Virology 420 (2011) 146–155

Contents lists available at SciVerse ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Use of Cre/loxP recombination to swap cell binding motifs on the adenoviral capsid protein IX

Kathy L. Poulin [a,b], Grace Tong [a], Olga Vorobyova [a], Madeline Pool [a,b,c], Rashmi Kothary [a,b,c,d], Robin J. Parks [a,b,d,e,*]

[a] Regenerative Medicine Program, Ottawa Hospital Research Institute, Ottawa, Ontario, Canada
[b] Centre for Neuromuscular Disease, University of Ottawa, Ottawa, Ontario, Canada
[c] Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, Ontario, Canada
[d] Department of Medicine, University of Ottawa, Ottawa, Ontario, Canada
[e] Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, Ontario, Canada

ARTICLE INFO

Article history:
Received 7 July 2011
Accepted 2 September 2011
Available online 2 October 2011

Keywords:
Adenovirus
Protein IX
Gene therapy
Cre recombinase
Infection
Virus retargeting

ABSTRACT

We used Cre/loxP recombination to swap targeting ligands present on the adenoviral capsid protein IX (pIX). A loxP-flanked sequence encoding poly-lysine (pK—binds heparan sulfate proteoglycans) was engineered onto the 3′-terminus of pIX, and the resulting fusion protein allowed for routine virus propagation. Growth of this virus on Cre-expressing cells removed the pK coding sequence, generating virus that could only infect through alternative ligands, such as a tyrosine kinase receptor A (TrkA)-binding motif engineered into the capsid fibre protein for enhanced infection of neuronal cells. We used a similar approach to swap the pK motif on pIX for a sequence encoding a single-domain antibody directed towards CD66c for targeted infection of cancer cells; Cre-mediated removal of the pK-coding sequence simultaneously placed the single-domain antibody coding sequence in frame with pIX. Thus, we have developed a simple method to propagate virus lacking native viral tropism but containing cell-specific binding ligands.

Introduction

Adenovirus (Ad) vectors are a popular tool for foreign gene delivery to mammalian cells, in part due to their ability to infect many different cell types and tissues (Amalfitano and Parks, 2002). There are over 50 different Ad serotypes, divided into 6 subgroups (A–F), which vary in the cell surface receptor they bind and the cell type which they preferentially infect (Havenga et al., 2002). The best characterized and most commonly used Ads are based on serotypes 2 and 5, which are very closely related genetically and biologically (Berk, 2007). Infection of Ad5 begins by binding of the capsid fibre protein to the Coxsackie-Adenovirus Receptor (CAR) on the cell surface (Bergelson et al., 1997; Tomko et al., 1997), and internalization is triggered by a secondary interaction between penton protein and $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins (Wickham et al., 1993). Ablation of CAR and/or integrin binding can dramatically reduce virus infection *in vitro* and alters tissue distribution of the vector *in vivo* (Einfeld et al., 2001). However, in mice, Ad5 has a very high efficiency of uptake by hepatocytes in the liver, which occurs through a unique mechanism: the virus hexon capsid protein binds to blood factor X, which then interacts with heparan sulfate on the surface of hepatocytes (Kalyuzhniy et al., 2008; Waddington et al., 2008). Importantly, swapping the hypervariable regions of the Ad5 hexon with those of Ad48, which does not interact with factor X, reduces hepatocyte uptake 600-fold, although much of the virus is still trapped extracellularly in the fenestrations in the liver, and is subsequently degraded (Kalyuzhniy et al., 2008). These studies suggest that effective detargeting of the vector can be achieved and, when combined with methods to re-target virus infection to cell-specific receptors, should lead to vectors with enhanced therapeutic potential and safety.

A number of Ad capsid proteins can be genetically modified to display novel ligands to allow Ad to bind specific cell types or improve transduction of CAR-deficient cells. These proteins include fibre, penton, hexon, and protein IX (pIX), and modification of each has met with varying success (Glasgow et al., 2006). pIX is a minor component of the Ad virion that associates with the hexons that make up the facets of the icosahedron (Boulanger et al., 1979). Although involved in stabilizing the virion, pIX is not essential for virion formation; however, capsids that are deficient of pIX are heat labile (Colby and Shenk, 1981). In addition to its role as a structural protein, pIX has been implicated as a transcriptional activator and may be involved in virus induced nuclear reorganization (Lutz et al., 1997; Rosa-Calatrava et al., 2001, 2003); however, these functions are not essential for virus replication, since viruses deleted of pIX grow to near wildtype levels (Sargent et al., 2004).

* Corresponding author at: Room 4A115, 501 Smyth Road, Regenerative Medicine Program, Ottawa Hospital Research Institute, Ottawa, Ontario, Canada K1H 8L6. Fax: +1 613 737 8803.
E-mail address: rparks@ohri.ca (R.J. Parks).

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doi:10.1016/j.virol.2011.09.004

pIX is also useful for display of large polypeptides on the surface of the Ad capsid. Auto-fluorescent proteins were fused to pIX of human, canine, and bovine viruses, and infection of the resulting virus could be tracked *in vitro* and *in vivo* (Le et al., 2004, 2005; Meulenbroek et al., 2004; Zakhartchouk et al., 2004). Other additions to pIX have included luciferase (Matthews et al., 2006), the herpes simplex virus thymidine kinase (HSV-TK) (Li et al., 2005), or complement-inhibiting polypeptides (Seregin et al., 2010a, 2010b). pIX has also served as a site for addition of a biotin acceptor peptide (BAP) for metabolic biotinylation of the Ad capsid, allowing for the subsequent avidin-mediated purification of the virus (Campos et al., 2004), or addition of avidin-linked targeting ligands (Campos and Barry, 2006). Indeed, several studies showed that pIX can be used as a cell attachment protein, provided suitable cell binding ligands are attached to the protein (Campos and Barry, 2006; de Vrij et al., 2008; Dmitriev et al., 2002; Poulin et al., 2010; Vellinga et al., 2004). One curious finding from these studies was that the nature of the targeting ligand can have a significant influence on the efficiency of retargeting using pIX. Although using transferrin as a targeting ligand could effectively enhance virus infection of C2C12 muscle cells, using an antibody directed towards CD71, the transferrin receptor, did not (Campos et al., 2004). In contrast, both transferrin and anti-CD71 functioned as efficient targeting ligands when conjugated to Ad fibre. Similar observations were made by Corjon et al. (2008) using a different combined genetic- and chemical-labeling system for pIX. Barry and coworkers postulated that, unlike Ad fibre, pIX (and its targeting ligand) may not dissociate from the capsid in the early endosome, resulting in the Ad particle remaining bound to the receptor in the endosome; thus, the virus is "trapped" in the endosome which prevents the viral DNA from reaching the nucleus (Campos et al., 2004). Thus, although pIX can be used to display targeting ligands on the surface of the Ad capsid allowing for efficient cell binding, the nature of the ligand may significantly influence whether it can successfully complete subsequent steps of infection.

Ad vectors that are detargeted (i.e. contain mutations within specific capsid proteins that prevent the virus from attaching to its normal receptors), cannot be easily propagated. Detargeting prevents the virus from utilizing the normal Ad infection route, and the cell surface epitope to which the virus may be retargeted is not necessarily expressed on the cells used for virus propagation (e.g. 293 cells; Graham et al., 1977). We thus investigated whether pIX could be employed as a transient cell attachment protein for propagation of vector with CAR-binding ablated/retargeted fibre proteins. In this scenario, a common, low-affinity cell binding motif (such as polylysine (pK) which binds heparan sulfate proteoglycans, HSPG) could be transiently displayed on pIX in order to allow for efficient propagation of vector; however, once pK is removed from the virus capsid, infection could only proceed from a second, cell type-specific targeting ligand present on a different Ad capsid protein, such as fibre. Development of this system was the goal of this study.

Results

pIX for transient display of cell attachment peptides

We constructed a virus that contained a floxed pK motif on the C-terminus of pIX, and tested its ability to act as a transient cell surface binding protein. pK binds HSPG on the surface of most cell types, and has been used successfully to enhance Ad transduction on a variety of cell types (Bramson et al., 2004; Dmitriev et al., 2002; Wickham et al., 1996). Several studies have shown that the Cre recombinase can act upon the viral DNA to excise elements contained between two tandemly arrayed loxP recognition sites (Anton and Graham, 1995; Parks et al., 1996). AdRP2428 encodes a pIX gene under endogenous regulation but containing a floxed pK-encoding motif on its 3′-terminus, and also encodes a murine secreted alkaline phosphatase (mSEAP) reporter gene expression cassette replacing the early region 1 (E1)-deletion (Figs. 1A and 2A). We initially examined the efficiency of Cre-mediated excision of the DNA element encoding the pK motif by analyzing DNA from infected 293 versus Cre-expressing 116 cells. In AdRP2428-infected 293 cells, we observed an MfeI/NotI DNA band of ~250 bp for pIX, which is as predicted based on the sequence of the virus (Fig. 1B). On 116 cells, Cre-mediated excision of the region encoding the pK motif from this virus should result in the disappearance of the 250 bp fragment and the formation of a unique 199 bp fragment; however, this region of the agarose gel was relatively indistinct. To better visualize the viral DNA bands, we performed Southern blot hybridization. Infection of 116 cells with AdRP2428 did result in the formation of the expected 199 bp fragment, but we also observed a significant amount of the unexcised 250 bp band (Fig. 1C). This data suggests that Cre-mediated excision of the pK motif in AdRP2428 was incomplete.

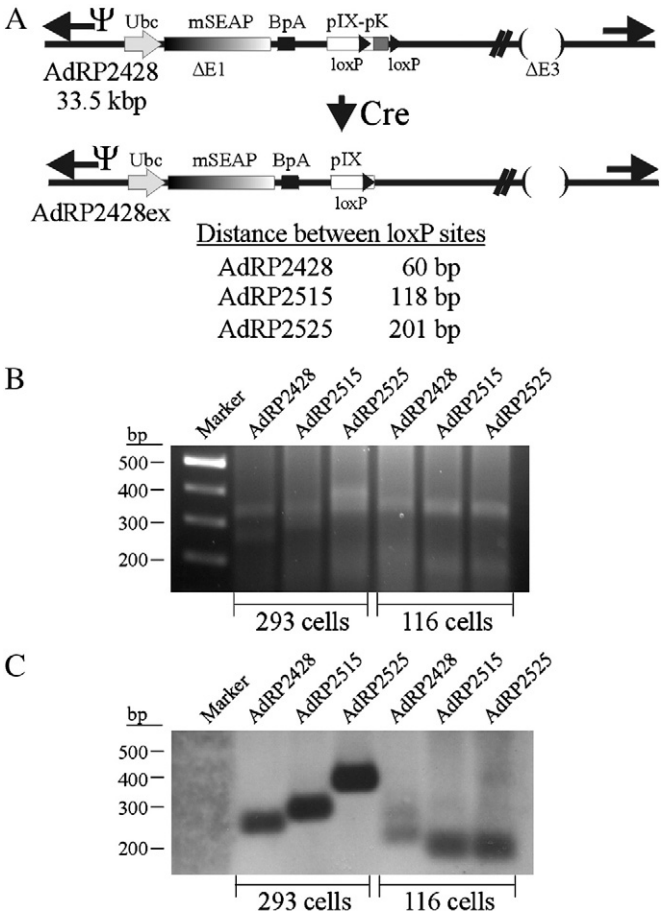

Fig. 1. The efficiency of Cre-mediated excision of a poly-lysine coding sequence from the 3′-terminus of pIX is dependent on the distance between the loxP sites. Panel A: AdRP2428 is deleted of early region 1 (E1) and E3 and contains the murine secreted alkaline phosphatase gene (mSEAP) under regulation by the human ubiquitin C promoter (Ubc) and bovine growth hormone polyadenylation sequence (BpA). AdRP2428 also encodes a pIX gene containing a C-terminal poly-lysine (pK) coding sequence flanked by loxP sites. Growth of AdRP2428 on Cre-expressing cells results in removal of the pK coding sequence. AdRP2515 and AdRP2525 are similar in structure to AdRP2428, but contain loxP sites separated by 118 and 201 bp, respectively (note: these distances are reported at the distance from the 5′ terminus of the first loxP site to the 5′ terminus of the second loxP site). The FLAG epitope tag is located immediately after the native pIX sequence, but before the first loxP site. Panel B and C: 293 or 116 cells were infected at an MOI of 3 with AdRP2428, -2515 or -2525 and, 72 h later, total DNA was isolated, digested with MfeI/NotI, and the resulting fragments separated by agarose gel electrophoresis. The resolved DNA was visualized by ethidium bromide staining of the gel (Panel B) or by Southern blot analysis using a probe specific for the pIX gene (Panel C).