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Novel vectors expressing anti-apoptotic protein Bcl-2 to study cell death in Semliki Forest virus-infected cells

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Abstract

Semliki Forest virus (SFV, *Alphavirus*) induce rapid shut down of host cell protein synthesis and apoptotic death of infected vertebrate cells. Data on alphavirus-induced apoptosis are controversial. In this study, the anti-apoptotic *bcl-2* gene was placed under the control of duplicated subgenomic promoter or different internal ribosome entry sites (IRES) and expressed using a novel bicistronic SFV vector. The use of IRES containing vectors resulted in high-level Bcl-2 synthesis during the early stages of infection. Nevertheless, in infected BHK-21 cells translational shutdown was almost complete by 6 h post-infection, which was similar to infection with appropriate control vectors. These results indicate that very early and high-level *bcl-2* expression did not have a protective effect against SFV induced shutdown of host cell translation. No apoptotic cells were detected at those time points for any SFV vectors. Furthermore, Bcl-2 expression did not protect BHK-21 or AT3-neo cells at later time points, and infection of BHK-21 or AT3-neo cells with SFV replicon vectors or with wild-type SFV4 did not lead to release of cytochrome *c* from mitochondria. Taken together, our data suggest that SFV induced death in BHK-21 or AT3-neo cells is not triggered by the intrinsic pathway of apoptosis.
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Keywords: Alphaviruses; Semliki Forest virus; Cell death; Bcl-2; AT3

1. Introduction

Semliki Forest virus (SFV) is a positive-stranded RNA virus in *Alphavirus* genus (family *Togaviridae*), a widely distributed group of human and animal pathogens (Strauss and Strauss, 1994). SFV genomic RNA (so-called 42S RNA) is approximately 11.5 kb long and encodes four non-structural proteins designated nsP1–4; these are involved in viral RNA synthesis. The remaining proteins form the virus capsid and envelope and are not essential for virus replication.

After virus entry, the 42S RNA is translated into a large non-structural polyprotein, which is processed to form an early and subsequently a late replicase complex (Strauss and Strauss, 1994). The early replicase mediates synthesis of the negative-stranded RNA complementary to the genomic 42S RNA. Minus strands are used by the late replicase as templates for the synthesis of new positive strand 42S RNA, and for transcription of subgenomic mRNAs encoding the structural proteins.

The structural genes of SFV are not required for replication and can be removed or replaced with a polylinker and/or with foreign gene sequences. This property forms the basis of the SFV-based replicon vector systems (Liljestrom and Garoff, 1991; Smerdou and Liljestrom, 1999). SFV-based replicon vectors mediate high-level expression of heterologous proteins. However, as with virus, vectors cause shutdown of cellular biosynthesis and induce apoptotic death (Glasgow et al., 1997, 1998; Scallan et al., 1997). This precludes long-term foreign gene expression, and several attempts to reduce the cytotoxicity of alphavirus vectors have been made (Fazakerley et al., 2002; Lundstrom et al., 2003, 2001; Perri et al., 2000).

The anti-apoptotic gene *bcl-2* is an antagonist of the intrinsic mitochondrial pathway of apoptosis (for reviews see Ashe and Berry, 2003; Cory and Adams, 2002; Tsujimoto and Shimizu, 2000). Bcl-2 can prevent release of cytochrome *c* from mitochondria, thus, precluding the apoptotic cascade (Kluck et al., 1997; Yang et al., 1997). Bcl-2 can block apoptosis induced by

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several viruses, including influenza virus and reovirus (Nencioni et al., 2003; Rodgers et al., 1997). Existing data on Bcl-2 in SFV- or Sindbis virus-induced apoptosis are contradictory. On one hand it has been shown that alphavirus-induced apoptosis of baby hamster kidney (BHK) cells, Chinese hamster ovary cells, rat insulinoma cells and rat prostatic adenocarcinoma (AT3) cells can be prevented by over-expression of Bcl-2 (Levine et al., 1993; Lundstrom et al., 1997; Mastrangelo et al., 2000; Scallan et al., 1997). Similarly, a Sindbis virus expressing Bcl-2 produces reduced encephalitis in infected mice (Levine et al., 1996). That Bcl-2 expression can block apoptosis, suggests involvement of intrinsic pathway of apoptosis. In contrast, other studies using rat embryo fibroblasts and monocyte cell lines overexpressing Bcl-2 failed to detect a protective effect against alphavirus-induced apoptosis (Grandgirard et al., 1998; Murphy et al., 2001).

The aim of this study was to determine whether expression of anti-apoptotic Bcl-2 directly from SFV-based replicon vectors in BHK-21 cells could be used to prolong co-expression of marker proteins from a bicistronic SFV replicon. Using the SFV1 vector system (Liljestrom and Garoff, 1991), the *bcl-2* gene was placed either under the control of a duplicated SFV subgenomic promoter or an internal ribosome entry site (IRES). It is possible that expression of Bcl-2 from the subgenomic promoter occurs too late to prevent cell death. Expression from an IRES element within the genomic RNA should be more rapid. We tested two different IRES elements, the Encephalomyocarditis virus IRES (EMCV-IRES) and the crucifer-infecting tobamovirus IRES (CR-IRES). The latter is a 148-nt element, which precedes the CR coat protein gene and displays IRES activity across all kingdoms (Dorokhov et al., 2002). Using this novel approach we demonstrate that early Bcl-2 expression does not protect SFV-infected BHK-21 cells from alphavirus-induced translational shutdown or cell death. Moreover, our results indicate that SFV-induced cell death in BHK-21 cells does not involve the release of cytochrome *c* from mitochondria, and most likely does not occur by the apoptotic intrinsic pathway.

2. Materials and methods

2.1. Plasmid construction

The BamHI-XmaI multicloning site of the pSFV1 replicon (Liljestrom and Garoff, 1991) was replaced with a BamHI, ApaI, ClaI, AvrII, NruI, NsiI and XmaI multicloning site; the resulting construct was designated as pSFV-PL. The spliced sequences encoding the mouse Bcl-2 alpha protein (locus AAA37282), the EMCV-IRES (pIRES2-EGFP; BD Clontech) and the 148 bp CR-IRES (Ivanov et al., 1997) were amplified by PCR, cloned and verified by sequence analysis. Each IRES was fused to the Bcl-2 coding sequence and cloned into NsiI-XmaI digested pSFV-PL vector; obtained constructs were designated as pSFV-EMCV-bcl2 and pSFV-CR-bcl2. To create constructs expressing Bcl-2 protein from the duplicated subgenomic promoter, the IRES from pSFV-EMCV-bcl2 was replaced by an oligonucleotide duplex representing the minimal SFV subgenomic

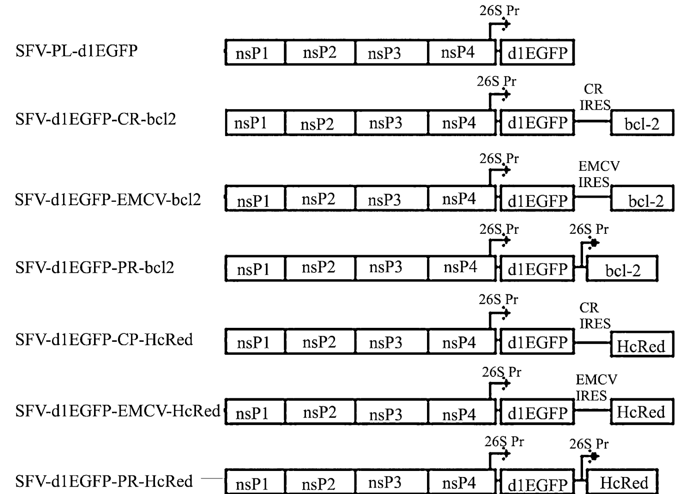

Fig. 1. Schematic presentation of replicon vectors.

promoter (Hertz and Huang, 1992); the resulting construct was designated pSFV-PR-bcl2. The d1EGFP reporter gene (BD Clontech) was amplified by PCR, sequenced and cloned into pSFV-PL, pSFV-EMCV-bcl2, pSFV-CR-bcl2 and pSFV-PR-bcl2 vectors treated with ClaI-NsiI. Resulting constructs were designated as pSFV-PL-d1EGFP, pSFV-d1EGFP-EMCV-bcl2, pSFV-d1EGFP-CR-bcl2 and pSFV-d1EGFP-PR-bcl2, respectively (Fig. 1). Sequences and primers are available upon request.

To construct SFV replicons expressing mutated chromoprotein HcRed (from the reef coral *Heteractis crispa*), *HcRed* was PCR amplified (from pHcRed1-N1; BD Clontech), cloned and sequenced. The sequence encoding Bcl-2 from pSFV-d1EGFP-EMCV-bcl2, pSFV-d1EGFP-CR-bcl2 and pSFV-d1EGFP-PR-bcl2 was replaced with *HcRed* to give constructs pSFV-d1EGFP-EMCV-HcRed, pSFV-d1EGFP-CR-HcRed and pSFV-d1EGFP-PR-HcRed (Fig. 1).

To obtain constructs used for viability analysis under puromycin selection, the sequence encoding d1EGFP from pSFV-PL-d1EGFP, pSFV-d1EGFP-EMCV-bcl2, pSFV-d1EGFP-CR-bcl2 and pSFV-d1EGFP-PR-bcl2 was replaced by that of puromycin acetyltransferase (*Pac*), and constructs were designated pSFV-PL-Pac, pSFV-Pac-EMCV-bcl2, pSFV-Pac-CR-bcl2 and pSFV-Pac-PR-bcl2.

To generate infectious RNA, constructs were linearised by SpeI digestion and in vitro transcription was carried out as previously described (Karlsson and Liljestrom, 2003).

2.2. Cells and viruses

BHK-21 cells were grown in Glasgow's Minimal Essential Medium containing 5% foetal calf serum, 0.3% tryptose phosphate broth, 0.1 U/ml penicillin and 0.1 μg/ml streptomycin. AT3-neo and AT3-bcl2 cells were grown in Roswell Park Memorial Institute-1640 medium containing 10% foetal calf serum, 0.1 U/ml penicillin and 0.1 μg/ml streptomycin. All cells were grown at 37 °C in a 5% CO_2 atmosphere. SFV4 was derived from the infectious cDNA clone pSP6-SFV4 (Liljestrom et al., 1991).

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