

## **INFORMATION TO USERS**

**This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.**

**The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.**

**In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.**

**Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.**

**Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.**

**ProQuest Information and Learning  
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA  
800-521-0600**

**UMI<sup>®</sup>**





Université d'Ottawa • University of Ottawa



**Pseudotyping the Moloney murine leukemia virus with  
engineered envelope glycoproteins**

Alexander C. Klimowicz

Thesis submitted to the Department of Biochemistry, Microbiology and Immunology in  
partial fulfilment of the requirements for the degree of Master's of Science

University of Ottawa

Ottawa, Ontario, Canada

©Alexander C. Klimowicz, Ottawa, Canada, 2001



**National Library  
of Canada**

**Acquisitions and  
Bibliographic Services**

**395 Wellington Street  
Ottawa ON K1A 0N4  
Canada**

**Bibliothèque nationale  
du Canada**

**Acquisitions et  
services bibliographiques**

**395, rue Wellington  
Ottawa ON K1A 0N4  
Canada**

*Your file Votre référence*

*Our file Notre référence*

0-612-66063-X

**The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.**

**The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.**

**L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.**

**L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.**

**Canada**

## **Abstract**

We were interested in generating an in vivo retroviral gene therapy vector based on the commonly used Moloney murine leukemia virus (MoMLV). This was accomplished by pseudotyping with an engineered influenza A hemagglutinin. Point mutations were introduced to abrogate hemagglutinin's wild type binding and a single chain variable domain antibody fragment (scFv) was added to its amino terminus to provide new binding specificity. The engineered hemagglutinin was able to mediate binding of pseudotyped retrovirus to a scFv specific peptide but was unable mediate infection of target cells. To rescue the infectivity of the pseudotyped retrovirus the role of lipid rafts in the lifecycle of the MoMLV was examined. Lipid raft isolation from transfected cells and virus particles revealed that retroviral proteins were not associated with lipid rafts. Using a panel of hemagglutinin mutants with reduced lipid raft affinity we also determined that this parameter did not affect pseudotyping efficiency.

## **Thesis Abstract**

Gene therapy holds great promise for the treatment of both genetic and acquired disease. Current delivery methods for therapeutic genes are non-specific. Consequently most gene therapy protocols call for *ex vivo* manipulation of target tissues. This is an invasive procedure and it yields poor success rates. We were interested in generating targeted retroviral gene therapy vectors, for use *in vivo*, based on the commonly used Moloney murine leukemia virus (MoMLV). Previous work on modifying and retargeting the retroviral envelope glycoprotein, which is responsible for binding and fusion to target cell membranes, has been largely unsuccessful.

The work in this thesis begins with the engineering of a foreign envelope glycoprotein, influenza A virus hemagglutinin (HA), to retarget MoMLV particles to cells expressing a specific target protein. Our lab has previously demonstrated that influenza A/PR/8/34 hemagglutinin (PR8HA) is incorporated into MoMLV particles. A series of mutations were made to PR8HA to retarget its binding properties. After each mutation the expression, cell fusion, retroviral incorporation, and retroviral infectivity were tested to ensure continued function of the engineered protein. PR8HA was first modified at its cleavage site to incorporate a factor Xa cleavage motif. Trypsin-like proteases are normally required to proteolytically activate the fusion ability of HA. This factor Xa modification was made to facilitate the proteolytic activation of HA in the presence of a novel binding domain. Two point mutations were then introduced into PR8HA's sialic acid binding pocket to destroy its normal tropism. Sialic acid is found as a terminal glycosylation on all mammalian cells and as such, it was necessary to abrogate PR8HA sialic acid binding in order to retarget this protein. Finally, a single chain

variable domain antibody fragment (scFv) binding domain was added to the amino terminus of the modified PR8HA to provide new binding specificity. This engineered envelope glycoprotein was able to mediate the binding of accordingly pseudotyped retroviral particles to a target peptide specific for the scFv binding domain. However, it was unable to mediate the infection of cells expressing the protein from which the target peptide was derived. In addition to the inability of the retargeted retrovirus particles to mediate infection, retrovirus particles containing the unmodified PR8HA gave very poor infectivity in a variety of cell types.

It was believed that if we could increase the infectivity of the wild type PR8HA bearing retrovirus the infectivity of the retargeted PR8HA pseudotyped particles could be rescued. The final chapter describes an attempt to increase the infectivity of the PR8HA pseudotyped retroviral particles by examining the biogenesis of the MoMLV. Data have demonstrated that HA is tightly associated with membrane microdomains enriched in cholesterol and sphingolipids termed lipid rafts. The influenza A virus uses these microdomains to concentrate viral proteins and ensure proper viral biogenesis. Recent data suggest that the type 1 human immunodeficiency virus and perhaps all retroviruses also use lipid rafts for the production of viral particles. We were interested in determining what role, if any, membrane microdomains played in the pseudotyping efficiency and in the lifecycle of the MoMLV. Isolation of rafts from transfected cells indicated that unlike HIV, MoMLV proteins are not strongly associated with lipid rafts. A series of PR8HA mutants with altered lipid raft affinity were also generated to verify that lipid rafts do not play a role in the MoMLV lifecycle. Retrovirus particles incorporated all of the PR8HA mutants with equal efficiency and accordingly infectivity

of the pseudotyped retrovirus particles was equivalent. All of these data demonstrate that lipid rafts are not used in the biogenesis of the MoMLV. This information will permit the generation of more infectious MoMLV pseudotypes by allowing for informed choices in the selection of foreign envelope glycoproteins.

## **Acknowledgements**

I would like to express my thanks to Dr. Ian Lorimer for his time and patience in mentoring me and for always providing me with just the right amount of supervision. I would also like to thank Maria Tsirigotis for all the great discussions and the late night company. Finally I would like to thank Elizabeth Long for helping me see my true potential and teaching me how much I could really accomplish.

## Table of Contents

Thesis abstract.....	i
Acknowledgements.....	iv
Table of contents.....	v
List of tables.....	viii
List of figures.....	ix
<b>Chapter 1: General Introduction.....</b>	<b>1-15</b>
1.1 Gene therapy.....	1
1.2 Non-viral gene therapy vectors.....	1
1.3 Viral gene therapy vectors.....	3
1.4 Retroviruses.....	3
1.5 Retroviral structure.....	4
1.6 The retroviral lifecycle.....	9
1.7 Pseudotyping retrovirus.....	12
1.8 The Moloney murine leukemia virus and gene therapy.....	14
1.9 Area of investigation.....	15
<b>Chapter 2: Retargeting the Moloney murine leukemia virus to cells expressing the mutant epidermal growth factor receptor EGFRvIII.....</b>	<b>17-58</b>
2.1 Summary.....	17
2.2 Introduction.....	18
2.2.1 EGFRvIII.....	18
2.2.2 MR1 scFV.....	19
2.2.3 Retargeting MoMLV with MR1.....	20
2.2.4 Influenza A hemagglutinin.....	21
2.2.5 Targeting influenza A hemagglutinin.....	21
2.2.6 Hypothesis.....	23
2.2.7 Specific objectives.....	24
2.2.8 Significance.....	24
2.3 Materials and methods.....	25
2.3.1 Cell culture.....	25
2.3.2 Plasmids.....	25
2.3.3 Retrovirus pseudotyping.....	28
2.3.4 Immunoblot analysis.....	29
2.3.5 Fusion assay.....	30
2.3.6 Infections.....	31
2.3.7 Sialic acid and EGFRvIII cell binding assays.....	31
2.3.8 EGFRvIII peptide binding assay.....	32
2.4 Results.....	32
2.4.1 Expression of influenza A/PR/8/34 HA with an altered cleavage site.....	32
2.4.2 PR8FXa incorporation into retroviral particles.....	34
2.4.3 PR8FXa mediates cell-cell fusion.....	37

2.4.4	PR8FXa pseudotyped MoMLV infection.....	37
2.4.5	Expression of PR8FXa with a mutated sialic acid binding domain.....	41
2.4.6	PR8FXaDo incorporation into retroviral particles.....	41
2.4.7	Reduced cell-cell fusion mediated by PR8FXaDo.....	41
2.4.8	PR8FXaDo cannot bind sialic acid efficiently.....	43
2.4.9	Introduction of scFv binding domains into influenza A HA.....	43
2.4.10	PR8MR1 pseudotyped MoMLV particles bind to an EGFRvIII peptide.....	47
2.4.11	PR8MR1 pseudotyped MoMLV particles bind to EGFRvIII expressing cells.....	49
2.4.12	Lack of syncytia formation and infectivity with PR8MR1.....	49
2.5	Discussion.....	51

<b>Chapter 3:</b>	<b>Retargeting the Moloney murine leukemia virus to cells expressing the mutant epidermal growth factor receptor EGFRvIII.....</b>	<b>59-90</b>
3.1	Summary.....	59
3.2	Introduction.....	60
3.2.1	Lipid Rafts.....	60
3.2.2	Enveloped viruses and lipid rafts.....	61
3.2.3	Hypothesis.....	62
3.2.4	Specific objectives.....	63
3.2.5	Significance.....	63
3.3	Materials and methods.....	64
3.3.1	Cell lines.....	64
3.3.2	Plasmids.....	64
3.3.3	Retrovirus.....	66
3.3.4	Crude lipid raft isolation.....	66
3.3.5	Lipid raft sucrose floatation.....	67
3.3.6	Immunoblot analysis.....	68
3.3.7	Infection assay.....	68
3.4	Results.....	68
3.4.1	Crude raft isolation of MoMLV proteins is inconclusive.....	68
3.4.2	Crude raft isolation of influenza A/PR/8/34 hemagglutinin and mutants.....	70
3.4.3	Crude lipid raft isolation from pseudotyped MoMLV particles.....	71
3.4.4	Sucrose floatation of lipid rafts demonstrate that the ecotropic envelope glycoprotein is not raft associated.....	75
3.4.5	Sucrose floatation of influenza A/PR/8/34 hemagglutinin and mutants.....	76

3.4.6	Sucrose floatation of envelope glycoproteins and retroviral structural proteins from MoMLV particles.....	76
3.4.7	Relative incorporation and infectivity of PR8HA pseudotypes.....	79
3.4.8	Infectivity levels of the PR8HA mutants remain unchanged.....	82
3.5	Discussion.....	85
	References.....	91

## **List of Tables**

<b>Table 2.1:</b>	<b>Cell to cell fusion assay.....</b>	<b>38</b>
<b>Table 2.2:</b>	<b>Infection of NIH 3T3 cells with PR8FXa pseudotyped MoMLV.....</b>	<b>39</b>
<b>Table 2.3:</b>	<b>Infection with HA pseudotyped MoMLV.....</b>	<b>40</b>
<b>Table 3.1:</b>	<b>Infection of NIH 3T3 cells with PR8HA cytoplasmic tail mutants.....</b>	<b>84</b>

## List of Figures

Figure 1.1:	Structure of a retroviral particle.....	5
Figure 1.2:	Lifecycle of a retrovirus.....	11
Figure 2.1:	Structure of HA monomer.....	22
Figure 2.2:	Schematic diagram of the PR8HA constructs.....	27
Figure 2.3:	Expression and trypsin cleavage of PR8HA and PR8Fxa.....	33
Figure 2.4:	Expression and incorporation of PR8FXa.....	36
Figure 2.5:	Expression and incorporation of PR8FXaDo.....	42
Figure 2.6:	Sialic acid binding assay.....	44
Figure 2.7:	Expression and incorporation of PR8MR1.....	46
Figure 2.8:	EGFRvIII peptide binding assay.....	48
Figure 2.9:	EGFRvII cell binding assay.....	50
Figure 3.1:	Schematic representation of the pseudotyping envelope glycoproteins...	63
Figure 3.2:	Crude raft localization of Eenv and gag in 293T cells.....	69
Figure 3.3:	Crude raft localization of PR8HA mutants in transfected cells.....	72
Figure 3.4:	Crude raft localization of envelope glycoproteins in MoMLV particles.....	73
Figure 3.5:	Crude raft localization of p65gag and p30 capsid in MoMLV particles.....	74
Figure 3.6:	Sucrose flotation of MoMLV proteins from transfected cells.....	77
Figure 3.7:	Sucrose floatation of envelope glycoproteins from transfected cells.....	78
Figure 3.8:	Sucrose floatation of envelope glycoproteins from MoMLV particles .....	80
Figure 3.9:	Sucrose floatation of p65gag from MoMLV particles.....	81
Figure 3.10:	Relative incorporation of envelope glycoproteins into MoMLV particles.....	83

## **Chapter 1:**

### **General Introduction**

#### **1.1 Gene therapy**

Modern treatment for genetic disease is sufficient to prolong the life of affected individuals; however, these treatments are unable to substantially improve their quality of life. Conventional medication can only mask disease symptoms for brief periods of time and consequently the patient must receive repeated treatment. Gene therapy is designed to treat all of the symptoms of the disease acting directly upon the underlying cause, genetic or acquired, by supplementing or replacing the genes at fault. Currently, clinical protocols are being tested for the treatment of a wide variety of genetic diseases including autoimmune, metabolic, and cardiovascular disorders. A variety of cancer treatments are also being examined, using therapeutic genes to elicit immune responses to the tumours or to directly kill the tumour cells and/or the vascular cells supplying them with blood. Research is also being carried out to use gene therapy to protect and or clear the body from viral infections such as HIV/AIDS (Reviewed in Larrick and Burck, 1991; Byrn and Roberts, 1996; Medin et al., 1996; Robbins, 1997).

#### **1.2 Non-viral gene therapy vectors**

To deliver the therapeutic genes to target cells and tissues, a variety of agents or vectors are used. These vectors can be divided into two distinct categories, viral and non-viral. Non-viral gene therapy vector strategies are quite numerous and diverse. Some of the most promising strategies either deliver therapeutic DNA into target cells by taking advantage of endogenous cellular pathways for the uptake of macromolecules or use lipid

encapsulation of DNA to fuse directly with the cellular membrane (Mulligan, 1993; Cooper, 1996; Robbins, 1997).

Strategies using macromolecular uptake mechanisms employ molecular fusions of rapidly internalized macromolecules. An example of this is a modified version of the iron transport protein transferrin, which has been engineered to contain a polycationic domain to safely and reversibly bind the therapeutic DNA (Robbins, 1997). Specific cell type targeting for this kind of system can be achieved through use of an internalized ligand specific for the target cell type. In theory this system seems ideal; however, in actual practice gene delivery is inefficient and has not had much success. Therapeutic DNA is exposed to the harsh environs of the endosome upon uptake and is often trapped in this compartment where it is unable achieve nuclear localization and subsequent gene expression (Mulligan, 1993; Cooper 1996).

While liposomal delivery is equally promising it has also failed to achieve practical success. This method delivers the therapeutic DNA to cells encapsulated in an artificially produced lipid bilayer. Targeting can be achieved broadly by modifying the lipid content of these vesicles or by incorporating targeting proteins within them. However, *in vivo*, liposomes are prone to rapid degradation by serum proteins (Mitchell, 1998). Similar to the strategies employing macromolecular uptake this methodology delivers genes to the cytoplasm and not to the nucleus were they are needed for gene expression. An additional drawback inherent with the macromolecular uptake and liposomal methodologies is that they both lead to transient gene expression. The therapeutic DNA that does manage to attain nuclear localization is quickly recognized as

foreign content and removed by the host cell (Mulligan, 1993; Cooper, 1996; Robbins, 1997).

### **1.3 Viral gene therapy vectors**

Viral gene therapy vectors take advantage of gene delivery systems that have been developing over millions of years to bypass host immunity and cellular defences. Viruses have developed a variety of strategies to efficiently insert their own genes into host cells and to then force these infected cells to express them. They offer a low risk/low side effect (Verma and Somia, 1997; Knoell and Yiu, 1998) form of gene delivery that has already been optimized by evolution. There are many candidate viruses for uses as delivery vectors; however, the majority do not integrate into the host cell genome and are therefore are also only transiently expressed in infected cells. Retroviruses hold great promise in this respect as these viruses, by their nature, permanently incorporate their genome within that of the host. In terms of gene therapy this is ideal. Theoretically the inserted gene of interest will be expressed for the entire lifetime of the infected cell. As well, if that cell should divide, all daughter cells will also express the therapeutic gene, opening the door to germ line correction of genetic faults. Retroviruses are also very simple viruses most being composed of no more than 10 proteins. Thus, these viruses are well suited for manipulation as viral vectors and can easily be made in a relatively safe replication incompetent form (Deisseroth et al., 1994).

### **1.4 Retroviruses**

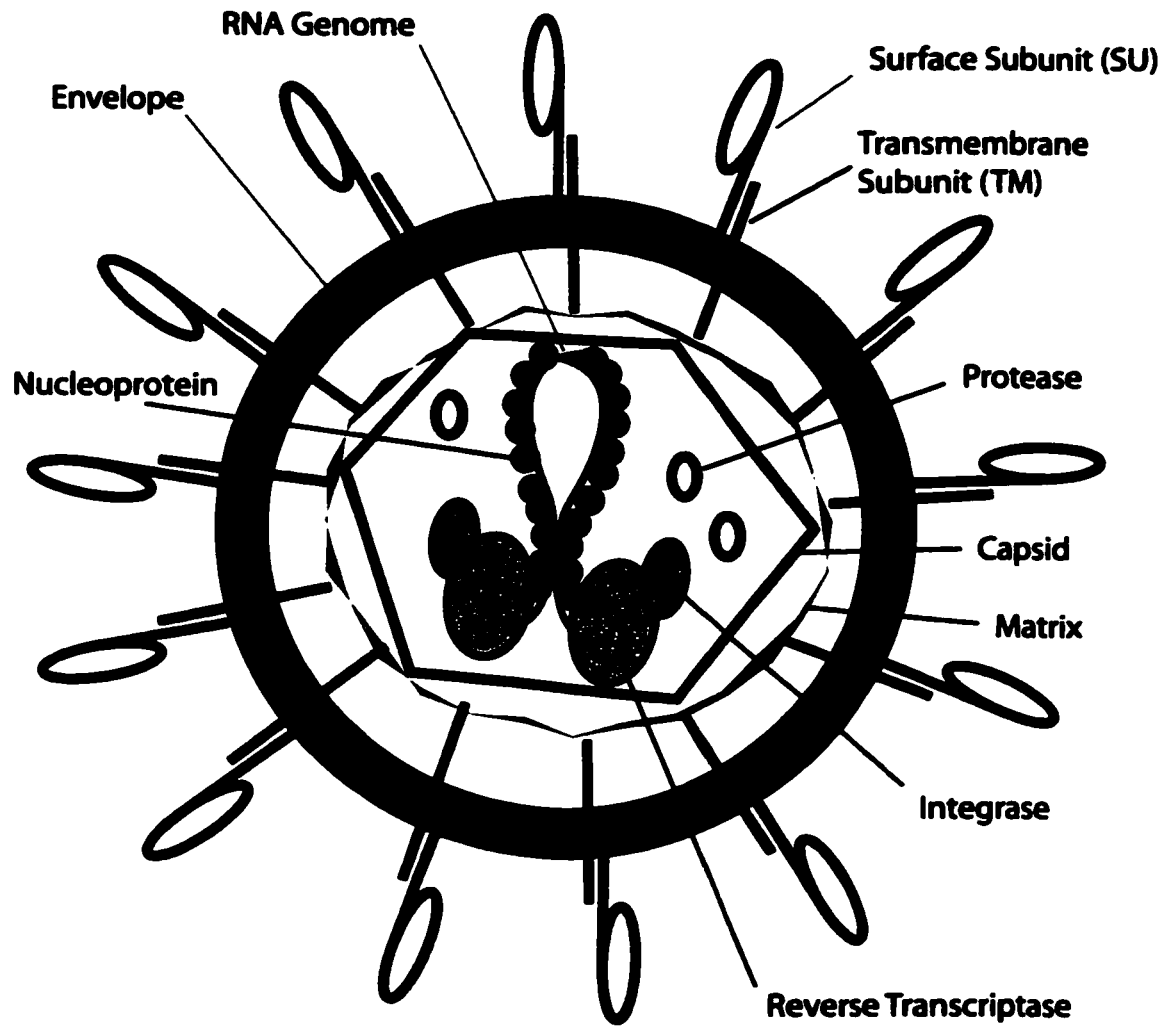
Retroviruses are a unique family of pathogens that primarily affect vertebrates (Reviewed in Coffin, 1996). Family members share a common structure, genetic

organization, and lifecycle. The three subfamilies, oncovirinae, lentivirinae and spumavirinae, are further classified by their structure, cell receptors, and oncogenicity. Retroviruses are the only group of diploid viruses, having two copies of their positive sense RNA genome. The name retrovirus is derived from the lifecycle of these viruses. 'Retro' refers to the reverse transcription that the viral genome undergoes before the production of viral progeny. Once inside a host cell, rather than immediately beginning the production of viral proteins as most other positive stranded RNA viruses do, retroviruses first convert their RNA genome to a DNA copy and integrate this DNA copy into the host genome. Only transcripts produced from the DNA copy, or provirus, are translated to produce viral proteins. Due to the integration of the provirus into the host cell genome, retroviral infections generally last for the rest of the host organism's life. This leads to a variety of disease symptoms quite different from other types of viral infections. While some retroviral infections have no associated pathologies (Reviewed in Meiring and Linial, 2001) the majority of disease states produced from infection are much more severe. Immunodeficiency is currently the most renowned pathology associated with retroviral infection; others include neurological disorders and malignancies.

### **1.5 Retroviral structure**

Retroviruses are enveloped viruses acquiring a membrane bilayer from infected host cells as they are produced. The source of the membrane bilayer varies. The spumavirinae bud from the endoplasmic reticulum (Clarke et al., 1969; Chopra et al., 1972), however, the majority of retroviruses bud from the plasma membrane. Enriched

**Figure 1.1**



**Figure 1.1 Structure of a retroviral particle**

**Schematic representation of a retroviral particle indicating the general location of the important structures and proteins.**

in the envelope, in addition to host cell membrane proteins, is the retroviral envelope glycoprotein (Figure 1.1). The polyprotein product of the env gene is cleaved by cellular proteases into two subunits, the surface subunit SU and the transmembrane subunit TM. The two subunits are each thought to perform a distinct function. The SU is responsible for the majority of the viral tropism. It recognizes and binds to specific cellular proteins allowing the retrovirus to attach to cells expressing these proteins on their surface (Gray and Roth, 1993; Morgan et al., 1993). The TM domain is responsible for the fusion of the viral membrane with that of the target cell (Gallaher, 1987), allowing the viral core access to the target cell's cytoplasm.

Directly underneath the plasma membrane envelope of immature retrovirus particles is the gag polyprotein (Figure 1.1). It is this protein that is responsible for all of the internal structure of the retrovirus. In the majority of retroviruses, gag is cleaved into four separate structural proteins. The first of these cleavage products is the matrix protein. This protein is responsible for the membrane association of gag. The majority of retroviral matrix proteins are modified by the addition of myristic acid at their amino terminus for this purpose (Rein et al., 1986). Mutation or removal of the matrix component of gag abrogates gag membrane localization (Facke et al., 1993; Zhou et al., 1994, Soneoka et al., 1997).

The next cleavage product of gag polyprotein varies in size among the different retroviruses. While it is the next structural layer underneath the matrix protein, it has no known structural function. Known as the late domain, this protein is involved with the budding process of the retrovirus. This function is accomplished while the late domain is still a part of the gag polyprotein. Recent work has determined that this region contains a

PY domain that is responsible for the mono-ubiquitination of gag (Strack et al., 2000). The PY domain is a proline rich motif that serves as a recognition site for E3 ubiquitin ligases (Goulet et al., 1998). Ubiquitination of gag is required for efficient virus particle formation. In the absence of ubiquitination viruses cannot be released from the cell leaving the cellular membrane littered with incompletely budded particles as is evidenced by the removal of the late domain, the modification of the PY motif or the depletion of cellular ubiquitin pools (Patnaik et al., 2000; Schubert et al., 2000).

Internal to the late domain is the capsid protein. This hydrophobic protein forms the core shell within the virus particle, recognizable by electron microscopy (Figure 1.1) (Weiss et al., 1984; Stewart et al., 1990). In its structural role very little is known about the function of the capsid. Within the context of the gag polyprotein it is responsible for the protein-protein interactions between gag molecules. Mutations within this protein can disrupt particle formation (Hansen et al., 1990; Jones et al., 1990; Wang et al., 1994; Hansen and Barklis, 1995; McDermott et al., 1996).

The last cleavage product of the gag gene is the nucleoprotein. Within the retrovirus particle this small protein is found associated with the retroviral RNA (Figure 1.1). Other than its basic nature the most prominent feature of the nucleoprotein is the presence of conserved zinc finger like domains of evenly spaced cysteine and histidine residues in all but the spumavirinae.

The other major proteins that are conserved among all retroviruses are the protease, reverse transcriptase (RT) and integrase (IN). The pro region of the retroviral genome encodes the protease while the pol region encodes RT and IN. The gag, pro, and pol products are all translated from full-length mRNA transcribed from the integrated

provirus. Neither pro nor pol can be considered separate genes as both proteins are produced as carboxy terminal extensions of the gag polyprotein (Reviewed in Hatfield et al., 1992). In some viruses, pro is in the same reading frame as gag and is produced directly as a carboxy terminal fusion, while in others, pro is in the pol reading frame, and in still others pro is in a distinct reading frame from both gag and pol. Depending on the virus there are two different translational strategies to produce pol or pro-pol. A frameshift of  $-1$  is required to read some of the pol or pro-pol regions generating either full or partial gag fusions with these proteins. In some cases two shifts are required, one to acquire pro, the other to acquire pol. If these regions are in frame with gag or gag-pro, readthrough extension of the stop codon is sufficient to produce the full gag-pro-pol product. This complex regulation allows for the stoichiometric production of the structural versus enzymatic proteins that make up the internal components of the retrovirus.

The pro gene product encodes the retroviral protease. It is sensitive to aspartic protease class inhibitors, yet unlike the aspartic proteases its active form is a dimer (Reviewed in Skalka, 1989). This unique feature is thought to keep the enzyme inactive until it is within the context of the fully formed retroviral particle. The tight packing between gag proteins allows for the dimerization and activation of the protease that then proceeds to cleave the gag polyproteins into their constituent components. This process is known as viral maturation and will be discussed later within the context of the retroviral lifecycle (Figure 1.2). The recognition site of the protease varies but it most commonly cleaves between a bulky hydrophobic residue and a proline.

The pol region codes for two proteins, the retroviral reverse transcriptase and integrase, both of which are loosely associated with the nucleoprotein. The amino terminal pol cleavage product is the reverse transcriptase protein. This protein contains two enzymatic functions; it has both RNA/DNA dependent polymerase and ribonuclease H activity. The RNA dependent DNA polymerase activity converts the retroviral RNA genome into DNA, while the RNase H activity degrades the RNA template as this process occurs. DNA dependent DNA polymerase activity synthesizes the complementary strand.

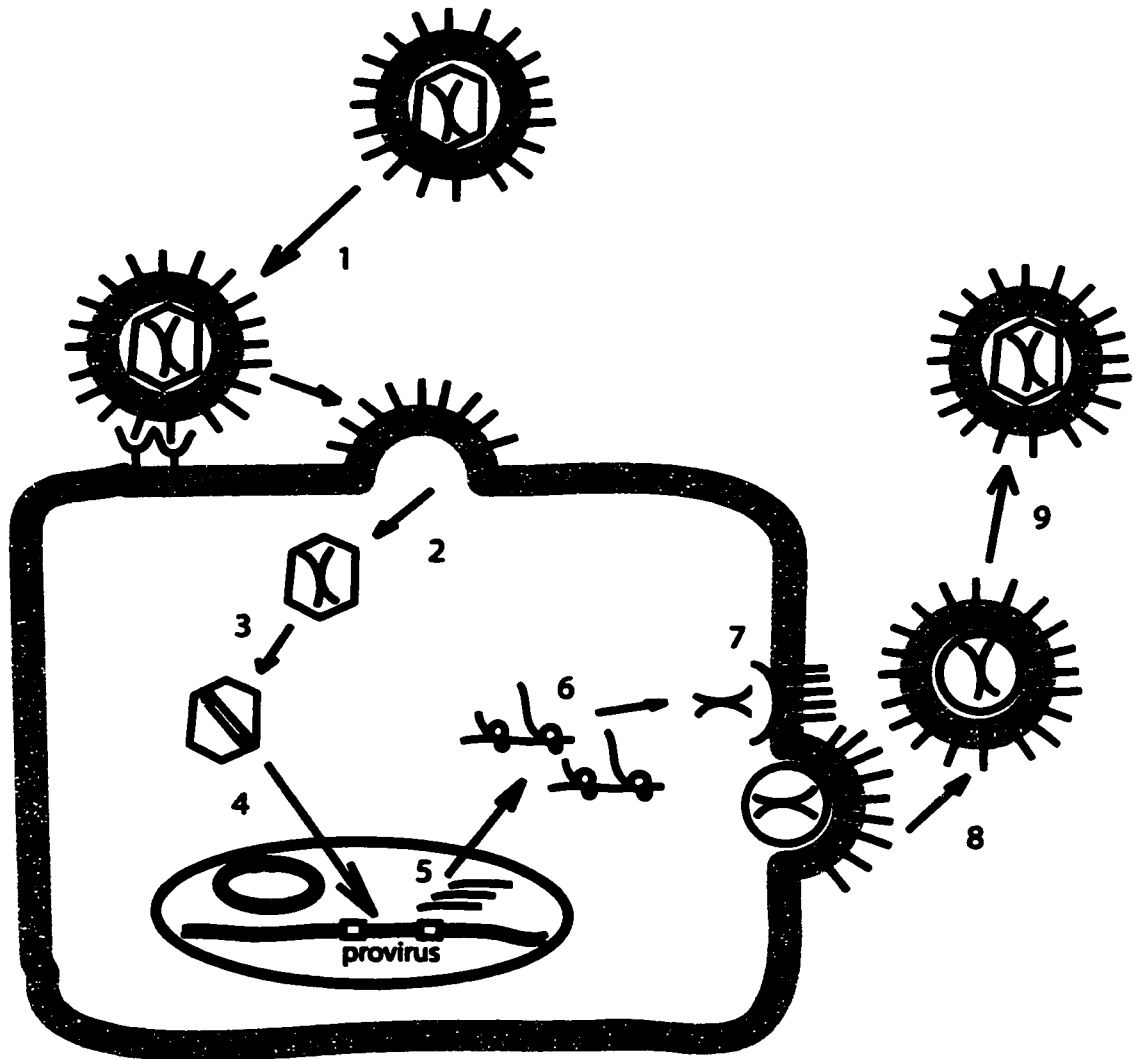
The carboxy terminal cleavage product of pol is the integrase protein. It is the smaller of the two pol proteins and it contains conserved DNA binding activity within zinc finger like domains. It is responsible for the integration of the proviral DNA into the target cell genome. This is accomplished by its two enzymatic functions. A tetramer of integrase proteins recognizes the 3' ends of the provirus where it catalyzes the removal of two 3' bases. It then catalyzes the joining of the free proviral 3' OHs to a random host DNA sequence (Brown et al., 1989). This creates an adjacent break in the target DNA that is recognized and filled in by host DNA repair mechanisms.

## **1.6 The retroviral lifecycle**

The retroviral lifecycle (Figure 1.2) begins outside the target cell where the SU domain of the envelope glycoprotein binds to its target receptor (Gray and Roth, 1993; Morgan et al., 1993). Binding triggers a conformational change within the SU domain, which translates into a conformational change within the TM domain. This exposes the previously buried hydrophobic fusion peptide of TM that, due to its close proximity,

inserts itself into the target cell plasma membrane initiating membrane fusion (Gallaher, 1987). The fate of the various structural components as the retroviral core is released into the host cell cytoplasm is not well understood; however, the processes occurring after the uncoating of the core are well studied. In the cytoplasm reverse transcription of the RNA genome generates a double stranded DNA copy whose ends are recognized by the integrase proteins. This complex must make its way into the nucleus for integration. Only lentiviruses have means by which they can actively transport their replication complexes across the nuclear envelope. The replication complexes of all other retroviruses passively enter the nucleus during mitosis. Once inside the nucleus integrase randomly inserts the retroviral DNA into the host cell genome. The provirus recruits cellular transcription machinery through the multiple enhancers and other transcriptional regulators found in its untranslated 5' and 3' ends (Speck and Baltimore, 1987). Two transcripts are produced, a genomic full-length transcript and a smaller transcript encoding the env gene. Env is translated in the rough endoplasmic reticulum where it trimerizes and acquires core glycosylation. With the exception of the spumavirinae env is then transported into the cis-Golgi. Subsequently, env is proteolytically processed into SU and TM, the core glycosylations are trimmed and modified, and the final product is transported to the cell surface. The full-length transcript, on the other hand, is associated with free ribosomes where it produces several different polyproteins. Initially only gag or gag-pro polyproteins are generated. Failure of the translational machinery 5% to 10% of the time leads to the generation of carboxy-terminal gag-pol fusions (Yoshinaka et al., 1985). The gag products make their way to the cell surface, possibly through association with the actin cytoskeleton as small amounts of actin (Ott et al., 1996; Nermut et al.,

**Figure 1.2**



**Figure 1.2    Lifecycle of a retrovirus**

A schematic representation of the retroviral lifecycle. 1, adsorption to the target receptor. 2, penetration into the cell. 3, reverse transcription. 4, integration of the provirus. 5 transcription of retroviral genes. 6, translation. 7, assembly of retroviral proteins and genome. 8, budding. 9, maturation.

1999) and actin binding proteins (Ott et al., 2000) are found in retrovirus particles, and gag has also been demonstrated to associate with actin (Rey et al., 1996, Liu et al., 1999). On their way to the surface, gag-gag interactions lead to the formation of small lateral associations of the polyprotein. Presumably, the more gag present within a cell, the less is translated as the association of full-length transcript with the nucleoprotein region of gag would compete with the translational machinery. At the cell surface lateral interaction between gag molecules initiates the budding process. This process can occur in the absence of env. In some retroviruses, there are distinct env-gag interactions, which serve to co-localize the two for the budding process (Yu et al., 1992; Lee et al., 1997). In others no such interaction has been demonstrated (Perez et al., 1987; Ragheb and Anderson, 1994) and it is thought that the envelope glycoprotein and gag can independently sort themselves to a common area of the cell membrane allowing the efficient incorporation of env into the budding retrovirus (Barklis et al., 1997; Yeager et al., 1998). Recognition of a subset of gag proteins by ubiquitin ligase proteins through the PY motif found in the late domain facilitates viral particle exit from the cell at which point the retroviral lifecycle can begin again.

As the viral genome is integrated into that of the infected host cell and retroviruses are not lytic viruses by nature, infections are generally life long. The long term nature of retroviral infections ideally suits them for use as therapeutic vectors.

## **1.7 Pseudotyping Retrovirus**

The tropism of the retrovirus is largely determined by its envelope glycoprotein and therefore it is easily modified by replacing the wild type envelope glycoprotein with

that of another enveloped virus. This process is known as pseudotyping. A variation of this process can occur in nature when two different enveloped viruses infect the same cell. If both viruses express their envelope glycoprotein at the cell surface, both will have the opportunity to pick up the other's envelope glycoprotein and thus broaden their tropism. In a laboratory setting pseudotyping is much easier to accomplish. Transfection of a plasmid that encodes a foreign envelope glycoprotein into a retroviral packaging cell line lacking env is sufficient to accomplish the task. The virion produced will have the tropism of the envelope glycoprotein used. This technique has been used to study the envelope glycoproteins of a variety of viruses. It is especially useful in the determination of the cellular receptor specificity for envelope glycoprotein from dangerous viruses such as the ebola virus (Wool-Lewis and Bates, 1998). This technique also lends itself quite readily to gene therapy. Pseudotyping is the simplest method of altering the tropism of a retroviral vector. In the search for newer and better retroviral vectors which can infect broader host ranges many groups have pseudotyped the Moloney murine leukemia virus (MoMLV) with a wide array of envelope glycoproteins. MoMLV is one of the most commonly used gene therapy vectors and gene transduction tools in current use. Its pseudotypes include viruses with the envelope glycoproteins from the Rous sarcoma virus (Landau and Littman, 1992), the ebola virus (Wool-Lewis and Bates, 1998), the human foamy virus (Lindemann et al., 1997), the fowl plague virus (Hatzioannou et al., 1998), a truncated form of the type 1 human immunodeficiency virus (Schnierle et al., 1997), the Ross River virus (Sharkey et al., 2001), and perhaps the most successful, the vesicular stomatitis virus (VSV) (Burns et al., 1993).

## **1.8 The Moloney murine leukemia virus and gene therapy**

The Moloney murine leukemia virus is ideally suited as a gene therapy vector for a number of reasons. For one, it is a type C retrovirus. While this classification refers to its particle morphology, type C retroviruses are amongst the most simple of their kind. Having the bare minimum complement of genes required for its function, it has only a small potential to generate harmful side effects relating to its use as a therapeutic. As with all retroviruses the question of where their genome, or in this case the therapeutic gene, is inserted in the host DNA is of some consequence. While the majority of insertions are harmless, insertion in or near proto-oncogenes could lead to tumour formation (Kung et al., 1991). Using a replication incompetent version of the retrovirus reduces this risk (Deisseroth et al., 1994).

As MoMLV is so simple, it is easily modified. For the purpose of developing *in vivo* gene therapy vectors, many groups have altered the tropism of this virus, as mentioned above, by pseudotyping with the envelope glycoproteins of other viruses. While this does impart new tropism, these tropisms are still generally too broad to be considered as an *in vivo* vector. To further refine the tropism of the virus, the retroviral envelope glycoprotein has been fused with a variety of distinct and specific binding domains. The titers obtained by a MoMLV modified with an anti-human MHC class I single chain variable domain antibody fragment (scFv) were very low (Marin et al., 1996). Single chain Fvs are the minimal binding unit of an antibody. Cosset et al. (1995) were equally unsuccessful with EGF as a binding domain. Somia et al. (1995) had more success with a scFv directed toward human low density lipoprotein receptor, which when fused to the ecotropic envelope glycoprotein generated virus particles able to infect

human cells with almost the same efficiency as wild type virus is able to infect murine cells. Similarly, Han et al. (1995) generated MoMLV with heregulin incorporated into the envelope glycoprotein, which could infect human tumour cells overexpressing the ERBB2 and ERBB4 proteins. Liu et al. (2000) were also successful in generating MoMLV that could infect human epithelial cells through the co-expression of env along with a targeting protein consisting of an env in which the SU domain had been replaced by a small targeting peptide motif.

Cosset et al. (1995) rationalize why a modified ecotropic envelope glycoprotein (Eenv), which is normally specific for the mouse cationic amino acid transporter 1 (mCAT1), cannot infect the target cells if they do not express mCAT1. The trigger for the fusion event of Eenv is the actual binding event between env and its cognate receptor, to replace that binding event is to bypass the fusion trigger. Lorimer and Lavictoire (2000) also support this notion with their scFv-env fusion, which could infect murine cells normally, could bind target human cells, but could not infect them. The fact remains that none of these strategies have succeeded in reaching clinical trials and as such leaves the area of altered tropism open to further investigation.

## **1.9 Area of investigation**

We were interested in generating a retargeted retroviral gene therapy vector, based on the MoMLV, which would be suitable for use *in vivo*. As altering the tropism this virus' envelope glycoprotein through the use of novel binding domains has met with mixed results, we were interested in using a foreign envelope glycoprotein as a model for retargeting retrovirus particles. Chapter 2 outlines the strategy used to modify influenza

**A virus hemagglutinin with a scFv binding domain for use as a MoMLV targeting and fusion protein. Due to the reduced level of infection of the influenza A hemmagglutinin pseudotyped MoMLV, Chapter 3 examines the role of lipid raft affinity in the MoMLV lifecycle and pseudotyping efficiency.**

## **Chapter 2:**

### **Retargeting the Moloney Murine Leukemia Virus to Cells Expressing the Mutant Epidermal Growth Factor Receptor EGFRvIII**

#### **2.1 Summary**

The Moloney murine leukemia virus (MoMLV) is a commonly used gene therapy vector. To expand the tropism of this retrovirus others have pseudotyped it with the envelope glycoproteins of a variety of other viruses including the vesicular stomatitis virus, the fowl plague virus and the type 1 human immunodeficiency virus. We are interested in modifying the tropism of the MoMLV to specifically target tumour cells for the purposes of cancer gene therapy. The MR1 single chain variable domain antibody fragment (scFv) binds specifically to a mutated epidermal growth factor receptor EGFRvIII. EGFRvIII is expressed in a variety of cancers including breast, ovarian and non-small cell lung cancer, as well as glioblastoma. We have generated an influenza A hemagglutinin (HA) pseudotyped MoMLV which binds to EGFRvIII. Two point mutations, Y98F and L194A, were introduced into the sialic acid binding pocket of A/PR/8/34 HA to remove normal binding. The proteolytic cleavage site, which generates the fusion active HA<sub>1</sub> and HA<sub>2</sub> subunits, was then altered to incorporate the factor Xa cleavage motif. The MR1 scFv was then incorporated at the N-terminus of the factor Xa double binding mutant HA. This construct, termed PR8MR1, was incorporated into MoMLV particles and efficiently directed the binding of these particles to EGFRvIII.

## **2.2 Introduction**

### **2.2.1 EGFRvIII**

The epidermal growth factor receptor family (reviewed in Moghal and Sternberg, 1999) is comprised of 4 members; epidermal growth factor receptor or erbB1, erbB2, erbB3 and erbB4. These type I transmembrane proteins bind to a number of small peptide ligands, which induces the dimerization of these receptor tyrosine kinases. Ligands for EGFR include EGF, transforming growth factor  $\alpha$  and amphiregulin. Betacellulin, epiregulin and heparin-binding EGF-like growth factor bind both EGFR and erbB4, while the neu differentiation factors bind only erbB3 and erbB4. No ligands have been found for erbB2 (Reise and Stern, 1998). Each family member has the ability to dimerize with a specific subset of its relatives depending on which ligand is initially bound (Tzahar et al., 1996; Graus-Porta et al., 1997). Regardless of which pairing occurs, the response to dimerization is the autophosphorylation of tyrosine residues on the cytoplasmic domain of the receptor. This activity further activates the tyrosine kinase activity of the dimerized receptors. Depending on the ligand and the members of the dimer, different autophosphorylation patterns are generated and these lead to the activation of different subsets of effector proteins (Olayioye et al., 1998). The regulated expression and localization of the EGFRs and their effectors in a particular cell type is also thought to affect the exact signals sent by the dimerized receptors (reviewed in Leof, 2000). As the name implies, EGFR signaling can be mitogenic and lead to cell division (Reviewed in Hackel et al., 1999). EGFR signaling can also lead to the upregulation of cell cycle inhibitors and pro-apoptotic proteins (Chin et al., 1996). Aberrant expression of this receptor and its related family members is associated with the formation and propagation

of many different forms of cancer. Overexpression of erbB2 is commonly associated with breast cancer while over expression of erbB3 is a poor prognostic marker for breast, ovarian and oral cancers. Several mutant forms of EGFR with abnormal activity have also been associated with cancer. One of the most common mutation is the deletion of a portion of the extracellular domain of EGFR (Sugawa et al., 1990; Ekstrand et al., 1992; Wong et al., 1992). This mutant receptor, EGFRvIII, is characterized by an 801 base pair in-frame deletion between exons 2 and 7, creating a novel glycine codon spanning the deletion. As a result of the loss of amino acid residues 6 through 273, EGFRvIII has constitutive tyrosine kinase activity that has been implicated with cell transformation (Haley et al., 1989). EGFRvIII, while absent in normal tissue, has been found in several forms of cancer including glioblastoma, breast, ovarian (Moscatello et al., 1995; Wikstrand et al., 1995) and non-small cell lung tumours (Garcia de Palazzo et al., 1993). The unique sequence of this protein and its selective localization makes it an ideal target for tumour specific cancer therapies.

### **2.2.2 MR1 scFv**

Single chain variable domain antibody fragments (scFv) consist of the two variable domains of an antibody, the  $V_H$  and the  $V_L$ , joined together by a flexible linker region. Together they comprise the minimal binding domain of an antibody. The MR1 scFv was selected by phage display to specifically target a peptide sequence of 14 amino acid residues surrounding the deletion in EGFRvIII (Lorimer et al., 1996). MR1 binds specifically to EGFRvIII, and not to wild type EGFR, as its epitope spans the deletion of exons 2 through 7 including the novel glycine residue (Landry et al., 2001). Injection of

$I^{125}$  labeled MR1 has demonstrated that it is preferentially localized to tumor tissue in mouse models (Kuan et al., 1999). When coupled with domains 2 and 3 of Pseudomonas exotoxin MR1 has been shown to be effective in the selective killing of cells and tumours expressing EGFRvIII (Lorimer et al., 1996).

### **2.2.3 Retargeting MoMLV with MR1**

Attempts have been made to retarget the MoMLV specifically to cancerous cells expressing EGFRvIII through the incorporation of MR1 into a surface loop of the ecotropic MoMLV envelope glycoprotein. Resultant retroviral particles were indeed targeted to EGFRvIII expressing cells but could not infect them (Lorimer and Lavictoire, 2000). The lack of infectivity may have been due to an inability of the modified envelope glycoprotein to undergo its fusogenic conformational change as a result of binding between the novel MR1 domain and EGFRvIII. It is thought that the virus-cell membrane fusion event is triggered by binding of the ecotropic envelope glycoprotein to its receptor mCAT1, which in this case was replaced by MR1 binding to EGFRvIII. Alternatively, the envelope protein may have been held too far from the target membrane. MR1 binds to the amino terminal residues of EGFRvIII, which are distal to the target membrane. The normal receptor for the ecotropic envelope glycoprotein, mCAT1, is a 14-transmembrane cationic amino acid transporter. This would place the envelope protein considerably closer to its target membrane. Pseudotyping the MoMLV with another viral envelope protein/MR1 fusion could circumvent these problems.

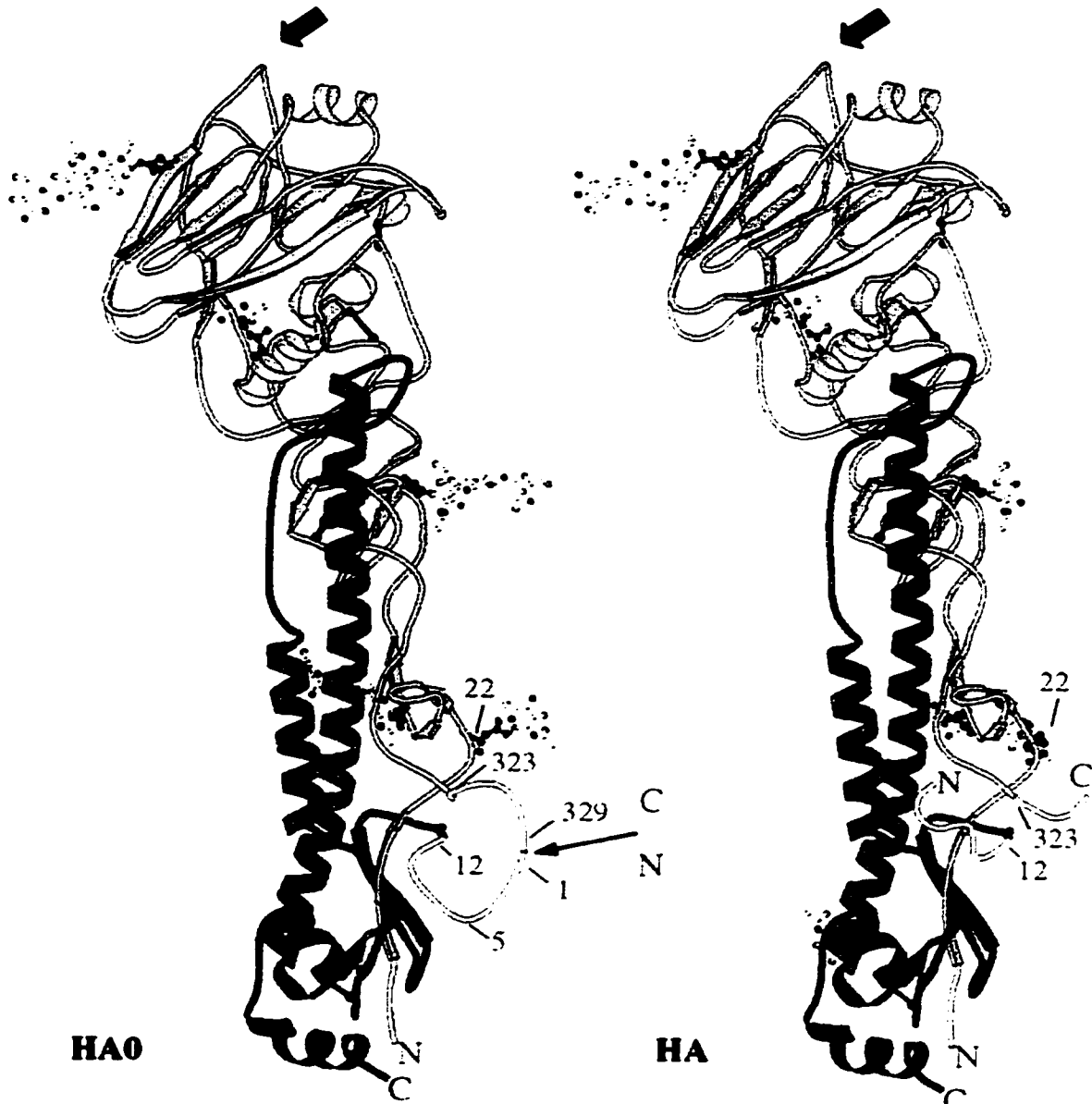
#### **2.2.4 Influenza A hemagglutinin**

Influenza A virus hemagglutinin (HA) is an ideally suited envelope glycoprotein for the purpose of MoMLV pseudotyping for several reasons. Firstly, its protein structure has been extensively studied; crystallography data has revealed the presence of several random coil regions within HA which could serve as potential sites for the insertion of a novel binding domain (Figure 2.1). Secondly, HA's binding specificity has been well characterized. The critical residues responsible for its binding to its natural target to sialic acid are known (Martin et al., 1998). Sialic acid is expressed as a terminal glycosylation on glycoproteins and glycolipids found on the surface of all mammalian cells. Thirdly, HA's fusion mechanism is perhaps the most studied and well understood of any fusion protein. This fusion mechanism is distinct from that of the MoMLV envelope glycoprotein. The HA<sub>0</sub> precursor protein is cleaved into the disulfide linked HA<sub>1</sub> and HA<sub>2</sub> subunits at the cleavage loop (Figure 2.1) by trypsin-like proteases to generate the fusion active form of HA. The activated protein is triggered by a pH-based mechanism that does not require binding to its natural receptor, sialic acid, to mediate membrane fusion (Wharton et al, 1986; Patterson et al., 1999). Previously, we have demonstrated that influenza A/PR/8/34 HA is successfully incorporated into MoMLV particles (Klimowicz and Lorimer, 1998). Others have demonstrated successful incorporation of the related A/Rostock/34 HA (Hatzioannou et al., 1998).

#### **2.2.5 Targeting influenza A HA**

The first attempt to add a scFv binding domain into influenza A HA was only partially successful. Patterson et al. (1999) added a scFv, specific for the haptan

**Figure 2.1**



**Figure 2.1 Structure of HA monomer**

The crystal structure of bromelain digested HA monomer adapted from Chen et al. (1998). The uncleaved HA<sub>0</sub> precursor protein is depicted on the left while the cleaved HA is depicted on the right. The yellow areas indicate the amino acids that are displaced after cleavage. HA<sub>1</sub> is indicated in blue while HA<sub>2</sub> is indicated in red. Note the flexible loop on the surface of HA<sub>1</sub> used by Patterson et al (1999) indicated with an arrow.

nitrophenyl, into a surface loop of the HA<sub>1</sub> subunit of influenza A/Aichi/2/68 HA. This chimeric protein was able to bind to its new target; however, it was unable to mediate membrane fusion. Upon co-expression of wild type HA transfected cells were able to mediate scFv specific fusion; demonstrating again that binding and fusion functions of this protein could be separated. More recently Hatzioannou et al. (1999) have fused a variety of scFvs at the amino terminus of FPV HA. These scFv fusion proteins were successfully expressed in MoMLV particles and could mediate the binding and fusion of these particles to target cells. In both of these cases wild type sialic acid binding of HA was not removed and so these envelope glycoproteins were not truly retargeted, rather their tropism was simply expanded.

#### **2.2.6 Hypothesis**

Effective strategies for targeting the infectivity of retroviral gene therapy vectors would enhance the efficacy and safety of gene therapy but previous strategies have been unsuccessful. The addition of binding domains to the retroviral envelope glycoprotein, while often lending new binding specificity to the retrovirus, does not lead to the production highly infectious particles. Through the use of a foreign envelope glycoprotein with a different fusion mechanism we hope to circumvent the retargeting problems that have been associated with the modification of the wild type retroviral envelope glycoprotein. We hypothesize that we can efficiently retarget the infectivity of a Moloney murine leukemia virus based retroviral vector to cells expressing EGFRvIII by pseudotyping with influenza A virus hemagglutinin that has been engineered to have new binding specificity.

### **2.2.7 Specific objectives**

- a) Alter the proteolytic cleavage site of influenza A HA
  - To protect both retroviral particles and inserted binding domains while proteolytically activating HA
  
- b) Knock out normal influenza A HA binding to sialic acid residues
  - To remove normal influenza A HA tropism
  
- c) Introduce scFv binding domain into influenza A HA
  - To modify the tropism of influenza A HA and pseudotyped retroviral particles

### **2.2.8 Significance**

The development of *in vivo* gene therapy vectors is necessary for this form of treatment to become a viable option in the combat against disease. While others have generated 'targeted' retroviral gene therapy vectors using foreign envelope glycoproteins engineered with various binding domains, these modified envelope glycoproteins maintained wild type binding and are thus not specific enough for use *in vivo*. It is unknown how a retargeted envelope glycoprotein, lacking its wild type binding specificity, would perform. We propose to generate such a retargeted MoMLV based retroviral gene therapy vector. This will be accomplished by first knocking out the wild type binding specificity of the envelope glycoprotein used followed by the addition of a novel scFv binding domain. This design should circumvent the problems known to be

associated with the targeting of the ecotropic MoMLV envelope glycoprotein due to the differences in fusion mechanism. It will also avoid the specificity problems of engineered foreign envelope glycoproteins, which have not had their wild type binding ability removed.

## **2.3 Materials and methods**

### **2.3.1 Cell lines**

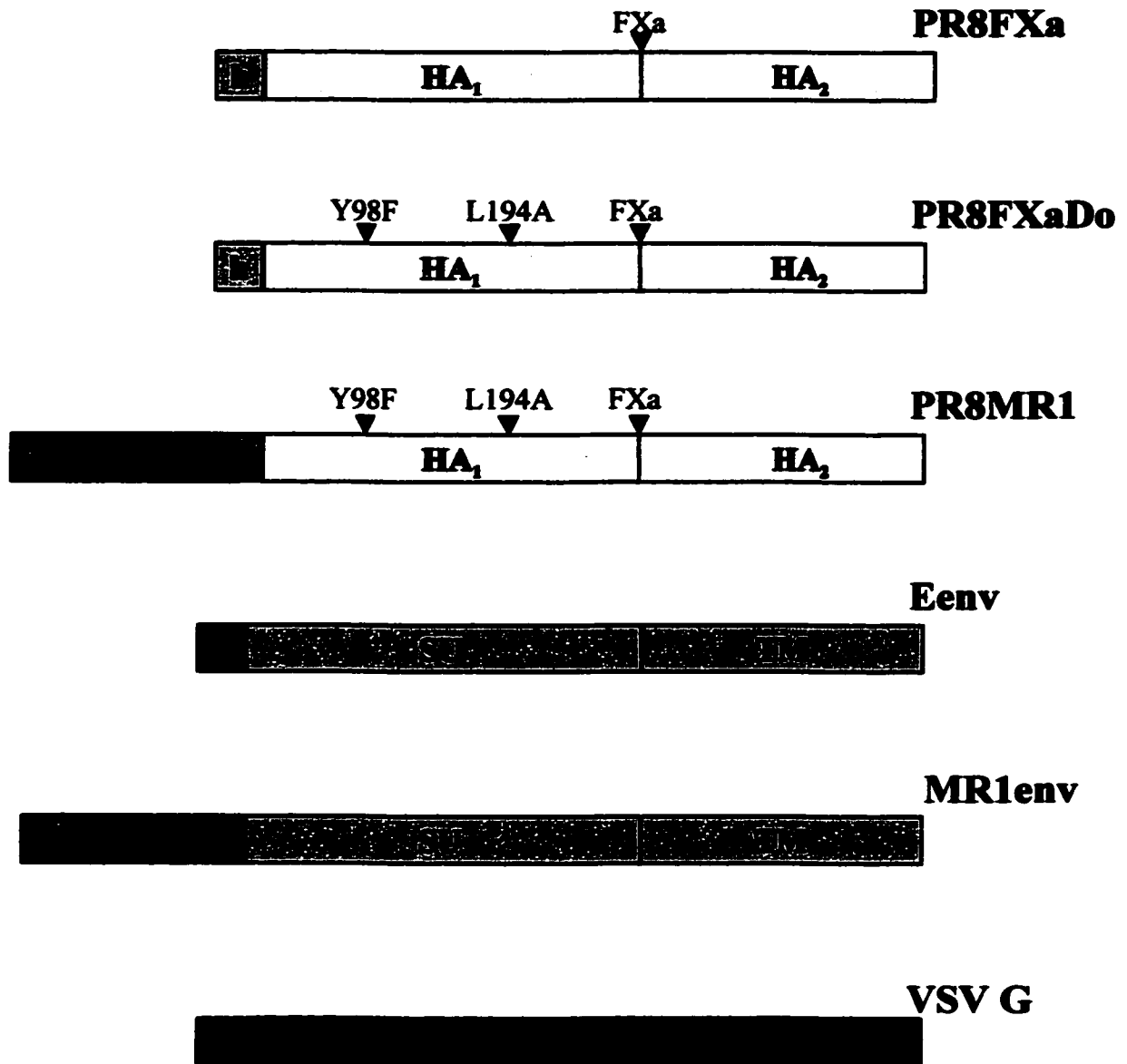
293T human embryonic kidney cells, NIH 3T3 mouse fibroblast cells, U87MG human neuroblastoma cells and the derivative U87MG $\Delta$ EGFRvIII cells were maintained at 37°C and 5% CO<sub>2</sub> in Dubelcco Modified Eagle's Medium with penicillin, streptomycin, glutamine (Gibco/Life) and 10% fetal calf serum (DMEM+). U87MG $\Delta$ EGFRvIII are U87MG cells that have been stably transduced with a retroviral vector that encodes the mutant epidermal growth factor receptor EGFRvIII.

### **2.3.2 Plasmids**

The Moloney murine leukemia virus retroviral plasmids pHIT60, pHIT111 and pHIT123 encoding *gag-pol*,  $\beta$ -gal retroviral vector and ecotropic *env*, respectively, were provided by Dr. Alan Kingsman. Influenza A/PR/8/34 hemagglutinin was obtained from the ATCC and was subcloned into pT7blue3 (Novagen) using BamHI and HindIII. The pT7blue/PR8HA construct was sequenced and subcloned into the pcDNA3.1+ eukaryotic expression vector (Invitrogen) using BamHI and HindIII. A factor Xa site was introduced into pT7blue/PR8HA using the Mutagene phagemid kit version 2.0 (Bio-Rad) and the primer PR8FXa (GGCAATGGCTCCAAATAGACCGCGGCCTTCGATTCCG

GATTGAATGGACGGAGTGTTTCCT). Mutant clones were analyzed for the presence of a SacII site introduced by the mutant oligo and positive clones were sequenced and subcloned into pcDNA3.1+. The Y98F and L194A double mutant, PR8FXaDo, was generated by the sequential mutation of pT7blue/PR8FXa with the HAY98F primer (GTCGATGAAATCTCCCGGGAAACATATTCCATTCTC) and the HAL194A primer (AGCATTTTCATTTTGATACGCGTTCTGTTGTTTCCTTAC). Mutant clones were analyzed for the presence of XmaI and MluI restriction sites respectively, and positive clones were sequenced and subcloned into pcDNA3.1+. To incorporate the MR1 scFv into PR8FXaDo, a 600bp fragment from the 5' end of pT7blue/PR8FXaDo was amplified and modified by 2 successive rounds of PCR to incorporate a NotI-SfiI linker using the primers HAL194A and PR8scFv (CAAGACCTTCCAGGAAATGACAACAGCACAGACACAATATGTATAGGCTACC) followed by HAL194A and PR8scFv2 (AAGCTTACTAGAGCGGCCGCAAGCGGTGGAGGCCAAGACCTTCCAGGAAATGAC). This approximately 600bp HindIII-MluI fragment of PR8FXaDo was cloned into pT7blue3 and sequenced. Clones with the correct sequence were subcloned into pT7blue/PR8FXaDo, in which the pT7blue3 backbone had previously been deleted of its MluI site, with HindIII and MluI. This construct PR8scFv was then subcloned into pcDNA3.1+ in which the NotI site had been deleted by blunt end ligation of a NotI digest. HindII and NotI were used to insert the MoMLV env leader sequence fused to the MR1 scFv from pMR1env(+1-+6) plasmid described elsewhere (Lorimer and Lavictoire, 2000) into pcDNA/PR8scFv generating PR8MR1. To generate PR8Tac, paTac containing the Tac scFv, which is specific for the interleukin-2 receptor, was modified by PCR to incorporate a SfiI site at its 5' end and a NotI site at its 3' end using the primers

**Figure 2.2**



**Figure 2.2 Schematic diagram of the PR8HA constructs**

Schematic representations of the envelope glycoprotein constructs used. Leader sequences are indicated at the far left of each diagram, **h** indicating the HA leader sequence, **e** indicating the Eenv leader sequence and **g** indicating the VSV G leader sequence. The subunits of each envelope glycoprotein are labeled: **HA<sub>1</sub>**, and the analogous **SU** or surface subunit as well as **HA<sub>2</sub>**, and the analogous **TM** or transmembrane subunit. The MR1 scFV is also identified along with the 7 amino terminal residues of influenza A/Hong Kong/2/68 (\*). Arrows specify sites that were mutated.

TacSfi (GCGGCCAGCCGGCCATGGCCCAGGTCCATCTGCAGCAGTCTGG) and TacNot (AGGTGCGGCCGCTTTGAGCTCCAGCTTGGTCC). This PCR product was blunt end cloned into pT7blue3 from which it was used to replace the MR1scFv in pcDNA/PR8MR1 using a SfiI/NotI digest. Figure 2.2 depicts the envelope glycoprotein constructs used.

### **2.3.3 Retrovirus Pseudotyping**

The three plasmid transfection system of Soneoka et al. (1995) was used to generate replication incompetent retrovirus that express beta-galactosidase. Briefly, 3.5µg of each retroviral plasmid, pHIT60, pHIT111 and pHIT123 was transfected into 60mm dishes of 293T cells using the calcium phosphate method. The pHIT123 plasmid was substituted for by pcDNA/PR8FXa (and mutants) to generate the corresponding MoMLV pseudotypes where indicated. 17 to 20 hours post transfection cells were washed once in PBS and changed into 2ml of Opti-Mem serum free medium (GIBCO). After another 24 hours of incubation another 0.5ml of Opti-Mem was added to the transfections. After a further 24-hour incubation retrovirus was harvested in 5ml syringes and filtered through a 0.45µM syringe filter (Nalgene). To generate fusion active PR8HA pseudotypes, 2µg/ml of factor Xa was also added to the initial 2ml of Opti-Mem medium. After 24 hours incubation another 0.5ml of Opti-Mem containing factor Xa, to make a final concentration of 4µg/ml, was added. Retrovirus that was not used immediately was stored at -80°C.

Concentration of retrovirus was done by ultracentrifugation. 10ml of filtered viral supernatant was diluted with PBS to fill a 14 x 89mm polyallomer ultracentrifuge tube

(Beckman). Samples were spun at 50 000 x g (25 000 rpm) at 4°C using an SW41-Ti rotor. Retroviral pellets were resuspended on ice in 500µl of cold TNE (50mM Tris-HCl pH7.4, 150mM NaCl, 5mM EDTA) buffer for 30 minutes.

#### **2.3.4 Immunoblot analysis**

Cell lysates were prepared from retrovirally transfected cells. After the removal of retroviral supernatant, transfected cells were washed twice in PBS. After the final wash 300 µl of boiling 2x SDS sample buffer (20% SDS, 10% glycerol, 5mM Tris pH 6.8, 0.2µM dithiothreitol) was added and cells were scraped off the plate. For lysates that were trypsin treated, cells dissociated from tissue culture dishes using 5ml of 1mM EDTA/ 1mM EGTA. Cells were pelleted by centrifugation at 1600rpm using a H-1000B swinging bucket rotor (Sorval). Following resuspension in 1ml of PBS, trypsin was added to make a concentration of 2µg/ml. Cells were then incubated at 37°C for 30 minutes. Next, cells were pelleted in a microfuge, the supernatant was removed and 300 µl of boiling 2x SDS sample buffer was added. All lysates were then boiled for 8 minutes, vortexed, cooled briefly and sonicated twice for 10 seconds at 35% relative output on a Fisher Sonic Dismembrator Model 300. Samples were boiled for 5 more minutes, vortexed, and stored at -80°C until use. Retroviral lysates were prepared by adding 4x Laemmli buffer with beta-mercaptoethanol to retroviral supernatants harvested as above. Alternatively, retroviral lysates were generated from concentrated retroviral pellets obtained by the ultracentrifugation of 3ml of retroviral supernatant in clear polycarbonate ultracentrifuge tubes (Beckman) at 25 000rpm and 4°C for one hour using a TLA 100.3 rotor (Beckman). Supernatants were then removed and the retroviral pellets resuspended

in 50 $\mu$ l of 1x Laemmli buffer with beta-mercaptoethanol. All samples were then vortexed, boiled for 5 minutes, vortexed again, and stored at -80°C until use. Either 10 $\mu$ g of cell lysate or 20 $\mu$ l of retroviral lysate was electrophoresed on a 12% SDS-polyacrylamide gel and transferred to Hybond-C nitrocellulose membrane (Amersham Pharmacia). Membranes were stained briefly in 0.025% amido black in 10% acetic acid and 50% methanol to ensure equal protein loading. Membranes were rinsed in ddH<sub>2</sub>O to remove excess amido black stain, photographed, and blocked overnight in 5% skim milk in TBST (10mM Tris pH 7.6, 150mM NaCl, 0.05% Tween-20). Blots were placed in fresh 5% milk in TBST with the primary antibody for 50 minutes. A rabbit anti-PR8 polyserum (donated by Dr. Earl Brown) and a goat anti p30 capsid polyserum (Quality Biotech) were used at dilutions of 1:5000 and 1:1000 respectively. Blots were washed 3 times for 8 minutes in TBST and then incubated in a 1:5000 dilution of secondary goat anti-rabbit HRP or swine anti-goat HRP conjugated antibodies (Cedarlanes) for 30 minutes. Membranes were again washed 3 times for 8 minutes in TBST and proteins were detected using the LumiGLO chemiluminescent substrate kit (Kirkegaard & Perry Laboratories).

### **2.3.5 Fusion assay**

293T cells in 6 well plates were transfected with 2 $\mu$ g of fusion protein (pSV5/VSV-G or pcDNA/PR8HA) using the calcium phosphate method. 17-20 hours post-transfection cells were washed once in PBS and replaced in DMEM+. 24 hours later, cells were changed into Opti-Mem serum free medium, with or without 2 $\mu$ g/ml factor Xa, and incubated for a further 6 hours. The pH of the medium was adjusted to 5.0

using 240µl of 1M sodium citrate and cells were incubate for 2 minutes at 37°C and 5% CO<sub>2</sub>. Cells were replaced in DMEM+ and further incubated for 3 hours. Cells were then fixed in 2.5% glutaraldehyde for 15 minutes at room temperature, washed in 50% ethanol for 1 minute and stained with Giemsa stain for a half-hour. Excess stain was removed with several washed in ddH<sub>2</sub>O, and cells were examined for syncytia formation.

### **2.3.6 Infections**

Retroviruses were titered as previously described (Soneoka et al., 1995). All infections were done with freshly prepared virus. NIH 3T3 cells were incubated with various concentrations of virus in the presence of 8µg/ml polybrene. After 2 hours the virus containing medium was replaced with fresh DMEM+. 48 hours post-infection cells were fixed in 0.2% glutaraldehyde and stained for β-galactosidase activity.

### **2.3.7 Sialic acid and EGFRvIII cell binding assays**

Cells were lifted off culture flask with 1mM EDTA/EGTA. 293T cells were used for the sialic acid binding assay, U87MGΔEGFRvIII and U87MG cells were used for the EGFRvIII binding assay. Suspended cells were then washed 3 times in 5ml of Opti-Mem serum free medium (Gibco/Life) and counted using a hemocytometer. Cells were divided into aliquots of 1x10<sup>6</sup> cells and dispensed into microfuge tubes on ice and the total volume was made up to 800µl with Opti-Mem. 200µl of concentrated filtered virus or 200µl of TNE was then added and gently mixed in. After 1hour on ice, cells were pelleted on a tabletop centrifuge at 4°C and 2000xg. Pellets were washed 3 times in 1% BSA in PBS and the final pellet was lysed in 80µl on 1x Laemmli buffer with beta-

mercaptoethanol. Lysates were prepared for Immunoblot analysis as above and stored at  $-80^{\circ}\text{C}$  until use.

### **2.3.8 EGFRvIII peptide binding assay**

Either 100 $\mu\text{l}$  of concentrated virus was made up to 1ml with DMEM+ or 1ml of unconcentrated virus grown in DMEM+ was aliquoted into microfuge tubes on ice in duplicate. To one of each set of duplicate tubes, biotinylated EGFRvIII peptide was added to a final concentration of 100nM. Both tubes were then left on ice for an hour. These viral solutions were then added to 0.5mg aliquots of Dynabeads M-280 streptavidin coated magnetic beads (Dyna) that had previously been washed 3 times in PBS and blocked in DMEM+ for 1 hour on ice. These solutions were incubated for 15 minutes on ice after which time the beads were pelleted in a magnetic tube rack. Pellets were washed 3 times in 1% BSA in PBS. After the final wash the pellet was lysed in 30 $\mu\text{l}$  of 1x Laemmli buffer with beta-mercaptoethanol. Lysates were prepared for immunoblotting as above. 20 $\mu\text{l}$  of the lysates were loaded for Immunoblotting.

## **2.4 Results**

### **2.4.1 Expression of influenza A/PR/8/34 HA with an altered cleavage site**

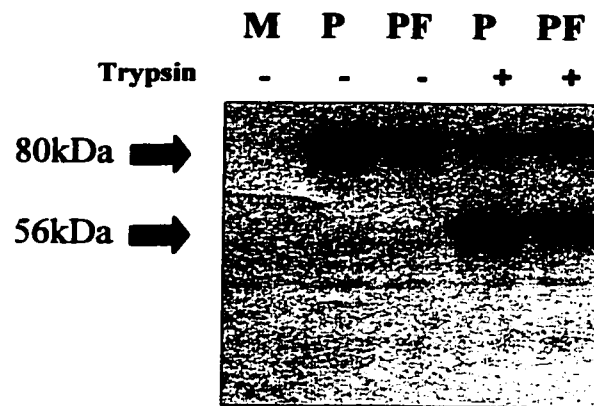
We were interested in pseudotyping the Moloney murine leukemia virus with influenza A PR/8/34 hemagglutinin (PR8HA) for the purpose of generating retargeted retroviral gene therapy vectors. As the final goal was to insert a scFv binding domain at the amino terminus of PR8HA, we needed to generate a version of PR8HA which could be proteolytically activated to its fusion active form without disrupting a novel binding

domain. The specificity of the hemagglutinin cleavage site was modified from its trypsin-like protease specificity, as the non-specific nature of trypsin could degrade the novel binding domain. HA is cleaved naturally by the factor Xa protease in infected chicken embryos through the recognition of a minimal cleavage motif (Gotoh et al., 1990). Factor Xa is normally involved in the proteolytic activation of prothrombin in the blood-clotting cascade and is a much more specific protease than trypsin. Factor Xa recognizes the motif IEGR↓T/I in prothrombin. This motif can be generalized to Q/E-X-R, where X is T, S or G (Winter et al., 1981; Blumberg et al., 1985; Toyoda et al., 1987). While PR8HA already contains a minimal factor Xa cleavage motif at the junction between HA<sub>1</sub> and HA<sub>2</sub>, we were interested in optimizing it to facilitate proteolytic cleavage. We altered the sequence around the tryptic cleavage site of PR8HA from QSR↓GL to QSGIEGR↓GL via insertional mutagenesis. This mutation was designed to assist the proteolysis of PR8HA by factor Xa by mimicking the optimal cleavage motif found in prothrombin, as well as facilitating protease access to the sequence by enlarging the cleavage loop by 4 amino acids. PR8FXa was expressed at similar levels to PR8HA when transfected into 293T cells (Figure 2.3) and both HAs were cleaved equally well by trypsin as demonstrated by the presence of the HA<sub>1</sub> subunit (Figure 2.3). The presence of the HA<sub>1</sub> subunit also indicates that the HA<sub>0</sub> precursor is properly expressed on the cell surface, as it is accessible to the externally applied proteases. Cell surface expression of the mutant was again confirmed by immunoblot analysis of transfected 293T cell lysates treated with either 2µg/ml trypsin or 2µg/ml factor Xa for 30 minutes (Figure 2.4 A). The level of HA<sub>1</sub> on this Immunoblot indicates that while both trypsin and factor Xa cleave PR8FXa, trypsin does so more quickly than factor Xa.

#### **2.4.2 PR8FXa incorporation into retroviral particles**

To generate retrovirus particles we employed the three-plasmid transfection system of Soneoka et al (1995). This system uses 293T cells with plasmids containing both the CMV promoter and the SV40 origin of replication to generate high titers of retrovirus. The three plasmids encode gag-pol, a retroviral vector, and an envelope glycoprotein. By switching the envelope glycoprotein plasmid, one is able to easily pseudotype the retrovirus particles produced. Immunoblot analysis with an anti-PR8 antibody demonstrated the presence of concentrated PR8HA in the supernatants harvested from PR8FXa transfected 293T cells that also received gag-pol and a retroviral vector compared to PR8FXa transfected 293Ts that did not receive the retroviral constructs (Figure 2.4 B). This indicates that PR8FXa was incorporated into retroviral particles. Cleavage of HA<sub>0</sub> into HA<sub>1</sub> in the retroviral supernatants by both trypsin and factor Xa, demonstrates that PR8FXa is properly folded in the retroviral particles.

**Figure 2.3**

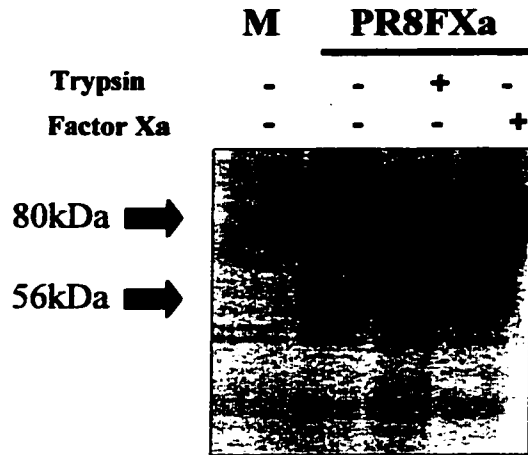


**Figure 2.3 Expression and trypsin cleavage of PR8HA and PR8FXa**

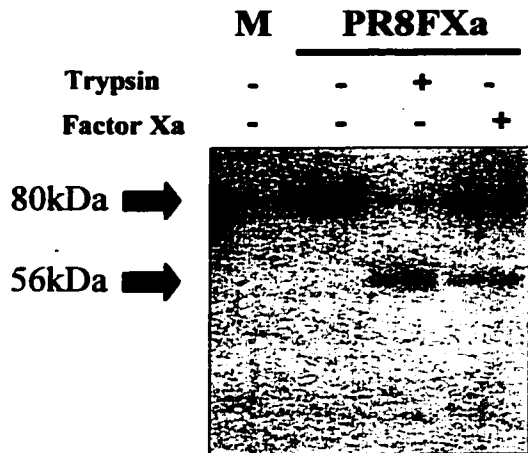
Immunoblot analysis comparing cell expression level and trypsin cleavability of PR8HA and PR8FXa hemagglutinin in 293T cells. Lysates were prepared from transfected cells that were resuspended in PBS containing 2µg/ml trypsin and incubated for 30 minutes at 37°C. No significant differences can be seen between the wild type PR8HA (P) and PR8FXa (PF). Blots were probed with an anti-PR8 polyserum. The control lysate (M) was mock transfected with empty pcDNA3.1+ vector. Trypsin cleavage of the HA<sub>0</sub> precursor (80kDa) generates the HA<sub>1</sub> (56kDa) and HA<sub>2</sub> (not detected by the polyserum) subunits.

**Figure 2.4**

**A**



**B**



**Figure 2.4 Expression and incorporation of PR8FXa**

Immunoblot analysis of PR8FXa hemagglutinin expression in 293T cells and incorporation into MoMLV particles. Blots were probed with an anti-PR8 HA polyserum. **A** Cell surface expression of PR8FXa is demonstrated by the ability of both trypsin and factor Xa to cleave the HA<sub>0</sub> precursor (80kDa) into HA<sub>1</sub> (56kDa) and HA<sub>2</sub> (not detected by the polyserum) subunits. **B** Retroviral incorporation of PR8FXa is demonstrated by the increased concentration of HA in supernatants from 293T cells transfected with retroviral plasmids. Equal volumes of retroviral supernatant was treated with 2µg/ml trypsin, 2µg/ml factor Xa or PBS for 30 minutes to demonstrate functional incorporation by the cleavage of HA<sub>0</sub> into HA<sub>1</sub>.

### **2.4.3 PR8FXa mediates cell-cell membrane fusion**

In order to test the function of the PR8FXa mutant, HA transfected cells were pretreated with factor Xa and then exposed briefly to low pH to trigger the HA fusion conformational change. The VSV G protein was used as a control in this function test as it does not require a cleavage event to activate its low pH fusion capability. After 3 hours extensive syncytia formation was present in both the VSV G control cells and the PR8FXa transfected cells, indicating the ability of the latter to successfully mediate cell-cell fusion (Table 2.1).

### **2.4.4 PR8FXa pseudotyped MoMLV infection**

The final functional test for the PR8FXa mutant was to determine if pseudotyped MoMLV particles could successfully infect target cells. NIH 3T3 cells were exposed to PR8FXa pseudotyped MoMLV particles that were either untreated or treated with factor Xa. Infectivity, determined by X-gal staining of the infected cells, pointed out increased infection efficiency for the virus treated with factor Xa compared to the untreated virus (Table 2.2). This indicates that the MoMLV virus particles were infecting target cells in a PR8FXa dependant manner as proteolytic activation of the fusion protein increases infectivity. It also indicates that PR8FXa could mediate virus-cell membrane fusion. However, infection efficiency for PR8FXa virus was minimal compared to the control infection with wild type ecotropic MoMLV (Table 2.3). While treatment of the PR8FXa virus with factor Xa nearly doubled retroviral titers from 15 Iu/ml to 25 Iu/ml, this was still dramatically lower than the ecotropic MoMLV titer of  $2.5 \times 10^5$  Iu/ml. The successful production of high titer ecotropic and amphotropic retrovirus indicates that the

**Table 2.1**

<b>Envelope Glycoprotein</b>	<b>Fusion</b>
None	-
VSV G	++
PR8FXa	++
PR8FXaDo	+
PR8MR1	-

**Table 2.1 Cell to cell fusion assay**

The relative amount of fusion observed after transfected 293T cells were treated with factor Xa and briefly exposed to acidic (pH = 5.0) conditions. Cells were considered fused if more than 5 nuclei were present in one distinct cell body. (++) Indicates high levels of fusion characterized by syncytia encompassing entire fields of view. (+) Indicates sparse levels of fusion characterized by small syncytia in most fields of view examined, while (-) indicates no syncytia were observed.

**Table 2.2**

<b>Retrovirus</b>	<b>Titer</b> ( <i>ltu/ml</i> )
PR8FXa	$1.5 \times 10^1$
PR8FXa + FXa	$2.5 \times 10^1$

**Table 2.2      Infection of NIH 3T3 cells with PR8FXa pseudotyped MoMLV**

**Titers of PR8FXa pseudotyped MoMLV treated with or without factor Xa. Infected NIH 3T3 cells were fixed and cells staining positive with x-gal were counted. Viral titers are expressed as lacZ transducing units per ml (ltu/ml). Infected cell counts were averaged from 10 fields of view, and corrected for dilution of virus, cell proliferation and magnification. Final titers were averaged from at least two different dilutions from two independent infections and background infectivity of envelope glycoprotein free virus like particles was subtracted.**

**Table 2.3**

<b>Retrovirus</b>	<b>Titer</b> ( <i>itu/ml</i> )		
	<b>NIH 3T3</b>	<b>U87MG</b>	<b>U87MG<sub>Δ</sub></b>
Ecotropic	$5.0 \times 10^5$	nd	nd
Amphotropic	nd	$3.3 \times 10^5$	$1.8 \times 10^6$
VSV G	$5.2 \times 10^4$	nd	nd
PR8FXa + FXa	$5.0 \times 10^1$	$7.2 \times 10^1$	nd
PR8MR1 + FXa	nd	~0	~0

### **Table 2.3      Infection with HA pseudotyped MoMLV**

**Titers of pseudotyped MoMLV infected cells determined by counting cells stained with x-gal and expressed as lacZ transducing units per ml (ltu/ml). Infected cell counts were averaged from 10 fields of view, and corrected for dilution of virus, cell proliferation and magnification. Final titers were averaged from at least to different dilutions from two independent infections and background infectivity of envelope glycoprotein free virus like particles was subtracted. (nd) indicates these infections were not done. Ecotropic and amphotropic retrovirus indicate the successful production of MoMLV using the three plasmid transfection system. The VSV G containing virus indicates the ability of this system to successfully pseudotype retrovirus.**

three plasmid system was working, while the VSV G pseudotype infectivity indicated that this method could also produce high titer MoMLV pseudotypes.

#### **2.4.5 Expression of PR8FXa with a mutated sialic acid binding domain**

The next step to retarget the binding of HA was to remove its wild type binding to sialic acid. Two point mutations, Y98F and L194A (using the numbering of Takemoto et al., 1996), were introduced via site directed mutagenesis into the factor Xa HA mutant. Separately, each of these mutations has been shown to dramatically reduce the binding affinity of influenza HA for sialic acid (Takemoto et al, 1996). Cell surface expression of the double mutant, PR8FXaDo, was verified by Immunoblot analysis of transfected cell lysates treated with either trypsin or factor Xa (Figure 2.5 A). Once again, the presence of HA1 indicates that this mutant is exposed to the exogenous proteases and is thus correctly expressed at the cell surface.

#### **2.4.6 PR8FXaDo incorporation into retroviral particles**

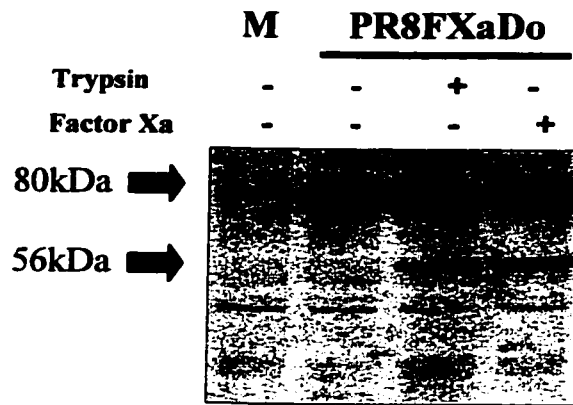
Immunoblot analysis of supernatants from transfected cells reveal that PR8FXaDo is concentrated in these supernatants only in the presence of the retroviral constructs (Figure 2.5 B). Factor Xa cleavage of PR8FXaDo demonstrates a functional incorporation into MoMLV particles.

#### **2.4.7 Reduced cell-cell fusion mediated by PR8FXaDo**

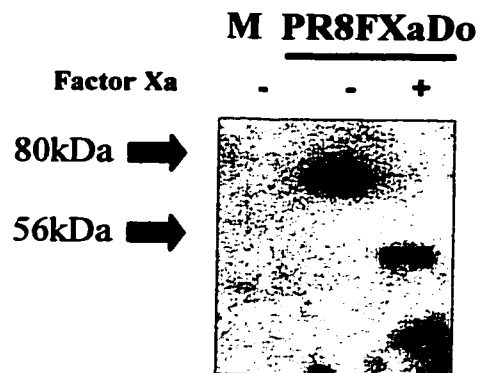
The syncytia-forming assay indicated that the ability of the PR8FXaDo construct to mediate cell-cell fusion was reduced compared to PR8FXa (Table 2.1). We assume

**Figure 2.5**

**A**



**B**



**Figure 2.5 Expression and incorporation of PR8FXaDo**

Immunoblot analysis of PR8FXaDo hemagglutinin expression in 293T cells and incorporation into MoMLV particles. Blots were probed with an anti-PR8 HA polyserum. **A** Cell surface expression of PR8FXa is demonstrated by the ability of both trypsin and factor Xa to cleave the HA<sub>0</sub> precursor (80kDa) into HA<sub>1</sub> (56kDa) and HA<sub>2</sub> (not detected by the polyserum) subunits. **B** Retroviral incorporation of PR8FXaDo is demonstrated by the increased presence of HA in supernatants from 293T cells transfected with retroviral plasmids. 2µg/ml factor Xa or an equivalent volume of PBS was added to retrovirally transfected cells for an overnight incubation at 37°C/ 5% CO<sub>2</sub> to demonstrate functional incorporation by the cleavage of HA<sub>0</sub> into HA<sub>1</sub>. Complete cleavage of HA<sub>0</sub> into HA<sub>1</sub> indicates that the overnight treatment is more effective than a 30 minute treatment.

that this decrease is not a reflection of a decrease in the ability of the double mutant to undergo the fusion conformational change rather it is due to the reduction in binding affinity for neighboring cells.

#### **2.4.8 PR8FXaDo cannot bind sialic acid efficiently**

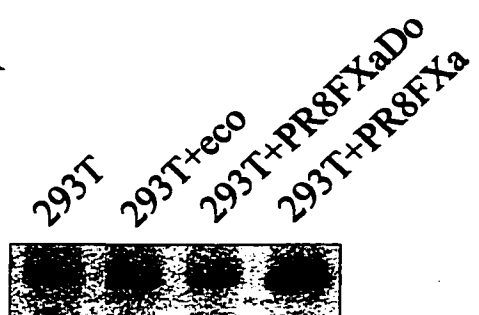
To test the success of the two point mutations that were introduced it was necessary to determine whether they were sufficient to abrogate HA's ability to bind sialic acid. This was examined through the use of a cell-binding assay as all mammalian cells express sialic acid residues as terminal glycosylations on a wide variety of glycolipids and glycoproteins. HA pseudotyped retroviral particles were incubated with 293T cells at 4°C to prevent the endocytosis of bound virus particles. Unbound virus particles were removed by washing and the resultant cell pellets were examined by immunoblot for the presence of the retroviral p30 capsid protein (Figure 2.6 A). Background levels of retrovirus, comparable to the ecotropic MoMLV negative control, were present in the pellet of the PR8FXaDo incubated cells. In contrast there was a strong band at approximately 30kDa indicating a large quantity of PR8FXa virus had bound to the 293T cells. Also indicative the loss of PR8FXaDo sialic acid binding was an increase in concentration, by approximately 10 fold, of PR8FXaDo virus compared to PR8FXa virus in cell supernatants (Figure 2.6 B).

#### **2.4.9 Introduction of scFv binding domains into influenza A HA**

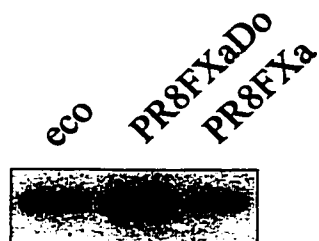
PCR was used to modify the amino terminus of PR8FXaDo to facilitate the introduction of both the MR1 scFv and the  $\alpha$ -Tac scFv into this construct. A flexible

**Figure 2.6**

**A**



**B**

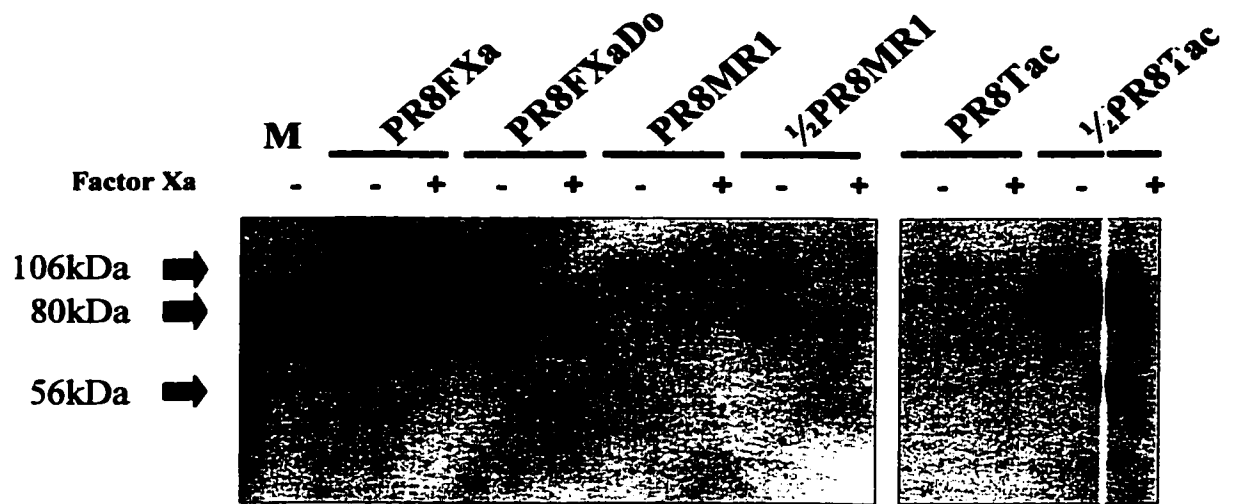


**Figure 2.6 Sialic acid binding assay**

Immunoblot analysis with an anti-p30 capsid protein antibody. **A** Lysates of 293T cells that were incubated in medium containing various pseudotyped retroviral particles, or in fresh medium. Ecotropic MoMLV, which only bind murine cells serve as a negative control. The PR8FXaDo MoMLV particles bind at background ecotropic MoMLV levels, while PR8FXa MoMLV bind strongly, indicating the loss of sialic acid binding ability in the double mutant. **B** The relative concentration of retrovirus produced and used in the assay. Much less PR8FXa MoMLV particles, compared to PR8FXaDo MoMLV particles are present in the cell supernatant as a result of their ability to bind the 293T cells in which they were produced.

spacer composed of three alanine, one serine and three glycine residues as well as the 7 amino terminal amino acids of influenza A/Hong Kong/2/68 HA, was placed between the scFvs and PR8FXaDo. Influenza A/Hong Kong/2/68 HA sequence was used in the spacer to minimize the foreign content at the amino terminus of the final construct since HA has been shown to be sensitive to changes in its amino terminus (Chao, 1992). PR8HA has a truncated amino terminus in comparison to most HA subtypes including A/Hong Kong/2/68 HA. As opposed to using a longer linker of glycines, alanines and serines, the more conservative A/Hong Kong/2/68 HA sequence was added to minimize any adverse affects of the amino terminal alteration. The spacer was used to promote proper folding of both HA and the binding domain as well as to allow sufficient flexibility for the scFv to position itself correctly to bind to its target. MR1 scFv binds EGFRvIII, while the  $\alpha$ -Tac scFv binds to the interleukin-2 receptor. As the  $\alpha$ -Tac scFv is a very stable protein it was used to compare expression levels of scFv modified influenza A HA. Cell surface expression of these mutants was verified by immunoblot analysis of transfected cell lysates treated with either trypsin or factor Xa. Cell surface expression levels were markedly lower than either the factor Xa or double mutant HA as revealed by immunoblot analysis. These data are not shown as the level of expression of the HA<sub>1</sub> cleavage products of the scFv modified PR8FXaDo constructs was difficult to distinguish from background bands. Instead, retroviral incorporation was used as the marker of cell surface expression of these proteins (Figure 2.7). One can clearly discern both HA<sub>0</sub> and HA<sub>1</sub> for PR8MR1 demonstrating proper cell surface expression and retroviral incorporation, however; the lowered expression levels of the scFv mutants were also

**Figure 2.7**



**Figure 2.7 Expression and incorporation of PR8MR1**

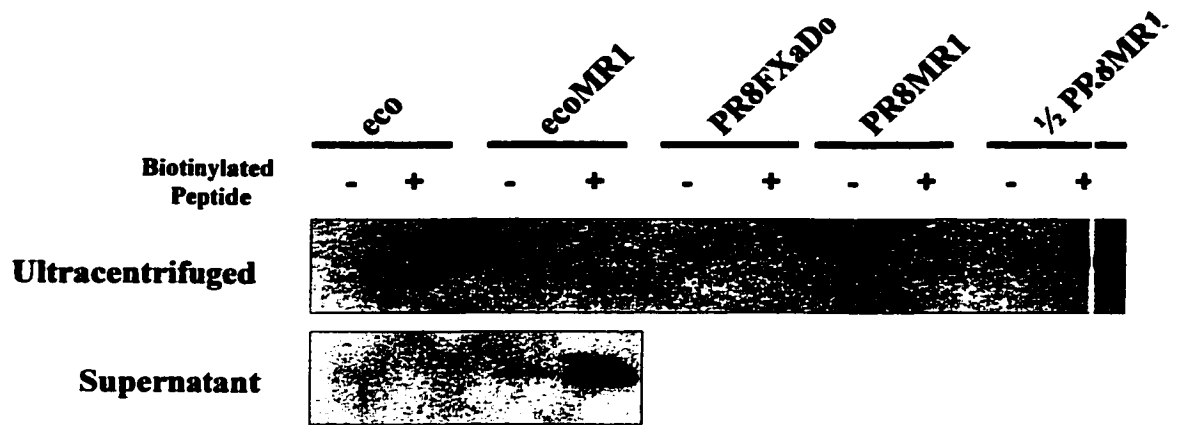
Immunoblot analysis of pseudotyped retrovirus particles concentrated by ultracentrifugation and treated with or without factor Xa. Blots were probed with an anti-PR8 polyserum. PR8MR1 expresses and generates a factor Xa cleavage product approximately 26kDa larger than the parental PR8FXaDo. Cell surface expression is demonstrated by the ability of PR8MR1 to be incorporated into MoMLV particles. When co-expressed with an equal amount of PR8FXaDo (1/2PR8MR1 lanes), expression levels of PR8MR1 are increased. Incubation of virus particles with factor Xa leads to the cleavage of PR8MR1 into its approximately 80kDa HA<sub>1</sub> subunit.

apparent as a reduction of envelope glycoprotein incorporated per virus particle (Figure 2.7). Co-expression of PR8FXaDo with either scFv construct improved the level of incorporation of the scFv construct by at least two fold (Figure 2.7).

#### **2.4.10 PR8MR1 pseudotyped MoMLV particles bind to an EGFRvIII peptide**

The peptide used to generate the MR1 scFv was derived from the 14 amino terminal amino acids of EGFRvIII (Lorimer et al., 1996). PR8MR1 pseudotyped MoMLV was incubated with and without a biotinylated version of the EGFRvIII peptide to determine if MR1 was functionally expressed as a fusion with PR8FXaDo on the surface of these particles. Streptavidin coated magnetic beads were used to pull down peptide bound virus particles. Immunoblot analysis of lysates from the magnetic beads, probed for the p30 capsid protein to identify the presence of retroviral particles, demonstrate the functional expression of MR1 in the hemagglutinin construct (Figure 2.8). Similar to the uncentrifuged ecoMR1 expressing MoMLV particles used as a positive control, there are p30 capsid bands in the lanes that contain both PR8MR1 and biotinylated peptide. Co-expression of PR8FXaDo with PR8MR1 (1/2 PR8MR1 lanes) increases the level of peptide bound retrovirus. Ultracentrifuged ecoMR1 virus was used as a negative control as the force generated by ultracentrifugation shears the disulfide bond linking the SU subunit of the ecotropic envelope glycoprotein to the membrane anchored TM subunit (Burns et al., 1993). The removal of the MR1 binding domain in this way abrogates peptide binding.

**Figure 2.8**



**Figure 2.8 EGFRvIII peptide binding assay**

Immunoblot analysis of streptavidin coated magnetic beads incubated with various pseudotyped retroviruses. Retrovirus samples were preincubated with and without biotin labelled EGFRvIII peptide. Blots were probed with an anti-p30 capsid polyserum to detect bound retroviral particles. Signal in the lanes with MR1 constructs incubated with target peptide indicate that MR1 is functionally expressed in these chimeric proteins. Ultracentrifugation of the PR8MR1 pseudotyped retrovirus has no deleterious effect on binding, while ultracentrifugation of the ecotropic-MR1 retrovirus destroys its ability to bind the EGFRvIII peptide.

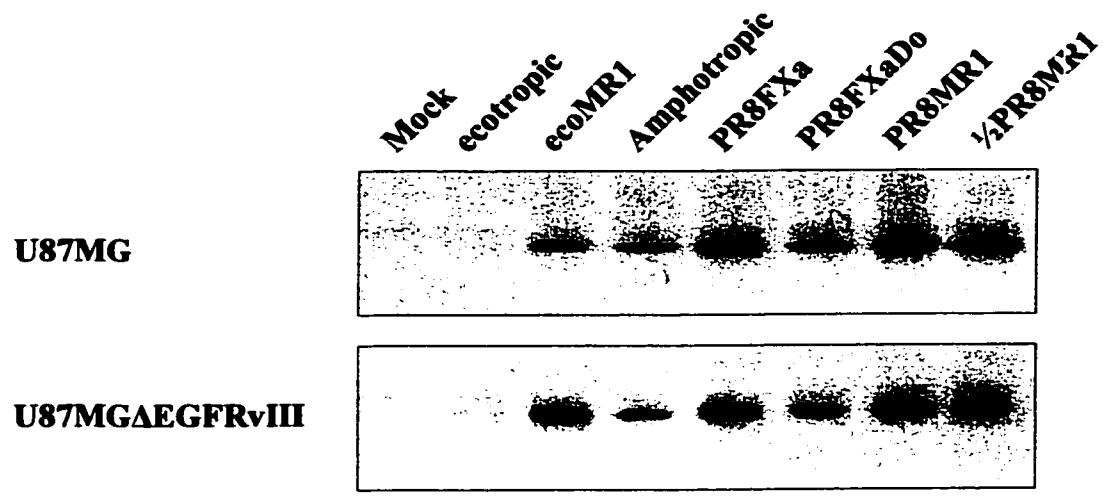
#### **2.4.11 PR8MR1 pseudotyped MoMLV binding to EGFRvIII expressing cells**

To test the binding ability of PR8MR1 in a more relevant context, pseudotyped retroviral particles were used in a cell-binding assay. PR8MR1 virus was incubated at 4°C with either the U87MGΔEGFRvIII cell line, which stably expresses EGFRvIII, or the parental U87MG cell line that does not express this mutant protein. After washing away the unbound virus particles, cell pellets were lysed and examined by immunoblotting for the presence of the p30 capsid protein, indicative of the presence of retrovirus particles. Comparing the lanes containing the MR1 binding domain as opposed to those without it, it is evident that the presence of the scFv leads to non-specific binding to both cell types. Despite the presence of non-specific binding the PR8MR1 virus may still have a slightly increased binding affinity for the EGFRvIII expressing cell line (Figure 2.9, compare U87MG to U87MGΔEGFRvIII). This increased binding is smaller than the levels of binding of the control virus particles containing the ecotropic envelope glycoprotein and the MR1 modified ecotropic envelope glycoprotein (Figure 2.9).

#### **2.4.12 Lack of syncytia formation and infectivity with PR8MR1**

The ability of PR8MR1 to induce cell-cell membrane fusion was tested in PR8MR1 transfected 293T cells. As the PR8FxaDo mutant could induce small pockets of syncytia despite its inability to bind to neighbouring cells, we were interested to see if PR8MR1 could do the same. No syncytia formation was observed leading us to believe that the low level of expression of PR8MR1 was insufficient to mediate non-specific cell-cell fusion. In order to increase the probability of cell-cell fusion PR8MR1 transfected

**Figure 2.9**



**Figure 2.9 EGFRvII cell binding assay**

Immunoblot analysis with an anti-p30 capsid protein antibody of U87MG and U87MGΔEGFRvIII cells incubated with various pseudotyped MoMLV. The first lane was mock treated with DMEM+ without virus while each successive lane was treated with ecotropic, ecoMR1, amphotropic, PR8FXa, PR8FXaDo, PR8MR1 and ½PR8MR1. Ecotropic MoMLV, which only bind murine cells serve as a negative control. EcoMR1 serves as a positive control. Comparison of the two cell lines with respect to this virus demonstrates a dramatic increase in binding in the presence of EGFRvIII. A less dramatic increase can be seen for both PR8MR1 and ½PR8MR1. Note the presence of MR1 seems to increase the non-specific binding of virus particles to cells as indicated by the binding of MR1 expressing MoMLV to U87MG cells.

cells were co-cultured with EGFRvIII transfected cells. Co-culture did not result in the formation of syncytia (Table 2.1). Infectivity data for PR8MR1 pseudotyped MoMLV particles with U87MG and U87MGΔEGFRvIII was also negative (Table 2.3).

## 2.5 Discussion

The Moloney murine leukemia virus (MoMLV) is a commonly used gene therapy vector. To expand the tropism of this retrovirus others have pseudotyped it with the envelope glycoproteins of a variety of other viruses. We were interested in modifying the tropism of the MoMLV to specifically target tumour cells for the purposes of *in vivo* cancer gene therapy.

The present study was designed to examine the feasibility of retargeting MoMLV particles by pseudotyping with an engineered influenza A HA. Others have used engineered HAs with novel binding domains to expand the tropism of pseudotyped MoMLV (Hatzioannou et al., 1999); however, these strategies simply expanded the host range of the virus and did not retarget its tropism and were thus of no real use as an *in vivo* therapy.

The first hurdle in generating a retargeted HA was to ensure the safety of the novel binding domain we intended on using. An optimized factor Xa site, corresponding to the prothrombin cleavage motif IEGR↓T/I, was added to alter the sequence of the normal PR8HA cleavage loop from QSR↓G to QSGIEGR↓G. It was hoped that both the optimization of the factor Xa recognition motif and the expansion of the HA cleavage loop would promote efficient cleavage of this construct, PR8FXa, by factor Xa; the former by improving the affinity of the protease for the HA and the latter by increasing

the accessibility of the cleavage loop to the protease. While the PR8FXa construct was expressed at similar levels on the cell surface as PR8HA, as demonstrated by the ease with which trypsin cleaves both PR8HA and PR8FXa (Figure 2.3), factor Xa did not cleave PR8FXa as efficiently as trypsin did given the same amount of time. As both enzymes cleave HA at the same site, it is unlikely that accessibility to the cleavage site was an issue. Factor Xa has been shown to be a very potent activator of HA (Goto et al., 1990; Ogasawara et al., 1992) and it is possible that the source of factor Xa was impure or had a low level of activation. As factor Xa is the proteolytically active form of factor X and commercial preparations require cleavage via Russell's viper venom in order to obtain full enzymatic function it is possible that an incomplete reaction between the enzymes resulted in sub-optimal activation. To overcome the problems posed by the sub-optimal factor Xa proteolytic activity HA transfected cells were incubated overnight with the enzyme. This modified procedure was more effective and did not have any adverse affect on the 293T producer cells (Compare Figure 2.4 B to Figure 2.5 B).

PR8FXa was successfully expressed on the cell surface, being cleavable by both trypsin and factor Xa (Figure 2.4 A). The PR8FXa mutant was also successfully incorporated into MoMLV particles, being concentrated in cell supernatants in the presence of retroviral components (Figure 2.4 B). Both cell surface expressed and virally incorporated PR8FXa were capable of membrane fusion as demonstrated by its ability to form syncytia in transfected cells and the ability of pseudotyped MoMLV virus particles to infect NIH 3T3 cells. While infection levels were poor, treatment of PR8FXa MoMLV with factor Xa increased the level of infectivity (Table 2.2) indicating that infection was proceeding via PR8FXa mediated membrane fusion. Still, background

levels of infection in virus untreated with factor Xa were high in comparison with factor Xa treated virus. It would be expected that untreated virus would not be able to infect target cells as they lack the ability to fuse the viral membrane with the cellular membrane. Infection of NIH 3T3 cells with envelope glycoprotein negative virus also demonstrates low levels of infection (data not shown) indicating that virus particles can infect target cells in the absence of a membrane fusion protein. The presence of an envelope glycoprotein, in the case of the untreated PR8FXa virus, simply serves as an anchor to increase the binding affinity of virus to target cells thereby increasing the chances of random infection through this fusion protein free mechanism. Alternatively, the infectivity of untreated PR8FXa virus could be attributed to endogenous proteases present in the Golgi apparatus of the retroviral packaging cells or within the early endosome of target cells. These proteases could proteolytically activate, albeit with very poor efficiency, PR8FXa and thus enable pseudotyped retrovirus to infect target cells.

Regardless of the low level of infectivity of PR8FXa pseudotyped MoMLV, we decided to continue modifying HA, thinking that the low infectivity could be solved later through the optimization of infection conditions. To permit the retargeting of PR8FXa, i.e. the removal and replacement of its normal tropism with a newer more specific tropism, two point mutations, Y98F and L194A, were introduced into the binding pocket of PR8FXa to abrogate normal binding to sialic acid. The double mutant PR8FXaDo was successfully expressed on the cell surface, incorporated into MoMLV particles, and was cleavable by factor Xa in both cases (Figures 2.5 A and B). This agrees with Takemoto et al. (1996) who generated influenza virus particles containing each of these mutations separately. Both single mutations severely reduced viral titers by decreasing binding

affinity. To test the success of the mutations, the ability of the PR8FXaDo pseudotyped virus to bind 293T cells was tested. As all mammalian cells express sialic acid on their surface any cell line would suffice; consequently 293T cells were chosen for ease of manipulation. Immunoblots of the cells exposed to the PR8FXaDo virus contained only background levels of retrovirus. This was comparable with the negative control ecotropic MoMLV which should not be able to bind the human 293T cells as it has a specific affinity for the cationic amino acid transporter mCAT-1 found only in murine cells. PR8FXa virus bound quite extensively to the 293T cells. The difference between the PR8FXa and PR8FXaDo binding abilities becomes more significant when the amount of each virus used is taken into consideration. Viral aliquots were not standardized for particle number content before use in the assay and immunoblotting reveals that a considerably greater amount of PR8FXaDo virus was used in this assay than both eco MoMLV and PR8FXa MoMLV. Despite an approximately 10-fold difference in the numbers of virus particles, the PR8FXaDo MoMLV could still not bind to the 293T cells compared to the less concentrated PR8FXa virus. That there were more PR8FXaDo virus particles present in the virus samples than there was PR8FXa MoMLV also confirms the loss of sialic acid binding in the former. Influenza A virus possesses a neuraminidase glycoprotein which is responsible for removing sialic acid residues off of the surface of infected cells (Seto et al., 1966). This permits the efficient release of influenza virus particles budding from the cell surface as their HA cannot bind to the sialic acid stripped glycoproteins and glycolipids present on the infected cell. The MoMLV does not have a neuraminidase function and thus budding virus particles with the PR8FXa envelope glycoprotein can recognize and bind to the myriad sialic acid residues present on the

surface of the producing 293T cells. The higher concentration of retroviral particles in the PR8FXaDo supernatant indicates that these particles are released from the producing 293T cells much more efficiently. This is consistent with a loss in the ability of these virus particles to bind to sialic acid on the cell surface.

The reduction in sialic acid binding helps to explain the reduction in the ability of PR8FXaDo to induce syncytia formation in transfected cells. As PR8FXaDo cannot bind to sialic acid it has no ability to anchor itself to neighboring cells. Once PR8FXaDo has been proteolytically activated and primed for fusion by low pH it has a much lower likelihood of being in the vicinity of a neighboring cell plasma membrane than PR8FXa, which can anchor itself to the sialic acid residues on these cells' surface. The small pockets of syncytia formation were most likely due to the high level of expression of HA in our 293T expression system and the tight packing of the 293T cells in tissue culture. Due to the reduced syncytia formation, no infection assay was performed with the PR8FXaDo pseudotyped virus.

The final step in engineering the retargeted PR8HA was the introduction of the novel binding domain, the MR1 scFv. Several locations were examined for the placement of this domain including both the amino terminus of HA<sub>1</sub>, and a prominent and flexible surface loop in HA<sub>1</sub> encompassing residues 139 to 145. Recent work from Patterson et al. (1999) demonstrated that this loop could be used to express a scFv; however, the fusion mechanism of such a chimera was compromised. This group inserted a scFv in this same loop in influenza A/Aichi/2/68 HA, and found that while it expressed properly and bound to its new target, it was unable to mediate cell-cell membrane fusion. Work by Hatzioannou et al. (1999) was more promising with respect to the amino

terminus of influenza A/Rostock/34 HA (FPV). All of their amino terminal targeting constructs could both bind to the novel target as well as infect cells expressing this target more efficiently than those without. Using an ecotropic-MR1 amino terminal fusion protein (Lorimer and Lavictoire, 2000), we removed the leader sequence and MR1 from this construct to place in PR8FXaDo to create PR8MR1. As cell surface expression of this construct and the PR8Tac control, which incorporated the very stable  $\alpha$ -Tac scFv, was very low in comparison to PR8FXaDo (data not shown), this translated into lower levels of incorporation into MoMLV particles (Figure 2.7). Several factors may have played a role in this reduction of expression. The addition of the bulky amino-terminal targeting domain may have reduced the efficiency with which the nascent HA trimerizes and folds in the ER. Proper trimerization of HA in the ER is thought to be necessary for HA folding and transport from the ER (Copeland et al., 1986). While Hatzioannou et al. (1999) did not experience this reduction of expression, our constructs may react differently due to the use of a different subtype of HA and different scFvs. PR8FXaDo was cotransfected in equal amounts with PR8MR1 (1/2 PR8MR1) to examine the possibility that folding and trimerization were impeding expression. The hope was that the formation of mixed trimers between the smaller PR8FXaDo and the bulky PR8MR1 would act to increase the efficiency of trimerization and folding. In fact, this seems to have been the case as cotransfection resulted in increased levels of cell surface expression of PR8MR1 and PR8Tac (data not shown) as well as a corresponding increase in the levels of their incorporation into MoMLV particles (Figure 2.7).

Another factor, which may have reduced the level of expression of the scFv containing HAs, is the modification of the amino terminus with the addition of the

ecotropic envelope glycoprotein leader sequence. Data suggests that the modification of the HA leader sequence may reduce the stability of HA. Others have also suggested that the modification of the amino terminal residues of the mature HA<sub>1</sub> subunit can also destabilize HA folding (Chao, 1992). While this does not seem to be the case with the related A/Rostock/34 HA, it may be a factor in the low level expression of PR8MR1. While PR8HA does have a high degree of homology with FPV HA, there are many regions with distinct differences, including the amino-terminus, which could account for the difference in behaviour.

Cell-cell fusion assays performed with the PR8MR1 construct as well as with cotransfected PR8FXaDo demonstrated an inability of these fusion proteins to induce syncytia formation in the presence of cells expressing EGFRvIII. Patterson et al. (1999) successfully used cotransfection of a scFv containing HA with the wild type counter part to induce fusion in erythrocyte ghosts expressing the antigen specific for the scFv. Two major differences distinguish these results. They had much higher levels of cell surface expression of their scFv construct. It has been demonstrated that the level of expression of HA directly affects the rate at which fusion pores form. Additionally, the use of erythrocyte ghosts may affect the perception of fusion. The exact mechanics of the HA fusion mechanism are not known and it has been observed that the requirements for successful cell-cell fusion and virus-cell fusion are different. Erythrocyte membrane structure and composition are quite different from those of cultured epithelial cells. These differences, which include membrane fluidity and membrane curvature, may also explain the lack of cell-cell membrane fusion observed using PR8MR1 with PR8FXaDo.

Despite the low level of incorporation of PR8MR1 into MoMLV particles, this was sufficient to direct the binding of these virus particles to both EGFRvIII peptide (Figure 2.8) and EGFRvIII expressing cells (Figure 2.9). PR8MR1 and ecoMR1 binding to EGFRvIII peptide was much cleaner than to U87MG and U87MGΔEGFRvIII cells. Most notably both scFv constructs bound with increased efficiency to the U87MG cells, which do not express EGFRvIII, as opposed to their precursor constructs that do not have the ability to bind this cell type. This indicates some form of non-specific interaction between the scFv and the cells in question. The use of the MR1 scFv as a binding domain in other studies has demonstrated that this scFv is very specific for its target (Lorimer et al., 1996; Kuan et al., 1999). The non-specific binding seen in Figure 2.9 may be due to improperly folded envelope glycoprotein and/or scFv. Consistent with this hypothesis, the poorly expressed PR8MR1 has an increased non-specific interaction compared with ecoMR1.

While PR8MR1 could successfully direct the interaction of pseudotyped MoMLV particles between target peptide and cells, it could not mediate the successful infection of these cells. This inability to infect target cells was attributed to the inability of the low levels of PR8MR1 to efficiently mediate virus-cell fusion, as they were also unable to mediate successful cell-cell fusion. The low levels of infectivity of the wild type PR8FXa MoMLV pseudotypes also played a significant role. Sufficient testing of the PR8MR1 construct will only be possible if the infectivity of PR8FXa pseudotypes can be increased.

## **Chapter 3:**

### **Lipid Disordered Regions of the Plasma Membrane Serve as the Location For Assembly and Budding of Nascent MoMLV Particles**

#### **3.1 Summary**

Several lines of evidence suggest that lipid rafts, membrane microdomains enriched in cholesterol and sphingolipids, serve as assembly and budding points for the influenza A virus. Several influenza virus proteins have been shown to associate with lipid rafts and the disruption of these interactions has been shown to reduce the incorporation of these proteins into viral particles, as well as to decrease the budding efficiency and reduce the titers of the resultant particles. Recently it has been suggested that the type 1 HIV retrovirus and perhaps other retroviruses also use these membrane microdomains for assembly and budding. We investigated the role of lipid rafts in the budding and assembly of the Moloney murine leukemia virus, a type C retrovirus. Neither of the major MoMLV proteins, the envelope glycoprotein or the p65gag structural polyprotein, expressed alone or in combination, associated preferentially with lipid rafts. To further verify that lipid rafts were not being preferentially used as budding sites for nascent MoMLV particles, we generated a series of mutant influenza virus hemagglutinins and evaluated their incorporation into MLV as well as the infectivity of the resultant pseudotyped particles. Mutations were introduced into the cytoplasmic tail of the PR/8/34 HA to remove one or all of the palmitoylation sites in this region. Consistent with previous work, the cytoplasmic tail mutations considerably reduce the association of PR/8/34 HA with raft domains in transfected cells; however, this had

negligible effects on the incorporation of HA into MoMLV particles, again suggesting that MLV budding does not occur from lipid rafts. These results demonstrate that the MoMLV does not discriminate between membrane subcompartments during assembly and budding. This indicates that not all retroviruses use lipid rafts during assembly as has been suggested. It was also found that of the two different methodologies that were used to isolate lipid rafts, sucrose gradient fractionation of lysates provides a clearer picture of lipid raft affinity than does the crude pelleting methodology, which confused detergent insolubility with raft affinity.

## **3.2 Introduction**

### **3.2.1 Lipid rafts**

Lipid rafts are small ordered domains within the plasma membrane enriched in cholesterol and sphingolipids (reviewed in (London and Brown, 2000)). The tight packing of acyl chains in these microdomains allows for strong lipid-lipid interactions, making them insoluble in non-ionic detergents at 4°C (Yu et al., 1973; Brown and Rose, 1992). This allows for their isolation via ultracentrifugation as these detergent insoluble glycolipid enriched complexes can be pelleted out of clarified cell lysates or collected from the low density fractions of sucrose gradients (Brown and Rose, 1992). Lipid rafts have been shown to sequester a variety of integral and peripheral membrane proteins; more specifically, many lipid-modified proteins containing myristoylation, palmitoylation or glycoposphatidylinositol anchors are associated with rafts (Simons and Ikonen, 1997; London and Brown, 2000). The ability to concentrate specific proteins has implicated lipid rafts in membrane trafficking and signal transduction. Caveolin-1

coated lipid rafts, or caveolae, have been shown to mediate an important non-clathrin coated pit mediated endocytic pathway (Schnitzer et al., 1994). A variety of receptor tyrosine kinases and their associated intracellular signaling partners including EGFR, ras and various src family members have been shown to associate with rafts forming signaling hubs (Mineo et al., 1996; Song et al., 1996).

### **3.2.2 Enveloped viruses and lipid rafts**

More recently, it has come to light that many enveloped viruses have also harnessed the selectivity of lipid rafts to concentrate their component proteins so as to promote efficient virus particle assembly. The influenza A virus (Scheiffele et al., 1997; Scheiffele et al., 1999; Zhang et al., 2000) has been shown to require lipid rafts for efficient particle production. Raft association of influenza A virus spike proteins hemagglutinin and neuraminidase, via conserved residues in their respective transmembrane domains and cytoplasmic tails, is necessary for them to be efficiently incorporated into viral particles. Their raft association is essential for the efficient release of viral particles (Jin et al., 1997) and is also thought to regulate their stoichiometry with respect to the viral M2 ion channel, which is generally excluded from rafts (Jin et al., 1997). It has also been suggested that the type 1 human immunodeficiency virus (Rousso et al., 2000; Nguyen and Hildreth, 2000) and the measles virus (Manie et al., 2000; Vincent et al., 2000) require rafts for efficient particle formation. It is thought that gp160 of type 1 HIV associates with rafts via the two palmitoylation sites in its cytoplasmic tail. Removal of both palmitoylation sites reduces its incorporation into viral particles as well as the infectivity of these particles (Rousso et al., 2000). Several cellular raft associated

proteins have also been shown to be preferentially incorporated into type 1 HIV particles (Nguyen and Hildreth, 2000). Much like the influenza A virus, the membrane composition of type 1 HIV particles is enriched in cholesterol and sphingolipids compared to the plasma membrane of infected cells (Aloia et al., 1993).

### **3.2.3 Hypothesis**

The poor level of infectivity determined for influenza A/PR/8/34 HA pseudotyped MoMLV particles was attributed to several variables. The level of incorporation of envelope glycoprotein into virus particles has been previously been demonstrated to play a major role in the ability of enveloped viruses to infect cells (Gunther-Ausborn et al., 2000; Bachrach et al., 2000). The idea that retroviruses are produced from lipid rafts is compromised by the inability of a heavily raft associated protein such as HA to mediate infection of pseudotyped MoMLV. It indicates that this retrovirus may not be incorporating sufficient quantities of HA for infection. We hypothesized that MoMLV particles do not bud from lipid raft rich regions of the cell membrane and thus do not efficiently incorporate proteins enriched in lipid rafts such as influenza A HA. By reducing the affinity of HA for lipid rafts we believed we could improve the incorporation of this protein into MoMLV particles and thus increase their level of infectivity.

### **3.2.4 Specific objectives**

- a) To determine if MoMLV proteins, env and p65gag, are associated with lipid rafts
  - in cell membranes
  - in virus membranes
  
- b) To determine if reducing the affinity of influenza A/PR/8/34 HA (PR8HA) for lipid rafts increases its incorporation into MoMLV particles and subsequently increases the infectivity of these particles.

### **3.2.5 Significance**

Determining whether or not the Moloney murine leukemia virus preferentially assembles and buds from lipid rafts is important for several reasons. This information will help to clarify the relationship between retrovirus family members. Recent evidence supporting the lipid raft association of HIV, suggests that all retroviruses are raft associated. Yet our infectivity data using raft associated HA indicates that the MoMLV does not resemble the lentivirus in this respect. More relevant to gene therapy, these experiments will help determine whether lipid raft affinity of an envelope glycoprotein is sufficient to increase or decrease its incorporation into MoMLV particles. This information could be used to select or engineer more effective retroviral vectors for gene therapy.

### **3.3 Materials and methods**

#### **3.3.1 Cell culture**

293T human embryonic kidney cells and NIH 3T3 mouse fibroblast cells were maintained at 37°C and 5% CO<sub>2</sub> in Dubelcco Modified Eagle's Medium supplemented with penicillin, streptomycin, glutamine (Gibco/Life) and 10% fetal calf serum.

#### **3.3.2 Plasmids**

Plasmids pHIT60, pHIT111, pHIT123, and pcDNA/PR8FXa have been described in Chapter 2 (Materials and Methods). The C6A mutation was generated in pT7blue/PR8FXa using the Mutagene kit described in Chapter 2 (Materials and Methods) with the C6A primer (CTAATCTCAGATGCATATGCGCGCCTGCAAAGATCCATTAGA). Mutant clones were analyzed for the presence of a BssHII restriction site and positive clones were sequenced and subcloned into pcDNA3.1+. The PR8FXa cytoplasmic tail was removed from pT7blue/PR8FXa by PCR using the primers PR8CT1 (CAATCCGGAATCGAAGGCCGCGGTCTA) and PR8CT2 (GGATCCTCAC TAAGACGCCATCCAGAACTGATTGCCCC). The PCR product was blunt end ligated into pT7blue3 using the Perfectly Blunt Cloning Kit (Novagen) and sequenced. The BamHI/SacII fragment was then cut out and used to replace the equivalent piece of pcDNA/PR8FXa to generate pcDNA/PR8CT-. Dr. Brian Lichty kindly donated the pSV5/VSV-G plasmid encoding the VSV fusion protein. A schematic representation of the cytoplasmic tails and transmembrane domains of the envelope glycoproteins used can be seen in Figure 3.1.

**Figure 3.1**

	<b>Transmembrane Domain</b>	<b>Cytoplasmic Tail</b>
<b>Eenv</b>	PWFTTLISTIMGPLIVLLMILLFGPCIL -	NRLVQFVKDRISVQALVLTQQFHQLKPIEYEP
<b>PR8HA</b>	ILAIYSTVASSLVLLVSLGAISFWMCS -	NGSLQCRICI
<b>PR8C6A</b>	ILAIYSTVASSLVLLVSLGAISFWMCS -	NGSLQARICI
<b>PR8CT-</b>	ILAIYSTVASSLVLLVSLGAISFWMAS -	
<b>VSV-G</b>	GWFSSWKSSIASFFFIIIGLIIGLFVL -	RVGIHL <sup>1</sup> CIKLKHTKKRQIYTDIEMNRLGK

**Figure 3.1 Schematic representation of the pseudotyping envelope glycoproteins**

Amino acid sequences of the transmembrane domains and cytoplasmic tails of the envelope glycoproteins used: ecotropic env, PR8HA, PR8C6A, PR8CT- and VSV-G. Residues that are known to be palmitoylated are underlined. Residues that have been mutated are indicated in bold.

### **3.3.3 Retrovirus**

The three plasmid transfection system of Soneoka et al. (1995) was used to generate retrovirus. Briefly, 3.5µg of each retroviral plasmid, pHIT60, pHIT111 and pHIT123, was transfected into 60mm dishes of 293T cells using the calcium phosphate method. The pHIT123 plasmid was substituted by pSV5/VSV-G and pcDNA/PR8FXa (and mutants) to generate the corresponding MoMLV pseudotypes where indicated. 17 to 20 hours post transfection, cells were washed once in PBS and changed into 2ml of Opti-Mem serum free medium (GIBCO) with 5mU/ml *Vibrio cholerae* neuraminidase (Sigma). After 24 hours of incubation 0.5ml of Opti-Mem was added to the transfections. After a further 24 hour incubation, retrovirus was harvested in 5ml syringes and filtered through a 0.45µM syringe filter (Nalgene). To generate fusion active PR8HA pseudotypes, 2µg/ml of factor Xa was also added to the Opti-Mem medium. After 24 hours incubation another 0.5ml of Opti-Mem containing factor Xa, to make a final concentration of 4µg/ml, was added. Retrovirus that were not used immediately were stored at -80°C.

### **3.3.4 Crude lipid raft isolation**

293T cells were transfected using the calcium phosphate method with 10µg of envelope glycoprotein plasmid and either 10µg of empty pcDNA3.1 vector or 10µg of the pHIT60 gag-pro-pol construct. 48 hours after transfection the cells were lysed in 500µl of ice cold TXNE buffer (1% w/v Triton X-100, 50mM Tris-HCl pH7.4, 150mM NaCl, 5mM EDTA), scraped into a syringe, passed through a 27 gauge needle several times, and incubated for 30 minutes on ice. Lysates were then centrifuged for 5 minutes at 4 C

and 6000 x g to remove bulk undissolved cellular material. These supernatants were then transferred into polycarbonate ultracentrifuge tubes and spun in the Beckman TL-100 ultracentrifuge with the TLA-100.3 rotor at 55 000rpm (125 000 x g) at 4°C for 30 minutes. Supernatants were removed and added to an equal volume of 2x SDS sample buffer (20% SDS, 10% glycerol, 5mM Tris pH 6.8, 0.2µM dithiothreitol) while pellets were dissolved in 100µl of TXNE buffer and added to an equal volume of 2x SDS sample buffer. All samples were then boiled for 8 minutes, briefly vortexed, and sonicated twice for 10 seconds at 35% relative output on a Fisher Sonic Dismembrator Model 300. Samples were stored at -80°C until use.

### **3.3.5 Lipid raft sucrose flotation**

Cells were lysed in the same manner as in the crude lipid raft isolation; however, cells were lysed in 1ml of TXNE buffer. After the final 30 minute incubation on ice, supernatants were mixed with 3ml of 65% sucrose in TXNE solution and transferred to a polyallomer ultracentrifuge tube to make a 48% sucrose in TXNE solution. Layered on top of this was 5.5ml of 30% sucrose in TXNE and 2.5ml of 5% sucrose in TXNE. Sucrose gradients were centrifuged at 265 000xg using the SW41Ti rotor (Beckman) at 4°C for 20 hours. Twelve 1ml fractions were carefully removed from the top of the ultracentrifuge tube by pipetting. The pellet was resuspended in 1ml of cold TXNE. All samples were then sonicated briefly and frozen at -80C until use. Immunoblot samples were prepared from 30µl aliquots of each fraction.

### **3.3.6 Immunoblot analysis**

Immunoblots were done as previously described in Chapter 2 (Materials and Methods). Both a mouse monoclonal antibody specific for the VSV G protein (Sigma) and a goat polyserum specific for the SU domain of MoMLV Eenv (Quality Biotech) were also used at dilutions of 1:1000 and 1:2000 respectively. A rabbit anti-mouse HRP conjugated secondary (Sigma) at a 1:5000 dilution was used to detect the mouse mAb.

### **3.3.7 Infection assay**

Retroviruses were titered as previously described in Chapter 2 (Materials and Methods). All infections were done with freshly prepared virus.

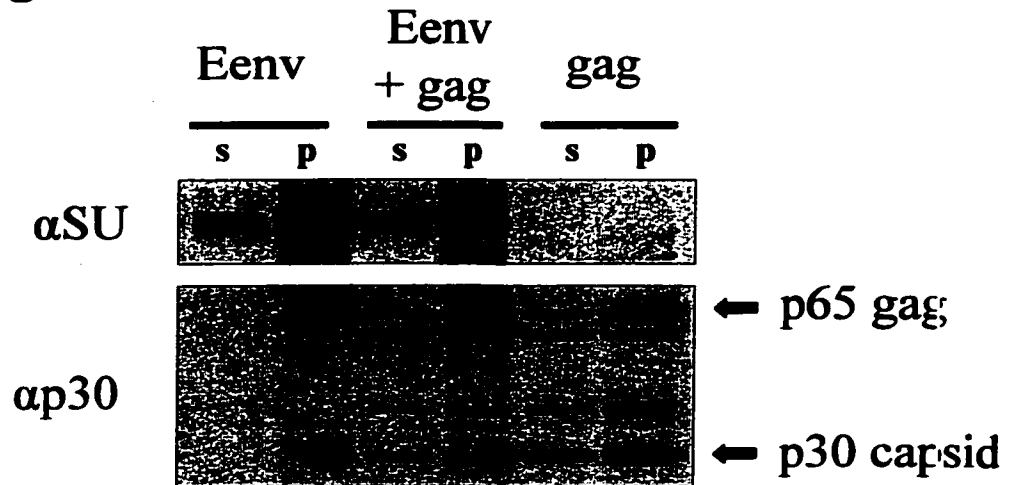
## **3.4 Results**

### **3.4.1 Crude raft isolation of MoMLV proteins is inconclusive**

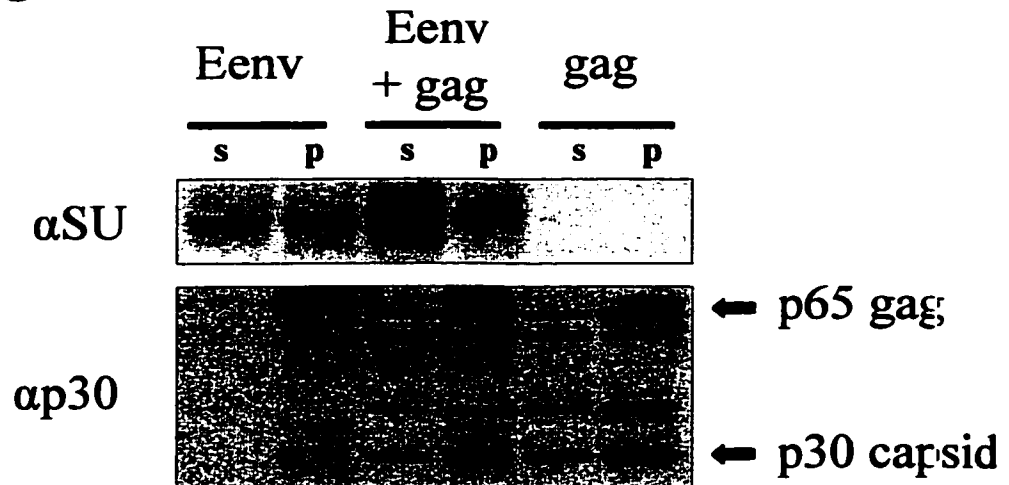
Lipid rafts have been associated with the production of the influenza A virus (Jin et al., 1997; Scheiffele et al., 1997; Scheiffele et al., 1999; Zhang et al., 2000a; Zhang et al., 2000b). Examination of other enveloped viruses indicates that lipid rafts might also play a role in the assembly of measles virus (Manie et al., 2000; Vincent et al., 2000) and the HIV type 1 virus (Nguyen and Hildreth, 2000; Rousso et al., 2000). It had been suggested that all retroviral particles are assembled in detergent insoluble glycolipid complexes (Rousso et al., 2000) after the recent finding that the HIV-1 gp160 is required to be associated with lipid rafts for efficient virus particle assembly. We were interested in determining the role of lipid raft association in the production of Moloney murine leukemia virus.

**Figure 3.2**

**4°C**



**37°C**



**Figure 3.2 Crude raft localization of Eenv and gag in 293T cells**

Cells were transfected with Eenv and/or p65gag and lysed in 1% Triton X-100 at 4°C or 37°C. Soluble (s) and pellet (p) fractions were isolated by ultracentrifugation of clarified lysates. An equal amount of protein from each fraction was loaded on a 10% SDS-PAGE gel and analyzed by immunoblotting. A goat  $\alpha$ SU polyserum was used to detect Eenv (top panel) and a goat  $\alpha$ p30 polyserum was used to detect p30 capsid and p65 gag (bottom panel).

A crude raft isolation technique was first used to determine if the ecotropic envelope glycoprotein (Eenv) was indeed associated with lipid rafts. Insoluble rafts were pelleted by ultracentrifugation of clarified lysates. Immunoblot analysis of the pellet and supernatant fractions from Eenv transfected 293T cells lysed in cold 1% triton X-100, indicated that Eenv was almost exclusively localized to the pellet fraction (Figure 3.2) as compared the vesicular stomatitis virus G protein (VSV-G), which localized almost exclusively to the soluble membrane fraction (Figure 3.3). VSV-G has been shown to be excluded from lipid rafts and can be used as a marker of detergent soluble membrane fractions (Scheiffele et al., 1999). The presence of p65gag in the transfected cells did not alter the detergent solubility of Eenv (Figure 3.2). The p65gag polyprotein was also localized primarily to the pellet fraction compared to one of its cleavage products the p30 capsid protein, which was more evenly distributed (Figure 3.2). While association with the pellet is consistent with lipid raft association, when the same experiment is performed at 37°C, the same result is obtained; the majority of the retroviral proteins are still localized to the pellet fraction. Lipid rafts are solubilized in triton X-100 at 37°C (Brown and London, 2000). This indicates that something other than lipid raft affinity is associating these proteins with the detergent insoluble pellet.

#### **3.4.2 Crude raft isolation of influenza A/PR/8/34 hemagglutinin and mutants**

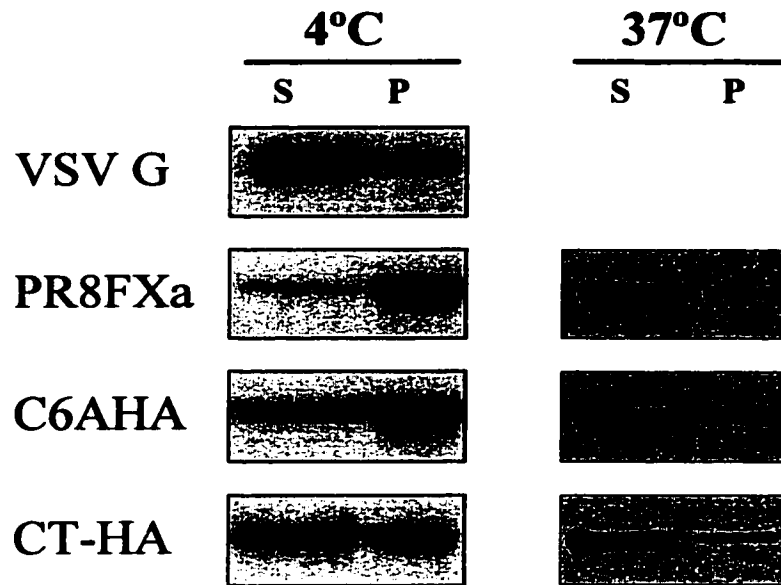
Lipid raft association of a panel of PR8HA mutants was examined to determine if it a reduction of lipid raft affinity could alter incorporation into MoMLV particles. Influenza A hemagglutinin is one of the envelope glycoproteins of the influenza A virus and has previously been shown to be tightly associated with lipid rafts through both the

sequence of its transmembrane domain (Scheiffele et al., 1997) as well as the palmitoylation of its cytoplasmic tail (Jin et al., 1997; Zhang et al., 2000a; Zhang et al., 2000b). We used a factor Xa optimized mutant of influenza A/PR/8/34 hemagglutinin (PR8FXa) as well as two cytoplasmic tail mutants derived from this protein for our experiments. The two mutants that were generated from PR8FXa, PR8C6A and PR8CT-, are deficient in either one or all of the cytoplasmic tail palmitoylation sites (Figure 3.1). The PR8C6A mutant has a point mutation to remove the middle palmitoylation site in its cytoplasmic tail by changing the cysteine at residue 544 to alanine. PR8CT- is missing its 10 carboxy-terminal amino acids, including two of the three palmitoylation sites, as well as having the remaining palmitoylation site at residue 537 (wild type PR8HA numbering) mutated from cysteine to alanine. Disruption of the palmitoylation sites in the cytoplasmic tail of influenza A HA has previously been shown to reduce the association of HA with lipid rafts (Jin et al., 1997; Zhang et al., 2000a; Zhang et al., 2000b). When assayed for lipid raft association using the crude lipid raft isolation technique, immunoblot analysis of transfected cell lysates revealed that reduction of the number of palmitoylation sites, reduced the relative amount of HA found in the pellet fractions compared with the VSV-G control (Figure 3.3). At 37°C association of the PR8HA mutants with the detergent insoluble pellet was abrogated, also consistent with lipid raft association (Brown and London, 2000).

### **3.4.3 Crude lipid raft isolation from pseudotyped MoMLV particles**

To determine what was happening in the virus particles, we next isolated lipid raft fractions from wild type and pseudotyped MoMLV virus particles using the crude lipid

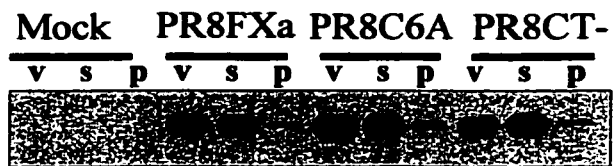
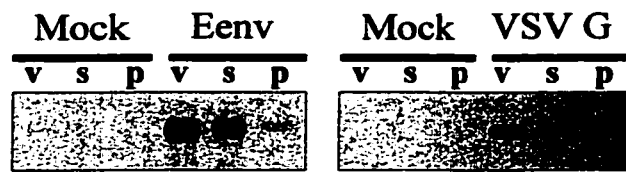
**Figure 3.3**



**Figure 3.3 Crude raft localization of PR8HA mutants in transfected cells**

Cells were transfected with PR8HA, C6AHA, CT-HA or VSV-G (control) and lysed in 1% Triton X-100 at 4°C or 37°C. Soluble (s) and pellet (p) fractions were isolated by ultracentrifugation of clarified lysates. An equal amount of protein from each fraction was loaded on a 10% SDS-PAGE gel and analyzed by immunoblotting. VSV-G was detected using a mouse monoclonal  $\alpha$ VSV-G antibody (upper panel) and HA was detected with a rabbit  $\alpha$ PR8 polyserum (lower panels).

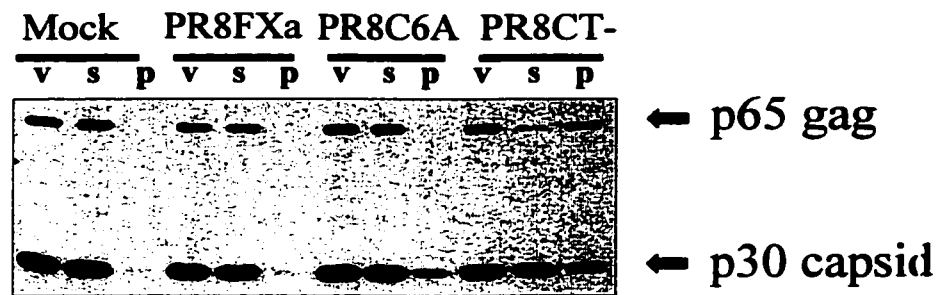
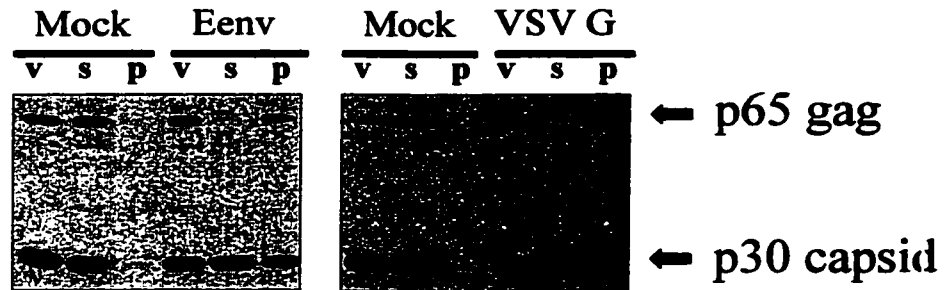
**Figure 3.4**



**Figure 3.4 Crude raft localization of envelope glycoproteins in MoMLV particles**

Retroviral supernatants harvested from transfected 293T cells were concentrated by ultracentrifugation and lysed in 1% Triton X-100 at either 4°C. Soluble (s) and pellet (p) fractions were isolated by ultracentrifugation of clarified lysates (v). The mock lane represents virus like particles that contain no envelope glycoprotein. An equal volume of each fraction was loaded on a 10% SDS-PAGE gel and analyzed by immunoblotting. A goat  $\alpha$ SU polyserum was used to detect Eenv. VSV-G was detected using a mouse monoclonal  $\alpha$ VSV-G antibody and HA was detected with a rabbit  $\alpha$ PR8 polyserum.

**Figure 3.5**



**Figure 3.5 Crude raft localization of p65gag and p30 capsid in MoMLV particles**

Retroviral supernatants harvested from transfected 293T cells were concentrated by ultracentrifugation and lysed in 1% Triton X-100 at 4°C. Soluble (s) and pellet (p) fractions were isolated by ultracentrifugation of clarified lysates (v). The mock lane represents virus like particles that contain no envelope glycoprotein. An equal volume of each fraction was loaded on a 10% SDS-PAGE gel and analyzed by immunoblotting. A goat  $\alpha$ p30 polyserum was used to detect p30 capsid and p65 gag.

raft isolation method. Virus particles were concentrated by ultracentrifugation and resuspended retroviral pellets were lysed in 1% triton X-100 and fractionated by ultracentrifugation. Immunoblot analysis indicated that in MoMLV virus particles all of the envelope glycoproteins, regardless of their lipid raft affinity within the context of the cellular membrane, were located in the detergent soluble fraction (Figure 3.4). Immunoblot analysis also revealed that the majority of the p30 capsid protein is in the detergent soluble fraction while the p65gag polyprotein is found in both the soluble and the pellet fractions (Figure 3.5). The p65gag polyprotein was always localized to the soluble fraction in the envelope glycoprotein negative virus particles (mock); however, with the incorporation of an envelope glycoprotein localization was distributed between soluble and pellet fractions.

#### **3.4.4 Sucrose floatation of lipid rafts demonstrates that the ecotropic envelope glycoprotein is not raft associated**

To further assess the raft affinity of Eenv, lipid rafts were also isolated by sucrose gradient floatation (Schieffele et al., 1999). Due to their insolubility in triton X-100, the buoyant lipid rafts float towards the top of the sucrose gradient, as opposed to being pelleted as in the crude isolation technique. Surprisingly, immunoblot analysis of the sucrose gradient fractions determined that Eenv, like VSV G, was found in the detergent soluble membrane fraction indicating that the protein is not raft associated (Figure 3.6 A compared to Figure 3.7). Again, the presence of p65gag did not noticeably affect the localization of Eenv (Figure 3.6 A). The p65gag polyprotein was primarily localized to the soluble fraction of the sucrose gradient; however, it did have a stronger presence in

the low density fractions than either Eenv or the p30 capsid protein (Figure 3.6 B). Plasma membrane isolation demonstrated that the majority of the p30 capsid protein was membrane associated (data not shown).

#### **3.4.5 Sucrose floatation of influenza A/PR/8/34 hemagglutinin and mutants**

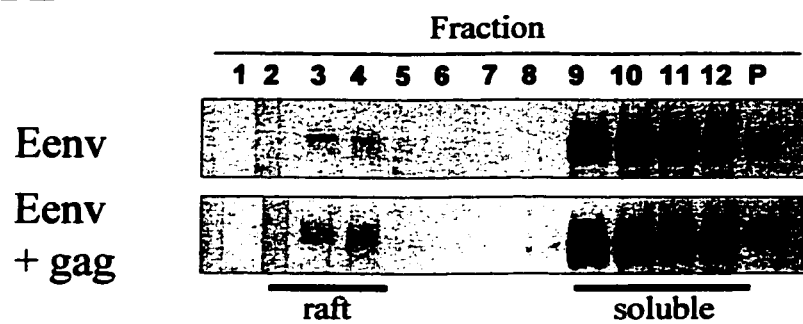
The panel of PR8HA mutants, PR8FXa, PR8C6A and PR8CT- were also assessed using sucrose gradient floatation. Fractions from gradients of transfected 293T cell lysates were analyzed by immunoblot for the localization of PR8HA. All of the PR8HA mutants had a stronger presence in the detergent insoluble raft fractions than the control VSV-G, which is almost absent from the raft fractions (Figure 3.7). Compared with each other PR8FXa, which has the most palmitoylation sites, has the strongest presence in the raft fractions. C6AHA, which is missing one palmitoylation site, has the next strongest presence followed by CT-HA, which has no palmitoylation sites. This is comparable to the results obtained with the crude raft isolation technique (Figure 3.3).

#### **3.4.6 Sucrose floatation of envelope glycoproteins and retroviral structural proteins from MoMLV particles**

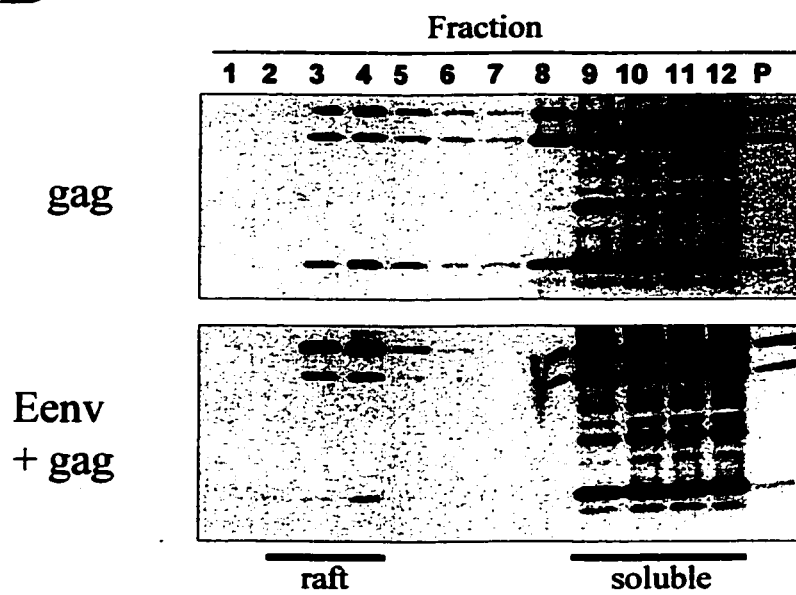
Next, the lipid raft affinity of virion incorporated proteins was assessed using sucrose gradient floatation of pseudotyped MoMLV. As with the crude isolation technique, virus particles were concentrated by ultracentrifugation prior to lysis in 1% triton X-100. Lysates were loaded into sucrose gradients and subjected to ultracentrifugation. Immunoblotting localized Eenv in the detergent soluble fractions along with the VSV-G control (Figure 3.8). These data are consistent with the

**Figure 3.6**

**A**



**B**



**Figure 3.6 Sucrose floatation of MoMLV proteins from transfected cells**

Sucrose gradient lipid raft localization of Eenv and gag in 293T cells. Cells were transfected with Eenv and/or p65gag and lysed in 1% Triton X-100 at 4°C. Clarified lysates were loaded on the bottom of a discontinuous sucrose gradient. After ultracentrifugation, 1ml fractions were removed by pipetting from the top of the gradient and the pellet was resuspended in 1ml of 1%Triton X-100 lysis buffer. Equal volumes of each fraction were loaded on a 10% SDS-PAGE gel and analyzed by immunoblotting. **A** a goat  $\alpha$ SU polyserum was used to detect Eenv and **B** a goat  $\alpha$ p30 polyserum was used to detect p30 capsid and p65 gag. Boyant lipid rafts float into the upper fractions (lanes 2-4) while soluble proteins remain in the lower fractions (lanes 9-12).



**Figure 3.7 Sucrose floatation of envelope glycoproteins from transfected cells**

Sucrose gradient lipid raft localization of PR8HA cytoplasmic tail mutants in 293T cells. Cells were transfected with PR8FXa, C6AHA, CT-HA or VSV-G (control) and lysed in 1% Triton X-100 at 4°C. Clarified lysates were loaded on the bottom of a discontinuous sucrose gradient. After ultracentrifugation, 1ml fractions were removed from the top of the gradient and the pellet was resuspended in 1ml of 1%Triton X-100 lysis buffer. Equal volumes of each fraction were loaded on a 10% SDS-PAGE gel and analyzed by immunoblotting. VSV-G was detected using a mouse monoclonal  $\alpha$ VSV-G antibody (upper panel) and HA was detected with a rabbit  $\alpha$ PR8 polyserum (lower panels). Buoyant lipid rafts float into the upper fractions (lanes 2-4) while soluble proteins remain in the lower fractions (lanes 9-12).

localization of Eenv from cell lysates using the sucrose gradient raft isolation method and supports the idea that the MoMLV is not preferentially associated with lipid rafts during assembly and budding. While the reduction of the number palmitoylation sites in HA reduced the amount of HA found in the low density sucrose fractions from lysates of pseudotyped MoMLV (Figure 3.8), the majority of each of the PR8HAs were found in the detergent soluble fractions. This is also consistent with the idea that the MoMLV is not preferentially associated with lipid rafts during assembly and budding. In addition it proposes that these processes do not discriminate between membrane subcompartments as both detergent soluble and insoluble PR8FXa is found in the viral particles. Immunoblotting for the p65gag and p30 capsid proteins from each of these pseudotyped viral lysates determined that all of the gag and the majority of the capsid protein were concentrated in the detergent soluble fractions (Figure 3.9). There were no significant differences in the localization of the retroviral structural proteins among the different pseudotypes.

#### **3.4.7 Relative incorporation and infectivity of PR8HA pseudotypes**

To determine whether or not the small shift of PR8HA mutants from lipid rafts to detergent soluble virus membranes had any bearing on overall incorporation, we looked at the relative incorporation of HA into pseudotyped MoMLV particles. The level of virus incorporation of the various PR8HAs, Eenv and VSV-G, was accomplished by generating pseudotyped retrovirus particles using the three-plasmid transfection system of Soneoka et al. (1995). Supernatants and cell lysates from the 293T cells cotransfected



**Figure 3.8 Sucrose floatation of envelope glycoproteins from MoMLV particles**

Sucrose gradient lipid raft localization of envelope glycoproteins in MoMLV particles. Retroviral supernatants harvested from transfected 293T cells were concentrated by ultracentrifugation and lysed in 1% Triton X-100 at 4°C. Clarified lysates were loaded on the bottom of a discontinuous sucrose gradient. After ultracentrifugation, 1ml fractions were removed from the top of the gradient and the pellet was resuspended in 1ml of 1%Triton X-100 lysis buffer. Equal volumes of each fraction were loaded on a 10% SDS-PAGE gel and analyzed by immunoblotting. A goat  $\alpha$ SU polyserum was used to detect Eenv. VSV-G was detected using a mouse monoclonal  $\alpha$ VSV-G antibody and HA was detected with a rabbit  $\alpha$ PR8 polyserum. Buoyant lipid rafts float into the upper fractions (lanes 2-4) while soluble proteins remain in the lower fractions (lanes 9-12).



**Figure 3.9 Sucrose floatation of p65gag from MoMLV particles**

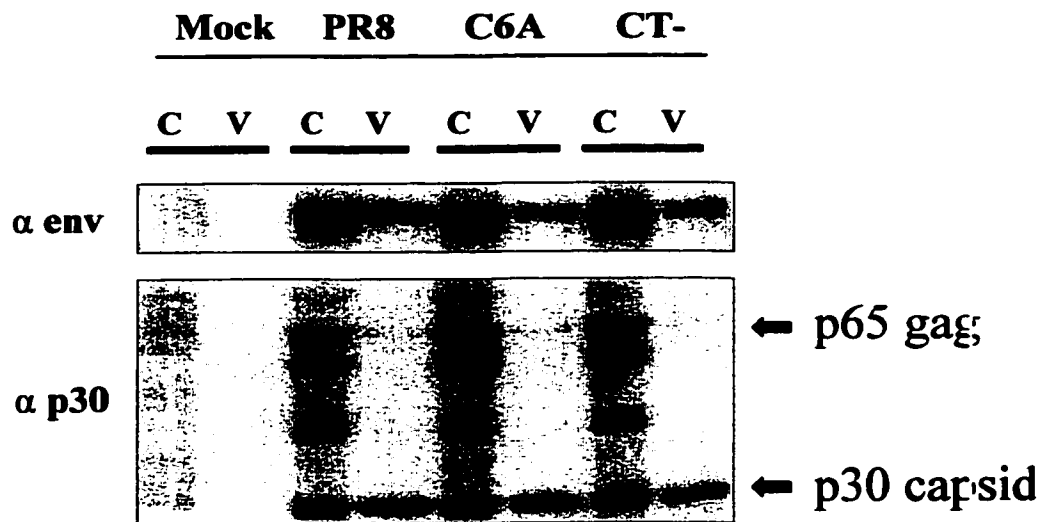
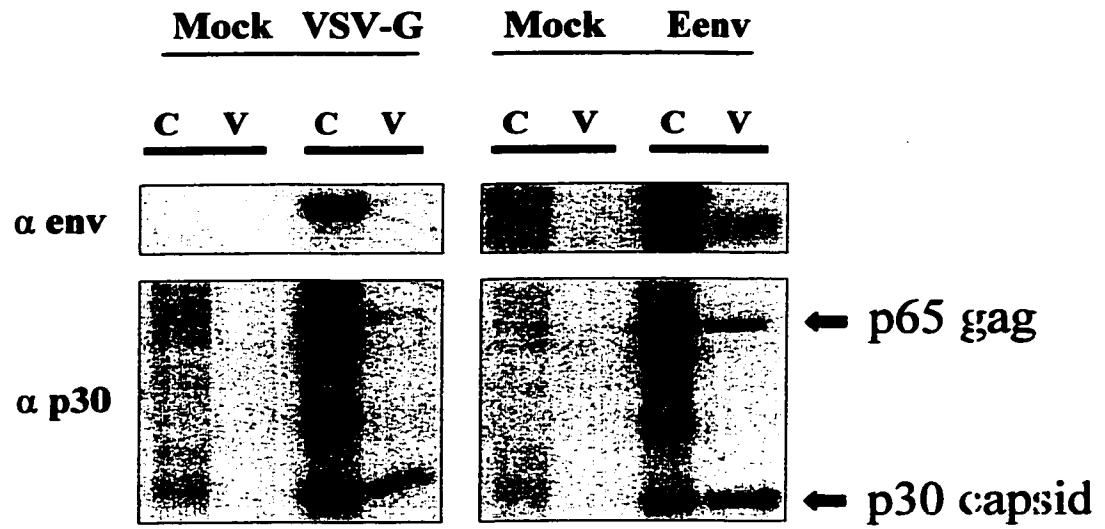
Sucrose gradient lipid raft localization of p65gag and p30 capsid in MoMLV particles. Retroviral supernatants harvested from transfected 293T cells were concentrated by ultracentrifugation and lysed in 1% Triton X-100 at 4°C. Clarified lysates were loaded on the bottom of a discontinuous sucrose gradient. After ultracentrifugation, 1ml fractions were removed from the top of the gradient and the pellet was resuspended in 1ml of 1%Triton X-100 lysis buffer. Equal volumes of each fraction were loaded on a 10% SDS-PAGE gel and analyzed by immunoblotting. A goat  $\alpha$ p30 polyserum was used to detect p30 capsid and p65 gag. Buoyant lipid rafts float into the upper fractions (lanes 2-4) while soluble proteins remain in the lower fractions (lanes 9-12).

with an envelope glycoprotein, gag-pol, and retroviral vector, were analyzed for pseudotyped MoMLV particles by immunoblotting. Analysis of the retroviral supernatants and the cell lysates with antibodies to both the p30 capsid protein of the retrovirus and to the appropriate envelope glycoprotein allowed for the determination of the relative levels at which these proteins were incorporated into particles (Figure 3.10). Comparing blots of the various cell lysates and viral supernatants, it was determined that the relative level of incorporation of PR8HA was lower than that of Eenv. The ratio of envelope glycoprotein to p30 capsid indicated that there was a net concentration of Eenv into MoMLV particles as compared to the producing cells while there was a net dilution of PR8FXa (Figure 3.10). None of the cytoplasmic tail mutations had any effect on the level of incorporation of PR8HA into retroviral particles. The fact that raft association had no bearing on the incorporation of a foreign glycoprotein into the retroviral envelope supports the results of Hammarstedt et al. (2000) who demonstrated that there was no discernable concentration or exclusion of cellular membrane associated proteins into MoMLV particles. The level of incorporation for all of the envelope glycoproteins used exceeded that of VSV-G control, which was very poorly incorporated into retrovirus (Figure 3.10).

#### **3.4.8 Infectivity levels of the PR8HA mutants remain unchanged**

To demonstrate that indeed there were no differences in the functional incorporation of the PR8HA constructs, pseudotyped retrovirus particles were generated containing each one. These viruses were treated with factor Xa to proteolytically activate the hemagglutinin into its fusion active form and then used to infect NIH 3T3 mouse

**Figure 3.10**



**Figure 3.10 Relative incorporation of envelope glycoproteins into MoMLV particles**

Immunoblot demonstrating the relative level of incorporation of envelope glycoproteins into MoMLV particles. Cell lysates (c) and retroviral supernatants (v) were harvested from transfected 293T cells treated with neuraminidase. Membranes were probed with antibodies directed against the appropriate envelope glycoprotein, stripped and reprobed with an  $\alpha$ -p30 capsid antibody. Comparison of the p30 capsid to envelope glycoprotein ratio between the cell lysate and the retroviral supernatant allows for the determination of the ability of the given envelope glycoprotein to incorporate into retroviral particles.

**Table 3.1**

<b>Retrovirus</b>	<b>Titer</b> (I <sub>tu</sub> /ml)
Ecotropic	$2.5 \times 10^5$
VSV G	$5.2 \times 10^4$
PR8FXa	$4.1 \times 10^0$
PR8C6A	$4.7 \times 10^0$
PR8CT-	$3.7 \times 10^0$

**Table 3.1 Infection of NIH 3T3 cells with PR8HA cytoplasmic tail mutants**

**Titers of pseudotyped MoMLV virus particles determined by x-gal staining of infected NIH 3T3 cells. Titers are expressed in Lac Z transducing units per ml (ltu/ml). Background infection from envelope glycoprotein free virus like particles was subtracted from all viral titers. PR8HA pseudotyped MoMLV were all treated with factor Xa prior to infection.**

fibroblasts. Titers from these cells, as determined by beta-galactosidase staining, indicated that there was no significant differences in the level of infectivity of each of the PR8HA viruses (Table 3.1).

### **3.5 Discussion**

Using two different isolation methods, we have analyzed detergent insoluble glycolipid complexes for lipid raft associated proteins in both cell and viral membranes to demonstrate that the ecotropic MoMLV envelope glycoprotein is not associated with lipid rafts. This differs from the localization of proteins from the related type 1 HIV (Nguyen and Hildreth, 2000; Rousso et al., 2000), and indicates that this virus does not preferentially bud from lipid rafts. Reduction of the lipid raft affinity of PR8HA, however, did not affect incorporation of the protein into MoMLV particles.

The crude lipid raft isolation data from transfected cell lysates indicated that both Eenv and p65gag were associated with the lipid raft containing pellets. All of the control envelope glycoproteins indicated that this isolation method was working, as VSV-G was associated with the detergent soluble supernatant (Scheiffele et al., 1999) and the panel of PR8HA mutants became more soluble as the number of palmitoylation sites in their cytoplasmic tails was reduced rafts (Jin et al., 1997; Zhang et al., 2000a; Zhang et al., 2000b). When the same extraction is performed at 37°C, the localization of all of the HA constructs shifted to the detergent soluble supernatant fraction while the MoMLV proteins remained in the insoluble pellets. Since lipid rafts are solubilized in the detergent used at this temperature (London and Brown, 2000), something other than lipid raft affinity was responsible for the localization of Eenv and gag to the pellet. When the

same method was used to extract these envelope glycoproteins from viral membranes, all of them were found in the detergent soluble supernatant. This is indicative of virus particles budding from detergent soluble areas of the membrane. Schieffele et al. (1999) demonstrated that VSV virus particles, which bud from detergent soluble areas of the plasma membrane, could acquire influenza A HA from the surface of transfected cells despite the fact that HA preferentially localizes to lipid raft microdomains. Examination of the detergent solubility of HA incorporated into the VSV particles revealed that, unlike the majority of the HA found in the cellular membrane, all of the incorporated HA was detergent soluble. This demonstrates that the detergent solubility of proteins incorporated into virus particles gives a good indication from what type of membrane the virus particle was produced. The contradictory raft localization data from both crude cell and virus extracts for MoMLV proteins would indicate that while all the retroviral proteins accumulate in lipid raft enriched areas of the plasma membrane, the assembled particles bud from detergent soluble membrane regions. While this is possible, it is unlikely. It is more likely that there is something interfering with the raft localization from cell lysates using this crude detection method.

Confirming this is the sucrose gradient floatation data. All of the retroviral proteins were demonstrated to be in the detergent soluble fractions for both cellular and viral membranes. With this methodology the buoyant insoluble lipid rafts float away from the rest of the cellular material. By using both insolubility and buoyancy to isolate lipid rafts, this assay generates much cleaner results than the crude pelleting method that simply separates detergent insoluble from soluble material (Simons and Ikonen, 1997). Pelleted insolubles contain not only lipid rafts but also protein aggregates, including

elements of the cytoskeleton, which are not removed by the low speed clarifying spin prior to ultracentrifugation. The retroviral gag protein has been shown to associate with cellular actin (Krauslich and Welker, 1996; Rey et al., 1996; Ott, 1997; Goto et al., 1998; Liu et al., 1999) and small amounts of actin have been found in MoMLV particles, suggesting that actin association/transport plays a role in the MoMLV lifecycle (Nermut et al., 1999). Eenv has also been associated with the actin cytoskeleton (Basgall et al., 1986; Soong and Tompkins, 1987). The crude pelleting method isolates lipid rafts based on their insolubility in detergent, which allows them to be pelleted, very similar to some cytoskeleton isolation techniques (Rey et al., 1996; London and Brown, 2000). Thus, detergent insoluble pellets isolated in this manner may also contain parts of the actin cytoskeleton and actin associated proteins including p65gag and possibly Eenv, resulting in the confusion lipid raft association. These data imply that the crude raft isolation technique, while of use for proteins such as HA and VSV G, is not stringent enough to distinguish between association with other detergent insoluble elements, including elements of the cytoskeleton, and true lipid raft affinity. Use of this assay alone to determine the ability of proteins to associate with lipid rafts is not reliable, and particular caution should be used in interpreting results for proteins that are known to be associated with the cytoskeleton.

The panel of influenza A hemagglutinin mutants altered in their ability to associate with lipid rafts were used to further examine of the role of lipid rafts in the incorporation of envelope glycoproteins into MoMLV particles. As has been previously reported by others, the reduction of the number of palmitoylation sites in the cytoplasmic tail of HA reduced its ability to associate with rafts (Jin et al., 1997; Zhang et al., 2000a;

Zhang et al., 2000b). Nevertheless, all of the hemagglutinin mutants were incorporated with similar efficiency into MoMLV particles, even though mutations altered their ability to associate with lipid rafts, and despite the fact that the majority of the wild type ecotropic envelope glycoprotein partitions into the detergent soluble membrane fractions. The bulk of the HA incorporated into MoMLV must be picked up in the detergent soluble membrane regions of the plasma membrane. Looking at the sucrose gradient floatation analysis of HA in MoMLV particles (Figure 3.8), only a small percentage of raft associated HA is incorporated into MoMLV. From this same analysis an even smaller percentage of viral structural proteins are present in the raft fractions. While incorporation of the PR8FXa into this small subset of raft associated particles was much higher than the CT-HA incorporation, this has no overall effect on the relative levels of incorporation of each PR8HA mutant into MoMLV particles. This suggests that unlike the related HIV-1 retrovirus, MoMLV particles do not bud selectively from lipid rafts, rather they bud indiscriminately from the cell surface. Why the VSV G protein, one of the most successful MoMLV pseudotypes, had by far the poorest incorporation of all of the envelope glycoproteins tested despite being localized to detergent soluble membrane fractions remains to be determined. However, it does indicate that only minimal G protein levels are required to mediate efficient virus-cell fusion.

By pseudotyping retroviral particles with influenza A hemagglutinin mutants with altered lipid raft affinities, we have also demonstrated that reducing the affinity for lipid rafts is not sufficient to increase the incorporation of a foreign envelope glycoprotein into MoMLV particles. Coupled with the poor incorporation of VSV G, an envelope glycoprotein that like the MoMLV proteins is not raft associated, this suggests that for

the efficient production of virus particles all of the components of the MoMLV virus must be localized to the same specific area of the detergent soluble plasma membrane. A similar idea was put forward by Barklis et al. (1997) after structural analysis of membrane bound MoMLV gag structures by electron microscopy and by Yeager et al. (1998) after their analysis of MoMLV particles using cryo-electron microscopy. Examination of the lattice formed by lateral association between gag molecules indicated that once these lattices are formed, it would be very difficult for transmembrane proteins to associate with them. Association would have to occur prior to formation and thus both env and gag would have to be localized in the same region of the plasma membrane. Given that both proteins were transported to the same area, no strong specific interactions between them would be necessary. In fact no specific interactions have been proven between Env and gag in MoMLV (Perez et al., 1987; Ragheb and Anderson, 1994a; Ragheb and Anderson, 1994b; Denesvre et al., 1996; Soneoka et al., 1997), and yet Env is enriched in retroviral particles. Widespread expression of both gag and Env at the cell surface could accomplish this same phenomenon. This situation would only exist using an experimental expression system as retroviruses produced in the wild tightly regulate the expression of their proteins. Even if this were the case in our experimental system one would expect that foreign envelope glycoproteins would be incorporated with a higher efficiency than they are.

These data indicate that MoMLV budding is indiscriminate, occurring from regions of membrane from within lipid rafts as well as regions of detergent soluble membrane. Our data indicate that the majority of virus particles originate outside of rafts. The ratio of lipid raft to non-raft virus particles may actually be indicative of the cellular

ratio of detergent insoluble to detergent soluble membrane; however, this ratio is not known. Depending on the cell type, lipid rafts can occupy as much as 40% of the plasma membrane (Simons and Toomre, 2000). If the ratios were in agreement this would agree with Hammarstedt et al. (2000) as they and found no gross differences between the protein content of overall cell membranes compared to isolated MoMLV membranes using SDS-PAGE. Relative incorporation of PR8HAs and VSV-G are reduced in comparison to Eenv in retroviral particles indicating that two separate mechanisms may exist for incorporation, as suggested by Suomalainen and Garoff (1994), one for homologous or retroviral proteins and another less efficient mechanism for heterologous foreign proteins.

## References

- Aloia, R.C., Tian, H., and Jensen, F.C. (1993) Lipid composition and fluidity of the human immunodeficiency virus envelope and host cell plasma membranes. *Proc. Natl. Acad. Sci. U S A* 90: 5181-5185.
- Bachrach, E., Marin, M., Pelegrin, M., Karavanas, G., and Piechaczyk, M. (2000) Efficient cell infection by Moloney murine leukemia virus-derived particles requires minimal amounts of envelope glycoprotein. *J. Virol.* 74: 8480-8486.
- Basgall, E.J., Soong, M.M., and Tompkins, W.A. (1986) Visualization of cytoskeletal elements and associated retroviral antigens by immunogold transmission electron microscopy of detergent extracted cells. *Scan. Electron Microsc.* Basgall EJ: 1419-1425.
- Blumberg, B.M., Giorgi, C., Rose, K., and Kolakofsky, D. (1985) Sequence determination of the Sendai virus fusion protein gene. *J. Gen. Virol.* 66: 317-331.
- Brown, D.A. and Rose, J.K. (1992) Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. *Cell* 68: 533-544.
- Brown, D.A., and London, E. (2000) Structure and function of sphingolipid- and cholesterol-rich membrane rafts. *J. Biol. Chem.* 275: 17221-17224.
- Brown, P.O., Bowerman, B., Varmus, H.E., and Bishop, J.M. (1989) Retroviral integration: structure of the initial covalent product and its precursor, and a role for the viral IN protein. *Proc. Natl. Acad. Sci. U S A* 86: 2525-2529.
- Burns, J.C., Friedman, T., Driever, W., Burrascano, M., and Yee, J.-K. (1993) Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and non-mammalian cells. *Proc. Natl. Acad. Sci. USA* 90: 8033-8037.
- Chao, C.C. (1992) A single amino acid deletion at the amino terminus of influenza virus hemagglutinin causes misfolding and blocks exocytosis of the molecule in mammalian cells. *J. Biol. Chem.* 267: 2142-2148.
- Chen, J., Lee, K.H., Steinhauer, D.A., Stevens, D.J., Skehel, J.J., and Wiley, D.C. (1998) Structure of the hemagglutinin precursor cleavage site, a determinant of influenza pathogenicity and the origin of the labile conformation. *Cell* 1998 95: 409-417.
- Chin, Y.E., Kitagawa, M., Su, W.C., You, Z.H., Iwamoto, Y., and Fu, X.Y. (1996) Cell growth arrest and induction of cyclin-dependent kinase inhibitor p21 WAF1/CIP1 mediated by STAT1. *Science* 272: 719-722.

- Chopra, H.C., Hooks, J.J., Walling, M.J., and Gibbs, C.J. Jr. (1972) Morphology of simian foamy viruses, with particular reference to virus isolated from spontaneous tumor of a rhesus monkey. *J. Natl. Cancer Inst.* 48: 451-463.
- Clarke, J.K., Gay, F.W., and Attridge, J.J. (1969) Replication of simian foamy virus in monkey kidney cells. *J. Virol.* 3: 358-362.
- Coffin, J.M. (1996) *In: Fundamental Virology 3<sup>rd</sup> Edition, Retroviridae: The Viruses and Their Replication, Eds.: B.N. Fields, D.M. Knipe, P.M. Howley, et al. (Lippincott-Raven Publishers, Philadelphia) pp 763-843.*
- Copeland, C.S., Doms, R.W., Bolzau, E.M., Webster, R.G., and Helenius, A. (1986) Assembly of influenza hemagglutinin trimers and its role in intracellular transport. *J Cell Biol* 103: 1179-1191.
- Deisseroth, A.B., Zu, Z., Claxton, D., Hanania, E.G., Fu, S., Ellerson, D., Goldberg, L., Thomas, M., Janicek, K., and Anderson, W.F. (1994) Genetic marking shows that Ph<sup>+</sup> cells present in autologous transplants of chronic myelogenous leukemia (CML) contribute to relapse after autologous bone marrow in CML. *Blood* 83: 3068-3076.
- Denesvre, C., Carrington, C., Corbin, A., Takeuchi, Y., Cosset, F.-L., Schulz, T., Sitbon, M., and Sonigo, P. (1996) TM domain swapping of murine leukemia virus and human T-cell leukemia virus envelopes confers different infectious abilities despite similar incorporation into virions. *J. Virol.* 70: 4380-4386.
- Ekstrand, A.J., Sugawa, N., James, C.D., and Collins, V.P. (1992) Amplified and rearranged epidermal growth factor receptor genes in human glioblastomas reveal deletions of sequences encoding portions of the N- and/or C-terminal tails. *Proc. Natl. Acad. Sci. U S A* 89: 4309-4313.
- Facke, M., Janetzko, A., Shoeman, R.L., and Krausslich, H.-G. (1993) A large deletion in the matrix domain of the human immunodeficiency virus gag gene redirects virus particle assembly from the plasma membrane to the endoplasmic reticulum. *J. Virol.* 67: 4972-4980.
- Gallaher, W.R. (1987) Detection of a fusion peptide sequence in the transmembrane protein of human immunodeficiency virus. *Cell* 50: 327-328.
- Garcia de Palazzo, I.E., Adams, G.P., Sundareshan, P., Wong, A.J., Testa, J.R., Bigner, D.D., and Weiner, L.M. (1993) Expression of mutated epidermal growth factor receptor by non-small cell lung carcinomas. *Cancer Res.* 53: 3217-3220.
- Gotoh, B., Ogasawara, T., Toyoda, T., Inocencio, N.M., Hamaguchi, M., and Nagai, Y. (1990) An endoprotease homologous to the blood clotting factor X as a determinant of viral tropism in chick embryo. *EMBO J.* 9: 4189-4195.

- Goulet, C.C., Volk, K.A., Adams, C.M., Prince, L.S., Stokes, J.B., and Snyder, P.M. (1998) Inhibition of the epithelial Na<sup>+</sup> channel by interaction of Nedd4 with a PY motif deleted in Liddle's syndrome. *J. Biol. Chem.* 273: 30012-30017.
- Graus-Porta, D., Beerli, R.R., Daly, J.M., and Hynes, N.E. (1997) ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. *EMBO J.* 16: 1647-1655.
- Gray, K.D. and Roth, M.J. (1993) Mutational analysis of the envelope gene of Moloney murine leukemia virus. *J. Virol.* 67: 3489-3496.
- Gunther-Ausborn, S., Schoen, P., Bartoldus, I., Wilschut, J., and Stegmann, T. (2000) Role of hemagglutinin surface density in the initial stages of influenza virus fusion: lack of evidence for cooperativity. *J. Virol.* 74: 2714-2720.
- Hackel, P.O., Zwick, E., Prenzel, N., and Ullrich, A. (1999) Epidermal growth factor receptors: critical mediators of multiple receptor pathways. *Curr. Opin. Cell Biol.* 11: 184-189.
- Haley, J.D., Hsuan, J.J., and Waterfield, M.D. (1989) Analysis of mammalian fibroblast transformation by normal and mutated human EGF receptors. *Oncogene* 4: 273-283.
- Han, X., Kasahara, N., and Kan, Y.W. (1995) Ligand-directed retroviral targeting of human breast cancer cells. *Proc. Natl. Acad. Sci. U S A* 21: 9747-51.
- Hatfield, D.L., Levin, J.G., Rein, A., and Oroszlan, S. (1992) Translational suppression in retroviral gene expression. *Adv. Virus Res.* 41: 193-239.
- Hatzioannou, T., Valsesia-Wittmann, S., Russel, S.J., and Cosset, F.-L. (1998) Incorporation of fowl plague virus hemagglutinin into murine leukemia virus particles and analysis of the infectivity of the pseudotyped retroviruses. *J. Virol.* 72: 5313-5317.
- Hatzioannou, T., Delahaye, E., Martin, F., Russel, S.J., and Cosset, F.-L. (1999) Retroviral display of functional binding domains fused to the amino terminus of influenza hemagglutinin. *Hum. Gene Ther.* 10:1533-1544.
- Jin, H., Lesser, G.P., Zhang, J., and Lamb, R.A. (1997) Influenza virus hemagglutinin and neuraminidase cytoplasmic tails control particle shape. *EMBO* 16: 1236-1247.
- Kuan, C.T., Reist, C.J., Foulon, C.F., Lorimer, I.A., Archer, G., Pegram, C.N., Pastan, I., Zalutsky, M.R., and Bigner, D.D. (1999) 125I-labeled anti-epidermal growth factor receptor-vIII single-chain Fv exhibits specific and high-level targeting of glioma xenografts. *Clin. Cancer Res.* 5: 1539-1549.

- Kung, H.J., Boerkoel, C., Carter, T.H. (1991) Retroviral mutagenesis of cellular oncogenes: a review with insights into the mechanisms of insertional activation. *Curr. Top. Microbiol. Immunol.* 171: 1-25.
- Landau, N.R., and Littman, D.R. (1992) Packaging system for rapid production of murine leukemia virus vectors with variable tropism. *J. Virol.* 66: 5110-5113.
- Landry, R.C., Klimowicz, A.C., Lavictoire, S.J., Borisova, S., Kottachchi, D.T., Lorimer, I.A.J., and Evans, S.V. (2001) Antibody recognition of a conformational epitope in a peptide antigen: Fv-peptide complex of an antibody fragment specific for the mutant EGF receptor, EGFRvIII. *J. Mol. Biol.* 308: 883-893.
- Lee, Y.M., Tang, X.B., Cimasky, L.M., Hildreth, J.E., and Yu, X.F. (1997) Mutations in the matrix protein of human immunodeficiency virus type 1 inhibit surface expression and virion incorporation of viral envelope glycoproteins in CD4+ T lymphocytes. *J Virol* 71: 1443-1452.
- Leof, E.B. (2000) Growth factor receptor signalling: location, location, location. *Trends Cell. Biol.* 10: 343-348.
- Lindemann, D., Bock, M., Schweizer, M., and Rethwilm, A. (1997) Efficient pseudotyping of murine leukemia virus particles with chimeric human foamy virus envelope proteins. *J Virol* 71: 4815-20.
- Liu, B., Dai, R., Tian, C.J., Dawson, L., Gorelick, R., and Yu, X.F. (1999) Interaction of the human immunodeficiency virus type 1 nucleocapsid with actin. *J. Virol.* 73: 2901-2908.
- London, E., and Brown, D.A. (2000) Insolubility of lipids in triton X-100: physical origin and relationship to sphingolipid/cholesterol membrane domains (rafts). *Biochim. Biophys. Acta* 1508: 182-195.
- Lorimer, I.A., Keppler-Hafkemeyer, A., Beers, R.A., Pegram, C.N., Bigner, D.D., and Pastan, I. (1996) Recombinant immunotoxins specific for a mutant epidermal growth factor receptor: targeting with a single chain variable domain isolated by phage display. *Proc. Natl. Acad. Sci. USA* 93: 14815-14820.
- Lorimer, I.A.J., and Lavictoire, S.J. (2000) Targeting retrovirus to cancer cells expressing a mutant EGF receptor by insertion of a single chain antibody variable domain in the envelope glycoprotein receptor binding lobe. *J. Imm. Methods* 1: 1-11.
- Martin, J., Wharton, S.A., Lin, Y.P., Takemoto, D.K., Skehel, J.J., Wiley, D.C., and Steinhauer, D.A. (1998) Studies of the binding properties of influenza hemagglutinin receptor-site mutants. *Virology* 241: 101-111.

- Meiering, C.D. and Linial, M.L. (2001) Historical perspective of foamy virus epidemiology and infection. *Clin. Microbiol. Rev.* 14: 165-176.
- Moghal, N., and Sternberg, P.W. (1999) Multiple positive and negative regulators of signaling by the EGF-receptor. *Curr. Opin. Cell. Biol.* 11: 190-196.
- Morgan, R.A., Nussbaum, O., Muenchau, D.D., Shu, L., Couture, L., and Anderson, W.F. (1993) Analysis of the functional and host range-determining regions of the murine ecotropic and amphotropic retrovirus envelope proteins. *J. Virol.* 67: 4712-4721.
- Moscatello, D.K., Holgado-Madruga, A.K., Ramirez, G., Gunn, G., Zoltick, P.W., Biegel, J.A., Hayes, R.L., and Wong, A.J. (1995) Frequent expression of a mutant epidermal growth factor receptor in multiple human tumors. *Cancer Res.* 55: 5536-5539.
- Mineo, C., James, G.L., Smart, E.J., and Anderson, R.G. (1996) Localization of epidermal growth factor-stimulated Ras/Raf-1 interaction to caveolae membrane. *J. Biol. Chem.* 271: 11930-11935.
- Mulligan, R.C. (1993) The basic science of gene therapy. *Science* 260: 926-932.
- Nermut, M.V., Wallengren, K., and Pager, J. (1999) Localization of actin in Moloney murine leukemia virus by immunoelectron microscopy. *Virology* 260: 23-34.
- Nguyen, D.H., and Hildreth, J.E.K. (2000) Evidence for budding of human immunodeficiency virus type 1 selectively from glycolipid-enriched membrane lipid rafts. *J. Virol.* 74: 3264-3272.
- Ogasawara, T., Gotoh, B., Suzuki, H., Asaka, J., Shimokata, K., Rott, R., and Nagai, Y. (1992) Expression of factor X and its significance for the determination of paramyxovirus tropism in the chick embryo. *EMBO J.* 11: 467-472.
- Olayioye, M.A., Graus-Porta, D., Beerli, R.R., Rohrer, J., Gay, B., and Hynes, N.E. (1998) ErbB-1 and ErbB-2 acquire distinct signaling properties dependent upon their dimerization partner. *Mol. Cell Biol.* 18: 5042-5051.
- Ott, D.E., Coren, L.V., Kane, B.P., Busch, L.K., Johnson, D.G., Sowder, R.C. 2nd, Chertova E.N., Arthur, L.O., and Henderson, L.E. (1996) Cytoskeletal proteins inside human immunodeficiency virus type 1 virions. *J. Virol.* 70: 7734-7743.
- Ott, D.E., Coren, L.V., Johnson, D.G., Kane, B.P., Sowder, R.C. 2nd, Kim, Y.D., Fisher, R.J., Zhou, X.Z., Lu, K.P., and Henderson, L.E. (2000) Actin-binding cellular proteins inside human immunodeficiency virus type 1. *Virology* 266: 42-51.
- Parton, R.G. (1996) Caveolae and caveolins. *Curr. Opin. Cell Biol.* 8: 542-548.

Patterson, S.M., Swainsbury, R., and Routledge, E.G. (1999) Antigen-specific membrane fusion mediated by the haemagglutinin protein of influenza A virus: separation of attachment and fusion functions on different molecules. *Gene Ther.* 6: 694-702.

Perez, L.G., Davis, G.L., and Hunter, E. (1987) Mutants of the Rous sarcoma virus envelope glycoprotein that lack the transmembrane anchor and cytoplasmic domains: analysis of intracellular transport and assembly into virions. *J. Virol.* 61: 2981-2988.

Ragheb, J.A. and Anderson, W.F. (1994a) pH-independent murine leukemia virus ecotropic envelope-mediated cell fusion: implications for the role of the R peptide and p12E TM in viral entry. *J. Virol.* 68: 3220-3231.

Ragheb, J.A. and Anderson, W.F. (1994b) Uncoupled expression of Moloney murine leukemia virus envelope polypeptides SU and TM: a functional analysis of the role of TM domains in viral entry. *J. Virol.* 68: 3207-3219.

Riese, D.J. 2<sup>nd</sup> and Stern, D.F. (1998) Specificity within the EGF family/ErbB receptor family signaling network. *Bioessays* 20: 41-48.

Rey, O., Canon, J., and Krogstad, P. (1996) HIV-1 Gag protein associates with F-actin present in microfilaments. *Virology* 220: 530-534.

Rouso, I., Mixon, M.B., Chen, B.K., and Kim, P.S. (2000) Palmitoylation of the HIV-1 envelope glycoprotein is critical for viral infectivity. *Proc. Natl. Acad. Sci. USA* 97: 13523-13525.

Scheiffele, P., Roth, M.G., and Simons, K. (1997) Interaction of influenza virus hemagglutinin with sphingolipid-cholesterol membrane domains via its transmembrane domain. *EMBO* 16: 5501-5508.

Scheiffele, P., Rietveld, A., Wilk, T., and Simons K. (1999) Influenza viruses select ordered lipid domains during budding from the plasma membrane. *J Biol. Chem.* 274: 2037-2044.

Schnierle, B.S., Stitz, J., Bosch, V., Nocken, F., Merget-Millitzer, H., Engelstadter, M., Kurth, R., Groner, B., and Cichutek, K. (1997) Pseudotyping of murine leukemia virus with the envelope glycoproteins of HIV generates a retroviral vector with specificity of infection for CD4-expressing cells. *Proc. Natl. Acad. Sci. USA* 94: 8640-8645.

Schnitzer, J.E., Oh, P., Pinney, E., and Allard, J. (1994) Filipin-sensitive caveolae-mediated transport in endothelium: reduced transcytosis, scavenger endocytosis, and capillary permeability of select macromolecules. *J. Cell. Biol.* 127: 1217-1232.

Seto, J.T., Brzeniek, R., and Rott, R. (1966) Isolation of a low molecular weight sialidase (neuraminidase) from influenza virus. *Biochim. Biophys. Acta* 113: 402-404.

Simons, K., and Ikonen, E. (1997) Functional rafts in cell membranes. *Nature* 387: 569-572.

Simons K., and Toomre, D. (2000) Lipid rafts and signal transduction. *Nat. Rev. Mol. Cell. Biol.* 1:31-39.

Skalka, A.M. (1989) Retroviral proteases: first glimpses at the anatomy of a processing machine. *Cell* 56: 911-913.

Smart, E.J., Graf, G.A., McNiven, M.A., Sessa, W.C., Engelman, J.A., Scherer, P.E., Okamoto, T., and Lisanti, M.P. (1999) Caveolins, liquid-ordered domains, and signal transduction. *Mol. Cell Biol.* 19:7289-7304.

Soneoka, Y., Cannon, P.M., Ramsdale, E.E., Griffiths, J.C., Romano, G., Kingsman, S.M., and Kingsman, A.J. (1995) A transient three-plasmid expression system for the production of high titer retroviral vectors. *Nucleic. Acids. Res.* 23: 628-633.

Soneoka, Y., Kingsman, S.M., and Kingsman, A.J. (1997) Mutagenesis analysis of the murine leukemia virus matrix protein: identification of regions important for membrane localization and intracellular transport. *J. Virol.* 71: 5549-5559.

Song, K.S., Li, S., Okamoto, T., Quilliam, L.A., Sargiacomo, M., and Lisanti, M.P. (1996) Co-purification and direct interaction of Ras with caveolin, an integral membrane protein of caveolae microdomains. Detergent-free purification of caveolae microdomains. *J. Biol. Chem.* 271: 9690-9697.

Soong, M.M. and Tompkins, W.A. (1987) Role of cell cytoskeleton in Mo-MuLV env transport and processing: implications in ts1 neuropathology. *Exp. Mol. Pathol.* 46: 294-311.

Speck, N.A. and Baltimore, D. (1987) Six distinct nuclear factors interact with the 75-base-pair repeat of the Moloney murine leukemia virus enhancer. *Mol. Cell Biol.* 7: 1101-1110.

Sugawa, N., Ekstrand, A.J., James, C.D., and Collins, V.P. (1990) Identical splicing of aberrant epidermal growth factor receptor transcripts from amplified rearranged genes in human glioblastomas. *Proc. Natl. Acad. Sci. U S A* 87: 8602-8606.

Takemoto, D.K., Skehel, J.J., and Wiley, D.C. (1996) A surface plasmon resonance assay for the binding of influenza virus hemagglutinin to its sialic acid receptor. *Virology* 217: 452-458.

Toyoda, T., Sakaguchi, T., Imai, K., Inocencio, N.M., Gotoh, B., Hamaguchi, M., and Nagai, Y. (1987) Structural comparison of the cleavage-activation site of the fusion glycoprotein between virulent and avirulent strains of Newcastle disease virus. *Virology* 158: 242-247.

Tzahar, E., Waterman, H., Chen, X., Levkowitz, G., Karunakaran, D., Lavi, S., Ratzkin, B.J., and Yarden, Y. (1996) A hierarchical network of interreceptor interactions determines signal transduction by Neu differentiation factor/neuregulin and epidermal growth factor. *Mol. Cell. Biol.* 16: 5276-5287.

Waugh, M.G., Lawson, D., and Hsuan, J.J. (1999) Epidermal growth factor receptor activation is localized within low-boyant density, non-caveolar membrane domains. *Biochem. J.* 337: 591-597.

Wharton, S.A., Skehel, J.J., and Wiley, D.C. (1986) Studies of influenza haemagglutinin-mediated membrane fusion. *Virology* 149: 27-35.

Wikstrand, C.J., Hale, L.P., Batra, S.K., Hill, M.L., Humphrey, P.A., Kurpad, S.N., McLendon, R.E., Moscatello, D., Pegram, C.N., Reist, C.J., Traweck, S.T., Wong, A.J., Zalutsky, M.R., and Bigner, D.D. (1995) Monoclonal antibodies against EGFRvIII are tumor specific and react with breast and lung carcinomas and malignant gliomas. *Cancer Res.* 55: 3140-3148.

Winter, G., Fields, S., Gait, M.J., and Brownlee, G.G. (1981) The use of synthetic oligodeoxynucleotide primers in cloning and sequencing segment of 8 influenza virus (A/PR/8/34). *Nucleic Acids Res.* 9: 237-245.

Wong, A.J., Ruppert, J.M., Bigner, S.H., Grzeschik, C.H., Humphrey, P.A., Bigner, D.S., and Vogelstein, B. Structural alterations of the epidermal growth factor receptor gene in human gliomas. *Proc. Natl. Acad. Sci. U S A* 89: 2965-2969.

Wool-Lewis, R.J. and Bates, P. (1998) Characterization of Ebola virus entry by using pseudotyped viruses: identification of receptor-deficient cell lines. *J Virol* 72: 3155-3160.

Yoshinaka, Y., Katoh, I., Copeland, T.D., and Oroszlan, S. (1985) Translational readthrough of an amber termination codon during synthesis of feline leukemia virus protease. *J. Virol.* 55: 870-873.

Yu, J., Fischman, D.A., and Steck, T.L. (1973) Selective solubilization of proteins and phospholipids from red blood cell membranes by nonionic detergents. *J. Supramol. Struct.* 1: 233-248.

Yu, X., Yuan, X., Matsuda, Z., Lee, T.H., and Essex, M. (1992) The matrix protein of human immunodeficiency virus type 1 is required for incorporation of viral envelope protein into mature virions. *J Virol* 66: 4966-4971.

Zhang, J., Lesser, G.P., Pekosz, A., and Lamb, R.A. (2000a) The cytoplasmic tails of the influenza virus spike glycoproteins are required for normal genome packaging. *Virology* 269: 325-334.

**Zhang, J., Pekosz, A., and Lamb, R.A. (2000b) Influenza virus assembly and lipid raft microdomains: a role for the cytoplasmic tail of the spike glycoproteins. J. Virol. 74: 4634-4644.**

**Zhou, W., Parent, L.J., Wills, J.W., and Resh, M.D. (1994) Identification of a membrane-binding domain within the amino-terminal region of human immunodeficiency virus type 1 gag protein which interacts with acidic phospholipids. J. Virol. 68: 2556-2569.**