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**Targeting Adenoviral Vectors to Cells Expressing EGFRvIII Using a Single Chain Antibody Fused to
pIX**

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Targeting Adenoviral Vectors to Cells Expressing EGFRvIII Using a Single Chain Antibody
Fused to pIX

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Submitted in partial fulfilment of the requirements for the degree of Masters of Science
Faculty of Graduate Studies

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Abstract

Current adenovirus retargeting strategies are unable to target the virus to specific cell types. In this study, we investigated whether fusion of a single-chain antibody, MR1, to the capsid protein IX (pIX) could target the virus to cancer cells expressing EGFRvIII. We show that addition of an endoplasmic reticulum signal peptide to pIX-MR1 significantly increased the ability of the fusion protein to bind its ligand. Use of the human CMV promoter rather than the native pIX promoter permitted a greater accumulation of the protein within the cell. Finally, addition of the HIV-1 Tat NLS caused pIX-MR1 to relocate to the nucleus, the site of capsid assembly. Taken together, these results provide a foundation to design Ad vectors targeted to specific cells through the use of single-chain antibodies. Ultimately, the development of a tropism modified Ad vector would result in a tailored treatment for a particular acquired genetic disease.

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List of Abbreviations

293 cells – human embryonic kidney cells
293 N3S cells – 293 suspension cells passaged through nude mice
Ad – adenovirus
ALV - subgroup A avian leukosis virus
Asp – asparagine
BGH – bovine growth hormone
C4BP – complement factor 4 binding protein
CAR – coxsackie-adenovirus receptor
CD20 – non-glycosylated phosphoprotein expressed on the surface of all mature B-cells
CD3-ε – component of the T-cell receptor
cDNA – complementary deoxyribonucleic acid
CDR – complementary determining region
CEA – carcinoembryonic antigen
CHO cells – Chinese hamster ovary cells
CMV – cytomegalovirus
CsCl – cesium chloride
dCMP – deoxycytosine monophosphate
DGK-ζ - diacylglycerol kinase zeta
DNA – deoxyribonucleic acid
DNP – dinitro phenol
dsRed – Discosoma coral red fluorescent protein
E1 – adenovirus early region 1
E2 – adenovirus early region 2
E3 – adenovirus early region 3
E4 – adenovirus early region 4
E4 ORF3 – early region 4 open reading frame 3
ECL – enhanced chemiluminescence
EDTA - ethylenediaminetetraacetic acid
EGFR – epidermal growth factor receptor
EGFRvIII – epidermal growth factor receptor version III
ENV – ecotropic envelope glycoprotein
ER – endoplasmic reticulum
ERBB2 – oncogene of the EGFR family named for its similarity to ErbB (avian erythroblastosis oncogene B)
FVII – factor VII
FIX – factor IX
FX – factor X
FBS – fetal bovine serum
FITC - fluorescein isothiocyanate
FLAG – epitope tag DYKDDDDK
GFP – green fluorescent protein
GON – group-of-nine
GTP – guanosine triphosphate
hdAd – helper-dependent adenovirus
HIV-1 – human immunodeficiency virus type 1

HS – horse serum
HSV-1 – herpes simplex virus type 1
HVR4 – hypervariable region 4
IFN – interferon
ITR – inverted terminal repeat
MEM – minimum essential media
Met – methionine
MLP – major late promoter
MLV – Moloney murine leukemia virus
M.O.I. – multiplicity of infection
MR1 – mutant receptor 1
mRNA – messenger ribonucleic acid
mSEAP – murine secreted alkaline phosphatase
MYC – myelocytomatosis epitope tag
NaCl – sodium chloride
NIH 3T3 cells – murine embryonic fibroblast cells
ND10 – nuclear domain 10 protein
NLS – nuclear localization sequence
NPC – nuclear pore complex
p21 – cyclin-dependent kinase inhibitor 1A
p130 CAS – protein 130 Crk associated substrate protein
PI3 – phosphatidylinositol-3
pIX – protein IX
pA – polyadenylation signal
PBS – phosphate buffered saline
PCR – polymerase chain reaction
PDZ – post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (DlgA), and zo-1 protein
PGK – phosphoglycerate kinase promoter
pK – polylysine
PML – promyelocytic leukemia protein
pTP – pre-terminal protein
PVDF – polyvinylidene difluoride
ras – rat sarcoma
RecA – recombination protein A
RGD – arginine-glycine-aspartic acid motif
RIPA – radioimmunoprecipitation buffer
scFv – single-chain variable fragment; single-chain antibody
SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis
Smac/DIABLO – proapoptotic factor
SP1 – signal peptide 1
SP2 – signal peptide 2
SP3 – signal peptide 3
SP4 – signal peptide 4
SRP – signal recognition particle
SU – surface component of MLV envelope glycoprotein
SV40 – simian virus 40

TBST – Tris buffered saline with Tween
TE – Tris buffered EDTA
TK – thymidine kinase
Tris – trishydroxymethylaminomethane buffer
TVA – gene locus name of subgroup A avian leukosis virus receptor
U87MG cells – human glioblastoma cells
Ubc – ubiquitin c
US11 – unique short region 11 of cytomegalovirus
V_L – variable region light chain
V_H – variable region heavy chain

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Chapter 1 - Introduction

1.1 General Introduction

Gene therapy is an approach to treat human disease through the introduction of therapeutic genetic material into an affected individual (Verma and Somia, 1997). Gene therapy can be used to alter or improve the function of a mutated gene through the introduction of a properly functioning copy of the gene, completely replace or repair a mutated gene, or introduce a gene intended to cause a diseased cell or tissue to undergo cell death or be targeted by the host immune system. The transfer of DNA can be achieved through the *in vivo* administration of viral vectors, leading to the infection of the tissue of interest. However, the *in vivo* administration of a viral vector results in the infection of numerous cell types since the viral tropism is frequently not limited to the tissue of interest. Infection of non-target tissues, such as immune cells (eg. macrophage or antigen presenting cells) can stimulate anti-vector or transgene protein immune responses. The primary function of the host immune response to a virus is to rapidly detect, limit spread and ultimately eradicate the infection (Liu and Muruve, 2003). Thus, the immune response to viral vectors can compromise the effectiveness of gene therapy.

In recent years, there has been intensive research conducted to determine the viability of using several viruses as vectors for gene therapy, including retroviruses, lentiviruses, adenoviruses, adeno-associated viruses and to a lesser extent herpes viruses and poxviruses. In order for viruses to be used as vectors for gene therapy, they must elicit nominal harm to a host, induce a minimal inflammatory response, and specifically infect a desired cell type. Current vector systems, however, have not reached a point where *in vivo* administration results in tissue specific infection and delivery of a therapeutic gene. Therefore it is critical to

explore means of specifically targeting viral vectors, thereby minimizing the inflammatory response and maximizing therapeutic potential.

As a means of facilitating the delivery of a therapeutic gene, adenovirus possesses numerous features that make them excellent vectors for gene therapy, including: a large cloning capacity, ease of genetic manipulation, ability to grow to high titer, and the capability of infecting numerous human cell types. However, it is the ability of adenovirus to infect numerous cell types that is also a disadvantage for targeted gene therapy. Therefore there has been significant interest in investigating various methods of retargeting Ad through capsid modifications, to attenuate the inflammatory response and increase specificity. In this thesis, I explore a novel method to specifically target adenovirus vectors to cancer cells.

1.2 Adenovirus Background

1.21 Classification

Adenoviruses (Ad) are non-enveloped DNA viruses with a linear, double-stranded genome encapsidated in an icosahedral protein shell measuring 70 to 100 nm in diameter. Ad are able to infect an array of cell types throughout the human body including both dividing and non-dividing cells, and normally cause only mild, self limiting illness (Horwitz, 1996). To date, over 50 human Ad serotypes have been identified and are divided into six subgroups (A to F) based on hemagglutination properties, oncogenicity in rodents, DNA homology, and genomic organization (Zhang and Bergelson, 2005). There is also a correlation between subgroup and tissue tropism – viruses in group B1, C and E cause respiratory infections; group B2 infects the urinary tract and kidneys; group F viruses lead to gastroenteritis; and some of the group D viruses have been associated with epidemic keratoconjunctivitis (Zhang and Bergelson, 2005).

The icosahedral structure of the Ad capsid was elucidated through a combination of electron microscopy and X-ray crystallography techniques (Horne et al., 1959; Valentine and Pereira, 1965). The major structural proteins that make up the capsid are named hexon, penton and fiber (Ginsberg et al., 1966). The facets of the icosahedron are composed primarily of the major capsid protein hexon. Projecting from each of the 12 fivefold vertices of the capsid is the fiber protein, which is bound at its proximal end to the capsid via the pentameric structure, the penton base (Valentine and Pereira, 1965). Other, so-called, minor capsid proteins include proteins IIIa, VI, VIII and IX, are thought to potentially stabilize the viral particle. Protein IIIa participates in viral assembly and maturation (Chroboczek et al., 1986) and is located on the inner capsid surface below the penton base (Saban et al., 2006). Protein VI is located on the inside of the capsid associated with the peripentonal hexons and the Ad DNA (Stewart et al., 1993). Protein VI regulates the import of hexon into the nucleus during Ad assembly (Wodrich et al., 2003), may participate in endosomal disruption (Wiethoff et al., 2005) and, when it is cleaved, the C-terminal portion serves as a cofactor for the Ad cysteine protease (Mangel et al., 1993; Webster et al., 1993). Protein VIII is located on the inner capsid surface associated with hexons and may serve to stabilize the Ad capsid (Fabry et al., 2005; Saban et al., 2006). Protein IX (pIX) serves as capsid cement, stabilizing the group of nine hexons located on each of the facets (Furcinitti et al., 1989). The N-terminus of pIX is buried below the capsid surface between hexon proteins, while the C-terminus of pIX is located along the hexon facet edge on the surface of the capsid (Marsh et al., 2006; Saban et al., 2006). Together, all of the Ad proteins form a very stable vehicle for the delivery of the Ad genome to an infected cell.

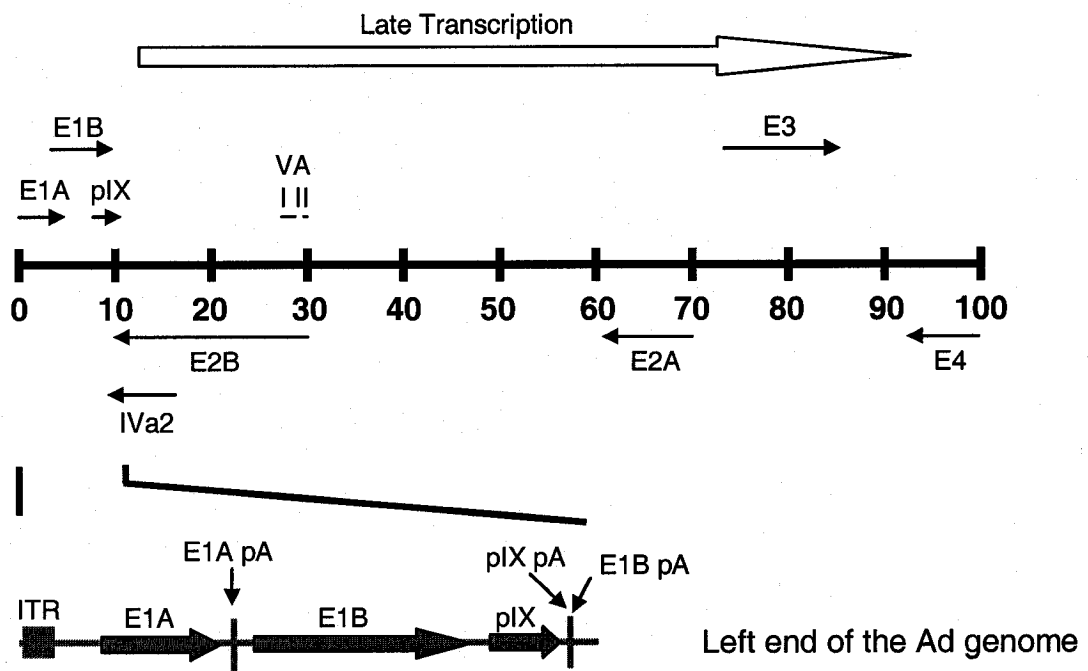
1.22 Genome

The Ad genome is approximately 36 kbp of linear double stranded DNA, bordered by inverted terminal repeats that serve as the origins of replication (Shenk, 2001). Next to the inverted terminal repeat is the packaging sequence, the minimum element necessary to package Ad DNA (Hearing et al., 1987; Hearing and Shenk, 1983). The Ad genome is divided into early and late genes, based on whether the genes are transcribed before or after viral genome replication (Figure 1). The early genes (E1, E2, E3, and E4) are expressed prior to DNA replication, while the late genes (the major late transcript is cleaved into five families of late mRNAs) are expressed after DNA replication. Unlike the early and late genes, pIX and protein IVa2 are transcribed at intermediate time points. The pIX coding region is located between the E1B coding region and the E1B poly adenylation signal. Due to this arrangement, pIX is not transcribed until after E1B transcription has subsided. The Ad early genes are responsible for promoting the expression of other Ad genes (E1A, E1B, and E4); inducing entry into and progression through the cell cycle (E1A); inhibition of apoptosis (E1B, E4), and double strand DNA break repair (E4); evasion of immune recognition (E3); and replication of the viral DNA (E2). In general, the late gene products encode viral structural proteins, such as fiber, hexon and penton and assemble to form the Ad capsid. For an excellent review of Ad biology, see Shenk (2001).

1.23 Entry

The classical view of Ad entry, as defined by cell culture experiments, is a two step process, initiated by the interaction of the virus fiber protein with the cell surface coxsackie-Ad receptor (CAR) (Bergelson et al., 1997)(Figure 2A). The Ad fiber protein mediates

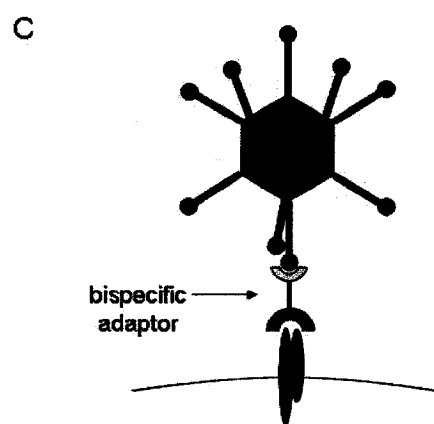
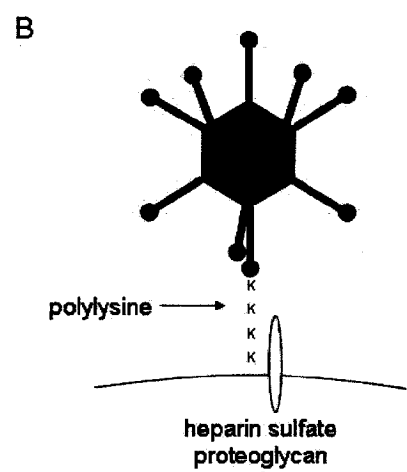
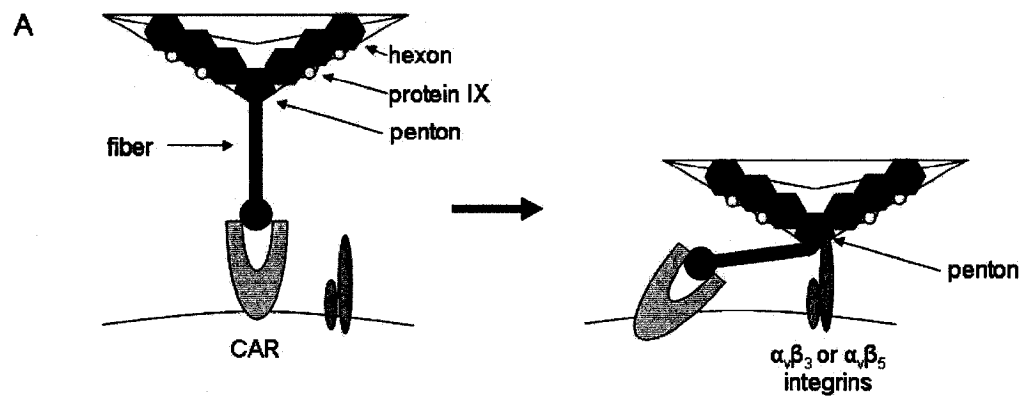
Figure1 : Simplified map of the Ad5 transcription units. Also shown is an enlargement of the left end of the Ad genome. Adapted from Sargent et al., (2004).



cellular binding, and consists of three distinct domains: the tail, the shaft, and the knob; each domain having a distinct function. The amino terminal tail anchors the fiber to the Ad capsid through association with the penton base (Weber et al., 1989). The shaft extends away from the virion surface and, in Ad5, is composed of 22 pseudorepeats of 15 amino acids in triple- β -spiral conformation (Green et al., 1983). The shaft repeats are reported to bind to cell surface heparin sulfate glycosaminoglycans, providing Ad with an additional receptor for entry (Dehecchi et al., 2001). The trimeric subunits of the carboxyl terminal knob domain are responsible for binding CAR (Henry et al., 1994; Louis et al., 1994). CAR is a 365-amino acid transmembrane protein with a short leader, a 222-amino acid extracellular domain, a membrane-spanning helical domain, and a 107-amino acid intracellular domain (Bergelson et al., 1997). Ads belonging to all subgroups, except subgroup B, utilize CAR as the primary attachment receptor for *in vitro* infection (Roelvink et al., 1998).

Following primary attachment to CAR, a secondary interaction occurs between an arginine-glycine-aspartic acid (RGD) motif present in the Ad penton base and cell surface $\alpha v \beta 3$ and $\alpha v \beta 5$ integrins (Wickham et al., 1994; Wickham et al., 1993). The engagement of integrins by penton induces intracellular signals, including the activation of PI3 kinase (Li et al., 1998b), p130CAS (Li et al., 2000) and Rho GTPases (Li et al., 1998a). These intracellular signals are important for rearranging the actin cytoskeleton and initiating virus internalization. After adsorption, virus-receptor complexes diffuse into clathrin coated pits and are internalized by receptor-mediated endocytosis – a process triggered by the penton-integrin interaction. For an excellent study of Ad disassembly following cell entry, see Greber et al. (1993).

Figure2: Mechanism of adenovirus entry and retargeting strategies. (A) Ad entry is a two step process involving the binding of the Ad fiber protein to the coxsackie adenovirus receptor (CAR) followed by a secondary interaction between an arginine-glycine-asparagine (RGD) motif present in the Ad penton protein and $\alpha\text{v}\beta 3$ or $\alpha\text{v}\beta 5$ integrins. (B) Retargeting strategies to broaden the tropism of Ad have involved the incorporation of targeting ligands, such as a polylysine (pK) motif into the fiber protein to facilitate entry through heparan sulfate proteoglycans. (C) Non-covalent modifications of Ad vectors for retargeting have employed the use of bi-specific adapters where one end binds to the Ad virion, while the other binds to the desired cell surface molecule.



Following Ad internalization, dissociation of the capsid follows and appears to require the acidification of the endosome (Greber et al., 1993). During the internalization process, the virion is sequentially disassembled beginning with the proteins at the vertices – fiber, penton and pIIIa. The pH-dependent dismantling liberates pVI from the capsid interior, promoting membrane lytic activity (Wiethoff et al., 2005). The rapidity of the movement to the cytosol suggests that the virus escapes from the early endosome prior to formation of a lysosome (Greber et al., 1993). Once it has escaped from the endosome, the Ad particle associates with microtubules via cytoplasmic dynein, and migrates towards the nucleus (Kelkar et al., 2004). Ultimately, the viral nucleocapsid, still containing the hexon proteins, docks at the nuclear pore complex (NPC) (Greber et al., 1993; Lonberg-Holm and Philipson, 1969). At the NPC the virus undergoes further uncoating, and protein IX, which had been stabilizing the hexon proteins, is lost. Finally, the viral DNA translocates into the nucleoplasm, where viral transcription and DNA replication occur (Greber et al., 1993; Whittaker et al., 2000).

1.24 DNA Replication

As the transcription of the Ad early genes progresses, the E2 gene products accumulate, allowing the virus to replicate its genome. Specific sequences within the inverted terminal repeats that border the genome serve as the origins of replication. A 5' dCMP attached to a β -hydroxyl group of a virally encoded pre-terminal protein (pTP) seryl residue serves as a primer for DNA replication (Ikeda et al., 1981). The pTP also helps to preserve the integrity of the viral terminal sequences during replication (Rekosh et al., 1977). DNA replication requires the presence of two virally encoded proteins, the DNA polymerase and single-stranded DNA binding protein, along with the cellular protein, nuclear factor II

(Nagata et al., 1983). Nuclear factor II protein acts through topoisomerase-like activity, overcoming DNA structural constraints to allow easy progression for the polymerase (Nagata et al., 1983). Ad DNA replication begins with the synthesis of a complementary DNA strand from either terminus of the genome in a continuous manner to the other end of the genome (Lechner and Kelly, 1977). Only one of the two parental DNA strands serves as a template for replication, therefore after each round of replication, the resulting DNA duplex consists of a parental and a daughter strand along with a displaced parental single-strand (Lechner and Kelly, 1977). The complementary inverted terminal repeats on the displaced single-strand are able to anneal with one another, forming a “panhandle” structure (Daniell, 1976). The formation of the panhandle restores a functional replication origin for the synthesis of a complementary strand. The DNA binding protein is thought to aid the polymerase by binding to the displaced single-stranded DNA, allowing the polymerase to travel the entire length of the viral genome following initiation (Lindenbaum et al., 1986). The end result of DNA replication is the production of multiple copies of the Ad genome that can be packaged to form progeny virus particles.

1.25 Virus Assembly, Maturation, Release

The replication of viral DNA, coupled with the production of large quantities of structural proteins, sets the stage for virus assembly. For an excellent review of Ad assembly, maturation and release, see Shenk (2001). Assembly of new Ad particles occurs in the nucleus, and is tightly connected to expression and nuclear import of partially pre-assembled viral structural proteins (Wodrich et al., 2003). Trimeric hexon capsomeres are rapidly assembled from monomers after their synthesis in the cytoplasm. Penton capsomeres and trimeric fiber assemble somewhat more slowly in the cytoplasm. Virus assembly involves

either insertion of the genome into an empty capsid (Sunquist et al., 1973) and/or assembly of the capsid around a viral nucleoprotein core (Zhang and Imperiale, 2003). The DNA-capsid recognition event is mediated by the packaging sequence, a cis-acting DNA element that is centered about 260bp from the left end of the viral chromosome. Following the assembly of the capsid, a maturation process occurs involving cleavage of several viral proteins by the viral cysteine protease (Anderson et al., 1973; Bhatti and Weber, 1978; Weber, 1995), rendering the capsid infectious. The complete viral life cycle takes between 24 to 36 hours to complete and results in the production of approximately 10^4 infectious virions per infected cell (Shenk, 2001).

1.26 Adenovirus as a Vector for Gene Therapy

Adenoviruses possess numerous features that make them excellent vectors for gene therapy applications. These include the ease of genetic manipulation, an ability to remove large portions of the genome and grow the virus to high titer, relative safety in infected patients, and the ability of the virus to transduce a wide range of cell types (Horwitz, 1996). Several classes of Ad vectors have been developed for gene therapy applications: first generation, second generation, helper-dependent and oncolytic.

Ad vectors deleted of E1 (with optional deletion of E3) are known as first-generation vectors (Yang et al., 1994). Deletion of the E1 region allows for approximately 5 kbp of genetic material to be cloned into the Ad backbone, thus allowing for the insertion of most transgenes (Bett et al., 1994). E1-deleted vectors can be propagated easily in cell lines that express the E1 gene products, such as 293 (Graham et al., 1977) and 293N3S cells (Graham, 1987). The E3 region is also dispensable for viral replication in tissue culture, and the removal of this region further increases the cloning capacity to 8 kbp (Bett et al., 1994).

Unfortunately administration of first-generation Ad vectors results in the activation of the innate and adaptive immune responses (Muruve et al., 1999; Zhang et al., 2001). The adaptive response is directed against Ad gene products expressed by first-generation vectors (Yang et al., 1994). Therefore, to reduce the virally induced immune response, the viral coding regions involved in DNA replication (E2 and E4) were removed, giving rise to second-generation Ad vectors. Deletion of E2 and E4 regions allows for insertion of expression cassettes up to 14 kbp. Various cell lines have been created to allow propagation of second generation Ad vectors (Krougliak and Graham, 1995; Lusky et al., 1998; Zhou et al., 1996). Newer generation Ad vectors, called helper-dependent Ad vectors (hdAd), are devoid of all viral proteins, and this has proven to reduce the cell-mediated immune response and improved the duration of gene expression *in vivo* (Amalfitano and Parks, 2002). hdAd vectors are deleted of all virally encoded proteins except the *cis*-acting sequences needed to replicate and package the viral DNA; theoretically, hdAd can accommodate up to 37 kb of DNA. Another application of Ad vectors include the development of viruses that preferentially replicate in and kill tumour cells, known as oncolytic Ad. Tumour specific replication can be achieved through the removal of viral functions dispensable in tumour cells, such as cell cycle regulation, or the regulation of viral genes with tumour specific promoters (Alemany et al., 2000). Oncolytic Ad vectors may also be used to specifically transfer suicide genes, such as HSV-1 thymidine kinase, that confers sensitivity to the drug ganciclovir to cancer cells, leading to cell death (Smythe et al., 1994). The Ad vectors utilized in this study are first generation vectors due to their ease of manipulation and propagation.

Although Ad vectors are able to infect numerous cell types, after intravenous application, most Ad particles are sequestered by the liver (Barr et al., 1995; Lieber et al.,

1997). The tissue distribution of intravenously administered Ad5, does not correlate with CAR expression (Fechner et al., 1999). The transduction of the liver, in mice (Vrancken Peeters et al., 1996) and non-human primates (Nunes et al., 1999), occurred as efficiently with Ads ablated for CAR binding as with unmodified vectors. Ad entry into the liver can lead to uptake by Kupffer cells - large liver macrophages located within the liver sinusoids. Kupffer cells represent the first line of defense against viruses entering the liver from the portal circulation (Lieber et al., 1997). Ad uptake into Kupffer cells is CAR-independent (Shayakhmetov et al., 2004). Recent, studies have suggested that Ad uptake into the liver may involve blood factors such as factor IX (FIX) and complement factor C4BP (Shayakhmetov et al., 2005) and vitamin-K dependent blood factors, FVII, FX and PC (Parker et al., 2006). FIX and C4BP can bind directly to fiber and facilitate Ad entry through heparin sulfate proteoglycans and/or low-density lipoprotein-receptor related protein (Shayakhmetov et al., 2005). FX is able to bind fiber in a calcium dependent mechanism, and mediates Ad transduction in a similar manner to FIX, through heparan sulfate proteoglycans (Parker et al., 2006). Therefore Ad transduction *in vivo* is a complicated process involving many factors, and is not mediated by CAR expression alone.

The sequestration of Ad in the liver might be beneficial for the treatment of liver diseases, or disorders where systemic secretion could be of benefit, but not for therapy targeted to other tissues or diseases involving other tissues. To advance the utility of Ad vectors *in vivo*, retargeting may be important to deliver the vector to the desired tissue as well as to reduce the immune response. One-quarter of all human gene therapy trials currently involve Ads, and their efficacy in the clinic would clearly be improved by the development of Ad vectors with selective tissue targeting capabilities (Hedley et al., 2006).

1.3 Retargeting Ad

Ads have a broad tropism and are able to enter numerous cell types, making them very promiscuous viral vectors. However, several cell types that are important targets for gene therapy are refractory to Ad infection. These include endothelial, smooth and adult skeletal muscle, brain tissue, primary tumours and hematopoietic cells (Bouri et al., 1999; Cantwell et al., 1996; Chillon et al., 1999; Miller et al., 1998; Wickham et al., 1996b). Therefore, to broaden the tropism of Ad to significant cellular targets and enhance the utility of Ad vectors, viral retargeting has been employed.

To increase the specificity of the Ad vectors, manipulations of Ad capsid proteins have been made to increase transduction of specific cell types. By replacing or modifying the native method of Ad entry with cell-specific ligands, vectors may be given enhanced cell specificity and activity (Ghosh and Barry, 2005). Researchers have explored several different methods to alter Ad transduction, including genetic incorporation of targeting ligands into capsid proteins, using linker molecules with bispecific activities to the viral capsid as well as the receptor, and the use of adaptor molecules, such as biotin, to non-covalently attach protein ligands. For a comprehensive review of Ad retargeting strategies, see Glasgow et al. (2006).

The majority of Ad retargeting studies have involved modification of the Ad fiber protein (Figure 2B). Since the traditional route of attachment for Ad involves interaction of the knob domain of fiber with CAR (Bergelson et al., 1997), several groups have studied the efficacy of Ad infection through the addition of targeting ligands to the knob domain. A polylysine motif (pK) was incorporated into the C-terminus of fiber in order to target the virus to heparan sulfate proteoglycans (Wickham et al., 1996a). Subsequently, a specific region of the knob domain, between the H and I loops, was shown to accommodate the

insertion of a FLAG epitope tag without compromising the trimerization and cell-binding ability of the fiber protein (Krasnykh et al., 1998). Several groups investigated the incorporation of an RGD motif, specific for α_v integrins, and pK, specific for heparan sulfate proteoglycans, into the HI loop of fiber and reported increased transduction of targeted vectors relative to native Ad (Dmitriev et al., 1998; Koizumi et al., 2003; Krasnykh et al., 2001; Mizuguchi et al., 2001; Wickham et al., 1997). Studies completed in our lab showed that the incorporation of RGD and pK motifs into the HI loop of fiber increased Ad transduction of mature muscle cells (Bramson et al., 2004). These strategies have been successful in expanding the tropism of Ad vectors, but have not addressed the issue of cell specificity; these vectors are still able to enter cell types other than the tissue of interest, thereby reducing their therapeutic potential.

Non-covalent modifications of Ad vectors for retargeting have used bi-specific adapters where one end binds to the Ad virion, while the other binds to the desired ligand (Figure 2C). Several examples include a bi-specific adapter composed of a single-chain antibody (scFv) – a single polypeptide chain that retains the binding capability of a monoclonal antibody – against fiber and an scFv directed against the epidermal growth factor receptor (EGFR) (Haisma et al., 2000) and an anti-fiber scFv and either an anti-CEA diabody or tandem scFv (Korn et al., 2004). A FLAG epitope incorporated into penton on the Ad capsid was used in conjunction with a bispecific antibody which binds to FLAG and to α_v integrins (Wickham et al., 1996b). Although these methods work effectively to alter Ad tropism, concerns have been raised regarding the degree of modification between different viral preparations, resulting in batch-to-batch variations. Thus modifying Ad using bispecific adapters may not be the most efficient retargeting method.

Another approach to retargeting Ad is to replace the Ad5 binding protein with that of other serotypes or from other viruses. The Ad5 fiber protein has been replaced with the fiber from subgroup B viruses, such as Ad3, 11 and 35 (Gall et al., 1996; Mizuguchi and Hayakawa, 2002; Shayakhmetov and Lieber, 2000; Stevenson et al., 1997; Zabner et al., 1999). Several groups have described the replacement of a majority of the fiber protein, leaving the tail domain, with the reovirus $\sigma 1$ protein, which targets the virus to cells expressing the junctional adhesion molecule 1 (Mercier et al., 2004; Schagen et al., 2006; Tsuruta et al., 2005). The replacement of the Ad5 fiber with the fiber from other serotypes and the reovirus $\sigma 1$ protein has retargeted Ad to new cellular receptors; however the receptors are expressed on numerous tissues, and therefore cannot target Ad to specific cell type of interest.

Modifications for retargeting have mainly focused on the Ad fiber protein and have been met with limited success. Some alterations to fiber have been able to increase transduction of certain tissue types, but they have yet to be cell type specific. Attempts to genetically incorporate more specific targeting ligands, such as scFv into the Ad capsid have been problematic to this point (Magnusson et al., 2002). Other Ad proteins, including protein IX may be good candidates for the incorporation of larger, more specific targeting ligands.

1.4 Protein IX

1.41 Protein IX Structure

Protein IX is a minor structural protein of the Ad virion (Furcinitti et al., 1989) and is approximately 14 kDa in size (Alestrom et al., 1980). The mRNA encoding pIX is not spliced (Alestrom et al., 1980) and is capped, poly-adenylated, and sediments at 9S (Pettersson and Mathews, 1977). E1B transcription across the pIX promoter inhibits pIX

gene expression early in infection (Vales and Darnell, 1989). Therefore, pIX is not transcribed until after E1B transcription has subsided. pIX polyadenylation is performed while the RNA is associated with the nuclear matrix (Mariman et al., 1982). The nuclear exit time of pIX-mRNA was determined to be about 4 minutes (Mariman et al., 1982).

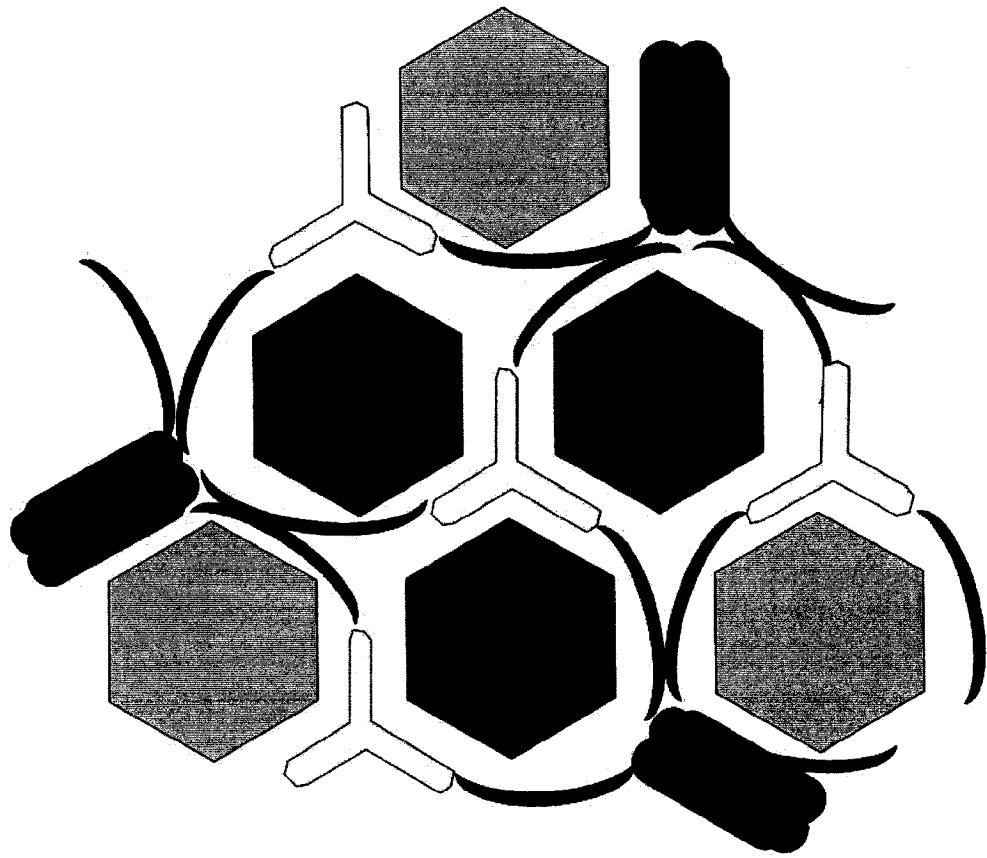
Comparison of sequences of pIX from a variety of human and animal species has identified three structural domains – a conserved amino-terminal domain, a central alanine-rich domain (found only in human Ads) and a conserved carboxy-terminal domain (Rosa-Calatrava et al., 2001). The amino-terminal region of pIX has been implicated in capsid incorporation (Rosa-Calatrava et al., 2001) and is predicted to form an α -helical bundle with the amino-termini of two other pIX molecules (Saban et al., 2006). The carboxy terminal domain has a leucine rich region (Rosa-Calatrava et al., 2001) and is predicted to form an α -helix that forms a four helix bundle with three other pIX proteins (Saban et al., 2006). One of the four α -helices in the bundle is predicted to interact with the hexon HVR4 loop (Saban et al., 2006).

pIX is a minor component of the planar group-of-nine hexons (GON) that form the central region of each facet of the icosahedral Ad capsid (Furcinitti et al., 1989). Stoichiometric measurements show that there are 12 copies of pIX per GON and 240 copies per virion (van Oostrum and Burnett, 1985). pIX is positioned in the four large cavities of the GON structure, based on scanning transmission electron microscopy, and cryogenic electron microscopy studies (Furcinitti et al., 1989; Saban et al., 2005). Using antisera specific for either the N or C-terminus of pIX along with immunogold electron microscopy, it was determined that only C-terminus specific antibodies bound to the intact virion (Akalu et al., 1999), suggesting that the C-terminal domain of protein IX is exposed to the surface of the capsid, while the N terminal region is embedded within the capsid. Recent studies by Saban

et al. (2006) have supported the idea that the N-terminal region of pIX is buried within the capsid, and have further deduced that the N-terminal region forms trimers. Earlier studies suggested that the C-terminal domain of pIX may adopt two different conformations, either binding on the capsid surface or extending away from the capsid (Saban et al., 2005). However, recent studies have revealed a density assigned to pIIIa along the hexon facet edge on the exterior of the capsid was most likely incorrect, and is in fact pIX (Saban et al., 2006)(Figure 3). pIX has a strong propensity for coiled coil formation, therefore the density at the facet edge was assigned to four α -helices of the C-terminal region of pIX (Saban et al., 2006). The two conformation model proposed by Saban et al. (2006) may still be valid; however, the model must be revised to state that the C-terminal α -helices of pIX may either cluster into helical bundles at the facet edges, sit at low occupancy sites on the capsid surface, or extend away from the capsid surface (Saban et al., 2006). At least one of the four α -helices in the bundle may come from pIX contained in a neighboring facet. If all four sites of all 60 α -helical bundles in the capsid are occupied, that would account for 240 (4 x 60) C-terminal domains of pIX, in agreement with the copy number for pIX in the Ad capsid (van Oostrum and Burnett, 1985).

Recent studies support the reassigned pIIIa density to pIX (Fabry et al., 2005; Marsh et al., 2006; Scheres et al., 2005). Fabry et al. (2005) showed that Ad deleted of pIX, regardless of whether the virus was subjected to freeze/thaw cycles, resulted in the presumed pIIIa density at the facet edge being lost. Scheres et al. (2005) reported that the deletion of pIX resulted primarily in a loss of pIIIa density, with minimal pIX density loss at the location within the hexon group of nine, suggesting that the assignments of pIIIa and pIX were, in fact, switched. Marsh et al. (2006) investigated the Ad capsid structure of viruses containing pIX linked to the green fluorescent protein (GFP) with a 45Å α -helical spacer

Figure 3: Predicted location of protein IX in the Ad capsid. The N-terminal domain of pIX is shown in yellow. The central region of pIX is shown in green. The C-terminal domain of pIX is shown in red. The hexon proteins that compose the facets of the Ad capsid are shown in blue. Adapted from Saban et al. (2006).



(pIX-45-GFP) and noted the densities attributed to pIX-45-GFP were located along the outer edges of the group of nine hexons, and not above the GON cavities. Taken together, these studies clearly suggest that the density previously assigned to pIIIa is correctly reassigned to pIX.

1.42 Protein IX Activity

When expressed in a cell, pIX forms distinct clear amorphous structures within the nucleus, and associates with promyelocytic leukemia protein (PML) (Rosa-Calatrava et al., 2003). pIX association with PML may impair PML function through sequestration. PML is a major component of nuclear structures known as nuclear domain 10 (ND10) or PML bodies (reviewed in (Maul, 1998; Maul et al., 2000). ND10 have been associated with many cellular processes including antiviral responses and several viruses disrupt ND10 as part of their natural life cycle (Chee et al., 2003). Since Ad E4 ORF3 disrupts ND10, the ability of pIX to sequester PML may represent a duplicate or backup mechanism to inactivate ND10 function (Parks, 2005).

The suggestion that pIX stabilizes the capsid (Maizel et al., 1968) was confirmed by Colby and Shenk (Colby and Shenk, 1981) who showed that a mutant of Ad5, deleted of most of the E1 region including portions of pIX, produced virions that were more heat labile than wild-type virions and do not form GON's when the virions are dissociated. This phenomenon suggests that as pIX is not necessary for virion formation, it is probably a capsid cement, functionally stabilizing the hexon proteins (Furcinitti et al., 1989). pIX expressing cells lines have even been created to stabilize pIX deficient viruses (Caravokyri and Leppard, 1995; Krougliak and Graham, 1995; Sargent et al., 2004b).

pIX is essential for packaging full length viral genomes (Ghosh-Choudhury et al., 1987). Capsid deficiency in pIX can only accommodate <35kb of viral DNA. The necessity for the presence of pIX in order to package full length genomes was utilized for the development of a size-restricted pIX-deleted helper virus for the amplification of helper-dependent Ad vectors; the rationale being that a large pIX-deficient helper virus would not be able to package its own genome, but would still be able to help package a small helper-dependent Ad into pIX-deficient capsids (Sargent et al., 2004b). However, the data indicated that helper viruses lacking pIX are still able to package genomes greater than full length. The virions, nonetheless, cannot complete a subsequent stage of the virus lifecycle, and are not detected in a plaque assay (Sargent et al., 2004b). Thus, the study implies that pIX may possess a yet unknown function in the Ad life cycle.

Protein IX has been implicated as a transcriptional activator, enhancing expression from the E1A, E4 and major late promoters as well as the β -globin and HSV-1 thymidine kinase promoters (Lutz et al., 1997). Mutagenesis of the central alanine-rich domain and the coiled-coil domain in the carboxy-terminus of pIX identified these regions as being involved in transcription regulation (Rosa-Calatrava et al., 2001). However, our lab has shown that pIX does not significantly influence expression from several viral promoters, including the E1A promoter, in transient assays *in vitro* as well as during normal viral replication (Sargent et al., 2004a). Deletion of pIX from an otherwise wild-type Ad results in only a small reduction in growth of the virus, suggesting that the ability of pIX to transcriptionally activate viral promoters is unimportant for viral growth (Sargent et al., 2004a).

1.43 Protein IX Modifications

To achieve modification of Ad vector tropism other than through alteration of the fiber protein, genetic and transient addition of targeting ligands have been made to pIX. Several motifs have been added to pIX for targeting purposes including polylysine, specific for heparan sulfate proteoglycans (Dmitriev et al., 2002), the epitope tag MYC and the integrin binding motif RGD (Vellinga et al., 2004). In the case of the addition of MYC and RGD to the C-terminus of pIX, the authors noted greater accessibility of antibodies to the ligands with the addition of an alpha-helical linker between the ligand and pIX (Vellinga et al., 2004). Additional modifications have included the incorporation of a biotin acceptor peptide onto pIX, which allowed for the *in vitro* addition of biotin (Parrott et al., 2003). Ad with pIX bound biotin provided a means of specifically purifying Ad through avidin affinity purification methods. Additionally, biotinylated Ad was coated with a bispecific adaptor consisting of an avidin-conjugated antibody against the transferrin receptor (Parrott et al., 2003). The modifications made to pIX have demonstrated enhanced transduction of Ad vectors; however, cell-type specific targeting has yet to be attained through ligand incorporation into pIX.

pIX has been used as a platform for the addition of large molecules. Work done in our lab has shown that GFP can be fused to pIX without affecting viral titer or infectivity (Le et al., 2004; Meulenbroek et al., 2004). We showed that that it was possible to track Ad infection *in vitro* and *in vivo* through the fluorescence of pIX-GFP. The addition of GFP to pIX of canine Ad or bovine Ad type 3 produced similar results (Le et al., 2005; Zakhartchouk et al., 2004). An alternate method of tracking Ad vector transduction following *in vitro* and *in vivo* administration was accomplished through the addition of an HSV-1 thymidine kinase (TK) or luciferase fusion protein to pIX (Li et al., 2005; Matthews et al., 2006). These studies demonstrated that pIX-luciferase and pIX-TK retain their enzymatic

activity while fused to pIX. Since pIX can tolerate the addition of large polypeptides, pIX may be a suitable platform for the attachment of a large targeting ligand, such as a single-chain antibody, allowing Ad to be targeted to specific cell types.

1.5 Antibodies and Cancer Therapy

1.5.1 Overview of Antibodies and Cancer Therapy

To date, many retargeting strategies have involved small modifications to the Ad capsid that do not necessarily increase the specificity of Ad infection. Antibodies and their derivatives represent a class of molecules with high specificity that are attractive as retargeting moieties.

Most cancers are due to mutation of a normal gene into transforming genes (Humphrey et al., 1990). The protein products from mutated genes are attractive targets for cancer therapy, as they should be found only in tumour tissues and not in normal tissues. Mutation results in altered protein structure and these altered regions may be a target for antibody based therapy (Humphrey et al., 1990). Antibodies have been used to disrupt the function of cancer specific molecules, such as the p21^{ras} protein (Biocca et al., 1990; Feramisco et al., 1985; Werge et al., 1990). The ras genes, collectively known as p21, code for highly related membrane associated GTP-bound proteins involved in proliferation and differentiation (Barbacid, 1987). When mutated, the p21^{ras} protein can lead to a loss of cell growth control. Feramisco et al. (1985) reported transient reversion of the mutant phenotype, following microinjection of an antibody specific for the p21^{ras} protein. Eight monoclonal antibodies have been approved for the treatment of cancer by the United States Food and Drug Administration including the anti-CD20 Rituxan[®] (Maloney et al., 1997) for the treatment of lymphoma and anti-ERBB2 Herceptin[®] (Carter et al., 1992) for the treatment of

breast cancer (Adams and Weiner, 2005). Thus, antibodies represent an approved anti-cancer therapeutic.

1.52 Single-chain Antibodies

Single-chain antibodies (scFv) represent advancement in the field of immunotherapy and have been utilized to specifically target intracellular molecules. ScFv's retain the binding specificity of the more complex monoclonal antibody, and are much smaller in size, since they are composed of only a single polypeptide chain. ScFv's are heterodimers, consisting of the variable light (V_L) and the variable heavy (V_H) chain of an antibody joined by a peptide linker (Bird et al., 1988; Huston et al., 1988). Each variable domain contains three hypervariable loops, known as complementary determining regions (CDR's), that form the antigen binding pocket (Wu and Kabat, 1970). Therefore scFv's are able to bind to an antigen with almost the same specificity and affinity as a full-size antibody. ScFv's have been used successfully to target molecules in viral infection, such as the HIV-1 Tat protein (Mhashilkar et al., 1995), various tumours, such as ErbB2 in breast cancer (Wright et al., 1997) and other diseases, such as the huntingtin protein in Huntington's disease (Lecerf et al., 2001). Numerous studies have demonstrated that intracellular scFv, or intrabodies, can function in a variety of anti-cancer and anti-viral treatments (reviewed by Lobato et al. (2003) and Stocks (2004)); however, for viral retargeting purposes, cell surface molecules are more accessible and represent a superior structural target. One of the tumour cell specific mutations which has been targeted by immunotherapy is the cell surface receptor EGFRvIII.

1.53 EGFRvIII

The most common rearrangement of epidermal growth factor receptor (EGFR) is deletion of exons 2-7 in the mRNA, which causes an in-frame deletion of 801bp in the extracellular domain of the molecule and the creation of a novel glycine residue (Nishikawa et al., 1994). Evidence from several studies demonstrates that this mutant receptor, EGFRvIII, is present in a large fraction of glioblastomas (Feldkamp et al., 1999; Frederick et al., 2000; Moscatello et al., 1995; Wikstrand et al., 1995; Worm et al., 1999). EGFRvIII has also been detected in other tumour types, including breast and ovarian cancers (Moscatello et al., 1995; Wikstrand et al., 1995), non-small cell lung cancers (Garcia de Palazzo et al., 1993) and prostate cancers (Olapade-Olaopa et al., 2000); however, further studies utilizing different techniques are needed to validate these results (Lorimer, 2002). Additionally, EGFRvIII does not appear to be found in normal tissue (Wikstrand et al., 1998). Introduction of a EGFRvIII cDNA into murine NIH 3T3 cells resulted in expression of a constitutively phosphorylated, membrane-associated 150kDa receptor species, which caused ligand-independent cell transformation (Yamazaki et al., 1990). Deletion of the protein region encoded by exons 2-7 appears to mimic the effect of ligand binding to the receptor, thereby inducing a change in the receptor to an active conformation. As a consequence, this change leads to constitutive activation of the intrinsic tyrosine kinase activity (Nishikawa et al., 1994).

Antibodies have been generated against the tumour-specific mutation found in EGFRvIII. Humphrey et al. (1990) synthesized a 14-amino acid peptide corresponding to the amino acid sequence at the fusion junction generated by the deletion of exons 2-7 in EGFRvIII. The anti-peptide antibody bound specifically to EGFRvIII *in vitro* and to tumour xenografts expressing EGFRvIII. The site-specific, anti-EGFRvIII peptide antibody was highly selective for the EGFRvIII protein and did not cross-react with EGFR protein

(Humphrey et al., 1990). Therefore it is possible to specifically exploit and target mutational abnormalities in cellular receptors found exclusively on cancer cells.

1.54 MR1

A scFv previously used in anti-cancer applications is MR1. MR1 was isolated through phage display technology, by “biopanning” on cancer cells overexpressing the mutant EGFRvIII receptor. MR1 has been utilized to target recombinant immunotoxin to glioblastoma cells expressing EGFRvIII (Lorimer et al., 1996). Immunotoxins are therapeutic agents for cancer therapy that consist of a targeting molecule linked to a cytotoxic agent (Lorimer et al., 1996). MR1 immunotoxin shows high-affinity binding to both EGFRvIII synthetic peptide and the mutant cell surface receptor, with dissociation constants (Kd) of 22nM and 11nM respectively (Lorimer et al., 1996). The similarity in binding affinities suggests that the scFv interacts with the peptide and the receptor N-terminal sequence in the same way. MR1 does not show any detectable binding to the wildtype receptor (Kuan et al., 1999). Therefore, MR1 represents a bio-reagent highly specific for tumour cells.

Mutational studies have investigated the importance of each of the first 13 amino acids of EGFRvIII to the binding of MR1. MR1 recognizes an eight amino acid residue epitope that spans the fusion junction at the novel glycine residue produced by the deletion of exons 2-7 (Landry et al., 2001). Further experimentation was performed to explain the complete lack of cross-reactivity of MR1 with wild-type EGFR, given that the normal receptor contains six of the eight residues of the MR1 epitope, including the three residues most involved in binding (Landry et al., 2001). Crystallographic analysis demonstrated that MR1 achieves its high level of specificity by forcing an interacting peptide to assume a

conformation that would be most favored with a glycine residue at the junction position (Landry et al., 2001). Since EGFRvIII has a glycine residue at the junction position produced by the deletion of exons 2-7, MR1 is able to specifically recognize and bind to this unique region of amino acids.

The specific binding of MR1 to EGFRvIII has been utilized for targeting viral vectors to cancer cells. MR1 was used to retarget Moloney murine leukemia virus (MLV), through fusion of MR1 into the ecotropic envelope glycoprotein (ENV) (Lorimer and Lavictoire, 2000). Normal infectivity of MLV is mediated by the envelope glycoprotein. The SU domain binds to a cell surface receptor, shown to be a 14-transmembrane-domain cationic amino acid transporter (Albritton et al., 1989; Kim et al., 1991; Wang et al., 1991). A fusion protein was constructed in which MR1 was inserted in a disulphide-bonded loop in the receptor binding loop of SU (Lorimer and Lavictoire, 2000). ENV-MR1 was efficiently incorporated into MLV and the resulting virus demonstrated specific binding to U87MG cells expressing EGFRvIII; however infectivity was not enhanced. The poor infectivity may be explained by the fact that MR1 recognizes an epitope at the amino-terminus of EGFRvIII that is distant from the cell membrane; MLV utilizes membrane fusion to facilitate entry, therefore the receptor may be too far from the cell membrane to permit cell entry (Lorimer and Lavictoire, 2000).

MR1 fused to the TVA receptor for subgroup A avian leukosis viruses, allowed for enhanced uptake of avian leukosis virus by EGFRvIII expressing cells (Snitkovsky et al., 2000). TVA-MR1 served as a bifunctional adaptor for ALV, and facilitated specific entry of ALV into 293T cells engineered to express a murine form of EGFRvIII. Nakamura et al. (2005) incorporated MR1 into the H protein of live-attenuated measles virus of the Edmonston lineage, to target oncolytic measles virus to cancer cells expressing EGFRvIII.

Measles virus with MR1 demonstrated specific infection of CHO cells expressing EGFRvIII (Nakamura et al., 2005). Taken together, these studies demonstrated that MR1 has been used successfully to retarget several viruses to cells expressing EGFRvIII, and suggest that incorporation of MR1 into the Ad capsid may allow for specific infection of cancer cells expressing EGFRvIII.

1.6 Rationale

The viral vectors currently used for gene therapy lack specificity with regard to the types of cells they infect (Verma and Somia, 1997). For cancer gene therapy, the mutant receptor EGFRvIII is a very promising target to confer cancer cell-specific infection. EGFRvIII is present with high frequency on several different tumour types and is not found on normal tissues (Kuan et al., 1999). Previous attempts at treating glioblastoma patients with intratumoural injection of replication deficient Ads resulted in limited infection (Puumalainen et al., 1998). Ad vectors that are able to specifically infect EGFRvIII-positive cancer cells through the incorporation of MR1 onto pIX could enhance the safety and efficacy of gene delivery in a therapeutic setting.

1.7 Objectives

The objectives of this study are to determine (i) whether MR1 can be fused to pIX, (ii) whether pIX-MR1 binds to EGFRvIII, (iii) whether Ads can be rescued containing pIX-MR1, and (iv) whether Ads containing pIX-MR1 preferentially infect EGFRvIII-expressing cells.

1.8 Hypothesis

The addition of the single-chain antibody MR1 to the Ad capsid protein IX, will target the virus specifically to cells expressing EGFRvIII.

Chapter 2 - Materials and Methods

2.1 Tissue Culture

2.11 Cell Culture Maintenance

293 cells (ATCC), were maintained in minimum essential media (MEM) (Invitrogen, Burlington, ON) supplemented with 10% fetal bovine serum (FBS)(Sigma Genosys, Oakville, ON), 1% antibiotic/antimycotic (Invitrogen, Burlington, ON), and 1% Glutamax-1 (Invitrogen, Burlington, ON) at 37°C and 5% CO₂. 293 cells were passaged using 1 x citric saline approximately every three days depending on cell confluency.

2.12 Cell Transfection

Twenty-four hours prior to transfection, 35 mm tissue culture dishes (Sarstedt, Montreal, QC) were seeded at a density of 0.3×10^6 cells per dish and allowed to reach confluency overnight. Two µg DNA was added to 100 µL MEM, and incubated with 4 µL SuperFect® transfection reagent (Qiagen, Mississauga, ON) for 10 min to allow complexes to form. During this incubation period, the media was removed from the 35 mm dishes and the monolayers were washed twice with 1 mL phosphate buffered saline (PBS) (Invitrogen, Burlington, ON). Following the 10 min incubation, 600 µL MEM was combined with the 100 µL reaction mix, placed on the 35 mm dish, and incubated at 37°C for 3-4 hours. After the allotted time, the reagent was removed and the cells were washed twice with 1 mL PBS. Two mL maintenance media was added to each dish and the cells were incubated at 37°C until the end of the experiment.

2.13 Virus Plaque Overlay

For the generation of virus, 293 cells were transfected as described above; however at the end of the transfection, cells were overlaid with media containing 0.5% agarose, 5% FBS, 1% antibiotic/antimycotic and 2% yeast extract and incubated at 37°C for 10 days to allow plaques to form.

2.14 Ad Infection

Twenty-four hours prior to infection, 35 mm tissue culture dishes were seeded at a density of 0.3×10^6 cells per dish and allowed to reach confluency overnight. For each 35 mm dish, 200 μ L of inoculum was used: an appropriate amount of virus stock was diluted in MEM to 200 μ L. Media was removed from the 35 mm dishes and the monolayers were washed twice with 1 mL PBS (Invitrogen, Burlington, ON). The 200 μ L diluted virus solution was applied to the 35 mm dishes and the cells/virus were incubated at 37°C for 1 hour with occasional rocking. Following the incubation, the virus solution was removed and the cells were washed twice with 1 mL PBS. Two mL maintenance media was added to each dish and the cells were incubated at 37°C until the end of the experiment.

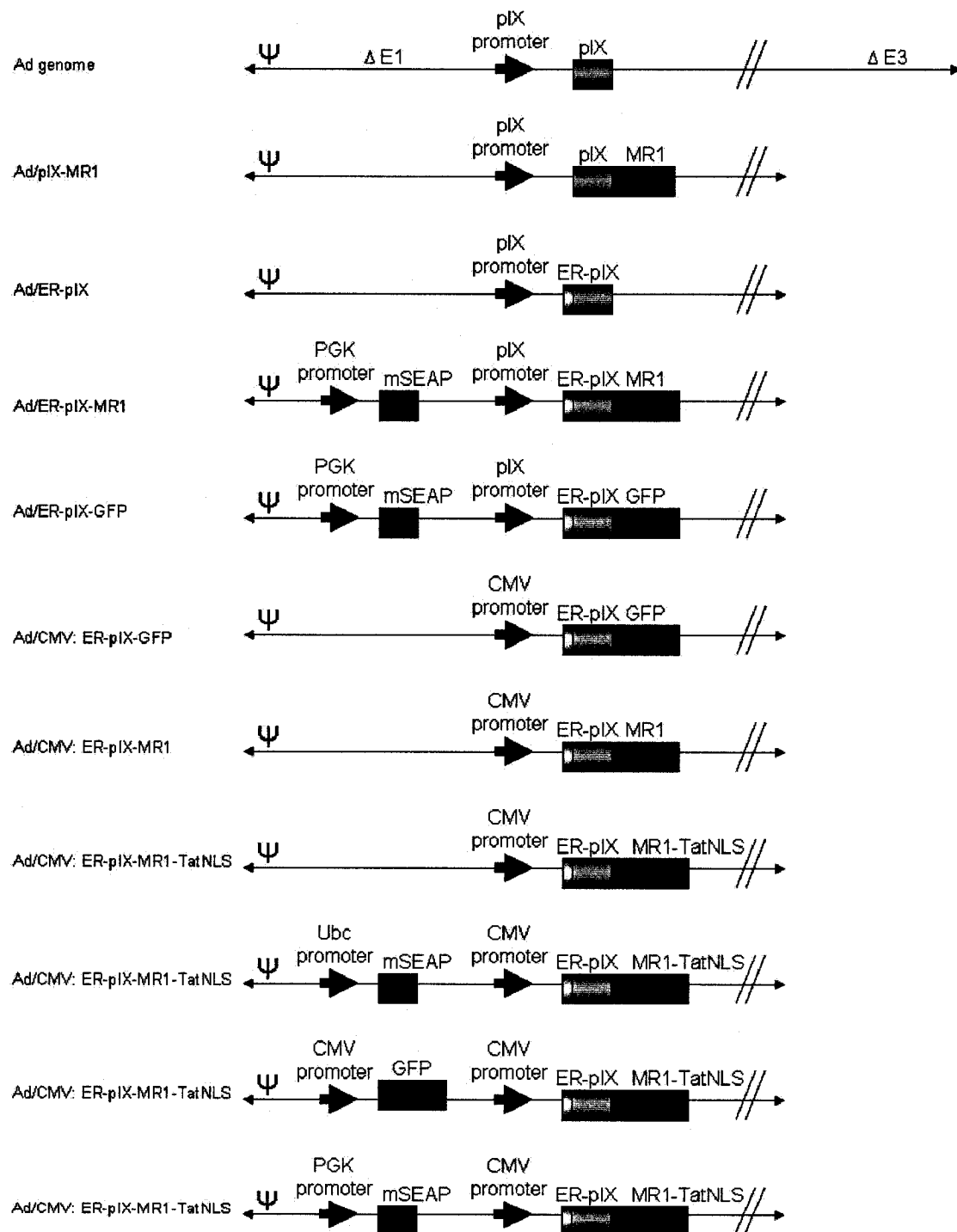
2.2 Cloning and Plasmid DNA Preparation

Restriction enzymes, T4 DNA ligase, T4 DNA polymerase, Klenow polymerase and calf intestinal phosphatase were obtained from NEB (New England Biolabs, Ipswich, MA), Invitrogen (Burlington, ON) or MBI Fermentas (Burlington, ON) depending on the availability of the enzyme. Small scale DNA preparations were performed by alkaline lysis following methods described by Birnboim and Doly (1979), while large scale DNA preparations were performed by alkaline lysis with purification by cesium chloride (CsCl) buoyant density centrifugation as described in Sambrook et al. (1989).

2.21 Construction of pIX-MR1

The MR1 scFv coding sequence was obtained through polymerase chain reaction (PCR) amplification using pcDNA3.1-fibreMR1scFv as the template (Kottachchi, 2002). The synthetic oligonucleotides used were 5'- gcggctagcggggcccagccggccatggcccag and 5'- gcgacgcgtggttcgaactatgcggccgctttgattccagc from Sigma Genosys (Oakville, ON). The PCR reaction utilized an annealing temperature of 58°C. The PCR product was digested with *NheI/MluI* and cloned into *NheI/MluI* digested pRP2288 (Meulenbroek et al., 2004), creating the pIX-MR1 fusion protein (plasmid denoted pRL4). The resultant pIX-MR1 fusion protein contains a FLAG epitope tag obtained from pRP2288 on the C-terminus of pIX. RL4 was subsequently digested with *NheI/BstBI* and cloned into *NheI/BstBI* digested pRP2306, generating the shuttle plasmid pRL5. The shuttle plasmid contains the Ad left ITR, the packaging sequence, a deleted E1 region, the pIX-MR1 expression cassette inserted into the native pIX locus, and several kbp of Ad DNA downstream of pIX (termed the right arm of homology). Homologous recombination between *Bst1107I* digested RL5 and *Bsu15I* digested pRP2014 (an Ad backbone plasmid containing the right arm of homology and the remainder of the Ad genome) transfers the pIX-MR1 expression cassette from the shuttle to the backbone. Recombination utilized RecA-proficient BJ5183 cells (Chartier et al., 1996; He et al., 1998) and resulted in the large infectious plasmid, pRL6. pRL6 was digested with *PacI* and transfected into 293 cells to generate viral plaques. The resultant plaques were isolated with a Pasteur pipette, amplified on 293 cells and purified by cesium chloride buoyant density gradient centrifugation (Ng and Graham, 2002). See Figure 4 for a schematic representation of the Ad vectors used in this study.

Figure 4: Schematic representation of Ad vectors used in this study. The Ad backbone used to generate the Ad vectors is deleted of the Ad early region 1 and 3 (E1 and E3). Ad/pIX-MR1 contains the pIX-MR1 expression cassette replacing the native pIX gene under endogenous regulation. Ad/ER-pIX contains the ER-pIX expression cassette replacing the native pIX gene under endogenous regulation (see Table 1 for description of ER signal peptides). Ad/ER-pIX-MR1 contains the PGK-mSEAP expression cassette with the SV40 polyadenylation signal (pA) replacing the E1 region and the ER-pIX-MR1 expression cassette replacing the native pIX gene under endogenous regulation. Ad/ER-pIX-GFP contains the PGK-mSEAP expression cassette with the SV40 pA replacing the E1 region and the ER-pIX-GFP expression cassette replacing the native pIX gene under endogenous regulation. Ad/CMV:ER-pIX-GFP contains the CMV immediate early promoter/enhancer replacing the native pIX promoter and the ER-pIX-GFP expression cassette replacing the native pIX gene. Ad/CMV:ER-pIX-MR1 contains the CMV immediate early promoter/enhancer replacing the native pIX promoter and the ER-pIX-MR1 expression cassette replacing the native pIX gene. Ad/CMV:ER-pIX-MR1-TatNLS contains either the Ubc-mSEAP expression cassette with the SV40 pA, CMV-GFP expression cassette with the BGH pA, PGK-mSEAP expression cassette with the SV40 pA or no expression cassette replacing the E1 region, the CMV immediate early promoter/enhancer replacing the native pIX promoter and the ER-pIX-MR1-TatNLS expression cassette replacing the native pIX gene.



2.22 Construction of ER-targeted pIX

To clone the ER signal sequences onto the 5' end of pIX, the following primers were used: SP1 5'- cgcggtatccgccgcatggtgccttgacattacttctgttactagctgccgcactagcaccaacgcaaacgcg agcgatgagcaccaactcgtttgatggaag; SP2 5'- cgcggtatccgccgcatggagacagacacactcctgctatgggtactg ctgctctgggtccaggtccactggtatgagcaccaactcgtttgatggaag; and SP3 5'- cgcggtatccgccgcatggaga cagacacactcctgctatgggtactgctgctctgggtccaggtccactggtgacagcaccaactcgtttgatggaag; SP4 5'- cgcggtatccgccgcatgaccaacaagtgtctctccaaattgctctcctgttgtgcttctccacgacagctcttccatgagcaccaac tcgtttgatg; along with the reverse primer 5'- cgctctagactatgggtcctctaccttgcgctcttttaggcggggat gctgcggccgctttgattccag to PCR amplify the pIX region of plasmid pRP2259. For a more detailed description of ER signal sequences SP1-SP4 see Table 1. All primers were obtained from Sigma Genosys (Oakville, ON). The PCR reactions utilized annealing temperatures of 58°C. The resultant PCR products were extracted with buffer-saturated phenol and chloroform:isoamyl alcohol, precipitated with isopropanol, resuspended in TE buffer and digested with *Bam*HI. The digested PCR products were separated by agarose gel electrophoresis and a gel slice containing each of the PCR products was isolated. The gel slices were heated at 65°C until complete melting occurred and diluted with an equal volume of TE buffer. The DNA was extracted with buffer-saturated phenol and chloroform:isoamyl alcohol, precipitated with isopropanol and resuspended in TE buffer. The isolated PCR products were cloned into *Bam*HI digested pcDNA3.1 (Invitrogen, Burlington, ON), creating plasmids pRL36-SP1, pRL36-SP2, pRL36-SP3, and pRL36-SP4. A 552 bp fragment encoding ER-targeted pIX from *Bam*HI digested pRL36 (SP1-SP4) was cloned into *Bgl*III digested pΔE1sp1AΔpIX, creating the shuttle plasmids pRL37-SP1, pRL37-SP2, pRL37-SP3 and pRL37-SP4. pRL37 (SP1-SP4) was digested with *Bst*1107I and recombined with *Bsu*15I digested pRP2014 in RecA+ competent bacteria as previously described. The

Table 1: Endoplasmic reticulum signal peptides used to target pIX-MR1 to the ER. Several ER signal peptides were utilized to traffic pIX-MR1 to the ER including HCMV US11 (SP1), Ig κ -chain from pSecTag2 (SP2), Ig κ -chain from pSecTag2 where the Met of protein IX was changed to Asp (SP3), and IFN- β (SP4).

<u>ER Signal Peptide</u>	<u>Origin</u>
SP1	US11 protein of CMV
SP2	Murine Ig κ -chain signal peptide from pSecTag2
SP3	Murine Ig κ -chain signal peptide from pSecTag2 (Met of pIX changed to Asp)
SP4	IFN- β

resultant plasmids, pRL39 (SP1-SP4) were digested with *PacI*, transfected into 293 cells, and recovered as viruses as described above.

2.23 Construction of ER-targeted pIX-MR1

The interferon- β ER signal peptide (SP4) was cloned onto the 5' terminus of pIX-MR1 through PCR amplification of pRL4 using the following primers: 5'-cgcgatccgccgcatgaccaacaagtgtctcctccaaattgctctcctgtgtgcttctccacgacagctctttccatgagcaccacacggttgatg and 5'-cgctctagactatgggtcctctaccttgcttcttttagg cgggatgctgcggccgctttgattccag (Sigma Genosys, Oakville, ON). Use of the reverse primer adds a nuclear localization sequence from the simian virus 40 large tumour antigen to the C-terminus of MR1 (Kalderon et al., 1984). The PCR reaction utilized an annealing temperature of 58°C. The resultant PCR product was digested with *BamHI/XbaI* and cloned into *BamHI/XbaI* digested pcDNA3.1, generating pRL14. pRL14 was digested with *BamHI/XbaI* and cloned into *BglII/AvrII* digested p Δ E1sp1A Δ pIX, creating the shuttle plasmid pRL20. The murine phosphoglycerate kinase (PGK) promoter driving expression of the murine secreted alkaline phosphatase (mSEAP) reporter gene and the simian virus 40 polyadenylation sequence (SV40 pA) was cloned upstream of pIX-MR1 through a *BamHI/NotI*(filled in) digestion product of pRP2202 cloned into *BamHI/EcoRV* digested pRL20, creating pRL26. pRL26 was digested with *Bst1107I* and recombined with *Bsu15I* digested pRP2014 in RecA+ competent bacteria as previously described, creating pRL32. Thus, pRL32 contains the PGK-mSEAP expression cassette replacing the E1 region and the ER-pIX-MR1 expression cassette replacing the native pIX gene. pRL32 was digested with *PacI*, transfected into 293 cells, and recovered as a virus as described above.

2.24 Construction of ER-targeted pIX-GFP

The interferon- β ER signal peptide was cloned onto the 5' terminus of pIX-GFP through PCR amplification of pRP2288 (Meulenbroek et al., 2004) using the following primers: 5'-cgcgatccgccgcatgaccaacaagtgtctcctccaaattgctctcgtgtgtgcttccacgacagctcttccatgagcaccacacgcgtttgatg and 5'-cgctctagactatgggtcctctaccttgcgcttcttttaggcggggatgcatcgcgtgtgtacagttcatc (Sigma Genosys, Oakville, ON). The PCR reaction utilized an annealing temperature of 58°C. The resulting PCR product was digested with *Bam*HI/*Xba*I and cloned into *Bam*HI/*Xba*I digested pcDNA3.1, generating pRL11. pRL11 was digested with *Bam*HI/*Xba*I and cloned into *Bgl*II/*Spe*I digested p Δ E1sp1A Δ pIX, creating the shuttle plasmid pRL17. The PGK-mSEAP expression cassette with the SV40 pA was cloned upstream of pIX-GFP through a *Bam*HI/*Not*I(filled in) digestion product from pRP2202 cloned into *Bam*HI/*Eco*RV digested pRL17, creating pRL23. pRL23 was digested with *Ehe*I and recombined with *Bsu*15I digested pRP2014 in RecA+ competent bacteria as previously described, creating pRL28. Thus, pRL28 contains the PGK-mSEAP expression cassette replacing the E1 region and the ER-pIX-GFP expression cassette replacing the native pIX gene. pRL28 was digested with *Pac*I, transfected into 293 cells, and recovered as a virus as described above.

2.25 Construction of ER-pIX-GFP regulated by the CMV promoter

To clone the cytomegalovirus (CMV) immediate early promoter/enhancer upstream of ER-pIX-GFP, the product from *Bgl*II/*Nhe*I digested RL11 was cloned into *Bam*HI/*Nhe*I digested RL17, creating the shuttle plasmid pRL70. pRL70 was digested with *Ehe*I and transformed into RecA+ cells with *Bsu*15I digested pRP2014 as previously described, generating pRL71. Thus, pRL71 encodes the CMV immediate early promoter/enhancer replacing the native pIX promoter and the ER-pIX-GFP expression cassette replacing the native pIX gene and the

remainder of the Ad genome. pRL71 was digested with *PacI*, transfected into 293 cells and recovered as described above.

2.26 Construction of ER-pIX-MR1 regulated by the CMV promoter

To clone the CMV promoter upstream of ER-pIX-MR1, the digestion product from *BglIII/NheI* digested RL14 was cloned into *BamHI/NheI* digested RL20, creating pRL72. pRL72 was digested with *Bst1107I* and transformed into RecA+ cells with *Bsu15I* digested pRP2014 as previously described, generating pRL74. Thus, pRL74 encodes the CMV immediate early promoter/enhancer replacing the native pIX promoter and the ER-pIX-MR1 expression cassette replacing the native pIX gene and the remainder of the Ad genome. pRL74 was digested with *PacI*, transfected into 293 cells and recovered as a virus as described above.

2.27 Construction of ER-pIX-MR1-TatNLS regulated by the CMV promoter

Cloning of amino acids 48 to 59 (the nuclear localization sequence (NLS) of the HIV-1 Tat protein onto the N terminus of MR1 was accomplished by PCR amplifying the pIX-MR1 fusion gene from pRL14 with synthetic oligonucleotides 5'-cgcgatccgcccgcacccaacaagtgtctctccaaattgctctctgtgtgtcttccacgacagctcttccatgagcaccaactcgtttgatg and 5'-gcgtctagactaaggagctcgtcgtcgtgtctccgcttctctgcctgcggccgctttgattccag (Sigma Genosys, Oakville, ON). The PCR reaction utilized an annealing temperature of 65°C. The PCR product was digested with *BamHI/XbaI* and cloned into *BamHI/XbaI* digested pcDNA3.1, generating pRL87. pRL87, was digested with *BamHI/XbaI* and cloned into *BglIII/SpeI* digested pΔE1sp1AΔpIX generating pRL90. pRL90 was digested with *Bst1107I* and transformed into RecA+ cells along with *Bsu15I* digested pRP2014, as previously described, generating pRL97. Thus,

pRL97 encodes the CMV immediate early promoter/enhancer replacing the native pIX promoter and the ER-pIX-MR1-TatNLS expression cassette replacing the native pIX gene and the remainder of the Ad genome. pRL97 was digested with *PacI* and transfected into 293 cells as described above. A virus was not recovered.

2.28 Construction of Ubc-mSEAP expression cassette with CMV: ER-pIX-MR1-TatNLS

To place the ubiquitin-c promoter regulated murine secreted alkaline phosphatase reporter gene (Ubc-mSEAP) expression cassette with the SV40 pA upstream of CMV:ER-pIX-MR1-TatNLS, a ligation/recombination reaction with pRL75 was performed. pRL90 was digested with *NheI/StuI* and ligated to *NheI/NotI* digested pRL75. The fragments are predicted to ligate at the *NheI* site, and the other regions contain overlap homology. Transformation of the ligation product into RecA+ competent bacteria, allowed homologous recombination to occur and generated pRL91. pRL91 encodes the Ubc-mSEAP expression cassette replacing the E1 region, the CMV immediate early promoter/enhancer replacing the native pIX promoter and the ER-pIX-MR1-TatNLS expression cassette replacing the native pIX gene. pRL91 was digested with *Bst1107I* and transformed into RecA+ cells along with *Bsu15I* digested pRP2014, as previously described, generating pRL92. Thus, pRL92 encodes the Ubc-mSEAP expression cassette replacing the E1 region, the CMV immediate early promoter/enhancer replacing the native pIX promoter and the ER-pIX-MR1-TatNLS expression cassette replacing the native pIX gene and the remainder of the Ad genome. pRL92 was digested with *PacI* and transfected into 293 cells as described above. A virus was not recovered.

2.29 Construction of CMV-GFP expression cassette with CMV:ER-pIX-MR1-TatNLS

To clone a GFP cDNA under regulation of the CMV immediate early promoter/enhancer and bovine growth hormone polyadenylation sequence (CMV-GFP) upstream of CMV:ER-pIX-MR1-TatNLS, the CMV-GFP expression cassette from pAVH5 was excised with *BglII/SspI* and cloned into *BglII/SspI* digested pRL87, creating pRL95. pRL95, was digested with *XhoI/XmnI* and cloned into *XhoI/EcoRV* digested pΔE1sp1AΔpIX, generating pRL96. pRL96 was digested with *EheI* and transformed into RecA+ cells along with *Bsu15I* digested pRP2014, as previously described, generating pRL98. Thus, pRL98 encodes the CMV-GFP expression cassette replacing the E1 region, the CMV immediate early promoter/enhancer replacing the native pIX promoter and the ER-pIX-MR1-TatNLS expression cassette replacing the native pIX gene and the remainder of the Ad genome. pRL98 was digested with *PacI* and transfected into 293 cells as described above. A virus was not recovered.

2.210 Construction of PGK-mSEAP expression cassette with CMV:ER-pIX-MR1-TatNLS

To clone the PGK-mSEAP expression cassette upstream of CMV:ER-pIX-MR1-TatNLS, the digestion product from *BamHI/NotI*(filled in) digested pRP2202 was cloned into *BamHI/EcoRV* digested pRL90, creating pRL99. pRL99 was digested with *Bst1107I* and transformed into RecA+ cells along with *Bsu15I* digested pRP2014, as previously described, generating pRL100. Thus, pRL100 encodes the PGK-mSEAP expression cassette replacing the E1 region, the CMV immediate early promoter/enhancer replacing the native pIX promoter and the ER-pIX-MR1-TatNLS expression cassette replacing the native pIX gene and the remainder of the Ad genome. pRL100 was digested with *PacI* and transfected into 293 cells as described above. A virus was not recovered.

2.3 EGFRvIII Binding Assay

The EGFRvIII binding assay used to assess binding of MR1 to an EGFRvIII like peptide was adapted from Lorimer and Lavictoire (2000). 293 cells were seeded at a density of 0.8×10^6 cells per 60 mm dish and, the next day, the cells were either transfected with the test plasmids or infected with a test virus. Twenty-four hours later, the cells were washed twice with cold PBS and incubated for 40 min at 4°C with 400 μ L of RIPA buffer (1 complete mini EDTA-free tablet with protease inhibitors (Roche, Mississauga, ON), 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 1% glycerol and 1% NP-40). The cells were scraped from the 60 mm dish into a microfuge tube and centrifuged at 14000 x g for 5 min to pellet the cell debris. During the centrifugation, Dynabeads M-280 streptavidin-coated magnetic beads (Dyna, Oslo, Norway) (20 μ L/sample) were washed 4 times with RIPA buffer. Following the centrifugation, the supernatant from the scraped cells was transferred to a new microfuge tube. A 20 μ L aliquot of the cell lysate was removed, and added to 20 μ L SDS-PAGE loading buffer. The remaining volume of supernatant was incubated with 20 μ L of streptavidin-coated magnetic beads for 1 hour in a rotating rack at 4°C as a pre-clearing step to reduce non-specific interactions with the magnetic beads. The pre-cleared samples were next placed in a magnetic rack (Dyna, Oslo, Norway) to pellet the magnetic beads and the supernatant was transferred to a new microfuge tube. The supernatant was then incubated with biotinylated peptide LEEKKGNVYVTDHSGGK-biotin (Lorimer et al., 1996) at a final concentration of 100 mM for 1 hour in a rotating rack at 4°C. Following the incubation, 20 μ L of streptavidin-coated magnetic beads was added to each sample and incubated for 15 min in a rotating rack at 4°C. The samples were placed in the magnetic rack and the supernatant was discarded. The magnetic beads were then washed 4 times with RIPA buffer, after each wash discarding the supernatant and retaining the beads. The beads were then

resuspended in 30 μ L SDS-PAGE loading buffer, and analyzed by immunoblot. See Figure 5 for a schematic of the EGFRvIII binding assay.

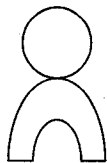
2.4 Immunoblot analysis

Prior to sample electrophoresis, experimental samples were heated for 10 min at 100°C, then centrifuged at 14000 x g for 3 min. An aliquot of the sample was separated by electrophoresis on an SDS-polyacrylamide gel using a Bio-Rad Mini-Protean II Cell (Bio-Rad, Hercules, CA, USA). Once the required resolution was attained, the separated proteins were transferred to a polyvinylidene difluoride membrane (Immuno-Blot PVDF Membrane, Bio-Rad) using a Bio-Rad Trans-Blot SD Semi-Dry Transfer Cell according to the manufacturers protocol. Protein transfer was allowed to proceed for 60 min when transferring pIX fusion proteins and fiber. However, for small proteins such as pIX, the separated proteins were transferred to a Millipore Immobilon-P^{SQ} membrane (Millipore, Mississauga, ON)(containing a 0.2 μ M pore size) for 30 min. The membrane was blocked overnight in 5% skim milk in TBST (10 mM Tris-HCL pH 8.0, 15 mM NaCl, 0.1% Tween). The membrane was probed with a monoclonal, FLAG, M2 antibody (Sigma Genosys, Oakville, ON; 1:15000) or fiber antibody (NeoMarker, Montreal, QC; 1:1000). Binding of the primary antibody was detected using a goat anti-mouse secondary antibody conjugated to horseradish peroxidase (Bio-Rad, Hercules, CA, USA). The membranes were visualized using the ECL plus Western Blotting Detection System (Amersham Biosciences) and autoradiography.

To examine the incorporation of pIX fusion protein into Ad capsids, 1×10^7 pfu of purified virus was combined with an equal volume of SDS-PAGE loading buffer to a final volume of 40 μ L. The resultant proteins were analyzed by immunoblot analysis.

Figure 5: A schematic of the EGFRvIII binding assay. The EGFRvIII binding assay is used to assess the ability of MR1 to bind to an EGFRvIII peptide as described by Lorimer and Lavictoire (2000). The experimental sample is incubated with the biotinylated peptide that represents the extracellular domain of EGFRvIII recognized by MR1. Following incubation with streptavidin-coated magnetic beads, protein bound to the peptide is isolated using a magnetic rack.

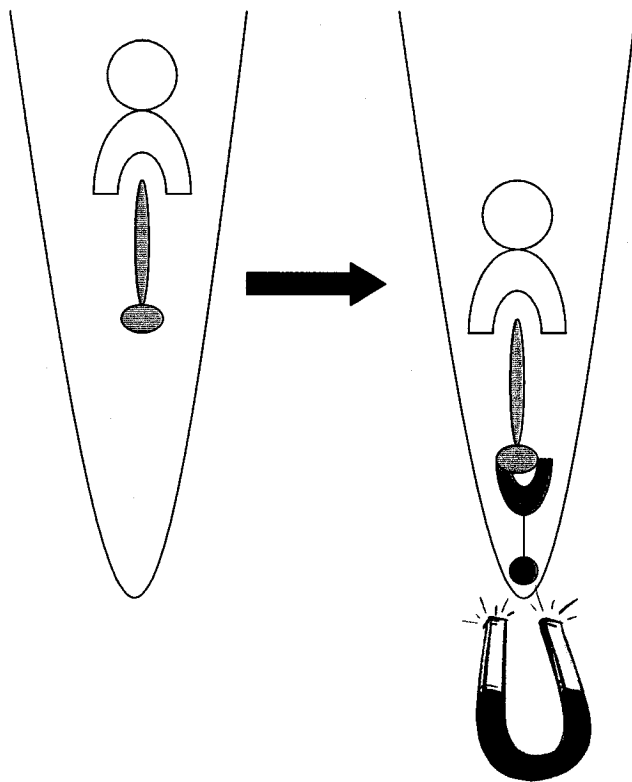
pIX-MR1



Biotinylated EGFRvIII peptide



Streptavidin coated magnetic bead



2.5 Immunofluorescence Analysis of pIX-MR1

Twenty-four hours prior to transfection or infection, 293 cells were seeded on 1 cm rounded glass cover slips (Fisher, Ottawa, ON) at a density of 0.1×10^6 cells per 35 mm dish. The next day, the cells were either transfected with the test plasmids or infected with the test viruses, as previously described. Twenty-four hours later, the media was removed and the cells were washed once with 1 mL cold PBS, fixed with 4% paraformaldehyde in PBS (BDH, Dorset, UK) for 15 min, and then washed immediately with 1 mL PBS containing 1% horse serum (HS) (Invitrogen, Burlington, ON). After two additional washes with PBS + 1% HS, the cells were permeabilized with PBS + 1% HS and 0.5% Triton X-100 (Fisher, Ottawa, ON) for 10 min. The cells were washed immediately with 1 mL PBS + 1% HS. Cells were then washed two more times for 5 min with 1 mL PBS + 1% HS. The coverslips were then placed in a humidified chamber and incubated with 100 μ L of mouse monoclonal, anti-FLAG, M2 (Sigma Genosys, Oakville, ON; 1:1500) in PBS + 1% HS for 1 hour at room temperature. The coverslips were washed 3 times for 5 min with PBS + 1% HS and placed in a humidified chamber and incubated with 100 μ L of FITC conjugated goat anti-mouse antibody (Jackson Immuno Research Laboratories, West Grove, PA ; 1:200) in PBS + 1% HS and containing 0.2 μ g/mL Hoechst (Sigma Genosys, Oakville, ON) for 1 hour at room temperature. The cover slips were washed 3 times for 5 min with PBS + 1% HS and were applied to glass slides (Fisher, Ottawa, ON) using a drop of DAKO fluorescent mounting medium (DAKO, Glostrup, Denmark). Immunofluorescent detection was performed using a Zeiss Axioplan 2[®] fluorescence microscope and the pictures were taken using an Axiocam HR[®] CCP camera. The image was processed using Adobe Photoshop[®] imaging software.

Chapter 3 - Results

3.1 pIX-MR1 Expression and Binding to EGFRvIII

After cloning the single-chain antibody MR1 onto the C-terminus of protein IX, we determined whether the fusion protein was expressed at similar levels to pIX-GFP and was of the appropriate size. The fusion protein pIX-GFP was used as a positive control since it is approximately the same molecular size as pIX-MR1 and is efficiently incorporated into the Ad capsid (Meulenbroek et al., 2004). 293 cells were transiently transfected with plasmids encoding pIX-GFP, pIX-dsRed and pIX-MR1 and visualized by Western blot. As shown in Figure 6, pIX-MR1 is expressed at similar levels to pIX-GFP and is the expected molecular weight, approximately 43 kDa.

To determine if MR1 fused to pIX was still capable of binding EGFRvIII, an immunoprecipitation assay was performed using a biotinylated EGFRvIII-like peptide (Lorimer and Lavictoire, 2000). The immunoprecipitation assay utilizes a biotinylated thirteen amino acid peptide that represents the region of the extracellular domain of EGFRvIII recognized by MR1 (Lorimer and Lavictoire, 2000). Incubation of an experimental sample with the biotinylated peptide, followed by incubation with streptavidin-coated magnetic beads, allows for isolation of bound protein using a magnetic rack (Figure 5). MR1 fused to the envelope glycoprotein (ENV) of Moloney Murine Leukemia Virus, was previously shown to efficiently bind EGFRvIII (Lorimer and Lavictoire, 2000) and served as a positive control for our assay. As shown in Figure 7A, ENV-MR1 was specifically retained in the binding assay. Using the binding assay, we can determine if pIX-MR1 is able to bind EGFRvIII as well. 293 cells were transiently transfected with plasmids encoding pIX-GFP, pIX-dsRed and pIX-MR1 and twenty-four hours later subjected to the pull-down assay as

Figure 6: pIX-MR1 expression. 293 cells were transiently transfected with plasmids encoding pIX-MR1, pIX-GFP, and pIX-dsRed. The fusion proteins pIX-GFP and pIX-dsRed were used as a positive control since they are approximately the same molecular size as pIX-MR1. Twenty-four hours later the cells were lysed in Laemmli sample buffer, subjected to SDS-PAGE and visualized by immunoblot with an anti-FLAG M2 specific antibody.

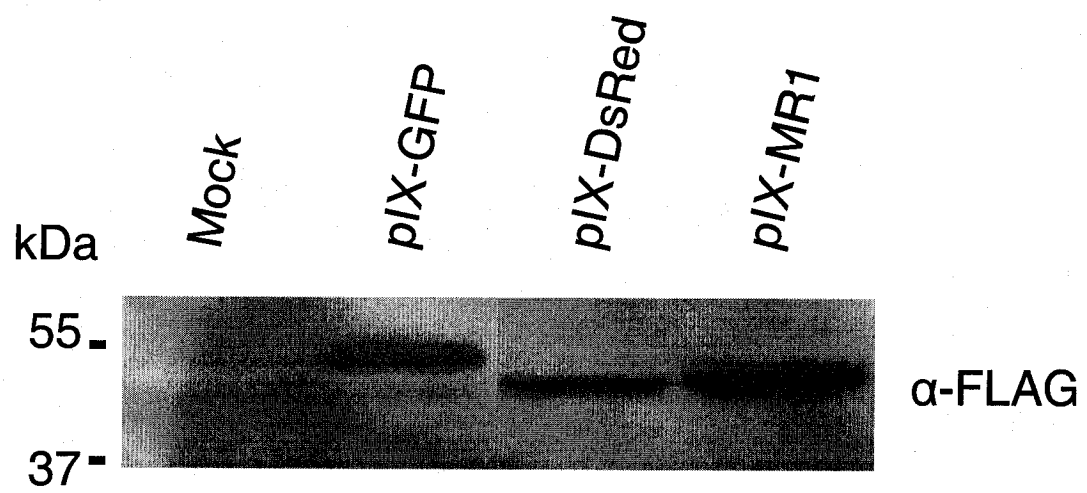
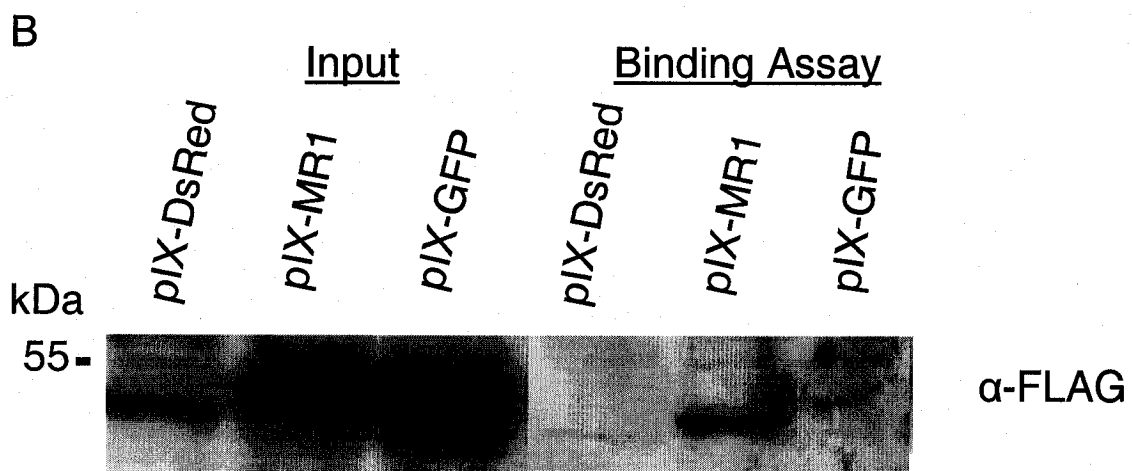
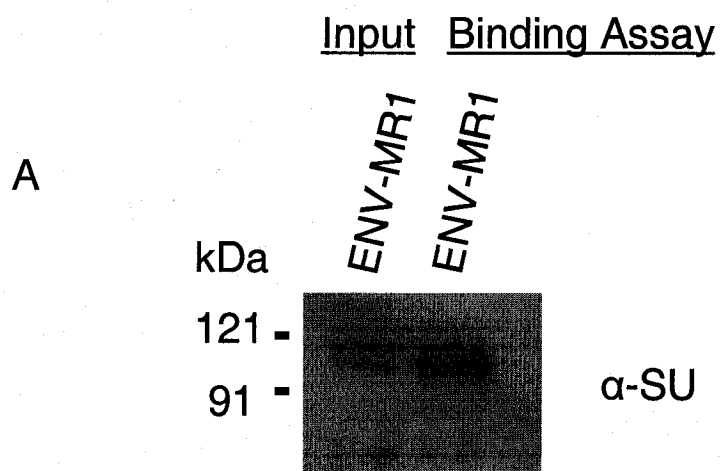


Figure 7: pIX-MR1 binding to EGFRvIII. 293 cells were transiently transfected with plasmids encoding (A) ENV-MR1 and (B) pIX-GFP, pIX-MR1 and pIX-dsRed. Twenty-four hours later, the cells were lysed in RIPA buffer and subjected to the EGFRvIII binding assay. Following the binding assay, the magnetic beads were resuspended in Laemmli sample buffer, subjected to SDS-PAGE and visualized by immunoblot. The captured material was analyzed in (A) with an anti-SU antibody and in (B) with an anti-FLAG M2 antibody. The lanes labeled input represent the cell lysate that was not subjected to the binding assay. The lanes labeled binding assay represent the protein able to bind to the EGFRvIII-like peptide, and immunoprecipitated with streptavidin coated magnetic beads.



described in the Materials and Methods. As shown in Figure 7B pIX-MR1 is able to specifically bind to the EGFRvIII peptide. Neither pIX-GFP nor pIX-dsRed is retained in the pull-down assay and, thus, do not bind to the EGFRvIII peptide. Even though pIX-MR1 is able to bind to the peptide, only a fraction of the total protein used in the experiment is able to do so. The amount of protein loaded in the input lanes represents about 5% of the total cell lysate, while the amount of protein loaded in the binding assay lanes represents approximately 50% of the total cell lysate. Thus, although pIX-MR1 is able to recognize and bind to the EGFRvIII peptide in a similar manner to ENV-MR1, only a fraction of the total pIX-MR1 was able to bind.

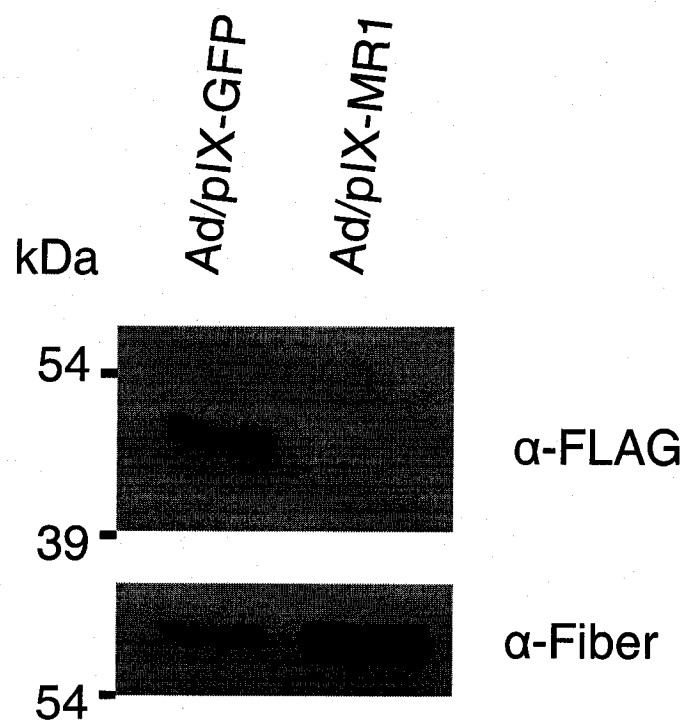
3.2 pIX-MR1 Incorporation into Ad virions

To determine whether pIX-MR1 can be incorporated into mature Ad virions, purified viruses Ad/pIX-GFP and Ad/pIX-MR1 were lysed in Laemmli sample buffer and analyzed by SDS-PAGE/immunoblot for capsid protein content. pIX-GFP is incorporated into Ad virions at a similar efficiency as native pIX and is therefore used as a positive control (Meulenbroek et al., 2004). As shown in Figure 8, pIX-MR1 is not incorporated into Ad virions. The blot was reprobed with an anti-fiber antibody to examine the relative amounts of each virus. A larger number of viral particles was loaded for Ad/pIX-MR1 relative to Ad/pIX-GFP; however even with the higher virion copy number, no band for pIX-MR1 is observed. Therefore, pIX-MR1 is not incorporated into Ad capsids.

3.3 ER-pIX Incorporation into Ad virions

pIX-MR1 bound poorly to EGFRvIII in our immunoprecipitation assay (Figure 7B) and is not incorporated into Ad capsids. During Ad infection, the translation of viral proteins

Figure 8: pIX-MR1 incorporation into adenovirus virions. Purified viruses Ad/pIX-GFP and Ad/pIX-MR1 were heated in Laemmli sample buffer and subjected to SDS-PAGE. The resulting proteins were transferred to a PVDF membrane and probed with an anti-FLAG M2 specific antibody. The membrane was stripped and reprobed with an anti-fiber specific antibody as a loading control (lower panel). The fiber loading control shows that a larger number of viral particles were loaded for Ad/pIX-MR1 compared to Ad/pIX-GFP.



occurs in the cytoplasm and is not associated with the endoplasmic reticulum (ER) (Thomas and Green, 1966; Velicer and Ginsberg, 1968). Thus the translation and folding of pIX-MR1 expressed from a plasmid or Ad/pIX-MR1 occurs in the cytoplasm. The redox state of the cytosol is potentially deleterious to MR1 due to the requirement for intra-chain disulfide bonds, found in the variable heavy and light chains, for proper MR1 folding (Ostergaard et al., 2004). Therefore there may be a need to traffic pIX-MR1 through the oxidative environment of the ER to allow for proper folding. Previous studies have shown that attachment of an ER signal peptide to an scFv can aid in folding of the protein, as judged by its ability to bind (Beerli et al., 1994). Shortly after its translation, the signal peptide interacts with the signal recognition particle (SRP) which causes translational arrest (Walter and Blobel, 1981; Walter and Johnson, 1994). The SRP-nascent polypeptide chain-ribosome complex is targeted to the ER membrane where SRP binds to the SRP receptor, the ribosome weakly interacts with the translocon, and translation resumes into the ER lumen (Gorlich et al., 1992; Kalies et al., 1994). Thus, inclusion of a signal peptide to a protein permits ER-mediated translation.

We investigated whether inclusion of an ER-signal peptide on the N-terminus of pIX-MR1 and subsequent translation within the ER might aid in the proper folding of pIX-MR1. Several ER signal peptides were tested to determine the best candidate: HCMV US11 (SP1), pSecTag2 (SP2), pSecTag2 Met changed to Asp (SP3), and IFN- β (SP4)(Table 1). HCMV US11 is a type I membrane glycoprotein that targets the major histocompatibility complex class I for destruction by the proteasome (Rehm et al., 2001). pSecTag2 is a commercially available expression plasmid, containing the murine Ig κ -chain signal peptide (Invitrogen, Burlington, ON). SP3 is a derivative of pSecTag2 signal peptide, but the methionine of pIX is changed to an asparagine to increase the likelihood of signal peptide cleavage. IFN- β is

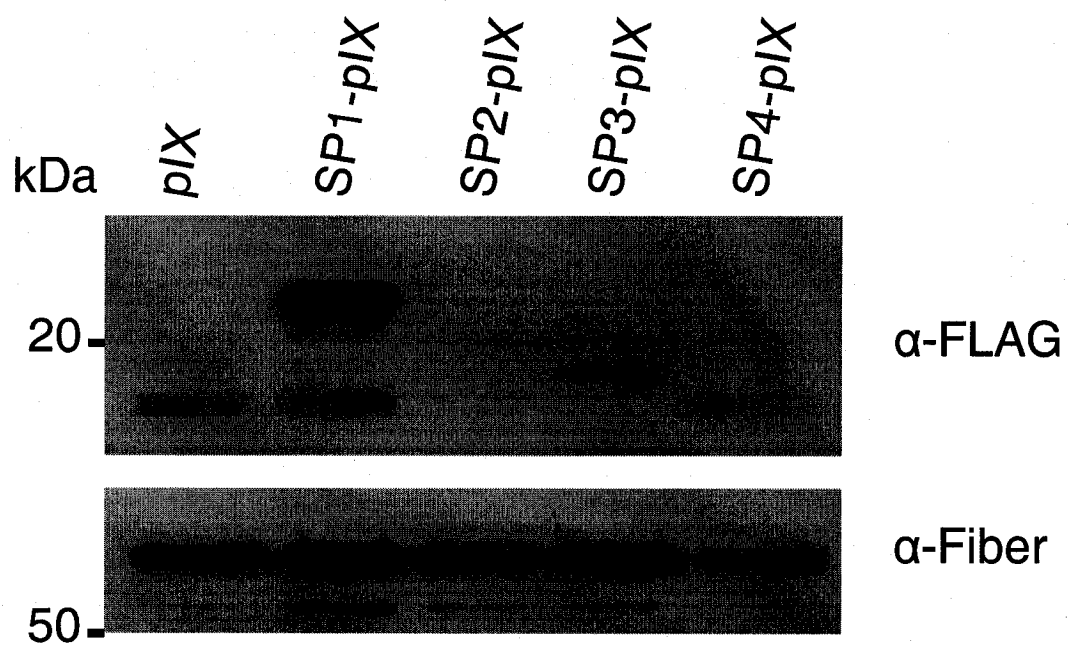
secreted by several cell types in response to viruses or foreign nucleic acids to elicit an inflammatory response, and is known to be post-translationally glycosylated in the ER (Conradt et al., 1987).

Following translation, the signal peptide should be cleaved from pIX by signal peptidase (Blobel and Dobberstein, 1975); this process is important for incorporation of pIX into virions since the N-terminus of pIX is buried within the Ad capsid. If the signal peptide is not cleaved from the protein, it may sterically prevent incorporation of pIX into the capsid. Therefore initial experiments were performed with ER-pIX (pIX with the ER signal peptides SP1-SP4 on the N-terminus and a FLAG tag on the C-terminus) to establish whether the protein was properly incorporated into Ad virions. Purified viruses Ad/pIX-FLAG, Ad/SP1-pIX, Ad/SP2-pIX, Ad/SP3-pIX and Ad/SP4-pIX were lysed in Laemmli sample buffer and visualized by immunoblot. As shown in Figure 9, SP1-pIX, SP3-pIX and SP4-pIX were incorporated into mature virus particles at a similar level to native pIX. However, a larger band is also present for SP1-pIX, which may be a post-translationally modified secondary species. SP3-pIX produces a band slightly larger than pIX-FLAG also potentially indicating a modification to pIX. SP2-pIX produces a band that is visible on over-exposure, and therefore is incorporated at a reduced level compared to pIX-FLAG. Taken together, these data suggest that the ER signal peptides are efficiently cleaved off pIX, leading to proper incorporation into virions. For all future experiments, the ER signal peptide SP4 from IFN- β was utilized as the ER-signal peptide.

3.4 ER-pIX-MR1 binding to EGFRvIII

As shown in Figure 9, rerouting pIX to the ER through inclusion of a signal peptide at the N-terminus still allows for its incorporation into mature Ad virions. We hypothesized

Figure 9: ER-pIX incorporation into adenovirus virions. Purified viruses Ad/pIX-FLAG, Ad/SP1-pIX, Ad/SP2-pIX, Ad/SP3-pIX and Ad/SP4-pIX were heated in Laemmli sample buffer and subjected to SDS-PAGE. The resulting proteins were transferred to a PVDF membrane and probed with an anti-FLAG M2 specific antibody. The membrane was stripped and reprobed with an anti-fiber specific antibody as a loading control (lower panel). The fiber loading control shows that a similar number of viral particles were loaded for each virus.



that inclusion of an ER signal peptide onto the pIX-MR1 protein might enhance the proper folding of MR1, improving its ability to bind to EGFRvIII. To test this hypothesis, 293 cells were transiently transfected with plasmids encoding pIX-GFP, pIX-dsRed, pIX-MR1 and ER-pIX-MR1, and 24hr later subjected to the EGFRvIII binding assay. As shown in Figure 10, ER-pIX-MR1 is retained following the binding assay indicating that it was able to bind to EGFRvIII. There are two bands present for ER-pIX-MR1 in the input lane potentially indicating protein with cleaved and uncleaved signal peptide; the band retained for ER-pIX-MR1 following the binding assay corresponds to the lower band in the input lane determined by a lower exposure immunoblot. In contrast, no protein is retained for non-ER targeted pIX-MR1, pIX-GFP or dsRed. Thus, the addition of an ER signal peptide to pIX-MR1 caused the protein to fold properly, presumably in the ER, and bind the EGFRvIII peptide. Once again, the amount of protein loaded in the input lanes represents about 5% of the total cell lysate, while the amount of protein loaded in the binding assay lanes represents approximately 50% of the total cell lysate. Therefore a large proportion of the total ER-pIX-MR1 protein present in the cell lysate is able to bind to the EGFRvIII peptide.

3.5 ER-pIX-GFP Incorporation into Ad virions

We have shown that inclusion of an ER signal peptide on pIX did not affect the incorporation of pIX in Ad capsids, suggesting the signal peptide is appropriately cleaved from pIX (Figure 9). We next evaluated whether the addition of the ER signal peptide to pIX-GFP would affect its ability to be incorporated into Ad virions. Purified viruses Ad/pIX-GFP and Ad/ER-pIX-GFP were lysed in Laemmli sample buffer and visualized by Western blot. As shown in Figure 11 (top panel), pIX-GFP is incorporated into Ad virions while ER-

Figure 10: ER-pIX-MR1 binding to an EGFRvIII-like peptide. 293 cells were transiently transfected with plasmids encoding pIX-GFP, pIX-MR1 and ER-pIX-MR1. Twenty-four hours later, the cells were lysed in RIPA buffer and subjected to the EGFRvIII binding assay. Following the binding assay, the magnetic beads were resuspended in Laemmli sample buffer, subjected to SDS-PAGE and visualized by immunoblot. The captured material was analyzed with an anti-FLAG M2 antibody. The lanes labeled input represent the cell lysate that was not subjected to the binding assay. The lanes labeled binding assay represent the protein able to bind to the EGFRvIII-like peptide, and immunoprecipitated with streptavidin coated magnetic beads.

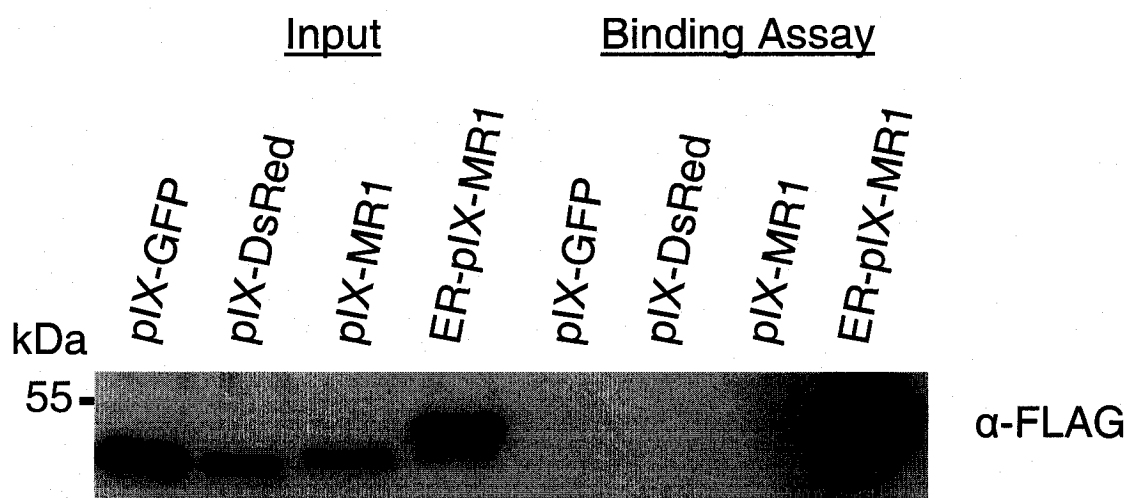
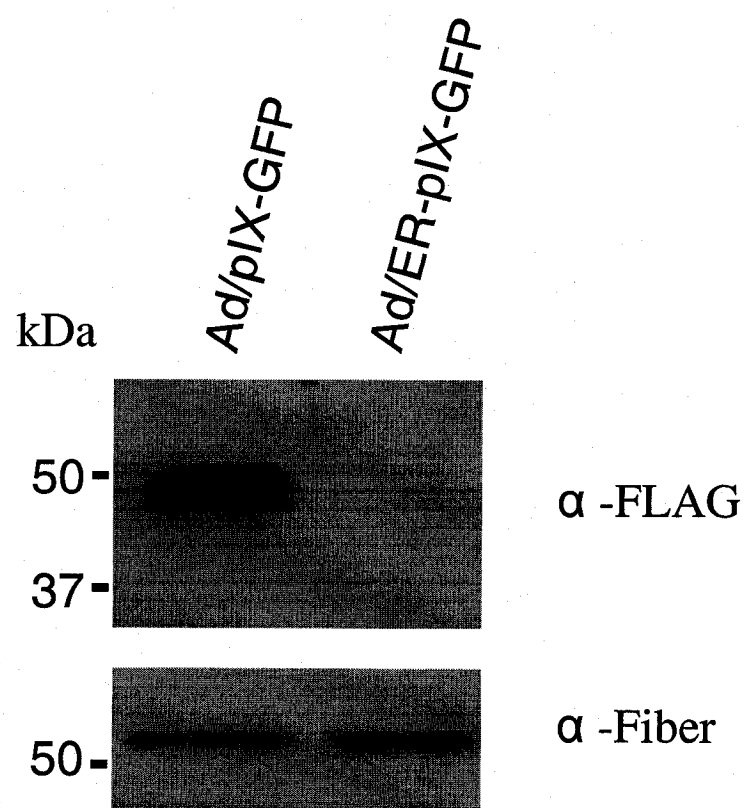


Figure 11: ER-pIX-GFP incorporation into adenovirus virions. Purified viruses Ad/pIX-GFP, and Ad/ER-pIX GFP were heated in Laemmli sample buffer and subjected to SDS-PAGE. The resulting proteins were transferred to a PVDF membrane and probed with an anti-FLAG M2 specific antibody. The membrane was stripped and reprobed with an anti-fiber specific antibody as a loading control (lower panel). The fiber loading control shows that a similar number of viral particles were loaded for each virus.



pIX-GFP is not. Therefore, while pIX incorporation into virions is not affected by a signal peptide, pIX-GFP is.

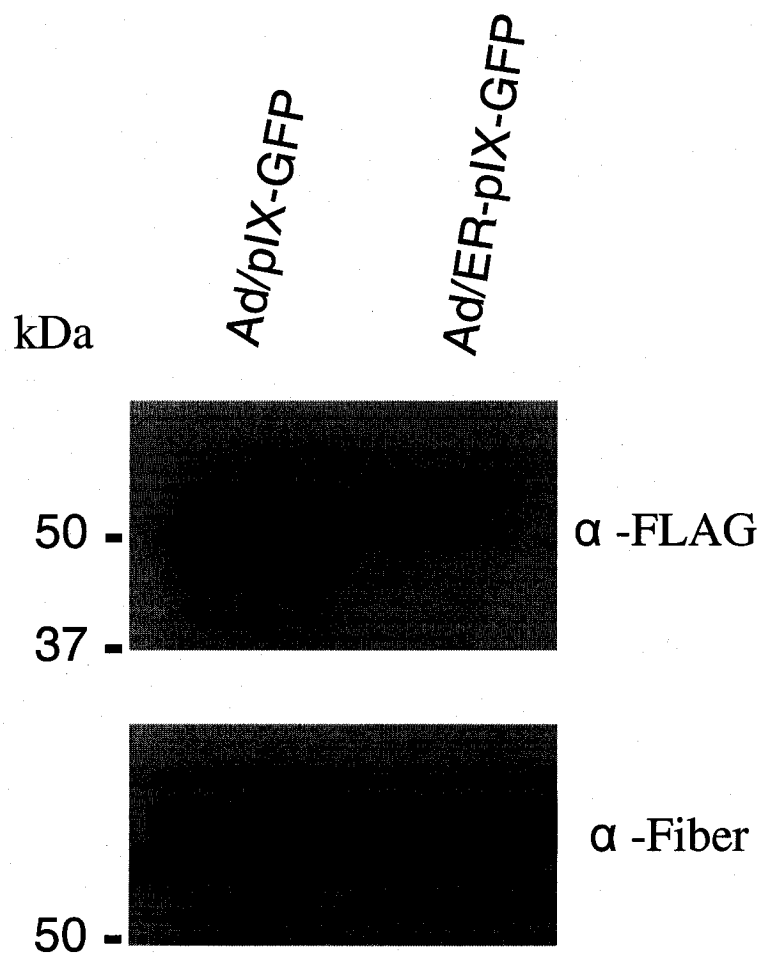
3.6 ER-pIX-GFP Expression During Infection

The addition of the ER signal peptide to pIX-GFP is in some way hindering its incorporation into mature virions. The signal peptide may be leading to an unwanted event such as causing the fusion protein to become trapped in the ER, post-translational modification in the ER, degradation of the fusion protein or secretion of the fusion protein out of the cell. We examined protein levels in 293 cells infected with Ad/pIX-GFP and Ad/ER-pIX-GFP 24 hour post infection. As shown in Figure 12, the relative abundance of ER-pIX-GFP is much lower than pIX-GFP in the infected cells. The expression of ER-pIX-GFP from the virus is only approximately 10% of that observed for Ad/pIX-GFP. The expression of other viral structural proteins from the two viruses was similar since the expression of fiber protein was identical for the two viruses. These data demonstrate that the addition of an ER signal peptide reduced the expression and/or accumulation of ER-pIX-GFP in an infected cell.

3.7 CMV:ER-pIX-GFP Incorporation into Ad virions

As shown in Figure 12, ER-pIX-GFP is present at lower levels than pIX-GFP in infected cells for reasons that are still unclear. ER-pIX-MR1 is also expressed at a lower level compared to pIX-GFP (data not shown). Reduced expression and/or accumulation of ER-pIX-GFP may affect its ability to be incorporated into Ad capsids. To increase the intracellular level of ER-pIX-GFP we placed the fusion gene under the regulation of the high activity CMV promoter. To determine whether increasing the intracellular level of ER-pIX-

Figure 12: ER-pIX-GFP expression during infection. 293 cells were infected at an M.O.I. of 1 with Ad/pIX-GFP and Ad/ER-pIX-GFP. Twenty-four hours later the cells were lysed in Laemmli sample buffer, subjected to SDS-PAGE and visualized by immunoblot with an anti-FLAG M2 specific antibody. The membrane was stripped and reprobed with an anti-fiber specific antibody as a loading control (lower panel). The fiber loading control shows that a similar number of viral particles were loaded for each virus.



GFP leads to increased incorporation of the protein into Ad virions, purified viruses Ad/pIX-GFP and Ad/CMV:ER-pIX-GFP were lysed in Laemmli sample buffer and analyzed by immunoblot. As shown in Figure 13, increasing expression of ER-pIX-GFP in the cell through the use of the CMV promoter results in improved incorporation. This incorporation is a dramatic improvement compared to the incorporation of ER-pIX-GFP without the CMV promoter (Figure 12). Therefore, replacement of the native pIX promoter with the CMV promoter significantly improved the incorporation of ER-pIX-GFP into Ad virions.

3.8 CMV:ER-pIX-GFP and CMV:ER-pIX-MR1 Expression During Infection

Replacing the protein IX promoter with the CMV promoter resulted in improved incorporation of ER-pIX-GFP into mature virions (Figure 13). We next investigated whether replacing the native pIX promoter with the CMV promoter also resulted in incorporation of ER-pIX-MR1. First, we determined whether use of the CMV promoter resulted in intracellular levels of ER-pIX-GFP and ER-pIX-MR1 similar to pIX-GFP. 293 cells were infected with Ad/pIX-GFP, Ad/CMV:ER-pIX-GFP or Ad/CMV:ER-pIX-MR1, lysed in Laemmli sample buffer and visualized by immunoblot. The quantity of CMV:ER-pIX-GFP and CMV:ER-pIX-MR1 is almost identical to the quantity of pIX-GFP (Figure 14). These data indicate that there is increased expression of ER targeted protein from the CMV promoter relative to the pIX promoter.

We have demonstrated that replacing the pIX promoter with the CMV promoter leads to intracellular levels of ER-pIX-MR1 similar to pIX-GFP. We next examined whether ER-pIX-MR1 produced from the CMV promoter was still able to bind EGFRvIII peptide in our binding assay since overexpression within the ER may adversely affect protein folding. 293 cells were infected at an M.O.I of 1 with Ad encoding CMV:ER-pIX-GFP or CMV:ER-pIX-

Figure 13: CMV:ER-pIX-GFP incorporation into Ad virions. Purified viruses Ad/pIX-GFP, and Ad/CMV:ER-pIX GFP were heated in Laemmli sample buffer and subjected to SDS-PAGE. The resulting proteins were transferred to a PVDF membrane and probed with an anti-FLAG M2 specific antibody. The membrane was stripped and reprobed with an anti-fiber specific antibody as a loading control (lower panel). The fiber loading control shows that a similar number of viral particles were loaded for each virus.

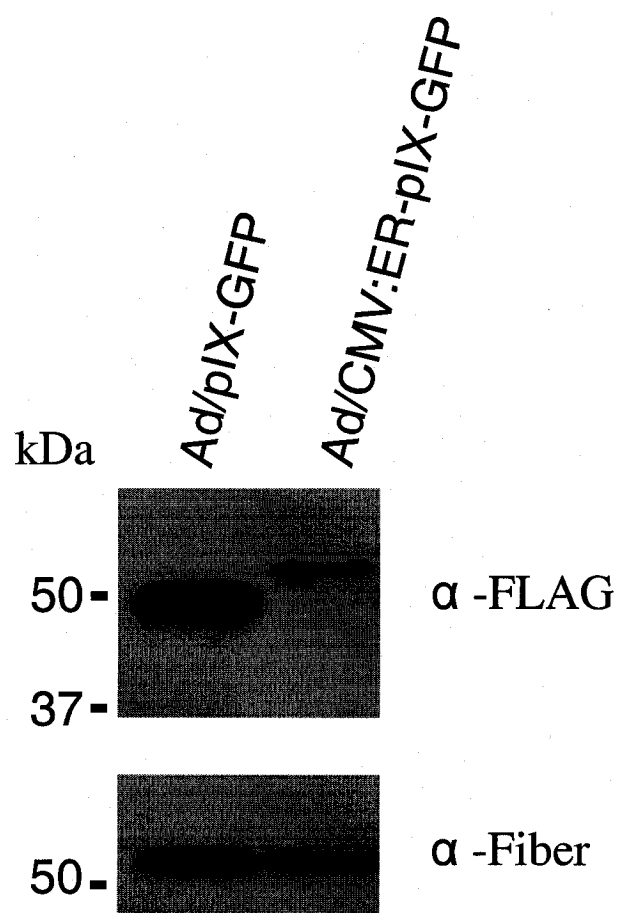
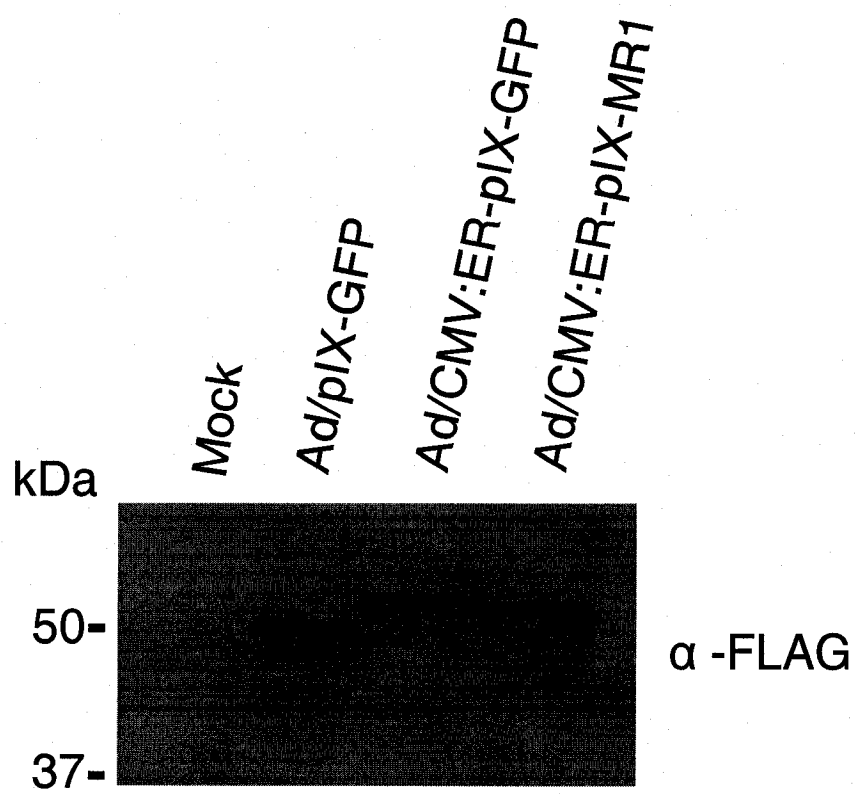


Figure 14: CMV:ER-pIX-GFP and CMV:ER-pIX-MR1 expression during Ad infection. 293 cells were infected at an M.O.I. of 1 with Ad/pIX-GFP, Ad/CMV:ER-pIX-GFP and Ad/CMV:ER-pIX-MR1. Twenty-four hours later the cells were lysed in Laemmli sample buffer, subjected to SDS-PAGE and visualized by immunoblot with an anti-FLAG M2 specific antibody.



MR1, and 24 hours later, subjected to the EGFRvIII binding assay as previously described. Figure 15 illustrates that CMV:ER-pIX-MR1 is able to specifically bind to the EGFRvIII peptide, while no binding is observed for CMV:ER-pIX-GFP. Taken together, these data indicate that use of the CMV promoter to drive expression of ER-targeted pIX-MR1 allows for higher level of protein expression/accumulation in the cell, and this protein is functional and able to bind EGFRvIII.

3.9 CMV:ER-pIX-MR1 Incorporation into Ad virions

We have shown CMV:ER-pIX-GFP and CMV:ER-pIX-MR1 are present at similar levels to pIX-GFP in infected cells and CMV:ER-pIX-GFP is incorporated into Ad virions (Figure 13 and 14). To determine whether CMV:ER-pIX-MR1 is incorporated into Ad virions, purified viruses Ad/pIX-GFP, Ad/CMV:ER-pIX-GFP and Ad/CMV:ER-pIX-MR1 were lysed in Laemmli sample buffer and visualized by immunoblot. As shown in Figure 16, CMV:ER-pIX-MR1 is not incorporated into Ad virions while pIX-GFP and CMV:ER-pIX-GFP are incorporated. CMV:ER-pIX-GFP and CMV:ER-pIX-MR1 utilize the same promoter to express the pIX fusion proteins and contain the same ER signal sequence. These data suggest there must be some other fundamental difference responsible for the exclusion of ER-pIX-MR1 from Ad virions.

3.10 CMV: ER-pIX-GFP and CMV: ER-pIX-MR1 Protein Localization

We have shown that CMV:ER-pIX-GFP is incorporated into mature Ad virions, while CMV:ER-pIX-MR1 is not (Figure 16). The nucleus is the site of virion assembly during Ad infection (Velicer and Ginsberg, 1970). If the pIX-MR1 fusion protein is unable to enter the nucleus, this may explain its absence from mature virions. Both ER-pIX-GFP and

Figure 15: CMV:ER-pIX-MR1 binding to EGFRvIII. 293 cells were infected with viruses Ad/CMV:ER-pIX-GFP and Ad/CMV:ER-pIX-MR1. Twenty-four hours later, the cells were lysed in RIPA buffer and subjected to the EGFRvIII binding assay. Following the binding assay, the magnetic beads were resuspended in Laemmli sample buffer, subjected to SDS-PAGE and visualized by immunoblot. The captured material was analyzed with an anti-FLAG M2 antibody. The lanes labeled input represent the cell lysate that was not subjected to the binding assay. The lanes labeled binding assay represent the protein able to bind to the EGFRvIII-like peptide, and immunoprecipitated with streptavidin coated magnetic beads.

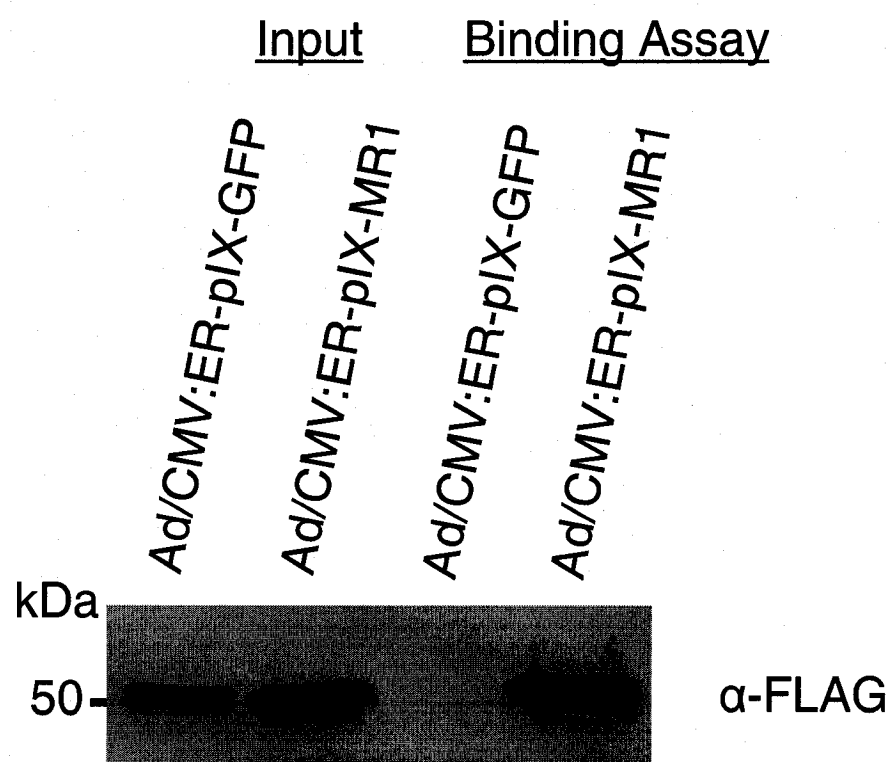
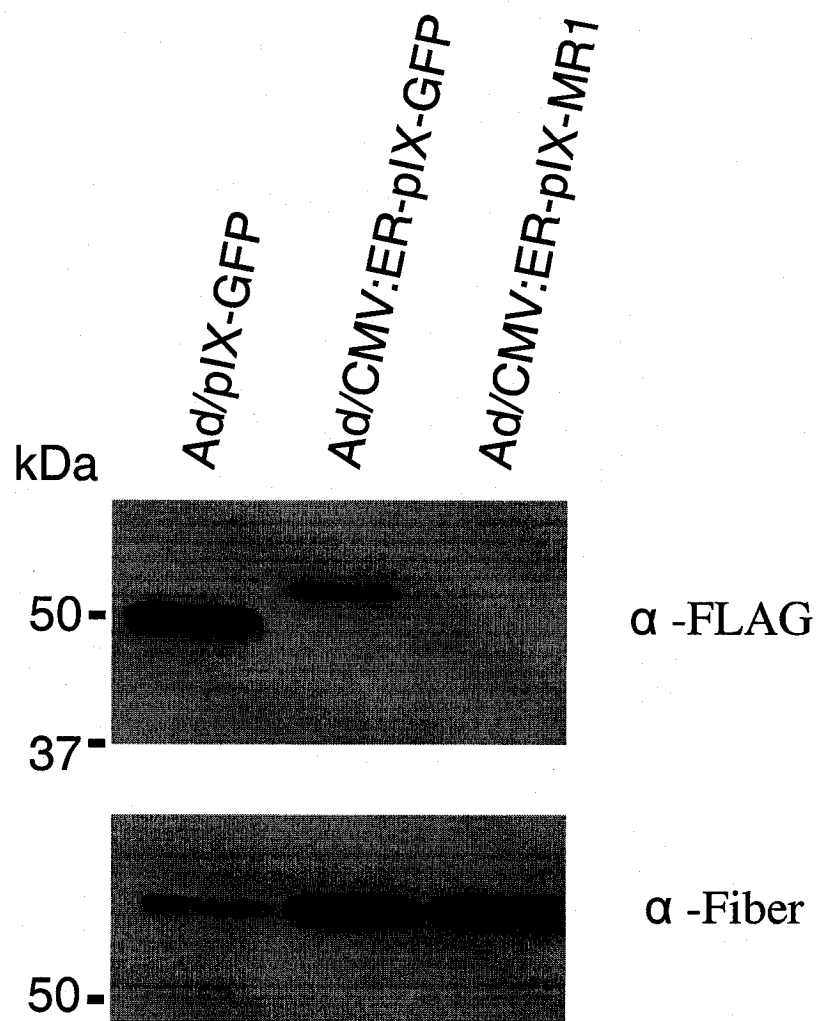


Figure 16: CMV:ER-pIX-MR1 incorporation into Ad virions. Purified viruses Ad/pIX-GFP, Ad/CMV:ER-pIX GFP and Ad/CMV ER-pIX-MR1 were heated in Laemmli sample buffer and subjected to SDS-PAGE. The resulting proteins were transferred to a PVDF membrane and probed with an anti-FLAG M2 specific antibody. The membrane was stripped and reprobed with an anti-fiber specific antibody as a loading control (lower panel). The fiber loading control shows that a larger number of viral particles were loaded for CMV:ER-pIX-GFP and CMV:ER-pIX-MR1 compared to pIX-GFP.



ER-pIX-MR1 contain NLS sequences, however, in the case of MR1, this might not be sufficiently strong to force nuclear localization of the pIX-MR1 protein. To determine the intracellular location of CMV:ER-pIX-GFP and CMV:ER-pIX-MR1 during infection, 293 cells were infected with CMV:ER-pIX-GFP and CMV:ER-pIX-MR1 at an M.O.I. of 10 and 24 hours later analyzed by immunocytochemistry. As shown in Figure 17, CMV:ER-pIX-GFP appears to be primarily located in the nucleus of infected cells; however, CMV:ER-pIX-MR1 appears to be excluded from the nucleus. These data suggest that the absence of ER-pIX-MR1 from the nucleus may be the cause of its exclusion from mature Ad virions.

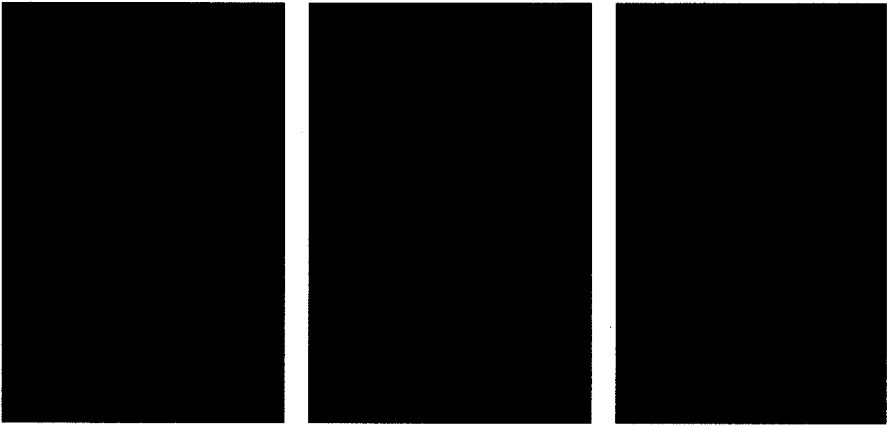
3.11 CMV: ER-pIX-MR1-TatNLS Expression and Protein Localization

The nucleus is the site of Ad assembly, and the attachment of an scFv to pIX may hinder its ability to enter the nucleus (Velicer and Ginsberg, 1970). During Ad infection, pIX is able to enter the nucleus (Rosa-Calatrava et al., 2003). However the attachment of an scFv to pIX will greatly increase its size and may hinder its nuclear translocation. As a result, the attachment of a nuclear localization sequence (NLS) to pIX-MR1 may be used to shuttle the fusion protein into the nucleus. Although our fusion proteins do contain a C-terminal NLS, it may not be sufficiently strong to force nuclear accumulation of pIX-MR1.

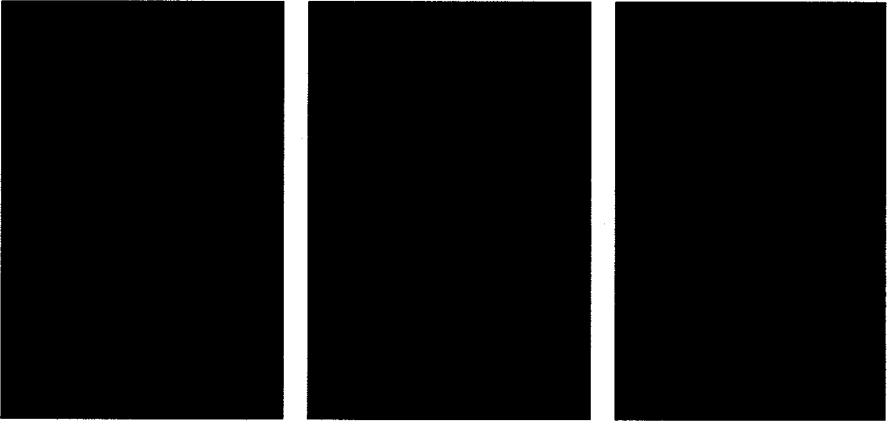
Previous studies have shown that the basic NLS of HIV-1 Tat is sufficient to allow efficient transduction of Tat-GFP to the nucleus of different cell types (Ryu et al., 2003; Stauber and Pavlakis, 1998; Yang et al., 2002). Additionally, the Tat protein transduction domain containing the NLS fused to HSV-TK, Bcl-rambo, and Smac/DIABLO allowed for efficient transduction of the fusion proteins when applied to the outside of cells (Yang et al., 2002). Tat-NLS has even been used to traffic an anti-Tat single-chain antibody to the nucleus (Theisen et al., 2006). We therefore generated a construct with the TatNLS on the C-

Figure 17: CMV:ER-pIX-GFP and CMV:ER-pIX-MR1 intracellular location during infection. 293 cells were infected at an M.O.I. of 10 with viruses Ad/CMV:ER-pIX-GFP and Ad/CMV:ER-pIX-MR1. Twenty-four hours later the cells were fixed, stained with Hoechst (blue) and the GFP fluorescence (green) was visualized by microscopy for CMV:ER-pIX-GFP. CMV:ER-pIX-MR1 location was visualized using an anti-FLAG M2 specific primary antibody and FITC-conjugated goat anti-mouse secondary antibody (red). FITC fluorescence was visualized by microscopy.

Ad/CMV:ER-pIX-GFP



Ad/CMV:ER-pIX-MR1



terminus of ER-pIX-MR1. To examine the expression of ER-pIX-MR1-TatNLS relative to our other pIX fusion proteins, 293 cells were transiently transfected with plasmids encoding pIX-GFP, pIX-MR1, ER-pIX-MR1 and ER-pIX-MR1-TatNLS, lysed in Laemmli sample buffer and visualized by immunoblot. Cotransfection of an RFP expressing plasmid confirmed equal transfection efficiency for all plasmids (data not shown). As shown in Figure 18, the addition of the Tat NLS did not adversely affect the expression of the fusion protein. There is however lower expression of ER-pIX-MR1 and ER-pIX-MR1-TatNLS relative to pIX-GFP and pIX-MR1. We have previously shown that the addition of an ER signal peptide to pIX-GFP resulted in lower expression and/or accumulation during infection (Figure 12). Therefore the presence of the ER signal peptide may be leading to lower expression and/or accumulation of ER-pIX-MR1 and ER-pIX-MR1-TatNLS compared to pIX-GFP and pIX-MR1.

We have shown that CMV:ER-pIX-MR1 appears to be excluded from the nucleus during Ad infection (Figure 17). To determine whether there was increased nuclear localization of CMV:ER-pIX-MR1-TatNLS, 293 cells were transiently transfected with plasmids encoding pIX-FLAG, pIX-MR1, ER-pIX-MR1 and ER-pIX-MR1-TatNLS, and analyzed by immunocytochemistry. pIX-FLAG is localized throughout the cell, including the nucleus, while pIX-MR1 and ER-pIX-MR1 appear to be almost exclusively cytoplasmic (Figure 19). ER-pIX-MR1-TatNLS appears to be localized primarily in the cytoplasm; however, inclusion of the TatNLS does allow some of the pIX-MR1 to reach the nucleus (compare ER-pIX-MR1 to ER-pIX-MR1-TatNLS, Figure 19). Thus, inclusion of the TatNLS on the C-terminus of ER-pIX-MR1 allows for at least a portion of the fusion protein to reach the nucleus. Therefore inclusion of TatNLS to CMV:ER-pIX-MR1 may allow enhanced incorporation in the Ad capsid. Taken together, the use of an ER signal peptide and Tat

Figure 18: CMV:ER-pIX-MR1-TatNLS expression. 293 cells were transiently transfected with plasmids encoding pIX-GFP, pIX-MR1, ER-pIX-MR1 and ER-pIX-MR1-TatNLS. Twenty-four hours later the cells were lysed in Laemmli sample buffer, subjected to SDS-PAGE and visualized by immunoblot with an anti-FLAG M2 specific antibody.

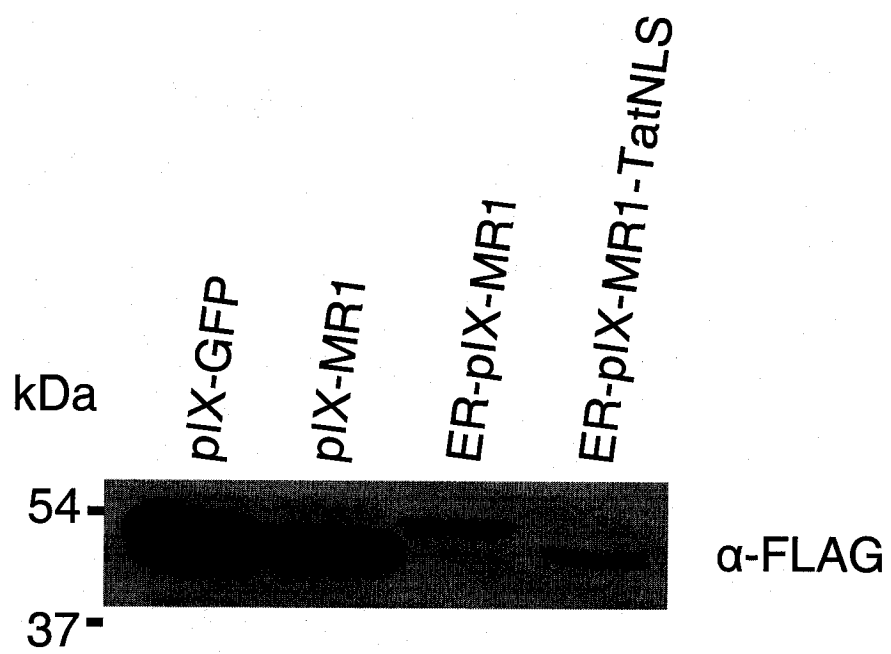


Figure 19: CMV:ER-pIX-MR1-TatNLS protein localization. 293 cells were transiently transfected with plasmids encoding pIX-FLAG, pIX-MR1, ER-pIX-MR1 and ER-pIX-MR1-TatNLS. Twenty-four hours later the cells were fixed, stained with Hoechst (blue) and incubated with an anti-FLAG M2 specific primary antibody and FITC-conjugated goat anti-mouse secondary antibody (green). FITC fluorescence was visualized by microscopy.

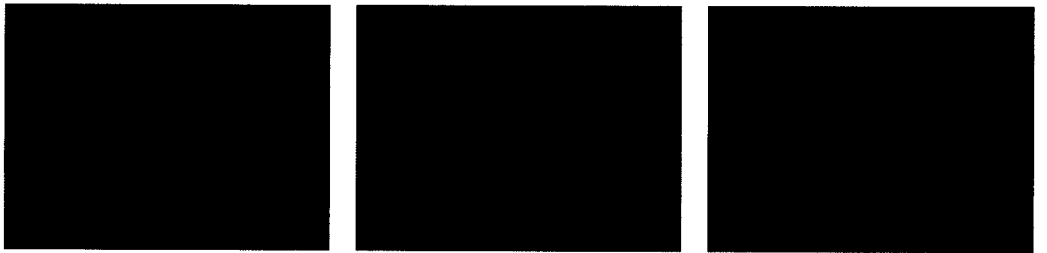
pIX-FLAG



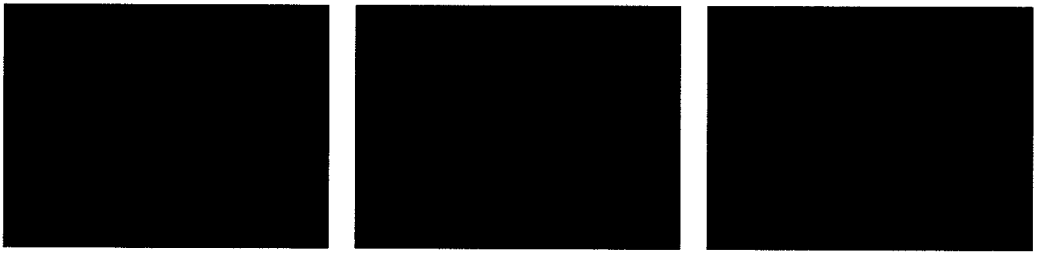
pIX-MR1



ER-pIX-MR1



ER-pIX-MR1-TatNLS



nuclear localization sequence may allow for the production of Ad viruses containing pIX-MR1 thereby allowing tumour specific infection.

Infectious plasmids were constructed with CMV:ER-pIX-MR1-TatNLS in combination with several transgenes: no transgene, Ubc mSEAP, PGK mSEAP or CMV-GFP. Unfortunately, numerous transfections of the plasmids into 293 cells failed to recover virus encoding CMV:ER-pIX-MR1-TatNLS. Therefore an element of the infectious plasmids containing CMV:ER-pIX-MR1-TatNLS may be toxic to the productive completion of the Ad life cycle.

Chapter 4 - Discussion

Gene therapy has the potential to aid in the treatment of a variety of disorders through the introduction of a therapeutic gene to a diseased tissue. There are two categories of gene delivery vehicles, non-viral and viral vectors. The non-viral vectors include direct injection of DNA into a cell, and mixing the DNA with an agent such as polylysine or cationic lipids to facilitate membrane permeabilization. Viral vectors include retrovirus, lentivirus, adeno-associated virus, adenovirus and to a lesser extent herpes virus and poxvirus. Viruses are excellent tools for gene delivery because they have evolved over millions of years to efficiently deliver their genome to cells and, in most cases, can be disabled of pathogenic effects. Ad possesses numerous features that make it an excellent vector for gene therapy, including: a large cloning capacity, ease of genetic manipulation, ability to grow to high titer, and the capability of infecting numerous human cell types. However, it is the ability of Ad to infect numerous cell types that is also a disadvantage for targeted gene therapy. At the present time the specific delivery of gene therapy vectors, including Ad, to a diseased cell type, such as cancer cells, cannot be achieved through systemic delivery of the virus. Therefore, in recent years there has been significant interest in investigating various methods of retargeting Ad through capsid modifications and the non-covalent attachment of targeting ligands.

The majority of Ad retargeting studies have involved modification of the Ad fiber protein. The native route of attachment for Ad involves the interaction of the knob domain of fiber with CAR (Bergelson et al., 1997). Therefore several groups have utilized the knob domain as a location for incorporation of targeting motifs. A specific region of the knob domain, between the H and I loops, has been shown to accommodate the insertion of

numerous ligands including FLAG, RGD and pK (Dmitriev et al., 1998; Koizumi et al., 2003; Krasnykh et al., 2001; Krasnykh et al., 1998; Mizuguchi et al., 2001; Wickham et al., 1997). Non-covalent modifications of Ad vectors for retargeting have employed the use of bi-specific adapters where one end binds to the Ad virion, while the other binds to the desired ligand. Bi-specific adaptors have been shown to work effectively in altering the Ad tropism (Haisma et al., 2000; Korn et al., 2004; Wickham et al., 1996b), however, concerns have been raised regarding the degree of modification between different viral preparations, resulting in batch-to-batch variations. Retargeting Ad through modifications to fiber have been successful; however, these modifications have involved small, non-specific ligands. Other Ad proteins, including pIX, may be good candidates for the incorporation of larger, more specific targeting ligands, such as single-chain antibodies (scFv).

Protein IX has been utilized for the retargeting of Ad through the incorporation of small targeting motifs, including pK, MYC and RGD (Dmitriev et al., 2002; Vellinga et al., 2004). Large molecules, including GFP and HSV-1 TK have been attached to pIX without affecting Ad titer or infectivity (Li et al., 2005; Meulenbroek et al., 2004; Zakhartchouk et al., 2004). Therefore, we investigated the use of pIX as a platform to incorporate the scFv MR1 into the capsid of Ad. MR1 is specific for EGFRvIII which is found only on cancer tissue, but never on normal tissue. Previous studies have used MR1 to successfully retarget MLV, ALV and measles virus (Lorimer and Lavictoire, 2000; Nakamura et al., 2005; Snitkovsky et al., 2000). We have demonstrated that pIX can be fused to MR1, the fusion protein is expressed and is the expected molecular weight (Figure 5). However pIX-MR1 binding to EGFRvIII was shown to be poor, and a virus was rescued, but no pIX-MR1 was present in the capsid (Figure 6 and 7).

The fusion of MR1 to pIX may affect the ability of MR1 to properly fold and bind EGFRvIII. Inserting ligands into viral capsid proteins may result in improper binding when the ligand is translated into the heterologous structure of the viral protein (Ghosh and Barry, 2005). Previous studies have shown that the addition of a C-terminal FLAG tag to DGK- ζ blocks its interaction with its binding partner, the syntrophin PDZ domain (Hogan et al., 2001). The addition of the FLAG epitope tag completely altered the binding domain of DGK- ζ . Therefore, the proper folding of MR1 may be hindered by the presence of pIX attached to its C-terminus, imposing constraints upon forming the correctly folded structure. However, when we included an ER peptide on the pIX-MR1 fusion protein, MR1 folded correctly as judged by its ability to bind an EGFRvIII peptide (see below).

The translation of Ad proteins occurs in the reducing environment of the cytoplasm and may not allow cysteine bonds to form during MR1 folding (Ostergaard et al., 2004). Thus, the non-native routing imposed on MR1 by Ad capsid incorporation likely confounds proper scFv folding, thus perturbing the structural configuration required for antigen recognition (Hedley et al., 2006). Studies involving the retargeting of Ad through fusion of an scFv to the knob domain of fiber have also reported a failure of the scFv to bind its ligand properly (Magnusson et al., 2002). The authors concluded that the reducing environment of the cytoplasm is likely deleterious to the proper folding of scFv. In subsequent studies, cytoplasmically stable ligands, called affibodies (Nord et al., 1997), were incorporated into fiber and successfully used for retargeting (Henning et al., 2002; Hong et al., 2003).

Previous studies utilizing MR1 for viral retargeting involved linkage of the scFv to envelope glycoproteins. Lorimer and Lavictoire (2000) fused MR1 to the envelope glycoprotein (ENV) of MLV in order to target the retrovirus to cancer cells expressing EGFRvIII. Snitkovsky et al. (2000) utilized a bi-specific fusion protein composed of MR1

and the extracellular domain of the TVA receptor for subgroup A avian leukosis virus (ALV) in order to enhance infection of cells expressing EGFRvIII by subgroup A ALV. Nakamura et al. (2005) incorporated MR1 into the H protein of live-attenuated measles virus of the Edmonston lineage, to target oncolytic measles virus to cancer cells expressing EGFRvIII. The translation of ENV, the TVA receptor, and the H protein occurs in the endoplasmic reticulum. All of the viruses were able to bind cells that expressed EGFRvIII (Lorimer and Lavioire, 2000; Nakamura et al., 2005; Snitkovsky et al., 2000). Therefore the translation of MR1 in the oxidative environment of the ER promoted proper folding and subsequently binding to EGFRvIII.

To promote proper folding of pIX-MR1, an ER signal peptide was utilized to send the fusion protein to the ER during translation. Previous studies have employed ER signal sequences to direct the expression and folding of scFv's to the ER. Three different scFv's specific for the hapten DNP, human transferrin receptor and the murine CD3- ϵ chain were expressed and secreted from the ER through the use of an ER-signal sequence (Jost et al., 1994). All three scFvs were able to specifically bind to their respective antigens in an immunoprecipitation experiment. The binding of the scFvs to their respective targets was inhibited by incubation with a competing antibody, confirming the specificity of interaction. (Jost et al., 1994). Similar findings were reported by Beerli et al. (1994) related to proper folding of two scFvs with ER-signal sequences specific for the cellular receptor ErbB2. The intracellularly expressed scFvs contained an N-terminal sequence directing their expression to the lumen of the ER in order to inhibit ErbB2 transport to the cell surface (Beerli et al., 1994). The addition of an ER signal peptide to pIX-MR1 resulted in increased binding to the EGFRvIII peptide relative to pIX-MR1 alone (Figure 9). Therefore sending pIX-MR1 to the ER allowed MR1 to fold correctly and bind to the EGFRvIII peptide. Future studies using

scFv fusion to pIX should therefore employ an ER signal peptide to facilitate proper folding of the scFv.

Our data with ER-pIX suggests that the ER signal peptide was cleaved off pIX since pIX was incorporated into the Ad capsid (Figure 8). However ER-pIX-GFP was not incorporated into mature virions (Figure 10). Subsequent experiments showed that the relative abundance of ER-pIX-GFP is much lower than pIX-GFP in the infected cells (Figure 11). ER-pIX-MR1 is also expressed at a lower level compared to pIX-GFP (data not shown). pIX is not normally trafficked through the ER, therefore the increased protein load may be causing stress on the cell leading to protein degradation; ER-pIX-MR1 and ER-pIX-GFP are much larger than ER-pIX and may become trapped in the ER, therefore unavailable for virion formation; additionally, shuttling out of the cell may be occurring, since many ER proteins are cell surface proteins.

To compensate for the loss of intracellular ER-pIX-MR1, the native pIX promoter was replaced with the highly active cytomegalovirus (CMV) immediate early promoter. CMV:ER-pIX-GFP and CMV:ER-pIX-MR1 were expressed at similar levels to pIX-GFP during Ad infection (Figure 13); however, CMV:ER-pIX-GFP was incorporated into mature virions, while CMV:ER-pIX-MR1 was not (Figure 15). Subsequent investigation of the intracellular location of CMV:ER-pIX-GFP and CMV:ER-pIX-MR1 during infection revealed that CMV:ER-pIX-GFP is able to enter the nucleus, while CMV:ER-pIX-MR1 is not (Figure 16).

The nucleus is the site of viral assembly and the lack of ER-pIX-MR1 in the nucleus may explain its absence from mature virions. It is necessary for all Ad proteins to enter the nucleus in order for the proteins to be incorporated into the Ad capsid. Native protein IX is able to enter the nucleus during normal virus infection, and forms distinct clear amorphous

structures (Rosa-Calatrava et al., 2001; Rosa-Calatrava et al., 2003). The addition of MR1 to pIX may be hindering its ability to enter the nucleus. pIX-MR1 contains a nuclear localization sequence (NLS) from the simian virus 40 large tumour antigen (SV40 T-antigen), however this was not sufficient to allow nuclear localization (Figure 16). Previous studies have reported the use of the SV40 T-antigen NLS to shuttle a cytoplasmically stable scFv bound to β -galactosidase from the cytoplasm to the nucleus (Sibler et al., 2003). Although the study by Sibler et al. (2003) did not involve an scFv fusion protein, the study demonstrated the NLS-mediated nuclear import of an scFv-protein complex. Sibler et al. (2003) reported the total relocation of the cytoplasmically expressed scFv bound to β -galactosidase to the nucleus, while our studies demonstrated minimal nuclear localization. The addition of the basic nuclear localization sequence from the HIV-1 Tat protein resulted in improved, but not complete, nuclear localization of CMV:ER-pIX-MR1-TatNLS relative to CMV:ER-pIX-MR1 (Figure 18); yet a virus could not be recovered with CMV: ER-pIX-MR1-TatNLS. Infectious plasmids were constructed with CMV:ER-pIX-MR1-TatNLS in combination with several transgenes: no transgene, Ubc mSEAP, PGK mSEAP or CMV-GFP. A virus was recovered containing the GFP expression cassette. Restriction digestion of the packaged genome, revealed that a DNA rearrangement had occurred. A recombination event occurred between the bovine growth hormone poly-adenylation signal (BGH-pA) following the GFP expression cassette and the BGH-pA following CMV: ER-pIX-MR1-TatNLS. In this rearranged genome, the protein IX fusion protein was excised from the genome, leaving only the GFP transgene. No virus was obtained from the other plasmids with CMV:ER-pIX-MR1-TatNLS, therefore no testing could be completed on the virus itself, or its packaged genome to determine why the viruses could not be recovered. A possible explanation for the inability to recover a virus with CMV:ER-pIX-MR1-TatNLS

may be that the fusion protein is hindering the nuclear import of other viral proteins, thereby impeding the formation of Ad virions. A small proportion of the expressed ER-pIX-MR1-TatNLS is able to enter the nucleus (Figure 18); however, the majority of the fusion protein remains outside of the nucleus. The non-nuclear ER-pIX-MR1-TatNLS may compete for the nuclear import machinery with other Ad proteins, thereby blocking their nuclear entry. Thus, the exclusion of Ad proteins from the nucleus may lead to the inability to recover Ad virions coding for CMV:ER-pIX-MR1-TatNLS.

4.1 Future Directions

To further the development of an Ad vector with pIX-MR1, several strategies may be employed. The expression cassette for CMV:ER-pIX-MR1-TatNLS could be placed in the Ad E3 region instead of the current pIX region, to express the fusion protein at a different point in the viral life cycle. Carette et al. (2005) inserted a firefly luciferase expression cassette containing a splice acceptor site in the Ad E3 region in order to express the transgene at a late point in the Ad lifecycle, under the regulation of the major late promoter (MLP). The authors verified through PCR and Western blot analysis that the expression of luciferase followed the pattern of the late gene penton, indicating proper splicing of the inserted splice-acceptor site. Additionally, the expression of luciferase with the introduced splice-acceptor was much higher during late stages of infection than a control virus containing the luciferase expression cassette in the E1 region under the control of the CMV promoter. Therefore, placing CMV:ER-pIX-MR1-TatNLS in the Ad E3 region along with the splice-acceptor may facilitate increased expression of the fusion protein at a later time in the Ad lifecycle, leading to incorporation of pIX-MR1 into mature Ad virions.

The MR1 scFv may also be changed to an intrinsically stable scFv. Utilizing an scFv that is stable in the cytoplasm would abrogate the need to use an ER-signal peptide to attain proper folding. We have shown that the intracellular levels of ER-pIX-MR1 and ER-pIX-GFP are lower than pIX-GFP during Ad infection (Figure 11 and data not shown). Therefore the relative abundance of the pIX-scFv fusion protein may be improved in the absence of an ER-signal sequence. Increased intracellular pIX-scFv may lead to increased incorporation into Ad virions. An intrinsically stable scFv was isolated for the anti-viral antibody F8 and was functionally expressed in the cytoplasm of *E.coli* and transgenic plants; the scFv was shown to possess a remarkable *in vivo* half-life and the ability to refold *in vitro* under reducing conditions (Tavladoraki et al., 1999). Using the scFv as a scaffold, Desiderio et al. (2001) created a phage display library by combinatorially mutating amino acid residues in the complementary determining regions of both the heavy and light chains of the scFv. The resultant library could be panned to isolate an scFv against EGFRvIII, or other cell-surface markers (Desiderio et al., 2001). Taken together, placing the pIX-MR1 expression cassette in the E3 region and intrinsically stable scFv may allow for the production of Ad viruses containing pIX-MR1.

4.2 Conclusions

Although an Ad containing pIX-MR1 was not rescued and shown to target the virus to cells expressing EGFRvIII, several advances have been made in the development of the vector. The addition of an ER signal peptide to pIX-MR1 resulted in increased binding to the EGFRvIII peptide relative to pIX-MR1 alone. The replacement of the native pIX promoter with the CMV promoter resulted in increased expression of ER-pIX-GFP and ER-pIX-MR1; the increased expression resulted in improved incorporation of ER-pIX-GFP into Ad virions.

However, use of the CMV promoter did not lead to incorporation of ER-pIX-MR1. Finally, the addition of the basic nuclear localization sequence from the HIV-1 tat protein increased proportion of ER-pIX-MR1 in the nucleus. Unfortunately, an Ad could not be rescued with ER-pIX-MR1-TatNLS.

If pIX can be used as a platform to retarget Ad through MR1 then, theoretically, other single-chain antibodies could be used for retargeting purposes. The technology exists to isolate single-chain antibodies specific for cell surface markers uniquely found on cancerous or other diseased cells. The result would be a tailored treatment for a particular acquired genetic disease.

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Chapter 6 - Appendix

6.1 General Solutions

6.11 General Chemicals and Reagents

1M Tris-HCL pH8.0

1 x TE: 10mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0

10M NaOH

5M NaCl

20% (w/v) SDS

0.5M EDTA pH 8.0

6.12 Cell Culture

Low passage 293 cells (Microbix, Toronto, Canada, Cat. No. PD-02-01)

Minimum essential medium (MEM)

Complete medium: MEM, 10% FBS, 1% antibiotic/antimycotic, 1% Glutamax

Maintenance medium: MEM, 5% FBS, 1% antibiotic/antimycotic, 1% Glutamax

1 x PBS pH 7.4: 1.06 mM KH_2PO_4 , 155.17mM NaCl, 2.97 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$

10 x Citric Saline: 1.35 M KCl, 150mM $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$

1x Citric Saline: 135mM KCl, 15mM $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$

SuperFect[®] transfection reagent (Qiagen)

Overlay Medium: 1 x MEM, 5% FBS, 1% antibiotic/antimycotic, 2% yeast extract, 0.5% agarose

4% sucrose (w/v) in PBS

6.13 Cloning and Plasmid DNA Preparation

Solution I: 50 mM Dextrose, 250mM Tris-HCl pH7.5, 10mM EDTA pH 8.0

Solution II: 200 mM NaOH, 1% Sodium dodecyl sulfate

Solution III: 3 M $\text{KC}_2\text{H}_3\text{O}_2$, 11.5% glacial acetic acid

10 x SSC: 1.5M NaCl, 50 mM $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$

0.1 x SSC: 15mM NaCl, 0.5 mM $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$

Loading Buffer 6x (Electrophoresis): 40% Sucrose, 0.125% bromophenol blue, 0.1M EDTA pH8.0

Loading Buffer + RNase: 40% Sucrose, 0.125% bromophenol blue, 0.1M EDTA pH8.0, 0.33 mg/mL RNase

50 x TAE: 2M Tris, 5.7% glacial acetic acid, 50mM EDTA

1 x TAE: 4mM Tris, 0.114% glacial acetic acid, 5mM EDTA

Fill-in Reaction: DNA from restriction digest, 100 μM dNTPs (4 μL of 1/100 dilution of 25mM dNTP stock solution), 1 unit T4 DNA Polymerase or Klenow per μg DNA, 1 x NEB2 buffer

PCR reaction: 1 x High Fidelity PCR Buffer, 0.2mM dNTP's, 2mM MgSO_4 , 100 μM Template DNA, 1 unit Platinum Taq High Fidelity

SDS-Proteinase K: 10 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0, 1% SDS, 1mg/mL Proteinase K

6.14 Bacterial Amplification of Cloned DNA

Luria Broth (LB) medium

LB Broth with Agar: 500mL LB Broth, 7.5g Agar

LB Broth with Agar and ampicillin: 500mL LB Broth, 200 μL 50mg/mL Ampicillin (50 $\mu\text{g}/\text{mL}$)

2x YT: 8g Tryptone-peptone, 5g Yeast extract, 2.5g NaCl, up to 500mL H_2O ; adjust to pH 7.0; filter sterilize

RF1: 100mM RbCl, 50mM $\text{MnCl}_2 \cdot 4 \text{H}_2\text{O}$, 30mM potassium acetate, 10mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 15% w/v Glycerol; adjust to pH 5.8 with 0.2M acetic acid

RF2: 10mM MOPS, 10mM RbCl, 75mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 15% w/v Glycerol; adjust to pH 6.8 with 2N NaOH

30% (w/v) glycerol

6.15 Immunoblot

2 x Laemmli Sample Buffer: 62.5 mM Tris-HCl pH 6.8, 25% Glycerol, 2% SDS, 0.01g bromophenol blue

10% (w/v) APS: 0.1g Ammonium persulfate, up to 1mL H₂O

2 x Stacking Gel Buffer pH 6.8: 250mM Tris-HCl pH 6.8, 0.2% SDS

2 x Separating Gel Buffer pH 8.8: 750 mM Tris-HCl pH 8.8, 0.2% SDS

Stacking Gel Solution: 2mL H₂O, 3mL 2x Stacking Gel Buffer, 1mL 30% Acrylamide, 100 μ L 10% APS, and 10 μ L TEMED.

Separating Gel Solution:

Reagents	7.5% SDS-PAGE	10% SDS-PAGE	12% SDS-PAGE
ddH ₂ O	2.5mL	2.0mL	1.0mL
2x Sep Gel Buffer	5.0mL	5.0mL	5.0mL
30% Acrylamide	2.5mL	3.0mL	4.0mL
10% APS	100uL	100uL	100uL
TEMED	10uL	10uL	10uL

Transfer Buffer: 48mM Tris-HCl, 0.293% glycine, 0.0375% SDS, 20% MeOH

Running Buffer: 50mM Tris-HCl, 1.44% glycine, 0.1% SDS

0.1% (v/v) TBST: 150mM NaCl, 10mM Tris-HCl pH8.0, 0.1% TWEEN-20

5% (w/v) Milk Blocking Solution: 5% skim milk powder in 0.1% TBST

6.16 Immunofluorescence

4% (w/v) paraformaldehyde

PBS with 1% Horse Serum

Triton-X100 (Fisher)

Anti-FLAG M2 antibody (Sigma)

Goat anti-mouse TRITC conjugated secondary antibody (Jackson Immune Research Laboratories)

Goat anti-mouse FITC conjugated secondary antibody (Jackson Immune Research Laboratories)

Hoechst (Sigma)

DAKO Fluorescent Mounting Media (DAKO)

Coverslips (Fischer)

Microscope Slides (Fischer)

6.2 Plasmid Constructs

pRL1	640bp PCR product digested with <i>NheI/MluI</i> cloned into <i>NheI/MluI</i> digested RP2288. This creates the pIX-dsRed fusion protein.
pRL2	685bp fragment from <i>NheI/BstBI</i> digested pRL1 cloned into <i>NheI/BstBI</i> digested pRP2306. This creates the pIX-dsRed fusion protein shuttle plasmid.
pRL3	<i>Bst1107I</i> digested RL2 recombined with <i>Bsu15I</i> digested RP2014. This creates a large infectious plasmid with the pIX-dsRed fusion protein.
pRL4	771bp PCR product from <i>NheI/MluI</i> digested pcDNA3.1-fibreMR1scFV cloned into <i>NheI/MluI</i> digested pRP2288. This creates the pIX-MR1 fusion protein.
pRL5	776bp fragment from <i>NheI/BstBI</i> digested pRL4 cloned into <i>NheI/BstBI</i> digested pRP2306. This creates the pIX-MR1 fusion protein shuttle plasmid.
pRL6	<i>Bst1107I</i> digested RL5 recombined with <i>Bsu15I</i> digested RP2014. This creates a large infectious plasmid with the pIX-MR1 fusion protein.
pRL7	2698bp fragment from <i>BamHI/MfeI</i> digested pRP2202 cloned into <i>BamHI/MfeI</i> digested pRL5. This places the PGK-mSEAP transgene upstream of the pIX-MR1 fusion protein.
pRL8	2698bp fragment from <i>BamHI/MfeI</i> digested pRP2202 cloned into <i>BamHI/MfeI</i> digested pRL2. This places the PGK-mSEAP transgene upstream of the pIX-dsRed fusion protein.
pRL11	1234bp PCR product from <i>BamHI/XbaI</i> digested pRP2288 cloned into <i>BamHI/XbaI</i> digested pcDNA3.1. This produces the fusion protein ER-pIX-GFP.
pRL14	1350bp PCR product from <i>BamHI/XbaI</i> digested pRP2288 cloned into <i>BamHI/XbaI</i> digested pcDNA3.1. This produces the fusion protein ER-pIX-MR1.
pRL17	1308bp fragment from <i>BamHI/XbaI</i> digested pRL11 cloned into <i>BglII/SpeI</i> digested pΔE1sp1AΔpIX. This creates the ER-pIX-GFP shuttle plasmid.
pRL20	1350bp fragment from <i>BamHI/XbaI</i> digested pRL14 cloned into <i>BglII/AvrII</i> digested pΔE1sp1AΔpIX. This creates the ER-pIX-MR1 shuttle plasmid.
pRL23	2292bp fragment from <i>BamHI/NotI</i> (filled in) digested pRP2202 cloned into <i>BamHI/EcoRV</i> digested pRL17. This creates the PGK-mSEAP ER-pIX-GFP shuttle plasmid.

- pRL25-30A** 84bp fragment from *SpeI* digested pIX-linker30A cloned into *NheI* digested pRL25. This creates the PGK-mSEAP ER-pIX-30A-MR1 shuttle plasmid.
- pRL25-45A** 123bp fragment from *SpeI* digested pIX-linker45A cloned into *NheI* digested pRL25. This creates the PGK-mSEAP ER-pIX-45A-MR1 shuttle plasmid.
- pRL25-75A** 84bp fragment from *SpeI* digested pIX-linker30A cloned into *XbaI* digested pRL25-45A. This creates the PGK-mSEAP ER-pIX-75A-MR1 shuttle plasmid.
- pRL26** 2292bp fragment from *BamHI/NotI*(filled in) digested pRP2202 cloned into *BamHI/EcoRV* digested pRL20. This creates the PGK-mSEAP ER-pIX-MR1 shuttle plasmid.
- pRL28** *EheI* digested RL26 recombined with *Bsu15I* digested RP2014. This creates a large infectious plasmid with PGK-mSEAP ER-pIX-GFP.
- pRL32** *Bst1107I* digested RL26 recombined with *Bsu15I* digested RP2014. This creates a large infectious plasmid with PGK-mSEAP ER-pIX-MR1.
- pRL34** *Bst1107I* digested RL7 recombined with *Bsu15I* digested RP2014. This creates a large infectious plasmid with the PGK-mSEAP pIX-MR1 fusion protein.
- pRL35** *Bst1107I* digested RL8 recombined with *Bsu15I* digested RP2014. This creates a large infectious plasmid with the PGK-mSEAP pIX-dsRed fusion protein.
- pRL36** (SP1toSP4) 448bp PCR product from *BamHI* digested pRP2259 cloned into *BamHI* digested pcDNA3.1. This produces the fusion protein ER-pIX.
- pRL37** (SP1toSP4) 522bp fragment from *BamHI* digested pRL36 cloned into *BglII* p Δ E1sp1A Δ pIX. This creates the ER-pIX shuttle plasmid.
- pRL38** (SP1toSP4) 1040bp fragment from *XmaI* digested pRL20 cloned into *XmaI* pRL37. This creates the ER-pIX-MR1-NLS shuttle plasmid.
- pRL39** (SP1toSP4) *Bst1107I* digested RL37 recombined with *Bsu15I* digested RP2014. This creates a large infectious plasmid with the ER-pIX fusion protein.
- pRL40** (SP1toSP4) 2292bp fragment from *BamHI/NotI*(filled in) digested pRP2202 cloned into *BamHI/EcoRV* digested pRL38. This creates the PGK-mSEAP ER-pIX-MR1-NLS shuttle plasmid.

- pRL40-30A** (SP1toSP4) 779bp fragment from *MfeI/KpnI* digested pRL25-30A cloned into *MfeI/KpnI* digested pRL40. This creates the PGK-mSEAP ER-pIX-30A-MR1-NLS shuttle plasmid.
- pRL40-45A** (SP1toSP4) 818bp fragment from *MfeI/KpnI* digested pRL25-45A cloned into *MfeI/KpnI* digested pRL40. This creates the PGK-mSEAP ER-pIX-45A-MR1-NLS shuttle plasmid.
- pRL40-75A** (linker is 30+45A)(SP1toSP4) 902bp fragment from *MfeI/KpnI* digested pRL25-30+45A cloned into *MfeI/KpnI* digested pRL40. This creates the PGK-mSEAP ER-pIX-30+45A-MR1-NLS shuttle plasmid.
- pRL40-75A** (linker is 45+30A)(SP1toSP4) 902bp fragment from *MfeI/KpnI* digested pRL25-75A cloned into *MfeI/KpnI* digested pRL40. This creates the PGK-mSEAP ER-pIX-75A-MR1-NLS shuttle plasmid.
- pRL41** 1455bp fragment from *XmaI* digested pAd5hexon cloned into *XmaI* digested pBluescriptKS(+). This places the hexon protein in a Bluescript backbone.
- pRL42** PCR amplification of RL41 with oligos creating a *BstBI* restriction site in the variable region of hexon.
- pRL43** Annealed pK (polylysine) oligos cloned into *BstBI* digested pRL42. This places pK in the variable region of hexon.
- pRL44** 1064bp fragment from *SpeI/PacI(chewed back)* digested pBHG10loxCre cloned into *SpeI/BstBI(filled in)* digested pRP2089. This creates a fiber deleted shuttle plasmid.
- pRL45** 1485bp fragment from *XmaI* digested pRL43 cloned into *XmaI* digested pRL44. This produces a hexon with pK and fiber deleted shuttle plasmid.
- pRL46** (SP1toSP4) *Bst1107I* digested RL40 recombined with *Bsu15I* digested RP2014. This creates a large infectious plasmid with PGK-mSEAP ER-pIX-MR1-NLS.
- pRL46-75A** (linker is 30+45A) (SP1toSP4) *Bst1107I* digested RL40-30+45A recombined with *Bsu15I* digested RP2014. This creates a large infectious plasmid with PGK-mSEAP ER-pIX-75A-MR1-NLS.
- pRL46-75A** (linker is 45+30A) (SP1toSP4) *Bst1107I* digested RL40-75A recombined with *Bsu15I* digested RP2014. This creates a large infectious plasmid with PGK-mSEAP ER-pIX-75A-MR1-NLS.

- pRL47** 1455bp fragment from *XmaI* digested pRL42 cloned into *XmaI* digested pRL44. This produces a hexon with *BstBI* restriction site and fiber deleted shuttle plasmid.
- pRL48** *Bst1107I* digested RP2312 recombined with *Bsu15I* digested RL45. This creates a large infectious plasmid with LacZ, pIX-GFP, hexon with pK and fiber deleted.
- pRL49** *Bst1107I* digested pCA35(RP) Δ PacI recombined with *Bsu15I* digested RL45. This creates a large infectious plasmid with LacZ, hexon with pK and fiber deleted.
- pRL52** 7541bp fragment from *AscI/AvrII* digested pMAD8 cloned into *AscI/AvrII* digested pRL49. This creates a large infectious plasmid with LacZ, hexon with pK and truncated fiber.
- pRL53** 7517bp fragment from *AscI/AvrII* digested pRP2014 cloned into *AscI/AvrII* digested pRL49. This creates a large infectious plasmid with LacZ, hexon with pK and full length fiber.
- pRL54-30A** 417bp fragment from *EcoRI/BsrGI* digested pRL25-30A cloned into *EcoRI/BsrGI* digested pRL14. This
- pRL54-45A** 456bp fragment from *EcoRI/BsrGI* digested pRL25-45A cloned into *EcoRI/BsrGI* digested pRL14. This
- pRL54-75A** 540bp fragment from *EcoRI/BsrGI* digested pRL25-75A cloned into *EcoRI/BsrGI* digested pRL14. This
- pRL63** (SP1toSP4) 3222bp fragment from *Clal/BglII* digested pShuttle-Ubc-mSEAP cloned into *Clal/BamHI* digested pRL38. This creates the Ubc-mSEAP ER-pIX-MR1-NLS shuttle plasmid.
- pRL65** (SP1toSP4) *Bst1107I* digested RL63 recombined with *Bsu15I* digested pRP2014. This creates a large infectious plasmid with Ubc-mSEAP ER-pIX-MR1-NLS.
- pRL66** 630bp fragment from *SstI/XbaI* digested pRL40-30+45A cloned into *SstI/NheI* digested pRP2339. This creates a plasmid with the fusion protein ER-pIX-30+45A-albumin.
- pRL67** 216bp fragment from *EcoRI/XbaI* digested pRL40-30+45A cloned into *EcoRI/NheI* digested pRP2344. This creates a plasmid with the fusion protein ER-pIX-30+45A-albumin.
- pRL68** 2112bp fragment from *MfeI* digested pRL66 cloned into *MfeI* digested pRP2350. This creates a plasmid with LacZ and ER-pIX-30+45A-albumin.

- pRL69** *Bst*1107I digested RL68 recombined with *Bsu*15I digested pRP2014. This creates a large infectious plasmid with LacZ and ER-pIX-30+45A-albumin.
- pRL70** 1441bp fragment from *Bgl*II/*Nhe*I digested pRL11 cloned into *Bam*HI/*Nhe*I digested pRL17. This produces the fusion protein CMV:ER-pIX-GFP shuttle plasmid.
- pRL71** *Ehe*I digested RL70 recombined with *Bsu*15I digested pRP2014. This creates a large infectious plasmid with CMV:ER-pIX-GFP.
- pRL72** 1441bp fragment from *Bgl*II/*Nhe*I digested pRL14 cloned into *Bam*HI/*Nhe*I digested pRL20. This produces the fusion protein CMV:ER-pIX-MR1 shuttle plasmid.
- pRL73** 3149bp fragment from *Mlu*I(filled in)/*Bst*EII digested pRL70 cloned into *Bgl*II(filled in)/*Bst*EII digested pShuttle-Ubc-mSEAP. This produces the Ubc-mSEAP CMV:ER-pIX-GFP shuttle plasmid.
- pRL74** *Bst*1107I digested RL72 recombined with *Bsu*15I digested pRP2014. This creates a large infectious plasmid with CMV:ER-pIX-MR1.
- pRL75** *Nhe*II digested RL72 ligated with *Nhe*I digested pRL73 and recombined. This produces the Ubc-mSEAP CMV:ER-pIX-MR1-NLS shuttle plasmid.
- pRL76** 390bp fragment from *Bam*HI/*Mfe*I digested pRL14 cloned into *Bam*HI/*Mfe*I digested pRP1070. This produces a plasmid with fusion protein intron-ER-pIX.
- pRL77** 1897bp fragment from *Eco*RI(filled in)/*Bst*EII digested pRL76 cloned into *Sna*BI/*Bst*EII digested pΔE1sp1AΔpIX. This produces the intron-ER-pIX shuttle plasmid.
- pRL78** *Bst*1107I digested RL75 recombined with *Bsu*15I digested pRP2014. This creates a large infectious plasmid with Ubc-mSEAP:CMV ER-pIX-MR1-NLS.
- pRL79** *Bst*1107I digested RL73 recombined with *Bsu*15I digested pRP2014. This creates a large infectious plasmid with Ubc-mSEAP CMV:ER-pIX-GFP.
- pRL80** 1040bp fragment from *Xma*I digested pRL20 cloned into *Xma*I digested pRL77. This produces the intron-ER-pIX-MR1 shuttle plasmid.
- pRL81** 3226bp fragment from *Cl*aI/*Bgl*II(filled in) digested pShuttle-Ubc-mSEAP cloned into *Cl*aI/*Eco*RV digested pRL80. This produces the Ubc-mSEAP intron-ER-pIX-MR1 shuttle plasmid.

- pRL82** *Bst1107I* digested RL81 recombined with *Bsu15I* digested pRP2014. This creates a large infectious plasmid with Ubc-mSEAP intron-ER-pIX-MR1.
- pRL83** 3819bp fragment from *EcoRI/Bst1107I* digested pRL68 cloned into *EcoRI/Bst1107I* digested pRL72. This produces the CMV:ER-pIX-30+45A-albumin shuttle plasmid.
- pRL84** *Bst1107I* digested RL83 recombined with *Bsu15I* digested pRP2014. This creates a large infectious plasmid with CMV:ER-pIX-30+45A-albumin.
- pRL85** 1635bp fragment from *XbaI/NheI* digested pRP1080 cloned into *XbaI* digested pHYGROCB. This produces mCMV-IRES-Hygro.
- pRL86** 3100bp fragment from *XbaI* digested pLernL cloned into *SpeI* digested pRL85. This produces mCMV-IRES-EGFRvIII-Hygro.
- pRL87** 1347bp PCR product from *BamHI/XbaI* digested pRL14 cloned into *BamHI/XbaI* digested pcDNA3.1. This produces the fusion protein CMV:ER-pIX-MR1-TatNLS.
- pRL90** 1347bp fragment from *BamHI/XbaI* digested pRL87 cloned into *BglII/SpeI* digested pΔE1sp1AΔpIX. This produces the CMV:ER-pIX-MR1-TatNLS shuttle plasmid.
- pRL91** 2558bp fragment from *NheI/StuI* digested pRL90 was ligated and recombined with *NheI/StuI* digested pRL75. This produces the Ubc-mSEAP CMV:ER-pIX-MR1-TatNLS shuttle plasmid.
- pRL92** *Bst1107I* digested RL91 recombined with *Bsu15I* digested pRP2014. This creates a large infectious plasmid with Ubc-mSEAP CMV:ER-pIX-MR1-TatNLS.
- pRL93** 3385bp fragment from *SgrAI/BglII* digested pShuttle-Ubc-mSEAP cloned into *SgrAI/BamHI* digested pRL5. This produces the Ubc-mSEAP pIX-MR1 shuttle plasmid.
- pRL94** *Bst1107I* digested RL93 recombined with *Bsu15I* digested pRP2014. This creates a large infectious plasmid with Ubc-mSEAP pIX-MR1.
- pRL95** 2662bp fragment from *SspI/BglII* digested pAVH5 cloned into *SspI/BglII* digested pRL87. This produces a plasmid containing CMV-GFP CMV:ER-pIX-MR1-TatNLS.
- pRL96** 5081bp fragment from *XhoI/XmnI* digested pRL95 cloned into *XhoI/EcoRV* digested pΔE1sp1AΔpIX. This produces the CMV-GFP and CMV:ER-pIX-MR1-TatNLS shuttle plasmid.

- pRL97** *Bst1107I* digested RL90 recombined with *Bsu15I* digested pRP2014. This creates a large infectious plasmid with CMV ER-pIX-MR1-TatNLS.
- pRL98** *EheI* digested RL96 recombined with *Bsu15I* digested pRP2014. This creates a large infectious plasmid with CMV-GFP and CMV ER-pIX-MR1-TatNLS.
- pRL99** 2292bp fragment from *BamHI/NotI*(filled in) digested pRP2202 cloned into *BamHI/EcoRV* digested pRL90. This produces the PGK-mSEAP and CMV:ER-pIX-MR1-TatNLS shuttle plasmid.
- pRL100** *Bst1107I* digested RL99 recombined with *Bsu15I* digested pRP2014. This creates a large infectious plasmid with PGK-mSEAP and CMV ER-pIX-MR1-TatNLS.

Robert Lanthier

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Education

Master's Degree: University of Ottawa
Microbiology and Immunology

2007

Bachelor of Science: University of Ottawa
Honours Biochemistry with Co-op

2004

Research Experience

Master's and Undergraduate Thesis: Targeting Adenoviral Vectors using a single-chain antibody fused to the capsid protein IX

Supervisor: Dr. Robin Parks

- Utilized restriction enzyme digestion and established cloning techniques to insert fusion proteins and reporter genes into adenoviral DNA
- Performed large and small scale plasmid DNA preparations utilizing bacterial transformation and recombination
- Mastered preparation of adenoviral vectors from plasmid DNA to purified virus
- Performed transfections, infections, and virus propagation using cultured cells
- Examined expression of protein using Western blot analysis
- Elucidated binding of single-chain antibody to ligand through immunoprecipitation
- Examined intracellular locations of proteins of interest through immunocytochemistry
- Analyzed and compiled data to deliver presentations regarding research

Work Experience

Laboratory Technician

2003

Vaccines Division: Health Canada, Ottawa, ON

- Studied various types of bifurcated needles in order to determine their efficacy in delivering vaccine
- Maintained tissue cultures for use in plaque and CAM assays
- Performed mock plaque assays and CAM assays in order to determine the potency of vaccinia virus lots
- Researched scientific literature for the development of a PCR protocol for the identification of components in smallpox vaccines
- Developed Real-time PCR protocol for the evaluation of mycobacteria contamination in smallpox vaccines

Assistant Cosmetic Safety Officer 2002
Cosmetics Division: Health Canada, Ottawa, ON

- Gathered, organized, verified and evaluated the comprehensive information relating to the behaviour of chemicals and toxicological data from a wide variety of sources
- Prepared written and oral reports on the findings
- Worked effectively in a team environment to complete the tasks presented
- Reviewed, commented on and discussed scientific papers completed by the Cosmetics division before their publication

Research and Development Scientist 2001
i-STAT Canada: Kanata, ON

- Safely performed testing of experimental material using blood as well as aqueous solutions
- Organized lifetime experiments consisting of numerous tests over extended periods of time
- Compiled, analyzed and graphed data found from testing
- Interpreted results and discussed them in a group setting
- Budgeted time in order to accomplish completion of new experiments and analysis of old under given time constraints

Additional Skills

Computers · Microsoft Word, Corel Wordperfect. Microsoft Excel, Microsoft Powerpoint and Lotus 123.
· Able to effectively use these programs to produce written documents, solve mathematical problems, as well as graphically display data

Analytical · Able to analyze collected data and come up with justified trends and conclusions relating the data through vigorous researching of the topic and use of problem solving skills

Communication · Able to prepare written documents and deliver presentations in an effective and concise manner.

Achievements

- Received entrance scholarship from the University of Ottawa 1999
- Received bursary from the University of Ottawa Graduate Studies Program 2005
- Acknowledged at Biochemistry, Microbiology and Immunology Poster Day 2005

Community Involvement

- Ottawa Race Weekend 2005-2006
- Muscular Dystrophy "Walk and Roll" 2005
- University of Ottawa Undergraduate Orientation 2003