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Alphavirus vectors as tools in neuroscience and gene therapy

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ABSTRACT

Alphavirus-based vectors have been engineered for *in vitro* and *in vivo* expression of heterelogous genes. The rapid and easy generation of replication-deficient recombinant particles and the broad range of host cell infection have made alphaviruses attractive vehicles for applications in neuroscience and gene therapy. Efficient delivery to primary neurons and hippocampal slices has allowed localization studies of gene expression and electrophysiological recordings of ion channels. Alphavirus vectors have also been applied for *in vivo* delivery to rodent brain. Due to the strong local transient expression provided by alphavirus vectors a number of immunization and gene therapy approaches have demonstrated both therapeutic and prophylactic efficacy in various animal models.

1. Introduction

Alphaviruses are enveloped viruses carrying a single stranded RNA genome of positive polarity belonging to the Togaviridae genus (Strauss and Strauss, 1994). The life cycle of alphaviruses consists of targeting a broad range of host animal cells through common surface receptors such as heparin and laminin, followed by fusion to the cell membrane and cell entry (Fig. 1). The genomic RNA is subjected to replication in the cytoplasm and subgenomic RNA serves as template for direct expression of the viral capsid and membrane proteins. Next full-length RNA molecules are incorporated into capsid protein resulting in nucleocapsid, which are transported to the cell surface. Simultaneously, the membrane proteins travel through Golgi and endoplasmatic reticulum before embedding nucleocapids and resulting in budding of mature viral particles.

Alphaviruses generally propagate in mosquitoes, but as they also can infect domestic animals and humans they have been associated with pathogenicity. For instance, Venezuelan equine encephalitis (VEE) virus has been linked to causing flu-like symptoms in humans and infection of the central nervous system (CNS) in horses (Sudia et al., 1975). Similarly, Semliki Forest virus (SFV) and Sindbis virus (SIN) have been associated with flu-like epidemics in Africa (Mathiot et al., 1990; Niklasson, 1988). More recently, Chikungunya virus (CHIK) caused an epidemic with painful rheumatic symptoms on the island of Reunion (Jansen, 2013), where the disease spread by the *Aedes albopictus* mosquito infected one third of the population of 800,000 (Renault et al., 2007). Likewise, millions of people were affected by a CHIK epidemic in northern India in 2006–2010 (Singh et al., 2012). Despite these biosafety concerns a number of alphavirus vectors have been engineered for gene expression both *in vitro* and *in vivo*. Generally, for this approach avirulent or attenuated strains of SFV (Liljestrom and Garoff, 1991), SIN (Xiong et al., 1989) and VEE (Davis et al., 1989) have been employed.

Alphavirus-based expression has generated high-level expression of different types of recombinant proteins in mammalian host cells (Liljestrom and Garoff, 1991; Lundstrom, 1997). Particularly, expression of integral membrane proteins from SFV vectors has generated large quantities of material for drug screening and structural biology (Lundstrom, 2003; Hassaine et al., 2006). Moreover, due to the broad host range, alphaviruses have proven efficient for transduction of primary neuronal cells in culture (de Hoop et al., 1994). Likewise, both SFV and SIN vectors have been used for highly efficient gene delivery to hippocampal slice cultures (Ehrengruber et al., 1999). Alphavirus vectors have also been applied for *in vivo* delivery to rodent brain (Altman-Hamamdzic et al., 1997; Lundstrom et al., 1999), which resulted in transient local reporter gene expression with no strong effect on motor function and general health of injected animals. Furthermore, alphavirus vectors have been subjected to studies on tumor regres-

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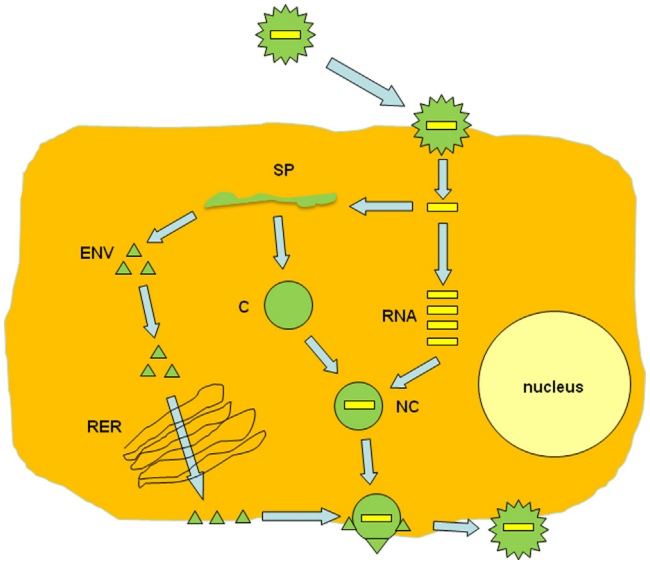

Fig. 1. Life-cycle of alphaviruses. Alphavirus particles infect host mammalian and insect cells, where genomic single-stranded RNA is released in the cytoplasm for immediate RNA replication. Full-length RNA is packaged into nucleocapsids. Simultaneously, the structural proteins are synthesized from subgenomic RNA molecules. The alphavirus envelope proteins are transported through the endoplasmatic reticulum and Golgi to the plasma membrane, where mature alphavirus particles are released by budding. C: capsid; ENV: envelope proteins; NC: nucleocapsid; RER: rough endoplasmic reticulum; SP: structural proteins.

sion after intratumoral and systemic administration of alphavirus particles to appropriate mouse tumor models (Lundstrom, 2009) and even for clinical trials on melanoma and kidney carcinoma patients (Ren et al., 2003).

2. Engineering of alphavirus vector systems

Different types of alphavirus vectors have been engineered to suit different needs. The most common strategy has been to generate replication-deficient alphavirus particles (Fig. 2A). This approach includes co-transfection of mammalian host cells with *in vitro* transcribed RNA from an expression vector carrying the alphavirus non-structural genes and the gene of interest and a helper vector including the structural genes. The presence of a packaging signal only in the expression vector sequence results in packaging preferentially RNA from this vector into nucleocapsid and generation of replication-deficient recombinant particles. However, these particles are capable of one round of host cell infection and can therefore provide high level of expression of the gene of interest. Alternatively, *in vitro* transcribed RNA from only the expression vector can be transfected, which results in no generation of recombinant particles, but transient expression of the gene of interest. The second type of vector system consists of generating replication-proficient alphavirus particles (Fig. 2B). This approach involves transfection of *in vitro* transcribed full-length RNA including an additional subgenomic promoter upstream of the gene of interest, which results in propagation of recombinant particles capable of recombinant protein expression and simultaneous generation of new viral progeny. The third option is a layered DNA/RNA expression vector (Fig. 2C), where the SP6 RNA polymerase promoter has been replaced by a CMV promoter to allow direct transfection of plasmid DNA for transient recombinant protein production. In this context, no viral particles are generated, but the expression success relies on the efficiency of transfection methods available for the host cell of interest.

In addition to standard vectors engineered for each expression system, plenty of effort has been invested in the development of improved vectors and expression systems. For instance, a number of point mutations in the non-structural genes of SFV (Lundstrom et al., 2001, 2003; Perri et al., 2000) and SIN (Perri et al., 2000; Dryga et al., 1997; Agapov et al., 1998) expression vectors has rendered reduced host cell cytotoxicity and enhanced recombinant protein expression levels. In this context, it was shown that a single point mutation in the SIN nsP2 gene caused persistent infection of transduced host cells (Dryga et al., 1997). Additionally, application of engineered expression vectors based on the non-structural genes from the avirulent SFV A7(74) strain resulted in reduced cytotoxicity in mammalian cell lines and primary neurons in comparison to the conventional SFV vector (Ehrengruber et al., 2003). Moreover, the SFV A7(74)-based vector showed a clearly temperature-dependent expression profile. Furthermore, enhanced expression in the range of 5–10 fold has been achieved from SFV vectors containing the translation enhancement signal from the capsid protein (Sjöberg et al., 1994; Rodriguez-Madoz et al., 2005). Introduction of the FMDV 2A protease sequence in the vector allowed the appropriate cleavage of the enhancer sequence from the final gene product. In another approach, introduction of point mutations in the capsid protein (Ser180/Gly183) produced larger viral particles of 205 nm in comparison to SIN wild type particles of 70 nm (Nanda et al., 2009). These particles could accommodate 18 kb RNA and theoretically up to 32 kb RNA should be able to be packaged into the capsid without any significant titer reduction.

Much attention has also been paid to development of improved helper vectors. To reduce the frequency of recombination between SFV helper and expression vector RNA, point mutations introduced in the p62 (precursor of E2 and E3) sequence resulted in production of conditionally infectious particles (Berglund et al., 1993). Moreover, engineering of split helper vectors with the capsid and envelope protein genes placed on separate vectors has provided additional safety as it prevents the generation of replication-competent particles (Smerdou and Liljestrom, 1999).

Related to recombinant virus particles and RNA efforts have been taken to improve and target delivery as well as prolong the duration of expression. For instance, replication-proficient vectors have been engineered for improved delivery and spread of gene expression *in vivo* (Heikkilä et al., 2010). Other approaches have included production of virus-like particles (VLPs) and encapsulation of viral particles. For instance, SIN and Ross River virus (RVV) nucleocapsids have been produced in a T7-based *Escherichia coli* expression system (Tellinghuisen et al., 1999). It was demonstrated that oligomerization of capsid protein occurred only in the presence of single-stranded RNA, but not double-stranded nucleic acid. SIN particles have been further applied for delivery of short sequences of single-stranded DNA and RNA, small fluorescent-labeled oligonucleotides and gold particles (Cheng et al., 2012; Mukhopadhyay et al., 2002). Electrostatic interaction has been demonstrated to driving VLP formation for packaging of different cargo although the total negative charge remained the same. However, negative charged L-Glu molecules could not be successfully packaged into VLPs. In other applications lipid-RNA complexes have been engineered for delivery of VEE RNA in vaccine development (Geall et al., 2012). Additionally, liposome formulations have been generated for SFV particles, which have demonstrated enhanced reporter gene expression in mice implanted with human LNCaP prostate tumor xenografts (Lundstrom, 2005). Furthermore, encapsulated SFV particles expressing interleukin-12 (IL-12) showed transient 5–10 fold increase in IL-12 serum levels after systemic administration in kidney carcinoma and melanoma patients in a phase I clinical trial (Ren et al., 2003).