin et al., 2010) resulting in decreased MMP, which induces the release of pro-apoptotic proteins causing cell death. Recent evidence shows that PB1-F2 is also able to form non-selective protein channel pores resulting in the alteration of mitochondrial morphology, the dissipation of MMP and the cell death (Henkel et al., 2010). The M2 protein of influenza virus, a viroprotein also causes the alteration of mitochondrial morphology, the dissipation of MMP and the cell death (reviewed by Gonzalez and Carrasco, 2003).

The p13II, an accessory protein encoded by x-II ORF of human T-lymphotropic virus (HTLV), a new member of the viroprotein family (Silic-Benussi et al., 2010), localizes to the mitochondria of the infected cells and increases the MMP leading to apoptosis (Ciminale et al., 1999) and mitochondrial swelling (Biasiotto et al., 2010; Ciminale et al., 1999; Silic-Benussi et al., 2004).

The Orf C protein of Walleye dermal sarcoma virus (WDSV) localizes to the mitochondria (Nudson et al., 2003), induce perinuclear clustering of mitochondria and loss of MMP (Nudson et al., 2003) leading to the release of pro-apoptotic factors thus causing apoptosis.

The 2B protein of Coxsackie virus decreases MMP by decreasing the Ca^{2+} concentrations in the infected cells (Nieva et al., 2003; van Kuppeveld et al., 2005)

1.2.5 Viruses regulate apoptosis

During the co-evolution of viruses with their hosts, viruses have developed several strategies to manipulate the host cell machinery for their survival, replication and the release from the cell. Viruses target the cellular apoptotic machinery at critical stages of the viral replication to meet their ends (White, 2006; Galluzzi et al., 2008). Depending upon the need, a virus may inhibit (Benedict et al., 2002) or induce (Hay and Kannourakis, 2002) apoptosis for the obvious purpose of replication and spread, respectively (Benedict et al., 2002; Galluzzi et al., 2008). Interference in the

mitochondrial function can cause either cell death due to the deregulation of the Ca^{2+} signaling pathways and ATP depletion or apoptosis due to the regulation of Bcl-2 family proteins. Apoptosis is a programmed cell death (Kerr et al., 1972) characterized by membrane blebbing, condensation of the nucleus and cytoplasm, and endonucleosomal DNA cleavage. The process starts as soon as the cell senses the physiological or the stress stimuli, which disturbs the homeostasis of the cell (Gulbins et al., 2003; Borutaite, 2010). Apoptotic cell death can be considered an innate response to limit the growth of microorganisms including viruses attacking the cell.

Two major pathways, namely the extrinsic and the intrinsic are involved in triggering apoptosis (Sanfilippo and Blaho, 2003; Borutaite, 2010). The extrinsic pathway is mediated by the signaling through death receptors (e.g tumor necrosis factor or Fas ligand receptor) causing the assembly of the death inducing signaling complex (DISC) with the recruitment of proteins like caspases leading to the mitochondrial membrane permeabilization. In the intrinsic pathway, the signals act directly on the mitochondria leading to the mitochondrial membrane permeabilization before caspases are activated causing the release of Cyt-C (Liu et al., 1996; Castanier and Arnoult, 2010), which recruits APAF1 (Zou et al., 1997; Karbowski, 2010) resulting in the direct activation of caspase 9 (Green and Reed, 1998; Sun et al., 1999). Both the extrinsic and the intrinsic processes congregate at the activation of the downstream effector caspases, (i.e.caspase-3) (Ashkenazi and Dixit, 1998), which is responsible for inducing the morphological changes observed in an apoptotic cell. In addition to Cyt-C, Smac/ DIABLO as well as caspase independent death effectors inducing factor (AIF) and endonuclease G (Ferri and Kroemer, 2001; Ravagnan et al., 2002; Ohta, 2003) act as activators of the the caspase.

The B cell lymphoma (Bcl)-2 family of proteins tightly regulate the apoptotic events involving the mitochondria (Danial et al., 2010; Soriano and Scorrano, 2010). More than 20 mammalian Bcl-2 family proteins have been described to date (Krishna et al., 2011; Llambi and Green, 2011). They have been classified by the presence of Bcl-2 homology (BH) domains arranged in the order BH4-BH3-BH2-BH1 and the C- terminal hydrophobic transmembrane (TM) domain, which anchors them to the outer the mitochondrial membrane (Scorrano and Korsmeyer, 2003). The highly conserved BH1

and BH2 domains are responsible for anti-apoptotic activity, and multimerization of Bcl-2 family proteins. The BH3 domain is mainly responsible for the pro-apoptotic activity and the less conserved BH4 domain is required for the anti-apoptotic activities of Bcl-2 and Bcl-X_L proteins (Scorrano and Korsmeyer, 2003; Danial et al., 2010). Most of the anti-apoptotic proteins are multidomain proteins, which contain all four BH domains (BH1 to BH4) and a TM domain. In contrast, pro-apoptotic proteins are either multidomain proteins, which contain three BH domains (BH1 to BH3) or single domain proteins, which contain one domain (BH3) (Galluzi et al., 2008). The Bcl-2 proteins regulate the MMP depending upon whether they belong to the pro or the anti-apoptotic group, respectively. The MMP marks the dead end of the apoptosis beyond which cells are destined to die (Crompton, 2000; Waterhouse et al., 2002; Belzacq et al., 2003; Zamzami and Kroemer, 2003; Paradies et al., 2009; Castanier and Arnoult, 2010; Villinger et al., 2010).

Viruses encode homologue of Bcl-2 (vBcl-2) proteins, which can induce (pro-apoptotic) or prevent (anti-apoptotic) apoptosis thus helping the viruses to complete their life cycle in the host cells (Borutait, 2010; Soriano and Scorrano, 2010; Kim et al., 2010). While the vBcl-2s and the cellular Bcl-2s share limited sequence homology, their secondary structures are predicted to be quite similar (Cuconati and White, 2002; Galluzzi et al., 2008; Danial et al., 2010). During primary infection, interplay between vBcl-2 and other proteins enhances the lifespan of the host cells resulting in efficient production of the viral progeny and ultimately spread of the infection to the new cells. It also favors viral persistence in the cells by enabling the latently infecting viruses to make the transition to productive infection. The pathways and strategies used by the viruses to induce/inhibit apoptosis have been reviewed earlier (Thomson, 2001).

Many viruses encode for the homologue of anti-apoptotic Bcl-2 proteins, which preferentially localize to the mitochondria and may interact with the other pro-apoptotic Bax homologue. The E1B19K encoded by human adenovirus (HAdV)-5 contains BH1 and BH3-like domains and block the TNF-alpha-mediated death signaling by inhibiting a form of Bax that interrupts the caspase activation downstream of the caspase-8 and upstream of the caspase-9 (Perez and White, 2000; Putzer et al., 2000). Like HAdV-5 E1B19K (Perez and White, 2000), some viruses encode Bcl-2 homologues lacking BH4

domain, which are thought to act by inhibiting the pro-apoptotic members of the Bcl-2 family proteins. The FPV309 protein encoded by fowl pox virus contains highly conserved BH1 and BH2 like domains and a cryptic BH3 domain, interacts with Bax protein and inhibit apoptosis (Banadyga et al., 2007). The A179L protein encoded by African swine fever virus (ASFV) contains BH1 and BH2 domains, interacts with Bax-Bak proteins and inhibits apoptosis (Brun et al., 1996; Revilla et al., 1997). The Bcl-2 homolog (vBcl-2) encoded by herpesvirus saimiri (HVS) contains BH3 and BH4 like domains and interacts with Bax, thus stabilizing the mitochondria against a variety of apoptotic stimuli preventing the cell death (Derfuss et al., 1998). The E4 ORF encoded by equine herpesvirus-3 contains BH1 and BH2 domains (Marshall et al., 1999), which may interact with Bax and be essential for the anti-apoptotic activity (Yin et al., 1994).

Viruses also encode homologue of pro-apoptotic Bcl-2 proteins. The HBV encodes protein X, a vBcl-2 protein containing BH3, which localizes to the mitochondria and interacts with VDACs inducing the loss of the MMP leading to apoptosis (Rahmani et al., 2000; Lu and Chen, 2005; Bhargava et al., 2010; Kim et al., 2010) or interacts with Hsp60 and induce apoptosis (Tanaka et al., 2004). In contrast, another study revealed the protective effects of HB-X in response to the pro-apoptotic stimuli (Fas, TNF and serum withdrawal) but not from chemical apoptotic stimuli (Diao et al., 2001). The protein X of HBV is known to stimulate NFκB (Kekule et al., 1993; Su and Schneider, 1996), SAPK (Benn et al., 1996; Henkler et al., 1998) and PI3K/PKB (Shih et al., 2000) to prevent apoptosis. It is possible that the diverse functions of HBV protein X occur at different times of virus replication cycle in the infected cells. The BALF1 protein encoded by EBV contains BH1 and BH4 domains (Komano et al., 1998), which interacts with the Bax - Bak proteins (Marshall et al., 1999) and inhibits the anti-apoptotic activity of the EBV BHRF1 and the Kaposi Sarcoma virus (KSV) Bcl-2 protein, both of which contain BH1 and BH2 domains (Bellows et al., 2002), and interact with BH3 only proteins (Flanagan and Letai, 2008).

The effects of viral Bcl-2 homologue are thus apparently centered around the mitochondria and include prevention or induction of the MMP loss. The induction of MMP loss leads to the release of Cyt-C and other pro-apoptotic signals into the cytosol, and the activation of downstream caspases leading to the cell death and dissemination of

viruses to the neighboring cells for further infection.

Viruses also encode pro/anti apoptotic proteins, which show no homology to the Bcl-2 proteins (Galluzzi et al., 2008). The E6 protein of human papilloma virus (HPV) down regulate Bax signal upstream of the mitochondria (Thomas and Banks, 1999; Jackson et al., 2000) and prevent the release of Cyt-C, AIF and Omi, thus preventing apoptosis (Leverrier et al., 2007). This E6 activity towards another Bcl2 family pro apoptotic protein Bak is a key factor promoting the survival of the HPV-infected cells, which in turn facilitates the completion of the viral life cycle (Jackson et al., 2000). Enterovirus (EV) 71 induces conformational changes in Bax and increases its expression in the cells following infection and induces the activation of caspase 3, 8 and PARP causing caspase dependent apoptosis (Sun, et al., 2011). On the contrary, rubella viral capsid binds to Bax, forms the oligo-heteromers and prevents the formation of pores on the mitochondrial membrane thus preventing Bax induced apoptosis (Ilkow, et al., 2011)

Viruses also encode proteins, which act as viral mitochondrial inhibitor of apoptosis (vMIA) thus protecting the cells. A splice variant of UL37 of HCMV acts as vMIA and protects the cells from apoptosis (Goldmacher et al., 1999) thereby helping viruses to complete their replication cycle. It localizes to the mitochondria and interacts with ANT (Goldmacher et al., 1999) and Bax (Arnoult et al., 2004; Poncet et al., 2004). HCMV vMIA has a N-terminal mitochondrial localization domain and a C-terminal anti-apoptotic domain (Goldmacher et al., 1999), which recruits Bax to the mitochondria and prevents the loss of MMP. It protects the cells against CD95 ligation (Goldmacher et al., 1999), oxidative stress induced cell death (Vieira et al., 2001; Boya et al., 2003) and prevents the mitochondrial fusion (McCormick et al., 2003) thus promoting cell survival.

vMIA does not inhibit the apoptotic events upstream of the mitochondria but can influence events like preservation of ATP generation, inhibition of Cyt-C release and caspase 9 activation following induction of apoptosis. However, the exact mechanisms of the events around vMIA still remain a question.

1.2.6 Viruses modulate mitochondrial antiviral immunity

Cells respond to virus attack by activating a variety of signal transduction pathways leading to the production of interferons (Katze et al., 2002), which limit or

eliminate the invading virus. The presence of the viruses inside the cell is first sensed by pattern recognition receptors (PRRs) that recognize the pathogen associated molecular patterns (PAMPs). PRRs include toll like receptors (TLRs), nucleotide oligomerization domain (NOD) like receptors (NLRs) and retinoic acid-inducible gene I (RIG-I) like receptors (RLRs). Mitochondria have been associated with the RLRs, which include retinoic acid-inducible gene I (RIG-I) (Yoneyama et al., 2004) and melanoma differentiation-associated gene 5 (Mda-5) (Andrejeva et al., 2004). Both are cytoplasm located RNA helicases that recognize dsRNA. The N-terminus of RIG-1 has caspase activation and recruitment domains (CARDs). The C-terminus has RNA helicase activity (Yoneyama et al., 2004), which recognizes and binds to uncapped and unmodified RNA generated by viral polymerases in ATPase dependent manner. This causes conformational changes and exposes its CARD domains to bind and activate the downstream effectors leading to the formation of enhanceosome (Maniatis et al., 1998) triggering NFκB production. RLRs have recently been reviewed in detail (Scott, 2010; Castanier and Arnoult, 2011; Wang et al., 2011).

A CARD domain containing protein [named mitochondrial anti-viral signaling (MAVS) (Seth et al., 2005; Seth et al., 2006), virus-induced signaling adaptor (VISA) (Xu et al., 2005), IFN-β promoter stimulator 1 (IPS-1) (Kawai et al., 2005) or CARD adaptor inducing IFN-β (CARDIF) protein (Meylan et al., 2005)] acts downstream of the RIG-I. Besides the presence of N-terminal CARD domain, MAVS contain a proline-rich region and a C-terminal hydrophobic transmembrane (TM) region, which targets the protein to the mitochondrial outer membrane and is critical for its activity (Seth et al., 2005). The TM region of the MAVS resembles the TM domains of many C-terminal tail anchored proteins on the outer membrane of the mitochondria including Bcl-2 and Bcl-xL (Seth et al., 2005). Recent reports indicate that MAVS has an important role in inducing the antiviral defenses in the cell. Over expression of MAVS leads to the activation of NFκB and IRF-3, leading to the induction of typeI interferon response. This interferon response is abrogated in the absence of MAVS (Seth et al., 2005) indicating the specific role of MAVS in inducing the antiviral response. MAVS has also been shown to prevent apoptosis by its interaction with VDAC (Xu et al., 2010) and preventing the opening of MPTP.

Some viruses induce cleavage of MAVs from the outer membranes of the mitochondria (Li et al., 2005; Meylan et al., 2005) thus greatly reducing its ability to induce interferon response. HCV persists in the host by lowering the host cell immune response including inhibiting the production of IFN-β by RIG-I pathway (Foy et al., 2003; Breiman et al., 2005; Foy et al., 2005). The NS3/4A protein of HCV co-localizes with the mitochondrial MAVS (Li et al., 2005; Meylan et al., 2005) leading to the cleavage of MAVS at amino acid 508. Since free form of the MAVS is not functional, the dislodging of MAV from the mitochondria inactivates MAVS (Meylan et al., 2005) thus helping in paralyzing the host defense against HCV. Interestingly, another member of family *Flaviviridae* GB virus B shares 28% amino acid homology with HCV over the lengths of their open reading frames (Beames et al., 2001). The NS3/4A protein of GB virus also cleaves MAVS in a manner similar to HCV, thus effectively compromising the host immune response by preventing the production of interferons (Chen et al., 2007). Other viruses like influenza A translocate RIG-I/MAVS components to the mitochondria of infected human primary macrophages and regulates the antiviral / apoptotic signals thus increasing the viral survivability (Ohman et al., 2009).

1.2.7 Viruses hijack host mitochondrial proteins

Over the years, viruses have perfected different strategies to establish complex relationships with their host with the sole purpose of preserving their existence. One such strategy involves the hijacking of the host cell mitochondrial proteins. The p32, a mitochondria associated cellular protein, is a member of a complex involved in the import of cytosolic proteins to the nucleus. Upon entry into the cell, adenovirus hijacks this protein and piggybacks it to transport its genome to the nucleus (Matthews and Russell, 1998), thereby increasing its chances of survival and establishment in the host cell. During HIV-1 assembly, $tRNA^{Lys}$ iso-acceptors are selectively incorporated into virions. The $tRNA_3^{Lys}$ binds to HIV genome and is used as the primer for reverse transcription (Cen et al., 2001). In humans, a single gene produces both cytoplasmic and mitochondrial Lys tRNA synthetases (LysRSs) by alternative splicing (Tolkunova et al., 2000). The mitochondrial LysRSs is produced as a pre-protein, which is transported into the mitochondria. The pre-mitochondrial or mitochondrial LysRS is specifically

packaged into HIV (Kaminska et al., 2007) and acts as a primer to initiate the replication of HIV-I RNA genome, which then binds to a site complementary to the 3'-end 18 nucleotides of $tRNA_3^{Lys}$. It is proposed that HIV viral protein R (Vpr) alters the permeability of the mitochondria (Jacotot et al., 2000) leading to the release of pre-mito or mito LysRS, which then interacts with Vpr (Stark and Hay, 1998) and gets packed into the progeny virions.

Viperin, an interferon inducible protein is induced in the cells in response to the viral infection (Qiu et al., 2009). This protein has been shown to prevent the release of influenza virus particles from the cells by trapping them in lipid rafts inside the cells thereby preventing its dissemination (Wang et al., 2007). During infection, HCMV induces IFN independent expression of viperin, which interact with HCMV encoded vMIA protein resulting in the relocation of viperin from the ER to the mitochondria. In mitochondria, viperin interacts with the mitochondrial tri-functional protein and decreases ATP generation by disrupting oxidation of fatty acids, which results in disrupting the actin cytoskeleton of the cells and enhancing the viral infectivity (Seo et al., 2011).

1.2.8 Viruses alter intracellular distribution of mitochondria

Viruses alter the intracellular distribution of the mitochondria either by concentrating the mitochondria near the viral factories to meet energy requirements during viral replication or by cordoning off the mitochondria within cytoplasm to prevent the release of mediators of apoptosis. The protein X of HBV causes microtubule mediated peri-nuclear clustering of the mitochondria by p38 mitogen-activated protein kinase (MAPK) mediated dynein activity (Kim et al., 2007). HCV non-structural protein 4A (NS4A) either alone or together with NS3, (in the form of the NS3/4A polyprotein) accumulates on mitochondria and change their intracellular distribution (Nomura-Takigawa et al., 2006). HIV-1 infection causes clustering of the mitochondria in the infected cells (Radovanovic et al., 1999). Interestingly, ASFV causes the microtubule mediated clustering of the mitochondria around virus factories in the cell providing energy for virus release (Rojo et al., 1998). Similar changes were observed in the chick

embryo fibroblasts infected with frog virus 3, where degenerate mitochondria surrounding the virus factories were found (Kelly, 1975).

1.2.9 Viruses mimic the host mitochondrial proteins

Molecular mimicry is "the theoretical possibility that sequence similarities between foreign and self-peptides are sufficient to result in the cross-activation of autoreactive T or B cells by pathogen-derived peptides" (Fujinami and Oldstone, 1985; Kohm et al., 2003). Since structure follows the function, viruses during their co-evolution with the hosts have evolved to mimic the host proteins to meet their ends during the progression of their life cycle inside the cell. Mimicking aids the viruses to gain access to the host cellular machinery and greatly helps in their survival in the hostile host environment.

Mimivirus, a member of the newly created virus family *Mimiviridae*, encodes an eukaryotic mitochondria carrier protein (VMC-I) (Monne et al., 2007), which mimics the host cell's mitochondrial carrier protein and thus controls the mitochondrial transport machinery in the infected cells. It helps to transport ADP, dADP, TTP, dTTP, and UTP in exchange for dATP, thus exploiting the host for energy requirements during the replication of its A+T rich genome (Monne et al., 2007). Besides VMC-I, mimivirus encodes several other proteins (L359, L572, R776, R596, R740, R824 L81, R151, R900, and L908) with putative mitochondria localization signals, which suggest that mimivirus has evolved a strategy to take over the host mitochondria and exploit its physiology to compensate for its energy requirements and biogenesis (Monne et al., 2007). Viral Bcl-2 homologues (vBcl-2) are other groups of viral proteins that mimic the host cell Bcl-2s and have been described elsewhere in this review.

1.2.10 Viruses cause host mitochondrial DNA (mtDNA) depletion

Mammalian mitochondria contain a small circular genome, which synthesizes enzymes for oxidative phosphorylation and mitochondrial RNAs (mtRNAs) (Burger et al., 2003). To increase the chance of survival, some viruses appear to have adopted the strategy of damaging the host cell mtDNA. Since mitochondria act as a source of energy and play an important role in antiviral immunity as well, it is possible that damage to the

mtDNA may help in evading mitochondrial antiviral immune responses (Saffron et al., 2007).

During productive infection of mammalian cells *in vitro*, HSV-1 induces the rapid and complete degradation of the host mtDNA (Saffran et al., 2007). The UL12.5 protein of HSV-1 localizes to the mitochondria and induces DNA depletion in the absence of other viral gene products (Saffran et al., 2007; Corcoran et al., 2009). The immediate early Zta protein of EBV interacts with mitochondrial single stranded DNA binding protein resulting in reduced mtDNA replication and enhanced viral DNA replication (Wiedmer et al., 2008). HCV causes the reactive oxygen species and nitrous oxide mediated DNA damage in the host mtDNA (Machida et al., 2006; Machida et al., 2010). Interestingly, depletion of mtDNA has also been observed in HIV/HCV co-infected humans (de Mendoza et al., 2007).

2.0 HYPOTHESIS AND OBJECTIVES

2.1. Rationale for the hypothesis

Mitochondria are key organelle in orchestrating various cellular functions including but not limited to ATP synthesis, regulation of apoptosis and induction of innate immune responses. Since viruses mainly survive by multiplying in the cells, viruses have to modulate mitochondrial functions during the process of virus replication (from entry till the release of progeny virus), in order to enhance their survival in the cell.

2.2. Hypothesis

I hypothesize that "BAdV-3 modulates the mitochondrial structure and function".

2.3 Objectives:

First objective of this work is to determine the structural and functional changes in mitochondria following BAdV-3 infection. Second objective is to find out if any of the BAdV-3 proteins localize to mitochondria and their effect on the mitochondrial function.

Specifically the objectives are

i) Effect of BAdV-3 infection on structure and function of mitochondria:

In step one, I propose to study the effect of viral infection on the structure of mitochondria by electron microscopy. We will infect the MDBK cells with BAdV-3 and observe changes at 6, 12 and 24 hrs post infection. In step two I will study the functionality of mitochondria by making observations on the vital mitochondrial processes viz., ATP generation, ROS/SO species generation, MMP alterations and the disturbances in Ca^{2+} homeostasis by functional assays.

ii) Effect of BAdV-3 proteins VII and 52K on the function of mitochondria:

In this objective I will determine what BAdV-3 specific protein(s) is/are involved in altering a specific mitochondrial function observed in objective Initially, I will

determine if any of the BAdV-3 proteins localize to the mitochondria. I will do that by determining the presence of BAdV-3 specific protein(s) in enriched mitochondrial fractions by Western blot after proteinase K assay, which eliminates the protein(s) from the outer mitochondrial membrane allowing me to observe the protein(s) localized within it. Using similar strategy, I will confirm the presence of the mitochondrial localization signal(s) in the identified BAdV-3protein(s) by determining the localization of a cytoplasmic protein fused to potential MLS of the identified BAdV-3 protein. I will then perform the functional studies (ATP generation, ROS/SO species generation, MMP alterations and disturbances in Ca^{2+} homeostasis) on mitochondria isolated from the cells transfected with genes expressing the identified BAdV-3 proteins

On the basis of my observations I should be able to determine the structural and functional changes in cellular mitochondria due to BAdV-3 infection and establish the identity of the BAdV-3 protein(s) responsible for these alterations.

3.0 EFFECT OF BOVINE ADENOVIRUS-3 ON MITOCHONDRIA

3.1 Introduction

Mitochondria are cytoplasmic organelles found in the eukaryotic cells. Their number and size varies from cell to cell depending upon the function and the metabolic state of the cell. Although mitochondria have their own genome and transcription-translation machinery, they also depend on the nuclear encoded gene products, which are indispensable for their normal function (Rapaport, 2003). Besides acting as powerhouse of the cell, they are also important in cellular metabolism and calcium regulation (Hackenbrock, 1966; Mannella et al., 2001; Chen and Chan, 2005) and play a central role in apoptosis (Antignani and Youle, 2006; Chan, 2006; McBride et al., 2006; Kroemer et al., 2007). Mitochondria have also been implicated in the process of aging (Wallace, 2005; Chan, 2006), cell-cycle control (Sesaki and Jensen, 1999; Hardie, 2005; Jones et al., 2005; Mandal et al., 2005), cell development (Bakeeva et al., 1978; Bakeeva et al., 1983; Honda and Hirose, 2003; Seth et al., 2005), antiviral responses (Seth et al., 2006) signal transduction (Bossy-Wetzel et al., 2003), and certain diseases (Olanow and Tatton, 1999; Van Den Eeden et al., 2003; Martin, 2006; McFarland et al., 2007). In short, they control the main processes critical for the survival of the cell. As such, they have developed a very intimate and complex relationship with the cell, some of which is still elusive to us.

A number of viruses can affect the structure and function of the mitochondria (Kaminska et al., 2007; St-Louis and Archambault, 2007; Ohman et al., 2009; Yang et al., 2009; Molouki et al., 2010). HAdV-5 causes the cathepsin dependent mitochondria mediated oxidative stress in the infected cells (McGuire et al., 2011). During African swine fever virus (ASFV) infection (Rojo et al., 1998) or frog virus (FV)-3 (Kelly, 1975) infection, the mitochondria accumulate near virus assembly sites to meet energy requirements of progeny virus production. Hepatitis B virus (HBV) (Kim et al., 2007), human immunodeficiency virus (HIV)-1 (Radovanovic et al., 1999), Waleye dermal sarcoma virus (WDSV) (Nudson et al., 2003) and hepatitis C virus (HCV) (Nomura-Takigawa et al., 2006) induce clustering of the mitochondria in the infected cells. During dengue virus type 2 infections, initially there is an increase in the number and the size of

the mitochondria in monocytes leading to the observation of cytoplasmic structures resembling diverse degrees of mitochondrial alterations during later stages of infection (Mosquera et al., 2005).

Viruses including Epstein-Barr virus (Gargouri et al., 2009), HIV-1 (Kruman et al., 1998; Baum et al., 2011; Gil et al., 2010) HCV (Ming-Ju et al., 2011) encephalomyocarditis virus (EMC) (Ano et al., 2010) and HBV (Kim et al., 2010) cause oxidative stress in the infected cells. A number of viruses including human T lymphotropic virus (HTLV) (Ciminale et al., 1999), HIV-1 (Sternfeld et al., 2009), WDSV (Nudson et al., 2003), influenza virus (IV) A (Danishuddin et al., 2010) and HCV (Machida et al., 2006) cause loss of the mitochondria membrane potential (MMP). Even some viruses including herpes simplex virus (HSV) -1 (Saffaron et al., 2007) and HCV together with HIV-1 (de Mendoza et al., 2007) cause depletion of the mtDNA.

Though adenovirus replicates in the nucleus of the cell, the possibility of its dependence on the mitochondria can't be ruled out. However, little is known about the role of mitochondria in adenovirus infections. Human adenovirus encoded early protein (E1Bs) localize to the mitochondria and prevent apoptosis (Lomonosova et al., 2005). Human adenovirus death protein (ADP) encoded by E3 region has been implicated in increasing the cellular respiration during the release of virus progeny from the infected cell suggesting the role of mitochondria in the process (Tollefson et al., 1996). Thus, mitochondria are a soft target for viruses, which use different mechanisms to modulate the mitochondrial activity so that they can survive, replicate and efficiently produce progeny virus in the infected cell.

Bovine adenovirus (BAdV)-3, a non-enveloped icosahedral particle of 75-nm diameter (Niiyama et al., 1975) replicates in the respiratory tract of cattle with mild or no clinical symptoms (Lehmkuhl et al., 1975; Thompson et al., 1981; Mattson et al., 1988; Lehmkuhl and Hobbs, 2008). The complete DNA sequence and the transcription map of BAdV-3 genome have been reported (Baxi et al., 1998; Lee et al., 1998; Reddy et al., 1998; Idamakanti et al., 1999). As little is know about BAdV-3- host interaction, the present study aims to identify the effect of BAdV-3 replication on the mitochondria of infected cells.

3.2 Materials and methods

3.2.1 Cell lines and virus

Madin Darby bovine kidney (MDBK) cells were grown in minimal essential medium (MEM; Invitrogen, Canada) (Cat # 11095) supplemented with 10% heat-inactivated fetal bovine serum (FBS). Wild-type BAdV-3 (WBR-1 strain) was propagated in MDBK cells in MEM supplemented with 2% FBS (Reddy et al., 1999a).

3.2.2 Antibodies

Production of polyclonal antibody specific to 19kDa E1Bs protein of BAdV-3 is described elsewhere (Reddy et al., 1999a). Antibody specific to 42 kDa β-actin protein was purchased from Sigma Canada (Cat # A5441). Alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG (Cat # 111-055-003) or AP-conjugated goat anti-mouse antibody (Cat # 115-055-003) was purchased from Jackson ImmunoResearch, USA.

3.2.3 Western blot

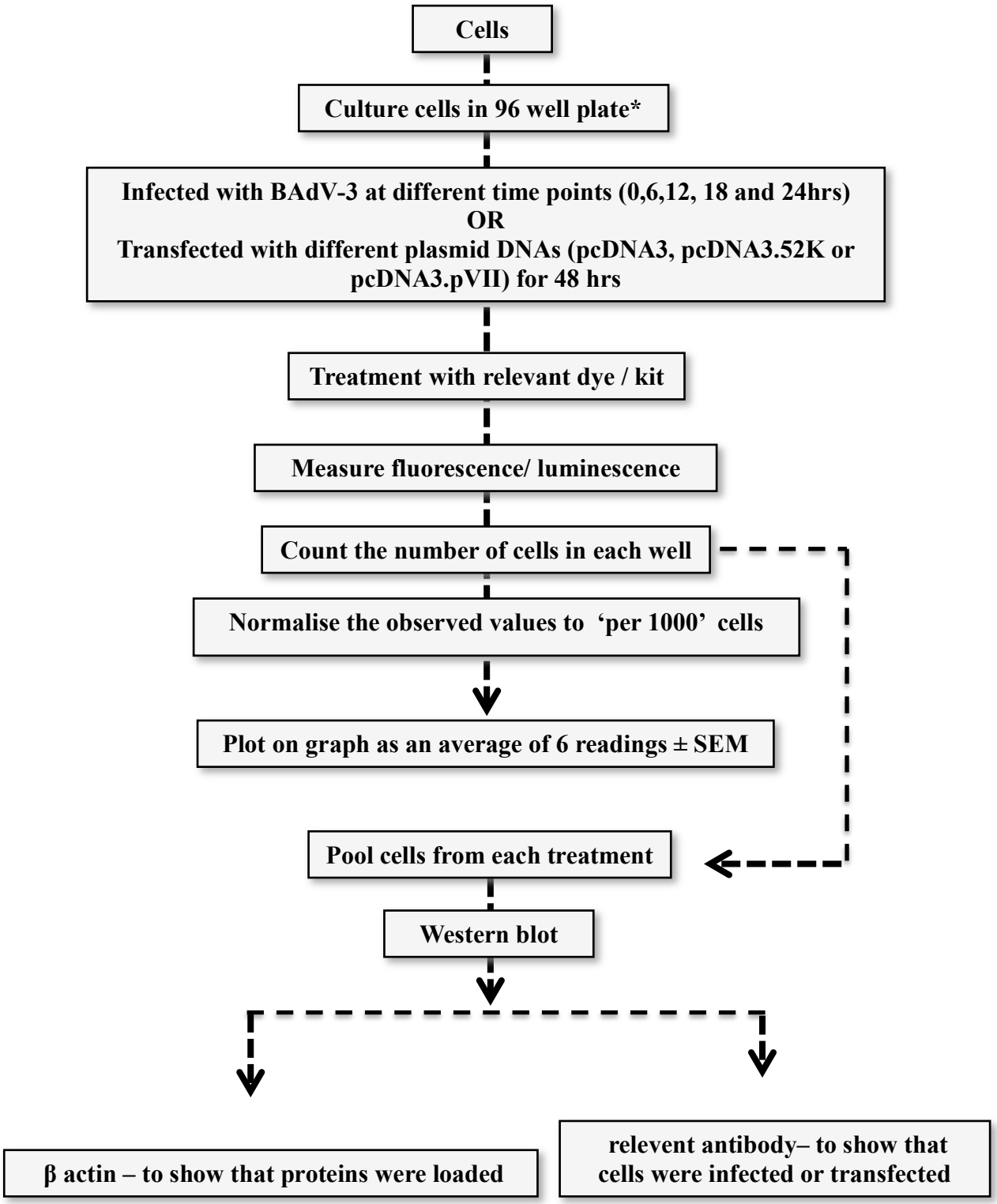
MDBK cells were grown in 96-well plates to 80% confluency before infecting with wild-type BAdV-3 at a multiplicity of infection (MOI) of 5. At indicated times post infection, the cells were harvested, counted and lysed by addition of RIPA buffer [0.15 M NaCl, 50 mM Tris-HCl (pH 8.0), 1% NP-40, 1% deoxycholate, 0.1% sodium dodecyl sulphate (SDS)] containing 1x anti-protease cocktail (Sigma Canada). Proteins from the lysates of the infected cells were separated by 10% sodium dodecyl sulphate (SDS) - polyacrylamide gel electrophoresis (PAGE) and transferred to a nitrocellulose membrane (Bio-Rad). The membrane was blocked with 5% or 10% skimmed milk powder (SMP: Nestle) in TBST [Tris-buffered saline (pH 8.0), 0.05% tween 20] buffer overnight at 4˚C and probed with protein specific antibodies diluted in TBST buffer containing 0.1% SMP for 1 hr at room temperature. Membranes were washed with TBST thrice before probing with alkaline phosphatase conjugated goat-anti rabbit or goat anti-mouse secondary antibodies diluted 1:10000 in TBST buffer containing 0.1% SMP for 1 hr at room temperature. Finally, the membranes were washed three times in TBST buffer and developed using a BCIP/NBT reagent (Sigma, Canada).

3.2.4 Transmission electron microscopy

MDBK cells were infected with wild-type BAdV-3 at an MOI of 5 and analyzed by electron microscopy at indicated times post infection. Briefly, the infected and uninfected MBDK cells were rinsed three times with MEM containing no serum before gently flooding with 3% glutaraldehyde in 0.1M sodium cacodylate buffer at room temperature. After gently swirling for few seconds, the fixative was discarded and replaced with fresh fixative for 15 min followed by three rinses of 0.1M sodium cacodylate buffer containing 0.22M sucrose. The cells were then fixed for 15 min in 0.8% osmium tetroxide (OsO_4) in 0.1M sodium cacodylate buffer without sucrose followed by 2- 5 minute, three washes with 0.1M sodium cacodylate buffer containing 0.22M sucrose. The fixed samples were stained for 1 hr in a solution [containing 1% uranyl acetate (UrAc) and 0.22M sucrose] and, dehydrated by removing part of the supernatant and adding absolute alcohol (ethanol) drop wise with gentle swirling until a concentration of 90% was achieved (10 min). This was followed by 3- 5 min changes using 100% ethanol. The cells were infiltrated with Epon 812 by adding the mixture drop wise with gentle swirling over a period of 20-30 min until a concentration of~80% plastic was achieved. The 80% plastic mixture was decanted and replaced with 100% Epon 812 mixture while gently rocking the chamber to improve the mixing. The resin was replaced with fresh 100% Epon 812 and samples were polymerized at 55°C for 24 hrs. The blocks were cut on a Reichert Ultracut E microtome and viewed using Philips 410LS transmission electron microscope.

3.2.5 Experimental design

All the experiments were performed as outlined in Figure 3.1. For normalizing the data, the cells from each well were counted after measuring the fluorescence. All luminescence / fluorescence data were normalized to “per 1000 cells” value observed in the assay. Since reagents used in ATP assay lyse the cells, two identical plates were made simultaneously. The counting of the cells in each well at three different times indicated that there was no significant difference in the number of cells in the plates prepared simultaneously. One plate was used for ATP assay. The other plate was used to



* Since reagents used in ATP assay lyse the cells, two identical plates were made simultaneously. 1st plate was used for assay and 2nd plate was used for counting the cells.

Figure 3.1 Scheme used to perform the luminescence and fluorescence experiments.

count the cells for normalizing the ATP data. The data represents the mean ± standard error of means (SEM) of two independent experiments each with three replicates.

3.2.6 Cellular ATP

MDBK cells were grown in 96 well plates and infected with wild-type BAdV-3 at a MOI of 5. At indicated times post infection, the cells were treated with ATP Lite^{TM} 1step kit reagents (Perkin Elmer) as per manufacturer's instructions. This assay system is based on the production of light caused by the reaction of available ATP with added luciferase and D-luciferin (Cree and Andreotti, 1997). The emitted light, which is proportional to the ATP concentration, was recorded using a multi label counter (Victor^3 - Perkin Elmer).

3.2.7 Mitochondrial and cytosolic Ca^{2+}

MDBK cells grown in 96 well plates were infected with BAdV-3 at a MOI of 5. At indicated times post infection, the cells were stained with 5µM mitochondrial calcium sensitive dye Rhod-2AM (Molecular Probes) (Cannell et al., 1994) or 10µM cytosolic Ca^{2+} sensitive dye Fluo-4AM (Molecular Probes) (Gee et al., 2000) for 30 min at 37^{O}C. The cells were washed three times in Ca^{2+} free PBS (Gibco) or KRH buffer [129mM NaCl, 5mM $NaHCO_3$, 4.8mM KCl, 1.2mM KH_2PO_4, 1mM $CaCl_2$, 1.2mM $MgCl_2$, 2.8mM glucose and 10mM Hepes (pH7.4)] and equilibrated for 10 min before taking reading. Fluorescence signals were acquired with multi label counter (Victor^3 -Perkin Elmer) using a 480/31 nm filter to excite the Fluo-4 AM and 531nm filter to excite Rhod-2 AM fluorescence. The signals were collected at 535 nM (Fluo-4 AM) and at 572 nm (Rhod-2 AM).

3.2.8 Mitochondrial membrane potential

MDBK cells were infected with wild-type BAdV-3 at a MOI of 5. At indicated times post infection, the cells were incubated with 100 nM T tetramethylrhodamine - methyl ester (TMRM; Molecular Probes) (Wong and Cortopassi, 2002) in KRH-glucose buffer containing 0.02% pluronic acid. After 30 min, the cells were washed with KRH buffer three times and allowed to equilibrate for 20 min. Fluorescence signals were

acquired with multi label counter (Victor^3 -Perkin Elmer) using a 531 nm excitation and 572 nm emission filter.

3.2.9 Mitochondrial reactive oxygen species (ROS) and superoxide (SO)

MDBK cells grown in 96 well plates were infected with wild-type BAdV-3 at an MOI of 5. At indicated times post infection, the cells were incubated with either 10µM of DCF-DA (Molecular Probes) (Degli Esposti, 2002) or 5µM of MitoSOX^{TM} red (Molecular Probes) (Patschan et al., 2008) and incubated for 30 min in KRH buffer. Finally, the cells were washed in KRH buffer three times and equilibrated for 10 min. Fluorescence signals were measured with multilabel counter (Victor^3- Perkin Elmer) using a 480/31 nm excitation and 535 nM (DCF-DA) and 580 nm (MitoSOX^{TM}) emission filters.

3.2.10 Statistical Analysis

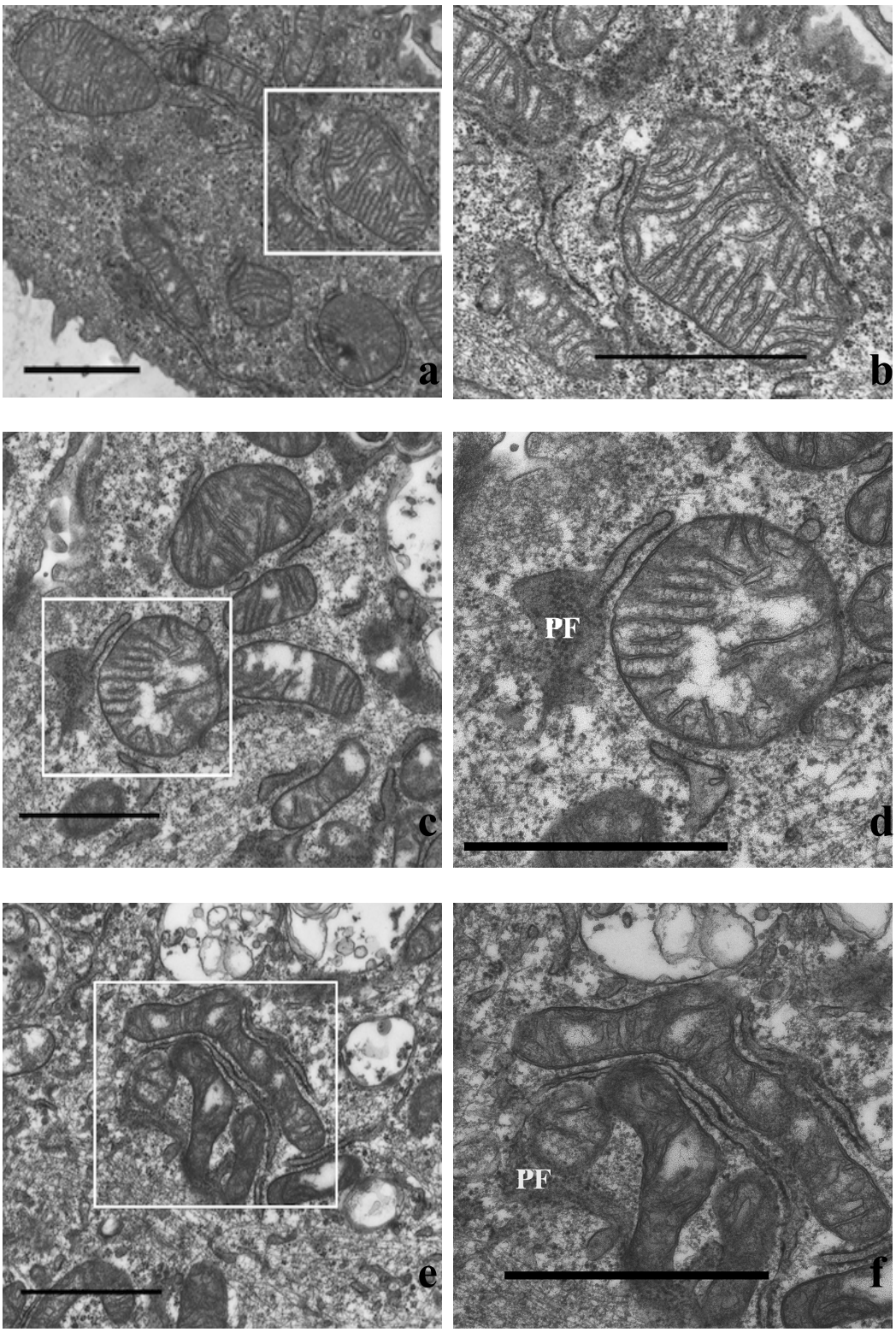
Data were analyzed by one-way analysis of variance (Anscombe, 1948), using a general linear model procedure (GLM; SAS Enterprise Guide 4.2 under SAS 9.2 environment for Windows XP; SAS Institute Inc., Cary, NC, USA) for effect of time (0, 6, 12, 18 and 24 hr). Probability values >0.05 were considered non-significant. Tukey's post-hoc tests for multiple comparisons were performed if main effect (i.e., time) was significant ($P \leq 0.05$). The values are expressed as mean ± SEM**.**

3. 3 Results

3.3.1 BAdV-3 damages mitochondrial architecture

To evaluate the effect of BAdV-3 on mitochondria during the course of infection, electron microscopy was used. Uninfected or BAdV-3 infected MDBK cells were collected at different times post infection and analyzed by transmission electron microscopy. Uninfected cells showed normal lamellar bodies with rough endoplasmic reticulum (rER) and many polyribosomes. Majority of the mitochondria were located in the peri-nuclear or central 2/3rd of the cytoplasm. Mitochondria were elongated or oval in shape containing typical cristae of protein producing cells (Figure 3.2 A, panel a,b). At 6

Figure 3.2 (A) Electron microscopy of BAdV-3 infected cells. MDBK cells mock infected (panel a and b) or infected with wild-type BAdV-3 (panel c,d,e,f) at a MOI of 5 were analyzed after 6 (panel c and d) and 12 (panel e and f) hrs post infection. Figures in the left panels (panel a, c and e) show cytoplasm in the vicinity of mitochondria. Area covered by the white rectangles is enlarged and shown in the panels on the right (panel b, d and f). Protein factories (PF) in the vicinity of damaged mitochondria at 6 (panel d) and 12 (f) hrs post infection. Bar = 0.25μ


a
b
c
PF
d
e
PF
f

hrs post infection (Figure 3.2 A, panel c, d), mitochondria still appeared to have normal morphology with intact outer and the inner membranes. Although there were increased cristae free zones in the mitochondria, the density of the cristae appeared to be normal.

At 12 hrs post infection (Figure 3.2 A, panel e, f), the mitochondria of the infected cells appeared to be smaller in size compared to the mitochondria of normal cells (Figure 3.2 A, panel a,b). Although morphology was intact, the cristae free zones increased in the virus infected cell mitochondria at 12 hr post infection compared to the infected cell mitochondria at 0 or 6 hrs post infection. At 24 hrs post infection (Figure 3.2 B, panel a,b) , the cells had longer microvilli on the surface and with fewer areas of a cell in contact with the other. The nuclear membrane was more indented with the appearane of nuclear inclusions. The progeny virus particles were observed inside the nucleus with the decrease in the number of nucleoli per cell. Moreover, the mitochondria appeared smaller and round / oval in shape. Interestingly, very few mitochondria were observed at this time and patches of protein synthesis mahinary were not visible around mitochondria.

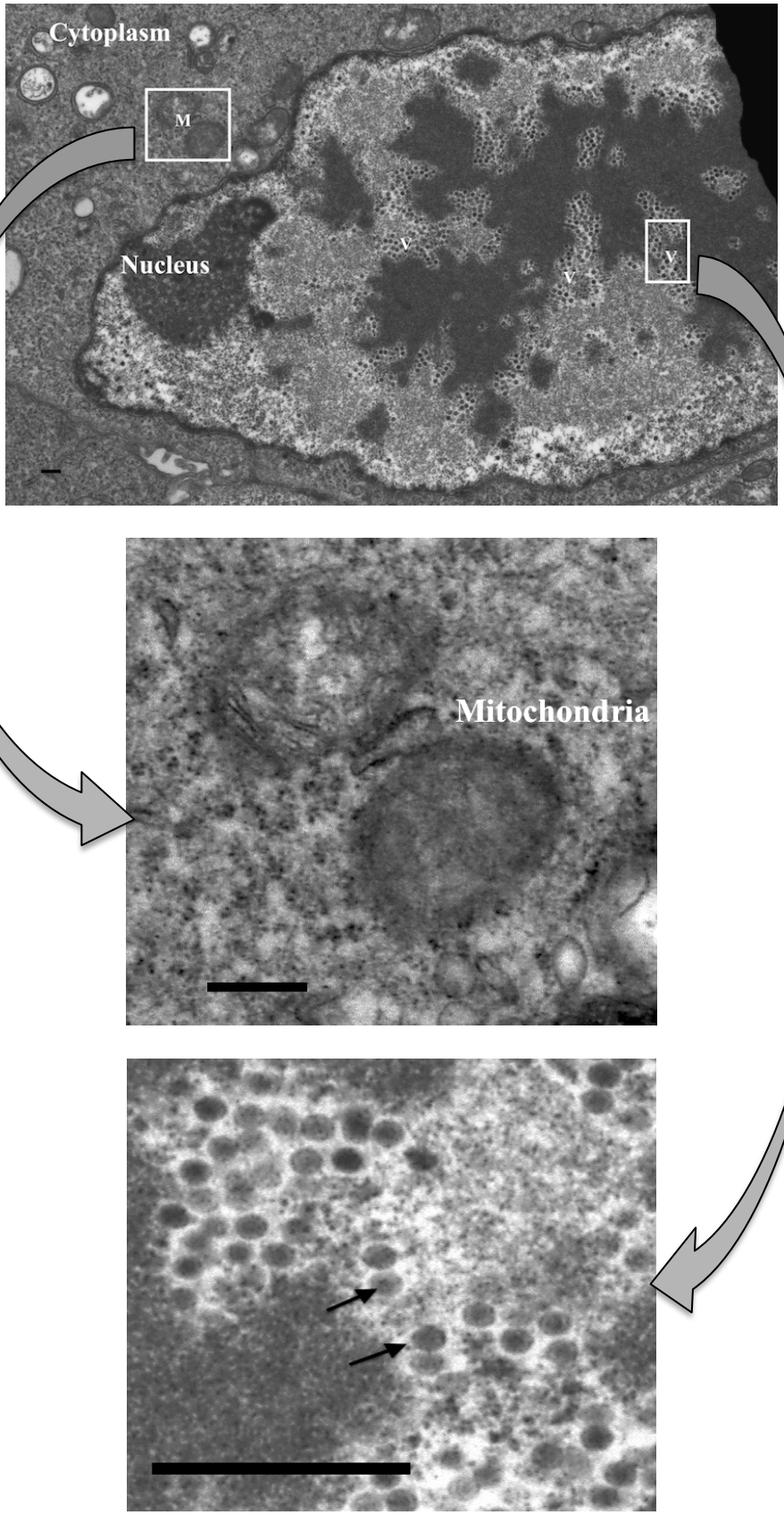
3.3.2 BAdV-3 regulates ATP production in the infected cells

Since BAdV-3 affects the mitochondrial morphology, we determined if ATP production capacity of the cells is compromised during the course of infection. ATP is present in all metabolically active cells and thus, is a marker for the cell viability. Whenever the cell is under stress, ATP concentration changes rapidly and thus, monitoring ATP is a good indicator of cell health. To observe this, MDBK cells were infected with BAdV-3 at MOI of 5 and the ATP production was measured at 6, 12, 18 and 24 hrs post infection using ATP Lite^{TM} 1step kit and multi-plate reader (Victor^3-Perkin Elmer). As seen in Figure 3.3, steady increase in ATP production was observed at 6, 12 and 18 hrs post infection. However, compared to 0, 6, 12 and 18 hrs, there was a significant ($P<0.0001$) decrease in the ATP production at 24 hrs post infection (Figure 3.3)

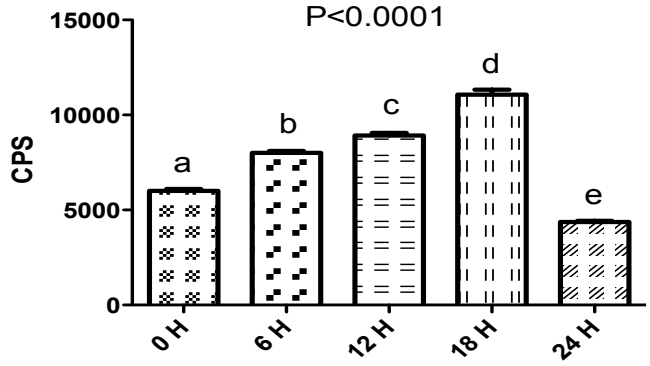
3.3.3 BAdV-3 infection causes loss in mitochondrial membrane potential in MDBK cells

As changes were observed in mitochondrial morphology and ATP production in

Figure 3.2. (B) Electron microscopy of BAdV-3 infected cells. MDBK cells at 24 hrs post infection. An infected cell showing virus particles (V) in the nucleus and damaged mitochondria (M) in the cytoplasm of the infected cell (panel a). The enlarged area indicated by a rectangle (M) in panel "a" showing mitochondria with amorphous internal structure in the cytoplasm of infected cell (panel b). The enlarged area indicated by a rectangle (V) in panel "a" showing virus particles (indicated by arrows) in the nucleus of the infected cell (panel c). Bar = 0.25μ.



(A)



(B)

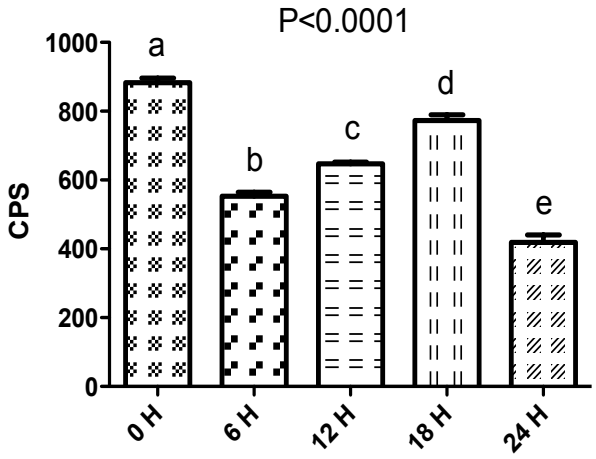
Figure 3.3 ATP production in BAdV-3 infected cells. (A) MDBK cells were mock infected or infected with BAdV-3 at a MOI of 5 and ATP levels were measured at 6, 12, 18 and 24 hrs post infection using ATP Lite^{TM} 1 step kit and multilabel reader (Victor^{3}-Perkin Elmer). Measurements are depicted in arbitrary units (CPS- counts per second). Data represents the mean of 2 independent experiments, each with 3 replicates. Means with the different letter are significantly different. * P<0.0001. To determine that the cells were infected, proteins from the lysates of BAdV-3 infected cells (from test plate as well as from parallel plate used to count the number of cells) were collected at indicated times post infection and analyzed by Western blot (B) using anti-β-actin serum (Sigma Canada) or anti -E1Bs serum (Reddy et al., 1999a**).**

BAdV-3 infected cells, we next determined if BAdV-3 alters the MMP in the virus-infected cells (Fig. 3.4). MDBK cells infected with BAdV-3 at a MOI of 5 were collected at 6, 12 18 and 24 hrs post infection, incubated with TMRM reagent and analyzed for the fluorescence. TMRM is a monovalent cationic mitochondrial selective probe, which exhibits fluorescence when it accumulates in MMP dependent manner. Under the conditions of the mitochondrial depolarization, TMRM diffuses out and becomes more evenly distributed throughout the cytoplasm. When dispersed, the fluorescence drops significantly, which can be quantified by multi-plate reader (Victor^3-Perkin Elmer). Quantification of the TMRM signals indicated that there was a significant decrease in the MMP in the cells from 0 to 6 hrs post infection. In contrast, compared to 0 and 6 hrs there was a significant ($P< 0.0001$) increase in the MMP in the cells from 6 to 18 hrs post infection. However, compared to 0, 6, 12 and 18 hrs, there was a significant ($P<0.0001$) decrease in the MMP in the cells at 24 hrs post infection.

3.3.4 BAdV-3 infection causes decrease in mitochondrial Ca^{2+} levels in MDBK cells

Since Ca^{2+} buffering capacity of the mitochondria is a good indicator of mitochondrial health and survival in the cells, the mitochondrial Ca^{2+} levels were measured after incubating BAdV-3 infected cells with Rhod-2AM, a high affinity mitochondrial Ca^{2+} indicator (Mothet et al., 1998). Rhod-2AM binds specifically with the mitochondrial Ca^{2+} and fluorescence can be quantified using a multi label reader. As seen in Figure 3.5A, the mitochondrial Ca^{2+} levels were found to be significantly higher at 6, 12 and 18 hrs post BAdV-3 infection. However, compared to 0, 6, 12 and 18 hrs, there was a significant decrease in the mitochondrial Ca^{2+} levels from 18 hrs post infection (Figure 3.5A). Similarly, cellular Ca^{2+} levels were measured by incubating BAdV-3 infected cells with Fluo-4AM, a highly specific fluorescent dye for measuring the cytosolic Ca^{2+} levels in the cells (Gee et al., 2000). Interestingly, there was no significant ($P<0.0001$) change in the cytosolic Ca^{2+} levels of the infected cells from 0 to 12 hrs post infection (Figure 3.5B). In contrast, compared to 0, 6 and 12 hrs, there was significant ($P<0.0001$) increase in the cytosolic Ca^{2+} levels of the infected cells at 18 and 24 hrs post infection (Figure 3.5B).

(A)



(B)

Figure 3.4 Mitochondrial membrane potential in BAdV-3 infected cells. (A) MDBK cells were mock infected or infected with BAdV-3 at a MOI of 5 and mitochondrial membrane potential was determined at 6, 12, 18 and 24 hrs post infection using TMRM and multilabel reader (Victor^3- Perkin Elmer). Data represents the mean of 2 independent experiments, each with 3 replicates. Means with the different letter are significantly different. *P<0.0001. **(B)** To determine that the cells were infected, proteins from the lysates of same BAdV-3 infected cells were collected at indicated times post infection and analyzed by Western blot using anti-β-actin MAb (Sigma Canada) or anti -E1Bs serum (Reddy et al., 1999a).

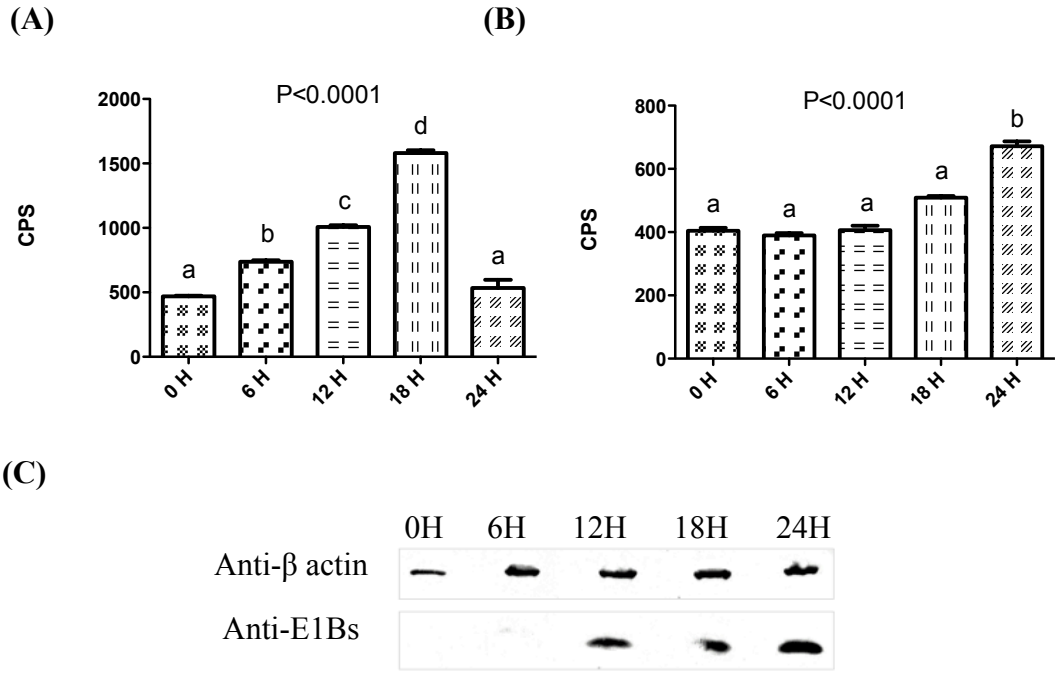


Figure 3.5 Ca^{2+} in BAdV-3 infected cells. MDBK cells were infected with BAdV-3 at a MOI of 5. At 0, 6, 12, 18 and 24 hrs post infection, the cells were treated with Rhod-2AM (Molecular Probes) to determine mitochondrial Ca^{2+}, (A) and Fluo-4AM (Molecular Probes), to determine cytosolic Ca^{2+}, (B). The cells were analyzed for fluorescence using Perkin Elmer multilabel reader (Victor^3- Perkin Elmer) as per manufacturer's instructions. Measurements are given in arbitrary units (CPS- counts per second). Data represents the mean of 2 independent experiments, each with 3 replicates. Means with the different letter are significantly different. Means with the same letter are not significantly. *P<0.0001. To determine that the cells were infected, proteins from the lysates of the same BAdV-3 infected cells were collected at indicated times post infection and analyzed by Western blot (C) using anti-β-actin MAb (Sigma Canada) or anti -E1Bs serum (Reddy et al., 1999a).

3.3.5 BAdV-3 infection aggravates the reactive oxygen species (ROS) and superoxide (SO) production in MDBK cells.

To assess the respiratory function of the cells infected with BAdV-3, both reactive oxygen species (ROS) and SO production was measured. MitoSOX™ Red reagent, a highly specific indicator of SO, is specifically targeted to the mitochondria and fluoresces when oxidized by SO but not by other ROS or reactive nitrogen species (RNS) generating systems (Mukhopadhyay et al., 2007) Similarly, DCF-DA is sensitive to all the other ROS except SO (Degli-Esposti, 2002). MDBK cells infected with BAdV-3 were collected at 0, 6, 12, 18 and 24 hrs post infection and incubated with DCF-2A which is sensitive to all the other ROS except SO (Degli-Esposti, 2002). MDBK cells infected with BAdV-3 were collected at 0, 6, 12, 18 and 24 hrs post infection and incubated with DCF-DA or MitoSOX™ red, and the fluorescence was quantified using multi-plate reader (Victor^3-Perkin Elmer). As seen in Figure 3.6A, compared to 0, 6 and 12 hrs, increased levels of SO were observed at 18 hrs post infection, which were significantly higher at 24 hrs post infection. Similarly, compared to 0, 6 and 12 hrs, increased levels of ROS were observed at 18 hrs, which were significantly higher at 24 hrs post infection (Figure 3.6B).

3.4 Discussion

Mitochondria perform various functions that make them absolutely indispensable for the cell (Chan, 2006). Besides acting as a powerhouse, they act as a common platform for the execution of a variety of cellular functions in the normal cells or in the cells under attack from the microorganisms including viruses (Seth et al., 2006). A number of viruses affect the structure and function of the mitochondria (Ohta and Nishiyama, 2011). Here, we report the effects of BAdV-3 on the mitochondria during the normal course of infection of bovine cells.

Electron microscopy of BAdV-3 infected cells revealed extensive damage to the inner mitochondrial membrane characterized by dissolution of cristae and amorphous appearance of the mitochondrial matrix while the outer mitochondrial membrane was observed to be intact. Various mitochondria specific lesions including degeneration of mitochondria and dissolution of mitochondrial cristae have been documented in Tobacco

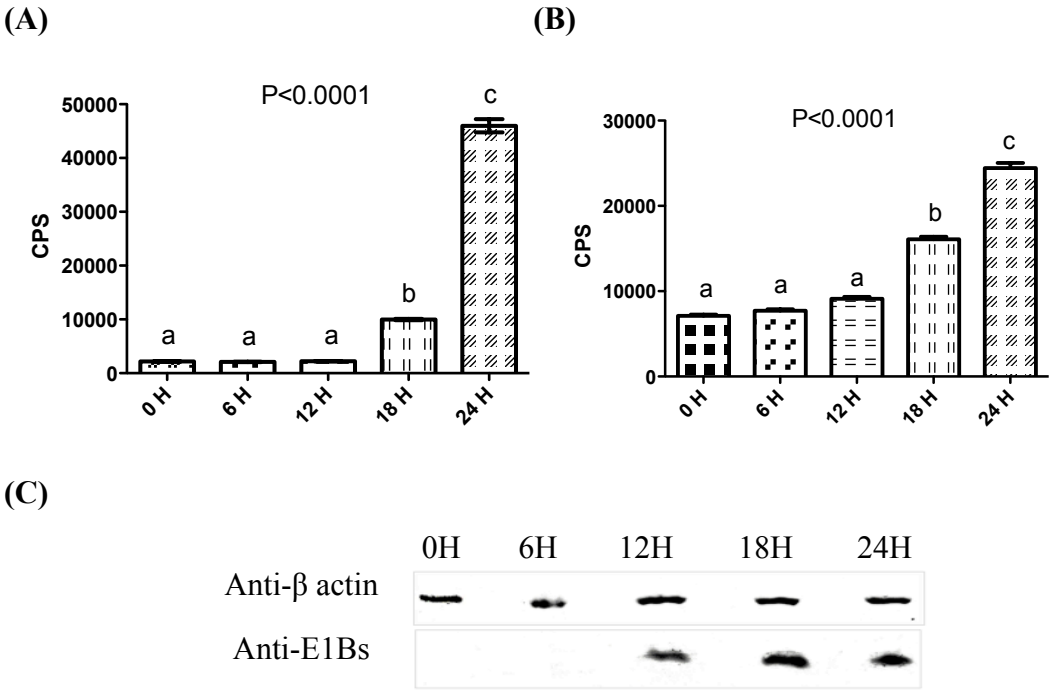


Figure 3.6 Induction of superoxide (SO) and reactive oxygen species (ROS) in BAdV-3 infected cells. MDBK cells were infected with BAdV-3 at a MOI of 5. At 0, 6 12, 18 and 24 hrs post infection, the cells were treated with MitoSox (Molecular Probes) to determine SO, (A) and DCF-2A (Molecular Probes) to determine ROS (B). The cells were analyzed for fluorescence using multilabel reader (Victor^3- Perkin Elmer) as per manufacturer's instructions. Measurements are given in arbitrary units (CPS- counts per second). Means with the different letter are significantly different. Data represents the mean of 2 independent experiments, each with 3 replicates. Means with the same letter are not significantly different. *P <0.0001. To determine that the cells were infected, proteins from the lysates of the same BAdV-3 infected cells were collected at indicated times and analyzed by Western blot (C) using anti-β-actin MAb (Sigma Canada) or anti -E1Bs serum (Reddy et al., 1999a).

mosaic virus (Weintraub and Ragetli, 1964), Western Equine Encephalomyelitis (WEE) virus (Morgan et al., 1961), vaccinia and fowl pox virus (Morgan et al., 1954) and Echo virus type 9 (Rifkind et al., 1961) infected cells. Such changes in the mitochondria have been attributed to the dissipation of mitochondria membrane potential (Nepomnyashchikh et al., 2001; Jaeschke et al., 2002; Kanno et al., 2002) due to opening of membrane permeability transition pores (Pessayre et al., 1999). Thus, invading viruses may be eliciting damage to cristae by decreasing the synthesis or blocking the transport of the mitochondria specific proteins responsible for the maintenance of the inner mitochondrial membrane.

Different steps in viral replication including DNA packaging and capsid maturation require ATP (Shuman et al., 1980; Dasgupta and Wilson, 1999; Hui and Nayak, 2001). Analysis of ATP production during the course of BAdV-3 infection showed a steady increase in the ATP production till 18^{th} hr post infection, when the production of progeny virus particles is at its peak. As expected, the ATP levels decline after 18 hrs post infection. This decline is in agreement with the culmination of the life cycle of BAdV-3. Variation in ATP production has also been associated with different stages of the viral life cycle indicating differential ATP requirements during the course of infection (Klumpp et al., 1998). Increased level of ATP increases the viral replication including the release of vaccinia virus (Chang et al., 2009) and virus budding in influenza A virus (Hui and Nayak, 2001) infected cells.

ATP is also required for the maintenance of most of the cellular and mitochondrial functions (Buttgereit and Brand, 1995; Hardie et al., 2003). Therefore, any change in cellular ATP production capacity will have direct impact on the membrane gradients inside the cell including the mitochondrial membrane potential (MMP). Our studies indicate that BAdV-3 modulates the MMP significantly at different times post infection. Initial transient decrease in MMP from 0-6 hrs followed by increase from 6-18 hrs may be due the expression of early adenoviral proteins. The role of adenovirus early proteins including E1A (Breckenridge and Shore, 2000; White, 2001), E1B 19K (Degenhardt et al., 2000) and E4orf4 (Kleinberger, 2000) in regulation of the longevity of the cell has been reported. A number of viruses including myxoma virus (Everett et al., 2000), HCV (Machida et al., 2006) and HIV-1 (Jacotot et al., 2000; Deniaud et al., 2004; Azuma et

al., 2006) modulate the MMP for their benefit by altering the activity of one or more components viz., the permeability transition pore (PTP) (Bernardi et al., 1999), the voltage dependent anionic channels (VDACs) (Colombini et al., 1996; Forte et al., 1996) and the membranes. As expected, at 24 hrs post infection, MMP showed significant decrease, which coincides with the observed damage to the mitochondria and decreased ATP levels. Thus, Increased ATP levels and prevention of the loss of MMP results in the prevention of the cell death, which is beneficial for the replication of BAdV-3. It is tempting to speculate that one or more BAdV-3 protein(s) may be involved in interactions with the mitochondria to help in increasing the ATP levels and the MMP.

Ca^{2+} is one of the most abundant and most universal signal carriers which acts as a second messenger to regulate many cellular processes (Berridge et al., 1998) including ATP synthesis (Balaban, 2009) and maintenance of MMP (Chorna et al., 2010; Liu et al., 2011). The ATP levels (Balaban, 2009) and the MMP regulate the Ca^{2+} homeostasis in the cells (Agudo-Lopez et al., 2011) and *vice versa* indicating a complex relationship between them. Our study didn't show a significant shift in cytosolic Ca^{2+} levels. In contrast, mitochondrial Ca^{2+} was observed to have peak retention at 18 hrs post infection, similar to what we observed for ATP and MMP. We believe that BAdV-3 causes the retention of Ca^{2+} in the mitochondria, which leads to increase in the ATP synthesis, thus helping in the maintenance of MMP. In addition to mitochondria, ER acts as a major source of Ca^{2+} in the cell. It is possible that whatever Ca^{2+} mitochondria uptake and retain during the process, ER releases the equivalent to make it up so that cytosolic Ca^{2+} concentration remains same. Thus, increase in mitochondrial Ca^{2+} leads to increase in ATP, MMP and decrease in ROS, which in turn may alter the apoptotic signalling (Pinton et al., 2001). Alterations in mitochondrial Ca^{2+} levels (Piccoli et al., 2006) have been reported during HCV or HSV -1 (Lund and Ziola, 1985) infection.

A variety of cellular defense mechanisms and enzymes including superoxide dismutase, catalases, lacto peroxidases, glutathione peroxidases and peroxiredoxins maintain the steady state concentration of the cellular oxidants at non- toxic levels (Brkic et al., 2010; Avery, 2011; Whaley-Connell et al., 2011). This delicate balance between oxidant generation and metabolism may be disrupted by various xenobiotics including the viral proteins. This imbalance between the oxidant (e.g ROS, SO) production and the

antioxidant cellular defences cause cell death. As expected, oxidative stress could be observed in the later phases of BAdV-3 infection, which may be the primary factor leading to the death of infected cells. A number of viruses including HAdV-5 cause the oxidative stress in the cells (McGuire et al., 2011, Hara et al., 2006; Piccoli et al., 2007; Nishina et al., 2008; Baum et al., 2011; de Mochel et al., 2010; Ming-Ju et al., 2011), which has been associated with the release of progeny virus (Arimoto et al., 2006).

Stimulus for mitochondria to perform beyond their usual capacity comes from various factors including stress caused to cell by ROS. It is known that oxidative stress causes an increase in mtDNA copy number and stimulates the nucleus to synthesize the proteins required for mitochondrial biogenesis (Lee et al., 2000). This scenario plays two roles in the affected cells. During initial stages (6-12 hrs post infection) of BAdV-3 infection, when cells are relatively healthy, they have higher antioxidant capacity and good quality of mtDNA. So, mild oxidative stress during this phase of infection may be causing an increase in mtDNA leading to proliferation of mitochondria with healthy cristae. This increases the total surface area available for ATP synthesis (cristae), which in turn compensates for the increased energy supply of the cell under given conditions. Such activity has also been loosely implicated to prevent apoptosis in HCMV infected cells (McCormick et al., 2003). These mitochondria will be able to cause increase in ATP synthesis, supply energy and participate in anti-apoptotic activities. Moreover, not all the cristae are damaged during initial phases of infection and remaining cristae may be producing ATP at increased levels along with healthy mitochondria produced as a result of biogenesis which explains the increase in ATP production when some of the cristae are observed to be damaged. At 18 hrs post infection, when oxidative stress has started to rise but it is still below threshold to produce faulty mtDNA and thus faulty mitochondria. At this time it is possible that there are some proportion of faulty mitochondria generating ROS. When ROS threshold is crossed, cells fall into ROS loop inflicting further damages to the mitochondria (as seen in 24hrs) and die releasing the progeny virus.

In conclusion, our study demonstrated that there is a delicate balance between the cellular functions, the mitochondrial physiology and BAdV-3 replication. Moreover, during early stages of BAdV-3 infection, retention of Ca^{2+} by mitochondria may prevent

the loss of MMP and, decrease the SO and ROS production by the infected cells, prolonging the cell survival for efficient production of the progeny BAdV-3 particles.

4.0 BOVINE ADENOVIRUS 3 PROTEINS VII AND 52K LOCALIZE TO MITOCHONDRIA AND CAUSE Ca^{2+} IMBALANCE AND ROS PRODUCTION

4.1 Introduction

Mitochondria are critically vital organelles of the cell that regulate the cellular functions and generate energy for all the molecular processes (Hackenbrock, 1966; Rapaport, 2003; Chen and Chan, 2005; Mannella, 2006). Besides energy production, mitochondria also play a central role in Ca^{2+} buffering, supply of metabolites, regulation of apoptotic factors, ageing and development (Hollenbeck and Saxton, 2005; Chan, 2006). Mitochondria, thus, regulate majority of the cellular processes. Many viruses can affect the structure and function of the mitochondria (Kaminska et al., 2007; St-Louis and Archambault, 2007; Ohman et al., 2009; Yang et al., 2009; Molouki et al., 2010), inducing oxidative stress (de Mochel et al., 2010; Hsieh et al., 2010; Lin et al., 2010; Machida et al., 2010; Ming-Ju et al., 2011), altering the mitochondrial membrane potential (MMP) and effecting the production of ATP (Monne et al., 2007; Chang et al., 2009; Su and Hong, 2010).

A number of viral proteins localize to the mitochondria and alter the mitochondrial functions (reviewed by Ohta and Nishiyama, 2011). Hepatitis C virus (HCV) proteins including E1, E2, and NS3 localize to the mitochondria, induce production of ROS, which causes the mtDNA damage and activation of STAT3 (Machida et al., 2010; Heish et al., 2010; Ming-Ju et al., 2011). The NS5A protein of HCV localizes to the mitochondria causing alterations in the oxidative stress-mediated Ca^{2+} homeostasis (Dionisio et al., 2009). The core protein of HCV targets the mitochondria and increases the Ca^{2+} dependent ROS production (Campbell et al., 2009).

Hepatitis B virus (HBV) protein X (Diao et al., 2001), human T-lymphotropic virus (HTLV) protein p13II (Ciminale et al., 1999), HIV proteins R (Vpr) (Azuma et al., 2006) and Tat (Macho et al., 1999), influenza virus proteins PB1-F2 (Danishuddin et al., 2010; Henkel et al., 2010) and M2 (Gonzalez and Carrasco, 2003), and Walleye dermal sarcoma virus (WDSV) protein encoded by Orf C (Nudson et al., 2003) localize to the mitochondria, decrease MMP and promote apoptosis. In contrast, human cytomegalovirus (HCMV) protein splice variant of UL37 (Goldmacher et al., 1999),

myxoma virus protein M11L (Everett et al., 2002) and vaccinia virus protein F1L (Wasilenko et al., 2005) inhibits the loss of MMP and inhibit apoptosis.

However, little is known about the role of the mitochondria in adenovirus infections. Human adenovirus (HAdV) has been reported to localize to the mitochondria in the cells infected with high titer virus (Alesci et al., 2008) inducing damage to the mitochondrial architecture. Adenoviral early proteins localize to the mitochondria and either prevent or induce apoptosis (Lomonosova et al., 2005). Adenovirus protein V interacts with p53 and localizes to the mitochondria (Matthews and Russell, 1998). Adenovirus death protein (ADP) encoded by E3 region of HAdV-5 induce oxidative stress and helps in the release of virus progeny from the virus-infected cell (Tollefson et al., 1996). Recently, HAdV-5 has been shown to cause the cathepsin dependent mitochondria mediated oxidative stress in the infected cells (McGuire et al., 2011).

Bovine adenovirus (BAdV)-3, a member of the genus *Mastadenovirus,* is a non-enveloped icosahedral virus, which is being developed and evaluated as a vaccine delivery vector for animals (Zakhartchouk et al., 1999) and humans (Rasmussen et al., 1999). Since mitochondria are a major cellular organelle performing various functions, study of mitochondria-virus interaction may provide new insights into the viral-host interactions. Earlier, we demonstrated that BAdV-3 infection affect the structure and function of mitochondria including ATP production, mitochondrial membrane potential, mitochondrial Ca^{2+} concentrations and oxidative stress (Chapter 3). In the present study, we determined the mitochondrial localization of BAdV-3 proteins and their role in inducing the observed changes in mitochondria.

4.2 Materials and methods

4.2.1 Cell lines and virus

Madin Darby bovine kidney (MDBK) cells were grown in minimal essential medium (MEM; Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS). Wild-type BAdV-3 (WBR-1 strain) was propagated in MDBK cells in MEM supplemented with 2% FBS (Reddy et al., 1999a). Vero cells were propagated in

Dulbecco's modified Eagle's medium (DMEM; Sigma Canada) supplemented with 10% FBS.

4.2.2 Antibodies

Polyclonal anti- 33K serum (Kulshreshtha et al., 2009) detects proteins of 42 kDa, 39kDa, 37kDa, 21kDa and19kDa in virus infected cells. Anti-52K serum detects a protein of 40kDa in virus infected cells (Paterson, 2010). Anti-penton serum and anti-hexon serum detects proteins of 62 and 98 kDa, respectively in virus infected cells (Kulshreshtha et al., 2004). Anti-pVII serum recognizes two proteins of 22 kDa and 20 kDa in virus infected cells (Paterson, 2010). Anti-IVa2 serum recognizes a protein of 55 kDa in BAdV-3 infected cells (Gaba and Tikoo unpublished). Monoclonal antibody (MAb) specific to hexokinase (sc-46695), polyclonal antibodies specific to ERK2 (Cat # sc-154) and fibrillarin (sc-25397 rabbit polyclonal) were purchased from Santa Cruz Biotechnology USA; MAb specific to cytochrome oxidase subunit (COX) -I (A6403) was purchased from Invitrogen, USA; MAb specific to heat shock protein 70 (anti-Hsp70, N27F3) was purchased from Stressgen, USA; MAb specific to mitochondrial complex II subunit (anti-cII, MS204) was purchased from Mitosciences, USA and MAb specific to β-actin (A5441) was purchased from Sigma, Canada. Alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG (#111-055-003) and AP-conjugated goat anti-mouse antibody (#115-055-003) were purchased from Jackson ImmunoResearch, USA.

4.2.3 Analysis of BAdV-3 proteins for potential mitochondrial localization signals

To determine the presence of potential mitochondrial localization signals (MLSs), selected BAdV-3 protein (Reddy et al., 1998) sequences were analyzed using PSORT and WolfPSORT (Nakai and Horton, 1999), SherLoc (Shatkay et al., 2007), PreDator (Rost et al., 2004), Target P (Emanuelsson et al., 2007) and MitoProt (Claros and Vincens, 1996) computer programs. Of the 29 BAdV-3 proteins analysed (GenBank accession # AF030154), potential MLS peptides were identified in N-terminal of pVII, 22K, 33K, and 52K. Only sequence with high reliability values were chosen for confirmation by Western blots.

4.2.4 Plasmid construction

(a) Construction of plasmid pcDNA3.52K: This plasmid was obtained from Dr C. Paterson (Paterson, 2010). Briefly, a 1,124-bp fragment containing the 52K open reading frame was amplified by PCR using primers CP1 (GAATTCATGCATCCCGC TTTACGGCAAATG) and CP2 (GGATCCACTCATTCGTCGACTTCAT) and plasmid pTG5435 (Rasmussen et al., 1999) as a DNA template. The PCR product was digested with *Eco*RI-*Bam*HI and ligated to *Eco*RI–*Bam*HI digested plasmid pcDNA3.1 (-) (Invitrogen) creating plasmid pcDNA.52K.

(b) Construction of plasmid pcDNA3.pVII: A 522-bp fragment containing the pVII open reading frame was amplified by PCR using primers XhoI-pVII-Fw (CGCCTCGAGATG GCCATTCTA ATCT CTCCTAG) and, XbaI-pVII-Rev (CGCTCTAGATCAA ACGGTGTTGCTGACCGTA GG) and plasmid pF304A (Zakhartchouk et al., 1998) as a DNA template. The PCR product was digested with *Xho*I-*Xba*I and ligated to *Xho*I-*Xba*I -digested plasmid pcDNA3.1 (-) (Invitrogen), creating plasmid pcDNA3.pVII.

(c) Construction of pcDNA3.33K: This plasmid was obtained from Dr V. Kulshreshtha (Kulshreshtha et al., 2004). Briefly, plasmid pGEX.33K (Kulshreshtha et al., 2004) was digested with *Nde*I-*Eco*RI and a 845 bp fragment was isolated, blunt end repaired with T4 polymerase and ligated to *Hind*III digested (blunt end repaired with T4 polymerase) plasmid pcDNA3 (Invitrogen), creating the plasmid pcDNA3.33K.

(d) Construction of pEYFP.52KMLS: A linker molecule (containing *Bam*HI and *Xho*I overhangs) containing the mitochondrial localization signal (MLS) of BAdV-3 protein 52K was created by incubating primers [52K-MLS-F (TCGAGATGCATCCCGCTT TACGGCAAATGAAGCCCCGATCGGCG) and 52K.MLS-R (GATCCGCCGATCG GGGCTTCATTTGCCGTAAAGCGGGATGCATC)] at 37^{o}C C for 1 hr. The linker was ligated to *Bam*HI-*Xho*I digested plasmid pEYFP.N1 (Clontech, USA) creating plasmid pEYFP.52KMLS.

(e) Construction of pEYFP.pVIIMLS. A 162-bp fragment containing the pVII MLS was amplified by PCR using primers [Xho-pVII-Fw (CGCCTCGAGATGGCCA TTCTAATCTCTCCTAG) and pVII-MLS-R.BamH1 (TTTGGATCCGCTGCGCGGCG ACCCACCCGAC)] and plasmid pcDNA3.pVII DNA as a template. The PCR product was digested with *Xho*I-*Bam*HI and ligated to *Xho*I-*Bam*HI digested plasmid pEYFP.N1

(Clontech) creating plasmid pEYFP.pVIIMLS. Plasmids pEYFP.pVII and pEYFP.52K were obtained from Dr C. Paterson (Paterson, 2010).

4.2.5 Isolation of mitochondria

MDBK cells were infected with BAdV-3 at a MOI of 5. Vero cells were transfected with individual plasmids (0.4μg/cm^2) using Lipofectamine™ 2000 (Invitrogen, USA) as per manufacturer's instructions. At 24 hrs post infection or 48 hrs post transfection, the cells were collected and used for isolation of mitochondria using the mitochondria isolation kit for mammalian cells (Pierce, USA), with following modifications. Approximately 2 x 10^7 MDBK cells (mock or infected) or Vero cells (mock or plasmid transfected) cells were dounce homogenized and pelleted at 300 x g to remove cell debris and nuclei. The supernatant 1 was collected and, the pellet containing cell debris and nucleus was dissolved in nucleus isolation buffer (NIB) [10mM KCl, 10mM $MgCl_2$, 10mM Tris.HCl (pH 7.4) and 10mM DDT], further homogenized in a dounce homogenizer and finally centrifuged at 212g to obtain nuclear fraction. The supernatant 1 was centrifuged at 3200x g to pellet mitochondria enriched fraction. The resulting supernatant 2 left after isolation of mitochondria enriched fraction was used as cytoplasmic fraction.

4.2.6 Western blot analysis

MDBK cells grown to 80% confluency (in 75cm^2 tissue culture flasks) were infected with wild-type BAdV-3 at a MOI of 5 and harvested 24 hrs post infection. Vero cells were transfected with individual plasmid DNA (0.4μg/cm^2) using Lipofectamine™ 2000 (Invitrogen, USA) as per manufacturer's instructions and harvested at 48 hrs post transfection. The cells were counted and equal numbers of cells were lysed by addition of RIPA buffer [0.15 M NaCl, 50 mM Tris-HCl (pH 8.0), 1% NP-40, 1% deoxycholate, 0.1% SDS] containing 1x anti-protease cocktail (Sigma). Similarly, isolated mitochondria or the nuclear fraction were lysed by addition of RIPA buffer [0.15 M NaCl, 50 mM Tris-HCl (pH 8.0), 1% NP-40, 1% deoxycholate, 0.1% SDS] with or without 1x anti-protease cocktail (Sigma, Canada).

Proteins from the lysates of the cells, the mitochondria, the cytoplasmic or the nuclear fractions were separated by 10% sodium-dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) and transferred to a nitrocellulose membrane (Bio-Rad, USA). The membrane was blocked with 5% or 10% skimmed milk powder (SMP: Nestle, Canada) in TBST [Tris-buffered saline (pH 8.0), 0.05% tween 20] overnight at 4˚C and probed with protein specific antibodies in TBST containing 0.1% SMP for 1 hr at room temperature. The membranes were washed with TBST thrice and probed with either alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG or (AP)-conjugated goat anti-mouse (Jackson ImmunoResearch, USA) diluted 1:10,000 in TBST containing 0.1% SMP for 1 hr at room temperature. Finally, the membranes were washed three times in TBST and developed using a BCIP/NBT reagent (Sigma, Canada).

4.2.7 Proteinase K treatment

The isolated mitochondria were dissolved in buffer C of the mitochondria isolation kit, (Pierce, USA) with or without Triton X-100 containing proteinase K at a final concentration 150µg/ml. The treated samples were incubated for 30 min on ice before centrifugation at 6,700 x g for 15 min at 4°C (Huh and Siddiqui, 2002). The pellet fraction(s) was subsequently analyzed by Western blotting using protein specific antibodies.

4.2.8 Experimental design

All the experiments were performed as outlined in Figure 3.1. For normalizing the data, the cells from each well were counted after measuring the fluorescence. All luminescence / fluorescence data were normalized to “per 1000 cells” value observed in the assay. Since reagents used in ATP assay lyse the cells, two identical plates were made simultaneously. The counting of the cells in each well at three different times indicated that there was no significant difference in the number of cells in the plates prepared simultaneously. One plate was used for ATP assay. The other plate was used to count the cells for normalizing the ATP data. The data represents the mean ± SEM of two independent experiments each with three replicates.

4.2.9 Cellular ATP

Vero cells grown in 96 well plates were transfected with 0.2 µg/well individual plasmid (pcDNA3, pcDNA3.pVII or pcDNA3.52K) DNA using Lipofectamine^{TM} 2000 (Invitrogen, USA) as per manufacturer's instructions. At 48 hrs post transfection, the transfected cells were treated with ATPLite^{TM} 1step kit reagents (Perkin-Elmer, Canada) as per manufacturer's instructions. This assay system is based on measuring the production of light caused by the reaction of cellular ATP with added luciferase and D-luciferin (Cree and Andreotti, 1997). The emitted light, which is proportional to the ATP concentration, was recorded using a multi label counter (Victor^3 - Perkin Elmer, Canada) from 30000- 40000 cells on an average and plotted as mean of 2 independent experiments, each with 3 replicates.

4.2.10 Mitochondrial and cytosolic Ca^{2+}

Vero cells grown in 96 well plates were transfected with 0.2 µg/ well of individual plasmid (pcDNA3, pcDNA3.pVII or pcDNA3.52K) DNA using Lipofectamine^{TM} 2000 (Invitrogen, USA) as per manufacturer's instructions. At 48 hrs post transfection, the transfected cells were incubated with 5µM mitochondrial Ca^{2+} sensitive dye Rhod-2AM (Molecular Probes, USA) (Cannell et al., 1994) or 10µM cytosolic Ca^{2+} sensitive dye Fluo-4AM (Molecular Probes, USA) (Gee et al., 2000) for 30 min at 37^{o}C. The cells were washed three times in Ca^{2+} free PBS (Gibco, Canada) or KRH buffer [129mM NaCl, 5mM $NaHCO_3$, 4.8mM KCl, 1.2mM KH_2PO_4, 1mM $CaCl_2$, 1.2mM $MgCl_2$, 2.8mM glucose and 10mM Hepes (pH7.4)], and equilibrated for 10 min. The fluorescence signals were measured using a multi label counter (Victor ^{3} - Perkin Elmer, Canada) using a 480/31 nm filter to excite the Fluo-4 AM and 531nm filter to excite Rhod-2 AM fluorescence. The signals were collected at 535 nM (Fluo-4 AM) and at 572 nm (Rhod-2 AM) from 30000- 40000 cells on an average and plotted as mean of 2 independent experiments, each with 3 replicates.

4.2.11 Mitochondrial membrane potential

Vero cells grown in 96 well plates were transfected with 0.2µg/well of individual plasmid (pcDNA3, pcDNA3.pVII or pcDNA3.52K) DNA using Lipofectamine^{TM} 2000

(Invitrogen, USA) as per manufacturer's instructions. After 48 hrs of transfection, the mitochondrial membrane potential was determined using tetramethylrhodamine methyl ester (TMRM) (Molecular Probes, USA) (Wong and Cortopassi, 2002). The transfected cells were incubated for 30 min with 100 nM TMRM in KRH-glucose buffer containing 0.02% pluronic acid, then washed and allowed to equilibrate for 20 min. The fluorescence signals were measured using a multi label counter (Victor^3 - Perkin Elmer, Canada) using a 531 nm excitation and 572 nm emission filter from 30000- 40000 cells on an average and plotted as mean of 2 independent experiments, each with 3 replicates.

4.2.12 Mitochondrial reactive oxygen species (ROS) and superoxide (SO)

Vero cells grown in 96 well plates were transfected with 0.2µg/well of individual plasmid (pcDNA3, pcDNA3.pVII or pcDNA3.52K) DNA with Lipofectamine^{TM} 2000 (Invitrogen, USA) as per manufacturer's instructions. At 48 hrs post transfection, the transfected cells were incubated with either 10µM of DCF-DA (Molecular Probes, USA) (Degli Esposti, 2002) or 5µM of MitoSOX™ red (Molecular Probes, USA) (Patschan et al., 2008) for 30 min in KRH buffer. The cells were washed in KRH buffer three times and equilibrated for 10 min until fluorescence equilibrated. The fluorescence signals were measured with multilabel counter (Victor^3- Perkin Elmer, Canada) using a 480/31 nm excitation and 535 nM (DCF-DA) and 580 nm (MitoSOX^{TM}) emission filters from 30000- 40000 cells on an average and plotted as mean of 2 independent experiments, each with 3 replicates.

4.2.13 Fluorescence microscopy

MDBK cells (2 x 10^5) seeded in each well of the 2-well glass slides (Nunc, USA) were infected with BAdV-3 at an MOI of 5. Twenty four hrs post infection, the cells were incubated with MitoTracker Red (Invitrogen, USA) at 500nM concentration for 15 mins in MEM. The cells were washed with phosphate buffered saline (PBS) [2.69 mM KCl, 2.47mM KH2PO4, 136.89 mM NaCl, 8.10 mM Na2HPO4] thrice and then fixed in 4% formaldehyde for 15 mins at room temperature. The cells were washed three times with PBS and permeabilized with ice cold acetone for 5 mins. The cells were blocked with 2% goat serum in PBS for 30 mins and incubated with primary antibody (either

anti-52K or anti-pVII serum, 1:50) in PBS containing 1% goat serum for 1 hr at room temperature. The antigen-antibody complex was detected by adding Cy2- conjugated goat anti- rabbit IgG (Jackson Immunoresearch; 1:400) in PBS containing 1% goat serum for 1 hr at room temperature. Finally, the cells were mounted in VectaShield mounting reagent (Vector Labs, USA) containing 4',6- diamidino-2-phenylindole (DAPI) and visualized using Leica confocal microscope (TCS-SP5).

Vero cells ($2x10^5$) seeded in each well of 2 well glass chamber slides (Nunc, USA) were co-transfected with 0.4 μg/well of plasmid (pEYFP, pEYFP.52K, or pEYFP.pVII) DNA using LipofectamineTM 2000 (Invitrogen, USA) as per manufacturers instructions. Twenty four post transfection, the cells were incubated with MitoTracker Red (500nM) for 15 min in MEM. Finally, the cells were washed and mounted with VectaShield (Vector Labs, USA) containing DAPI. In separate experiments, transfected cells were treated with cycloheximide (Sigma, USA) at 2 μg/ml in MEM at 6 hrs after transfection for 18 hrs. Finally, the cells were washed thrice with MEM to remove cycloheximide and visualized every two hrs to observe the expression of the proteins using Leica confocal microscope (TCS-SP5).

For SO measurements, Vero cells seeded in each well of 2-well glass slides were transfected with 0.4 μg/well of individual plasmid (pcDNA3, pcDNA3.pVII or pcDNA3.52K) DNA using Lipofectamine^{TM} 2000 (Invitrogen, USA) as per manufacturer's instructions. At 48 hrs post transfection, the cells were incubated for 30 min in MEM without phenol red containing 5μM of MitoSOX^{TM} Red (Molecular Probes, USA). After three washes with PBS, the cells were mounted in Citifluor mounting reagent (Citifluor, Ltd., Leicester, U. K.) and visualized using Leica confocal microscope (TCS-SP5).

4.2.14 Apoptosis assay

Vero cells grown in one well (10^5 cells/well) of 24 well plate were co-transfected with 0.8μg/well of plasmid phRL-Renilla Luciferase DNA and 0.8μg/well of individual (pcDNA3, pcDNA3.pVII or pcDNA3.52K) or combined (pcDNA3.pVII- pcDNA3.52K) plasmid DNA using LipofectamineTM 2000 (Invitrogen, USA) as per manufacturer's instructions. After 48 hrs post transfection, the selected samples were treated with

500nM Staurosporine (Sigma, Canada) for 4 hrs The treated cells were washed with PBS, lysed and incubated for 1 hr in reaction buffer containing DEVD-AFC substrate to determine the cleavage of caspage-3. Caspase-3 assay (Clontech, USA) employs a specific caspase-3 substrate, N-Ac- DEVD-N'-AFC, which upon cleavage by active caspase-3, generates a fluorescent product that can be measured using excitation and emission wavelengths of 400 and 505 nm, respectively using a multi label counter (Victor3 - Perkin Elmer, Canada). Expression of Renilla luciferase was measured using Dual-Luciferase® Reporter Assay System (Promega, USA) and a multi label counter (Victor^3 - Perkin Elmer, Canada).

4.2.15 Statistical Analysis

Data were analyzed by one-way analysis of variance (Anscombe, 1948), using a general linear model procedure (GLM; SAS Enterprise Guide 4.2 under SAS 9.2 environment for Windows XP; SAS Institute Inc., Cary, NC, USA) for effect of treatment (pcDNA3, pcDNA3.52K and pcDNA3.pVII). Probability values >0.05 were considered non-significant. Tukey's post-hoc tests for multiple comparisons were performed if main effect (i.e., time) was significant ($P \leq 0.05$). The values are expressed as mean ± SEM.

4.3 Results

4.3.1 Isolation of mitochondria from bovine cells

The mitochondria rich fraction was purified from mock or BAdV-3 infected MDBK cells. To check the purity, the isolated mitochondrial fraction was analyzed by Western blot. Equal amount of the proteins from the mitochondrial fraction, the cytoplasmic fraction and the thenuclear fraction of mock infected or BAdV-3 infected MDBK cells were separated by 10% SDS-PAGE, transferred to nitrocellulose membrane and probed with anti-COX1 serum recognizing mitochondria specific protein, anti-ERK serum recognizing cytoplasm specific protein and anti-fibrillarin serum recognizing nucleus specific protein. Anti-COX serum recognized a specific protein in the mitochondrial fractions but not in the nuclear fraction or the cytoplasmic fraction of

mock or BAdV-3 infected cells (Figure 4.1). Anti-ERK serum recognized a specific protein in the nuclear fraction but not in the mitochondrial fraction or the cytoplasmic fraction of mock infected or BAdV-3 infected cells. However, anti-fibrillarin serum recognized a specific protein in the nuclear fraction and the cytoplasmic fraction but not in the mitochondrial fraction of mock or BAdV-3 infected cells. Anti- Hsp-70 serum detected a specific protein in all fractions of mock or BAdV-3 infected cells and was used as a loading control. These results suggest that that the mitochondrial fraction purified from mock or BAdV-3 infected MDBK cells was highly enriched in mitochondria.

4.3.2 Analysis of proteins for potential mitochondrial localization signal

To determine if BAdV-3 proteins localize to the mitochondria rich fraction, initially, selected protein sequences of BAdV-3 (Reddy et al., 1998) were analyzed for the presence of potential mitochondrial localization signal(s) (Habib et al., 2007) with available software(s) (Table 4.1) using default parameters except TargetP, which was used in the 'winner-takes-all' mode without setting a specificity cut-off for targeting. As seen in table 1, BAdV-3 proteins pIII, pVII, pX, 22K, 33K and 52K were predicted to contain a strong mitochondria localization signal (MLS) whereas pIVa2 and E1B 19K were found to contain a weak MLS. In pIII, the MLS was not predicted by MitoPort, but PSORT, WolfPSORT and TargetP indicated high probability of its localization in mitochondria. The proteins 33K, 52K and pVII with predicted high probability of localization to mitochondria and proteins hexon, penton and IVa2 with no predicted probability of localization to mitochondria were chosen for further analysis by Western blot. Since 33K and 22K have same MLS, we choose 33K instead of analyzing both.

4.3.3 Bovine adenovirus-3 proteins associate with mitochondria

To determine if any of these BAdV-3 proteins are localized to the mitochondria, proteins from different cellular (mitochondrial, nuclear and cytoplasmic) fractions purified from the mock or BAdV-3 infected cells were separated by 10% SDS-PAGE analyzed by Western blot using protein specific antibodies. As seen in Figure 4.2, anti-

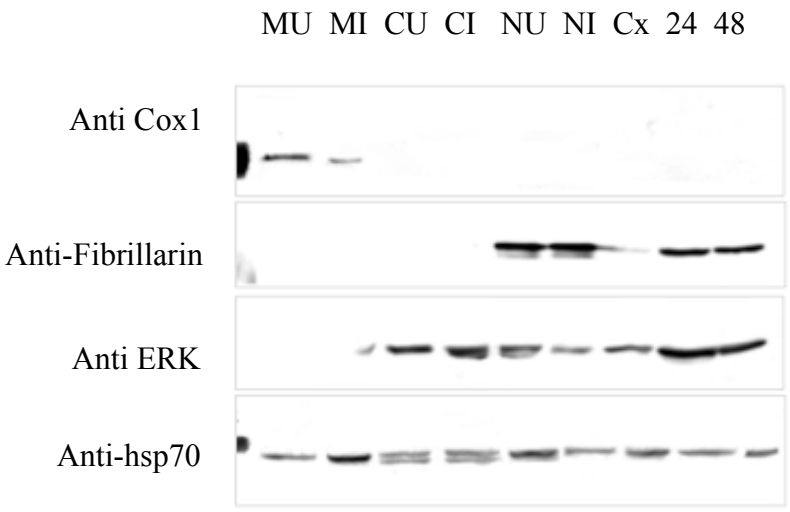


Figure 4.1 Western blot analysis of cellular fractions. Proteins from the lysates of the indicated cellular fraction isolated from mock infected or BAdV-3 infected cells were separated by 10% SDS-PAGE, transferred to nitrocellulose membranes and probed in Western blot using anti-Cox1 serum (mitochondrial marker), anti-fibrilarin serum (nuclear marker), anti-ERK serum (cytoplasmic marker) and anti-Hsp70 serum (loading control). Mitochondrial fraction (MU); cytosolic fraction (CU) and nuclear fraction (NU) from the uninfected cells. Mitochondrial fraction (MI); cytosolic fraction (CI) and nuclear fraction (NI) from the infected cells. Uninfected MDBK cells (Cx); BAdV-3 infected MDBK cells collected at 24 (24) or 48 (48) hrs post infection.

Table 4.1 Sequence analysis of BAdV-3 proteins to predict mitochondrial localization signal (MLS).

Gene ID	MitoProt	PSORT	WolfPSORT	SherLoc	Predator	TargetP1.1
AP_000022.1	E1B 19K	.05	**M**	M	M	-
AP_000025.1	IVa2	.026	M	M	-	-
AP_000028.1	III	.839	-	M	M	M
AP_000029.1	pVII	**.762 (S)**	M	M	-	-
AP_000035.1	33K	**.802 (S)**	M	M	-	-
AP_000036.1	22K	**.677 (S)**	M	M	-	-
AAD09721	52K	**0.88 (S)**	M	M	M	-

M: Indicates the predicted mitochondrial localization by different computer programs. S: Predicted putative mitochondrial localization signal (MLS). Numbers in MitoProt OR Psort column indicate the probability of the localization of a protein in mitochondria. The value with (s) denotes high /significant probability Gene IDs are from GenBank and BAdV-3 genome sequence (GenBank accession # AF030154). Sequences were analyzed using available web based protein analysis programs MitoProt (Claros and Vincens, 1996), PSORT and WolfPSORT (Nakai and Horton, 1999), SherLoc (Shatkay et al., 2007), PreDator (Rost et al., 2004) and Target P (Emanuelsson et al., 2007).

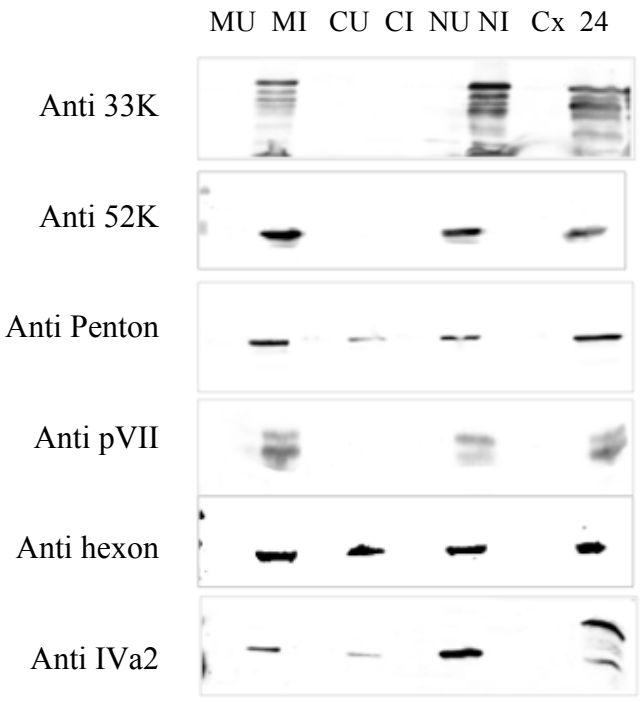


Figure 4.2 Western blot analysis of cellular fractions. Proteins from the lysates of the indicated cellular fraction isolated from mock infected or BAdV-3 infected cells were separated by 10 % SDS-PAGE, transferred to nitrocellulose and probed in Western blot using anti-33K serum (Kulshrestha and Tikoo, 2009), anti-52K serum (Paterson, 2010), anti-penton serum (Kulshrestha et al., 2004), anti-pVII serum (Paterson, 2010) , anti-hexon serum (Kulshrestha and Tikoo, 2009) or anti-IVa2 serum (Gaba and Tikoo, unpublished). Mitochondrial fraction (MU); cytosolic fraction (CU) and nuclear fraction (NU) from uninfected cells. Mitochondrial fraction (MI); Cytosolic fraction (CI) and nuclear fraction (NI) from infected cells. Uninfected MDBK cells (Cx); BAdV-3 infected MDBK cells collected at 24 (24) hrs post infection.

33K serum recognized specific proteins in BAdV-3 infected MDBK cells. Similar proteins were detected in purified mitochondrial and nuclear fractions of BAdV-3 fractions of uninfected MDBK cells. Similarly, anti-52K serum or anti-pVII serum recognized specific proteins in BAdV-3 infected MDBK cells or purified mitochondrial and nuclear fractions of BAdV-3 infected cells. No such proteins could be detected in purified cytoplasmic BAdV-3 infected cells, uninfected MDBK cells or purified mitochondrial and nuclear fractions of uninfected MDBK cells. In contrast, anti-hexon serum, anti-penton serum and anti-IVa2 serum recognized specific proteins in BAdV-3 infected MDBK cells or purified mitochondrial, cytoplasmic and nuclear fractions. No such proteins could be detected in uninfected MDBK cells or purified mitochondrial, cytoplasmic and nuclear fractions of uninfected MDBK cells.

4.3.4 pVII and 52K proteins localize to mitochondria in virus infected cells

Preliminary studies could not discriminate whether the viral proteins localize to the mitochondria on their own due to the presence of the mitochondrial localization signal (integral membrane proteins and soluble proteins located in the inter-membrane space or matrix) or they are loosely attached to outer mitochondrial membranes. To resolve the issue, mitochondria rich fractions from BAdV-3 infected MDBK cells were treated with proteinase K. Treated and untreated mitochondria were analyzed by Western blot using protein specific antibodies. Proteinase K treatment should degrade hexokinase protein (inserted in outer mitochondrial membrane) but will not degrade complex II protein (inserted in inner mitochondria membrane). As seen in Figure 4.3, anti-hexokinase serum detected hexokinase (Mulichak et al., 1998) specific protein in untreated mitochondria and BAdV-3 infected MDBK cells but not in proteinase K treated mitochondria (Figure 4.3). As expected, anti-cII serum detects a complex II specific protein in untreated mitochondria or BAdV-3 infected MDBK cells and also in proteinase K treated mitochondria (Figure 4.3). To demonstrate that the isolation procedure did not damage mitochondria integrity, the purified mitochondria were treated with both proteinase K and 0.1% Triton X-100. This treatment renders proteins contained within the mitochondria (inner membrane and matrix) susceptible to protease treatment (Sardanelli et al., 2006). Treated and untreated mitochondria were analyzed by Western blot using

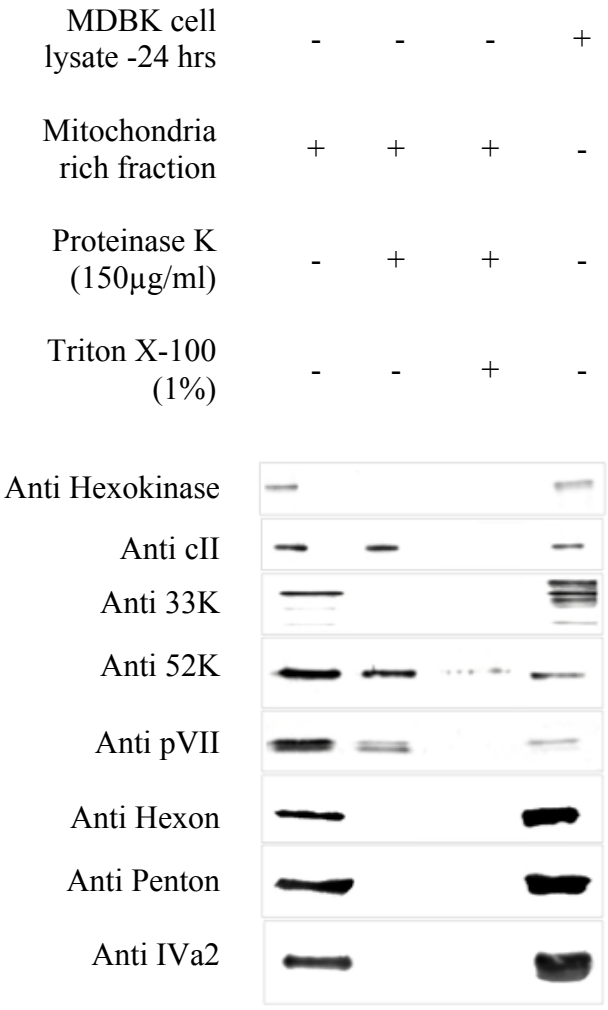


Figure 4.3 Proteinase K treatment of mitochondrial fractions of BAdV-3 infected cells. Proteins from the lysates of the indicated cellular fraction isolated from BAdV-3 infected cells were separated by 10% SDS-PAGE, transferred to nitrocellulose and probed in Western blot using anti-hexokinase MAb (Santa Cruz Biotechnologies, USA), anti-cII MAb (MitoSciences, USA), anti-33K serum (Kulshrestha and Tikoo, 2009), anti-52K serum (Paterson, 2010), anti-pVII serum (Paterson, 2010), anti-hexon serum (Kulshrestha et al., 2004), anti-penton serum (Kulshrestha et al., 2004) and anti-IVa2 serum (Gaba and Tikoo, unpublished).

protein specific antibodies. As seen in Figure 4.3, anti-hexokinase serum did not detect hexokinase specific protein in proteinase K or proteinase K-Triton X-100 treated mitochondria. In contrast, anti-cII serum detects a complex II specific protein in proteinase K treated mitochondria (degrades proteins inserted only in the outer mitochondrial membrane) but not in proteinase K-Triton X-100 treated mitochondria (degrade proteins inserted both in the outer and inner membranes). These results reconfirmed and established that proteinase K treatment degrades the proteins exposed on the outer mitochondrial membrane but has no effect on the proteins inside the outer mitochondrial membrane. Next, mitochondrial fraction isolated from BAdV-3 infected cells was treated with proteinase K in the absence or presence of Triton X-100. Treated and untreated mitochondria were analyzed by Western blot using BAdV-3 protein

4.3.5 pVII and 52K proteins localize to mitochondria in transfected cells

To determine if pVII and 52K could localize to the mitochondria, initially BAdV-3 infected MDBK cells or individual plasmid DNA transfected Vero cells were visualized for fluorescence as described in section 4.2.13. In both cases, the expression of pVII and 52K was observed all over the cells (data not shown) making it difficult to determine if protein is also localizing to the mitochondria of the infected /transfected cells.

To control the expression of proteins in transfected cells, the cells were treated with cycloheximide, which inhibits the process of translation but has no effect on transcription of mRNA from DNA. Translation block was removed 24 hrs post transfection and expression was monitored every 2 hrs. To our surprise, we still could not determine if proteins were localizing to the mitochondria of transfected cells due to their distribution all over the cell (data not shown). It is possible that the small quantities of these proteins localize in the mitochondria, which could not be detected by fluorescence assay. We repeated these experiments several times without any success and decided to proceed with proteinase K assay as discussed below.

To determine if pVII and 52K could independently localize to the mitochondria, Vero cells were transfected with 0.4μg/cm^2 with individual plasmid (pcDNA3.33K, pcDNA3.pVII, or pcDNA3.52K) DNAs. After 48 hrs of transfection, mitochondria rich fractions were isolated from transfected cells, treated with proteinase K or proteinase K-

Triton X-100 and analysed by Western blot using protein specific antibodies. As seen in Figure 4.4, both 52K and pVII were detected in proteinase K treated mitochondria but not in proteinase K-Triton X-100 treated mitochondria. These results confirmed earlier observations and suggested that BAdV-3 52K and pVII proteins localize inside the mitochondria (inner membrane or matrix) by virtue of their endogenous MLS independent of any other viral protein.

4.3.6 pVII and 52K contain functional mitochondrial localization signal

Earlier, protein analysis predicted the presence of potential mitochondrial localization signal (MLS) at the N terminus of both 52K (amino acid 1-14) and pVII (amino acid 1-54) proteins. To determine if these signals were targeting these proteins to mitochondria, these sequences were individually fused in -frame to EYFP and Vero cells were transfected with individual plasmid DNAs. After 48 hrs of transfection, mitochondria rich fractions were isolated from the transfected cells, treated with proteinase K or proteinase K – Triton X-100 and analyzed by Western blot using protein specific antibodies. As seen in Figure 4.4, both MLS-EYFP fusion proteins were resistant to proteinase K treatment but degraded after proteinase K-Triton X-100 treatment. These results suggest that targeting of 52K and pVII proteins to the mitochondria involve N- terminus amino acid 1-14 and amino acid 1-54, respectively.

4.3.7 BAdV-3 protein VII regulates ATP production

Since ATP is present in all metabolically active cells and is a very good marker of cell's health and viability, we next determined if localization of proteins VII and 52K to the mitochondria has any effect on the production of ATP in the transfected cells. To observe this, Vero cells were transfected with plasmid pcDNA3, pcDNA3.pVII or pcDNA3.52K DNA and ATP production was measured 48 hrs post transfection. ATP concentration was found to be significantly higher ($P<0.0001$) in the cells transfected with pVII compared to the cells transfected with plasmid pcDNA3 or pcDNA3.52K DNA (Figure 4.5). This indicates that pVII induces the ATP production in the cells and plays some role in ATP synthesis.

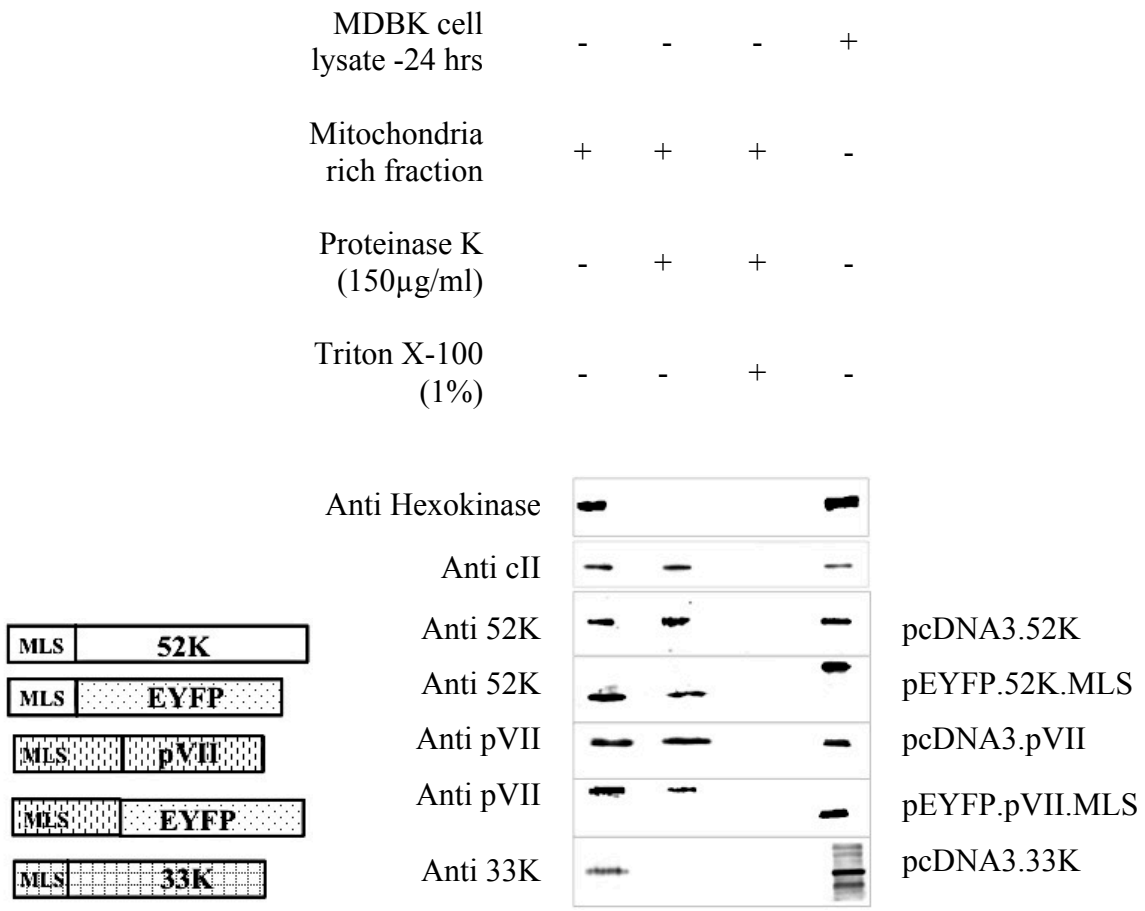
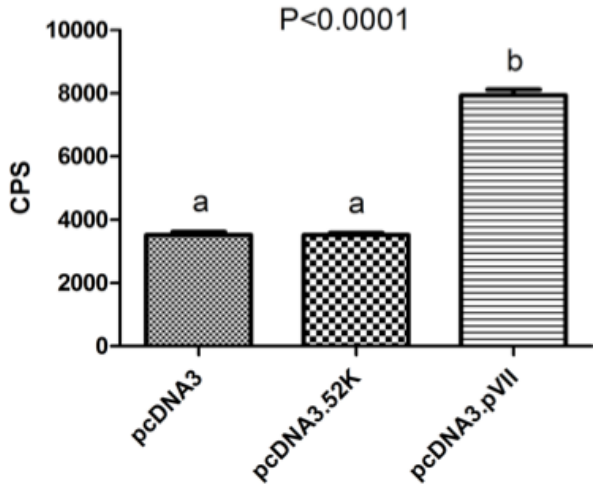


Figure 4.4 Proteinase K treatment of mitochondria rich fraction from transfected cells. Proteins from the lysates of the mitochondrial fraction isolated from the cells transfected with indicated plasmid DNAs were separated by 10% SDS-PAGE, transferred to nitrocellulose membrane and probed in Western blot using anti-hexokinase MAb (Santa Cruz Biotechnologies, USA), anti-cII MAb (Mitosciences, USA), anti-52K serum (Paterson, 2010), anti-pVII serum (Paterson, 2010), anti-33K serum (Kulshrestha and Tikoo, 2009). Schematic diagram of plasmids shown on the left of the panel. The name of the plasmids depicted on the right of the panel.

(A)



(B)

Figure 4.5 ATP production in the cells expressing 52K and pVII. (A) Vero cells were transfected with individual plasmid (pcDNA3, pcDNA3.52K or pcDNA3.pVII) DNAs and ATP production was measured 48 hrs post transfection using ATP Lite^{TM} 1 step kit and multilabel reader (Victor^{3}- Perkin Elmer). Measurements are depicted in arbitrary units (CPS- counts per second). Data represents the mean of 2 independent experiments, each with 3 replicates. Means with the different letter are significantly different. * P<0.0001. **(B)** To determine that the cells expressed respective proteins, proteins from the lysates of the transfected cells were also separated by 10% SDS-PAGE, transferred to nitrocellulose membrane and probed in Western blot using anti-β actin MAb (Sigma Canada), anti-52K serum (Paterson, 2009) and anti-pVII serum (Paterson, 2010).

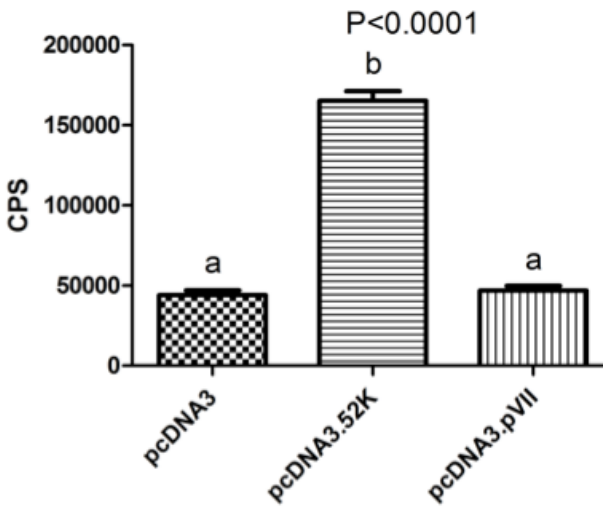
4.3.8 BAdV-3 protein 52K increase ROS and SO production in Vero cells

To assess the mitochondrial function in Vero cells expressing BAdV-3 pVII and/or 52K protein(s), we measured mitochondrial ROS production. Vero cells were transfected with individual plasmid (pcDNA3, pcDNA3.pVII, or pcDNA3.52K) DNA and ROS production was measured 48 hrs post transfection. The cells were incubated with DCF-DA [sensitive to the cellular ROS (Zhang et al., 2009)] and the fluorescence was quantified (Figure 4.6 A). The cells transfected with pcDNA3.52K showed increased levels of ROS production in comparison to the cells transfected with pcDNA3, or pcDNA.pVII indicating that expression of the pVII did not increase oxidative stress levels in Vero cells. To verify the above findings (as measured with DCF-DA), we used MitoSOX Red to determine mitochondrial SO levels in Vero cells transfected with same set of plasmid (pcDNA3, pcDNA3.pVII, pcDNA3.52K) DNAs. MitoSOX™ Red reagent is a highly specific SO indicator, which is rapidly and specifically targeted to the mitochondria and fluoresces when oxidized by SO but not by other ROS or RNS generating systems (Cassina et al., 2008). Vero cells transfected with pcDNA3.52K showed higher levels of mitochondrial superoxide as compared to the cells transfected with pcDNA3 or pcDNA3.pVII (Figure 4.7 A, C). These results suggest that theexpression of 52K increases both the mitochondrial ROS and SO production indicating that this protein could impair mitochondrial function. Expression of BAdV-3 protein VII did not alter the oxidative state of the transfected cells.

4.3.9 BAdV-3 protein VII regulates mitochondrial Ca^{2+} levels

Next, I determined the Ca^{2+} buffering ability of mitochondria in these cells. Ca^{2+} buffering capacity of the mitochondria is a good indicator of mitochondrial health and survival in the cells. Vero cells were transfected with individual plasmid (pcDNA3, pcDNA3.pVII or pcDNA3.52K) DNAs. At 48 hrs post transfection, mitochondrial and cytosolic Ca^{2+} levels were measured using Fluo-4AM and Rhod-2AM which are highly specific indicators of cellular and mitochondrial Ca^{2+} respectively. The cells expressing the BAdV-3 protein 52K did not show any significant increase in the mitochondrial Ca^{2+} buffering activity (Figure 4.8 A) whereas cells expressing pVII showed a significant increase in the mitochondrial Ca^{2+} levels (Figure 4.8 A). Thapsigargin treatment of the

(A)



(B)

Figure 4.6 Induction of ROS in the cells expressing 52K and pVII. (A) Vero cells were transfected with individual plasmid (pcDNA3, pcDNA3.52K or pcDNA3.pVII) DNAs. At 48 hrs post transfection, the cells were treated with DCF-DA (Molecular Probes) and analyzed for fluorescence using multilabel reader (Victor^3- Perkin Elmer) as per manufacturer's instructions. Measurements are given in arbitrary units (CPS- counts per second). Means with the different letter are significantly different. Data represents the mean of 2 independent experiments, each with 3 replicates. Means with the same letter are not significantly different. *P <0.0001. **(B)** To determine that the cells were transfected and expressed respective proteins, proteins from the lysates of these transfected cells were also separated by 10% SDS-PAGE, transferred to nitrocellulose membrane and probed in Western blot using anti-β actin MAb (Sigma Canada), anti-52K serum (Paterson, 2010) and anti-pVII serum (Paterson, 2010).

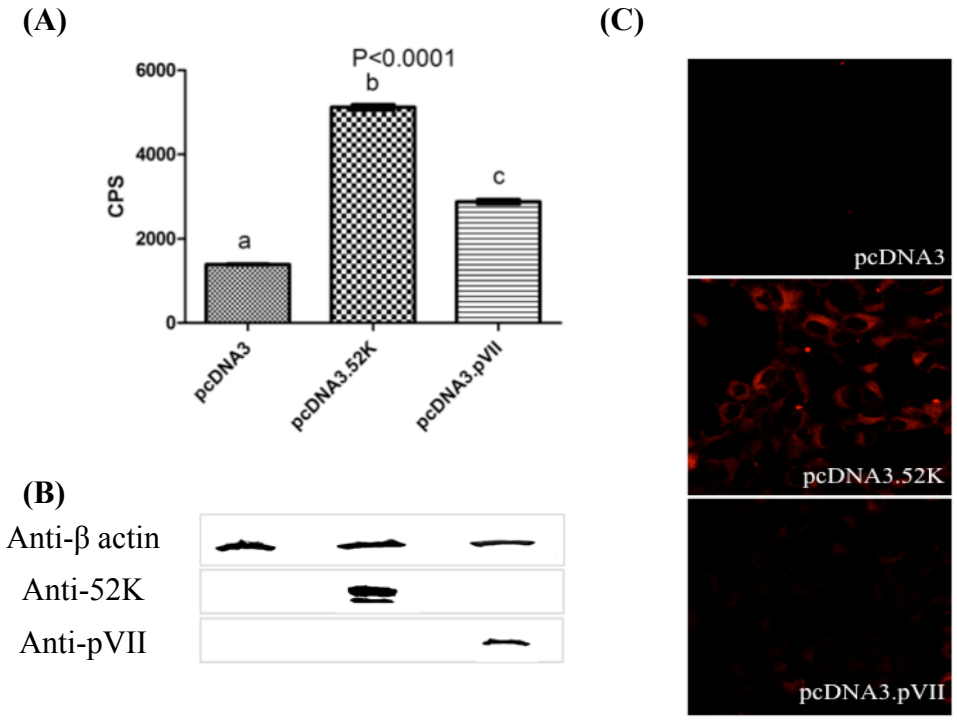


Figure 4.7 Induction of SO in the cells expressing 52K and pVII. (A) Vero cells were transfected with plasmid (pcDNA3, pcDNA3.52K or pcDNA3.pVII) DNA. At 48 hrs post transfection, the cells were treated with MitoSox (Molecular Probes) and analyzed for fluorescence using multilabel reader (Victor^3- Perkin Elmer) as per manufacturer's instructions. Measurements are given in arbitrary units (CPS- counts per second). Means with the different letter are significantly different. Data represents the mean of 2 independent experiments, each with 3 replicates. Means with the same letter are not significantly different. *P <0.0001. **(B)** To determine that the cells were transfected and expressed respective proteins, proteins from the lysates of these transfected cells were also separated by 10% SDS-PAGE, transferred to nitrocellulose membrane and probed in Western blot using anti-β actin MAb (Sigma Canada), anti-52K serum (Paterson, 2010) and anti-pVII serum (Paterson, 2010). **(C)** Vero cells transfected with indicated plasmid DNA were treated 48 hrs post transfection. with MitoSox Red and visualized by Leica confocal microscope (TCS-SP5).

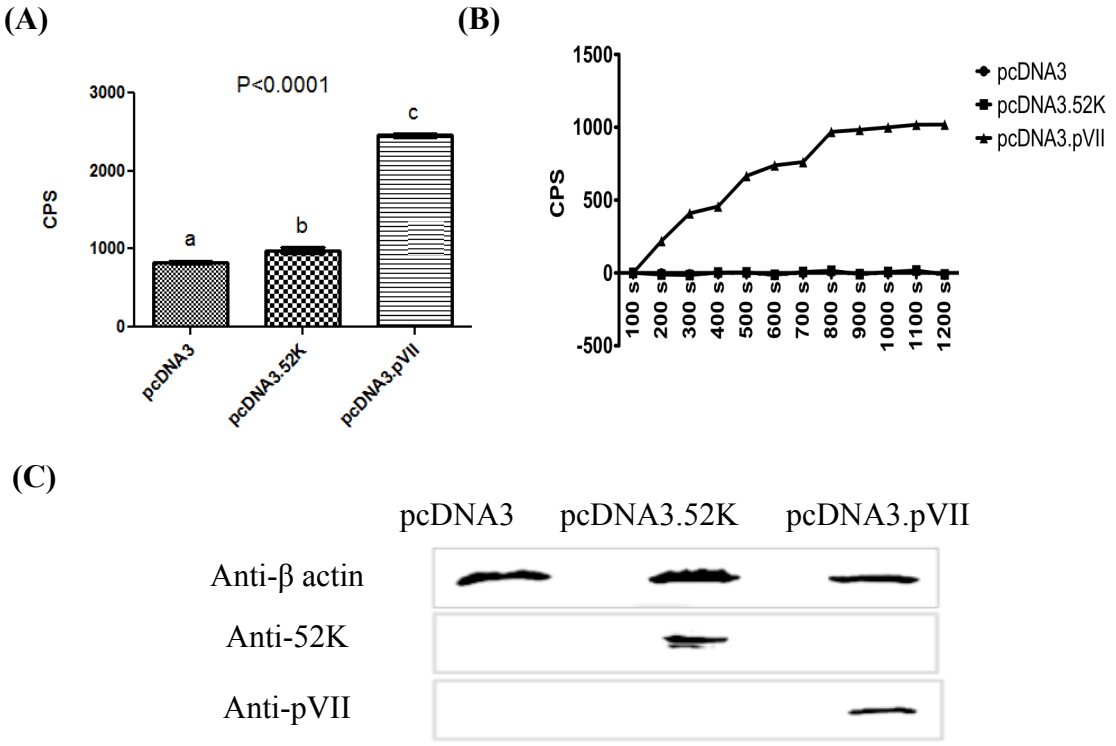


Figure 4.8. Mitochondrial Ca^{2+} in the cells expressing 52K and pVII. (A) Vero cells were transfected with individual plasmid (pcDNA3, pcDNA3.52K, pcDNA3.pVII) DNA. At 48 hrs post transfection, the transfected cells were treated with Rhod-2AM (Molecular Probes) and analyzed for fluorescence using Perkin Elmer multilabel reader (Victor^3- Perkin Elmer) as per manufacturer's instructions (left). Means with the different letter are significantly different. Means with the same letter are not significantly. *P<0.0001. **(B)** The same cells were treated with 1μM thapsigargin for 30 mins and fluorescence measurements were taken for 1200 seconds post treatment at 100 second intervals. Measurements are given in arbitrary units (CPS- counts per second). Data represents the mean of 2 independent experiments, each with 3 replicates. **(C)** To determine that the cells expressed respective proteins, proteins from the lysates of the same transfected cells were also separated by 10% SDS-PAGE, transferred to nitrocellulose membrane and probed in Western blot using anti-β actin MAb (Sigma Canada), anti-52K serum (Paterson, 2010) and anti-pVII serum (Paterson, 2010).

cells results in a global and transient increase in cytosolic calcium levels (Ong and Hausenloy, 2010), thus helping in examining the ability of the mitochondria to effectively uptake and sequester Ca^{2+}. Thapsigargin (1μM) treatment of the cells expressing pVII showed a significant sequestration and retention of mitochondrial Ca2+ even after 20 mins post treatment (Figure 4.8 B) whereas the cells expressing 52K showed a significant decrease in the mitochondrial calcium uptake (Figure 4.8 B). In addition, thapsigargin treatment of Vero cells expressing pVII and 52K showed no significant change in the cytosolic Ca^{2+} levels over the period of treatment (Figure 4.9 A, B). This shows that the expression of pVII induces mitochondria to sequester and retain Ca^{2+}.

4.3.10 BAdV-3 proteins pVII and 52K regulate mitochondrial membrane potential

To verify if alterations in the mitochondrial Ca^{2+} causes any changes in the mitochondrial membrane potential (MMP), we measured MMP using TMRM. The MMP is a critical indicator of the mitochondrial function and allows for an accurate determination of mitochondrial bioenergetics and cellular metabolism. Vero cells were transfected with individual plasmid (pcDNA3, pcDNA3.pVII or pcDNA3.52K) DNAs and MMP changes were measured 48 hrs post transfection. As seen in Fig. 4.10A, the TMRM fluorescence levels decreased significantly in the cells expressing 52K after thapsigargin treatment for 30 min but not in the cells expressing pVII (Figure 4.10B). Even after thapsigargin treatment, there was significant MMP loss in the cells expressing 52K but not in the cells expressing pVII (Figure 4.10B). This indicates that the expression of BAdV-3 protein pVII helps the cells to maintains the MMP whereas 52K has little or no effect on the maintenance of MMP.

4.3.11 BAdV-3 protein 52K causes apoptosis

To confirm if proteins VII and 52K induce or inhibit apoptosis, caspase-3 assay was performed. Caspase-3 is an active cell-death protease involved in the execution phase of the apoptosis (Porter & Janicke, 1999; Zou et al., 1999) and gets activated in response to various signals. Vero cells were transfected with plasmid (pcDNA3, pcDNA3.pVII or pcDNA3.52K) DNAs. At 48 hrs post transfection, indicated cells were

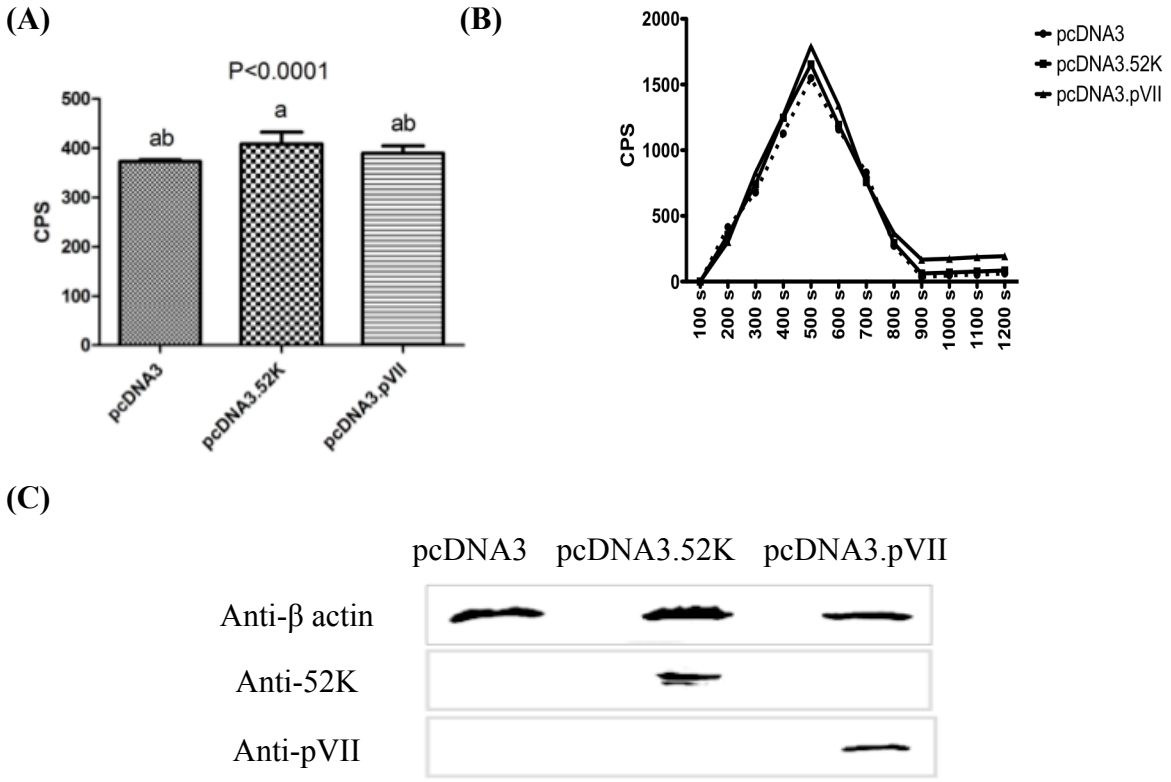


Figure 4.9 Cytosolic Ca^{2+} in cells in the cells expressing 52K and pVII. (A) Vero cells were transfected with plasmid (pcDNA3, pcDNA3.52K or pcDNA3.pVII) DNA and the cytosolic Ca^{2+} was measured 48 hrs post transfection. The cells were treated with Fluo-4AM (Molecular Probes) and analyzed for fluorescence using a Perkin Elmer multi plate reader Victor3 as per manufacturer's instructions. Means with the same letter are not significantly different. *P<0.0001. **(B)** Same cells were treated with 1μM thapsigargin for 30 min and fluorescence measurements were taken for 1200 seconds post treatment at 100 sec intervals (right). Measurements are given in arbitrary units (CPS- counts per second). Data represents the mean of 2 independent experiments, each with 3 replicates. **(C)** To determine that the cells were transfected and expressed respective proteins, proteins from the lysates of these transfected cells were separated by 10% SDS-PAGE, transferred to nitrocellulose membrane and probed in Western blot using anti-β actin MAb (Sigma, Canada), anti-52K serum (Paterson, 2010) and anti-pVII serum (Paterson, 2010).

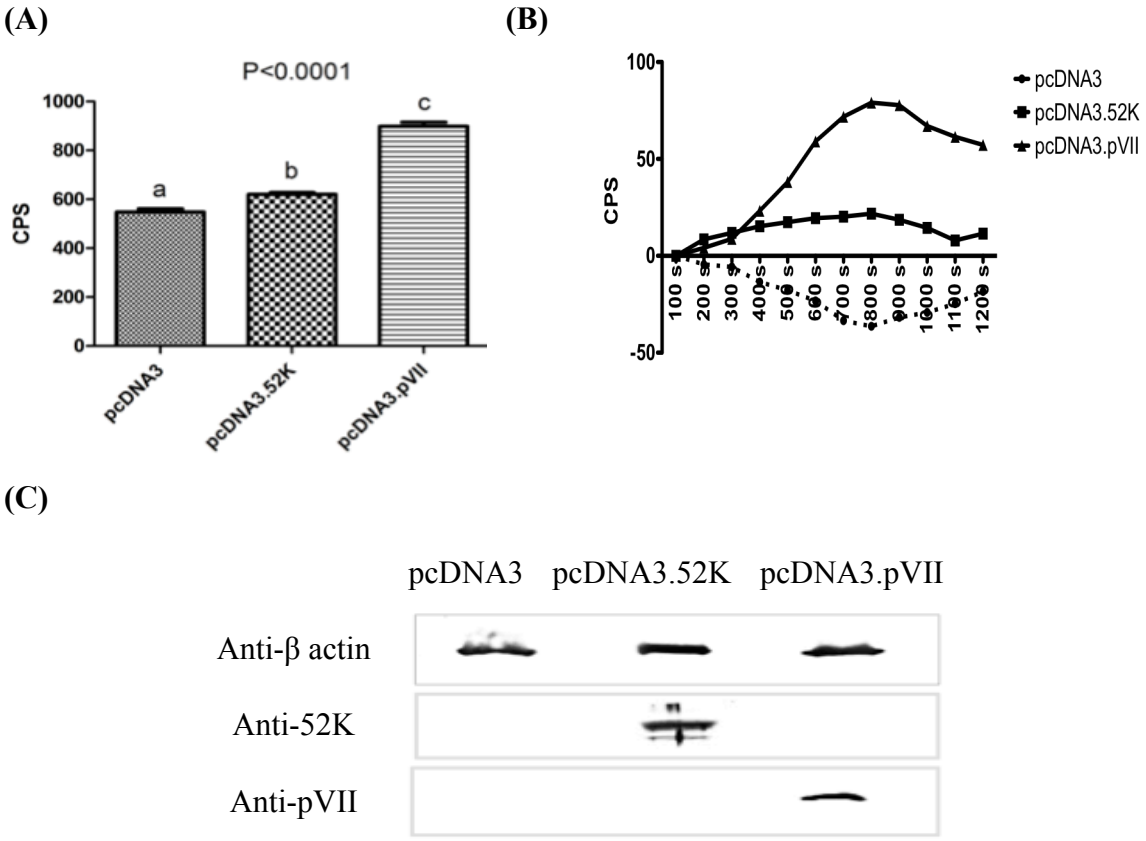


Figure 4.10 Mitochondrial membrane potential in the cells expressing 52K and pVII (A) Vero cells were transfected with individual plasmid (pcDNA3, pcDNA3.52K, pcDNA3.pVII) DNA and the MMP was measured 48 hrs post transfection using TMRM and multilabel reader (Victor^3- Perkin Elmer). Means with the different letter are significantly different. *P<0.0001. **(B)** The same cells were treated with 1μM thapsigargin for 30 minutes and measurements were taken for 1200 seconds post treatment at 100 sec intervals. Data represents the mean of 2 independent experiments, each with 3 replicates. **(C)** To determine that the cells were transfected and expressed respective proteins, proteins from the lysates of these transfected cells were separated by 10% SDS-PAGE, transferred to nitrocellulose membrane and probed in Western blot using, anti-β actin MAb (Sigma, Canada), anti-52K serum (Paterson, 2010) and anti-pVII, serum (Paterson, 2010).

treated with staurosporine for 4 hrs and caspase 3 activity was measured using caspase 3 assay kit (Clontech). Expression of 52K increased the activation of caspase-3 in transfected cells **(**Fig 4.11 A) indicating its involvement in inducing apoptosis. In contrast, expression of pVII did not lead to the activation of caspase-3. Interestingly, expression of pVII significantly reduced the activation of caspase-3 in staurosporine treated cells or the cells expressing 52K protein indicating the anti-apoptotic role of pVII.

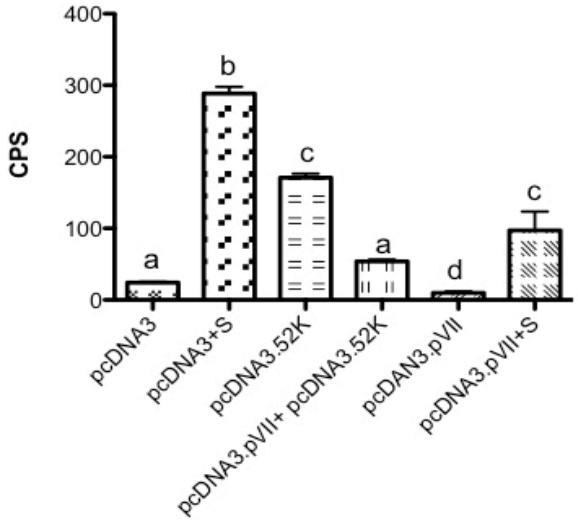
4.4 Discussion

A number of viruses target mitochondria during infection process and alter its functions (Ohta and Nishiyama, 2011). This usually involves the transport of specific viral proteins to the mitochondria leading to the modulation of mitochondrial functions. Our previous report suggested that BAdV-3 interacts with the mitochondria and alters mitochondrial structure and function (Chapter 3). In the present study, we demonstrate that BAdV-3 core protein VII and a non-structural protein 52K localize into the mitochondria and modulate the mitochondrial physiology.

Earlier, amino acid sequence analysis identified few BAdV-3 proteins, which appeared to contain potential mitochondrial localization signals. However, Western blot analysis of mitochondrial fraction isolated from infected cells suggested that the all tested proteins appeared to be associated with the mitochondria. It is possible that some of these proteins non specifically associate with the mitochondria due to the effect of virus replication on distribution of mitochondria in the infected cells. Support for this comes from the fact that electron microscopic analysis of infected cells at 12 hrs post infection shows the presence of mitochondria in the close vicinity of protein synthesis factories in the infected cells. These protein(s) synthesizing factories might be synthesising viral proteins, which might have been purified with mitochondrial fraction during purification process

Interestingly, some adenovirus proteins 33K, 22K and IVa2 predicted to contain MLS do not localize to the mitochondria. On the other hand BAdV-3 proteins like pV which appear not to have a potential MLS localize to the mitochondria. Similarly,

(A)



(B)

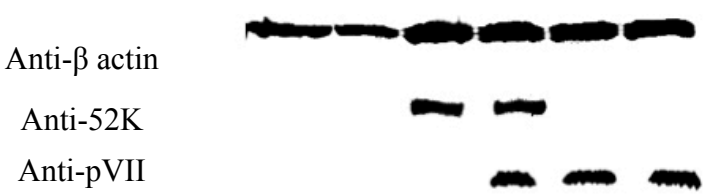


Figure 4.11 Caspase-3 assay in the cells expressing 52K and pVII. (A) Vero cells were transfected with individual plasmid DNA (pcDNA3, pcDNA3.52K, pcDNA3.pVII) and caspase-3 was measured 48 hrs post transfection using multilabel reader (Victor^3-Perkin Elmer). Data represents the mean of 2 independent experiments, each with 3 replicates. Means with the different letter are significantly different. *P<0.0001. S= Staurosporine. **(B)** To determine that the cells were transfected and expressed respective proteins, proteins from the lysates of the transfected cells were separated by 10% SDS-PAGE, transferred to nitrocellulose and probed in Western blot using anti-β actin MAb (Sigma, Canada), anti-52K serum (Paterson, 2010) and anti-pVII serum (Paterson, 2010).

adenoviral proteins, which appear not to contain a potential MLS, have been reported to localize in the mitochondria (Lomonosova et al., 2005; Matthews and Russell, 1998). Thus, the presence/absence of a MLS in a protein does not guarantee that a protein will localize/not localize to the mitochondria.

Of the six BAdV-3 proteins, which were found to be associated with mitochondria, only two namely 52K and pVII appear not to be loosely attached, but localized to the mitochondria. Several lines of evidence support the suggestion that 52K and pVII localize to the mitochondria due to the presence of a functional MLS. First, 52K and pVII specific proteins could be detected in Western blots of mitochondrial fractions of infected/transfected cells treated with proteinase K. Secondly, potential MLS of 52K (amino acid 1-14) or pVII (amino acid 1-54) could localize the cytoplasmic protein EYFP to the mitochondria of transfected cells. Third, MLS-EYFP fusion proteins could be detected in Western blots of mitochondrial fractions of transfected cells treated with proteinase K.

Localization of viral proteins in mitochondria (Ohta and Nishiyama, 2011) has been implicated in altering various cellular processes including the host defense mechanisms (Yokota et al., 2010; Castanier and Arnoult, 2011), the Ca^{2+} homeostasis (Zhou et al., 2009), cellular metabolism (Maynard et al., 2010) and apoptosis (Everett and McFadden, 2001; Danthi, 2011) to establish themselves and replicate. Mitochondrial localization of BAdV-3 52K protein had little or no effect on ATP synthesis and cytosolic or mitochondrial Ca^{2+} retention in transfected cells. Interestingly, it significantly increased the ROS, particularly SO, levels in transfected cells resulting in stress, which may cause disturbances in cellular homeostasis. SO has been demonstrated to alter oxygen sensing capacity of the cell (Chandel et al., 2000; Loor et al., 2011), alter cell cycle control (Sauer et al., 2001) and induce inflammation ultimately resulting in apoptosis (Simon et al., 2000). ROS and SO production have been implicated in leading to Bcl2 mediated apoptosis (Cai and Jones, 1998). The significant amount of ROS/SO production at 24 hrs post infection coincides with the release of progeny virus suggesting that 52K may be involved in inducing apoptosis at later stages of infection cycle. The detection of activation of caspase 3 in the cells expressing 52K supports this conclusion.

Mitochondrial localization of BAdV-3 VII protein induces a significant increase in the levels of ATP indicating a positive role this protein appears to play during the course of infection. Increased ATP is involved in the maintenance of ion gradients and thus MMP across the mitochondrial membranes (Buttgereit and Brand, 1995; Hardie et al., 2003). Protein VII also appears to help in the retention of mitochondrial Ca^{2+}. Retention of Ca^{2+} in the mitochondria and consistent ATP generation help maintain the MMP (Halestrap, 2009; Agudo-Lopez et al., 2010; Halestrap, 2010). Since Ca^{2+} is a physiological stimulus for ATP synthesis and is one of the positive effectors of oxidative phosphorylation (Balaban, 2009), it is conceivable that mitochondrial Ca^{2+} retention helps cells to maintain steady supply of ATPs, thus helping to maintain the MMP. Based on these observations, protein VII appears to be an antiapoptotic protein that prolongs the life of the cells thus helping the viruses to complete their life cycle. Consistent with these observations is the fact that pVII has little or no effect on ROS and SO generation in the cells, which have been attributed to cause oxidative stress in the cell and considered proapoptotic. In addition, expression of pVII in cells treated with staurosporine or expressing 52K significantly decreases the activation of caspase 3.

Although pVII helps in the increase and retention of the mitochondrial Ca^{2+}, it has little or no effect on the cytosolic Ca^{2+}. This may be due to fact that other Ca^{2+} storages like the endoplasmic reticulum, which acts as a main store house of the Ca^{2+} in the cells, may be releasing enough Ca^{2+} to maintain the cytosolic levels in spite of significant portions of Ca^{2+} being retained by the mitochondria.

In conclusion, our results suggest that there is a complex correlation between ATP generation, Ca^{2+} regulation, SO and ROS generation, and modulation of the membrane potential in the cell when they are under the influence of viral proteins 52K and pVII (Figure 4.12). Although pVII enhances the vital mitochondrial processes and prolongs the longevity of the cell, 52K seems to enhance the ROS and SO generation leading the cells towards stress and ultimately cell death. It is not clear from our studies which protein takes precedence during the course of the infection. We speculate that during initial stages of infection when virus needs viable cells, pVII a component of mature virion helps to maintain the life of the cell. However, during later stages of the viral life

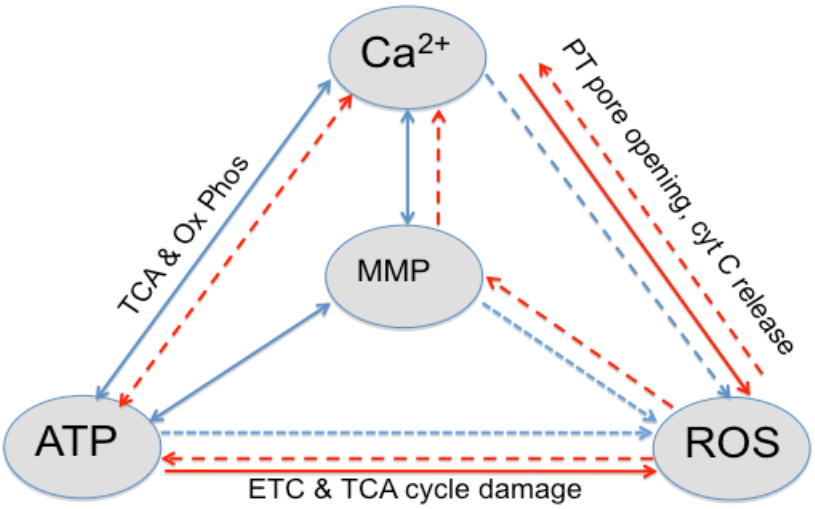


Figure 4.12 Schematic diagram showing the effect of pVII and 52K on various mitochondrial processes. Solid lines indicate activation and broken lines indicate inhibitory effect. Arrows determine the direction. ATP: Adenosine Trophosphate, MMP: Mitochondrial Membrane potential, ROS: Reactive Oxygen Species, ETC: Electron transport chain, TCA: Tricaoboxylic/ Krebs cycle, PT Pore: Permeability transition pore. pVII (blue lines); 52K (red lines).

cycle , 52K a non structural late protein potentially induces apoptotic changes helping the release of the progeny virus from the cell.

5.0 GENERAL DISCUSSION AND CONCLUSIONS

Mitochondria are dynamic organelles and perform various functions that make them indispensable for the cell (Chan, 2006). They act as a common platform for the execution of a variety of cellular functions in normal cells and in the cells under attack from microorganisms like viruses (Seth et al., 2006). A number of viruses have been shown to affect the structure and the function of the mitochondria. In the studies presented in this thesis, I attempted to determine the correlation between Ca^{2+} homeostasis, ATP and ROS production during the course of BAdV-3 infection and also determined the potential role(s) of proteins VII and 52K independently in orchestration of these processes.

Complete loss of internal architecture of mitochondria at 24 hrs post infection indicated that the organelle was one of the main targets of the BAdV-3 during infection. This prompted me to ask a few questions about the role of the mitochondria during the course of infection. It is generally accepted that the structure follows the function. The functional analysis revealed that the functions of the mitochondria were not compromised till late during the infection. This is consistent with the fact that the completion of virus cycle requires supply of ATP. Interestingly, at late times post infection, the mitochondria were located near protein factories (possibly virus factories). Taken together, these results suggested that the mitochondria are active till late times post infection, when they lose the internal architecture consistent with providing energy required for the release of the virus. These observations allow us to speculate that virus takes over the vital mitochondrial processes under its control early during the infection and abandons the organelle once the life cycle is over.

The cell fractionation studies identified BAdV-3 proteins associated with the mitochondria. Moreover, the cell fractionation studies coupled with proteinase K assay confirmed the identity of the BAdV-3 proteins localized inside the mitochondria. These results were further confirmed by detecting the localization of EYFP (a cytoplasmic protein) fused to MLS of identified proteins to mitochondria using cell fractionation with proteinase K assay. Interestingly, none of these proteins could be detected by analysis of infected / transfected cells using immunofluorescence. It is possible that small amount of these BAdV-3 proteins are localized in the cells, which cannot be detected by the

immunofluorescence assay. However, isolation of cellular fractions helps to concentrate the specific proteins in mitochondrial fractions, which could be detected by Western blot analysis. Thus, it is clear that more than one assay should be used for determining the localization of proteins to different cellular organelles including mitochondria.

Ca^{2+} is one of the universal signalling molecules on which depends almost every aspect of cellular processes. Viruses during the course of evolution have developed various strategies to modulate this universal messenger and BAdV-3 is no exception. BAdV-3 causes the sequestration of Ca^{2+} to the mitochondria. Even though the mitochondrial Ca^{2+} increases, there is no appreciable change in the cytoplasmic Ca^{2+}. Interestingly, BAdV-3 protein pVII, which localizes to the mitochondria, shows similar effects in transfected cells. Such changes can be attributed to the release of Ca^{2+} from storage organelles to maintain homeostasis. The Ca^{2+} retained in the BAdV-3 infected or pVII transfected cell mitochondria is utilized for various processes including but not limited to ATP synthesis and maintenance of the MMP. Since there is no predicted Ca^{2+} binding motif in pVII, how pVII helps mitochondria retain Ca^{2+} is still unclear. Further work is needed to understand this interplay of virus and Ca^{2+} signalling and to understand the entire mechanism,

Mitochondrial membrane potential (MMP) determines the fate of the cell. MMP pattern changes continually during BAdV-3 infection. This physiological function of the mitochondria plays an important role in the survival of the cell and in the regulation of innate antiviral immunity. Steady state of MMP also maintains the cytochrome C locked in the inter membrane space. Increase in MMP in the cells expressing pVII, and decrease in the caspase 3 activation in cells treated with staurosporine confirm that pVII is an anti-apoptotic protein and is involved in the maintenance of life cycle of the cell.

Although increase in ROS was observed upto 18 hrs post BAdV-3 infection, no such increase was observed in SO production. It is possible that ROS produced upto 18 hrs post infection may be helpful for the cell. A low grade production of ROS is generally helpful where ROS act as signal molecules (Stowe and Camara, 2009) and help in modulation of various cellular and mitochondrial functions including but not limited to oxidant scavenging, cell cycle, and cellular repair (D'Autreaux and Toledano, 2007). However, significant amount of ROS / SO was detected at 24 hrs post infection, which

coincides with the culmination of BAdV-3 life cycle, the loss of MMP and decrease in the mitochondrial Ca^{2+} accompanied by loss of ATP synthesis. This also suggests the inability of the cell to scavenge the free radicals from the cells and thus repairing the resulting damage. Since all these processes involve mitochondria, I propose that the virus takes over the function of organelle

Interestingly, BAdV-3 52K protein is specifically involved in increased production of ROS particularly SO radicals in transfected cells resulting in oxidative stress leading to mitochondria dysfunction. Since mitochondria is the source of all the SO produced in the cell (Han et al., 2001; Koopman et al., 2010) we propose that majority of the stress during terminal stages of infection is caused by 52K dependent mitochondrial dysfunction, which may help in the release of the virus from the infected cell. Induction of caspase 3 activation in the cells expressing 52K support the notion that 52K protein is a proapoptotic protein and may help in the release of BAdV-3 from the infected cells. The proapototic effect may be exerted by the production of SO as SO has been suggested to be involved in Bcl2 mediated release of Cyt-C from the mitochondria that leads to apoptosis (Cai and Jones, 1998).

One of the major issues in gene therapy is the shortened longevity of the transduced cells, resulting in the elimination of the vector-transduced cells. Therefore, the potential use of anti-apoptotic proteins in gene therapy is of major interest. The clinical application of this technology could lead to a higher delivery of therapeutic genes in the treated cells for extend periods. One way to achieve this is by designing virus-based vectors expressing multiple copies of pVII (an antiapoptotic protein), which could increase the life span of the transduced cells. This may help in the use of virus vector based gene delivery for the treatment of degenerative diseases such as Alzheimer's disease, where vectors could target apoptosis-promoting proteins in specific tissues.

On the contrary, in cancer therapy, we desire to have vectors, which are highly apoptotic and efficiently kill the cancerous cells. In most of the cancers, one or more of pro apoptotic processes has been compromised leading to the uncontrolled growth of cells. So vectors with deletions in anti-apoptotic genes or insertion of multiple copies of pro-apoptosis genes can be very helpful in inducing effective oncolysis. Thus, viral

based vectors expressing multiple copies of 52K (a proapoptotic protein) may be engineered to increase the oncolytic capability of a viral vector.

In the present work, I only dealt with two proteins (pVII and 52K) localizing inside the mitochondria. I am not sure whether these proteins localize in the mitochondrial matrix (enclosed by inner mitochondrial membrane) or in the inter-membrane space (space between outer and inner mitochondrial membrane) or both. Further experiments are required to confirm the localization of these proteins in mitochondrial compartments. We could also explore the BAdV-3 protein(s)- mitochondrial protein(s) interactions during the process of localization.

One can also explore the functions of BAdV-3 proteins localizing on the outer mitochondrial membrane like 22K, 33K and pVIII, which may allow us to understand if any of these proteins is playing a role to support the life of BAdV-3 by modulating the functions of mitochondrial proteins. To further confirm our results, comparative proteomic (by mass spectrometry) studies of mitochondria isolated from BAdV-3 infected or mock infected cells can be performed which may reveal the localization of some other BAdV-3 proteins in the mitochondria.

These studies will not only increase our understanding of basic biology of BAdV-3 but also may lead to the development of potentially beneficial vectors, which may be of therapeutic value.

6.0. REFERENCES

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