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Targeting of Adenovirus Serotype 5 infectivity to EGFRvIII expressing cells using a bispecific CAR-scFv

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Submitted in partial fulfillment

of the requirements for the degree of

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ABSTRACT

A mutant form of the Epidermal Growth Factor Receptor (EGFR), known as EGFR version III, is over-expressed exclusively on various forms of cancer cells. MR1, a single chain antibody variable domain fragment (scFv), is able to bind with high affinity and specificity to EGFRvIII and not to the wild-type receptor. The purpose of this study was to investigate a possible means of developing an adenoviral vector (based on subclass C viruses) that can utilize MR1's targeting ability towards EGFRvIII to infect only cancer cells that express this receptor. Adenovirus infectivity is primarily mediated by binding of the globular carboxyl terminus of its fiber protein, known as the knob domain, to the coxsackievirus and adenovirus receptor (CAR), which is expressed on various cell types. Although we attempted three different approaches to try and target the virus using MR1, including direct incorporation of the scFv into the knob domain of adenovirus, it was the creation of a fusion protein consisting of the extracellular domain of CAR linked to MR1 that suggested increased infectivity to EGFRvIII expressing cells. According to ELISA analysis, the CAR/MR1 fusion protein is able to retain the two independent binding affinities of its composite subunits (bispecificity) as strongly as the two independent proteins do for the knob domain and EGFRvIII, respectively. Additionally, a U87MG EGFRvIII transduced cell line, as opposed to its parental U87MG cell line, showed increased infectivity by adenovirus when incubated with CAR/MR1. These findings suggest that CAR/MR1 is able to bind to and re-direct infection of adenovirus to EGFRvIII expressing cells.

Key Words: EGFR, MR1, adenovirus, fiber protein, knob domain, CAR, fusion protein.

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LIST OF ABBREVIATIONS

aa – amino acid

Ab – Antibody

Ad – Adenovirus, also Ad2/Ad5 refer to adenovirus serotypes 2 and 5 respectively

ATCC - American Type Culture Collection

ATP - Adenosine triphosphate

BSA – Bovine Serum albumin

CAR – Coxsackievirus and Adenovirus Receptor

DMEM - Dulbecco's modified Eagle's medium

DNA – Deoxyribonucleic acid

dsFv – Disulfide linked antibody variable domain fragment

DTT - 1,4-Dithio-DL-threitol

EDTA - Ethylenediaminetetraacetic acid

EGF – Epidermal Growth Factor

EGFR – Epidermal Growth Factor Receptor

EGFRvIII – Mutant EGFR (version III)

ELISA – Enzyme linked immunabsorbancy assay

FCS – Fetal Calf Serum

FPLC – Fast Protein Liquid Chromatography

HRP - Horseradish peroxidase

Ig – Immunoglobulin

IPTG – isopropyl-β-D-thiogalactopyranoside

kDa – kiloDaltons

NaOH - Sodium hydroxide

ONPG - o-nitrophenyl-β-D-galactopyranoside

PBS – phosphate buffered saline

Pep3 – A synthetic peptide consisting of the first amino terminal 13 amino acids.

scFv – Single chain antibody variable domain fragment

SDS-PAGE - Sodium dodecyl sulphate polyacrylamide gel electrophoresis

TBS – Tris buffered saline

X-gal – 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

INTRODUCTION – Chapter 1

1.1 Review of the Literature

1.1.1 Cancer – A Brief Overview

Cancer is a disease that is characterized by multiple genetic mutations within a normal cell's homeostatic regulatory pathways, resulting in the cell's transformation into a malignant derivative. If left untreated, the eventual outcome of cancer can result in the death of the organism.[1] Each type of cancer must therefore be examined and treated individually with surgery, radiation, chemotherapy or any combination of the three, depending on the stage of the disease or the extent of spread of the cancer from its origin. Although there has been a progressive decline in the incidence and mortality rates for most cancers over the past decade, the ageing population may reverse this trend in the near future. The decline in mortality from cancer has mainly been attributed to improvements in prevention, screening, and treatment.[2] If, however, both prevention and screening are insufficient to avert a late diagnosis of cancer, advances in the treatment and understanding of cancer's development will be needed to compensate for the changing genetic makeup of this insidious disease. It is due to this diverse genetic dysfunction in cancer that a repertoire of therapeutics is required for each of the more than 100 types of known cancers. Research must therefore continue to develop different strategies to target defective cellular factors for each specific type of cancer.

1.1.2 Epidermal Growth Factor Receptor

Structure and Function

An attractive vehicle for examining the progression of cancer cells has been growth promoting cellular receptors. More specifically, the epidermal growth factor receptor, which belongs to a subfamily of closely related tyrosine kinase receptors: EGFR (or ErbB-1), Her 2/neu (ErbB-2), Her 3 (ErbB-3) and Her 4 (ErbB-4), has been shown to be overexpressed on various types of cancer cells. The normal, mature cell surface EGFR is composed of a single polypeptide chain of 1,186 amino acid residues (~130-kDa) and has a substantial amount (~ 40 kDa) of *N*-linked oligosaccharide.[3] This 170 kDa EGFR transmembrane glycoprotein consists of an extracellular ligand-binding domain, a helical transmembrane domain, and two intracellular domains; one regulatory and the other a tyrosine kinase.[4] Inactive EGFR exists as a monomer, but upon binding to a signalling ligand, such as EGF, amphiregulin, or TGF-α, the receptor dimerizes with either another EGFR monomer (homodimer) or another related family member (heterodimer). This results in the transphosphorylation of five tyrosine residues by each subunit's intracellular tyrosine kinase domain. The resultant signalling cascade delivers a message through various pathways that causes cell growth and division.[5] Under normal circumstances, a cell is able to modulate the effects of EGFR signalling, however, the obvious conclusion of unregulated EGFR overstimulation on cellular growth would be the uncontrolled differentiation of cells as that found in cancer.

Unfettered proliferative signaling by EGFR has been implicated in the progression of cancers to a metastatic state.[6] Additionally, the role of EGFR in cancer development can be extended by the fact that its inhibition can result in the apoptosis of

cells,[7] and its activation results in the release of vascular endothelial growth factor (VEGF) that is required in angiogenesis - a major requirement for the proliferation of high oxygen demanding tumour cells.[8] However due to EGFR's wide tissue distribution and its diverse function in different cell types, the use of this receptor as a tumour-specific marker for targeted drug delivery raises many safety concerns, especially when therapies threaten the accidental destruction of normal cell populations.

Mutant Epidermal Growth Factor Receptor version III

A more specific target for oncogenesis is a truncated form of the epidermal growth factor receptor designated EGFR version III (EGFRvIII) or also known as deltaEGFR, which is only expressed on tumour cells. EGFRvIII arises from a spontaneously occurring in frame deletion of exons 2-7 of the epidermal growth factor receptor (EGFR) gene, resulting in the loss of 601 coding bases and deletion of amino acids 6-273. The outcome of this deletion is a truncated extracellular domain that results in spontaneous homodimerization and constitutive protein kinase activity in the absence of EGF and other activating factors.[9,10] The downstream events resulting from EGFRvIII activity are still poorly understood and may vary from those of the wild-type receptor.[11,12]

This mutant form of the receptor has been found to be overexpressed on 50-60% of glioblastoma cells, 70-80% of breast and ovary carcinomas, and 16% of non-small cell lung cancers.[13-16] EGFRvIII was recently also shown to be expressed on prostate cancers and is linked to all these cancers' gradual progression towards malignancy and poor prognosis in patients.[17] Last year, these five forms of solid tumour cancers accounted for

over half a million new cases of cancer diagnosed in the United States alone. Fatalities from these types of cancer can also reach over half a million people per year according to cancer statistics from 1999.[18] Due to the high incidence of these forms of cancer, and the link this mutant EGFR shares with them, research into therapeutics targeting this oncogenic receptor have become attractive.

1.1.3 MR1 scFv

Immunoglobulins (Igs), also referred to as antibodies (Abs), have been extensively exploited to specifically bind to target ligands in the past. Not only are the affinities of antibodies relatively high, but also the specificity can be such that a single amino acid substitution in the target molecule can result in the complete loss of antibody-ligand binding. Coupled with other molecular biology techniques, an antibody raised to a ligand can be further engineered to act directly as a therapeutic agent. An example of such an engineered antibody is the linking directly of only the Ig's variable domains, creating what is termed a single chain variable domain fragment (scFv).

Such an engineered scFv protein was isolated previously to exclusively bind to EGFRvIII and not EGFR. Designated MR1, it was initially developed as an immunotoxin, which is a targeting immunoglobulin linked to a cytotoxic agent (specifically Pseudomonas exotoxin).[19] Since the immunotoxin aspect of MR1 is not relevant in the context of the work undertaken here, the development and actual physical characteristics of MR1 will be detailed further instead.

The creation of MR1 involved the raising of mouse immunoglobulins against both EGFRvIII and a synthetic peptide (Pep3) spanning the first 13 amino acids of the newly

truncated amino terminus of the mutant receptor. The epitope for the antibody would therefore includes a unique glycine amino acid not found in wild-type EGFR (see Figure 1). Phage display was then used to improve the specificity of the resulting scFv library by screening the scFv phage library with successively lower quantities of Pep3 to isolate the strongest binders, including MR1. MR1 was shown to have a high affinity for EGFRvIII, with a dissociation constant of 11nM, while simultaneously showing a lack of specificity for wild-type EGFR. MR1 is also a thoroughly studied targeting molecule; with the crystal structure of MR1 complexed to Pep3 being recently elucidated in conjunction to alanine scanning mutational analysis of the 13 amino acids of Pep3 that comprise MR1's epitope.[20] Additionally, a newly modified version of MR1 has been shown to have a 15-fold increased binding affinity for EGFRvIII.[21]

The major focus of this thesis, however, involves the application of MR1 as a viral tropism modifying protein. Previous work has demonstrated the utility of MR1 as a re-targeting ligand by directly incorporating it into the surface loop of the Murine Moloney leukemia virus envelope glycoprotein.[22] Although, infectivity of the MR1 incorporated viral particles was poor, targeting of the virus to EGFRvIII expressing cells was highly successful. The obvious evolution of this concept would be to encompass various viral based gene therapy vectors beyond the retrovirus used previously. The strongest candidate for further retargeting studies would be the most widely used and studied gene therapy vector: adenovirus.

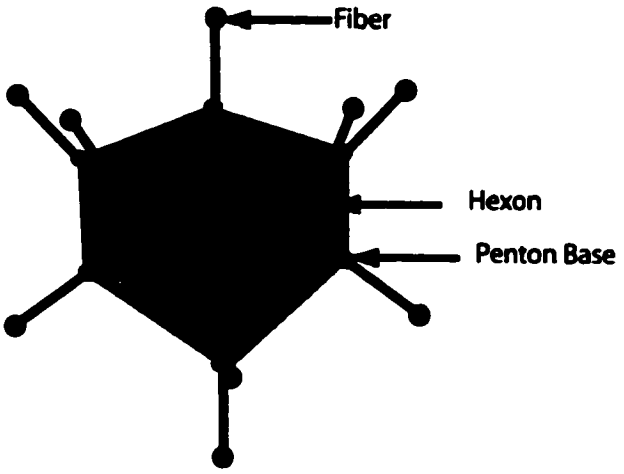
Figure 1. Illustration of MR1 Targeting the mutant Epidermal Growth Factor Receptor version III. The deletion of amino acids 6 to 273 (region between white arrows labeled "5" and "273") results in the formation of a glycine codon at the junction (shown in yellow) within the newly truncated extracellular domain. The amino acid epitope MR1 recognizes is coloured in red and enclosed within a red box. Box A emphasizes that MR1 does not target wild-type EGFR.

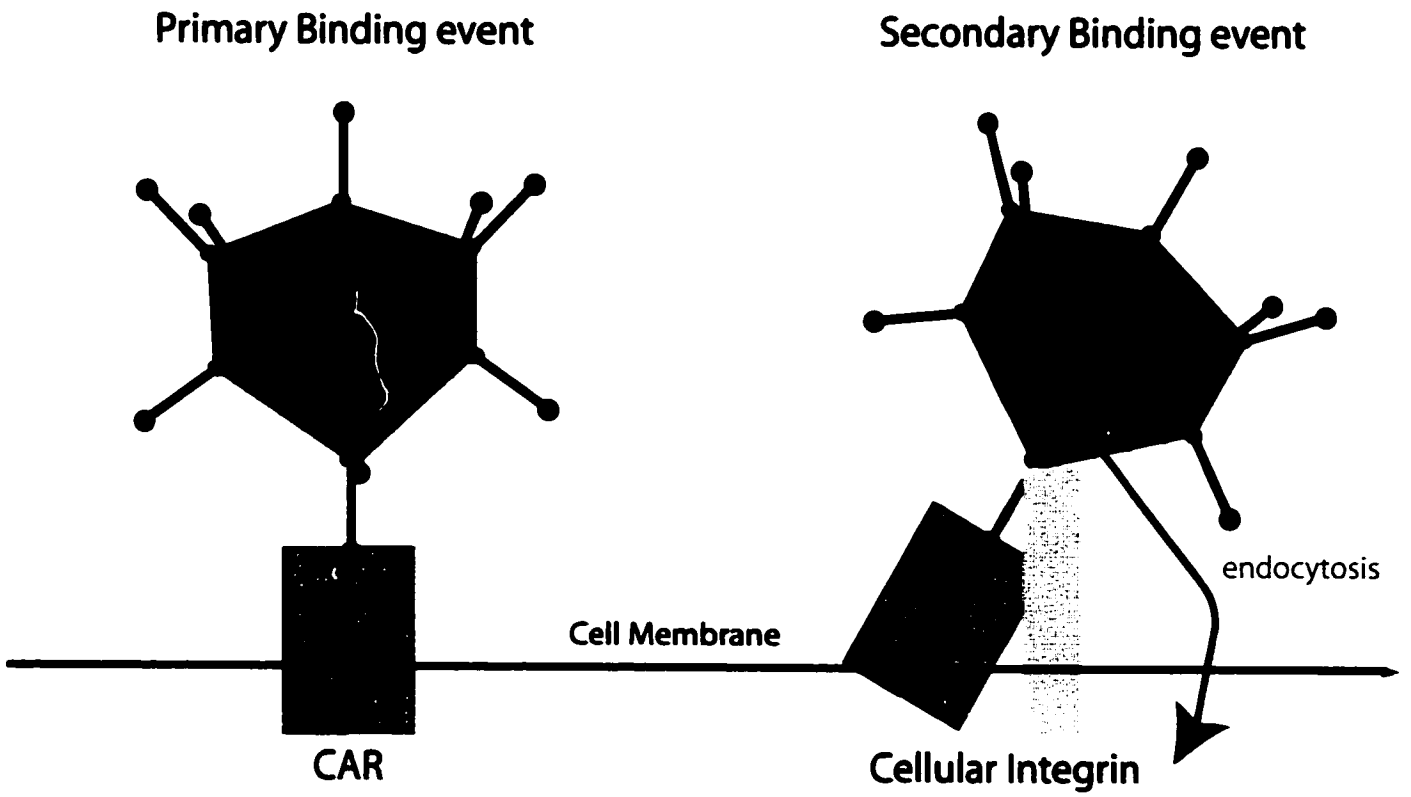
1.1.4 Adenovirus (Serotypes 2 and 5)

Structure – A Brief Overview

Human adenoviruses (Ads) serotypes 2 and 5, which belong to the *Mastadenvorius* genus, are non-enveloped, double stranded DNA, protein encapsulated viruses that cause mild respiratory infection, conjunctivitis, and infantile gastroenteritis in humans.[23] Structurally, these adenoviruses are icosahedral particles mainly composed of three major capsid proteins forming twenty triangular faces and twelve vertices. The major capsid constituent is a trimer of polypeptide II proteins termed the hexon capsomeres, which associates with three other polypeptides to provide a stabilizing lattice for the complex.[24] At each of the twelve vertices are five copies of polypeptide III, which form the penton base protein, and projecting outward from each of the penton base proteins are a trimer of polypeptide IV, known as the fiber protein (see Figure 2).[25] Both penton base and fiber proteins will be discussed in further detail later, as they are primarily responsible for mediating binding and infection of the virus. The other proteins encoded by the adenovirus genome have other diverse functions including structural stability of the virion, viral replication and cell cycle regulation, and attachment and packaging of the genome. The viral life cycle is well understood and for further details the reader is referred to : "*Adenoviridae*: The viruses and their replication" by T. Shenk, in Fields Virology (3rd Edition, 1996). Both the replication cycle and other viral proteins are less relevant to the body of work undertaken and will be excluded for the sake of brevity.

Figure 2. Illustration of a subclass C adenovirus and the binding events required for infection of a susceptible cell. Three proteins compose the majority of the adenovirus capsid: the hexon protein, the penton base protein, and the fiber protein. The primary binding event is mediated by the fiber protein to its cognate cellular receptor the coxsackie and adenovirus receptor (CAR). A second binding event involving the adenovirus's penton base protein's RGD motifs to cellular integrins is then required for endocytosis of the whole virion.


Fiber
Hexon
Penton Base


Primary Binding event
Secondary Binding event
endocytosis
Cell Membrane
CAR
Cellular Integrin

Adenoviral Vectors in Gene Therapy

Initially the major interest in adenoviruses stemmed from their ability to transform cultured cells, however the large body of information from this research resulted in the realization of the virtues of adenoviruses as an experimental gene therapy system.[26] The complete sequencing of Ad2's genome,[27] and the subsequent understanding of the virus's replication cycle and transcriptional regulation, allowed for the further refinement of the virus as a viable vector for gene therapy use.[28,29-31]

Several significant advantages for the employment of adenoviruses as a cancer therapeutic are: 1) the high levels of *in vivo* transduction of target cells; 2) the ability to produce high titres of virus for *in vivo* gene delivery; 3) the ready manipulation of the viral genome to package foreign DNA; and 4) the recent elucidation of the virus's binding mechanism.[32]

There are, however, certain limitations for their use as cancer gene therapy vectors that must first be overcome for their successful application in this field. The first is adenovirus's high immunogenicity in humans, mainly due to late viral proteins, which is prohibitive for re-administration of the virus. This problem has been of considerable concern in relation to the development of both first and second generation adenovirus vectors, since both were created primarily to alleviate safety considerations. These types of vectors contain critical deletions in the adenoviral genome that prevent productive replication outside a complementary cell line that is able to provide the missing gene *in trans*. For example, first generation adenoviruses lack early 1 region genes (ΔE1,),[33] and second generation vectors lack E1 genes and either contain an additional temperature

sensitive DNA polymerase mutation in early 2A region genes (ΔE1/ts E2A,),[34] or also contain deleted E4 genes.[35] Since there is a delicate balance between expression and regulation of late viral genes by early transcripts of the viral genome, it is important to maximize the amount of modification/deletions to the viral genome to ensure safety while minimizing instability in propagating the virus. Therefore, there have been significant advances in the production of third generation "gutted" adenoviral vectors that contain the minimum number of viral genes necessary to propagate the virus in a complementing cell line with the aid of a helper virus.[36] These latter generation vectors not only ensure safety for *in vivo* gene delivery but also show much less immunogenicity. However, it is important to note that immunogenicity concerns may not be a serious drawback in certain applications, since transient gene expression may be adequate to elicit the desired response in cancer cells, such as through the use of a suicide gene. Also, induction of immune responses against cancer cells solely due to their infection by adenovirus is also an attractive side affect of using this vector for this form of therapy.

However, the most important concern for the use of adenoviruses in cancer gene therapy is the promiscuous tropism of the virus. The repercussions involved in poorly administering non-targeted adenoviruses, albeit replication-incompetent, virus in relatively high doses have already been felt within the gene therapy field.[37] Limiting the tropism of adenovirus to specific cell populations would increase the safety for adenovirus's use in clinical trials. Therefore, a major new focus in advancing adenoviral vectors is by thoroughly understanding the method of virus binding and entry into the cell, and thereby modifying those elements involved, to specifically target infectivity to only certain cell populations.[38] To accomplish these goals, research has been able to

elucidate the two key proteins involved in viral attachment and absorption, and their cognate cellular receptors.

Adenovirus Binding and Infectivity Mechanism

Fiber Protein and Coxsackievirus and Adenovirus Receptor

As mentioned previously, the 581 amino acid fiber protein (polypeptide IV) exists as an extremely thermo stable and detergent resistant homotrimer at the apex of each of the twelve vertices of the icosahedral virion capsid.[39] There are very few differences between Ad2 and Ad5 fiber proteins (very high sequence similarity), and since they are structurally identical, all references will henceforth concern only Ad5 fiber but are still applicable to Ad2 fiber with only slight modifications (e.g. Ad2 fiber is 582 amino acids). The fiber protein is structurally divided into three domains: the amino-terminal 'tail' domain (residues 1-43), the central 'shaft' domain (residues 44-400), and finally the carboxy-terminal 'knob' domain (residues 401-581).[40]

The tail domain projects into the penton base of the virus and anchors the fiber protein through non-covalent interactions to the capsid. This domain also contains the nuclear localization signal for proper shuttling of the fiber to the nucleus for viral packaging.[41]

The amphipathic β-sheet structure of the shaft domain is composed of at least 22 15-amino acid repeats each containing two β-strands and two β-bends.[42] The shaft's overall structure forms a triple β-spiral with highly conserved inter- and intra-chain main-chain hydrogen bonding whose stability is supplemented by hydrophobic interactions.[43] The last 15 carboxy-terminal amino acids in the shaft domain, prior to the globular knob

domain, may help to stabilize the trimeric fiber protein.[44] The fiber protein's shaft domain is also post-translationally modified by the addition of O-linked N-acetylglucosamine,[45] which may also play a role in trimeric fiber stability.

Finally, the knob domain is structurally composed of two antiparallel β-sheets, whose β-strands are linked together by fairly long flexible loops. The overall folding pattern forms a β-sandwich structure, that when trimerized, resembles a three-bladed propeller.[46,47] The knob domain trimerizes independent of the other fiber domains, and is therefore extremely important in the trimerization of the whole adenovirus's fiber protein. In fact, perturbations in the knob domain's structure (i.e. deletions or peptide insertions at the carboxy-terminus, amino-terminus, and within the polypeptide chain) results in impaired, or complete loss of, proper trimer folding. The importance for accurate knob domain trimerization is related to the functional significance it plays in adenovirus infection – is responsible for the primary binding interaction of the virus to its cognate cellular receptor, the coxsackie and adenovirus receptor (CAR).[48-50]

It has been known for quite some time that the fiber protein mediates attachment of adenovirus to cells, and antibodies against the knob domain were most efficient at neutralizing adenovirus infection (Figure 2).[51,52] Initial fiber receptor candidates included the MHC class I α2 domain, but this speculation is now believed to be false.[53,54] It was not until very recently that the actual adenovirus fiber receptor protein was irrevocably proven to be what is now referred to as the coxsackie and adenovirus receptor (CAR).[55]

CAR is a 365 aa transmembrane (46 kDa) glycoprotein composed of a 107-aa intracellular domain, a membrane spanning helical domain and a 222-aa extracellular domain. The latter domain is composed of two Ig-related structural domains, termed the

D1 (IgV-like) and D2 (IgC2-like) domains.[55] The amino-terminal D1 IgV-like domain is sufficient to promote viral attachment through binding of the knob domain of fiber.[56] It is important to note that although CAR is speculated to be involved in cellular adhesion, it still has no known physiological function even though it has a relatively wide tissue distribution.[57,58]

The actual structural motifs and amino acids involved in the knob-CAR binding interaction were highly speculated and heavily researched. Extensive mutational analysis offered many critical knob domain candidate residues along with regions on CAR that might mediate binding.[59-62] The crystal structure of the Ad12 knob domain complexed to the D1 domain of CAR offered the most revealing evidence for contact residues between the two molecules and also clarified that each knob monomer bound to one CAR D1 domain.[63,64] Ad12 is also in the same subclass (C) adenoviruses as 2 and 5 and has a very similar knob domain to Ad5 with the only major difference being in the HI loop structure. The major binding determinants on knob for CAR exist in the AB loop, which is positioned in the cleft between monomer subunits in the trimer, and also within the DG loop. Whereas for CAR, the knob interacting amino acids occur within strands C, C', C'', and the second half of strand F in the D1 domain.[63] The binding affinity for trimeric knob to cell surface CAR is relatively high with a dissociation constant (K_d) of approximately 5nM.[65]

The importance of the fiber-CAR interaction was illustrated when fiberless virions were successfully constructed through the use of a 293-Fiber complementing cell line. These fiberless virions showed diminished absorption by target cells.[66] In the absence of fiber, the relative transduction efficiency of adenovirus was less than 0.1% of

wild-type virus, and the amount of viable fiberless adenovirus particles was significantly less than that obtained with wild-type virus.[67] These observations led to the conclusion that not only is fiber protein extremely important in terms of mediating the primary cell binding event for the virus, but may also play a role in the morphological maturation and stabilization of the virion itself.[68]

Further studies examining how adenoviruses infect cells revealed several unusual discrepancies in the theory that the fiber protein was solely responsible for mediating infectivity. When high multiplicities of infection (M.O.I.) of fiber neutralized adenoviruses (i.e. through the use of anti-knob mAbs) were used, transduction of target cells was still quite successful. Also, the fact that adenoviruses can bind to certain cells (expressing CAR) but are still unable to be internalized provided strong evidence that another binding protein or receptor interaction may be required.[69]

Penton Base Protein and Cellular Integrins

Studies have shown that an adenovirus-encoded protein was able to cause the dissociation of cells from glass or plastic surfaces *in vitro*. This protein was subsequently shown to be the penton base protein.[70] Sequencing of the polypeptide III (penton base) encoding gene resulted in the discovery that an Arg-Gly-Asp (RGD) peptide sequence is present within the polypeptide.[71] Since there are five copies of the penton base protein present at each vertex of the adenovirus, it was speculated that these RGD motifs might act analogous to cell adhesion molecules that have similar RGD sequences, and which bind to heterodimeric cell surface receptors known as integrins.[72]

Integrins exist as non-covalently associated heterodimers composed of an alpha and beta subunit. It was known, prior to the discovery of CAR, that cellular integrins promoted adenovirus internalization through binding to the penton base but were not responsible for the initial attachment event.[73] Since there are many types of alpha and beta subunits, and many possible combinations for αβ associations, the specific integrins adenoviruses may use for internalization are still being discovered. Thus far, $\alpha_v\beta3$, $\alpha_v\beta5$, $\alpha M\beta2$ and $\alpha_v\beta1$ have been named as coreceptors for adenovirus infection.[73-75] What is known for certain is the penton base binding to integrin event is absolutely necessary for endocytosis of adenoviruses. However, the mechanism of how the penton base comes into proximity of cellular integrins following fiber-CAR association is still uncertain.

Although the penton base binding to cellular integrins can act as an attachment event for adenovirus infection, the affinity is approximately 30 fold less strong than that of fiber protein for CAR. Therefore, when devising techniques to modify the tropism of adenovirus, the focus has been limited to modifying the fiber protein and, more specifically, the knob domain.

1.1.5 Tropism Modification of Adenovirus Serotypes 2 and 5 Fiber protein

Direct Genetic Modification of the Fiber protein

Generally, three different approaches have been successfully used in retargeting adenovirus through covalently linking a re-targeting peptide to an adenoviral capsid protein, predominantly the knob domain. Although all four methods have shown limited tropism modification, none have achieved exclusive targeting to the desired receptor, or when CAR binding is abolished infectivity was never on par with unmodified virus through its natural binding to CAR.

The first approach for modification of fiber was addition of various peptides at the carboxy end of knob.[76-78] Although limited re-targeting of adenovirus was achieved through insertion of a poly-lysine tract and an RGD motif, the carboxy-terminus was proven to be less than optimum for foreign peptide insertions. The upper size limit tolerated by the carboxy terminus was 25-30 amino acids where larger inserts would interfere with the trimeric stability and incorporation of fiber protein into virus.

The second approach was through insertion of small peptides in the HI loop of the knob domain. Examination of the crystal structure of knob revealed that the HI loop is highly flexible, projects outward from the fiber protein, and is very hydrophilic; three key requirements for insertion of foreign peptides through genetic manipulation, and therefore the best candidate region to tolerate such additions. Various small peptide insertions were indeed successfully tolerated by the HI loop. The most notable of these were the additions of a poly-lysine tract to target heparin sulfphate, an RGD motif to target cellular integrins, the HA epitope, and a FLAG octapeptide for stability analysis.[77,79-82] Our laboratory has also successfully inserted a myc-epitope within the HI

loop and has shown that it does not interfere with trimerization, while still being accessible for binding to an anti-myc monoclonal antibody. All these insertions, however, did not prevent adenovirus from infecting cells through CAR and therefore, are not suitable for true *in vivo* tropism modification applications. Also, similar to inserting peptides at the carboxy terminus of knob, it appears that large insertions in the HI loop destabilize trimerization, resulting in the inability to rescue viable virus. The upper limit for peptide insertion within the HI loop without inciting deleterious effects in the quaternary structure of Ad fiber has subsequently been found to be 63 aa.[83] Therefore the HI loop is not as good of a target for tropism modification as originally thought; especially when attempting to insert a large molecule such as MR1.

The third, and most drastic method is the complete replacement of the knob domain with a heterologous trimerization motif and targeting peptide.[84,85] Both groups accomplished something akin to pseudotyping of adenovirus by the complete replacement of the knob domain of fiber protein with another virus's trimerizing motif fused to a targeting protein. Krasnykh, *et al.*, replaced the knob domain with the bacteriophage T4 fibritin protein while van Beusechem, *et al.*, used the Moloney Murine Leukemia Virus's alpha-helix trimerization domain. These methods have shown the most potential for covalently linking a large re-targeting protein to the fiber protein while simultaneously abrogating native binding to CAR. As opposed to the method of coating the virion with a polymer complex, the removal of the knob domain does not require further treatment of adenovirus and allows for the direct propagation of the tropism modified virus. However, since neither group has demonstrated the feasibility for fusion of a large protein complex, such as MR1, to their chimeric proteins, the simplest method

for re-targeting adenovirus with MR1 to EGFRvIII is through non-covalent binding of a bispecific re-targeting molecule.

Non-Genetic Modification

Two non-covalent re-targeting efforts have focused on creation of bispecific conjugates, which are fusion proteins with two independent binding domains, each specific for a unique target. Two types of bispecific conjugates have been constructed and have successfully demonstrated tropism modification. The first is the linking or direct fusion of the antigen binding fragement (Fab) of an anti-knob antibody to a re-targeting protein. Examples of this are Fab-folate, Fab-Fibroblast Growth Factor 2, Fab-anti-EGFR mAb, Fab-anti-EpCAM, and Fab-anti-CD70 mAb. [86-91] A slight modification of this re-targeting principle was used to create a scFv against knob fused to a scFv against EGFR.[92]

The second non-covalent technique is fusing the extracellular domain of CAR, known as the ectodomain (domains D1 and D2 mentioned earlier) with a re-targeting protein. This theory has been successfully utilized to create CAR-EGF and CAR-Fcγ fusion proteins.[93,94] This system is the more attractive, of the two non-covalent techniques, for our attempts to re-target adenovirus via MR1 to EGFRvIII because there are no further requirements for creating a highly specific anti-knob antibody since CAR already has an extremely high affinity for the knob domain.

A third method coats the whole virion with a polymer complex. This technique is unique and requires the manipulation of the virus after it is fully assembled. The creation of a polymer-coated virion where targeting peptides are incorporated in the matrix

showed to not only evade immune neutralization, but demonstrated an ability to selectively infect the peptide's conjugate receptor positive cells.[95] The requirements for pre-treating the virus with additional chemistry, and variability in the efficiency of integrating retargeting peptides within a capsid-coated polymer are drawbacks to this method.

Due to the following two additional requirements: a) an interaction between two proteins; and b) the required expression of a second protein, non-covalent methods of re-targeting adenovirus raise safety and efficiency questions as opposed to covalent re-targeting strategies. However, since there is no precedent set for the insertion of a molecule the size of MR1 scFv (~24 kDa) into the knob domain (~24 kDa) without disturbing the tertiary and quaternary structure of the fiber protein, non-covalent techniques are more appealing due to their higher success rates in establishing a successful re-targeting system. Additionally, it is hoped that methods for chemically cross-linking adenovirus fiber protein to a bispecific conjugate will circumvent the *in vivo* safety concerns that may arise due to the possible dissociation of the fiber-bispecific conjugate complex. However, pursuit of a covalent strategy of re-targeting adenovirus using MR1, especially through collaboration with a group that has demonstrated substitution of the knob domain, remains a viable option for the future.

1.2 Objective

Originally, the main objective of the project was to develop a 293T cell based system for the expression of adenoviral fiber proteins with insertions in the HI loop. As mentioned earlier, this is a method of direct covalent modification of the knob domain in the hopes of re-targeting fiber binding. Specifically, two unique restriction sites flanked by sequences encoding highly flexible amino acids were incorporated within the HI loop and various peptides were inserted and tested for expression. Unfortunately the size tolerance of the HI loop prevented the successful incorporation of MR1. However, as mentioned earlier, an eight amino acid myc-epitope was acceptable.

Prevented from utilizing the HI loop, we next examined the potential of a twenty amino acid peptide (icosapeptide) dubbed MH20 that appeared to bind to the knob domain with a very high affinity.[53,96,97] To circumvent any problems involving the structural stability of any proteins involved in this re-targeting strategy, we proposed that the icosapeptide should be safely fused to the amino terminus of MR1 without any detrimental effects to its structure. Bacterial expression, refolding and FPLC purification, however, proved our premise to be incorrect, as the protein appeared to aggregate. Refolding of just the light chain domain of MR1 (V_L) was able to demonstrate limited binding to knob protein via Enzyme-Linked Immunosorbent Assay (ELISA), but not nearly as well as claimed in the literature. Therefore, this re-targeting strategy was abandoned.

Finally, the last strategy attempted appears to have successfully increased infectivity of adenovirus to EGFRvIII cells. The rationale and details are provided in the hypothesis.

1.3 Hypothesis

We propose that the creation of a fusion protein consisting of the extracellular domain of CAR (eCAR) linked to MR1 scFv (Figure 3) would be able to:

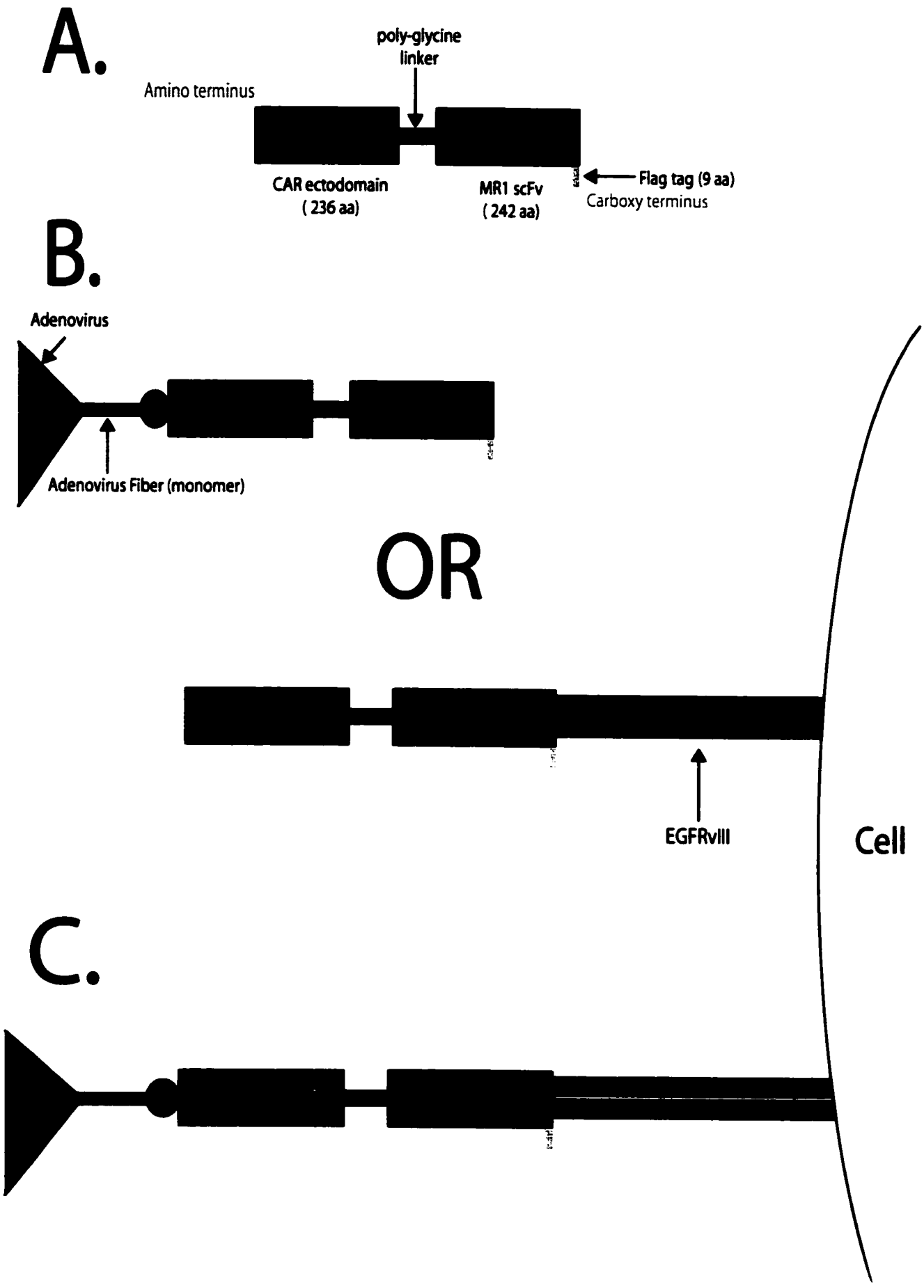
1. Retain the binding affinity of CAR for the Ad5/Ad2 knob domain while simultaneously being able to bind to EGFRvIII through its MR1 subunit; and

2. Increase infectivity of adenovirus serotype 5 to cells expressing EGFRvIII over that of wild-type Ad5 virus alone.

We intend to demonstrate this by:

- Expressing an amino terminal FLAG tagged eCAR-MR1 protein (~ 54.1 kDa) in 293T cells and purifying it using anti-FLAG affinity agarose gel.
- Confirming by ELISA the ability of the fusion protein to independently bind Ad knob protein through its eCAR subunit, and to also bind the EGFRvIII epitope through MR1.
- Determine the relative binding affinity, using competitive ELISA analysis, of the eCAR-MR1 fusion protein for its respective targets as detailed in the previous goal.
- Assessing the ability of the eCAR-MR1 protein to increase infectivity of adenovirus to cells expressing EGFRvIII, but competitively inhibit infectivity of adenovirus to the same cells lacking EGFRvIII expression.

Figure 3. Illustration of the eCAR/MR1 fusion protein and the proposed mechanism of increasing adenovirus infection to cells expressing EGFRvIII. (A) The eCAR/MR1 fusion protein is composed of the extracellular domain of the coxsackie and adenovirus virus receptor's extracellular adenovirus knob-binding domain fused, via a glycine linker to the scFv MR1, which binds EGFRvIII. A carboxy terminal FLAG tag is also added into the coding sequence to facilitate detection and purification of the ~54 kDa eCAR/MR1 fusion protein. (B) The eCAR/MR1 fusion protein may either: bind to the knob domain of adenovirus and then attach to EGFRvIII through the MR1 subunit; or bind to EGFRvIII and act as an addition CAR receptor to allow adenovirus binding. (C) Regardless of which mechanism eCAR/MR1 uses in (B), the ultimate outcome is that it acts as an intermediary bridging molecule to assist in increasing adenovirus infection to EGFRvIII expressing cells.


A.
poly-glycine linker
Amino terminus
CAR ectodomain (236 aa)
MR1 scFv (242 aa)
Flag tag (9 aa)
Carboxy terminus
B.
Adenovirus
Adenovirus Fiber (monomer)
OR
EGFRvIII
Cell
C.

MATERIALS AND METHODS - Chapter 2

Materials and Methods

Cell culture

All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 100 units/ml penicillin, 100 mg/ml streptomycin, and 4mM L-glutamine at 37°C in a 5% CO_2 atmosphere. 293T (human embryonic kidney) cells were obtained from the American Type Culture Collection. U87MG (human glioblastoma) and U87MG transduced with EGFRvIII (U87MGΔ EGFR) were from W. Cavenee (University of California at San Diego).[98] CHO (chinese hamster ovary) cells were kindly provided by Dr. K. Dimock (University of Ottawa).

Bacterial Cultures

Chemically competent Novablue *Escherichia coli* for the propagation of pT7Blue3 vectors were purchased from Novagen. Ultra competent TOP10F' *Escherichia coli* cells for the propagation of all other plasmids were purchased from Invitrogen. Calcium chloride competent BL21λ (DE3) *Escherichia coli* for the expression of proteins was prepared by inoculating 100 ml of Luria-Bertani broth (LB) with 1 ml of an overnight bacterial culture and incubating with constant shaking at 37 °C until an OD_{590} of 0.4-0.5 was reached. The culture was then chilled on ice for 10 minutes and centrifuged at 4°C at 2000 xg for 6 minutes. The bacterial pellet was then resuspended in 20 ml of ice-cold $CaCl_2$ solution (sterilized 60 mM $CaCl_2$, 10 mM PIPES pH7, and 15% glycerol) and centrifuged again at 4°C at 2000 xg for 6 minutes. Following resuspension of the pellet in 10 ml of $CaCl_2$ solution, the solution was left on ice for 30 minutes,

pelleted and resuspended in 2 ml of $CaCl_2$ solution. Aliquots of 100 µl were frozen at –80°C for future use.

Construction of pCDNA3.1-eCAR/MR1.

The pEF6/V5-CAR plasmid, containing the entire coding sequence of human CAR, was kindly provided by A. Haddid (University of Ottawa). The pCANTAB-MR1 (scFv) plasmid was originally constructed by I. Lorimer (University of Ottawa).[19] This plasmid contains the complete 741-bp coding sequence for the single chain Fv version of MR1.

The sequence encoding the first 236 amino acids of the extracellular domain (ecto-domain) of human CAR (eCAR), including the Kozak sequence[99] and the amino terminal signal peptide sequence, was amplified from the pEF6/V5-CAR plasmid using sequence specific synthetic oligonucleotides on a Perkin Elmer GeneAmp PCR 2400 System with the Expand High Fidelity PCR System (Boehringer Mannheim). The eCAR PCR product, using an upstream primer containing a HindIII site (underlined) (5'CGTAAGCTTGCCACCATGGCGCTCCTGCTGTG CTTCGTG 3') and a downstream primer containing a NotI site (underlined) (5'TGAGAGCGGCCGCCTCCACCTCCAGCTTTATTTGAAGGAGG 3') was gel purified, following excision of the DNA band from a 1.8% agarose gel, using the Gelex DNA Purification Kit from Calgen. The purified PCR product was then blunt end ligated into pT7Blue3, to yield pT7B3-eCAR, using the "Perfectly Blunt Cloning kit" (Novagen) following the manufacturer's protocol. The ligation mixture was then incubated overnight at room temperature, and the next day, 2 µl of ligation mixture was transformed into Novablue *Escherichia coli* cells. Briefly, the transformation protocol involves incubation

of 50-100 µl of ultracompetent bacteria for 30 minutes on ice after the addition of the plasmid to be propagated, followed by a 30 second heat shock and 1 hour outgrowth at 37°C in a horizontal shaker. Transformants were then plated on 100 mm LB agar plates containing 100 µg/ml ampicillin, 100 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and 40 mg/mL X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) for blue/white screening at 37°C. White colonies were picked the next day and allowed to grow overnight at 37°C in a horizontal shaker in 2ml LB containing 200 µg ampicillin. Plasmid isolation and purification followed the next day using the QIAprep Spin Miniprep Kit (Qiagen) using the manufacturer's protocol. Restriction analysis confirmed the presence of an insert of the appropriate size. The eCAR insert was then sequenced using pT7Blue3 specific primers (M13For and M13Rev) on an ABI Prism 377 DNA Sequencer. The eCAR insert was excised from pT7B3 through restriction digesting and then subcloned, for eukaryotic expression, into HindIII/NotI digested pCDNA3.1(+) (Invitrogen) to yield pCDNA-eCAR following similar methodology as previously described. Two attractive characteristics of this expression plasmid include the use of the human cytomegalovirus immediate-early (CMV) promoter for high transcription levels of the inserted gene, and the simian virus 40 (SV40) origin of replication for episomal replication of the plasmid in transfected cells containing the SV40 large T antigen (e.g. 293T cells).

Similarly, a PCR was run to amplify out the complete MR1 scFv sequence using pCANT-MR1 as a template with an upstream primer containing a NotI site (underlined) (5'GGAGGCGGCCGCTCTCAGGTGAAACTGCAGCAGTCT 3'), and a downstream primer encoding a carboxy terminal FLAG tag and also containing a XbaI site

(underlined)

(5'CTCTAGATTACTTGTCATCGTCATCCTTGTAATCTTTGATTTCCAGCTTG 3'). This PCR product was blunt end ligated into pT7Blue3, sequenced and subcloned into NotI/XbaI digested pCDNA-eCAR to yield the final expression vector, pCDNA-eCAR/MR1. TOP10F' bacteria were transformed with this vector and plated as outlined previously. Colonies were picked for overnight cultures, and the next day a large culture was begun for the purification of large quantities of pCDNA-eCAR/MR1 using the Qiafilter Plasmid Maxi Kit (Qiagen) following the manufacturer's protocol. Concentrations of purified pCDNA-eCAR/MR1 plasmids were then determined from A_{260} values using a Beckman DU-640 spectrophotometer.

Construction of pET22b-Ad2Knob and Ad5Knob.

The Ad2 (pFG28) and Ad5 (pRP2019) Fiber plasmids were kindly provided by Dr. Robin Parks (Ottawa Health Research Institute). Using these as templates, synthetic oligonucleotides were designed to amplify the sequence encoding the Ad2 Knob domain and the Ad5 Knob domain, both containing a newly encoding 6xHistidine tag at the amino terminus. For the Ad2 Knob protein, the upstream primer (GAGCATATGCACCACCATCACCACCATGGGGCCATTACAATAGGAAAC) containing a NdeI site (underlined) and encoding the amino terminal 6xHis-tag was used in conjunction with a downstream primer (CAAGCTTCATTATTCCTGGGCAATGTAGG) containing a HindIII site (underlined). Similarly for Ad5 Knob, the upstream primer (GAGCATATGCACCACCATCACCACCATGGTGCCATTACAGTAGGAAAC)

containing a NdeI site (underlined) and encoding the amino terminal 6xHis-tag was used in conjunction with a downstream primer (CAAGCTTCATTATTCTTGGGCAATGTATG) containing a HindIII site (underlined). Both PCR products were cloned into pT7Blue3, sequenced, and subcloned into pET22b(+) for prokaryotic expression in BL21(DE3) bacteria. For large-scale expression of the Ad2 knob protein, at least 1 μg of the pET22b-Ad2 knob plasmid was transformed into 50 μl BL21 (λ DE3) *Escherichia coli* cells. The transformations were plated on 10 100mm agar plates containing 100 μg/ml ampicillin. The next day, a 1L bacterial culture was prepared by scraping the colonies from the 10 plates into a growth solution consisting of 1L of Superbroth media, 0.2% glucose, 0.025% $MgSO_4$, and 100 mg ampicillin. The cultures were grown to an A_{600} of 2.5, as measured on a Beckman DU640 spectrophotometer, at 37 °C with constant shaking then induced with 10 ml of 100 mM isopropyl β-D-thiogalactoside (IPTG) for 1.5 hours. The cultures were then pelleted, using a Beckman J2-21M/E centrifuge, and resuspended in 100 ml 50mM Tris pH 7.4. The cells were lysed with 3 ml of 5 mg/ml lysozyme at room temperature with constant shaking for 30 minutes. Incubation with 2.5 ml 10% NP40 and 2.2 ml 5M NaCl on ice to dissociate bacterial membrane was then followed by digestion of endogenous DNA with 11 μl DNase I solution (100 mg pancreatic Dnase I in 10 ml of 20 mM Tris-HCl pH 7.4, 40 mM NaCl, 1 mM dithiothreitol, 0.1 m/ml bovine serum albumin, in 50 % glycerol) and 115 μl $MnCl_2$ (Sigma). The supernatant, containing all soluble protein, was retained following centrifugation on a Beckman J2-21M/E centrifuge. The soluble His-tagged knob protein was then bound to a Nickel-NTA agarose column (Qiagen) that had been pre-washed in Buffer A (10 mM Tris pH 7.4, 100mM NaCl). Non-specifically

bound proteins were washed off the column with 20% of Buffer B (10mM Tris pH 7.4, 100mM NaCl, and 500 mM Imidazole) in buffer A, and then the knob protein was eluted in 1 ml fractions with 300mM imidazole. All purifications were done on a Pharmacia Biotech FPLC system and peak protein fractions were determined through absorbance at 280nm with a Pharmacia LKB-W-MII Spectrophotometer. Fifteen µl of each peak fraction was added to 5 µl of 4X Laemmli buffer (0.004% Bromophenol Blue, 8% SDS, 20% glycerol, 2.5mM Tris pH 6.8, 20% β-mercaptoethanol), boiled for ten minutes and then separated via sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (12% polyacrylamide) in SDS-PAGE running buffer (250 mM Tris, 2.5M glycine pH 8.3, 1% SDS in water). The presence of a knob protein band was visualized through staining with Coumassie Brilliant Blue stain (0.025% Coomassie Brilliant Blue dye, 10% Acetic Acid, 50% methanol in water).

PAGE and Western Blotting.

For the analysis of expression of eCAR/MR1, 293T cells were transiently transfected with pCDNA-eCAR/MR1 using the calcium phosphate method.[100] The day prior to transfection, $2x10^6$ cells were plated on 100mm tissue culture dishes in 10 ml of DMEM media. Also, 10 µg of pCDNA-eCAR/MR1 and 10 µg of pCDNA vector, to be used as a negative control, were precipitated and sterilized with ethanol overnight at -20°C. The next day, the DNA was pelleted, re-suspended in sterile ddH_2O, precipitated in 2M $CaCl_2$ and 2X HeBS (42 mM N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES), 274 mM NaCl, 10 mM KCl, 1 mM $Na_2HPO_4 \bullet 2H_2O$, 11 mM glucose, adjusted to pH 7.2), and added to the 293T cells. To reduce the presence of unrelated

proteins, twenty-four hours post-transfection, the 293T cells were washed and switched to 3ml of DMEM (pen./strep./glut.) containing only 0.5% FCS for an additional day. Both supernatants of transfected cells and cell lysates were harvested. Whole cell lysates were isolated by scraping transfected cells with boiling 2X Western Blot lysis buffer containing 5mM Tris pH 6.8, 4% sodium dodecyl sulfate (SDS), 2% glycerol, and 0.3 g 1,4-Dithio-DL-threitol (DTT). The cell lysates were then boiled for 10 minutes at 95°C, sonicated for 20 seconds at an intermediate setting, and boiled for a further 8 minutes. Supernatants and lysates were quantified using Biorad's Coomassie Protein Assay reagent, based on the Bradford dye-binding procedure.[101] 40 μg of boiled protein from each sample was separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (10% polyacrylamide) after diluting in 4X Laemmli Buffer containing β-mercaptoethanol. The separated proteins were transferred electrophoretically to nitrocellulose membranes in transfer buffer (16.8 mM Tris, pH 8.6, 192 mM glycine, and 20% methanol) for a period of 1 hour at 100 V. The membranes were stained with Amido Black (10% acetic acid, 50 % Methanol, 0.25% Amido Black in water) to confirm transfer of all proteins and then blocked overnight in TBST (100mM Tris pH 7.6, 150mM NaCl, 0.05% Tween) containing 4% skim milk. The next day, the membranes were incubated with 5000X diluted M2 mouse monoclonal anti-FLAG antibody (Sigma-Aldrich) in TBST containing 4% skim milk for 1h. The membranes were then washed 3x with TBST, and probed with 10,000X diluted horseradish peroxidase-conjugated goat anti-mouse IgG (Biorad) in TBST containing 4% skim milk for 1h. Following an additional 3 washes with TBST, immunoreactivity was determined

using the SuperSignal® West Pico enhanced chemiluminescence Western blotting detection system (Pierce) on Kodak X autoradiography film.

Purification of eCAR/MR1.

Harvested supernatant from 293T cells transfected with pCDNA-eCAR/MR1 were incubated overnight with constant shaking at 4 °C in a 40:1 volume ratio with 1 ml of anti-Flag M2 Affinity gel (Sigma-Aldrich) pre-washed in TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.4). The beads were then spun down at 3500 rpm for 3 minutes and then washed with TBS. Washings were repeated two more times and then bound eCAR/MR1 was eluted with 2 ml of 100 ng/ml Flag peptide (Sigma-Aldrich) in TBS by incubating overnight with constant shaking at 4 °C. Beads were again spun down at 3500 rpm for 3 minutes and the supernatant was retained. The purified eCAR/MR1 supernatant was passed through a PD10 desalting column to remove any excess FLAG peptide. The column was washed with 5 bed volumes of TBS and then the 2ml of eCAR/MR1 supernatant was added to the column. When all excess solution was drained from the column, 3.5 ml of TBS was added and 1ml fractions of eluent were collected. The purity of the eCAR/MR1 protein was confirmed by SDS-PAGE followed by transfer to nitrocellulose and staining with Amido Black. To determine the concentration of purified eCAR/MR1, 20 µl of the purified eCAR/MR1 volume was run alongside BSA standards on a 12% SDS-PAGE. This gel was transferred to a nitrocellulose membrane and stained with Amido Black. The relative band intensities for the BSA standards were then assessed using a UVP Biochemi System, and the intensity of the eCAR/MR1 band was related to these.

Competitive ELISA.

Binding of eCAR/MR1 to EGFRvIII epitope (Pep3).

A 96 well polystyrene plate was coated overnight at 4 °C with 200 ul of 1 μg/ml streptavidin in PBS (137 mM NaCl, 3 mM KCl, 4 mM Na_2HPO_4, 1.5 mM KH_2PO_4). The next day, the plate was washed three times with 0.05% Tween 20/PBS (v/v) and incubated with 1 μg/ml biotinylated EGFRvIII peptide (Pep3) for 20 minutes at room temperature. All subsequent washing steps were done by rinsing all the wells with 0.05% Tween 20/PBS (v/v) three times. Blocking for 1 hour with 200 μl per well 2% skim milk powder in PBS (w/v) was then followed with the competitive ligand binding assay. To determine the dissociation constant for eCAR/MR1 binding to Pep3, 100 ul of 10 μg/ml of eCAR/MR1 crude supernatant was incubated with 100 ul of 2000nM, 200nM, 60nM, 20nM, 6nM, 2nM, 0.2nM, or 0nM non-biotinylated Pep3 for 1 hour at room temperature in 4% skim milk powder in PBS. The wells were washed and incubated with anti-Flag antibody (Sigma-Aldrich) in 2% skim milk powder in PBS (1:1000 dilution) for 1 hour at room temperature. The wells were washed and incubated with goat anti-mouse HRP conjugated antibody (Biorad) in 2% skim milk powder in PBS (1:5000 dilution) for 1 hour at room temperature. After washing the wells again, 200 μl per well of hydrogen peroxide added 2,2′-azinobis-[3-ethylbenzothizoline-6-sulfonic acid] (ABTS) substrate solution (sterilized 0.05 M Citric acid pH 4, 220 mg ABTS in water) was added. The plates were incubated at room temperature for 20 minutes and absorbance was assayed on a Dynex Technologies ELISA plate reader at 405nm.

Binding of eCAR/MR1 to Ad2Knob. ELISA analysis of the binding affinity of eCAR/MR1 to Ad2Knob was done in a similar manner to that of eCAR/MR1 to Pep3 with the following exceptions: 1) The polystyrene plate was directly coated with 1 μg/ml Ad2knob the day prior to the ELISA and incubated overnight at 4 °C and 2) the competitive ligand used was recombinant Ad2knob protein rather than Pep3 at the same concentrations.

Prior to competitive binding ELISA analysis, several experiments with sufficient controls were conducted to ensure that non-specific binding interactions were not accounting for the observed green colour. These included primary and secondary antibody controls, mock transfected supernatant reactivity and possible reactivity of streptavidin or Pep3 by the antibodies used.

Ad5LacZ U87MG/U87MGΔEGFRvIII Infectivity Assays – Cell Staining.

First generation Ad5 virus genetically manipulated to contain the β-galactosidase gene under the regulation of the cytomegalovirus promoter was kindly provided by Dr. Robin Parks (Ottawa Health Research Institute). 2.5×10^5 cells from each cell line were added to separate tissue culture treated 6 well polystyrene plates the day prior to the infection. The following day, cells were washed and three wells from each plate were incubated for 1 hour at 37 °C/5% CO_2 with 2 μM Ad2knob in PBS, whereas the remaining wells received just PBS. Addition of sufficient CsCl purified Ad5-LacZ virus to achieve a theoretical infectivity of 500 particles per cell (MOI of 5 pfu/cell) was added to each well. Simultaneously a well on each plate that was pre-incubated with knob and one that was incubated with only PBS, received a volume corresponding to 150 μg of

CAR/MR1 crude supernatant. Similarly, a well on each plate that was pre-incubated with knob and one that was incubated with PBS received a volume corresponding to 150 μg of protein from supernatant from mock transfected cells. The final volume in each well for these infections was made up to a total of 400 μL; any differences in volume were accounted for by the addition of PBS. The plates were then incubated at 37 °C/5% CO_2 for 1 hour with agitation every 15 minutes. Following the one-hour incubation, 2 ml of DMEM were added to each well and the plates were allowed to be incubated for 18 hours at 37 °C/5% CO_2. Cells were washed with 1ml of PBS, fixed with 1ml of 0.2% glutaraldehyde, 2% para-formaldehyde, and 2mM $MgCl_2$ for 10 minutes at room temperature, washed again with PBS, and then stained for β-galactosidase activity with 1ml of 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 2mM $MgCl_2$, 1mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) in PBS. Plates were then incubated overnight in the dark at room temperature. Following purification of the eCAR/MR1 fusion protein, these experiments were repeated and the crude eCAR/MR1 supernatant was substituted with 100 μl of purified eCAR/MR1 protein. This volume amounts to 2 μg (3.7 μM) of the fusion protein per well.

Ad5LacZ U87MG/U87MGΔEGFRvIII Infectivity Assays – ONPG Assays.

To measure β-galactosidase activity of transduced cells, viral infectivity assays for both cell lines were done in a similar manner as the infections involving β-galactosidase activity staining but by incubating with 2 μg of purified eCAR/MR1 as indicated. Following the 18 hour incubation post-infection, infected cell monolayers were washed with 1ml PBS, and incubated with 300 μL of 1X Reporter Lysis Buffer

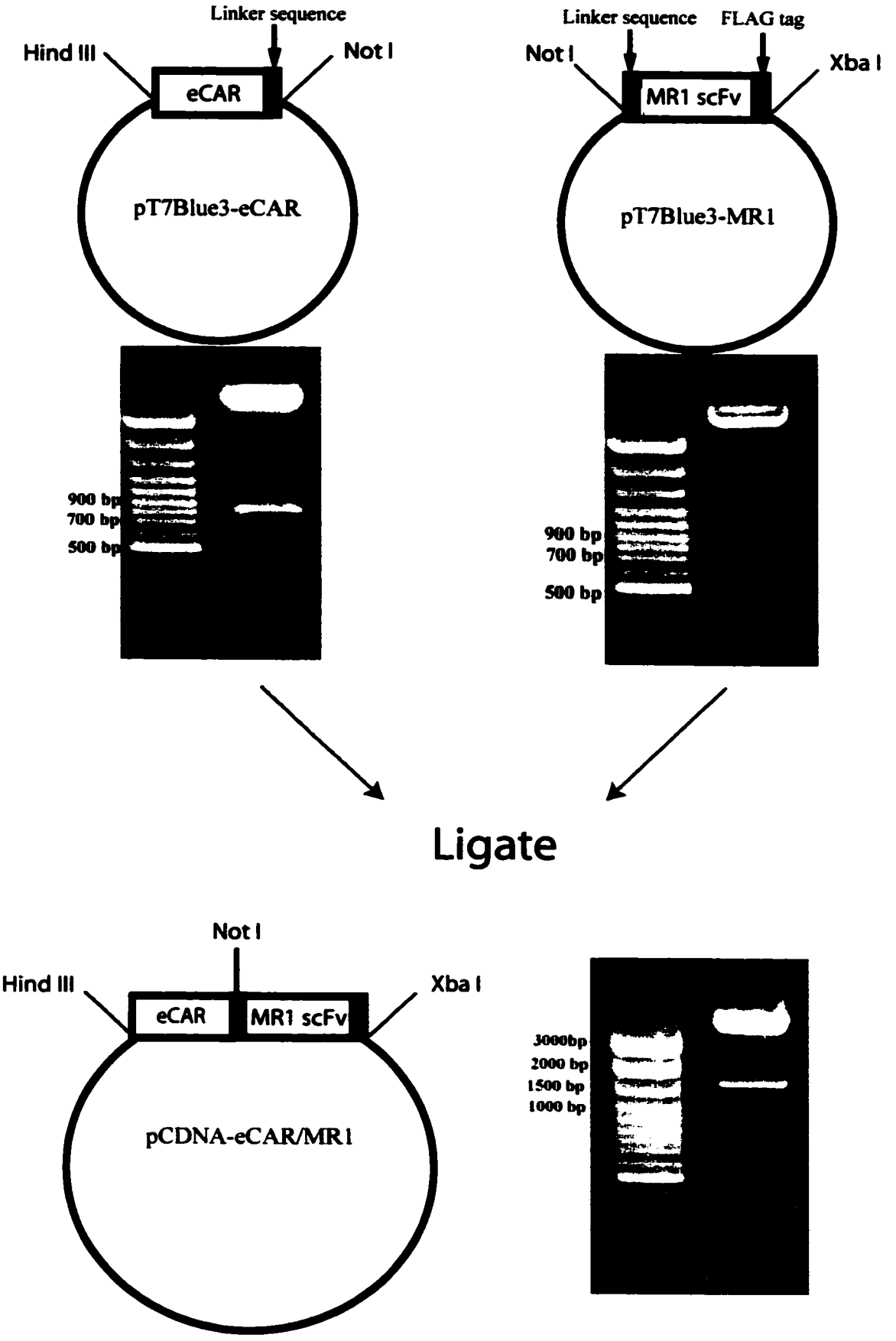
(Promega) for 10 minutes. The cells were then scraped with a rubber policeman, transferred to a microfuge tube and frozen at –20 °C. The samples were then thawed, centrifuged at 14000 rpm for 3 minutes, and 10 µL of supernatant was added to an assay solution composed of 748 µL ddH_2O, 240 µL 5X reaction buffer (0.06M $Na_2HPO_4 \bullet 7H_2O$, 0.04 M $NaH_2PO_4 \bullet H_2O$, 0.01 M KCl, 0.05 M $MgSO_4 \bullet 7H_2O$, 0.05M β-mercaptoethanol), and 2.7 µL β-mercaptoethanol. The assay began upon addition of 200 µL 4mg/ml ONPG (o-nitrophenyl-β-D-galactopyranoside). The samples were then incubation at room temperature in the dark until a yellow colour formed in the highest activity sample (20-60 minutes). Concurrently, standard solutions of β-galactosidase were run to ensure that the measured optical density remained within the linear range of the spectrophotometer. When sufficient colour was observed the reactions were stopped by addition of 500 µL of 1M Na_2CO_3, and the absorbance measured at 420 nm on a Beckman DU-600 spectrophotometer.

RESULTS – Chapter 3

Results

Expression and purification of the eCAR/MR1 fusion protein. Construction and expression of a gene encoding the ectodomain of human CAR fused to the scFv MR1 was created in the hope that the expressed bispecific protein would increase Ad infectivity to cells that express EGFRvIII. A PCR-amplified DNA sequence corresponding to amino acids 1 to 236 of the human CAR along with the endogenous Kozak consensus sequence and 14 aa endoplasmic reticulum (ER) cleaved N-terminal signal sequence was cloned into the HindIII/NotI restriction enzyme digested multiple cloning site of pCDNA3.1, resulting in the vector known as pCDNA-eCAR. The extracellular, or ectodomain, of CAR is composed of two Ig-like domains D1 and D2, where the D1 is primarily responsible for binding to group C adenoviruses (in addition to other subclasses). The MR1 scFv gene (726 nucleotide, 242 aa) encoding the variable heavy chain (V_H) joined to the variable light chain (V_L) with a poly-glycine linker was PCR-amplified along with additional, primer embedded, sequence for a C-terminal 9 aa FLAG epitope and cloned into NotI/XbaI restriction enzyme digested pCDNA-eCAR plasmid (Figure 4). The mature (secreted) fusion protein is expected to consist of 493 aa with a calculated molecular mass of 54 kDa. Expression of the fusion protein was under the control of the CMV promoter and an SV40 origin of replication, allowing for overexpression and secretion of eCAR/MR1 from transiently transfected 293T cells using the well-established calcium phosphate protocol. Eukaryotic cells were used for the expression of eCAR/MR1 since it has been well known that bispecific constructs form as insoluble aggregates (inclusion bodies) when expressed in prokaryotic cells. This would therefore require an additional refolding step where optimizing the conditions can waste a

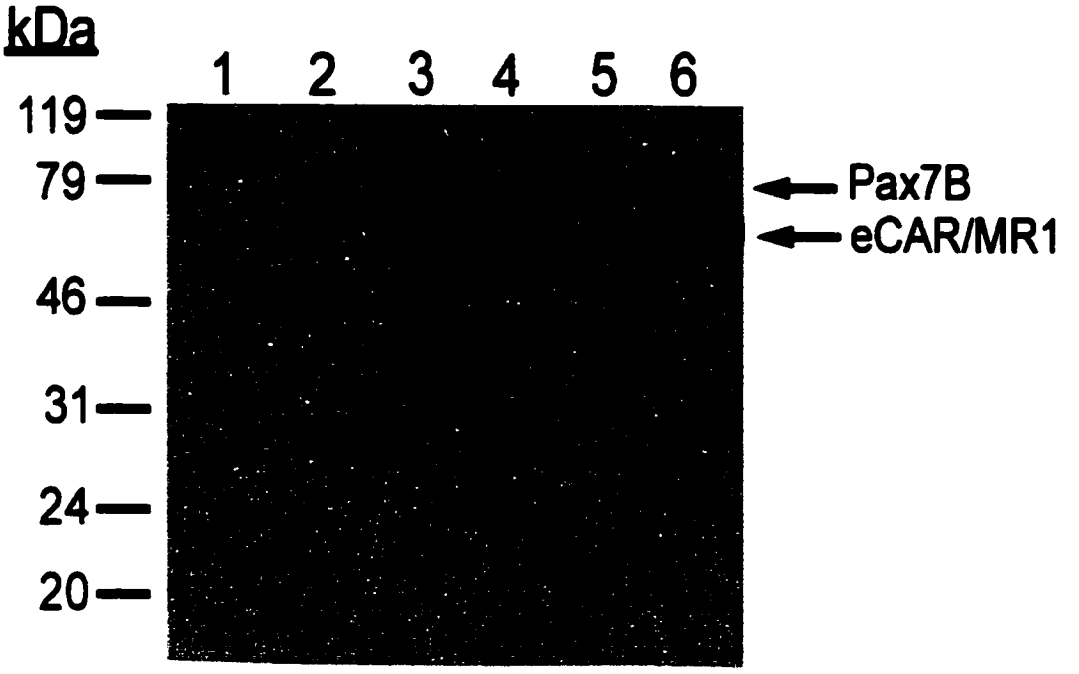
Figure 4. Creation of the eCAR/MR1 fusion protein expression plasmid. Two separate PCRs were conducted to create pT7Blue3 constructs containing either the sequence encoding the extracellular domain of CAR (eCAR) (total size 746 nucleotides) or the scFv MR1 with a carboxy terminal FLAG tag (total size 775 nucleotides) flanked by uniquely occurring restriction sites. The sequence for the internal poly-glycine linker also contains an internal NotI site which facilitated ligation of the two sequences together in the final expression vector pCDNA-eCAR/MR1.


Linker sequence
Hind III
Not I
eCAR
pT7Blue3-eCAR
900 bp
700 bp
500 bp
Linker sequence
FLAG tag
Not I
Xba I
MR1 scFv
pT7Blue3-MR1
900 bp
700 bp
500 bp
Ligate
Not I
Hind III
Xba I
eCAR
MR1 scFv
pCDNA-eCAR/MR1
3000bp
2000 bp
1500 bp
1000 bp

lot of time. The one detrimental effect of relying on eukaryotic expression is the limited amount of protein obtained – microgram quantities as opposed to the milligram quantities obtainable from prokaryotic expression. The supernatants (media) of transfected 293T cells and whole cell lysates of the same cells were analyzed by SDS-Page under reducing conditions and by Western blotting using an anti-FLAG mouse monoclonal primary antibody and an HRP-conjugated monoclonal goat anti-mouse secondary antibody. Amido Black staining of the nitrocellulose membrane prior to commencing the Western blot analysis demonstrated equal loading of the quantity of protein in each lane. A specific signal on autoradiography film of a band migrating as a ~54 kDa species was found in crude supernatants of 293T cells transfected with pCDNA-eCAR/MR1 expression plasmid but not mock transfected 293T cells. 293T cells were also transiently transfected with a pHIT-Pax7B plasmid as a positive control (provided by Dr. Michael Rudnicki, Ottawa Health Research Institute), and a diffuse band of ~ 90 kDa corresponding to Pax7 was observed as expected. Comparison of whole cell lysates versus supernatant of pCDNA-eCAR/MR1 transiently transfected 293T cells also demonstrates a higher amount of eCAR/MR1 being secreted as opposed to being retained within the cell (Figure 5). The presence of detectable amounts of eCAR/MR1 within the cell can be accounted for by the high levels of expression of the protein, due to the CMV promoter, leading to saturation of secretory mechanisms of the cell and therefore causing retention of excess protein within the ER and Golgi.

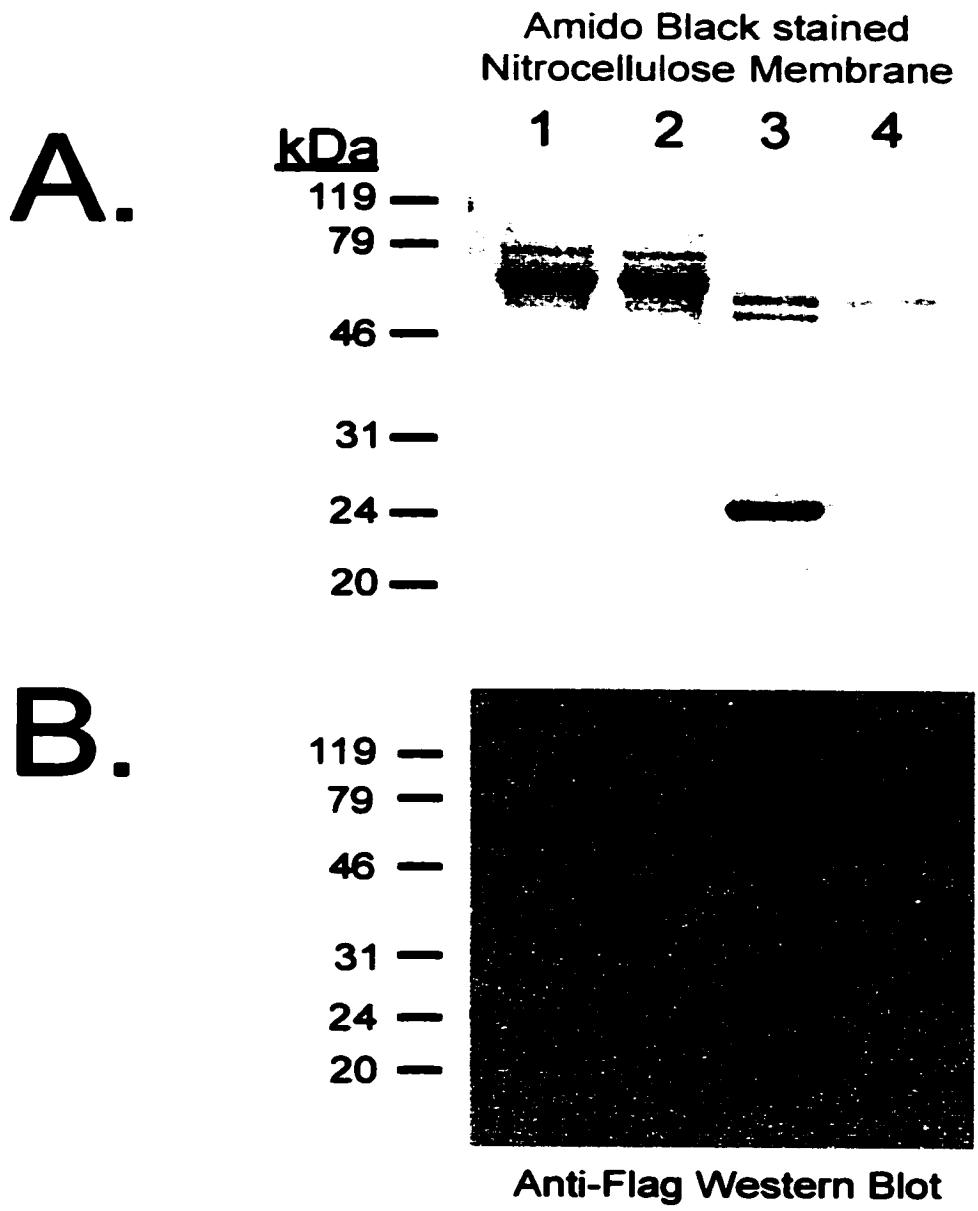
Crude supernatants from pCDNA-eCAR/MR1 transfected 293T cells were incubated in TBS washed anti-FLAG M2 affinity gel overnight. This was done to allow for thorough binding of eCAR/MR1 to the resin. To reduce non-specific interactions of

Figure 5. Expression of eCAR/MR1 fusion protein in transiently transfected 293T cells. 20 μg of cell lysates from 293T transiently transfected with either pCDNA-eCAR/MR1, pCDNA3.1, or pHIT-Pax7B plasmids were electrophoresed on a 12% acrylamide SDS-PAGE gel alongside 20 μg of protein from media harvested from the same cells. The protein gel was then electrophoretically transferred to a nitrocellulose membrane and the presence of FLAG-tagged proteins was detected via Western blotting with an anti-FLAG mouse monoclonal antibody (Sigma-Aldrich). The picture is a representative autoradiography film of the Western blot. The lanes are as follows: 1) pCDNA3.1 transfected 293T cell lysates; 2) pCDNA3.1 transfected 293T media; 3) pHIT-Pax7B transfected 293T cell lysates; 4) pHIT-Pax7B transfected 293T media; 5) pCDNA-eCAR/MR1 transfected 293T cell lysates; and 6) pCDNA-eCAR/MR1 transfected 293T media. The expected MW of eCAR/MR1 fusion protein is approximately 54 kDa, whereas for Pax7B a molecular weight of approximately 60 kDa is expected.


kDa
1
2
3
4
5
6
119
79
46
31
24
20
Pax7B
eCAR/MR1

unwanted proteins to the affinity gel, the eCAR/MR1 bound resin was washed with TBS and then the bound eCAR/MR1 fusion protein was competitively eluted with FLAG peptide. To remove any excess FLAG peptide remaining in the eluted eCAR/MR1 fraction, and thereby allowing for a more accurate determination of the concentration of purified fusion protein, the eCAR/MR1 was loaded onto a TBS pre-washed PD10 desalting column. The FLAG peptide was eluted in the first elution fraction and the next two fractions contained the eCAR/MR1 fusion protein. The whole purification process was followed via Western blotting of supernatant fractions at various stages of the protocol to ensure that only a minimal amount of eCAR/MR1 was lost (Figure 6). Examining figure 6, a single 54 kDa species is detected with the mouse anti-FLAG monoclonal M2 antibody used in all lanes with the least amount present in supernatant following incubation with the anti-FLAG affinity gel (as expected). The two additional lower bands in lane 3 of the blot correspond to the reactivity of the secondary goat anti-mouse with the mouse anti-FLAG monoclonal M2 coupled resin also loaded on the gel. Crude estimation of the fold purification is possible by examining the Western blot. By comparing lanes 1 and 2 of the blot, approximately 90% of the eCAR/MR1 from the crude supernatant becomes bound to the resin. Then comparing lane 3 to lane 4, approximately 90% of the eCAR/MR1 bound to the resin is competitively eluted; therefore approximately 80 % of the eCAR/MR1 from the crude supernatant is purified. Assuming that this is the theoretical maximum efficiency for the purification under the conditions used, and since the eCAR/MR1 is in a final volume of 2 ml from an original volume of 40, the fold purification would be 16.2. Running an SDS-PAGE gel of 20 µl of eCAR/MR1 alongside BSA standards of known concentrations and electrophoretically

Figure 6. Purification of eCAR/MR1 fusion protein from pCDNA-eCAR/MR1 transfected 293T media. The purification of eCAR/MR1 is outlined in the Materials and Methods section. eCAR/MR1 fusion protein from 40 ml of media from 293T cells transiently transfected with pCDNA-eCAR/MR1 was bound to 1 ml of anti-FLAG M2 affinity gel. This was then competitively eluted with 100 µg/ml FLAG peptide. The lanes are as follows: 1) 20 µl of media harvested from 293T cells transfected with pCDNA-eCAR/MR1 (note: the major protein stained is bovine serum albumin); 2) following incubation of the media with the anti-FLAG affinity gel, the gel beads were pelleted via centrifugation and 20 µl of the supernatant (media) was run in this lane; 3) 20 µl of the eCAR/MR1 bound anti-FLAG affinity gel; and 4) 20 µl of eCAR/MR1 fusion protein following competitive elution off the anti-FLAG affinity gel with FLAG peptide. The top panel (A) is the Amido Black stained nitrocellulose membrane of the transferred protein fractions listed above. It illustrates the degree of purity of the eCAR/MR1 fusion protein (~ 54 kDa) following elution off the anti-FLAG resin (last lane [4]). The bottom panel (B) is the autoradiography film following Western blotting of the nitrocellulose membrane from A. The secondary goat anti-mouse HRP conjugated antibody used in the Western blot reacts with the anti-FLAG mouse monoclonal antibody coupled to the beads (lower bands on lane 3).


A.
Amido Black stained
Nitrocellulose Membrane
kDa
1
2
3
4
119
79
46
31
24
20
B.
119
79
46
31
24
20
Anti-Flag Western Blot

transferring the gel to a nitrocellulose membrane and staining with Amido Black, the concentration of eCAR/MR1 fusion protein purified was determined by densitometry to be 19.4 μg/ml, amounting to 38 μg eCAR/MR1 purified from 40 ml of transfection supernatant.

Expression and purification of Ad2Knob protein. Construction and expression of a gene encoding Ad2 knob protein was conducted to provide a means to block adenovirus infection to target cells *in vitro* and to test the ability of the eCAR/MR1 fusion protein to bind to adenovirus knob. A PCR-amplified DNA sequence corresponding to aa 400 to 582 of Ad2 fiber protein (knob domain) including an additional sequence encoding a N-terminal 6X His-tag was cloned into the NdeI/HindIII restriction enzyme digested bacterial expression plasmid pET22b(+) to generate pET22b-Ad2Knob. This plasmid places the expression of Ad2Knob under the regulation of T7 RNA polymerase. The pET22b-Ad2Knob plasmid was transformed into the *E.coli* strain (BL21 DE3) that has the T7 RNA polymerase under the regulation of the *lacUV5* promotor, which is inducible by addition of IPTG to the media. Bacterial cultures were grown, induced for protein expression, and all soluble protein was isolated as described in the materials and methods.

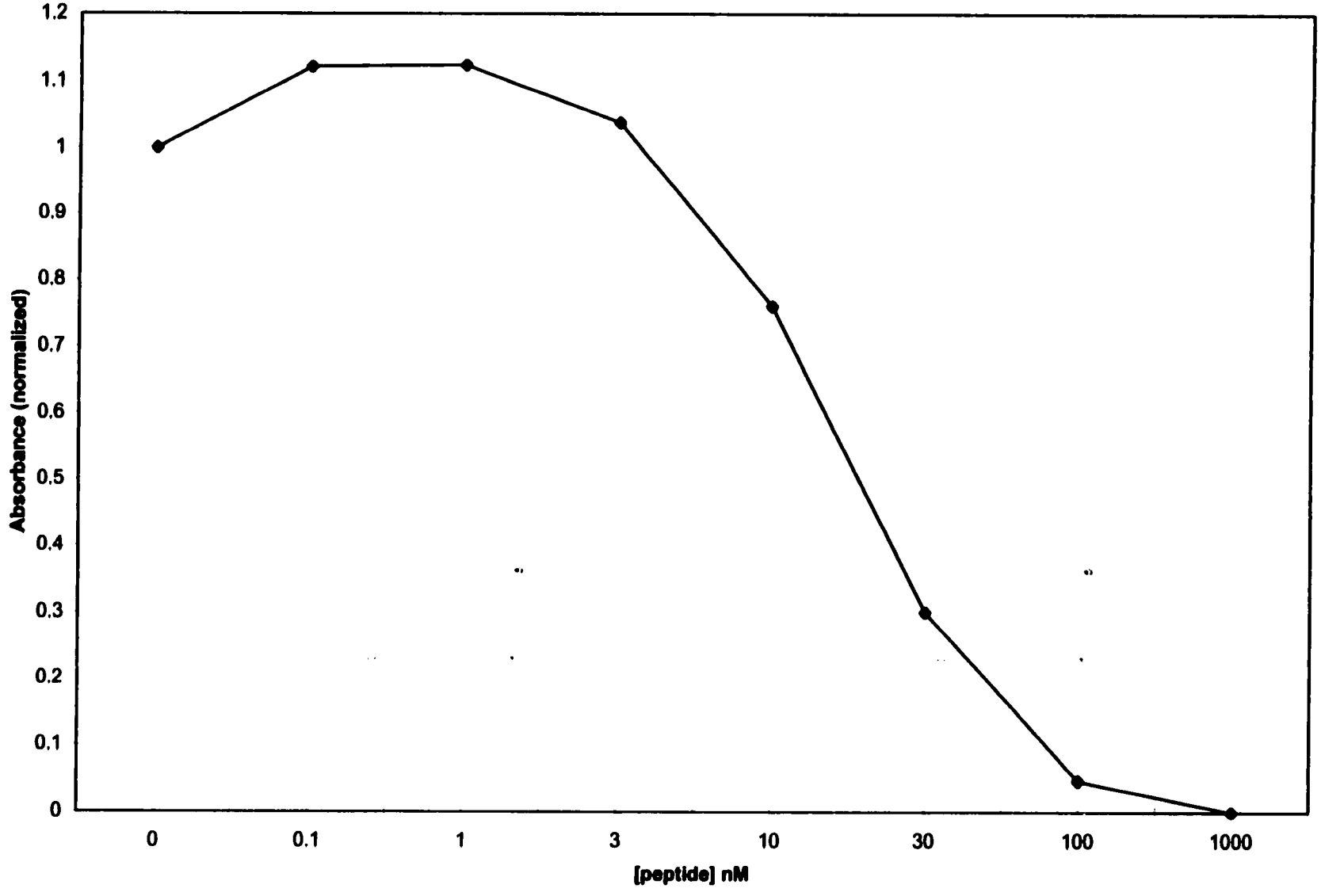
Crude soluble supernatant was then loaded onto a Nickel-NTA agarose column where the majority of Ad2 knob protein became bound through interactions of its 6X His tag with the nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography matrices of the resin. Washing the column with 100mM imidazole dissociated and eluted non-specifically bound or weakly interacting proteins on the column, and the Ad2 knob protein was eluted in 1ml fractions with an increasing gradient of imidazole from 300

mM up to 500 mM. The majority of Ad2 knob protein was eluted when the imidazole concentration reached 300 mM. The presence of Ad2 knob protein in each fraction was assayed by SDS-PAGE by running 15 µl of each eluted fraction on the gel and staining with Coumassie Brilliant Blue. A representative gel can be examined in Appendix A. Peak Ad2 knob protein fractions were pooled and the concentration was determined by using a Pierce assay based on the Bradford method, which relies on a BSA standard curve to determine the amount of Ad2 knob protein purified. The yield of Ad2 knob protein purified with this method varied, however an average of 2 mg (@ 0.3 mg/ml) Ad2 knob protein was usually obtainable from a 1 L culture.

Determining eCAR/MR1 fusion protein's binding capability for Ad2 Knob and EGFRvIII peptide. Competitive ELISA was used to determine the relative binding affinity of the eCAR/MR1 fusion protein for its two target molecules, adenovirus knob and EGFRvIII, and to compare whether the strength of these affinities were comparable to CAR binding Ad2 knob and MR1 binding to EGFRvIII. To ensure the antibody reagents being used in these assays were not erroneously cross reactive, they were tested for non-specific interactions with streptavidin, Pep3, and Ad2 knob. Controls to make certain eCAR/MR1 was not interacting with the streptavidin were also done. Additionally, all binding assays were done with Mock transfected supernatant to show that the interacting protein responsible for the colorimetric signal was eCAR/MR1 fusion protein and not some endogenous protein present in the media. Ad2 knob coated 96 well polystyrene plates were competitively bound with eCAR/MR1 fusion protein in the presence of increasing concentrations of free Ad2 knob protein. As the amount of competing free Ad2 knob protein that binds to the eCAR/MR1 fusion protein increases,

the amount of eCAR/MR1 protein able to bind to the Ad2 knob protein coated on the wells decreases. This was detected by indirect colorimetry, whereby the anti-FLAG M2 mAb bound to any eCAR/MR1 present in the well after washing of the wells with a slight detergent to remove any non-bound eCAR/MR1 fusion protein. A second HRP-conjugated antibody against the anti-FLAG M2 mAb was then used. Incubation with hydrogen peroxide in ABTS cleaves the HRP, which then results in the formation of a green colour. The degree of green colour indicated the relative amount of eCAR/MR1 bound to the wells containing Ad2 knob protein. The signal obtained for the binding of eCAR/MR1 fusion protein to Ad2 knob protein in the absence of competing Ad2 knob protein was taken as 100% binding, and all the other samples' absorbance values, with increasing concentrations of competitive Ad2 knob, were related to that. The point at which 50% of the eCAR/MR1 fusion protein was inhibited from binding Ad2 knob protein, by competing Ad2 knob protein, was considered the dissociation constant for the eCAR/MR1-Ad2 knob complex. Competitive ELISA analysis for the binding affinity of eCAR/MR1 fusion protein to a synthetic peptide, consisting of the 13 aa epitope of EGFRvIII that MR1 recognizes, was done in a similar manner as the binding assay for eCAR/MR1 binding to Ad2 knob protein. The differences in the protocols were noted in the Materials and Methods section. A dissociation constant for eCAR/MR1 fusion protein bound to Ad2 knob protein was determined to be approximately 3 nM (Figure 7), compared to a literature value of between 4.5-5 nM, which is within error of the experimental approach used.

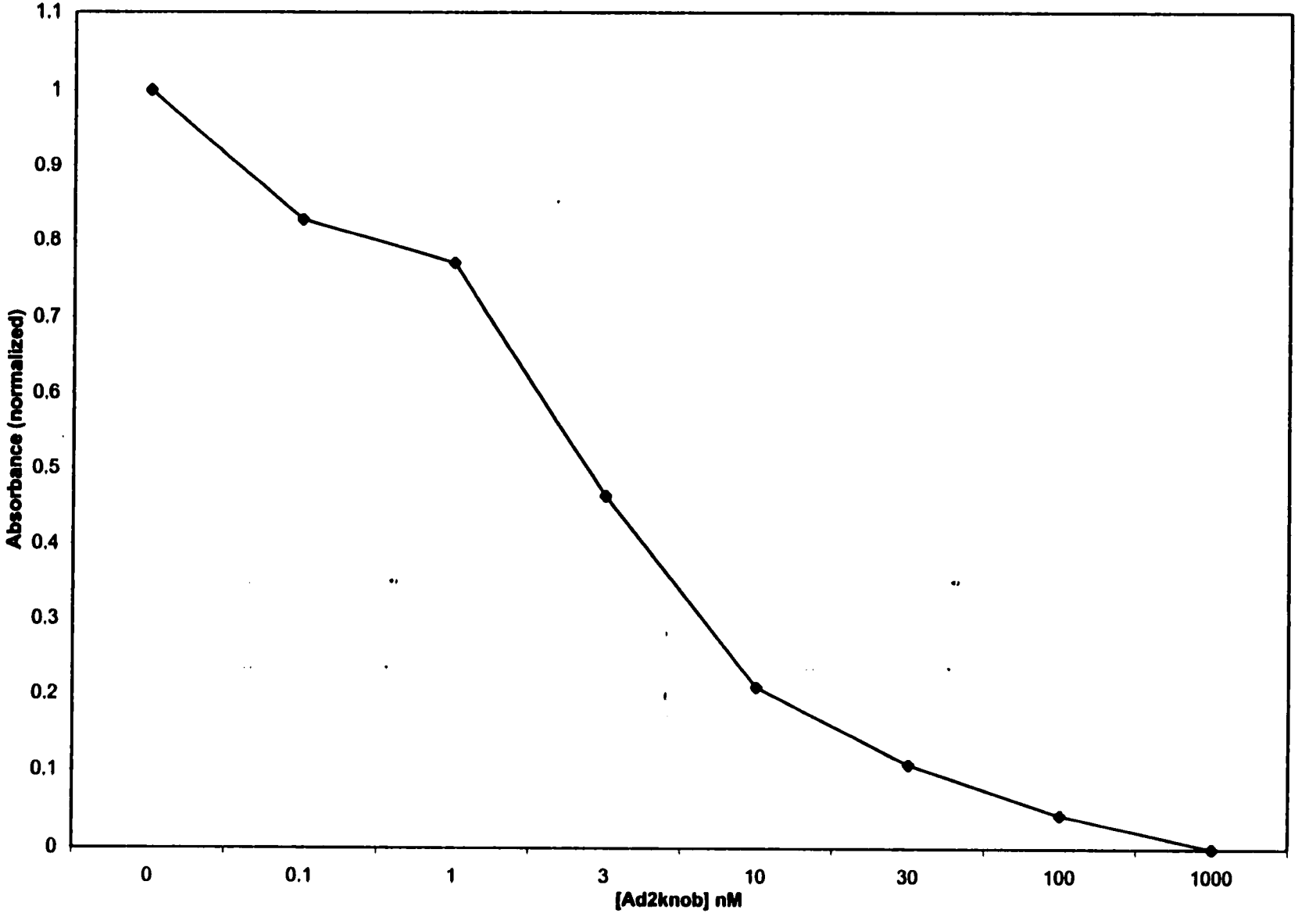
Figure 7. Relative Binding affinity of eCAR/MR1 fusion protein to Ad2 knob protein. Competitive ELISA analysis confirmed that the CAR subunit of the fusion protein is able to retain high affinity binding to the adenovirus knob domain. The absorbance values obtained with increasing concentrations of competitive Ad2 knob protein are normalized to the absorbance obtained in the absence of Ad2 knob protein (0 nM). A dissociation constant (K_d) of approximately 3 nM is obtained for the binding of eCAR/MR1 fusion protein to Ad2 knob protein.


1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Absorbance (normalized)
0
0.1
1
3
10
30
100
1000
[peptide] nM

The dissociation constant for eCAR/MR1 bound to Pep3 was determined to be approximately 20 nM, as opposed to 17 nM in the literature (Figure 8). Again, this is also within an acceptable error range for the experimental approach used.

β-galactosidase activity of U87MG and U87MGΔEGFRvIII transduced with Ad5LacZ virus in the presence of eCAR/MR1 fusion protein. Adenovirus serotype 5 with the gene encoding the β-galactosidase enzyme regulated by the CMV promoter in the E1 region of the virus's genome was used to measure the influence on infectivity eCAR/MR1 fusion protein has on U87MG and U87MGΔEGFRvIII cells. U87MG ceils are a human glioblastoma cell line and the U87MGΔEGFRvIII is the same cell line that stably expresses the mutant form of EGFR to which MR1 binds. One reason for using these two cell lines is that they allow for a direct comparison of infection efficiency of adenovirus in the presence of eCAR/MR1 fusion protein and whether it has the ability to increase transduction of Ad5LacZ to EGFRvIII expressing cells. Another reason is that there is strong evidence that human glioma cells have relatively low levels of CAR expression compared to more adenovirus susceptible cell lines such as 293 and A549 cells. Therefore an increase in Ad5LacZ infectivity of U87MGΔEGFRvIII cells by eCAR/MR1 at a low multiplicity of infection (MOI) should be more discernable than when using a more susceptible cell line. All infections were done at an MOI of 5 or approximately 500 viral particles per cell in a final volume of 400 μl of solution. This volume was used to maintain a high concentration of virus in a low volume since this is the optimal condition for efficient transduction of targets cells.[102] Cells were either incubated with virus alone, to establish standard levels for infection, or in the presence of

Figure 8. Relative Binding affinity of eCAR/MR1 fusion protein to the EGFRvIII eptitope. Competitive ELISA analysis confirmed that the MR1 subunit of the fusion protein is able to retain high affinity binding to the EGFRvIII epitope (Pep3). The absorbance values obtained with increasing concentrations of competitive EGFRvIII peptide are normalized to the absorbance obtained in the absence of EGFRvIII peptide (0 nM). A dissociation constant (K_d) of approximately 20 nM is obtained for the binding of eCAR/MR1 fusion protein to EGFRvIII peptide.


1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Absorbance (normalized)
0
0.1
1
3
10
30
100
1000
[Ad2knob] nM

2000 nM Ad2 knob protein, to block infection of adenovirus through binding via CAR, or with just 2 μg of eCAR/MR1. In the preliminary experiments, prior to purification of eCAR/MR1, a specific volume of crude transfection supernatant corresponding to 150 μg of total protein was incubated. Supernatants from both Mock transfected and eCAR/MR1 plasmid transfected cells were tested. The cells were incubated for 18 hours to allow for expression of β-galactosidase and then fixed and stained for the enzyme's activity (Figures 9, 10, 11, 12). In both cell lines, an MOI of 5 was sufficient to infect the majority of cells, and when incubated with Ad2 knob to block accessibility of the virus for CAR, infectivity was almost completely lost. This low MOI was used to ensure that Ad infection was solely based on fiber mediated binding to cell surface CAR. If MOIs of 100 or greater are used, penton base binding to integrins can mediate initial binding events of virus to cells. When eCAR/MR1 fusion protein was simultaneously incubated in the media during the infectivity assays, U87MG infection was impaired whereas infections of U87MGΔEGFRvIII appeared to increase in comparison to levels of staining when virus was used alone. Additionally, as the amount of eCAR/MR1 fusion protein was increased, a dose dependent inhibition of Ad5LacZ infection of U87MG cells was observed. However, staining of U87MGΔEGFRvIII at these concentrations demonstrated that the lowest concentration of eCAR/MR1 fusion protein used (0.5 μg) appeared sufficient to cause comparable levels of Ad5LacZ infection to the highest concentration (2 μg) used. Following the initial success of using eCAR/MR1 to increase Ad5 infection to U87MGΔEGFRvIII cells, more quantitative assessments, through the use of ONPG assays, were conducted to measure the relative infectivity of Ad5LacZ for

Figure 9. U87MG cells stained for β-galactosidase activity following infection with adenovirus serotype 5. Media containing 150 μg of protein from either pCDNA-eCAR/MR1 or pCDNA3.1 transiently transfected 293T cells were incubated with Ad5LacZ at an MOI of 5 during U87MG infections (panel C and D, respectively). Panel A represents staining of Ad5LacZ infected U87MG in the absence of any other proteins. Panel B demonstrates the reduction in Ad5LacZ infection when incubating with 2 μM Ad2 knob protein. Finally the bottom two panels (E and F) are infections represented in panels C and D done in the presence of 2 μM Ad2 knob protein.

U87MG

Virus Alone
Virus + Knob
Virus + Car/MR1
Virus + Mock
Virus + Car/MR1 + Knob
Virus + Mock + Knob
A
B
C
D
E
F

Figure 10. U87MGΔEGFRvIII cells stained for β-galactosidase activity following infection with adenovirus serotype 5. Media containing 150 μg of protein from either pCDNA-eCAR/MR1 or pCDNA3.1 transiently transfected 293T cells were incubated with Ad5LacZ, expressing the β-galactosidase gene, at an MOI of 5 during U87MGΔEGFRvIII infections (panel C and D, respectively). Panel A represents staining of Ad5LacZ infected U87MGΔEGFRvIII in the absence of any other proteins. Panel B demonstrates the reduction in Ad5LacZ infection when incubating with 2 μM Ad2 knob protein. Finally the bottom two panels (E and F) are infections represented in panels C and D done in the presence of 2 μM Ad2 knob protein. It is apparent the Ad2 knob competitively binds the eCAR/MR1 fusion protein as it is present in significant excess.

U87MGEGFRvIII

Figure 11. The effects of eCAR/MR1 dose on Ad5LacZ infection of U87MG cells. U87MG cells were stained for β-galactosidase activity following infection with recombinant Ad5LacZ, expressing the β-galactosidase gene, at an MOI of 5. Panel A is Ad5LacZ infection of U87MG in the absence of any additional proteins in the incubation solution. Panel B is Ad5LacZ infection of U87MG in the presence of 2 μM Ad2 Knob. Panel C is Ad5LacZ infection of U87MG in the presence of 0.5 μg of eCAR/MR1 fusion protein. Panel D is Ad5LacZ infection of U87MG in the presence of 1 μg of eCAR/MR1 fusion protein. Panel E is Ad5LacZ infection of U87MG in the presence of 2 μg of eCAR/MR1 fusion protein. An eCAR/MR1 dose dependent decrease in Ad5LacZ infection of U87MG cells is apparent with increasing eCAR/MR1 fusion protein amount.

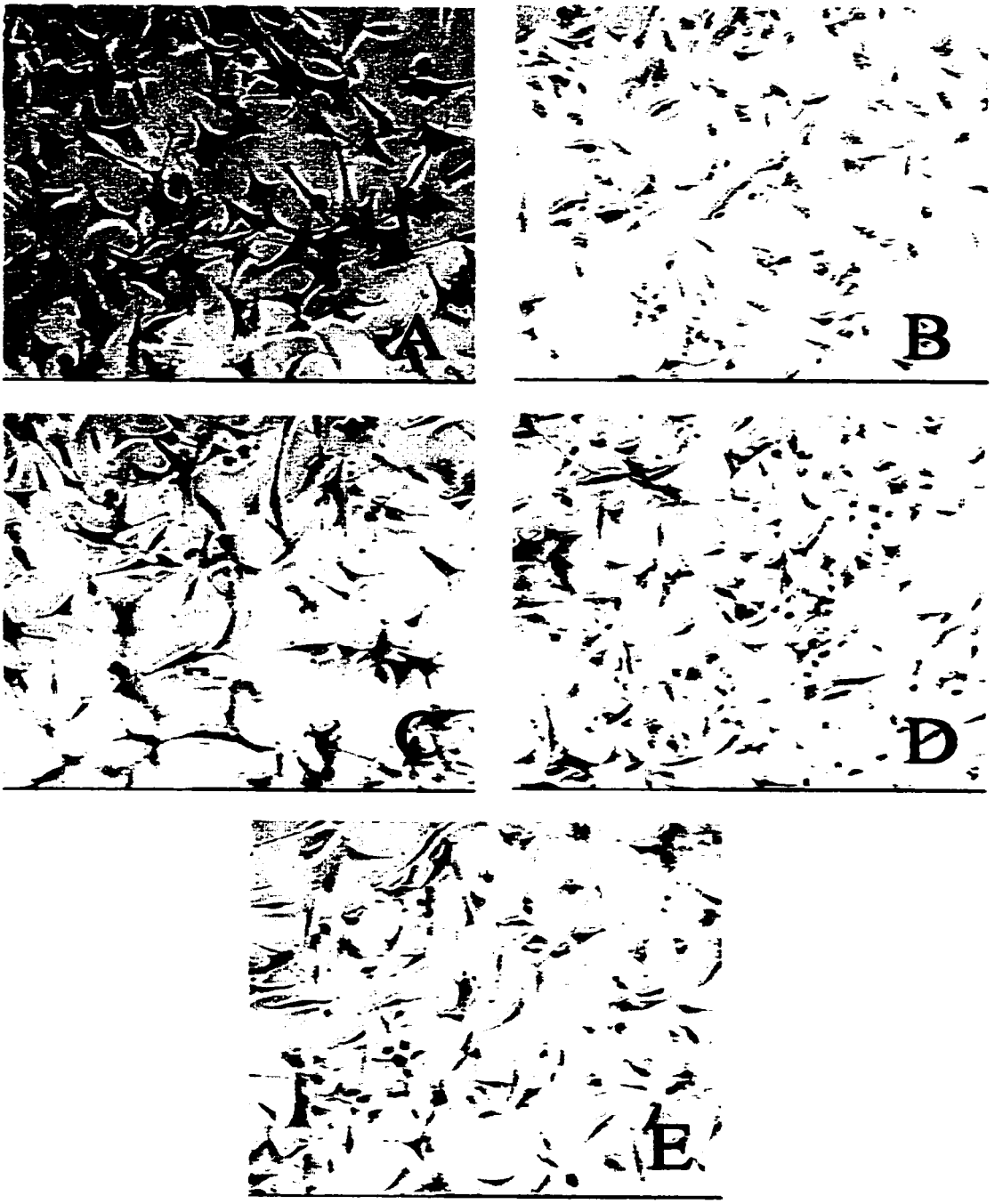
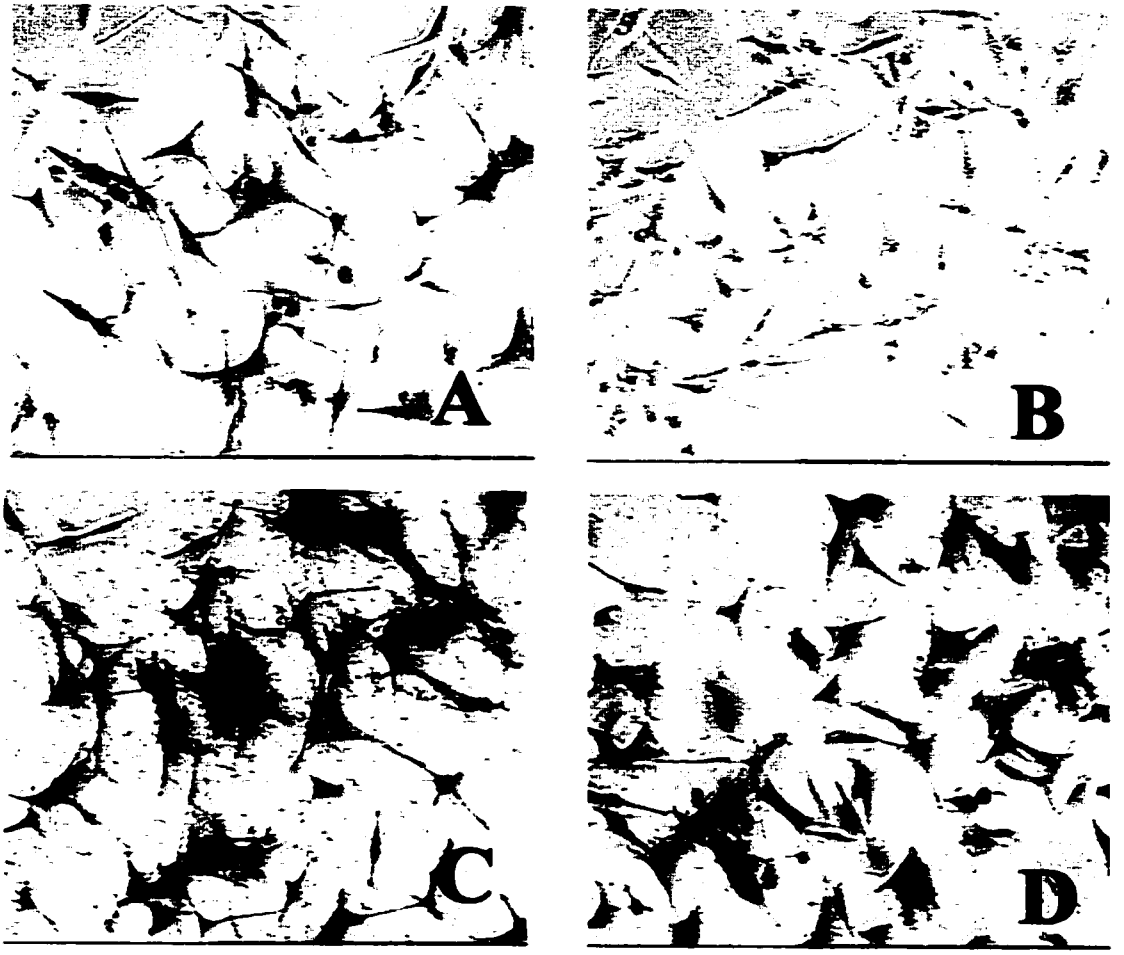
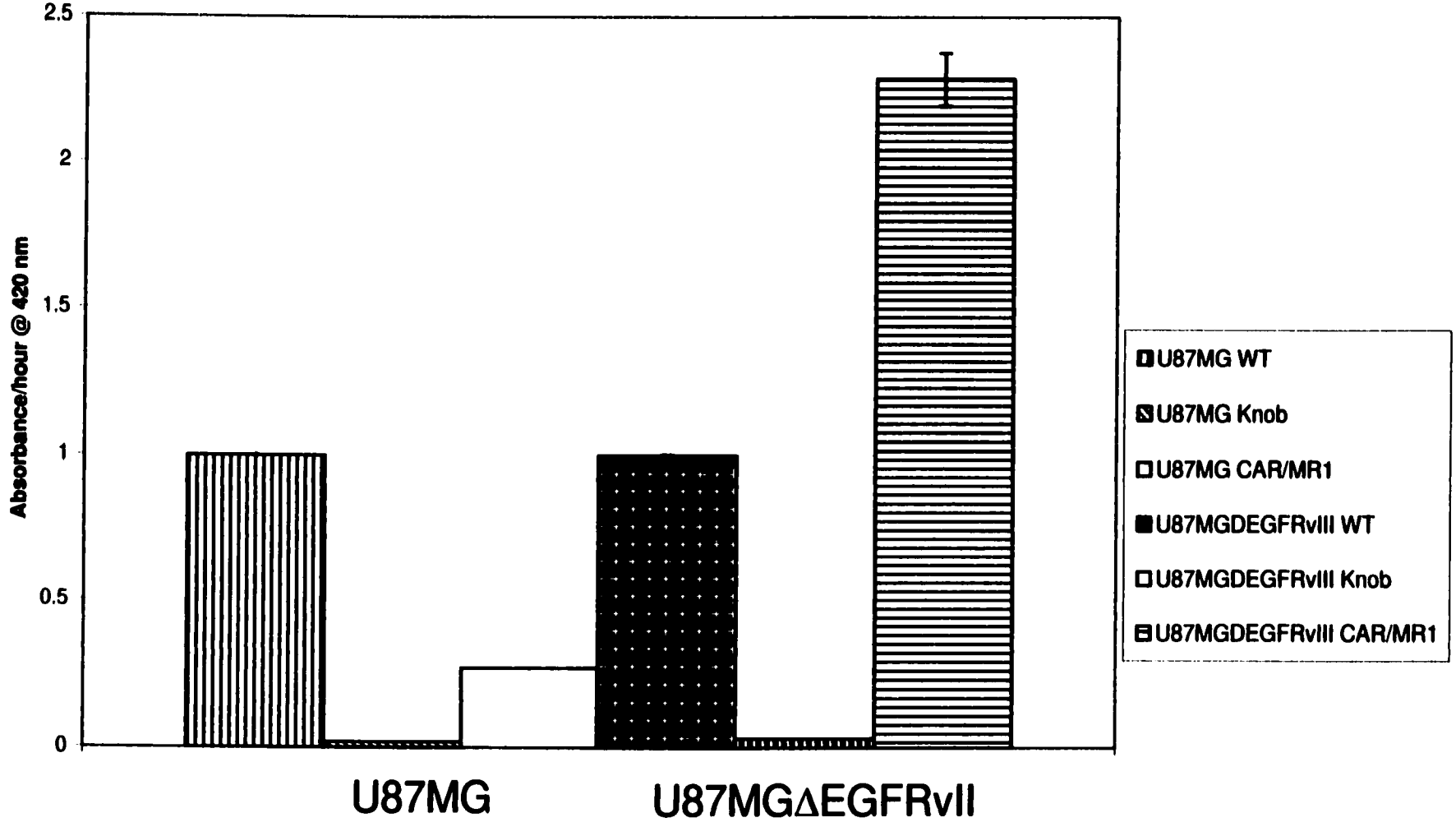

A
B
C
D
E

Figure 12. The effects of eCAR/MR1 dose on Ad5LacZ infection of U87MGΔEGFRvIII cells. U87MGΔEGFRvIII cells were stained for β-galactosidase activity following infection with Ad5LacZ at an MOI of 5. Panel A is Ad5LacZ infection of U87MGΔEGFRvIII in the absence of any additional proteins in the incubation solution. Panel B is Ad5LacZ infection of U87MGΔEGFRvIII in the presence of 2 μM Ad2 Knob. Panel C is Ad5LacZ infection of U87MGΔEGFRvIII in the presence of 0.5 μg of eCAR/MR1 fusion protein. Panel D is Ad5LacZ infection of U87MGΔEGFRvIII in the presence of 1 μg of eCAR/MR1 fusion protein. Panel E is Ad5LacZ infection of U87MGΔEGFRvIII in the presence of 2 μg of eCAR/MR1 fusion protein. Addition of more than 0.5 μg of eCAR/MR1 does not appear to significantly increase transduction of U87MGΔEGFRvIII cells by Ad5LacZ.


A
B
C
D
E

both these cell lines at the highest concentrations of eCAR/MR1 fusion protein (2μg) previously used. This colorimetric experiment measures the activity of β-galactosidase, in 10 μl of Ad5LacZ infected cell lysates, to catalytically convert o-nitrophenyl-β-D-galactopyranoside (ONPG) to ONP (yellow colour) by measuring the absorbance at 420 nm. The measured absorbance obtained when visible formation of yellow colour occurred, which took an average of 20 minutes, was expressed as (units) per hour. Infections of U87MG and U87MGΔEGFRvIII were repeated as done for the cell staining assays, but only with the highest concentration of eCAR/MR1 fusion protein (2 μg) previously used. The ONPG assays indicate that incubation with Ad2 knob protein reduced infection of Ad5LacZ to both cell lines to as little as 2% the levels observed for virus alone (Figure 13). In U87MG cells, incubation of eCAR/MR1 fusion protein reduced infectivity approximately four times relative to the activity in cells incubated with Ad5LacZ virus alone. In U87MGΔEGFRvIII cells, eCAR/MR1 increased transgene expression to twice the level of cells incubated with Ad5LacZ virus alone. In other assays this 2-fold increase was observed to be the minimum factor for the increase in Ad transduction for U87MGΔEGFRvIII cells – four-fold increases in transduction have also been attained with 2μg of eCAR/MR1 (data not shown). This level of Ad infection could possibly be increased if more eCAR/MR1 is used. Therefore, the limitations become the amount of eCAR/MR1 fusion protein obtainable from eukaryotic expression, and the stability of either the EGFRvIII-eCAR/MR1 complex or the Ad5-eCAR/MR1 complex.

Figure 13. ONPG assay assessing the influence of eCAR/MR1 fusion protein on the relative infectivity of Ad5LacZ for U87MG and U87MGΔEGFRvIII cells. Ad5LacZ virus infections of U87MG and U87MGΔEGFRvIII cells were conducted at an MOI of 5 either in the presence of 2 μg of eCAR/MR1, or 2 μM Ad2 knob, or alone without any additional protein. The absorbance values obtained for each of the two former incubation conditions was normalized relative to the absorbance value obtained when infections were carried out with virus alone for each cell lines. Assays were done in triplicate for the same infection, the average taken, and the standard deviation is marked on the graph.


2.5
2
1.5
1
0.5
0
Absorbance/hour @ 420 nm
U87MG
U87MGΔEGFRvII
U87MG WT
U87MG Knob
U87MG CAR/MR1
U87MGDEGFRvIII WT
U87MGDEGFRvIII Knob
U87MGDEGFRvIII CAR/MR1

DISCUSSION - Chapter 4

Discussion

The present utility of adenoviral vectors for cancer gene therapy is severely handicapped by the promiscuous tropism of the virus and the inability to target alternative cellular (tumour-specific) receptors. These two facets of present generation adenoviruses prevents their fulfillment as an ideal gene transfer vector; the ability to selectively transduce a target cell in a highly effective manner. To accomplish this elusive goal, techniques that result in the abrogation of Ad fiber binding to its cognate cellular receptor, CAR, while simultaneously targeting the virus to an exclusively tumour expressed receptor needs to be developed.

Several strategies to selectively increase infectivity of adenovirus to specific cell types by binding to an alternative primary receptor other than CAR have met with success in the past. Insertion of heterologous peptides within the HI loop[77,79-82] of the fiber protein or at its C-terminus.[76-78] have demonstrated that this limited tropism modification of adenovirus is a viable approach to increasing *in vitro* transduction of normally poorly transduced tissue. However, these modified viruses still suffered from the promiscuous tropism bestowed upon them through binding via the fiber protein to CAR, since natural tropism was not abolished. Additionally the HI loop is not able to tolerate large protein insertions that could increase the specificity of the adenovirus to alternative target receptors while simultaneously sterically obstructing native binding to CAR. Therefore alternative approaches to retargeting adenovirus have to be pursued to increase the efficacy for adenoviral use as a gene therapy vector.

In pursuit of this goal, we have designed a bispecific fusion protein that is simple to express and purify for testing purposes. We have demonstrated that a fusion protein composed of the extracellular knob-binding domain of human CAR fused to a scFv that is highly specific for a mutant EGFR improves the transduction efficiency of EGFRvIII expressing cells by adenovirus by as much as 4-fold. The choice to use the ectodomain of CAR in our fusion protein is based on the availability of a construct with the gene encoding CAR and the protein's innate ability to provide a high-affinity binding domain for adenovirus fiber protein. The binding affinity of CAR for Ad fiber protein (5 nM) is comparable to that of even the highest-binding anti-knob scFvs (3 to 12 nM).[103] If eCAR/MR1 binds a sufficient number of the capsid bound fiber protein through the CAR subunit, then it is expected that adenovirus would preferentially bind to EGFRvIII rather than native cell surface CAR.

Measurements for the affinities of eCAR/MR1 using competitive ELISA implies that each subunit of the fusion protein is able to independently bind its target molecule with the same affinity as the individual free proteins. Although the observed dissociation constants are not 100% accurate, they do demonstrate the utility of eCAR/MR1 to act as a bispecific conjugate. The CAR subunit in our fusion construct has an estimated dissociation constant of 3 nM in reproducible trials, whereas the linked MR1 molecule can bind EGFRvIII with a dissociation constant of 20 nM. These findings also hold additional significance for future bispecific fusion constructs using MR1 since it was unclear whether MR1 could still function properly as a C-terminus fusion subunit. This study has clearly demonstrated MR1's EGFRvIII binding capability is not impaired when fused at the carboxy terminus of a fusion protein.

Additional proof for the ability of eCAR/MR1 to bind Ad fiber protein is demonstrated by the reduction in Ad infection of U87MG cells upon incubating Ad5LacZ simultaneously with eCAR/MR1. Transgene expression, as visualized by X-gal staining, is significantly reduced in comparison to eCAR/MR1's absence; therefore showing that less adenovirus is able to successfully infect cells. Additional proof can be garnered, if necessary, by competitively inhibiting the observed increased Ad5LacZ infection of EGFRvIII cells, facilitated by eCAR/MR1, with the soluble CAR protein. Since the ectodomain of CAR can be readily expressed in E.coli as a soluble protein,[56] large-scale purifications can be undertaken similar to that used for Ad2 knob. This protein can then be used to competitively inhibit not only Ad infection, but also Ad knob binding to eCAR/MR1 in competitive ELISA analyses. This is just another possible approach to provide further evidence of eCAR/MR1's binding ability to Ad fiber protein.

Conversely, proof for the MR1 subunit's ability to bind to EGFRvIII is provided by two of the experimental results of this study. The first evidence is the ability of Pep3 to competitively inhibit binding of eCAR/MR1 to bound EGFRvIII peptide in the ELISA assays. The second is when there is no increase in Ad infection, as measured by transgene expression, of cells lacking EGFRvIII even when eCAR/MR1 is present in the incubation media, but the converse is true when infections of U87MGΔEGFRvIII cells are conducted.

Regardless of the successful demonstration for eCAR/MR1's utility in mediating EGFRvIII-based infection with Ad, a considerable problem that still needs to be answered concerns how many eCAR/MR1 fusion protein molecules bind to each of the twelve fiber trimers on the virus capsid. The peptide linker between the eCAR domain

and the MR1 domain should provide enough flexibility to prevent interference of additional eCAR/MR1 binding to trimeric knob. Ideally three eCAR/MR1 molecules would bind to a single trimer of fiber proteins, as native CAR binds to each monomeric knob domain. However, a valid argument against such an occurrence, is the steric effects a single molecule of eCAR/MR1 would bring about upon binding to a trimer of fiber proteins. Since eCAR/MR1 is approximately twice the size of the extracellular domain of CAR alone, it can, ostensibly, interfere with additional eCAR/MR1 binding to any unbound knob domains composing a single trimer. The stoichiometric ratio of eCAR/MR1 bound to fiber protein would, therefore, provide a better understanding of whether steric hindrance is a barrier in maximizing the number of retargeting molecules that are accessible on the virus capsid for EGFRvIII binding. This could be a major limiting factor for retargeting Ad infection to EGFRvIII through eCAR/MR1.

An important point mentioned in the study conducted by Haisma, et al. concerning increased infectivity of adenovirus via EGFR pathways rather than through CAR, as demonstrated in their A549 transduction experiments with their anti-EGFR fusion protein, could be applicable to eCAR/MR1 as well. It is possible that adenovirus bound to EGFRvIII through eCAR/MR1 may enter the cell through an RGD-independent mechanism and this could account for some of the increased infectivity of U87MGΔEGFRvIII observed. CAR expression on the cell surface is relatively stable with very little turnover, however EGFRvIII has a half-life of 4-6 hours for cell surface expression. The higher level of turnover of EGFRvIII, as opposed to CAR, can account for the higher levels of Ad transduction of these cells if eCAR/MR1 acts as an intermediary molecule and the Ad-eCAR/MR1-EGFRvIII complex is then endocytosed

due to natural turnover of EGFRvIII. This theory can easily be assessed by comparing standard infection data already obtained for eCAR/MR1 with infectivity assays using either an adenovirus that has its RGD motifs in the penton base capsomere blocked or mutated out, or by using a kinase dead mutant EGFRvIII expressing cell line, since this mutation should decrease turnover of the receptor. If the infectivity levels, relative to levels seen for eCAR/MR1 with normal first generation Ad5LacZ, do not decrease significantly when using the adenovirus lacking its RGD motif, or do decrease significantly when using the kinase dead EGFRvIII, then our theory that the eCAR/MR1-mediated increase in infectivity observed for U87MGΔEGFRvIII cells is due, in part, to turnover of EGFRvIII.

However, another more simplistic explanation for the increased Ad transduction is that the presence of eCAR/MR1 just increases the density of available adenovirus receptors, by providing another receptor (EGFRvIII) in addition to CAR, thereby making it easier for adenovirus to bind to cells. As opposed to the theory put forth regarding EGFRvIII turnover resulting in increased infectivity of U87MGΔEGFRvIII cells, examining evidence involving the signalling pathway of EGFRvIII can strengthen this second theory. Since α_V integrin-based internalization of adenovirus, through binding the virus's penton base, requires activation of a phosphoinositide-3-OH kinase (PI3K),[104] and it has been shown that EGFRvIII constitutively signals through activation of PI3K,[11,12] it can be speculated that endocytosis is not a rate limiting step in the infection of adenovirus in EGFRvIII expressing cells but rather the binding event is. Therefore, receptor turnover of EGFRvIII alone would not provide a faster alternative entry for EGFRvIII bound eCAR/MR1-Ad5 since penton base binding may not even be required to

initiate endocytosis in U87MGΔEGFRvIII cells. By incubating with eCAR/MR1, EGFRvIII is simply converted to another adenovirus receptor, and increases the probability of the virus binding to the cell and therefore increasing transduction efficiency. This is the simplest and most straightforward explanation for the increased levels of adenovirus infection observed in U87MGΔEGFRvIII when incubated with eCAR/MR1 fusion protein. This theory can easily be tested by simply incubating either U87MGΔEGFRvIII cells with eCAR/MR1 for one hour prior to infection or incubating Ad5LacZ with eCAR/MR1 for one hour prior to infection. If receptor turnover of EGFRvIII is a major factor, then the infections using U87MGΔEGFRvIII cells pre-incubated with eCAR/MR1 would be lower than the infections using Ad5LacZ pre-incubated with eCAR/MR1, since some of the EGFRvIII bound eCAR/MR1 in the former experiment should have been internalized during the one hour incubation.

An important precautionary note should be raised when comparing our strategy of retargeting with other techniques published in scientific literature. There is a large degree of variability in the experimental approaches used to test bispecific conjugates that aid in the retargeting of adenovirus. This is a common problem among all the different retargeting strategies involving bispecific conjugates already attempted.[90,93,94,105] The major variations between each laboratory's protocols are due to their use of different infectivity protocols and differences in their assays to measure infection efficiency. For example, a previous study using a fusion protein composed of a neutralizing anti-adenovirus fiber scFv fused to a scFv directed against EGFR has also shown to markedly increase, by as much as 10X, infection of Ad5 to cells expressing (wild-type) EGFR.[92] Their infections were conducted on various cell lines expressing high or low levels of

CAR; no correlations were given for the level of EGFR expression on the cell lines used. They even observed significantly higher levels of transduction (25X higher) with their fusion protein for a very high CAR expressing, and easily Ad transduced, A549 cell line. However, unlike their approach we have been able to increase infection with eCAR/MR1, to cells expressing EGFRvIII, by 2-4X, than that of virus alone, while using 200X less virus than they used their experiments (1000 MOI versus 5 MOI). The variability in the experimental testing of our two targeting fusion proteins therefore does not allow for direct comparisons between the two, unless further experiments on the same cell lines are conducted. One important observation is that eCAR/MR1 functions extremely well even at low MOIs of virus, even on hard to transduce (low CAR expressing) cell lines such as U87MGΔEGFRvIII. This is an attractive characteristic, as it should assuage safety concerns since large amounts of virus are not necessary to obtain an effect on transduction efficiency. This example of another fusion protein with a similar function accentuates how difficult it is to directly compare how beneficial eCAR/MR1 is in facilitating increased adenovirus infection relative to other fusion proteins that have been created before. A common system for assessing the value of a bispecific conjugates in adenovirus targeting would greatly increase the speed at which improvements can be made to eCAR/MR1 for further preclinical studies.

Two observations from previous studies using bispecific conjugates should increase the retargeting value of eCAR/MR1 further, and these should have been incorporated into the methodology used in this research. In hindsight it would be more rationale to incubate the eCAR/MR1 fusion protein with Ad5LacZ prior to infection. As opposed to incubating virus and eCAR/MR1 with cells simultaneously, pre-incubation

for an hour would increase the percentage of eCAR/MR1 bound virus particles. This may lower viral entry into the cell through its native receptor and concurrently increase targeting to EGFRvIII. In addition to this modification, it would also be valuable to test the effects of using less eCAR/MR1 to promote Ad infection than the 2 μg amount used in the present ONPG assays. Ebbinhaus, et al. were the first to discover that dose escalation of their bispecific fusion protein beyond an ideal concentration resulted in inhibition of virus infection. This was theorized to occur due to binding competition between excess CAR fusion protein (CARex-Fc) and virus-bound CARex-Fc for the Fc receptor on cells. Although this effect was not detectable in the cell staining shown in Figure 12, more definitive evidence regarding possible inhibition of our EGFRvIII-based infections can be attained simply by repeating the variable eCAR/MR1 dose experiment and measuring β-galactosidase activity using the ONPG assay.

On the other hand, an argument for having excess amounts of eCAR/MR1 can also be made. In Dimitriev et al. study using a fusion protein composed of the ectodomain of CAR fused to EGF (termed sCAR-EGF), they determined an ideal ratio of fusion protein to virus particle. Using ^{3}H-radiolabeled Ad, they measured the maximal Ad binding relative to sCAR-EGF concentration and determined the best ratio to be 12 pmol of sCAR-EGF per $6x10^{9}$ viral particles.[93] However, upon accounting for the larger molecular weight of our construct and the amount of protein used in our assays, our ratio is only 0.89 pmol per 6 billion virus particles. Therefore further assays using greater amounts of eCAR/MR1 are also warranted. Regardless of whether we assayed with too little eCAR/MR1 or too much, an ideal molar ratio should be determined and utilized in

future investigations of eCAR/MR1, since each CAR based bispecific conjugate can vary in both structure and affinity.

Unfortunately, our experimental approach does possess one major difference in comparison to other bispecific conjugate based targeting strategies. A similar study, whereby a knob domain neutralizing scFv was fused to EGF demonstrated a 16-fold increase in Ad transduction resulted in a key observation. The exact contribution of their fusion protein was difficult to determine since the cell lines also expressed CAR. [103] Like the majority of other studies using bispecific conjugates, the precise contribution of our MR1 based retargeting of adenovirus is also difficult to assess since the cell lines we tested do express low levels of CAR. Although the glioblastoma cells tested are more clinically relevant for eCAR/MR1 based retargeting of Ad, the best model to assess accurate increases in infectivity of EGFRvIII expressing cells would be to test the effects of our eCAR/MR1 fusion protein on Ad transduction of non-CAR expressing cell lines following the same principle as Ebbinghaus, et al.'s study.[94] Their group created a fusion protein composed of the ectodomain of CAR joined to constant fragment (Fc) of a G1 immunoglobulin protein and demonstrated up to a 250-fold increase in transduction to CAR negative, Fcγ receptor I (Fc binding) positive cells such as THP-1 (acute monocytic leukemia) cells. These cells have no detectable CAR expression as determined by both FACS and Western analysis. To provide a stronger measurement for the influence of eCAR/MR1 on targeting Ad we are in the process of creating a stable CHO cell line that expresses EGFRvIII. CHO cells are known to lack any detectable CAR expression. In addition to this cell line, experiments conducted with the U87MG and U87MGΔEGFRvIII cells will be repeated with a NIH 3T3 cell line that has been

retrovirally transduced to stably express EGFRvIII. NIH 3T3 cells also do not express murine or human CAR.[57] By examining the effects of eCAR/MR1 mediated Ad infection of these two cell lines, a more accurate measurement for the increase in transduction efficiency through EGFRvIII can be calculated.

In summary, we have demonstrated that a fusion protein composed of the ectodomain of the Ad fiber binding CAR protein fused to MR1 can increase viral transduction of glioblastoma cells that express EGFRvIII. This non-covalent retargeting technique provides an attractive alternative to pursue further, especially following the failure to genetically modify Ad fiber protein with MR1. The preferential infectivity eCAR/MR1 confers Ad, for EGFRvIII, and the increased transduction of cancer cells partially fulfills the goal of developing an ideal Ad based cancer gene therapy vector. Furthermore, by using CAR as our Ad binding molecule we have bestowed the freedom to switch Ad serotypes if necessary in the future, since CAR is recognized as the primary receptor for five of the six Ad subgroups. Although the use of Ad retargeting with eCAR/MR1 may not be clinically viable due to safety concerns regarding the requirement for an additional (non-covalent) binding event, and the difficulty in expressing and purifying large quantities of eCAR/MR1 for clinical study, this strategy has demonstrated that preferential targeting of Ad to EGFRvIII can be achieved. With the ultimate goal of directly coupling eCAR/MR1 to Ad through genetic or covalent methods, it may soon be possible to develop true retargeting strategies against cancer.

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Appendix I

Peptide insertions in the HI loop of Ad2 knob domain

Brief overview

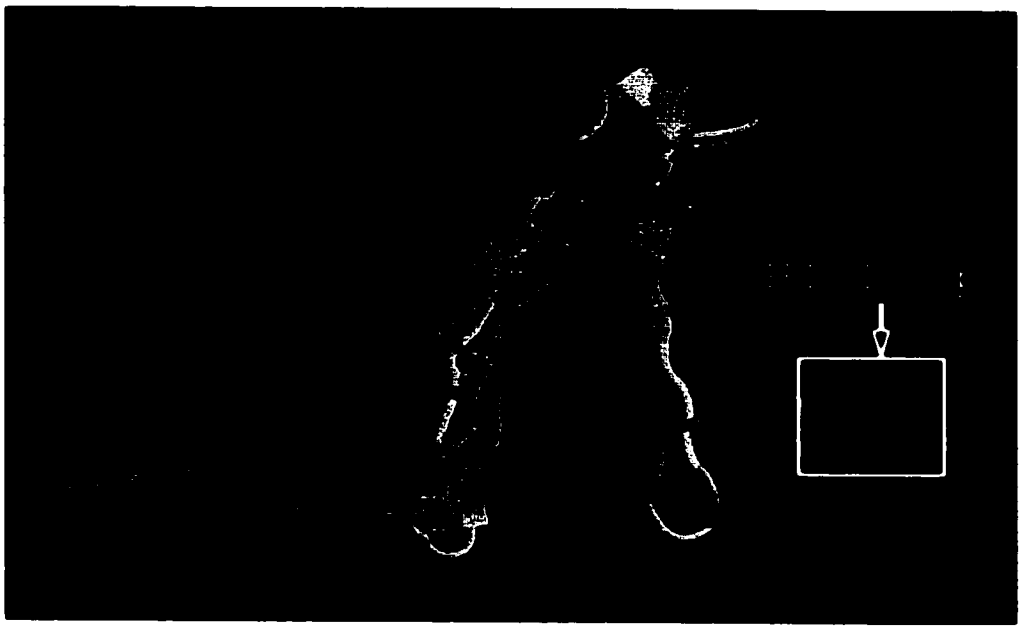
As mentioned in the introductory section of the main body of work presented in this thesis, our initial attempt to retarget Ad infection was based on genetic (covalent) strategies by heterologous peptide insertions within the HI loop of Ad fiber protein's knob domain. The ultimate goal was the insertion of a relatively large protein, namely MR1, within the HI loop to mediate binding of Ad to EGFRvIII. Success in using this approach was based on the justification that the HI loop was a highly flexible, hydrophilic region of the Ad knob domain, that did not appear to be involved in the trimer's structural stability.[1] A large body of ongoing research from other groups was also being compiled regarding the feasibility for HI loop-based peptide insertions, which further supported this approach for successful Ad retargeting.[2-6] Armed with the previous success of incorporating MR1 within the surface loop of Murine Moloney Leukemia virus glycoprotein, and retargeting binding of the virus to EGFRvIII,[7] we were fairly optimistic of our chances of translating our previous approaches towards applications with adenovirus.

Objective

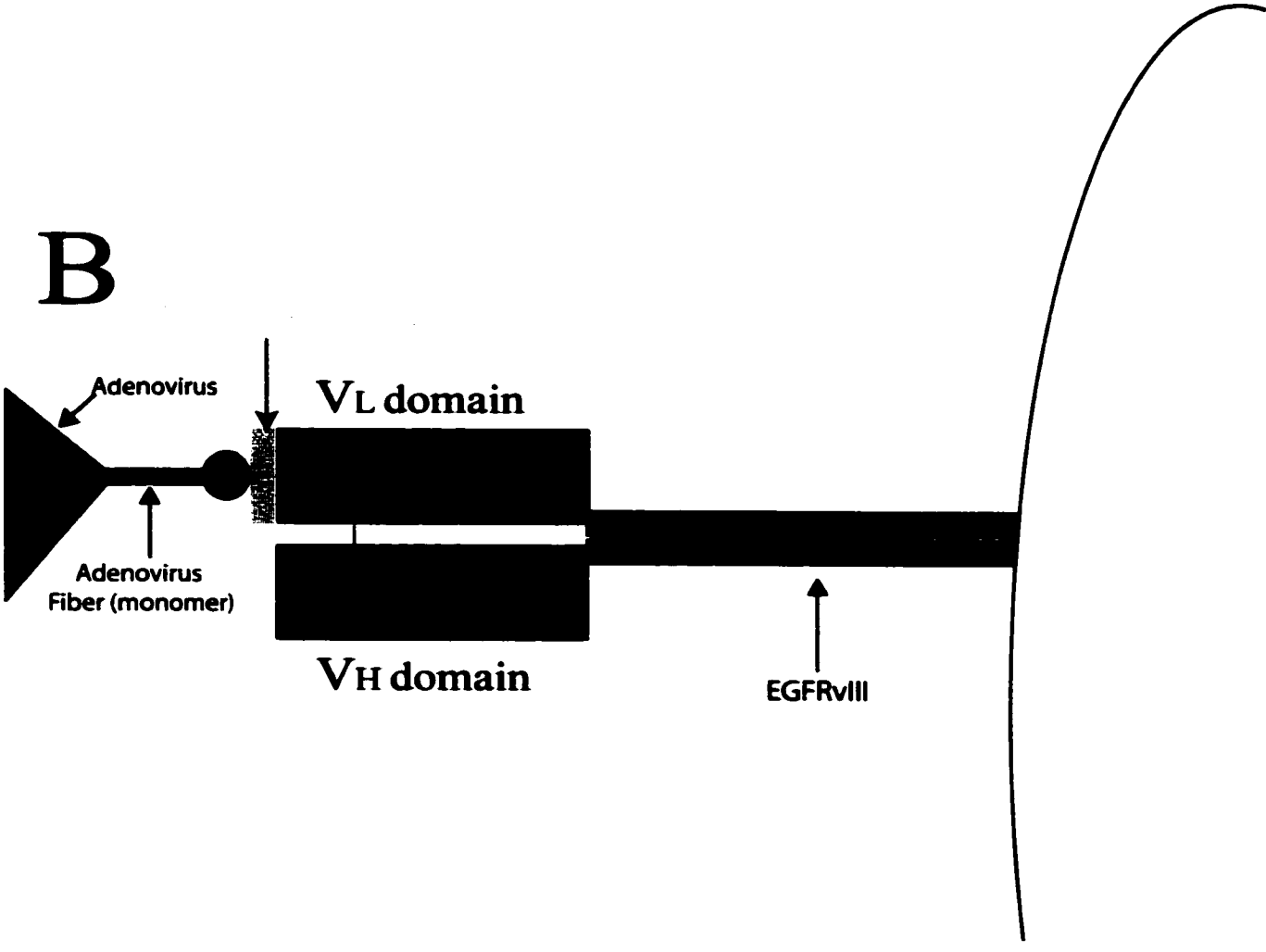
The overall objective of the work was to develop a plasmid-based sytem to directly incorporate targeting molecules into the HI loop of adenovirus type 2 fiber protein, more specifically the knob domain (see Figure 14a). This would be accomplished through insertion of two unique restriction sites flanked by flexible linker

Figure 14. The two alternative Ad retargeting strategies that will be discussed in Appendices I and II. (A) This is a cartoon diagram of the crystal structure for monomeric Ad knob domain with the HI loop emphasized. Notice the projection of the HI loop outwards relative to the rest of the structure. This is the region of Ad knob that we had initially hypothesized would be a suitable location for insertion of MR1 to retarget Ad. (B) Alternately, upon realizing the HI loop would not be a feasible location for MR1 insertion, we designed a fusion protein consisting of the MH20 icosapeptide joined at the amino terminus of MR1's V_L domain (Appendix II). This approach relied on MH20 binding to Ad knob and then the Ad-MR1 complex would bind to EGFRvIII through MR1.

A



B


Adenovirus
VL domain
Adenovirus
Fiber (monomer)
VH domain
EGFRvIII

sequences within the sequence encoding the Ad2 knob HI loop; thereby allowing the direct ligation of exogenous sequences encoding retargeting molecules simply and efficiently into Ad knob. Ultimately, the strategy would entail the incorporation of MR1 scFv into the HI loop to modify the binding affinity of adenovirus away from its native receptor and towards EGFvIII.

Indirectly, the failure to incorporate MR1 within the HI loop led to an examination of the structural instability resulting from HI loop incorporated peptides, and testing of various retargeted adenoviruses to specific cell lines. . Dr. Robin Parks (Ottawa Health Research Institute (OHRI)), who had also been involved in these initial attempts at Ad retargeting, provided us with two first generation Ad5 viruses with peptides within their HI loops he had constructed. One virus had an RGD motif and the other a poly-lysine (pK) tract incorporated within their fibers' HI loops. These viruses had their Ad5 fiber proteins substituted, through homologous recombination, with Ad2 fiber protein that had the sequences for the new peptides directly inserted into their HI loop using an endogenous SpeI restriction site.[8] We tested these two viruses on various cell lines to determine whether they were able to infect certain cell types better than unmodified virus.

Materials and Methods

Protocols already described in the main body of the thesis will simply be referred to.

Cell culture

293 cells (human embryonic kidney cells), C2C12 (mouse myoblasts), and differentiated C2C12 (mouse myotubes) were provided by Dr. Robin Parks (OGHRI). C2C12 myoblast cells were differentiated to myotubes for infection by Dr. Robin Parks.

Bacterial Cultures

Please refer to the main body of the thesis for strains of *E.coli* used.

Construction and expression of Ad2 knob protein, for eukaryotic expression, with internal sequence modifications in the HI loop

Three PCRs, using Taq polymerase (MBI Fermentas), were used to create the sequence encoding the Ad2 knob domain containing the Sfi/Not ligation site within the HI loop. The first PCR encoding the upstream portion of the knob domain from the HI loop was created using a sense oligonucleotide Ad2E1 (GAAGCTTGCCGCCACCATGCACCACCATCACCACCATGGGGCCATTACAATA GGAAAC) with a HindIII site (underlined) that also contained nucleotides encoding a 6xHis epitope. This was coupled with an antisense primer Ad2E4 (GCGGCCGCGGCCATGGCCGGCTGGGCCCCACCTCCTGTGGATTCACTAGTGC CATTAAG) with the NotI site (underlined). The second PCR was used to encode the remaining downstream portion of the knob domain past the HI loop and involved the use of a sense Ad2E3 (CCGGCCATGGCCGCGGCCGCAGGAGGCGGTGGAGAAACTAGCGAGGTAAGC AC) primer with a SfiI site (underlined), and an antisense primer Ad2E2 (CTCTAGATTATTCCTGGGCAATGTAGG) with a Xba I site (underlined) and also containing a stop codon. A third PCR was required to join the two fragments together using primer Ad2E1 and primer Ad2E2 incubated simultaneously with PCR products from the first two reactions. This resulted in the creation of the full Ad2 knob domain containing internal SfiI/NotI sites within the HI loop, in addition to the flexible linker regions. This PCR product was purified, ligated into pT7Blue3, propogated, sequenced and subcloned into pCDNA3.1 as previously described. Large plasmid preparations of

the final pCDNA-Ad2KnobwSfi/Not were then performed. This plasmid was then restriction digested with SfiI and NotI, and similarly digested MR1 sequence (from pMR1FxPE38K plasmid) was ligated into this plasmid, using standard molecular biology protocols, to generated pCDNA-Ad2KnobwMR1. This plasmid was propogated and purified, and the concentration was determined as previously described. All plasmids throughout this thesis have also been thoroughly tested for proper genetic inserts by restriction analysis. 293T cells were transiently transfected with 10 μg of pCDNA-Ad2KnobwMR1 plasmid using the calcium phosphate protocol as previously described. Additionally, a plasmind encoding Ubiquitous Nuclear Protein (UNP), a kind gift of Maria Tsirigotis (University of Ottawa), and pCDNA vector alone were similarly transfected. The UNP plasmid encodes a RGS6xHis tagged UNP protein. This tag is the same as the 6XHis tag with the addition of the three amino acids resulting in a more sensitive epitope for detection, that is still cross reactive with anti-6XHis antibodies and reagents. Following the harvesting of cell lysates, 20 μg of protein was loaded onto a 12% acrylamide SDS gel and western blot analysis was conducted as previously described. The detection of 6XHis-tagged protein, via Western Blotting, in the cell lysates was done using a 1000X diluted mouse monoclonal anti-Penta·His antibody (Qiagen). A secondary 5000X diluted goat anti-mouse HRP conjugated antibody was then used to probe for the Penta·His antibody. An additional blot using 5000X diluted mouse monoclonal primary RGS·His antibody (Qiagen) was also done to prove that the transfection protocol worked successfully.

Construction and expression of Ad2 fiber protein with internal sequence modifications in the HI loop for eukaryotic expression

To create the complete sequence encoding the 582 amino acid fiber protein containing the Sfi/Not linker sequence, Pwo/Taq PCR reactions (Expand High Fidelity PCR System) were conducted with sense primer FB1RGS (GGGATCCGCCGCCACCATGCGCGGTAGCCACCACCATCACCACCATAAACG), with a BamHI restriction site (underlined) and a N-terminal RGS6xHis tag, and antisense primer FB2 (ATGAATTCTGCAGTTAGGAGATGG), with a EcoRI restriction site (underlined). This resulted in the amplification of nucleotides encoding the tail and shaft domain of Ad2 fiber protein and also creating a more sensitive RGS6xHis epitope than the 6xHis tag present in the Ad2 knob domain plasmids. The amplified PCR product was sequenced and then ligated into EcoRI digested pCDNA-Ad2KnobwSfi/Not to generate the full Ad2 fiber gene in plasmid pCDNA-Ad2FiberwSfi/Not. The gene encoding standard Ad2 fiber protein was also PCR amplified with the same flanking restriction sites as the Ad2FiberwSfi/Not gene, then sequenced and subcloned into pCDNA3.1(+). Since the knob domain is located at the C-terminus of the fiber protein, and there is an endogenous EcoRI site in the knob domain prior to the sequence for the HI loop, this ligation abolished the 6xHis tag and incorporated the RGS6xHis epitope at the N-terminus of the fiber gene (tail domain). MR1 was ligated into the Sfi/Not sites as previously described, and large plasmid preparations of all constructs were done. Transient transfections of 293T cells were carried out, cell lysates harvested, and Western blotting conducted as previously described. The only exception from the Ad2 knob

domain blotting was the use of a 5000X diluted mouse monoclonal primary RGS·His antibody (Qiagen).

Construction and detection of Ad2 Knob proteins for bacterial expression with an internal Sfi/Not linker sequence and Myc epitope

The gene encoding Ad2 Knob was prepared via PCR using a sense primer (GAGCATATGCACCACCATCACCACCATGGGGCCATTACAATAGGAAAC) with an underlined NdeI site, and an antisense primer (CAAGCTTCATTATTCCTGGGCAATGTAGG) with an underlined HindII site, on the pT7Blue-Ad2KnobwSfi/Not previously created for eukaryotic expression. This insert was purified, ligated into pT7Blue, sequenced, and subcloned into pET22b(+) for bacterial expression as previously described. Complementary synthetic oligonucleotides, Ad2Myc1 (CGGCCGAGCAGAAGCTGATCAGCGAGGAGGACCTGGC) AND Ad2Myc2 (GGCCGCCAGGTCCTCCTCGCTGATCAGCTTCTGCTCGGCCGGCT), encoding the human c-myc epitope (EQKLISEEDL), were cassette ligated into Sfi/Not digested pET22b-Ad2KnobwSfi/Not plasmid following duplex formation. This was done as follows: the two oligonucleotides, incubated in an equamolar concentration in 20mM Tris pH7.4, were heated to 65°C for 3 minutes and allowed to cool to room temperature in a water bath initially at 55 °C. This annealed the two oligonucleotides together. Since the proper overhangs are already encoded within their sequences, the annealed DNA was simply ligated into SfiI/NotI digested pCDNA-Ad2KnobwSfi/Not. This plasmid, along with pET22b-Ad2KnobwSfi/Not and pET22b-Ad2Knob, were separately transformed into BL21 (DE3) *E.coli* and soluble Ad2 knob protein was purified as previously

described. Western blotting detection of the 6xHis tag at the amino terminus of each protein was conducted. Additionally, the Ad2 knob protein with the myc tag within the HI loop was detected using a 5000X diluted primary rabbit monoclonal anti-myc antibody (9E10), generously provided by Dr. Harry Atkins (Ottawa Regional Cancer Center), and a 5000X diluted secondary goat anti-rabbit HRP conjugated antibody (Biorad), via Western blotting.

Infectivity assays of C2C12 myoblasts and differentiated myotubes with tropism enhanced Ad5LacZ constructs

Two first generation tropism modified Ad5 viruses genetically manipulated to encode the β-galactosidase gene under the regulation of the cytomegalovirus promoter was kindly provided by Dr. Robin Parks (Ottawa Health Research Institute), along with a similar unmodified Ad5LacZ virus. Viral infectivity assays, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) cellular staining and ONPG assays for β-galactosidase activity were done as previously described. Infections of both cell types were done at 10^6 bfu/well (blue forming units per well). Dr. Robin Parks provided appropriate values for bfu used in these infections by standardizing the infection efficiency of the three viruses for 293 cells and relating them to lacZ-transducing units per ml.

Relative incorporation of tropism enhanced Ad5 fiber proteins into virus particles

To compare the incorporation, into virions, of the modified fiber proteins, consisting of the inserted 7 lysine residues (poly-lysine tract [pK]) and RGD motif ([GS_5ACDCRGDCFCG) relative to wild-type fiber, the above viruses used in the infectivity assays were examined via Western blotting for their level of detectable fiber protein. All samples were diluted to a volume of 10 µl in 4X Laemmli buffer and boiled for 10 minutes at 95°C. Preliminary SDS-PAGE gels were run using $1x10^9$ virus particles per lane, transferred to nitrocellulose membranes and stained with Amido Black. The particle count was then corrected for any discrepancies in titer values by standardizing the virus number to staining of hexon protein, which is the only detectable virion protein on the stained gel at the amount of virus. For the Western Blot analysis, $1x10^9$ Ad5 virus particles of wild-type fiber and pK incorporated fiber virus were loaded alongside $1.5x10^9$ Ad5 particles of RGD pK incorporated fiber virus onto a 10% SDS-PAGE gel, and the proteins were then transferred to a nitrocellulose membrane. This membrane was then probed with a 5000X diluted mouse monoclonal anti-fiber primary antibody (Medicorp), which was detected for binding to fiber as previously described.

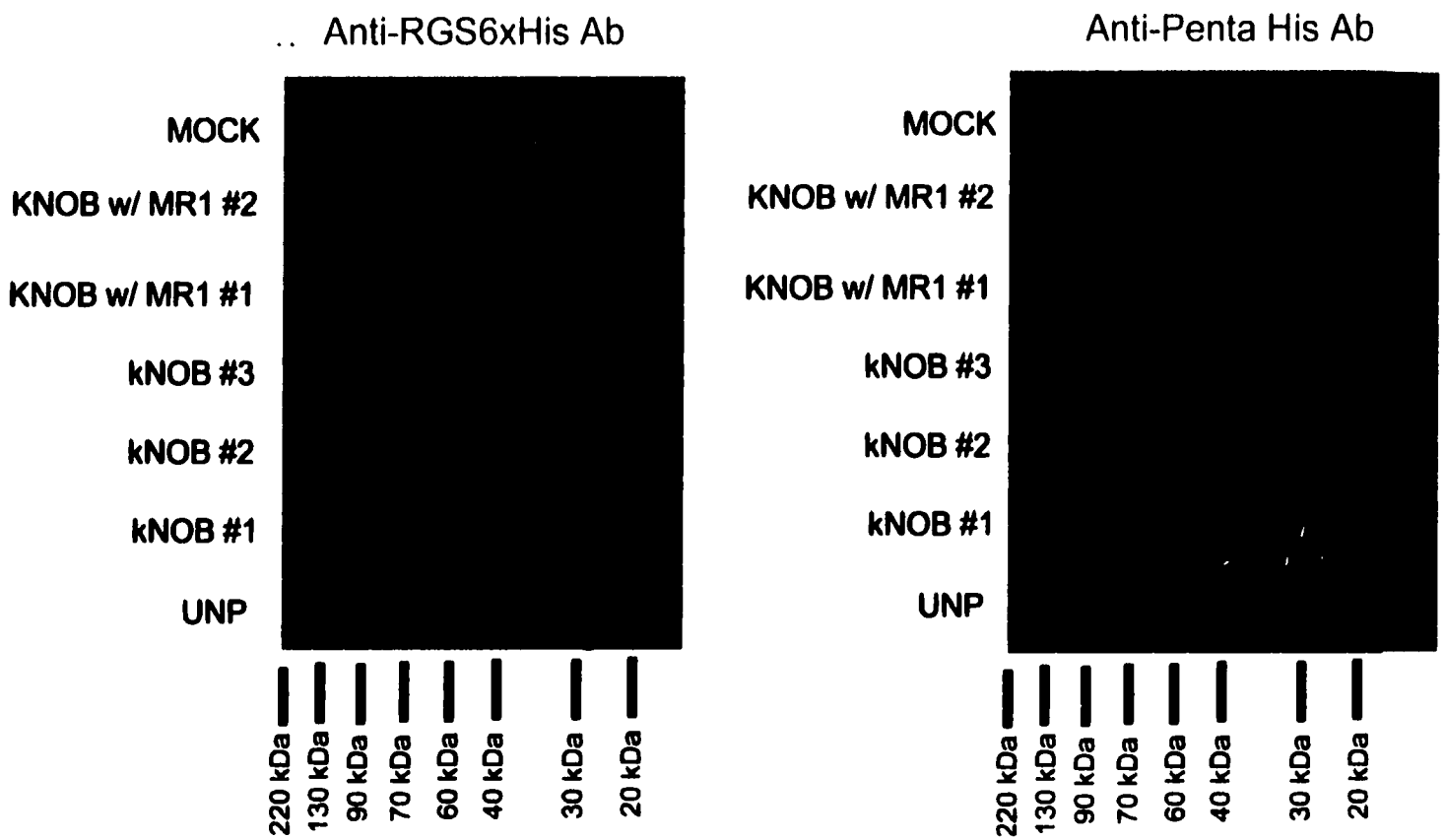
Results and Discussion

Creation, eukaryotic expression and detection of Ad2 Knob protein

To incorporate MR1 into the HI loop, unique restriction sites were introduced into the Ad2 knob domain HI loop sequence by PCR splicing together two separate portions of the Ad2 knob gene. These newly established ligation points within the HI loop for MR1 were, in turn, flanked by sequences encoding flexible linker regions that were also primer encoded. A Sfi I site was introduced upstream of the MR1 insertion point, and a NotI site was incorporated into the sequence downstream. Overall, the ensuing sequence results in the translation of amino acids such that the primary structure is encoded as: GTSEST-*GGGAQPAMA*-**MR1**-*AAAGGGG*-ETSEVS; using single letters designation used in amino acid nomenclature. Normal text indicates endogenous amino acids belonging to the HI loop, whereas italicized text corresponds to the newly introduced linker. The position where MR1 amino acids are located is indicated with a bold, underlined "MR1." Another feature of the expression construct was the primer-encoded addition of a N-terminal 6XHis tag to facilitate purification and detection of the Knob/MR1 protein.

Several pCDNA3.1-based eukaryotic expression plasmids containing the Ad2 knob protein constructs, including one encoding Ad2 knob with MR1, were transiently transfected into 293T cells, and cell lysates were assayed for the desired proteins' expression via Western blotting (see Figure 15). The top blot consists of 20 μg of cell lysates probed with the anti-RGS·His antibody, whereas the bottom blot corresponds to the same samples probed with the anti- Penta·His antibody. Detectable levels of UNP are present on both blots. As is apparent, the RGS·His tag reveals its sensitivity in

Figure 15. Western blots of Ad2 knob protein expression in transfected 293T cell lysates. For each blot, 20 μg of cell lysates were loaded on 12% SDS-PAGE gels and transferred to nitrocellulose membranes for Western blotting. The top blot is a duplicate of the bottom but probed with an anti-RGSHis antibody. The lanes are marked according to the expected protein to be expressed (plasmid transfected), and numbers following each protein corresponds to a separate transfection of different plasmid copies. The MOCK lane corresponds to empty pCDNA vector transfected 293T cell lysates. The bands from a protein molecular weight marker run in the first lane are clearly marked on the left side of each blot.


Anti-RGS6xHis Ab
MOCK
KNOB w/ MR1 #2
KNOB w/ MR1 #1
kNOB #3
kNOB #2
kNOB #1
UNP
220 kDa
130 kDa
90 kDa
70 kDa
60 kDa
40 kDa
30 kDa
20 kDa
Anti-Penta His Ab
MOCK
KNOB w/ MR1 #2
KNOB w/ MR1 #1
kNOB #3
kNOB #2
kNOB #1
UNP
220 kDa
130 kDa
90 kDa
70 kDa
60 kDa
40 kDa
30 kDa
20 kDa

comparison to the Penta·His tag, as the former blot is much cleaner than the latter. The smeared band on the UNP lane for the Penta·His probed blot most likely corresponds to degradation products of RGS·His tagged UNP. Also on the Penta·His blot, there is a band corresponding to the appropriate size of trimeric Ad2 knob protein (~69 kDa), however, this protein is also present on the Mock transfected cell lysates, therefore it must be an artifact of the antibody. Additional evidence against this band being Ad2 knob protein is its presence on the Ad2 knob with MR1 transfection lanes (Ad2 knob with MR1 would be ~141 kDa), and also the fact that these samples were boiled, which is known to dissociate knob trimers to monomeric form. Therefore, expression of knob protein could not be detected with the epitope tag used in these experiments. This statement is most applicable for normal knob protein and Ad2 knob with the internal Sfi/Not linker. This is the only justifiable explanation since the exact same construct is expressible in bacterial cells (see below). It is highly improbable that the Ad knob construct containing MR1 is expressed at all. This is based on the results by David Curiel's laboratory that was referred to in the introduction section of the main thesis. To reiterate their claims, the upper threshold for amino acid insertion into the HI loop is 63 amino acids without causing detrimental effects to the trimeric structure of Ad fiber protein.[9] Further evidence for this was provided by the concurrent discovery that an adenoviral recombination plasmid, based on the same construct as Ad2 knob with MR1, was unsuccessfully used by Dr. Robin Parks to try and rescue viable virus with MR1 in its fiber protein.

Creation, eukaryotic expression and detection of Ad2 Fiber protein

Initially our strategy focused on the eukaryotic expression of Ad2 knob only, since the tail and shaft domains are not necessary to obtain stable trimers. However, following the initial failure to detect any Ad2 knob protein, the gene encoding the whole fiber protein was constructed and tested for expression in 293T cells. The ease in accomplishing this task was greatly facilititated by the presence of an endogenously encoded EcoRI site within the knob domain of the fiber gene. This permitted us to only have to amplify out nucleotides encoding the N-terminal tail domain, the central shaft domain, and a small portion of the Ad2 knob domain to simply ligate this into the Ad2 knob constructs that were previously created. In addition to the standard pCDNA-Ad2FiberwSfi/Not and with MR1 plasmids, the fiber genes were also transferred to pCDNA vectors with the adenovirus tripartite leader (TPL) sequence upstream of the multiple cloning site (data not shown). Incorporation of this nucleotide sequence can significantly enhance eukaryotic gene expression.[10] Unfortunately, expression of these Ad2 fiber proteins was also elusive, and we were unable to even obtain detectable expression of normal Ad2 fiber protein. Through discussions with Dr. Andrea Amalfitano (Duke University), it was hypothesized that inadequate expression may be caused by the toxicity of Ad2 fiber protein in the transfected 293T cells.

Regardless of why we were not able to detect expression, the outcome led us to theorize that the HI loop was not as attractive a location for peptide insertion as originally thought. Small peptide insertions appear to be tolerated, but large molecules were most likely not going to result in viable virus. We, therefore, during the process of developing other strategies for Ad tropism modification, decided to examine the structural

significance of the HI loop and how its modification can influence fiber incorporation into virus.

Construction and detection of Ad2 Knob proteins for bacterial expression with an internal Sfi/Not linker sequence and Myc epitope

These experiments were conducted to determine whether the Ad2 knob gene constructs initially designed for eukaryotic protein expression were actually viable, or whether we had blundered in their design. The pCDNA-Ad2Knob constructs (unmodified Ad2 knob and Ad2 knob with Sfi/Not linker) were used as templates for the amplification of the Ad2 knob genes flanked with restriction sites specifically designed for the ligation into the bacterial expression plasmid, pET22b(+). In addition to the normal Ad2 knob protein and one with an internal Sfi/Not linker, a vector with nucleotides encoding a myc-epitope within the HI loop region was also created.

All these vectors were transformed, expressed, extracted, and purified on Nickel-NTA columns as described previously (see Figure 16 for representative Ad knob gel). Western blots of the purified proteins demonstrated that the genes meant for eukaryotic expression were not erroneously designed, as the various Ad2 knob domains were not only able to be expressed, but also existed as trimers (Figure 17A). Boiling of the proteins subsequently dissociated the Ad2 knob to monomeric protein. Additionally, the myc insertion within the HI loop did not interfere in the protein's expression or trimerization and was detectable via Western blotting (Figure 17B). The myc epitope was also shown to be accessible to binding in ELISA assays of plated Ad2 knob protein (data not shown).

Figure 16. A representative Coumassie Gel of Ad2 knob protein fractions purified on a Nickel-NTA column. A volume of 10 µl from Ad2 knob protein fractions that were purified on a Ni-NTA column by FPLC were loaded on a 12% SDS-PAGE gel following boiling. There is an abundant amount of 23 kDa knob protein obtained from prokaryotic expression as visualized by Coumassie Brillian Blue staining of the gel. The bands from a protein molecular weight marker run in the first lane are clearly marked on the left side of each blot.

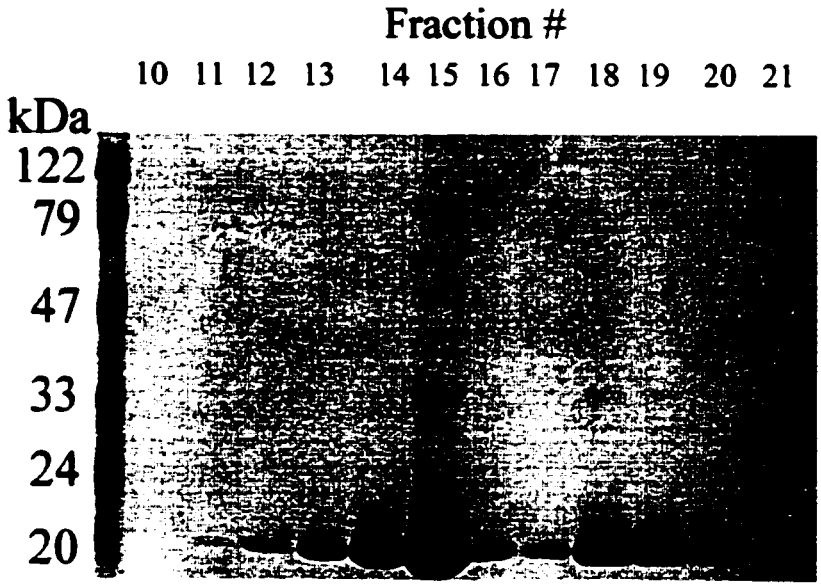
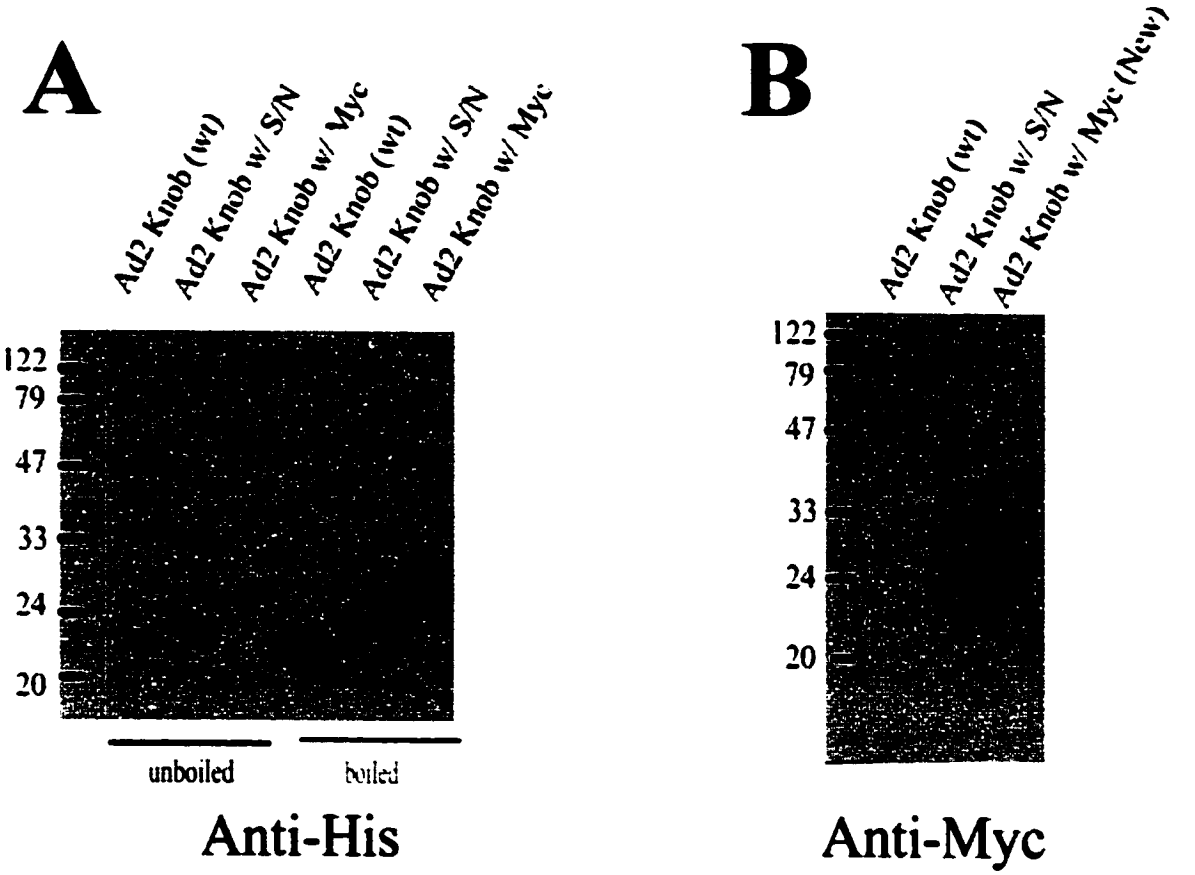

Fraction #
10 11 12 13 14 15 16 17 18 19 20 21
kDa
122
79
47
33
24
20

Figure 17. Western blot of various Ad2 knob proteins. (A) This 6xHis probed blot displays the various Ad2 knob proteins (designated above each lane and loaded at 10 µl per lane) as existing in trimeric form, prior to boiling, and monomeric form, following boiling. (B) The same Ad2 knob proteins as in (A) but this blot was probed with anti-myc antibody demonstrating that the Ad2 knob protein with myc does in fact contain the epitope. The bands from a protein molecular weight marker run in the first lane are clearly marked on the left side of each blot.


A
Ad2 Knob (wt)
Ad2 Knob w/ S/N
Ad2 Knob w/ Myc
Ad2 Knob (wt)
Ad2 Knob w/ S/N
Ad2 Knob w/ Myc
122
79
47
33
24
20
unboiled
boiled
Anti-His
B
Ad2 Knob (wt)
Ad2 Knob w/ S/N
Ad2 Knob w/ Myc (New)
122
79
47
33
24
20
Anti-Myc

Having proven that the difficulty was not due to underlying problems in the fabrication of the various Ad2 knob genes, and knowing that MR1 insertion would not be tolerated within knob domain, we then focused our attention on whether incorporation of HI loop modified fiber protein is somehow impaired. Our investigation was aided by the availability of Ad5 viruses that already had various peptides inserted within the (substituted) Ad2 fiber protein.

Testing infectivity of Ad5LacZ viruses with a poly-lysine tract and a RGD motif in their fiber protein's HI loop.

Dr. Robin Parks provided us with first generation Ad5LacZ virus that had its fiber gene substituted with Ad2 fiber, which is virtually the same as its native gene. The major difference between these viruses and wild-type virus, therefore, is the fact that the fiber protein contains foreign peptides within the HI loop. One construct contained a poly-lysine tract, and another contained an RGD-motif. These types of viruses had already been created previously, and his viruses were very similar with only slight modifications.[2,6]

To prove the retargeting value of these viruses, we first compared their ability to transduce terminally differentiated mouse muscle cells (myotubes), which express nominal amounts of CAR receptor, relative to wild-type Ad5LacZ virus. These assays were done in the presence of excess Ad2 knob protein to provide further evidence that an alternative receptor was being used to mediate the primary binding events, as recombinant Ad knob protein should block the majority of available CAR receptors.

The cell staining of 293 cells, for transgene expression, shows that β-galactosidase activity is lost for all three viruses when the cells are pre-incubated with 2μM of knob (Figure 18). A similar observation can be made for the transduction of myoblast cells (Figure 19); knob is able to completely block all three Ad5LacZ viruses from infecting these cells. It is known that both cell types express CAR and are readily transduced by Ad. As myoblasts differentiate, however, CAR expression is down regulated and these cells become refractory to Ad infection.[11] The Ad5LacZ virus with a pK tract in its fiber protein's HI loop appears to be able to transduce these cells even in the presence of excess knob (see Figure 20). The ONPG assays, to measure β-galactosidase activity, of these cells fortify the findings of the cell staining experiments (Figure 21), and clearly demonstrates that the poly-lysine tract is aiding the transduction of these cells quite possibly through attachment to heparan sulfate proteoglycan, as previously determined.

One observation, prior to the commencement of these studies, was the unexplainably poor correlation in these virus's titer numbers and their level of observed transduction. There is an ongoing debate regarding whether virus particle number or the viral plaque forming (pfu) unit should be used in adenovirus infectivity assays. Support for the latter is based on the fact that, on average, only 1 out of every 100 viral particles is viable for infection. Therefore pfu is more useful since it gives an accurate estimate of the number of viable virus particles infecting a cell. Support for using virus number is based on the fact that viable (infectious) virus is still present in viral supernatant even after all viable virus, as estimated by pfu, has infected the target cells. We had to resort to standardizing the virus particle count to the relative blue forming units (bfu) of

Figure 18. 293T infectivity assays of Ad5LacZ wild-type virus and the two other β-galactosidase encoding viruses, one containing a pK tract and another an RGD motif in the HI loop of their fiber proteins. Each pair of slides corresponds to a representative picture of a well of 293T cells transduced with 10^6 bfu of either Ad5 (wt), or Ad5 (pK), or Ad5 (RGD) as designed to the left, in the presence (+ Knob [right column]) or absence of 2μM of recombinant Ad2 knob protein. Cell staining for β-galactosidase activity was done as described in the Materials and Methods section of the main thesis.

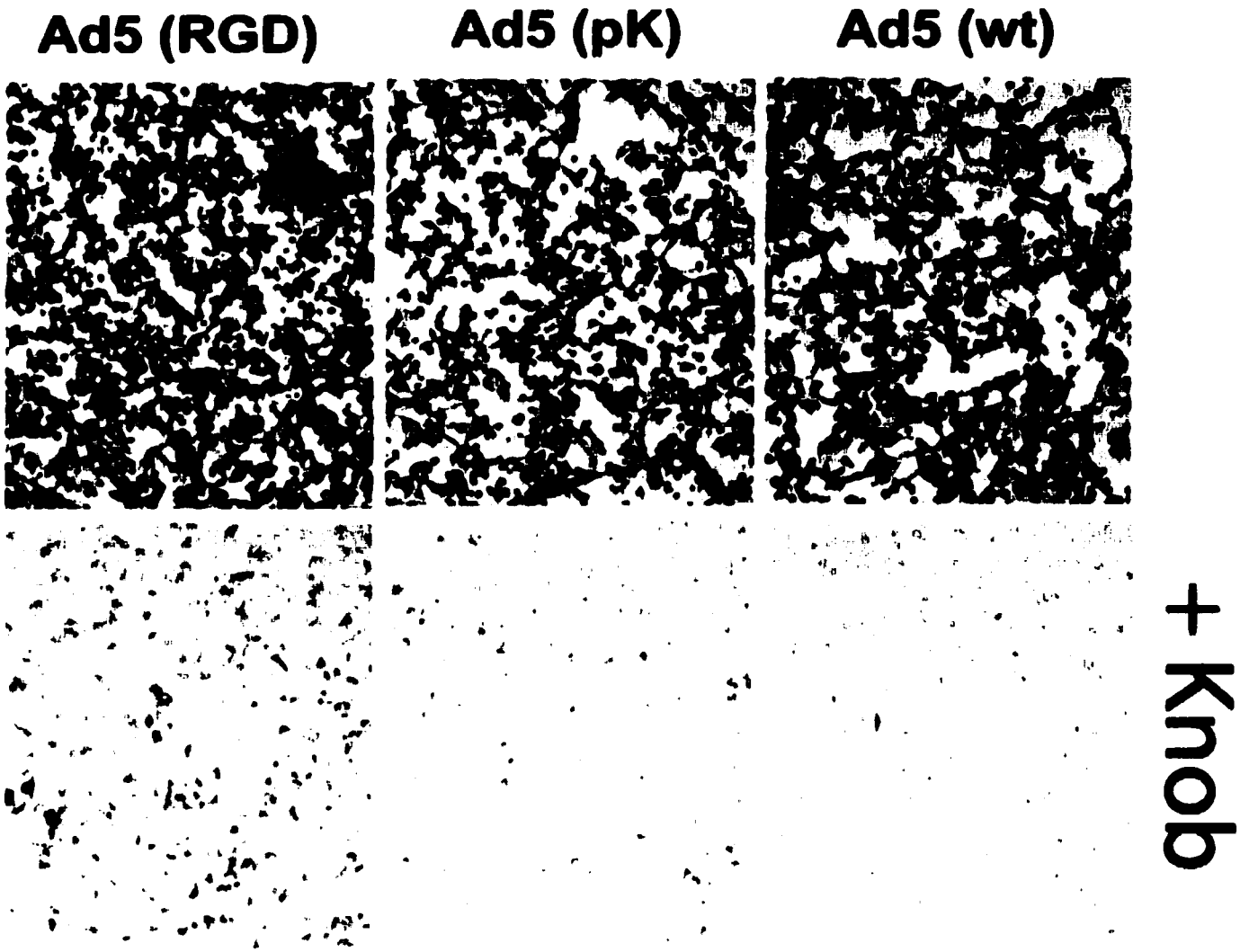

Ad5 (RGD)
Ad5 (pK)
Ad5 (wt)
+ Knob

Figure 19. Undifferentiated C2C12 (myoblast) infectivity assays of Ad5LacZ wild-type virus and the two other β-galactosidase encoding viruses, one containing a pK tract and another an RGD motif in the HI loop of their fiber proteins. Each pair of slides corresponds to a representative picture of a well of myoblast cells transduced with 10^6 bfu of either Ad5 (wt), or Ad5 (pK), or Ad5 (RGD) as designed to the left, in the presence (+ Knob [right column]) or absence of 2μM of recombinant Ad2 knob protein. Cell staining for β-galactosidase activity was done as described in the Materials and Methods section of the main thesis.

+ Knob

Ad5 (wt)

Ad5 (pK)

Ad5 (RGD)

Figure 20. Differentiated C2C12 (myotubes) infectivity assays of Ad5LacZ wild-type virus and the two other β-galactosidase encoding viruses, one containing a pK tract and another an RGD motif in the HI loop of their fiber proteins. Each pair of slides corresponds to a representative picture of a well of myotubes cells transduced with 10^6 bfu of either Ad5 (wt), or Ad5 (pK), or Ad5 (RGD) as designed to the left, in the presence (+ Knob [right column]) or absence of 2μM of recombinant Ad2 knob protein. Cell staining for β-galactosidase activity was done as described in the Materials and Methods section of the main thesis.

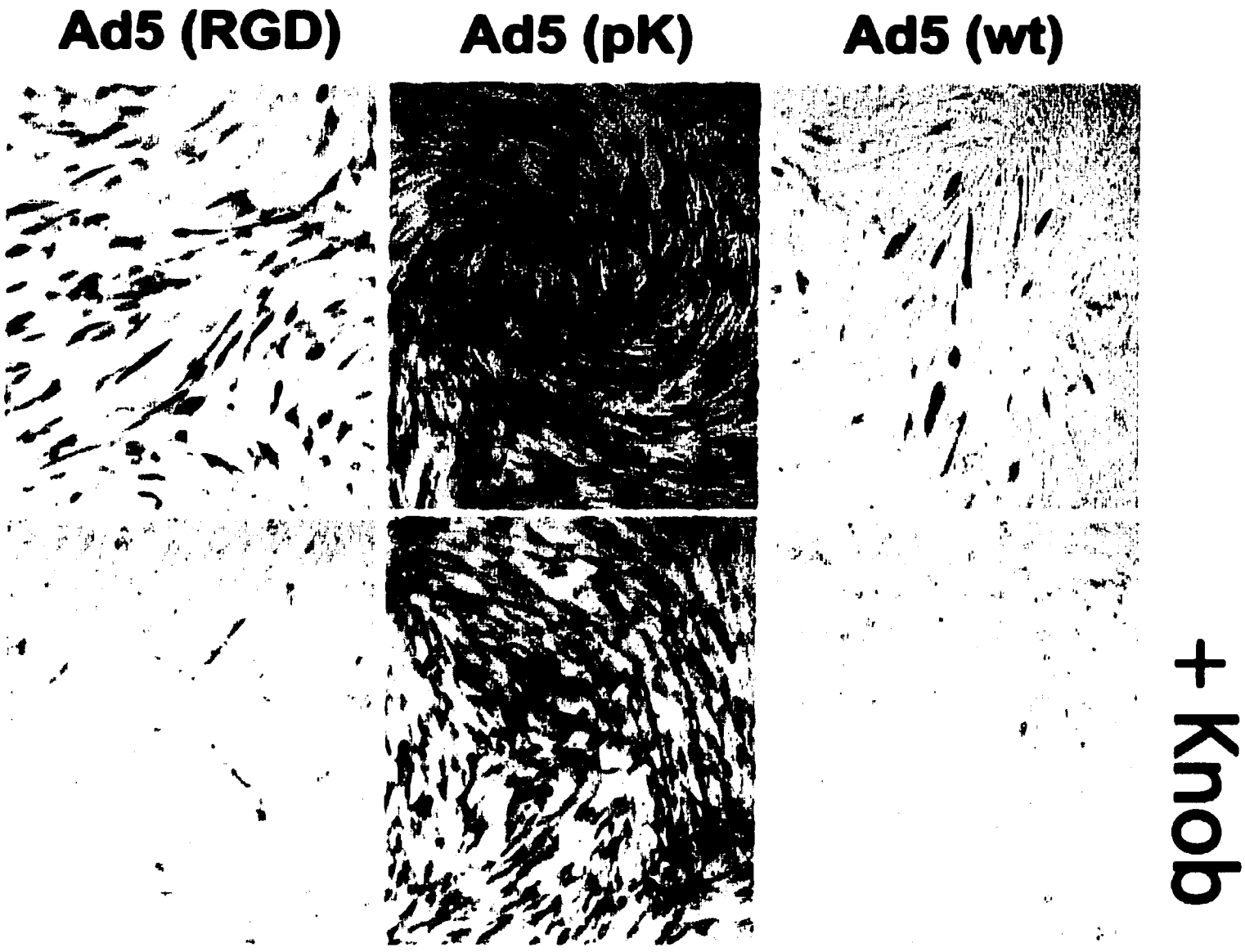
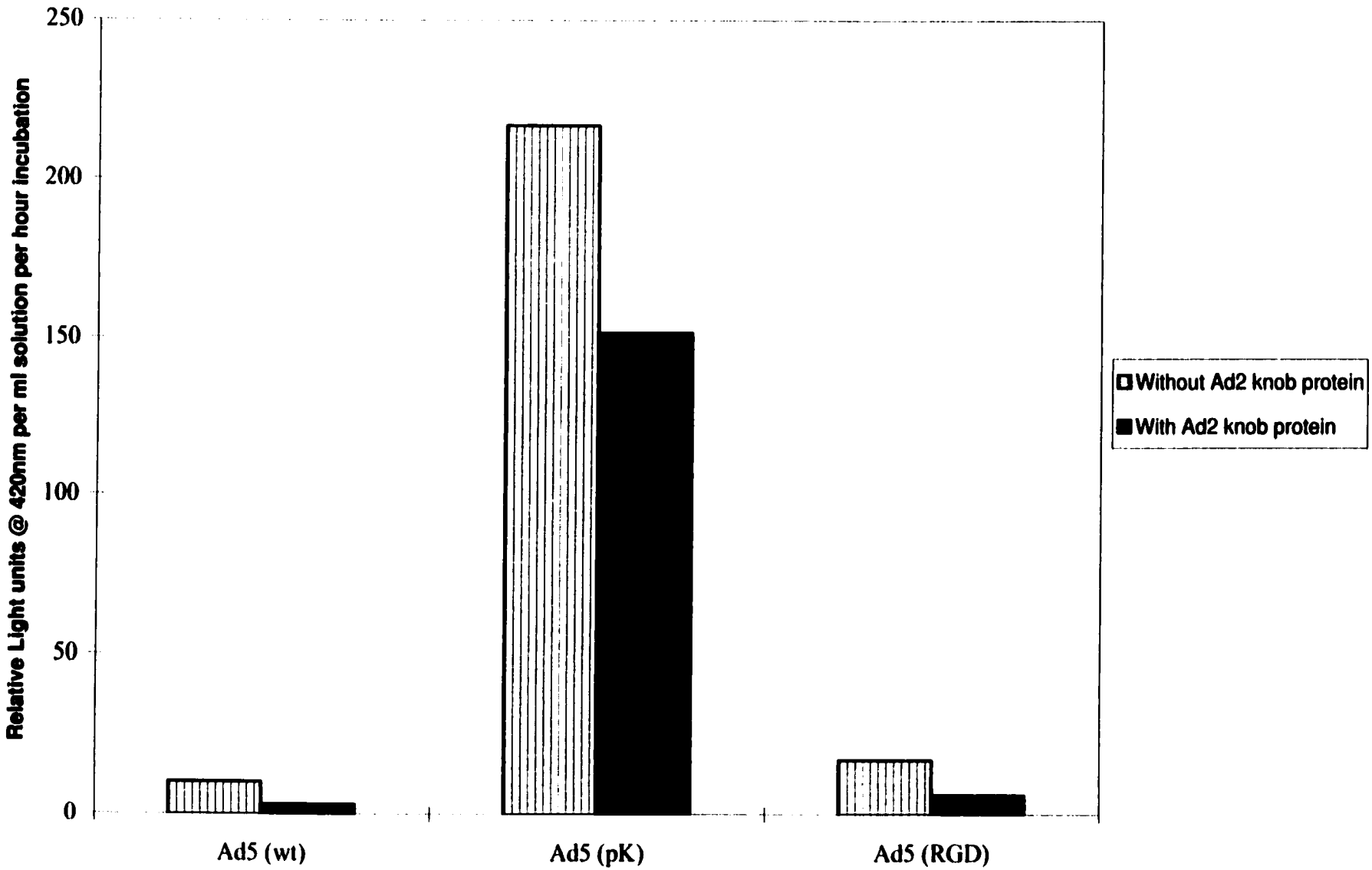

Ad5 (RGD)
Ad5 (pK)
Ad5 (wt)
+ Knob

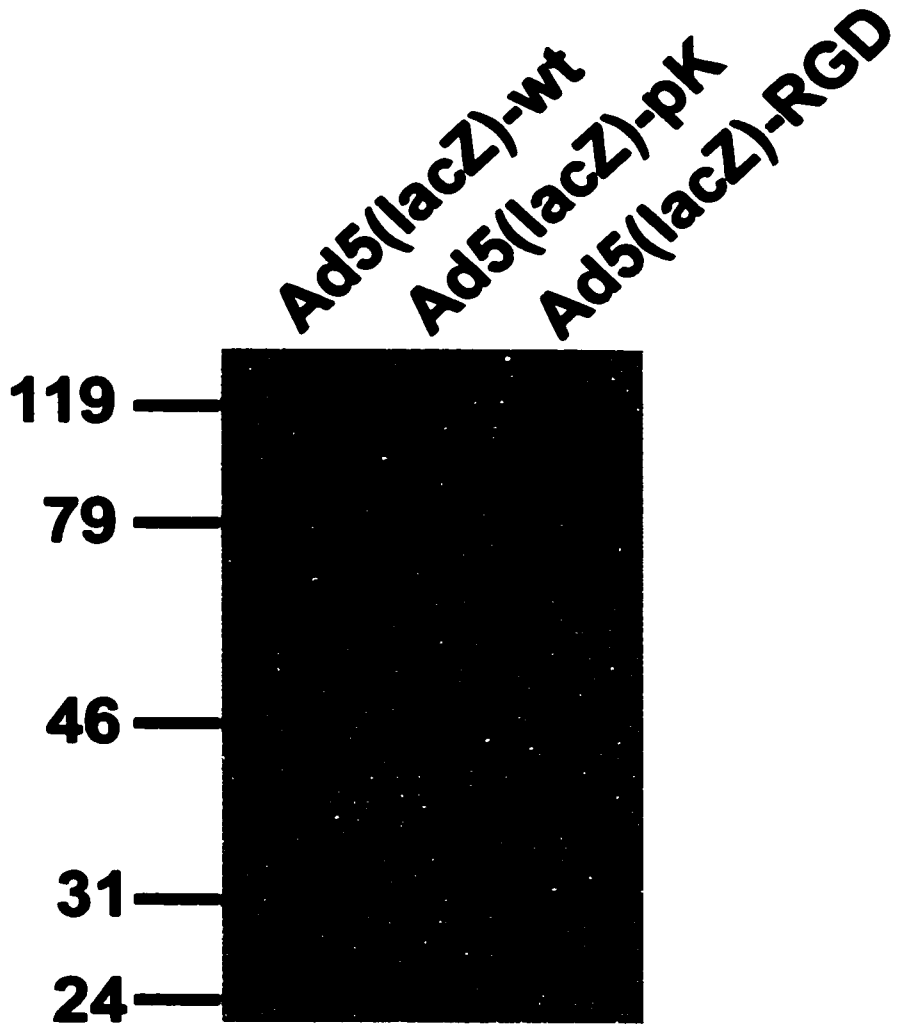
Figure 21. ONPG assays of differentiated C2C12 (myotubes) infectivity assays with Ad5LacZ-pK. Measurements of β-galactosidase activity in Ad5LacZ-pK infected myotubes was done to obtain quantitative measurements of transgene expression observed in Figure 20. The measured relative light units (RLU) were standardized for a theoretical one-hour incubation time of the assay and per 1 ml of cell lysates. The light purple column corresponds to the respective virus's natural ability to infect myotubes, whereas the dark purple column corresponds to the designated virus's transduction efficiency in the presence of 2 μM Ad2 knob protein. In the presence of Ad2 knob protein, wild-type Ad5LacZ's infections were reduced to 30% of the corresponding infections in its absence. Similarly, Ad5LacZ-pK infection was only reduced to 70%, and Ad5LacZ-RGD to 36% by Ad2 knob protein.


250
200
150
100
50
0
Relative Light units @ 420nm per ml solution per hour incubation
Ad5 (wt)
Ad5 (pK)
Ad5 (RGD)
Without Ad2 knob protein
With Ad2 knob protein

transduced 293 cells with the three viruses (similar to pfu), since the actual number of viable HI loop modified virus was lower than the virus particle count indicated. SDS-PAGE gels were used to run boiled first generation virus particles from the three viruses: Ad5LacZ (unmodified fiber gene), Ad5LacZ-pK, and Ad5LacZ-RGD. The number of virus particles was normalized to the Amido Black staining of hexon protein, and then following appropriate adjustments for differences in apparent virus titer, the nitrocellulose membrane was probed for the presence of fiber protein (see Figure 22). Ultimately, Western blotting proved that the observed discrepancies between each of the virus's titers and the bfu values were due to poor incorporation of fiber protein in the HI loop modified viruses. The pK and RGD viruses had much less fiber protein incorporated into their virion capsids than wild-type Ad5, and therefore a larger volume of these CsCl purified viruses were used in the infections.

An argument could therefore be made that the observed increase in myotubule transduction by the pK virus was due to a higher volume of virus used relative to the other two. This could result in the higher transduction being mediated by cellular integrins binding penton base protein, the second binding event in Ad infection. Examining the volumes for each of the viruses used in the transduction experiments (following normalization to bfu) and the original virus titers however, shows that there was approximately 70X more pK virus particles and 80X RGD virus particles used in each well than unmodified Ad5LacZ. If there are more RGD virus particles than even pK virus, then this modified virus should have just as readily infected the myotube cells as the pK virus if an integrin based attachment event was facilitating the increased infection of pK virus. This, however, is not the case.

Figure 22. Western blot of the relative incorporation of HI loop modified fiber proteins into Ad5LacZ virus. The three different LacZ encoding viruses used in the infectivity assays are labeled above each lane. The number of viral particles for each virus was standardized based on hexon protein's amido black staining profile, as described in the Materials and Methods. The bands from a protein molecular weight marker run in the first lane are clearly marked on the left side of the blot.


Ad5(lacZ)-wt
Ad5(lacZ)-pK
Ad5(lacZ)-RGD
119
79
46
31
24

In summary, adenovirus tropism modification by inserting retargeting molecules within the HI loop of Ad fiber protein is probably only a viable option for small peptides. The caveat to this statement is that even small peptide insertions, such as a poly-lysine tract and an RGD motif, can drastically reduce the incorporation of modified, trimeric fiber protein into the virion capsid. The reasons behind this are still unclear, but it is possible that the HI loop does in fact play a role in the structural stability of the Ad fiber protein and even slight disturbances can impair proper protein folding.

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Appendix II

Fusion of an Ad binding peptide with MR1 dsFv to retarget virus

Brief overview

Upon realizing the HI loop was not an appropriate location to mediate retargeting of adenovirus by insertion of MR1, we changed our focus from the genetic manipulation of Ad fiber protein to a non-covalent targeting strategy. The incompatibility of the HI loop for large molecular insertions forced us to adopt a minimalistic approach in our next strategy.

Examination of the literature led to the discovery that a twenty amino acid peptide can directly bind to the knob domain. This peptide motif was isolated by biopanning a random hexapeptide phage library on immobilized Ad5 knob protein and competitively eluting bound phage with an anti-knob monoclonal antibody. Four phagotopes (receptor mimotopes) were recognized that shared homologous sequences with each other, and this common span of amino acids was, in turn, found to be homologous to MHC class I $\alpha 2$.[1] The MHC class I $\alpha 2$ consensus sequence was used in the creation of a twenty amino acid peptide RAIVGFRVQWLRRYFVNGSR, termed MH20 icosapeptide. Scatchard analysis of binding assays determined that the dissociation constant for binding of MH20 to Ad5 fiber was 3.0 nM, which is comparable to binding of fiber to CAR. This peptide was used in an additional study as an artificial receptor for Ad infection and demonstrated a limited increase of Ad5 infection to CAR-negative cells.[2] Therefore, they claimed to have discovered a relatively simple means of binding Ad5 fiber protein with a single peptide.

Objective

We felt that this MH20 peptide offered an alternative means of binding to Ad fiber protein without resorting to using larger, more cumbersome, anti-knob monoclonal antibodies to assist MR1 in binding the knob domain. To accomplish binding of MR1 to knob we proposed that fusion of the MH20 icosapeptide to the C-terminus of MR1's V_L domain would allow binding to Ad5 knob protein (Figure 14b). Previous successes in fusing small peptides, such as a myc epitope and a 6XHis tag, to this region of the V_L domain leant strength to the feasibility of this approach.

Since bacterial expression of MR1 results in the formation of inclusion bodies, that are difficult to purify, we already possessed constructs that contain the sequence for MR1 fused to domains II and III of pseudomonas exotoxin (PE) to mediate greater levels of expression and simplify the purification process. This is due to the ability of PE to bind ionically to the Q Sepharose column resin, thereby allowing purification of MR1 protein via FPLC. This point is mentioned because the V_H domain of MR1 contains a Factor Xa digestion site linking it to PE. Following purification of MR1 the PE is usually cleaved off. Since MH20 also contains amino acid sequences that may be mistakenly cleaved by Factor Xa, we had to genetically substitute a Genenase I site where the factor Xa site was located. Another point to clarify is that a disulfide linked Fv, as opposed to a scFv of MR1, was created because better yields of MR1 dsFv are obtained upon refolding. Cysteine residues had been previously incorporated into MR1's V_H and V_L domains at positions 44 and 100 respectively to facilitate the formation of the disulfide bond, upon oxidation of the two domains during refolding.

Construction of MR1 dsFv with N-terminal icosapeptide

To create the gene encoding MR1's V_L fused to the MH20 icosapeptide, a "piggyback" PCR reaction was required. This entailed two separate PCR reactions to sequentially add the nucleotides for the icosapeptide at the 3' end of the V_L domain sequence. Using the pMR1VL plasmid as template DNA, the first PCR added 30 nucleotides encoding the first 10 amino acids of the icosapeptide to the V_L domain. A sense primer VL5NEW (GAGTCACATATGGACATCGAGCTCACTCAGTCT) with a NdeI site (underlined) and an antisense primer ADVL1 (CCACTGAACACGGAAACCAACGATAGCACGTCCGATTTCCAGCTTGGTGCCG CA) with the sequence for the icosapeptide, were used in the first PCR. The PCR product from this reaction was incubated alongside a second set of primers to complete the addition of the icosapeptide sequence using a second PCR. These were, the VL5NEW primer used previously, and a new antisense primer ADVL2 (AGAATTCAACGAGAACCGTTAACGAAGTAACGACGCAGCCACTGAACACGG AAACCAACG) with an EcoRI site (underlined) and the remaining sequence for the encoding of the icosapeptide. This PCR product was purified, ligated into pT7Blue3, sequenced, excised and ligated into NdeI/EcoRI digested pMR1PE38K to generate pULI7-$V_L$icosa.

The V_H domain of MR1 was much simpler to create and required only a single PCR. Sense primer MR1Nde (GAGTCACATATGCAGGTGAAACTGCAGCAGTCTGG) with a NdeI site (underlined) and antisense primer VHGFN (AGAAGCTTCAGCGTAGTGAGCAGCACCCGGTGAGGAGACGGTGACCGTGG)

with a HindIII site (underlined), which also contained the sequence for the Genenase I cleavage motif, were used to amplify out the V_H domain of MR1 from pMR1FxPE38K template DNA. This PCR product was purified, ligated into pT7Blue3, sequenced, excised, and ligated into NdeI/HindIII digested pMR1PE38K to generate pMR1-V_HGen.

Inclusion Body (IB) Preparations

For large scale expression of the pULI7-$V_L$icosa constructs, 2 µl of the plasmid was transformed into 50 µl BL21 (λ DE3) *Escherichia coli* cells along with a separate transformation of 2 µL of the Genenase I V_H domain containing plasmid pMR1-V_HGen. The transformations were plated on 10 100mm agar plates containing 100 µg/ml ampicillin. Two separate 1L growth cultures, one for the V_H domain and one for the V_L domain, were prepared by adding the colonies from the 10 plates to 1L of LB media, 10 ml 20% glucose, 5 ml 5% $MgSO_4$, and 2 ml 1mg/ml ampicillin. The cultures were grown to an A_{600} of 2.5, as measured on a Beckman DU640 spectrophotometer, at 37 °C with constant shaking, then induced for protein expression with 1mM isopropyl β-D-thiogalactoside (IPTG). The culture was incubated for a further 1.5 hours at 37 °C with constant shaking. The cultures were then pelleted, using a Beckman J2-21M/E centrifuge, and resuspended in 180 ml 50mM Tris pH 7.4/ 20mM (TE 50/20). The cells were lysed with 8 ml 5 mg/ml lysozyme in ddH_2O at room temperature with constant shaking for 30 minutes. Bacteria were lysed at room temperature with 20 ml 5M NaCl and 20 ml 25% Triton X-100 for 30 minute with constant shaking. The inclusion bodies were pelleted again, drained, and resuspended in 200 ml TE 50/20 and 1% Triton X-100 using a tissuemizer (Kinematica C8-6010). This wash step was repeated 3 more times

with 200 ml TE50/20 without the Triton X-100. The inclusion body pellets were then solubilized in a denaturing solution of 4ml Guanidine/Cl soltuion (6M Gnd/Cl, 0.1M Tris (HCl) pH 8.0, and 2mM EDTA) per gram IB. These solutions were centrifuged and the supernatants, containing denatured IBs, were retained. IB concentrations were assayed using Pierce Coomassie Reagent, via a BSA standard curve, and then diluted to 10 mg/ml.

Refolding and Purification of dsFvs

The oxidation of the V_L and wild-type V_H domains required the addition of 50mg DTT to 4 ml of the 10 mg/ml V_H-Gen domain and 1 ml of the 10 mg/ml V_L-icosa domain, and incubation at room temperature for at least two hours. This solution was then slowly added to 500 ml of refolding buffer (0.1M Tris pH 9.0, 0.5M Arginine•HCl, and 2mM EDTA) at 10^{O}C containing 275.5 mg oxidized glutathione (GSSG). The refolding solution was placed at 10^{O}C for two days. Dialysis of the dsFvs required incubation in washed size exclusion dialysis tubing (GibcoBRL) placed in two 50L buffered tanks (0.02M Tris pH 7.4, 0.005 M EDTA pH 8.0, 300g urea). Following the dialysis, any particulate matter in the dialyzed dsFvs solutions were spun down, and the supernatants removed for Fast Protein Liquid Chromatography (FPLC). A 10 ml Q Sepharose Fast Flow HR 10/10 column was first used to separate the proteins based on ionic properties. To elute bound protein, a gradient elution was used that consisted of the mobile phase (10mM Tris pH7.4, 1mM EDTA) and increasing ionic solution (10mM Tris pH7.4, 1mM EDTA, and 1M NaCl). SDS-PAGE analysis of the protein fractions confirmed the presence of properly folded dsFv. The resulting protein fractions were

pooled, diluted 5 fold, and then applied to a Resource Q 1ml column, where the PE subunit fused to the V_HGen domain readily bound to this ionic resin. Elution was done in a similar manner as the Q Sepharose column. Eluted fractions were loaded on a 12% SDS-PAGE gel and stained with Coumassie Brilliant Blue to visualize protein as described previously.

Refolding and Purification of $V_L$icosa followed by ELISA analysis

Refolding of only the $V_L$icosa domain of MR1 was done in a similar manner as previously described for the whole dsFv, but in the absence of any V_HGen protein. Concurrently, a refolding under the same condition for the $V_L$myc domain was conducted for later comparisons in ELISA analyses. The only major exception in this time was that no FLPC purification of the dialysed protein was conducted, but instead, the protein was run through a PD10 column, as described previously. The amount of refolded $V_L$icosa and $V_L$myc was then determined using a Pierce standard curve assay and each protein was spotted onto a 96-well polystyrene plate for ELISA. The $V_L$myc was incubated at a concentration of 1 μg/ml overnight at 4°C, whereas the $V_L$icosa was incubated at 10 μg/ml. The ELISA was done in a similar manner as previously described. $V_L$myc protein was directly assayed by incubating with 1000X diluted anti-myc (9E10) primary antibody, and was only used as a positive control. To detect binding of $V_L$icosa protein to Ad5, $1x10^9$ particles/ml of virus was incubated initially, and then 1000X diluted mouse anti-sera to Ad5 was bound to any present virus. Secondary antibody detection for $V_L$myc wells was facilitated by 5000X diluted goat anti-rabbit HRP conjugated antibody, whereas for $V_L$icosa wells, 5000X diluted goat anti-mouse HRP conjugated antibody was

used. All assays were done in triplicate.

Results and Discussion

Construction, expression, and purification of $V_L$icosa-V_HGen MR1 dsFv

The 786 bp coding sequence of the newly designed MR1 dsFv with a C-terminal MH20 icasapeptide was tested for expression in *E.coli* as separate protein domains. Expression of each gene, the 381 bp $V_L$icosa and 357 bp V_HGen subunits, appeared to result in the formation of inclusion bodies, or aggregates of insoluble protein, that corresponded to the appropriate size for each domain. The $V_L$icosa domain migrated at ~14 kDa, and the V_HGen domain fused to PE (~38 kDa) migrated at ~51 kDa. Refolding of these proteins together, however, resulted in aggregated oxidized dsFv protein, as observed on SDS-PAGE following purification on the Resource Q column.

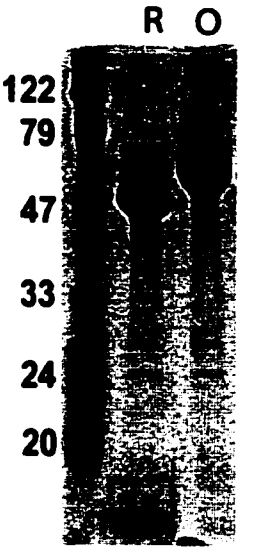
Upon observing these abnormalities in the proper folding of the MR1 dsFv protein consisting of the $V_L$icosa-V_HGen domains, other MR1 dsFvs with various combinations of different V_L and V_H domains were also created. This was done to determine whether the newly introduced icosapeptide or Genenase I site in these two proteins was interfering in their refolding. Previously, a MR1 dsFv construct was created with a V_L domain fused to a C-terminal myc epitope ($V_L$myc), which was disulfide linked to a V_H domain with a Factor Xa site (V_HFactXa). This is dubbed "wild-type" MR1 dsFv in this study, as it readily refolds and functions exceptionally well. This protein was expressed along with various combinations of its domains to those of the $V_L$icosa-V_HGen dsFv. To examine the structural influence of the $V_L$icosa domain, it was refolded with the V_HFactXa domain, and similarly the $V_L$myc domain was refolded with the V_HGen. Upon comparing these proteins to wild-type dsFv, however, they also did not refold properly which led to the conclusion that either, or both, the icosapeptide fused

to V_L, or the newly introduced Genenase I site in V_H, was interfering with the refolding of the dsFvs (Figure 23).

Realizing that eukaryotic expression was the only alternative to obtaining properly refolded $V_L$icosa-V_HGen MR1 dsFv, we wanted to first examine the claim that the icosapeptide was able to bind strongly to Ad5 fiber protein. To accomplish this, we refolded just the $V_L$icosa domain and used it for ELISA binding assays with Ad5 knob protein and Ad5 virus. Preliminary experiments showed that the icosapeptide fused to the V_L domain of MR1 was unable to bind to His tagged Ad5 knob protein. Not completely defeated in our resolve, we hypothesized that multivalent binding of icosapeptide to fiber may be required and we attempted the binding assays again, this time with Ad5 virus. A weak signal was consistently obtained for Ad5 virus binding to ELISA spotted $V_L$icosa at a concentration of 10 μg/ml. To assuage any concerns that this binding was occurring due to interactions of the virion with the hydrophobic face of the V_L domain, $V_L$myc at a concentration of 20 μg/ml was simultaneously tested and failed to show any affinity for Ad5. The observed signal for Ad5 binding to $V_L$icosa was extremely low, approximately 8X less compared to the signal obtained when $1x10^9$ virions/ml were plated in a well and detected with mouse anti-sera (Figure 24). This led us to conclude that further studies utilizing the icosapeptide would not be valuable in trying to retarget Ad virus, and therefore eukaryotic expression of the MR1 dsFv construct was not pursued.

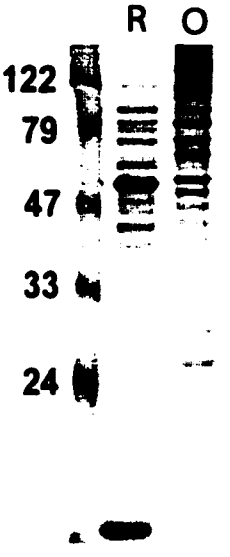
Figure 23. Coomassie gels of MR1 dsFv constructs. 20 µl of each refolded, Q-Sepharose purified MR1 dsFv had its disulfide bond either reduced by the addition of β-mercaptoethanol in the loading buffer (letter R above the lane) or left in its oxidized form by this chemical's exclusion (letter O above the lane). These samples were then loaded and run on a 12% SDS-PAGE gel and then stained with Coumassie Brilliant Blue (A) Wild-type MR1 dsFv protein composed of the V_HFactXa domain and the $V_L$myc domain.. (B) MR1 dsFv protein composed of the V_HFactXa domain and the $V_L$icosa domain. (C) MR1 dsFv protein composed of the V_HGen domain and the $V_L$icosa domain. The bands from a protein molecular weight marker run in the first lane are clearly marked on the left side of each blot. Notice that only wild-type, oxidized, MR1 dsFv does not aggregate and a relatively large, clean band of the appropriate ~51 kDa sized protein is observed.

A



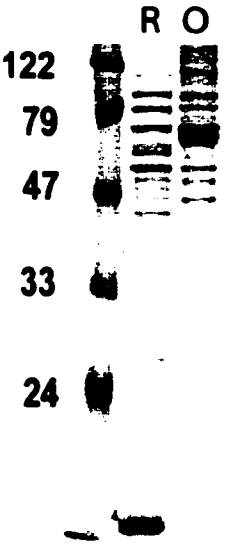
V_H(factorXa)-V_L(myc)

B



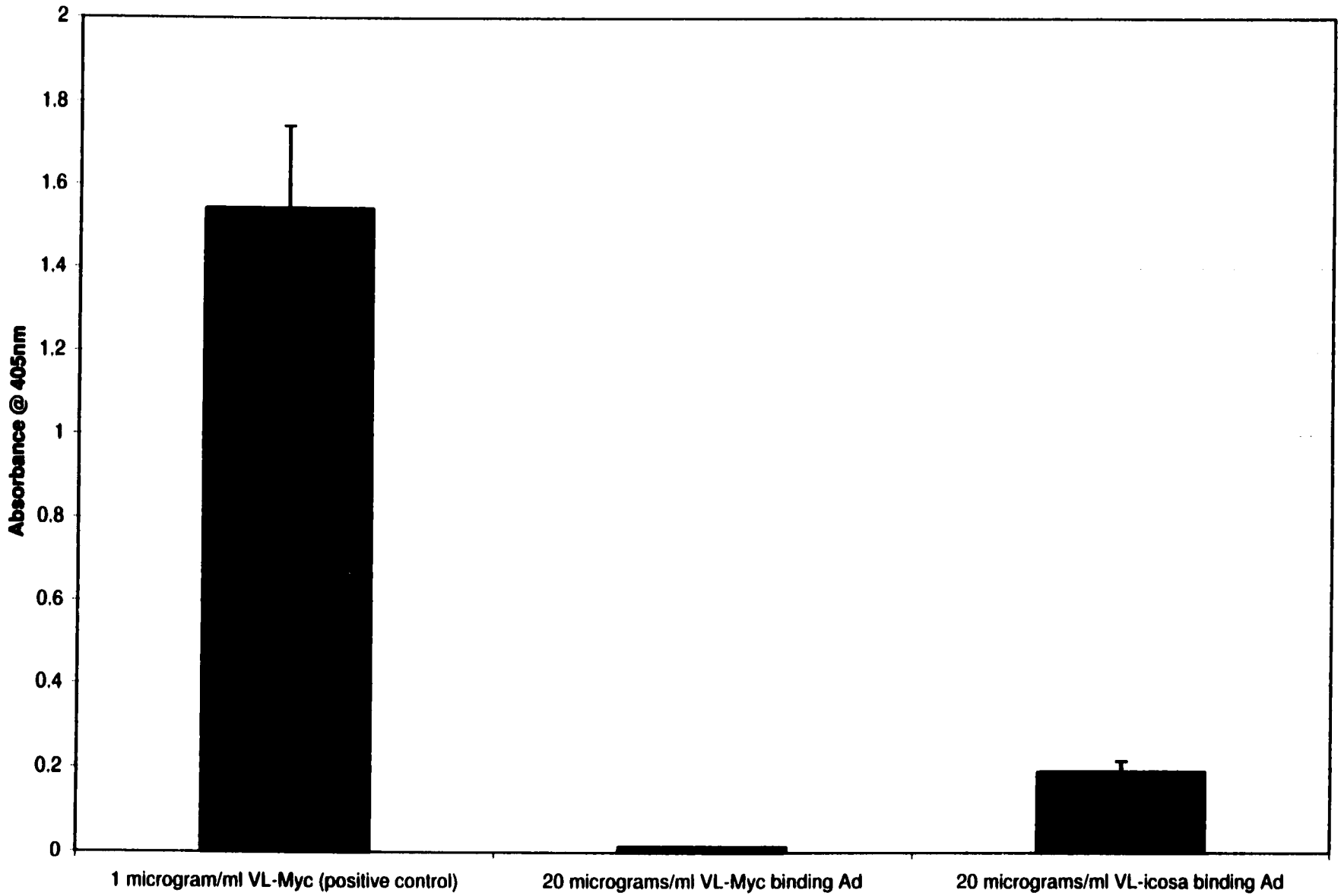
V_H(factorXa)-V_L(icosa.)

C



V_H(Gen.)-V_L(icosa.)

Figure 24. ELISA of Ad5 virus binding assay to $V_L$icosa protein. To determine whether Ad5 virus was able to bind to the MH20 icosapeptide fused to MR1's V_L domain, 10 μg./ml of $V_L$icosa was spotted in ELISA plate wells. A very weak signal for Ad5 binding was obtained when probed with anti-Ad5 mouse anti-sera (far right column). The first column corresponds to the signal obtained from 1 μg/ml $V_L$myc protein probed with anti-myc antibody. The second column represents the signal obtained from 20 μg/ml $V_L$myc protein that was incubated with Ad5 virus and then probed with anti-Ad5 mouse anti-sera.


Absorbance @ 405nm
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1 microgram/ml VL-Myc (positive control)
20 micrograms/ml VL-Myc binding Ad
20 micrograms/ml VL-icosa binding Ad

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- Obtained my M.Sc. in Biochemistry at the University of Ottawa.

1995 - 1998 University of Ottawa Ottawa, Ontario

Honours in Biochemistry

- Completed my Honours degree in Biochemistry.

1991 - 1995 Evan Hardy Collegiate Saskatoon, Saskatchewan

Senior Matriculation

Work experience

Sept. 1999-Present University of Ottawa Ottawa, Ontario

Volunteer with Let's Talk Science (LTS)

Supervisor: Dr. Barbara Vanderhyden

- Description of Activity :
  - As a member of LTS, I go around to high schools within the community and give presentations or hands-on demonstrations on science topics. The ultimate goal of the program is to increase scientific research awareness and assist high school teachers in conveying science related subject matter more effectively to their students.
  - During the Summer 2000, I was fortunate to TA the Professional Development course in Biotechnology for High School Teachers, where I gave lectures and monitored laboratory experiments. Teachers from throughout the city participated to update their molecular biology techniques.
  - I have also recently completed a newly designed Let's Talk Science Website for the University of Ottawa Partnership Program. It should replace the current one very soon.

Sept. 1999-2001 University of Ottawa Ottawa, Ontario

Teaching Assistant, Course Lecturer, and Exam Proctor

Supervisor: Dr. Miguel Rodriguez

- Description of Work :
    - I was a laboratory TA for third year biochemistry labs at the University of Ottawa. For one semester out of the year I taught and marked the laboratory component of the third year biochemistry labs.
    - I gave lectures on Spectrophotometry and pH determination to second year students in Biochemistry and Biopharmacy. I also participated in the writing up of questions for the final examination of the class.
    - I have proctored various courses in Biochemistry at the University of Ottawa, including General Metabolism and Molecular Genetics I.

Sept. 1999-2001 Ottawa Regional Cancer Center Ottawa, Ontario

Master's Research

Supervisor: Dr. Ian Lorimer

- Description of Work :
    - Adenovirus tropism modification was the focus of my Master's research. This included techniques for non-covalent modifications of adenovirus fiber protein, and competitive infectivity assays with other tropism modified viruses. A manuscript based on the thesis work will be written up for submission.

Sept. 1998-May 1999 Ottawa Regional Cancer Center Ottawa, Ontario

Honour's Project

Supervisor: Dr. Ian Lorimer

- Description of Work :
    - My Honours thesis work involved mutational analysis of three residues in a scFv's (MR1) V_H domain CDR2. This scFv was designed as an immunotoxin against an oncogenic EGFR receptor, and the research was meant to complement X-ray crystallography data of the molecule bound to its epitope. From this work, I was a co-author on a paper submitted to (and accepted by) the Journal of Molecular Biology.

Summer 1998 Ottawa Regional Cancer Center Ottawa, Ontario

Summer Researcher

Supervisor: Dr. Ian Lorimer

- Description of Work :
    - My primary objective was to investigate the feasibility of initiating a tumour specific immune recponse to EGFRvIII expressing cancer cells. The hypothesis for the work was that transduction of CD80 (B7-1), a T-cell co-stimulatory molecule expressed on APCs, into cancer cells would initiate a tumor specific immune response against these cells.

Winter 1998 Natural Resources Canada Ottawa, Ontario

Researcher

Supervisor: Dr. Shantha De Silva

- Description of Work :
    - Researching and compiling terminology pertaining to environmental contamination sites.
    - Production of a comprehensive dictionary of definitions on environmental contamination sites to be published and distributed by Natural Resources Canada.

May 1997- January 1998 Carleton University Ottawa, Ontario

Volunteer Researcher

Supervisor: Dr. Nimal De Silva

- Description of Work :
    - Developing new analytical methods using a novel technique to analyze powders directly by Inductively Coupled Plasma Spectrometry. Work included, preparation of samples, analysis by Direct Powder Introduction technique (eg. Sediments, glass powders for boron), operation of ICP spectrometer and data processing using spreadsheets; teaching the other students operation of the spectrometer and sample introduction system.
    - Calibration, alignments of ICP optical spectrometers.
    - Building and maintaining commercial computer hardware (i.e. Computer systems).
    - Evaluation of a new software package for ICP data acquisition which is being developed in collaboration with an industrial partner.