INVESTIGATION OF THE ROLE OF TARGET CELL FACTORS IN RETROVIRUS TRANSDUCTION

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Delfi Krishna

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INVESTIGATION OF THE ROLE OF TARGET CELL FACTORS IN RETROVIRUS TRANSDUCTION

Approved by:

Date: 10/25/05

Dr. Joseph M. Le Doux, Chairman
Department of Biomedical Engineering
Georgia Institute of Technology

Dr. Mark R. Prausnitz
School of Chemical & Biomolecular Engineering
Georgia Institute of Technology

Dr. Timothy Wick
Department of Biomedical Engineering
University of Alabama at Birmingham

Dr. Harish Radhakrishna
School of Biology
Georgia Institute of Technology

Dr. Richard Compans
Department of Microbiology and Immunology
Emory University School of Medicine

Dr. Athanassios Sambanis
School of Chemical & Biomolecular Engineering
Georgia Institute of Technology

To Mummy and Papa

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SUMMARY

Gene therapy is the intracellular delivery of genetic material for a therapeutic effect. One of the fastest growing areas of experimental medicine it is currently being used in clinical trials for the treatment of cancer, inherited or acquired monogenic disorders, AIDS, vascular diseases and neurodegenerative disorders such as Alzheimer's and Parkinson's disease. Recombinant retroviral vectors are one of the most commonly used gene delivery vectors in clinical trials because they can permanently integrate the therapeutic gene into the genome of the target cell resulting in persistent gene expression. The success of gene therapy in clinical programs is primarily dependent on the degree of vector design development. Recombinant retroviral vectors suffer from several limitations as gene transfer vectors. The gene transfer efficiency is not high enough to produce the desired therapeutic effect and the vectors lack the ability to genetically modify target tissue without producing unpredictable side effects on healthy bystander tissue. The focus of this thesis is to determine target cell factors that affect the efficiency and specificity of gene transfer of recombinant retroviruses. Successful gene transfer by recombinant retroviruses is a multi-step process and we have focused our efforts on those target cell factors that affect virus entry into the target cell.

We have developed an experimental system to study the effect of pathway of virus entry and the intracellular trafficking itinerary of the targeted receptor, on the efficiency of gene transfer of targeted retroviruses. Our results indicate that interaction with a targeted receptor affects the efficiency of gene transfer of a targeted retrovirus by altering the residence time of the virus on the cell surface, by changing the

region of the cell surface that the virus is exposed to, with respect to its natural receptor or by changing the pH that the virus is exposed to during intracellular transport.

We have examined if recombinant retroviruses are capable of inducing signaling events in target cells to overcome barriers to efficient gene transfer. We have found that retroviruses are capable of activating actin-regulating-GTPase Rac1 while entering target cells. We have found that retroviruses use non-envelope and non-receptor molecules to induce Rac1 activation. Rac1 activity is important for efficient fusion and intracellular trafficking of the virus and blocking mediators of Rac1 activity on target cells affects the efficiency of gene transfer of recombinant retroviruses. The implications of our findings on efficient retrovirus-cell interactions are discussed.

CHAPTER 1

BACKGROUND AND OBJECTIVES

1.1 Gene therapy

Gene therapy is the intracellular delivery of genetic material for a therapeutic effect. One of the fastest growing areas of experimental medicine, it is currently being used in clinical trials for the treatment of cancer, inherited or acquired monogenic disorders, AIDS, vascular diseases and neurodegenerative disorders such as Alzheimer's and Parkinson's disease (33-35, 42). Vehicles used for gene delivery are termed gene delivery vectors and can be classified as viral or non-viral. Viral vectors are replication-incompetent derivates of wild type viruses and have been exploited for the natural ability to deliver their genetic material to target cells. Gene delivery using non-viral methods comprises administration of DNA, RNA or oligonucleotides by mechanical or electrical means or by complexation with polymers, proteins or lipids. While some viral vectors offer the possibility of long-term gene expression due to permanent integration of their therapeutic gene into the target cell, non-viral vectors are not infectious, exhibit low toxicity and have no limit for the size of DNA that can be delivered. However non-viral vectors are non-specific, have low gene transfer efficiency and transient gene expression (5, 15).

Gene delivery can be conducted in two settings: *in vivo* or *ex vivo*. In the *in vivo* setting, gene delivery vectors are directly injected into specific tissues or administered systemically into the patient while in the *ex vivo* setting the target tissue is removed from the patient, the cells cultured *in vitro*, genetically modified, selected for the modification, expanded and reimplanted back into the patient. *Ex vivo* gene delivery can be tightly controlled and optimized but is not practical for large scale applications as it is expensive

and labor intensive and not applicable to tissues that cannot be removed and reimplanted (e.g. brain, heart and lung). On the other hand, effective *in vivo* gene delivery is only possible with vectors that have high specificity for the tissue of interest (1, 13, 38).

1.2 Recombinant retroviruses as gene delivery vectors

Recombinant retroviral vectors are one of the most commonly used gene delivery vectors in clinical trials because they can permanently integrate the therapeutic gene into the genome of the target cell resulting in persistent gene expression. They can be produced to relatively high titers (10^7-10^8 cfu/ml), have a broad cell tropism, no toxic effects in infected cells and a total insert (transgene + transfer vector) capacity of 8-10kb. Recombinant retroviral vectors based on Moloney murine leukemia virus (MLV) are capable of infecting only dividing cells. However vectors based on human immunodeficiency virus type 1 (HIV-1) can infect non-dividing cells, an important property for *in vivo* gene delivery since many tissues in the human body are not actively dividing (2, 7, 25, 35).

Retroviruses are spherical in shape with a diameter of 100nm. Their genetic material consists of two identical copies of single stranded RNA enclosed within an icosahedral matrix of proteins called the virus capsid. The capsid proteins are surrounded by a lipid bilayer into which are inserted proteins termed envelope glycoproteins (11). Recombinant retroviruses are structurally identical to wild-type viruses but carry an engineered genome (called the recombinant vector) that encodes for therapeutic genes of interest but contains no genetic information required for virus replication. Such manipulations retain the ability of the virus to transfer therapeutic genes but disable virus replication in the target cell.

Recombinant retroviruses are produced by a two-part system composed of a packaging cell line and a recombinant vector (Figure 1.1). The packaging cell line is engineered to express all the viral genes (*gag*, *env* and *pol*) required for the formation of infectious virus particles. *Gag* encodes for viral capsid protein, *env* encodes for viral envelope proteins and *pol* encodes for viral enzymes, reverse transcriptase and integrase (11). The recombinant vector contains the therapeutic gene, regulatory sequences that control its expression, and a packaging sequence (ψ) that enables the recognition and packaging of the vector into a virus particle by viral structural proteins. Recombinant retroviruses are produced by transfecting packaging cells with the recombinant vector. Within the cell, the recombinant vector is transcribed from DNA to RNA, the viral proteins recognize the ψ sequence on the vector and assemble around it. The virus is then transported to the plasma membrane of the packaging cell that expresses viral envelope proteins. During budding, viruses acquire the lipid bilayer from the packaging cell surface and in the process incorporate envelope proteins on their surface. The virus-laden cell culture medium is then collected and used to transfer genes to target cells.

The process of gene transfer (transduction) consists of exposing target cells to recombinant retrovirus stocks. Transduction involves a series of complex events that begin with the transport of the virus to the cell surface, non-specific adsorption to the cell surface followed by the interaction of virus envelope proteins with the cognate cell surface receptor that permits fusion of the virus and the cellular membrane. Upon fusion, the RNA genome of the virus and the associated virus proteins and enzymes are released into the cytoplasm of the cell. Next, the RNA genome is reverse transcribed to DNA using viral reverse transcriptase and integrated into the chromosomal DNA of the

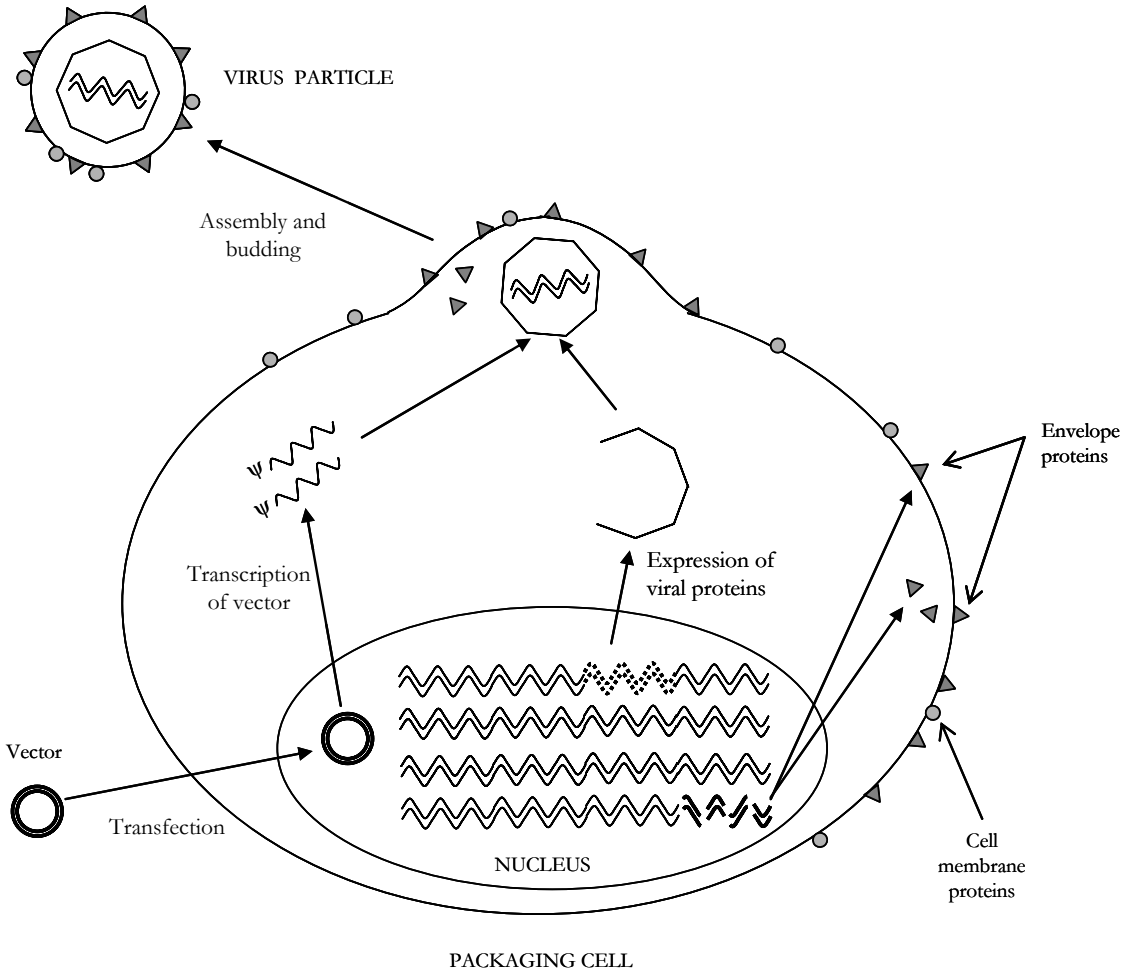

Figure 1.1 Production of recombinant retroviruses by packaging cell lines. Recombinant retroviruses are produced by transfecting packaging cells, genetically modified to express all the structural proteins of a retrovirus particle, with the recombinant vector. The recombinant vector encodes the therapeutic gene of interest, the regulatory sequences that drive its expression, and a packaging sequence (ψ) that ensures vector recognition by viral structural proteins. Virus capsid proteins recognize the vector and assemble around it. The virus then buds from the surface of the cell incorporating a part of the lipid bilayer of the cell that contains viral envelope proteins.

target cell using viral integrase. The target cell machinery then expresses the therapeutic gene of interest from the site of chromosomal insertion (Figure 1.2).

1.3 Limitations of gene therapy

The success of gene therapy in clinical programs is primarily dependent on the degree of vector design development. Four factors in the area of vector development have hampered progress (25). (1). The gene transfer efficiency of vectors is not high enough to produce the desired therapeutic effect. (2). It is necessary to enhance the specificity of vectors to avoid unpredictable side effects on healthy tissue. (3). Regulation and control of therapeutic gene expression is highly desirable and has not been successfully achieved. (4). The vectors should be safer and more reliable in order to avoid oncogenic transformation of target cells, cytotoxicity and immunogenicity. This thesis is focused on determining target cell factors that affect the efficiency and specificity of gene transfer of recombinant retroviruses. Successful gene transfer by recombinant retroviruses is a multi step process and we have focused our efforts on understanding virus entry into the target cell.

Specificity of gene transfer

Gene transfer mediated by recombinant retroviruses is specific if the therapeutic gene is delivered only to target cells of interest without any effects on healthy by stander cells. Retroviruses are typically classified in terms of their host range or the species they can infect (11). The host range of retroviruses is largely determined by the interaction between envelope proteins on the surface of the virus and virus receptors on the surface of the cell (11). Amphotropic and ecotropic murine leukemia viruses (MLV-A, E) are two classes of retroviruses that are commonly used in gene therapy protocols. Amphotropic

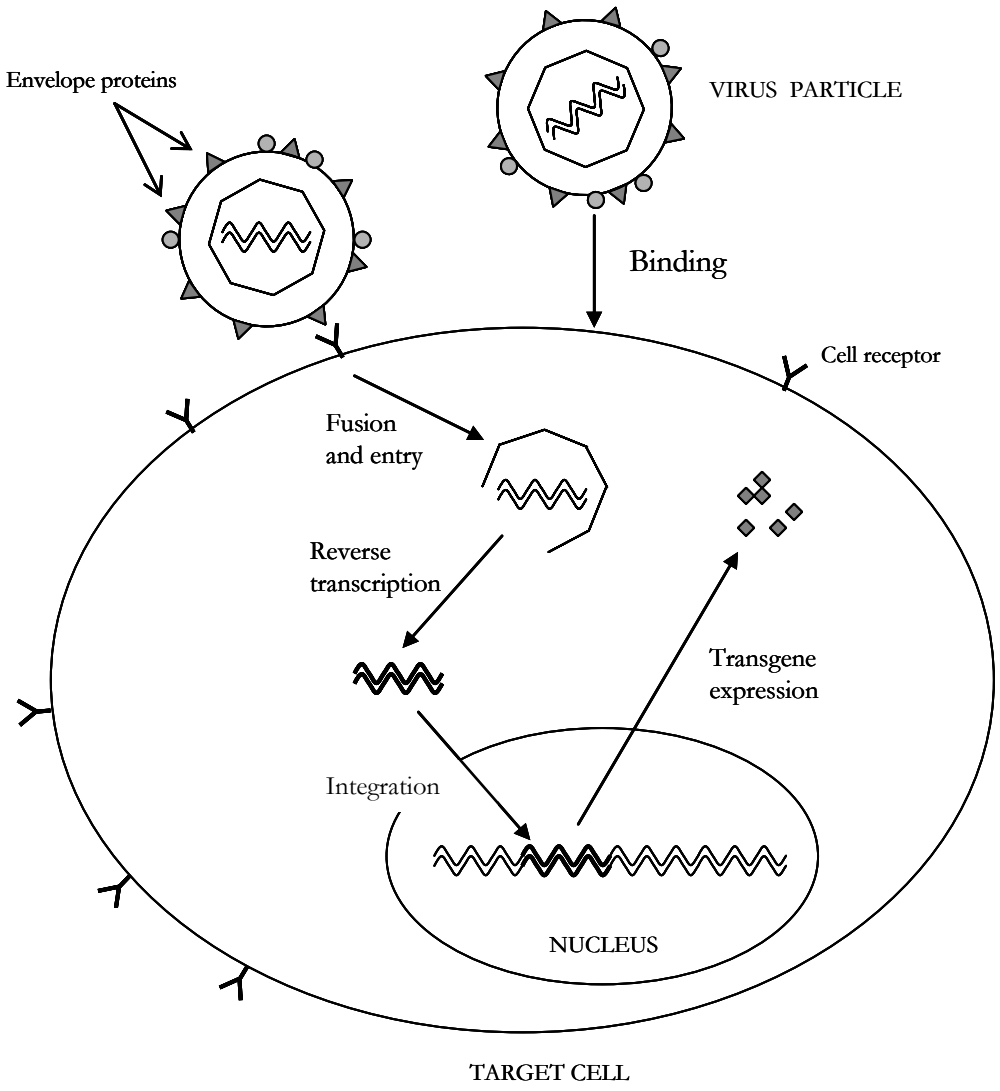

Figure 1.2 Transduction of target cells by recombinant retroviruses. Transduction begins with the transport of the virus to the cell surface, non-specific adsorption to the cell surface followed by the interaction of virus envelope proteins with the cell surface receptor and the subsequent fusion of the virus and the cellular membranes. Upon fusion, the RNA genome of the virus and associated virus proteins and enzymes are released into the cytoplasm of the cell. Next, the RNA genome is reverse transcribed to DNA using viral reverse transcriptase and integrated into the chromosomal DNA of the target cell using viral integrase. The target cell machinery then expresses the therapeutic gene of interest from the site of chromosomal insertion.

retroviruses are of primary interest in human gene therapy protocols because they can infect human cells whereas ecotropic retroviruses infect only rodent cells. The cell surface receptor for MLV-A is a sodium-dependent phosphate transporter called Pit2, and it is a multi-membrane-spanning protein that is expressed in almost every human cell type (4, 6). As a result, amphotropic retroviruses can infect almost all human cells. This lack of cell type specificity is a major disadvantage for *in vivo* gene therapy applications.

Retroviruses enter target cells by interacting with the cell surface receptor through their envelope glycoproteins. In virus packaging cells, the envelope glycoprotein is initially translated as a precursor, Pr85, and then assembled as an oligomer in the endoplasmic reticulum. Oligomerized protein is transported to the golgi compartment where it is proteolytically cleaved into two subunits, surface protein (SU; gp70, 70kDa) and transmembrane protein (TM; p15E, 15kDa) by a cellular protease. At the time of virus budding or shortly thereafter, p15E is further processed by the viral protease to release a 16-amino acid peptide (R-peptide) from the carboxyl terminus. Both p15E and the processed p12E forms of TM coexist in the mature virion. Binding of the envelope protein to the receptor is a property of the SU subunit that contains the receptor binding domain, whereas the post binding functions that lead to fusion between the viral and cellular membranes are largely properties of the TM subunit. Current models of retroviral entry predict that following the interaction of the SU subunit with its receptor, a conformational change is triggered in the associated TM subunit leading to adoption of a fusogenic conformation that enables the fusion of the viral and cellular membranes.

Since viral envelope proteins fulfill critical functions of receptor binding and fusion that enable the virus to penetrate into the cytoplasm, they have been the primary focus of retroviral targeting strategies. Retroviral envelope proteins can tolerate a variety of genetically encoded modifications (41). Chimeric envelope proteins have been designed

by inserting several types of ligands such as growth factors, hormones, peptides or single chain antibodies in different regions of the envelope gene (22). Such insertions modify the host range of the viruses expressing the chimeric envelope proteins by allowing the virus to bind to cell surface molecules different from the retrovirus natural receptor. The modifications of the envelope proteins include replacement of the receptor-binding domain (19), peptide insertion into prefolded domains (3, 40, 43) and display of polypeptides as additional folded domains (12, 20, 24, 37). For most N-terminally modified chimeric envelope proteins, the efficiency of viral incorporation is low due to suboptimal folding, assembly or transport of the envelope protein to the surface of the virus packaging cell. Many other chimeric envelope proteins fold correctly, are stably incorporated on viruses and allow efficient retargeted virus binding to the receptor of interest. However upon binding most of these chimeras are unable to induce membrane fusion and the subsequent penetration of the viral core into the cytoplasm. This is not because the envelope proteins become non-fusogenic after the modification, in fact such chimeric envelope proteins fuse readily with the natural viral receptors. The block in fusion is attributed to the presence of non-viral target receptor and its inability to trigger fusion of the chimeric envelope glycoprotein (12, 36, 44).

To overcome the limitations imposed by the use of a non-viral target receptor in targeting strategies, methods have been designed where chimeric envelope proteins recognize non-viral target receptors as well as the natural viral receptor on the cell surface. Thus after the initial phase of interaction with a specific target cell surface molecule, cellular entry of the retroviral vectors carrying the chimeric envelope protein relies on the interaction with the natural viral receptor, which permits efficient membrane fusion. One such strategy consists of inclusion of cell-specific binding motifs on viral envelope proteins in regions that do not affect its ability to promote binding and fusion with the natural viral receptor (14, 26) . Alternatively, co expression of the wild-type

envelope protein with a second “escort” glycoprotein that carries cell-specific binding determinants and that is usually defective for fusion, also permits virus binding to tissues that abundantly express the target molecules (17, 18). However, retrovirus targeting by insertion of cell-specific binding motifs into their envelope proteins or lipid bilayers also has limitations. For instance, although viruses expressing chimeric amphotropic envelope proteins that display the epidermal growth factor (EGF) are targeted to EGF receptors on cells expressing both EGF and amphotropic receptors (12, 30), the efficiency of transduction is low, possibly due to a non-productive entry pathway into the target cell (23). A better understanding of the rate limiting steps in retrovirus targeting strategies will enable the design and development of efficient targeted retroviruses.

Efficiency of gene transfer

Successful gene transfer by retroviruses begins with the adsorption of the virus to the surface of the target cell followed by the interaction of the viral envelope proteins with their natural receptor that enables fusion of the viral and cell membranes and entry of the virus core into the cytoplasm (32). Following membrane penetration, retroviruses encounter the cortical actin network that poses a barrier against the inward movement of viral capsids into the cytoplasm (10, 39). Diffusion is not a viable option for the transport of the viral capsid in the cytoplasm given its large dimensions and the fact that the environment of the cytoplasm is crowded, packed with organelles and consists of a highly structured cytoskeletal network (9, 39).

Emerging evidence suggests that viruses exploit intracellular proteins for active transport within the cytoplasm. Viruses from several families activate cell-signaling cascades that facilitate their entry and intracellular transport (16). By activating tyrosine kinases in caveolae, SV40 triggers the normally dormant caveolar endocytosis to enter target cells (31). By clustering its entry receptors, adenovirus-2 activates protein kinases

and small GTPases that lead to actin cytoskeleton rearrangement and microtubule mediated intracellular transport of the virus (16, 28, 29). Recent studies suggest that retroviruses also use the target cell cytoskeleton for active transport. It has been shown that a functional actin network is required for productive retrovirus entry (21) and dynein motor proteins facilitate retrovirus travel along microtubules towards the nucleus (27). Several target cell GTPases regulate the function of the cytoskeletal network (8) however it is not clear if retroviruses are capable of signaling to the GTPases to facilitate active transport. A better understanding of cell signaling events that aid in the entry process will be helpful in the design of retrovirus vectors especially suited for target cells refractory to retrovirus gene transfer.

1.4 Thesis objectives

The objectives of this thesis were to identify and characterize the target cell factors that affect the efficiency and specificity of gene transfer of recombinant retroviruses. To accomplish these objectives we pursued the following aims.

1. Construct targeted retroviruses that express chimeric envelope proteins with a cell specific binding motif and cell lines that express the receptor to the binding motif (targeted receptor) and the natural MLV-A receptor. Examine the role of pathway of virus entry and the intracellular trafficking itinerary of the targeted receptor on the efficiency of gene transfer of retroviruses expressing the chimeric envelope proteins.
2. Determine if retroviruses are capable of signaling to target cell proteins in order to overcome barriers to successful gene transfer.
3. Determine the role of signaling proteins in retrovirus gene transfer.

1.5 Organization of the thesis

In chapter 1 we briefly introduce gene therapy and the use of recombinant retroviruses as gene delivery vectors. We identify the limitations of retroviruses as gene delivery vectors and discuss the goals that we formulated to address them.

In chapter 2 we develop an experimental system to examine the effect of pathway of virus entry and the intracellular trafficking itinerary of the targeted receptor on the efficiency of gene transfer of targeted retroviruses. We examine the effect and discuss implications for designing targeted retroviruses.

In chapter 3 we examine if retroviruses are capable of signaling to target cell proteins to overcome barriers to successful gene transfer. We identify a signaling GTPase that retroviruses activate, the molecules on the virus and the cell surface that induce the activation and discuss implications of our findings for retrovirus-cell interactions.

In chapter 4 we examine the role of the GTPase identified in chapter 3 in retrovirus transduction. We identify the step of virus gene delivery that is affected by the GTPase.

In chapter 5 we summarize our major conclusions and present suggestions for future work.

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CHAPTER 2

TARGETED RECEPTOR TRAFFICKING AFFECTS THE EFFICIENCY OF RETROVIRUS TRANSDUCTION

2.1 Abstract

We describe the development of an experimental system to test the hypothesis that the efficiency of retrovirus transduction is dependent on the pathway of virus entry into the host cell and the intracellular trafficking itinerary of the cellular receptor with which it interacts. The experimental system consists of three model target cell lines, derived from HeLa cells, that stably express one of three interleukin-2 receptor alpha chain (CD25) chimeras – TAC, TAC-CD16, and TAC-DKQTLL - that have identical extracellular domains but different intracellular trafficking itineraries, and a targeted amphotropic murine leukemia retrovirus whose envelope proteins were modified to include a binding site for TAC at their N-termini. We found that the efficiency of retrovirus transduction was affected by the distribution and trafficking itinerary of the TAC receptors. Transduction of cells that expressed TAC-DKQTLL was nearly 4-fold lower than transduction of control cells that did not express any of the TAC receptors. In contrast, transduction of cells that expressed TAC was 1.6 fold higher than transduction of control cells, whereas transduction was not significantly affected by the expression of TAC-CD16. Our results suggest that in the course of designing a targeted retrovirus it may be prudent to target only those receptors that internalize retroviruses via pathways that most efficiently support post-binding steps of infection.

2.2 Introduction

Recombinant retroviruses are frequently used for experimental and clinical gene transfer because they stably modify cells. Stable modification is important for the treatment of chronic or inherited disease. Recombinant retroviruses have had limited success in human gene therapy

clinical trials to date, however, in part because it has proven difficult to ensure that gene transfer is specific for the cell type of interest while at the same time efficient enough to achieve the desired therapeutic effect (1, 29, 36). The specificity with which retroviruses transduce cells is primarily determined by the envelope glycoproteins they display on their outer surface (2). Retrovirus envelope proteins mediate binding of the virus to its cell-surface receptors, and initiate fusion between the membranes of the virus and the cell.

Due to their central role in retrovirus infection, most efforts to improve the specificity of retrovirus transduction have focused on manipulating the structure of the envelope proteins of the retrovirus (22-24). One common strategy has been to insert cell-specific binding motifs into the viral envelope proteins to increase the likelihood the virus will bind to the targeted cell type (13, 39, 48). For example, insertion of collagen-binding peptides into retrovirus envelope proteins increased the accumulation of amphotropic retroviruses at sites of vascular injury, and in a different set of studies lentiviruses that displayed CD-3 and interleukin-7 receptor-binding domains on their surfaces successfully transduced T-cells (8, 25, 46). The efficiency with which these targeted viruses transduced cells was low, however, and must be improved before they are likely to prove useful for clinical gene transfer applications.

The results of some recent studies suggest that efficient transduction may require retroviruses to enter cells through a specific intracellular trafficking pathway. For example, retrovirus avian sarcoma and leukosis viruses (ASLV) appear to transduce cells more efficiently when they enter cells through lipid rafts, microdomains on the surfaces of cells that are enriched in sphingomyelin and cholesterol, than when they enter cells through other endocytic pathways (31). After entry, retroviruses appear to require an intact actin network as evidenced by the fact that disassembly of the network impairs virus entry and infection but not the physiological function of the receptor or its ability to bind virus (14). Disassembly of microtubules also blocks retrovirus infection, possibly because microtubules play an important role in the transport of retroviruses to the nucleus after they have entered the cytosol of the host cell (14). Moreover,

retroviruses targeted via receptor-specific antibodies to the insulin receptor, or MHC class I molecules were able to infect cells whereas viruses that targeted the transferrin receptor were not able to infect cells (6, 9). Taken together, these studies prompted us to hypothesize that the efficiency of retrovirus transduction is, in part, dependent on the pathway of virus entry into the host cell and the intracellular trafficking itinerary of the cellular receptor with which it interacts.

To test this hypothesis, we developed an experimental system composed of a 1) targeted amphotropic murine leukemia virus (MLV) that is capable not only of binding to and fusing with its wild-type virus receptor, Pit-2, but is also capable of binding to a model virus binding protein, TAC (CD25, the interleukin-2 receptor alpha chain), and 2) three cell lines derived from HeLa cells that stably express one of three TAC receptor chimeras (TAC, TAC-CD16, and TAC-DKQTLL), each of which contain identical extracellular domains but different cytoplasmic domains that cause them to be internalized via distinct intracellular trafficking pathways (4, 11, 18, 32, 35). We chose to use TAC as a model virus binding protein because it can be easily altered through modifications of its cytoplasmic tail, and because TAC is not endogenously expressed in our model cell line (HeLa cells), which simplified the interpretation of our experimental results (12, 30). Our results show that the trafficking itineraries of cellular proteins that bind retroviruses affects the efficiency of retrovirus transduction and suggest that it may be prudent, in the course of designing a targeted retrovirus, to consider targeting only those proteins that internalize viruses via pathways that most efficiently support post-binding steps of infection.

2.3 Materials and methods

Chemicals, plasmids, and antibodies. 1,5-dimethyl-1,5-diazaundecamethylene polymethobromide (Polybrene, PB), 3'-azido-3'-deoxythymidine (AZT), Igepal CA-630 (NP40), poly vinyl alcohol (PVA 30,000-70,000), glycerol, saponin, horseradish peroxidase conjugated goat anti-rabbit immunoglobulin G, and horseradish peroxidase conjugated rabbit anti-goat

immunoglobulin G were purchased from Sigma Chemical Co. (St. Louis, MO). Polyoxyethylene 20-sorbitan monolaurate (Tween 20) and sodium azide were purchased from Fisher Scientific (Fair Lawn, NJ). Non-fat dry milk (blotting grade) was purchased from Bio-Rad Laboratories (Hercules, CA). 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was purchased from Denville Scientific, Inc. (Metuchen, NJ). Mouse anti-gp70 and anti-TAC monoclonal antibodies were purified from the supernatants of the hybridoma cell lines 83A25 and 7G7, respectively (ATCC, Rockville, MD). Goat anti-gp70 (79S834) and anti-p30 (78S221) antisera were purchased from Quality Biotech Inc. (Camden, NJ). Anti-TAC function blocking antibody Zenapax (Daclizumab) was a kind gift from Roche Pharmaceuticals. Rabbit anti-TAC antisera for immunoblots was purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Horseradish peroxidase conjugated rabbit anti-mouse immunoglobulin G, Cy2 conjugated donkey anti-rat, Cy3 conjugated donkey anti-mouse and donkey sera were purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). PolyFect was purchased from Qiagen (Valencia, CA). Lipofectamine-2000 was purchased from Invitrogen Corporation (Carlsbad, CA). Alexa-488 conjugated concanavalin A and 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) were purchased from Molecular Probes (Eugene, OR). cDNA encoding folate receptor-α (FR-α) or its mutant FR-MCP (in which the COOH-terminal GPI anchor signal (19 amino acids) had been replaced with the sequence for the COOH-terminal TM domain (85 amino acids) of the complement membrane cofactor protein (MCP)) cloned into the mammalian expression vector pZeoSV2 (+), and anti-folate receptor antisera, were kind gifts from V.L. Stevens (Emory University School of Medicine, Atlanta, GA) (5). Plasmids encoding TAC (CD25), TAC-CD16 and TAC-DKQTLL were kind gifts of Harish Radhakrishna (School of Biology, Georgia Institute of Technology, Atlanta, GA) (4, 18, 44). pGFP-Vpr (26) was a kind gift of T.J Hope (University of Illinois, Chicago).

Cell lines. HeLa (human cervical cancer) cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone Labs Inc., Logan, UT) with 10% fetal bovine serum (FBS; Hyclone Labs Inc.), 100 U/mL of penicillin, 100 μg/mL of streptomycin (Hyclone Labs Inc.), and 110 μg/mL of sodium pyruvate (Hyclone Labs Inc.) (DMEM/FBS). TELCeB6 cells (a kind gift from F.L. Cosset) expressing Moloney MLV Gag and Pol, and the retroviral vector MFGnlsLacZ, Te671 (human rhabdomysarcoma) (3) and 293T/17 (human embryonic kidney epithelial) cells were cultured in DMEM/FBS. CHO-K1 (Chinese hamster ovary) cells were cultured in F12K (Hyclone Labs Inc.) with 10% FBS, 100 U/mL of penicillin, 100 μg/mL of streptomycin and 200mM L-glutamine.

To construct target cell lines that uniquely express one of three TAC chimeras, HeLa cells (2×10^6 cells per 10 cm dish) were co-transfected, using the calcium phosphate method (43) with 1 μg pcDNA3.1+/Neo and 10 μg of an expression plasmid for TAC, TAC-CD16, or TAC-DKQTLL. Three days after transfection cells were cultured for two weeks in medium that contained G418 (500 μg/mL) to select for stably modified cells. G418 resistant clones were isolated and those that expressed the highest levels of TAC receptor identified by immunofluorescence microscopy, then expanded and frozen for later use. To obtain cells that co-expressed a TAC chimera and FR-α or FR-MCP, PolyFect (25 μL) was used to transiently transfect TAC-expressing HeLa cells (8×10^5 cells per 60 mm dish) with 3 μg of an expression plasmid for FR-α or FR-MCP, and then cultured 2 d before use in the lipid raft assay.

Immunofluorescence microscopy. All cells were immunostained by plating them on coverslips (#1.5, 12 mm, Fisher Scientific; Suwanee, GA). First the cells were fixed with 2% paraformaldehyde (1 mL/well) for 10 min, and then blocked with PBS/sera (1 mL/well) (5% donkey sera in PBS (PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_47H_2O$, 1.4 mM KH_2PO_4, pH 7.3)) for 15 min on a shaker. Next, the cells were incubated with primary antibody for 1 h at room temperature, washed 3 times with PBS, incubated with the secondary antibody

for 1 h at room temperature, washed 3 times with PBS and once with double distilled water, and then their nuclei stained with DAPI (300nM in PBS) for 1 min at room temperature. The cells were subsequently washed with double distilled water (1 mL/well) and the coverslips mounted on glass slides with gelvatol (42). All cells were visualized by confocal microscopy (Zeiss LSM 510, 40X oil objective).

To visualize the expression of retrovirus envelope proteins on the surface of cells that were producing virus, TELCeB6 cells, transiently transfected 24 h earlier with 2 μg of pCAGGS-AScFvTAC or pCAGGS-A using Lipofectamine-2000 were plated on coverslips in a 12 well plate (50,000 cells/well) and cultured for 12 h. The cells were then exposed to rat anti-gp70 (83A25) primary antibody (diluted 12.5 fold in PBS/sera) and Cy2 conjugated donkey anti-rat secondary antibody (diluted 200 fold in PBS/sera). To visualize the expression of TAC receptors, HeLa cells expressing TAC chimeras were plated on coverslips in 12 well plates (50,000 cells/well) and exposed to mouse anti-TAC primary antibody (7G7) (diluted 64 fold in PBS/sera/0.2% saponin) and Cy3 conjugated donkey anti-mouse secondary antibody (diluted 800 fold in PBS/sera/0.2% saponin).

To determine if the subcellular localization of viruses was affected by the intracellular trafficking itinerary of the TAC-DKQTLL receptor, HeLa cells expressing TAC-DKQTLL were plated on cover slips in 12 well plates (50,000 cells/well). Twenty-four hours later the cells were chilled to 4^{o}C for 1 hr to block endocytosis, placed in medium containing 1 mL GFP-labeled lentivirus pseudotyped with the A-ScFv-TAC or amphotropic envelope protein and brought to 20 μg/mL Polybrene, centrifuged (2100 × g, 30 min, 4^{o}C), placed in fresh cell culture medium pre-warmed to 37°C and incubated for a range of times (0 to 60 min). To visualize the TAC-DKQTLL receptors, the cells were incubated sequentially with mouse anti-TAC primary antibody (7G7) (diluted 64 fold in PBS/sera/0.2% saponin), and Cy3 conjugated donkey anti-mouse secondary antibody (diluted 800 fold in PBS/sera/0.2% saponin), and then visualized by

confocal microscopy. For each experimental condition tested, the pinhole, objective magnification, zoom, optical slice thickness, scan averaging and pixel resolution were kept constant between the red and the green channels, and eight cells were chosen at random and analyzed for the extent of co-localization of the fluorescent probes using Metamorph Imaging System Software (Universal Imaging Corp., West Chester, PA) (47).

Lipid raft isolation. HeLa cells that co-expressed one of the three TAC chimeras and either FR-α or FR-MCP were lysed with 1 mL ice-cold 1% Triton-X in TNE buffer (25mM Tris-base, 0.15M sodium chloride, 5M EDTA, pH 7.5). Lysates were homogenized and adjusted to 40% sucrose by adding an equal volume (900 μL) of 80% sucrose in TNE buffer. The samples were placed in ultracentrifuge tubes and then overlaid with a gradient of 5 mL of 38% sucrose (in TNE) followed by 3.5 mL of 5% sucrose (in TNE). The gradients were centrifuged at 30,000 rpm for 15 h at 4°C in an SW41 rotor (Beckman Coulter, Palo Alto, CA). The lipid raft fraction at the interface of the 5% and 38% sucrose solutions was isolated, brought to 12 mL with cold TNE buffer, and then centrifuged at 30,000 rpm for 1 h at 4°C in an SW41 rotor. The pelleted lipid raft fraction was resuspended in 100 μL of cold TNE buffer, and then analyzed by Western blot using anti-TAC receptor and anti-folate receptor antibodies.

Immunoblotting. Lipid raft fractions were prepared for analysis by immunoblotting as described above. To prepare virus samples for analysis by immunoblotting, viral supernatants (10 mL) were brought to 80 μg/mL of polybrene and 80 μg/mL of chondroitin sulfate C at 37^{o}C for 20 min (17), centrifuged for 5 min at 10000 $\times$ g, and then the pelleted virus resuspended in 500 μL of IP buffer (20 mM sodium phosphate, 500 mM sodium chloride, 0.1% SDS, 1% NP40, 0.02% sodium azide) and frozen (-80°C) for later use. Equal amounts of protein (for analysis of lipid raft fractions) or p30 (for analysis of virus samples) from each sample were combined 1:2 (vol/vol) with sample buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue and 5% β-mercaptoethanol), vortexed and boiled for 5 min, separated by size

by electrophoresis (4-20% Tris-HCl gel), transferred to a PVDF membrane (0.2 μm, BIO-RAD, Hercules CA), and then the proteins of interest identified with specific antibodies and a chemiluminescent detection system (Super Signal West Femto kit, Pierce Chemical Company, Rockford, IL). The primary antibodies were used in blocking buffer (5% non-fat milk in PBS-T (PBS, 0.1% Tween 20, pH 7.4)) at the following dilutions: goat anti-gp70 antisera (1:1000), goat anti-p30 antisera (1:10000), rabbit anti-TAC antisera (1:1000), and rabbit anti-folate antisera (1:5000). Bound Ig was detected by incubation with rabbit anti-goat or goat anti-rabbit horseradish peroxidase conjugate (diluted $1:10^6$ in blocking buffer).

Measurement of internalization rates. HeLa cells expressing one of the three TAC chimeras were plated on coverslips in a 12 well dish at 30,000 cells per well. The next day cells were incubated on ice for 30 min to block endocytosis, incubated for 1.5 h at 4°C with anti-TAC primary antibody (diluted 64 fold in cold media (DMEM/FBS)), and then incubated with pre-warmed media at 37°C for 0 to 3 h. Next the cells were incubated for 30 min on ice with Alexa-488 conjugated concanavalin-A (400 μg/mL in PBS), fixed with 2% paraformaldehyde (1 mL) for 10 min, blocked with PBS/sera for 15 min with gentle shaking, and then incubated for 1 h at room temperature with Cy3 conjugated donkey anti-mouse secondary antibody (diluted 800 fold in PBS/sera). Labeled cells were washed three times with PBS, once with double distilled water, mounted on glass slides using gelvatol and visualized by confocal microscopy. For each experimental condition tested, eight cells were chosen at random and analyzed for the extent of co-localization of the fluorescent probes using Metamorph Imaging System Software.

Cell based ELISA. To quantitatively compare TAC receptor expression on the surface of HeLa cells, we used a cell based ELISA similar to one previously described (49). HeLa cells expressing one of the three different TAC chimeras and, as a negative control HeLa cells that did not express any of the TAC receptors, were plated in a 96 well dish (15,000 cells/well). The next day the cells were washed once with PBS (100 μL/well), fixed for 10 min with 2%

paraformaldehye (100 μL/well), and blocked for 15 min with PBS/sera (100 μL/well). Next, the cells were incubated with anti-TAC primary antibody (7G7) (32 ng in 100 μL PBS/sera per well) for 1 h at room temperature. Cells were then washed 4 times with PBS (100 μL/well) and incubated with HRP conjugated rabbit anti-mouse secondary antibody (diluted 5000 fold in PBS/sera) for 1 hr at room temperature. Following four additional washes with PBS the wells were developed for 5 min with OPD solution (100 μL/well) (10 mg OPD, 10 μl H_2O_2 in 25 mL of substrate buffer (24 mM citric acid-monohydrate, 51 mM Na_2HPO_4-$7H_20$, pH 5.0)). The reaction was stopped with 8N sulfuric acid (50 μL/well) and the optical density at 490 nm (OD_{490}) measured using an absorbance plate reader and the non-specific background at 650 nm (OD_{650}) subtracted. Values for each point are the average of at least triplicate wells.

Construction of modified *env* gene. A pcDNA3.1+/Neo derived expression plasmid (pAScFvTAC) encoding for a TAC-binding amphotropic envelope protein with the structure 5' – ecotropic envelope signal peptide – *Nhe*I site – ScFv TAC (single chain antibody against TAC) – *Not*I site – *Fse*I site – amphotropic envelope protein – 3' was constructed as follows: (1) a 3000 bp fragment containing the ecotropic Moloney-MLV envelope protein was generated by digestion of pMOV9.2 (a kind gift of J.G. Sutcliffe (40)) with *Nhe*I and *Hind*III, and then ligated to pcDNA3.1+/Neo (Invitrogen, Carlsbad, CA) digested with *Xba*I and *Hind*III. (2) Non-coding sequences 5' of the start codon of the ecotropic Moloney-MLV envelope protein were removed from the resulting plasmid by digestion with *Xba*I and *Nhe*I to form pEcoStart, an ecotropic envelope expression vector. (3) To append the restriction sites *Nhe*I, *Not*I, and *Fse*I to the 3' end of the signal peptide sequence of pEcoStart, we generated two amplicons, EcoUpstream and EcoDownstream, with 18 overlapping (complimentary) bases at their 3' and 5' ends, respectively. We used PCR of pEcoStart with the primers FWD1 (CAAGAGTTACTAACAGCC) and REV1 (GGCCGCATTGGCGGCCGCAGCATTGCTAGCAGTACTGACCCCTCTGAG) to generate EcoUpstream, an amplicon that contained the restriction sites *Nhe*I, *Not*I and *Fse*I at

the 3' end of the signal peptide sequence. Similarly, we used PCR of pEcoStart with the primers FWD2 (GCGGCCGCCAATGCGGCCGGCCAGGCTTCGCCCGGCTCCAGT) and REV2 (TGAGTCCGATCCCAAATG) to generate EcoDownstream. The amplicon EcoUpDown was then generated by PCR amplification of EcoUpstream and EcoDownstream using the primers FWD1 and REV2. EcoUpDown and pEcoStart were digested with *Xba*I and *BamH*I, and then the resulting fragments ligated to create pSP*Nhe*I*Not*I*Fse*IEco. (4) To replace the ecotropic envelope sequence of pSP*Nhe*I*Not*I*Fse*IEco with the amphotropic envelope sequence, we PCR amplified pFB4070ASALF, a plasmid that encodes for the amphotropic envelope protein (a kind gift of Stephen Russell (3)) with the primers AAATATGGCCGGCCAGATGGCAGAGAGCCCCC and AGCGATGTTTAAACCTCATGGCTCGTACTCTATGG, to generate an amplicon that consisted of the amphotropic envelope sequence with the restriction sites *Fse*I at the 5' end and *Pme*I at the 3' end. We digested this amplicon and pSP*Nhe*I*Not*I*Fse*IEco with *Fse*I and *Pme*I (*Pme*I was a unique restriction site at the 3' end of the sequence for the ecotropic envelope), then ligated the resulting fragments to form pSP*Nhe*I*Not*I*Fse*IAmpho. (5) Next, we PCR amplified pRK78 (a plasmid the encodes TAC ScFv, a kind gift of Dr. Pastan (15)) with the primers CAAGAGGCTAGCATGCAGGTCCATCTGCAG and AGTTCAGCGGCCGCTTTGAGCTCCAGCTTGGT to generate an amplicon that consisted of the TAC ScFv sequence with the restriction site *Nhe*I at its 5' end and *Not*I at its 3' end. We digested this amplicon and pSP*Nhe*I*Not*I*Fse*IAmpho with *Nhe*I and *Not*I, then ligated the resulting fragments to form pAScFvTAC. The structure of pAScFvTAC was verified by DNA sequencing. (6) Finally, using primers GCAGAAGGTACCATGGCGCGTTCAACGC and GGCCGCAGATCTTCATGGCTCGTACTCTATGGG we PCR amplified pAScFvTAC to generate an amplicon that consisted of the AScFvTAC sequence with the restriction site *Asp718*I at its 5' end and *Bg*III at its 3' end. This amplicon and the viral envelope expression vector pCAGGS.MCS (33) were digested with *Asp718*I and *Bg*III and then ligated to each other to form

pCAGGS-AScFvTAC. As a control we PCR amplified pFB4070ASALF with primers GCATAAGGTACCATGGCGCGTTCAACGC and GGCCGCAGATCTTCATGGCTCGTACTCTATGGG to generate an amplicon that consisted of the amphotropic envelope sequence with the restriction sites Asp*718*I and *Bg*III. This amplicon and pCAGGS.MCS were subsequently digested with Asp*718*I and *Bg*III and ligated to each other to form pCAGGS-A.

Virus production. To produce MLV retroviruses pseudotyped with the amphotropic or modified amphotropic (A-ScFv-Tac) envelope protein, TELCeB6 cells were plated in a 10 cm dish (1.7 x 10^7 cells/dish), and the next day transiently transfected with Lipofectamine-2000 and 24 μg of pCAGGS-AScFvTAC or pCAGGS-A, respectively. Thirty-six, forty-eight, and sixty hours after transfection, virus supernatants were collected, filtered (0.45 μm), and frozen (-80°C) for later use. To produce GFP-labeled lentiviruses, 293T/17 cells were plated in a 10 cm dish (1.7 x 10^7 cells/dish) and co-transfected with Lipofectamine-2000 and 6 μg each of plasmid DNA encoding the lentiviral packaging construct pCMVΔR8.91 (kind gift of Scott S. Case), lentivirus vector pTY-EFnlacZ (AIDS Research and Reference Reagent Program, Bethesda, MD), GFP-Vpr (26) and pCAGGS-AScFvTAC or pCAGGS-A. Virus laden tissue culture medium was harvested 36, 48, and 60 h after transfection, filtered (0.45 μm), and frozen (-80°C) for later use.

Soluble envelope protein binding assay. Te671 cells were plated in a 12 well dish (10^6 cells/well) and transfected with 2 μg pCAGGS-AScFvTAC or pCAGGS-A using Lipofectamine 2000. Conditioned cell culture medium, which contained soluble envelope proteins shed from the surfaces of the transfected cells, was harvested 36, 48, and 60 h after transfection, filtered (0.45 μm) and frozen (-80°C) for later use. Target CHO cells were plated the previous day on coverslips in a 12 well dish (2.4 X 10^5 cells/well), and then transiently transfected with 2 μg of an expression plasmid for TAC-CD16 using Lipofectamine 2000. Forty-

eight hours later the cells were placed in conditioned medium (1 mL/well) that contained either A-ScFv-TAC or amphotropic soluble envelope proteins, centrifuged for 30 min at 37°C and 2100 × g (Allegra 6R; Beckman Coulter, Palo Alto, CA), incubated an additional 30 min at 37°C, washed with PBS, fixed with 2% paraformaldehyde for 10 min, and then blocked with PBS/sera for 15 min. To detect bound envelope proteins, cells were immunostained with a rat monoclonal antibody (83A25) against gp70 (diluted 250 fold in PBS/sera) for 1 h at room temperature, washed 3 times with PBS and then incubated with Cy2 conjugated donkey anti-rat (diluted 200 fold in PBS/sera) for 1 h at room temperature. Following 3 additional washes with PBS the cells were fixed (2% paraformaldehyde) and blocked with PBS/sera for 15 min. To detect TAC-CD16 expression, cells were immunostained with a mouse monoclonal antibody (7G7) against TAC (diluted 64 fold in PBS/sera) for 1 h at room temperature, washed 3 times with PBS and incubated with Cy3 conjugated donkey anti-mouse secondary antibody (diluted 800 fold in PBS/sera) for 1 h at room temperature. Following 3 additional washes with PBS the cells were mounted on glass slides using gelvatol and visualized by confocal microscopy. As a control, parallel experiments were conducted in which cells were incubated with Zenapax (40 μg/mL) for 1 h at 4°C, prior to their exposure to conditioned medium that contained soluble A-ScFv-TAC.

Diluted virus titer assay. Serial dilutions of virus stock were made in DMEM/FBS and Polybrene (20 μg/mL). A total of 1 mL per well was used to transduce HeLa cells seeded the previous day in a 12-well dish (1 x 10^5 cells/well). Virus was centrifuged onto the cells for 30 min at 37°C and 2100 × g. The transduced cells were incubated for 2 d at 37°C until confluent, fixed and stained for *lacZ* activity with X-Gal, colonies of *lacZ*+ cells counted, and the titer (CFU/mL) calculated as previously described (28). To verify that the virus titers were due to bonafide retrovirus transduction events that required reverse transcriptase activity, we transduced cells in the presence of AZT (2 μM). To verify that the differences we observed in virus titers were due to interactions with TAC receptors, we incubated cells with anti-TAC

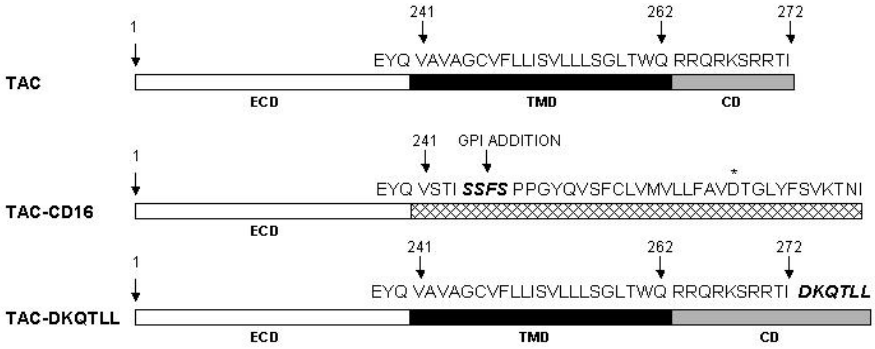
function blocking antibody (Zenapax, 40 μg/mL) 1 h before and during transduction in the virus titer assay.

Statistics. To analyze the data statistically, we performed one-way analysis of variance for repeated measurements of the same variable. We then use the Tukey multiple comparison test to conduct pairwise comparisons between means. We considered differences significant at $p < 0.05$.

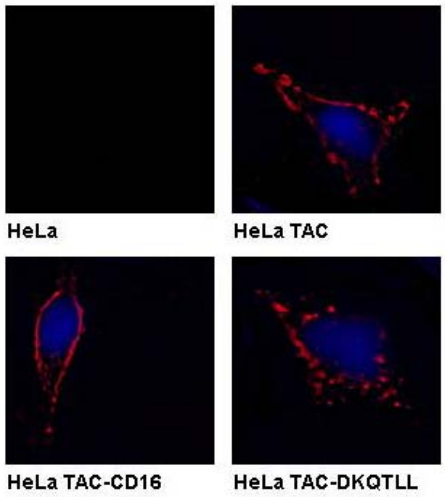
2.4 Results

Generation and characterization of HeLa cells that stably express chimeric TAC receptors. As a first step towards investigating the effects that the trafficking itinerary of virus-binding proteins has on retrovirus transduction, we isolated three HeLa cell lines, each of which stably expressed a chimeric TAC (CD25) receptor with a distinct intracellular trafficking itinerary. HeLa cells were transfected with expression vectors that encoded TAC, TAC-CD16, and TAC-DKQTLL (Figure 2.1a), plated at clonal density, and then cultured with neomycin (500 μg/mL) to eliminate cells that were not stably transfected. Stably transfected clonal cell lines that expressed the highest levels of each TAC chimera were identified by immunostaining with an anti-TAC monoclonal antibody (7G7), then expanded and frozen for later use.

To characterize differences in the cellular localization and intracellular trafficking itineraries of the TAC chimeras in these clonal cell lines, we examined their cell-surface distribution, including whether or not they localized to lipid rafts, and measured their rates of internalization. We fixed, permeabilized, and stained the cells with 7G7 and a fluorescently-labeled secondary antibody against 7G7, then visualized the cells by confocal microscopy (Figure 2.1b). Cells that expressed TAC and TAC-CD16 showed characteristic cell surface staining, whereas cells that expressed TAC-DKQTLL showed discrete vesicular staining that was consistent with localization of the receptor to the lysosomes.



a.



b.

Figure 2.1 HeLa cell lines stably express TAC chimeric proteins. (a) Summary of the COOH-terminal sequences of TAC, TAC-CD16 and TAC-DKQTLL. All three constructs have identical extracellular domains. TAC-CD16 has a signaling motif for GPI addition (indicated with an arrow). The C-terminal 5 amino acids of TAC-DKQTLL target the protein to the clathrin coated pit internalization pathway. The amino acids are numbered from the N-terminal methionine. An asterisk denotes the charged amino acid in the GPI signal. ECD: extracellular domain; TMD: transmembrane domain; CD: cytoplasmic domain (b) Immunofluorescence localization of TAC chimeric proteins in stably transfected HeLa cells. HeLa cells stably expressing TAC chimeric proteins were fixed and stained with a mouse monoclonal antibody to TAC (7G7), followed by Cy3 conjugated donkey antibodies (red) to mouse IgG. Nuclei were stained with DAPI (blue). Micrographs show representative cells visualized by confocal microscopy with a 40x objective (Zeiss LSM 510). TAC and TAC-CD16 are localized to the plasma membrane whereas TAC-DKQTLL is predominantly distributed within cytoplasmic vesicles, which is consistent with its expected localization to the lysosomes.

To determine if the TAC chimeras localized to lipid rafts, we transfected the HeLa cell lines expressing the three TAC chimeras with the wild-type folate receptor-α, an established marker of lipid rafts, or MCP, a mutant of the folate receptor that does not localize to lipid rafts. Two days later, detergent-resistant microdomains (lipid rafts) were extracted from the transfected cells with 1% Triton X-100 and then purified by equilibrium flotation centrifugation. Western blot analysis revealed that TAC-CD16, but not TAC or TAC-DKQTLL, localized to lipid-rafts (Figure 2.2). As expected, the wild-type folate receptor-α, but not MCP, was detected in the detergent-resistant fractions. In addition, Western blots of whole-cell lysates showed that all the cell lines tested expressed detectable levels of the TAC and folate receptors, which confirmed that our inability to detect TAC and TAC-DKQTLL in the lipid-raft fractions was because those receptors do not localize to lipid rafts, and not because their expression levels were too low to be detected (data not shown).

We also measured the internalization rates of the TAC chimeras using the fact that with fluorescence microscopy it is possible to detect the presence of two molecules in the same location at the same time in a cell (co-localization). Cell-surface TAC receptors were labeled with 7G7 for 1.5 h at 4°C, then the cells rapidly warmed and incubated at 37°C for up to 3 h to allow the TAC receptors, and the antibodies that were bound to them, to become internalized. The cells were fixed and stained with a Cy3 conjugated anti-mouse antibody to label the 7G7 antibody and with fluorescently labeled concanavalin A to label the surfaces of the cells. Images of the cells were taken by confocal microscopy and analyzed to quantify the rate at which the level of TAC receptors on the cell surface changed with time (Figure 2.3). We found, as expected, that the levels of cell-surface TAC and TAC-CD16 were stable whereas the levels of cell-surface TAC-DKQTLL declined rapidly with a half time of less than 10 minutes. Given the differences in the internalization rates of the TAC receptors, we wondered if there were also significant differences in the numbers of TAC receptors expressed on the cell surface. Using a

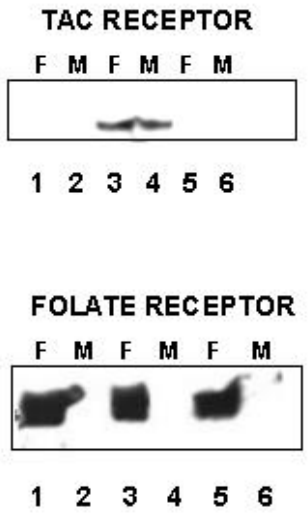
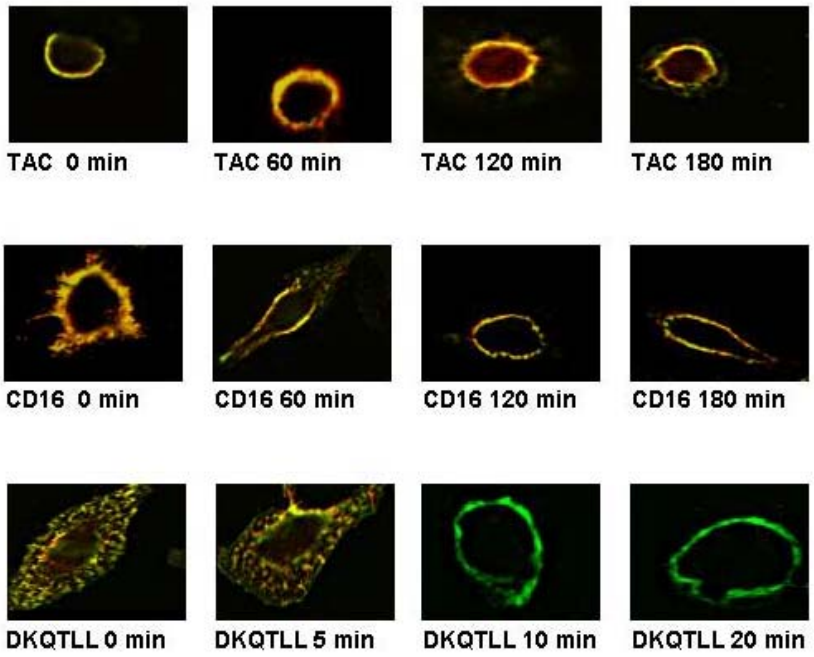
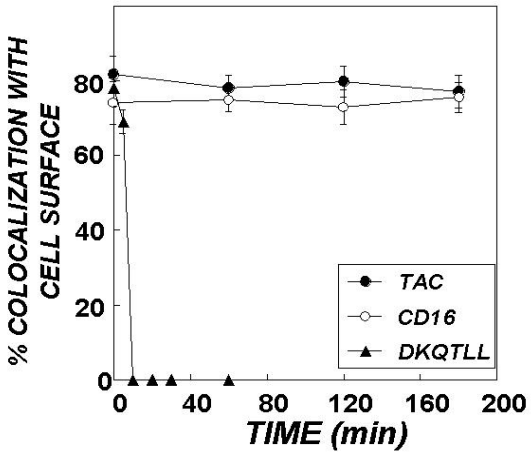


Figure 2.2 TAC-CD16 is associated with lipid rafts. Detergent resistant domains (lipid rafts) were isolated from HeLa cells expressing one of the three TAC chimeras and either the wild type folate receptor-α (F), which localizes to lipid rafts, or the mutant folate receptor MCP (M), which does not localize to lipid rafts. Cells were lysed with ice-cold Triton X-100 (1% in TNE), subjected to equilibrium flotation centrifugation, the lipid raft fraction pelleted by centrifugation, then the pellet analyzed by Western blot for the presence of TAC (top panel), and folate receptor-α and MCP (bottom panel). Lipid raft fractions from HeLa cells expressing TAC and folate receptor-α (lane 1) or MCP (lane 2), TAC-CD16 and folate receptor-α (lane 3) or MCP (lane 4), or TAC-DKQTLL and folate receptor-α (lane 5) or MCP (lane 6) were visualized by chemiluminescence. As expected, folate receptor-α was detected in the lipid raft fractions (bottom panel, lanes 1, 3 and 5) whereas MCP was not (bottom panel, lanes 2, 4 and 6).



a.



b.

Figure 2.3 Internalization kinetics of TAC chimeras. HeLa cells expressing a TAC chimeric protein were chilled on ice for 30 min to block endocytosis, incubated with a mouse monoclonal antibody to TAC (7G7) for 1.5 h at 4^{o}C, rapidly warmed and incubated at 37^{o}C for 0 to 3 h to allow the antibody-labeled TAC proteins to internalize, and then stained on ice with Alexa-488-conjugated concanavalin A (green). Concanavalin-stained cells were fixed and stained with Cy3 conjugated antibodies (red) against mouse IgG to detect the TAC proteins. Cells were (a) visualized by confocal microscopy and (b) the extent to which the TAC proteins co-localized (yellow) with the cell-surface marker concanavalin A quantified using image analysis software (Metamorph 6.0). No significant level of internalization of TAC or TAC-CD16 was detected, whereas TAC-DKQTLL was rapidly internalized with a half-time of less than 10 min.

cell based ELISA for TAC, we found that the same number of TAC and TAC-CD16 receptors, but 2.4-fold fewer TAC-DKQTLL receptors, were expressed on the surfaces of the cells (Figure 2.4). As expected, the parent HeLa cell line did not express any TAC receptors.

Taken together, our results show that we had successfully developed HeLa cell lines that stably expressed TAC receptors with distinct intracellular trafficking itineraries and subcellular distributions. HeLa-TAC cells express TAC receptors with long residence times on the cell surface that do not preferentially localize to lipid rafts, HeLa-TAC-CD16 cells express TAC receptors with long residence times on the cell surface that primarily localize to lipid rafts, and HeLa-TAC-DKQTLL cells express TAC receptors that are rapidly internalized from the cell surface.

Construction of retrovirus envelope proteins targeted to TAC. To examine the effect of the trafficking itinerary of virus-binding proteins on retrovirus transduction, we constructed a modified envelope protein designed to bind to the TAC receptors by fusing the sequence for a single-chain antibody (ScFv) against TAC to the N-terminus of the amphotropic envelope protein (Figure 2.5). To produce retrovirus that bind to TAC, plasmids encoding the modified envelope protein were transiently transfected into TELCeB6 cells, which express MLV Gag-Pol core particles and an nlslacZ retroviral vector. Envelope expression by these cells was examined by fixing and staining them with an antibody (83A25) against the amphotropic envelope protein (Figure 2.6a). As controls, cells that expressed no envelope protein and cells that expressed the wild-type envelope protein were also examined. Cell surface expression of the modified envelope proteins was clearly visible in transfected cells, which suggested that the proteins were correctly expressed and processed, although they were present at lower levels as compared to cells that expressed the wild-type amphotropic envelope protein.

Modified envelope proteins are incorporated into virus particles and bind to TAC. To determine if the modified envelope proteins were incorporated into retrovirus particles, virus supernatants were harvested, concentrated, separated by size by gel electrophoresis, and then

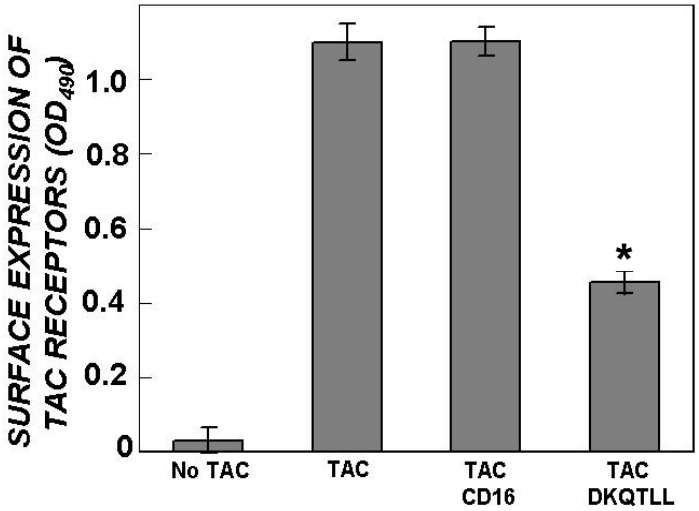


Figure 2.4 Surface expression levels of TAC chimeras. HeLa cells expressing one of the three different TAC chimeras and, as a negative control HeLa cells that did not express any of the TAC receptors, were plated the previous day in a 96 well dish (15,000 cells per well), fixed (2% paraformaldehye), blocked (PBS/sera), and then sequentially stained with anti-TAC primary antibody (7G7) and an HRP-conjugated rabbit anti-mouse secondary antibody. Stained cells were developed with a solution of OPD and the optical density at 490 nm (OD_{490}) measured using an absorbance plate reader and the non-specific background at 650 nm (OD_{650}) subtracted. Values for each point are the average of at least triplicate wells. (*) denotes statistically significant differences ($p < 0.05$).

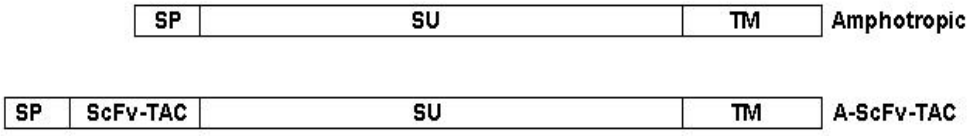
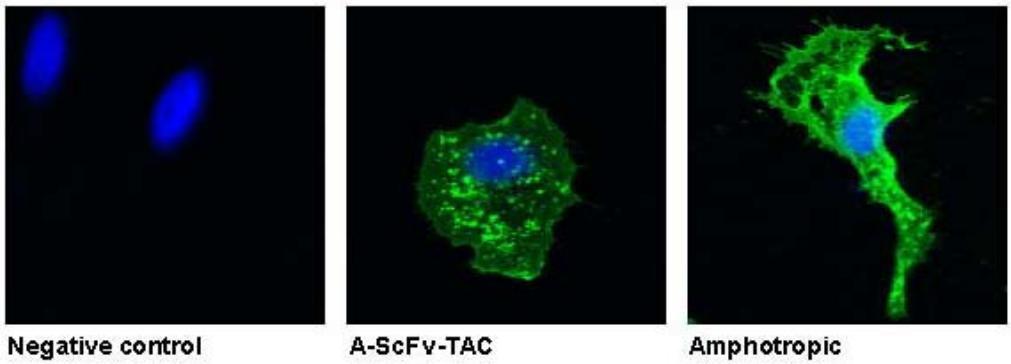


Figure 2.5 Schematic diagram of envelope proteins. A schematic diagram of the structure of the wild-type amphotropic envelope protein (amphotropic) and the anti-TAC ScFv amphotropic envelope fusion protein (A-ScFv-TAC) are shown. The positions of some functional regions are indicated: SP, signal peptide; SU, surface protein; TM, transmembrane protein.



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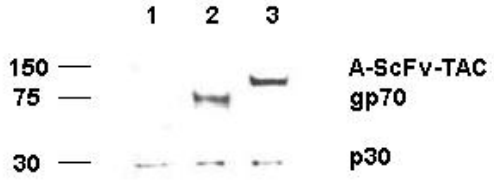


b.

Figure 2.6 TAC-binding amphotropic envelope proteins are expressed on the surface of virus producer cells and incorporated into virus particles. (a) Envelope protein expression on the surface of virus producer cells. TELCeB6 cells were transiently transfected to express the TAC-binding amphotropic envelope protein (A-ScFv-TAC), the wild-type amphotropic envelope protein (amphotropic), or no envelope protein (negative control), and then fixed but not permeabilized, immunostained with a rat monoclonal antibody (83A25) against the gp70 envelope protein and donkey anti-rat Cy2 conjugated secondary antibody (green), and visualized by confocal microscopy. (b) TAC-binding envelope proteins are incorporated into virus particles. Supernatants from virus producer cells were concentrated 18-fold, separated by size by SDS-PAGE electrophoresis, transferred to a PVDF membrane, and the retrovirus proteins detected using goat anti-sera against gp70 (79S834) and p30 (78S221) and a chemiluminescent detection system. Supernatants from TelCeB6 cells that produced retroviruses with no envelope proteins (lane 1), amphotropic envelope proteins (lane 2), and TAC-binding amphotropic envelope proteins (lane 3) are shown. The numbers on the left are molecular weights in kilodaltons.

the Gag (p30; CA) and envelope protein content (Figure 2.6b) quantified by Western blot. As controls, supernatant from cells that produced viruses pseudotyped with the wild-type amphotropic envelope or with no envelope protein were also examined. Envelope proteins were detected in the wild-type (70 kD) and TAC-binding (113 kD) retrovirus stocks, but not, as expected, in the virus stocks produced by cells that did not express any envelope protein.

To determine if the modified envelope proteins (A-ScFv-TAC) bind to TAC as expected, we compared the extent to which the soluble form of A-ScFv-TAC bound to CHO cells that were transiently transfected with an expression plasmid encoding for the TAC-CD16 receptor versus the extent to which they bound to unmodified CHO cells which do not normally express either TAC or the amphotropic retrovirus receptor (Pit-2). CHO cells were placed in medium that contained the soluble form of the modified or amphotropic envelope proteins, centrifuged for 30 min at 37°C, cultured an additional 30 minutes at 37°C, fixed and stained for envelope protein and TAC-CD16, and then visualized by confocal microscopy (Figure 2.7). As a control, we pretreated parallel cultures of cells with Zenapax (40 μg/mL), an antibody that blocks the ability of anti-TAC ScFv to bind to TAC. Binding was only observed when the modified envelope proteins were incubated with CHO cells that expressed TAC-CD16. Zenapax blocked binding of the modified envelope, which confirmed that binding was due to a specific interaction between the anti-TAC ScFv domain of the modified envelope and the TAC-CD16 receptor. As expected, no binding was observed when the unmodified amphotropic envelope protein was incubated with the cells. Taken together, these data showed that the modified envelope proteins were properly expressed, processed, and incorporated into retrovirus particles, and were able to bind to cell surface TAC receptors.

Transduction with retroviruses targeted to TAC receptors with different intracellular trafficking itineraries. To determine if the different cellular distributions and trafficking itineraries of the TAC receptors affects the ability of the TAC-binding retroviruses to

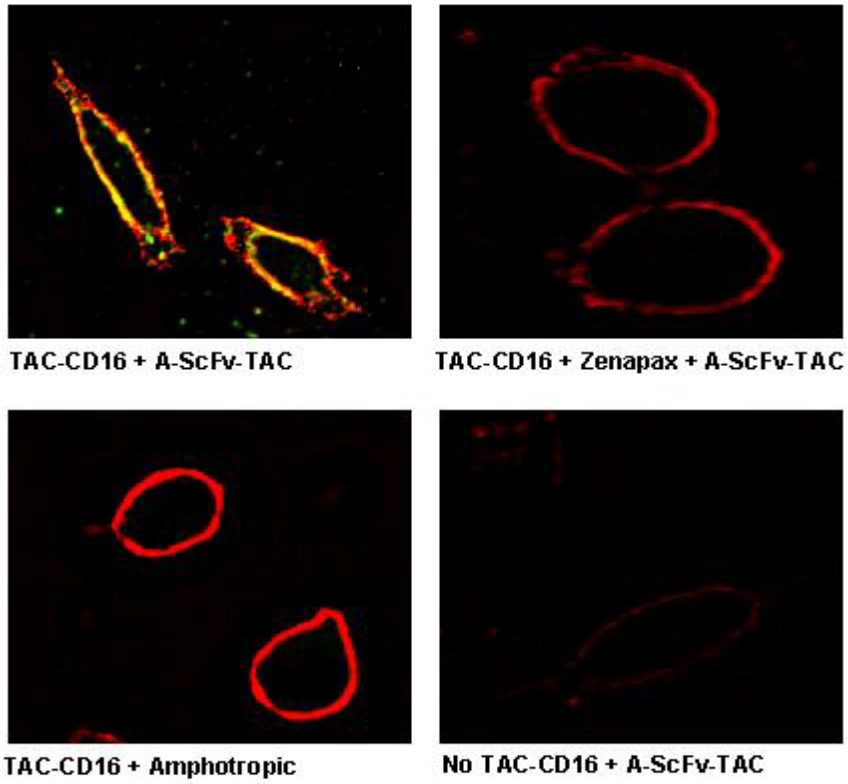


Figure 2.7 Modified envelope protein (A-ScFv-TAC) binds to TAC-CD16. CHO cells, transiently transfected to express TAC-CD16, were placed in medium that contained soluble A-ScFv-TAC (upper left panel), soluble A-ScFv-TAC and Zenapax (upper right panel), or soluble amphotropic envelope protein (lower left panel). As a control, CHO cells that were not transfected to express TAC-CD16 were incubated with soluble A-ScFv-TAC (lower right panel). Cells were centrifuged (2100 x g, 30 min, 37°C), incubated an additional 30 min at 37°C, then fixed and immunostained for envelope and TAC-CD16. To detect envelope proteins, cells were incubated with a monoclonal antibody against gp70 (83A25), and then with a secondary Cy2 conjugated antibody. To detect TAC-CD16, cells were incubated with a monoclonal antibody against TAC (7G7), and then with a secondary Cy3 conjugated antibody. The cells were visualized by confocal microscopy. Overlays of green (envelope) and red (TAC-CD16) fluorescence are shown. Colocalizations appear as yellow.

transduce cells, we measured the titer of a stock of TAC-binding viruses on each of the HeLa cell lines that uniquely expressed one of the three TAC chimeras. As a control, we also measured the titer of the virus stock on the parent HeLa cell line that did not express any of the TAC chimeras. We found that virus titer was affected by expression of TAC (Figure 2.8). The virus titer on HeLa cells that expressed TAC-DKQTLL was nearly 4-fold lower than on the control HeLa cells that did not express any of the TAC chimeras. In contrast, the virus titer on HeLa cells that expressed TAC was slightly higher (1.6 fold) than on the control cell line, whereas TAC-CD16 expression had no effect on titer.

To confirm that these differences in titers were due to interactions between the retroviruses and the TAC receptors, we examined the effect on transduction of a function-blocking antibody against TAC. Cells, pretreated with the anti-TAC antibody for one hour, were incubated with virus stocks that contained the anti-TAC antibody, grown to confluence, then fixed and stained for beta-galactosidase activity in the virus titer assay. Transduction in the presence of the anti-TAC antibody virtually eliminated the differences in the virus titers on the different cell lines. To verify these differences were not due to differences in the growth rates of the cell lines, we used the MTT assay (27) to measure the growth rates of the cells and found that there were no statistically significant differences among the cell lines (Figure 2.9). In addition, transduction was completely blocked when AZT (2 μM) was added to the cell culture medium, which confirmed that the virus titers were due to bonafide retrovirus transduction events and were not due to pseudo-transduction of the cells with the beta-galactosidase enzyme.

To determine if the intracellular trafficking itinerary of the viruses was affected by interactions with TAC-DKQTLL, we established a cohort of GFP-labeled lentiviruses, pseudotyped with either A-ScFv-TAC or the amphotropic envelope protein, on the surfaces of HeLa cells that expressed the TAC-DKQTLL receptor by centrifuging the virus onto the cells at 4°C for 30 min at 2100 $\times$ g. After centrifugation, we placed the cells in medium pre-warmed to

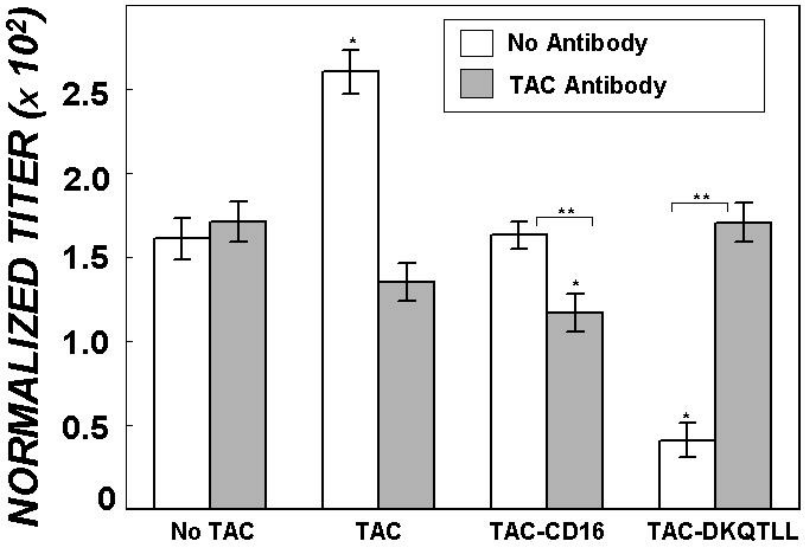


Figure 2.8 Transduction of HeLa cells that express TAC receptors with different intracellular trafficking itineraries. HeLa cells that do not express TAC (No TAC), or that stably express the wild-type TAC receptor (TAC), or chimeras of the TAC receptor that are localized to lipid rafts (TAC-CD16), or that internalize via the clathrin-coated pit pathway (TAC-DKQTLL), were plated in a 12 well-dish (100,000 cells/well), and transduced the next day with TAC-binding amphotropic retrovirus (1 mL/well) that was brought to 20 μg/mL Polybrene and 0 μg/mL (white bars; No antibody) or 40 μg/mL (shaded bars; TAC antibody) of an anti-TAC function blocking antibody (Zenapax). Transduced cells were incubated for 2 d at 37^0C until confluent, fixed and stained for *lacZ* activity with X-gal, colonies of *lacZ*$^+$ cells counted, and the titer (CFU/mL) calculated as described in the Materials and Methods. To control for TAC independent differences among the HeLa cell lines in their susceptibility to transduction, we normalized titers to the titer of amphotropic virus on HeLa cells. (*) denotes statistically significant differences ($p < 0.05$) from the virus titer on HeLa cells that do not express TAC in the absence of Zenapax. (**) denotes statistically significant differences ($p < 0.05$) between the virus titers indicated by the brackets.

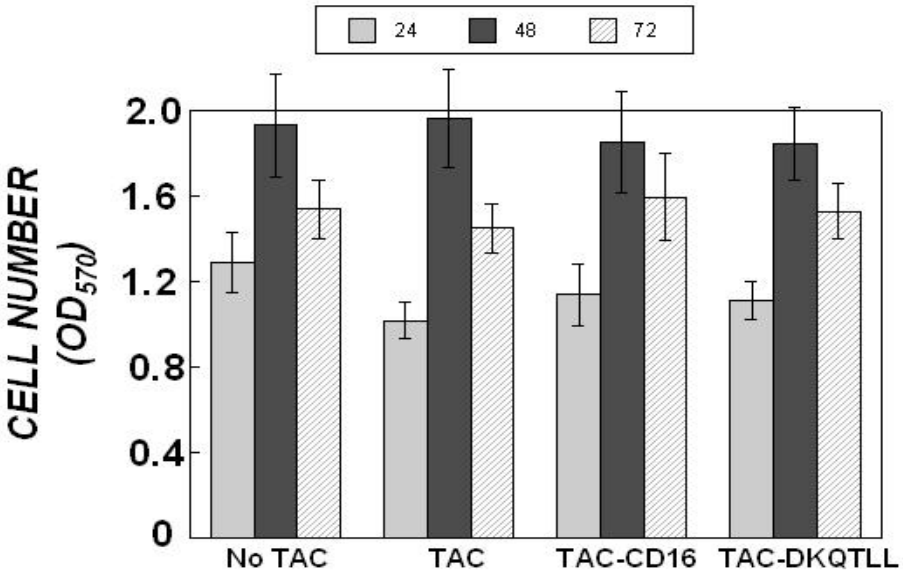
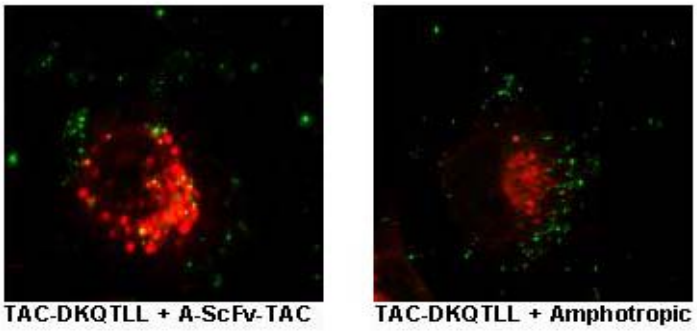


Figure 2.9 Growth rate of HeLa cell lines. HeLa cells that do not express TAC (No TAC), or that stably express the three different TAC chimeras were plated at 10,000 cells per well and at indicated time points post plating assayed for cell viability. 10 μL per well of MTT solution (100 mg of MTT in 1 mL of PBS) were added per well to cells in a 96-well plate. The plate was incubated for 4 hours at 37°C, then 150 μL of 10% SDS were added per well and the plate incubated overnight. The optical density at 570 nm was measured using an absorbance plate reader and the non-specific background at 650nm subtracted. Values for replicate wells without cells were subtracted as background. Values for each point are the average of triplicate wells.

37°C, allowed the viruses to internalize for 1 hour, and then fixed and immunostained the cells for TAC-DKQTLL. We visualized virus (green) and TAC-DKQTLL (red) by confocal microscopy (Figure 2.10a) and quantified the degree to which they were co-localized (Figure 2.10b). Lentiviruses that were pseudotyped with the TAC-binding envelope protein (A-ScFv-TAC) co-localized with TAC-DKQTLL to a much greater extent than lentiviruses that were pseudotyped with the amphotropic envelope protein. Taken together, these results suggest that the differences we observed in the virus titers were the result of interactions between the viruses and the TAC receptors, and that the likelihood a virus will successfully transduce a cell is influenced in part by the cellular distribution and trafficking itinerary of the cellular proteins with which the virus interacts.

2.5 Discussion

We have developed an experimental system to examine the effect of retrovirus trafficking on transduction. Our system consists of three model target cell lines, derived from HeLa cells, that stably express one of three chimeras of the interleukin-2 receptor alpha chain (CD25/TAC) that have identical extracellular domains but different intracellular trafficking itineraries, and a targeted murine leukemia virus whose amphotropic envelope proteins were modified to include a binding site for TAC at their N-termini. Similar to other viruses pseudotyped with N-terminal modified envelope proteins, our TAC-modified viruses required the presence of amphotropic receptors to transduce cells (the titer of TAC-modified virus on CHO cells that do not express the amphotropic receptor but were transfected to express TAC-CD16 was not detectable), most likely because interactions between viruses and TAC receptors were not sufficient to induce the viruses to fuse with the cells.



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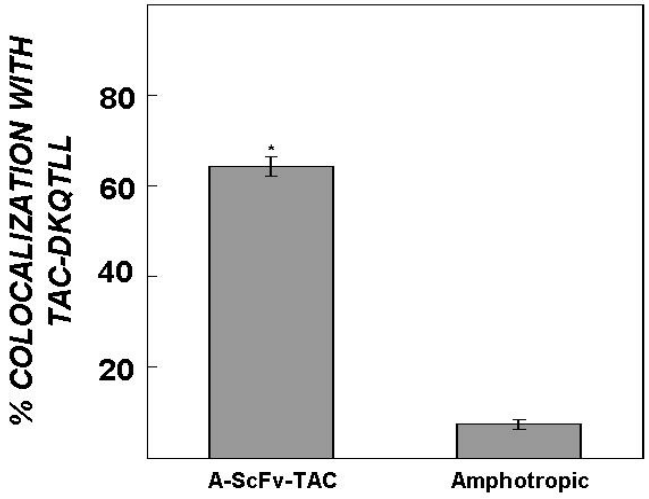

b.

Figure 2.10 Virus pseudotyped with A-ScFv-TAC colocalizes with the TAC-DKQTLL receptor. HeLa cells expressing TAC-DKQTLL were cultured at 4°C for 1 h to block endocytosis, and then placed in cold (4°C) viral supernatant that contained GFP-labeled lentiviruses pseudotyped with the TAC-binding envelope protein (A-ScFv-TAC) or the amphotropic envelope protein. Cells were centrifuged (2100 x g, 30 min, 4°C), transferred to fresh cell culture medium that had been pre-warmed to 37°C, incubated for 1 h, and then fixed, permeabilized, and immunostained for TAC-DKQTLL using a monoclonal antibody against TAC (7G7) and a Cy3 conjugated secondary antibody. (a) Cells were visualized by confocal microscopy. Overlays of green (virus) and red (TAC-DKQTLL) fluorescence are shown. Colocalizations appear as yellow. (b) For each experiment eight cells were chosen at random and the extent of co-localization of virus and TAC-DKQTLL quantified using Metamorph Imaging System Software. (*) denotes statistically significant differences ($p < 0.05$).

Using our novel experimental system, we found that the efficiency with which recombinant retroviruses transduce cells appears to be a function of the distribution and trafficking itinerary of virus-binding proteins. Transduction of cells that expressed TAC-DKQTLL, which is rapidly internalized from the cell surface, was nearly 4-fold lower than transduction of control cells that did not express any of the TAC receptors. In contrast, transduction of HeLa cells that expressed TAC, which has a long residence time on the cell surface, was slightly higher (1.6 fold) than transduction of control cells. Transduction was not significantly affected by the expression of TAC-CD16, which has a long residence time on the cell surface but which is preferentially localized to lipid rafts. In addition, we found that TAC-binding viruses were more likely to co-localize with TAC-DKQTLL receptors located within the cell than were viruses that did not bind to TAC, which suggests that targeted receptors can significantly alter the intracellular fate of the viruses with which they interact.

The influence of virus-binding proteins on the trafficking of recombinant retroviruses designed for human gene transfer has not been previously systematically examined, but our findings are consistent with studies that have explored the effects of receptor trafficking on transduction. For example, a recent study found that retroviruses pseudotyped with avian sarcoma and leukosis subgroup A envelope proteins (ASLV-A) transduce cells more efficiently when the cells express a GPI-anchored form of the virus receptor than when they express a transmembrane form of the receptor, apparently because the viruses are subjected to different intracellular fates depending upon which receptor they interact with (31). Similarly, another study has shown that binding of virus-anchored ICAM-1 to its cellular counterligand lymphocyte-function associated antigen 1 (LFA-1) enhances HIV-1 binding, uptake and infection. Apparently, interactions between ICAM-1 and LFA-1 redirects virus entry toward a more productive infection pathway (45). Taken together, these studies and our results strongly suggest that virus-binding proteins can alter the pathway of virus entry and the efficiency of infection, although further studies are needed to confirm this hypothesis.

We do not know the mechanism by which the trafficking itinerary of the TAC receptors affected virus transduction. One possibility is that virus interactions with TAC changed the amount of time the viruses resided in locations of the cell that contained amphotropic (fusion-competent) receptors. Viruses that bind to TAC receptors that move rapidly through the regions of the cell that contain amphotropic receptors might be less likely to fuse with and infect cells than viruses that are bound to TAC receptors that remain in those regions for longer periods of time. Since amphotropic receptors reside almost exclusively on the cell surface (37), we considered it a possibility that transduction would be less efficient with cells that expressed TAC-DKQTLL, which has a short residence time on the cell surface, than with cells that expressed TAC receptors with long residence times such as TAC and TAC-CD16. We found that transduction was, in fact, several-fold less efficient in HeLa cells that expressed TAC-DKQTLL than in control cells that expressed no TAC, or in cells that expressed TAC or TAC-CD16. Although we can only speculate, transduction may have been reduced even more in the cells that expressed TAC-DKQTLL if these receptors had been expressed on the cell surface at the same levels as were the TAC and TAC-CD16 receptors in the other HeLa cell lines.

Virus interactions with TAC might also have affected transduction by altering the cellular distribution of the viruses to regions of the cell that differed significantly in the local concentration of the amphotropic receptor. The efficiency of retrovirus infection increases with increasing receptor concentration, most likely because the presence of more receptors facilitates the formation of the multivalent receptor-envelope protein complexes that are needed for retroviruses to fuse with cells (16, 34, 41). Therefore, we would expect transduction to be low in cells that expressed chimeras of the TAC receptor that are located in regions of the cell that contained few or no amphotropic receptors, and unchanged or possibly higher in cells that expressed chimeras of the TAC receptor that are located in regions of the cells that contained many amphotropic receptors. Interestingly, we found that cells expressing TAC, a cell surface receptor that does not preferentially localize to lipid rafts, were transduced 1.6-fold more

efficiently than cells that expressed TAC-CD16, a cell surface receptor that is primarily localized within lipid rafts, results that may indicate that the distribution of amphotropic receptors on the cell surface is not random. Moreover it is possible that the mechanism by which TAC-DKQTLL decreases gene transfer efficiency of TAC-binding virus is through unfavorable positioning of the virus on the cell surface, resulting in virus sequestration away from the amphotropic receptors. This suggests that the amphotropic and TAC-DKQTLL receptors do not colocalize on the cell surface.

It is also possible that interactions with TAC caused viruses to become localized to regions of the cell that differed in aspects of the microenvironment that were important for virus infection. For example, some viruses (e.g., influenza) require passage through a low pH subcellular compartment in order to infect cells, whereas others do not, and may, in fact, even be impeded in their ability to infect cells by passage through a low pH subcellular compartment (e.g., HIV-1) (7, 10, 21, 38). Infection also appears to be affected by the lipid composition of the cellular membrane with which the viruses interact. For example, several enveloped viruses, including murine leukemia virus and HIV-1, require intact lipid rafts to be present on the cell surface in order to infect cells (19, 20). If alterations in the microenvironment were the primary mechanism by which the TAC molecules affected transduction, then our results suggest that amphotropic viruses, similar to HIV-1, are less likely to infect cells if they are transported through low pH compartments of the cell (by receptors such as TAC-DKQTLL). In addition, it is interesting to note that transduction was not enhanced in cells that expressed TAC-CD16, a molecule that is primarily localized in lipid rafts, which suggests that transport to cell-surface lipid-raft domains is not a major rate-limiting step of transduction in our experimental system.

Regardless of the mechanism by which the virus-binding TAC chimeras affected transduction, our results show that the distribution and trafficking itinerary of cellular virus-binding proteins can significantly affect the efficiency of transduction. Our findings suggest that it may be important to consider the subcellular distribution and trafficking itinerary of virus-

binding proteins when designing a targeted retrovirus, although additional studies in other cell lines are needed to confirm the generality of our findings. Unfortunately, little is known about the mechanism by which the trafficking itinerary of virus-binding proteins affects virus infection. Experimental systems such as the one developed in this study should prove useful for the quantitative analysis of how viruses and other gene delivery vehicles traffic within cells, information that is likely to prove critical for the development of clinically relevant targeted gene transfer vectors.

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CHAPTER 3

MURINE LEUKEMIA VIRUS PARTICLES ACTIVATE RAC1 IN HELA CELLS

3.1 Abstract

A number of viruses, when they bind to cells, activate intracellular signals that facilitate post-binding steps of infection. To determine if retroviruses activate intracellular signaling, we transduced HeLa cells with amphotropic retroviruses produced by TelCeB6 cells and examined cell lysates for activated Rac1. We found that retroviruses activate Rac1. Rac1 activation was blocked when cells were depleted of cholesterol, cultured in suspension, or incubated with an anti-β_1 integrin antibody, and when viruses were treated with heparinase III. Retrovirus activation of Rac1 did not require the amphotropic envelope protein. The implications of these findings with respect to retrovirus-cell interactions are discussed.

3.2 Introduction

Retrovirus infection is a multistep process that begins with adsorption of the virus particle to the cell surface (4). Adsorbed retroviruses bind to cellular receptors, which appear to be localized to specific regions of the plasma membrane, presumably by diffusing to these receptor-rich sites, or by recruiting the receptors to the site where the virus is bound (1, 20, 28). Multivalent complexes form between the envelope proteins of the virus and the cell surface receptors, which induce structural changes in the viral envelope glycoproteins that ultimately lead to fusion of the viral and cellular membranes (31). Cytochalasin D treatment blocks virus entry, which indicates that early steps of retrovirus infection require a functional actin cytoskeleton (13).

Recent studies in a number of different experimental systems suggest that viruses facilitate early steps of infection by activating signaling events within their host cells (10).

Adenoviruses activate Rac1 and Cdc42, which stimulates actin rearrangements that boost virus entry (10, 18, 21). The intracellular mature form of vaccinia virus activates Rac1 and RhoA, which leads to the formation of actin-containing protrusions at the plasma membrane that internalize the viruses (19). Recent work by Pontow et al suggests that retroviruses may also be capable of activating signals within their host cells (27). Pontow et al showed that Rac1 was activated when cells that expressed HIV-1 Env were cocultured with cells that expressed the cellular receptors CD4 and CCR5. Fusion from without, induced by culturing HIV-1 particles with cells that co-expressed CD4 and CCR5, was inhibited by RacN17, a dominant negative mutant of Rac1. Based on these observations, we hypothesized that retrovirus particles, during an early event in infection, activate intracellular signals within their host cells. To test this hypothesis, we used a model recombinant amphotropic retrovirus produced by TelCeB6 cells to determine if Rac1 is activated in HeLa cells within the first hour of infection.

3.3 Materials and Methods

Reagents, antibodies and plasmids. Rac activation assay kit was purchased from Upstate Signaling Technologies (Upstate, NY). Polybrene (PB), chondroitin sulfate C (CSC), methyl β cyclodextrin and heparinase III were purchased from Sigma Chemical Co. (St. Louis, MO). Retronectin, Bovine serum albumin (BSA), Fraction V, Hydrogen peroxide 30%, and Polyoxyethylene 20-Sorbitan Monolaurate (Tween 20) were from Fisher Scientific (Fair Lawn, NJ). Non-fat dry milk (blotting grade) was from Bio-Rad Laboratories (Hercules, CA). o-Phenylenediamine Dihydrochloride (OPD) was from Pierce (Rockford, IL). Mouse anti-p30 antibodies were purified from the supernatant of the CRL-1219 hybridoma cell line (ATCC, Rockville, MD) following standard procedures. The goat polyclonal anti-p30 antibody (78S221) was from Quality Biotech (Camden, NJ). The horseradish peroxidase conjugated rabbit anti-goat immunoglobulin G polyclonal antibody was from Zymed Laboratories (South San Francisco, CA). Peroxidase conjugated rabbit anti-mouse immunoglobin G was purchased from

Jackson Immunoresearch (West Grove, PA). Phosphatidylinositol-specific phospholipase C was purchased from Invitrogen Corporation (Carlsbad, CA). Anti β_1 integrin antibody AIIB2 and isotype control R26.4.C were purchased from Developmental Studies Hybridoma Bank (University of Iowa, Iowa). Super Signal West Femto chemiluminescent detection system was purchased from Pierce (Rockford, IL). Plasmid encoding TAC-CD16 was a kind gift of Harish Radhakrishna (School of Biology, Georgia Institute of Technology, Atlanta, GA).

Cell culture. HeLa (human cervical cancer) cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone Labs Inc., Logan, UT) with 10% fetal bovine serum (FBS; Hyclone Labs Inc.), 100 U/mL of penicillin, 100 μg/mL of streptomycin (Hyclone Labs Inc.), and 110 μg/mL of sodium pyruvate (Hyclone Labs Inc.) (DMEM/FBS). TELCeB6 cells (a kind gift from F.L. Cosset) expressing Moloney MLV Gag and Pol, and the retroviral vector MFGnlsLacZ, and 293T/17 (human embryonic kidney epithelial) cells were cultured in DMEM/FBS.

Virus production. An amphotropic packaging cell line (TELCeB6-A) was generated by stable transfection of TELCeB6 cells. Five micrograms of the plasmid FB4070ASALF, an expression plasmid that encodes for the amphotropic envelope glycoprotein (a kind gift of Stephen Russell), was dissolved in 400 μL of 0.25M $CaCl_2$, mixed with 400 μL of 2X HEPES buffered saline (274 mM NaCl, 42 mM Hepes acid, 10 mM KCl, 1.4 mM Na_2HPO_4 and 12 mM dextrose), incubated at room temperature for 20 minutes, then added to a 50% confluent T75 flask of TELCeB6 cells. Twelve hours after transfection the cells were washed with PBS, and then the medium replaced with fresh DMEM/FBS. Two days later the cells were trypsinized, pelleted, and resuspended. Two hundred microliters of the resuspended cells were diluted in 10 mL of selective medium (DMEM, 10% FBS, 50 μg/mL of phleomycin, and 7 μg/mL of blasticidin), and plated in a T75 flask. Fourteen days later pooled clones of stably transfected cells were frozen for later use. To generate retrovirus stocks, virus-producing cells were grown to confluence in T175 tissue culture flasks, and then incubated for 24 h with 35 mL of cell

culture medium. The virus-laden tissue culture medium was harvested, filter sterilized (0.45-μm), then frozen (-80°C) for later use. Envelope deficient virus stocks were produced by culturing TELCeB6 cells to confluence. The cell culture supernatant was harvested, filter sterilized (0.45-μm), then frozen (-80°C) for later use. Viruses expressing TAC CD16 were produced by transient transfection of 293T/17 cells. The cells were plated in a 10 cm dish (1.7 x 10^7 cells/dish) and co-transfected with Lipofectamine-2000 and 6 μg each of plasmid DNA encoding the lentiviral packaging construct pCMVΔR8.91 (kind gift of Scott S. Case), lentivirus vector pTY-EfnlacZ and plasmid expressing TAC CD16. Virus laden tissue culture medium was harvested 36, 48, and 60 h after transfection, filtered (0.45 μm), and frozen (-80°C) for later use.

Concentration using polymers. Samples were brought to 80 μg/ml of PB and CSC, incubated at 37°C for 20 min, centrifuged at room temperature for 5 min at 10,000 x g, the supernatant decanted and the pelleted material resuspended in a desired volume of DMEM.

Rac activation assay. HeLa cells were plated at 260,000 cells/well in a 6 well dish. Concentrated retrovirus stocks or conditioned medium (CM) were added to 3 wells of HeLa cells (washed 2X with DMEM prior to exposure) at 2 ml per well. Virus or CM was centrifuged onto the cells for 30 min at 37°C and 2100 x g, the cells incubated for an additional 30 min at 37°C and lysed using 1X buffer MLB (Upstate Signaling Technologies). The cell lysates were then precleared with glutathione agarose and activated Rac1 (Rac1-GTP, 21kDa) isolated using glutathione agarose beads (bound to GST fusion protein corresponding to the p21 binding domain of human PAK-1) that selectively bind Rac1-GTP. Equivalent quantities of cell lysates were separated by size by SDS-PAGE (4-20% Tris-HCl) gel electrophoresis, transferred to a PVDF membrane, probed with anti-Rac1 primary antibody (1 μg/ml), HRP conjugated secondary antibody (4 ng/ml) and visualized using a chemiluminescent detection system.

ELISA for p30. We used an enzyme-linked immunosorbent assay (ELISA) to determine the concentration of virus capsid protein (p30) in cell lysates. ELISA plates (Nunc immuno

Maxisorp 96-well plates, Nalge Nunc International, Rochester, NY) were coated overnight at 4°C with 10 μg/mL of mouse anti-p30 antibody (100 μL/well) in PBS. The next day, the antibody solution was removed and blocking buffer (PBS, 0.05% Tween-20, 5% non-fat milk) added (200 μL/well) for 2 h at 37°C to block non-specific binding sites. Samples were brought to 0.5% Triton-X to expose the p30 antigen, then added to the ELISA plate (100 μL/well) and incubated for 1 h at 37°C. Bound p30 was sandwiched by the addition of the goat polyclonal anti-p30 antibody diluted 1:1000 in blocking buffer, and incubated for 1 h at 37°C. The horseradish peroxidase conjugated polyclonal rabbit anti-goat immunoglobulin G was diluted 1:5000 in blocking buffer then added to the ELISA plate (100 μL/well) for 1 hour at 37°C to enable detection and quantitation of the sandwiched p30 antigen. The plates were developed for 5 min using hydrogen peroxide (H_2O_2) and OPD (100 μg/well) from a solution of 10 mg of OPD and 10 μl H_2O_2 in 25 mL of substrate buffer (24 mM citric acid-monohydrate, 51 mM Na_2HPO_4-$7H_20$, pH 5.0). 8N sulfuric acid (50 μL/well) was used to stop the reaction and the optical density at 490 nm (OD_{490}) measured using an absorbance plate reader and the non-specific background at 650nm subtracted. Values for replicate wells without virus were subtracted as background. Values for each point are the average of at least triplicate wells.

Retronectin ELISA. 96 well ELISA plates were coated with 20 μg/ml (in 1 x PBS) retronectin at 60μl per well for 2 hrs at room temperature. Next, the wells were blocked with PBS/BSA (2% BSA) at 100 μl per well for 30 min at room temperature. Following one wash with 1 X PBS (100 μl per well), virus samples previously digested with heparinase III were allowed to bind to the wells for 4 hr at 37°C. Following virus binding, the wells were washed three times with 1 X PBS (100 μl per well) to remove any unbound virus, and the bound virus was lysed to expose virus capsid p30, with 125 μl per well of lysis buffer (1% Triton X-100/150mM NaCl/0.02% sodium azide) for 1 hr at 37°C. The lysate was then transferred to an ELISA to

quantify p30. The amount of p30 corresponds to the amount of virus bound on retronectin, which is proportional to the number of heparin sulfate proteoglycans on the virus surface.

3.4 Results

Retrovirus activates Rac1 in an envelope independent manner. To determine if retrovirus binding induces Rac1 activation, we used a model recombinant amphotropic retrovirus (MFGnlsLacZ, produced by the TelCeB6 packaging cell line), HeLa cells, and an assay to detect activated Rac1 (Rac1-GTP, 21 kDa) in cell lysates. HeLa cells, plated the previous day in 6-well dishes (260,000 cells per well) were incubated with stocks of amphotropic retrovirus (1.47×10^7 CFU/ml $\pm$ 0.25×10^7), that had been centrifuged (16 hr, 4°C, 6000 x g), and resuspended in fresh DMEM that contained 20 µg/ml PB. In addition, we incubated parallel cultures of HeLa cells with conditioned medium (i.e., the supernatant that remained after virus particles were pelleted from virus stocks by centrifugation at 30,000 rpm in a Beckman SW41 rotor for 2 h at 4°C), which had been concentrated and processed in the same way as the virus stocks, to determine if substances in virus stocks other than virus particles were able to induce Rac1 activation in cells. Next, we centrifuged (2100 $\times$ g) the cultures for 30 min at 37°C to maximize virus binding, incubated the cultures an additional 30 min at 37°C, and then incubated lysates of the cells (1 hr, 4°C) with agarose beads functionalized to bind activated Rac1 (Rac1-GTP). Proteins captured on agarose beads were separated by size using SDS-PAGE, transferred to a PVDF membrane, and then visualized using an anti-Rac1 primary antibody (1 µg/ml, clone 23A8), an HRP-conjugated secondary antibody (4 ng/ml), and a chemiluminescent detection system. We found that retroviruses, but not conditioned medium, stimulated Rac1 activation in HeLa cells. As expected, Rac1-GTP was detected in cell lysates that had been preloaded with GTPγ, a non-hydrolysable form of GTP that activates Rac1, but not in lysates that had been preloaded with GDP, which prevents Rac1 activation (Figure 3.1a).

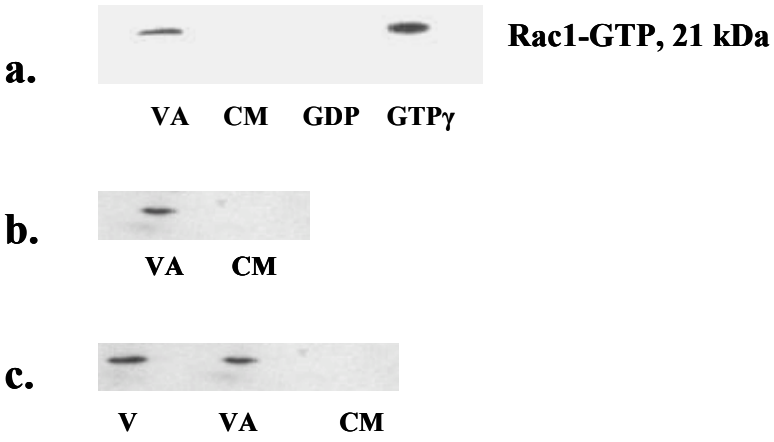

Figure 3.1 Retrovirus activates Rac1 in an envelope independent manner. (a). HeLa cells were plated at 260,000 cells/well in a 6 well dish. Retrovirus stocks (72 ml) or CM (72 ml) were concentrated 12-fold by centrifugation (6000 x g, 4^{o}C, 16 hr). After centrifugation, the supernatant was decanted, the pelleted material resuspended in 6 ml fresh DMEM, the solutions brought to 20 µg/ml PB and added to 3 wells of HeLa cells (washed 2X with DMEM prior to exposure) at 2 ml per well. Virus or CM was centrifuged onto the cells for 30 min at 37^{o}C and 2100 x g, the cells incubated for an additional 30 min at 37^{o}C and lysed using 1X buffer MLB (Upstate Signaling Technologies). In parallel, cell lysates were loaded with GDP or GTPγ at 1 mM and 100 µM concentrations respectively. The cell lysates were then precleared with glutathione agarose and activated Rac1 (Rac1-GTP, 21kDa) isolated using glutathione agarose beads (bound to GST fusion protein corresponding to the p21 binding domain of human PAK-1) that selectively bind Rac1-GTP. Equivalent quantities of cell lysates were separated by size by SDS-PAGE (4-20% Tris-HCl) gel electrophoresis, transferred to a PVDF membrane, probed with anti-Rac1 primary antibody (1 µg/ml), HRP conjugated secondary antibody (4 ng/ml) and visualized using a chemiluminescent detection system. Lanes VA and CM depict HeLa cells exposed to virus and CM respectively. Lanes GDP and GTPγ depict HeLa cell lysates loaded with GDP and GTPγ respectively. (b). HeLa cells were plated at 260,000 cells/well in a 6 well dish. Retrovirus stocks (18 ml) or CM (18 ml) were concentrated 3-fold by complexation with PB and CSC. The samples were brought to 80 µg/ml of PB and CSC, incubated at 37^{o}C for 20 min, centrifuged at room temperature for 5 min at 10,000 x g, the supernatant decanted, the pelleted material resuspended in 6 ml fresh DMEM and exposed to 3 wells of HeLa cells (washed 2X with DMEM prior to exposure) at 2 ml per well. Rac1-GTP was isolated as described above. Lanes VA and CM depict HeLa cells exposed to virus stocks and CM respectively. (c). Envelope deficient retrovirus particles (18 ml), virus stocks (18 ml) or CM (18 ml) were concentrated 3-fold using PB and CSC, added to HeLa cells and Rac1-GTP isolated as described above. Lanes V, VA and CM depict HeLa cells exposed to *env*- retroviruses, virus stocks and CM respectively.

To verify that Rac1 activation was not dependent on the methods used to process the virus stocks, we repeated these experiments using virus that was rapidly concentrated and purified by a different means: complexation with CSC and PB. We have previously shown that retroviruses concentrated using this method are highly purified (i.e., they contain about 80% of the virus particles, but less than 0.3% of all other proteins, that were present in the original virus stock), and bind to cells much more rapidly than virus that is not part of a complex (14, 16). Retrovirus stocks, and conditioned medium as a control, were brought to equal weight concentrations (80 μg/mL) of CSC and PB, incubated for 20 min at 37°C, pelleted by centrifugation (5 min, 10, 000 x g), resuspended in fresh DMEM, and then incubated with HeLa cells as described above. We found that Rac1 was activated in HeLa cells exposed to retrovirus but not in HeLa cells exposed to conditioned medium (Figure 3.1b). Since these results show that PB and CSC do not activate Rac1, and given the advantages of the complexation method for concentrating and purifying retrovirus stocks, we used polymer-complexed virus for subsequent experiments.

We wondered, given their central role in retrovirus binding and fusion, if retrovirus envelope proteins were required for Rac1 activation. To examine this possibility, retroviruses that did not contain any envelope proteins (*env*- retrovirus), amphotropic retroviruses, and conditioned medium generated from amphotropic retrovirus stocks, were concentrated and incubated with HeLa cells. Interestingly, we found that retroviruses activated Rac1 whether or not they contained envelope proteins. As expected, conditioned medium did not activate Rac1 (Figure 3.1c). Taken together, our results show that retroviruses, whether or not they contain envelope proteins, activate Rac1 in HeLa cells. As a result, we used *env*- retrovirus in the remainder of our experiments.

Retroviruses do not activate Rac1 in HeLa cells when the cells are cultured in suspension or when their plasma membranes have been depleted of cholesterol. Since Rac1-GTP preferentially localizes to lipid rafts, and targeting of Rac1 to the plasma membrane

is dependent on cell adhesion (5), we wondered if retrovirus activation of Rac1 would be affected by a change in the adhesion state of the cells or in the integrity of their lipid rafts. To examine this possibility, we incubated a single-cell suspension of HeLa cells, detached with versene, with *env*- retrovirus for 1 hr at 37^{o}C. As controls, we incubated monolayers of adherent HeLa cells with *env*- retroviruses or conditioned medium. Interestingly, we found that *env*- retrovirus did not activate Rac1 in detached HeLa cells. Consistent with our previous experiments, *env*- retrovirus, but not conditioned medium, activated Rac1 in adherent HeLa cells (Figure 3.2a). We also tested the effect of disrupting lipid rafts on Rac1 activation. We treated Hela cells with methyl β cyclodextrin (MBCD; 5 mM, 2.5 h, 37°C) to extract cholesterol from their plasma membranes, incubated the cells with *env*- retrovirus, and then probed lysates of the cells for activated Rac1. As controls, we incubated *env*- retrovirus or conditioned medium with HeLa cells that had been mock-treated (2.5 h, 37°C) with cell culture media that did not contain any MBCD. Interestingly, Rac1 was not activated in cells that had been pretreated with MBCD. Consistent with our other experiments, *env*- retrovirus, but not conditioned medium, activated Rac1 in HeLa cells that were not treated with MBCD (Figure 3.2b). These results suggest that cell adhesion and cholesterol-rich microdomains in the plasma membrane are required for retroviruses to activate Rac1 in HeLa cells.

Retrovirus activation of Rac1 is blocked by an anti-β_1 integrin antibody. Given that cell adhesion is mediated by integrins, and that engagement of β_1 integrins by extracellular ligands activates Rac1 (2), we decided to determine if β_1 integrins were required for retrovirus activation of Rac1. HeLa cells were pretreated with an anti-β_1 integrin antibody (10 μg/ml, AIIB2, Development Studies Hybridoma Bank (DHSB), University of Iowa), or an isotype control (10 μg/ml, R26.4C, DHSB), for 1 hr at 37°C, incubated with *env*- retrovirus and the antibody for an additional 1 hr at 37°C, and then lysates of the cells probed for activated Rac1. We found that

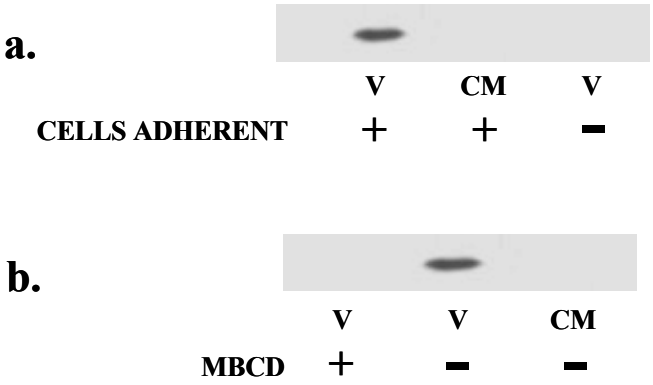

Figure 3.2 Retroviruses do not activate Rac1 in HeLa cells when the cells are cultured in suspension or when their plasma membranes have been depleted of cholesterol. (a). HeLa cells (3 wells of a 6 well dish plated at 260,000 cells/well the previous day and washed 2X with DMEM prior to detachment) were detached from tissue culture plates using 2 ml versene (0.5 M EDTA in 1X PBS) per well. Next, the cells were pelleted, the supernatant discarded, resuspended in 6 ml fresh DMEM containing *env*- retroviruses (18 ml) that had been previously concentrated 3-fold using PB and CSC, and agitated for 1 hr at 37^{o}C. In parallel, adherent HeLa cells were exposed as described above to *env*- retroviruses (18 ml) or CM (18 ml) concentrated 3-fold using PB and CSC. Rac1-GTP was isolated as described above. Lanes V+ and CM+ depict adherent HeLa cells exposed to *env*- retroviruses and CM respectively and Lane V- depicts non-adherent HeLa cells exposed to *env*- retroviruses. (b). HeLa cells (3 wells of a 6 well dish plated at 260,000 cells/well the previous day) were treated with MBCD (5 mM in cell culture media) for 2.5 hr at 37^{o}C and then incubated with *env*- retroviruses (18 ml) concentrated 3-fold using PB and CSC. As controls, HeLa cells were mock treated with cell culture media for 2.5 hr at 37^{o}C and exposed as described above, to *env*- retroviruses (18 ml) or CM (18 ml) concentrated 3-fold using PB and CSC. Rac1-GTP was isolated as described above. Lanes V- and CM- depict mock treated HeLa cells exposed to *env*- retroviruses and CM respectively. Lane V+ depicts HeLa cells pretreated with MBCD and exposed to *env*- retroviruses.

Rac1 activation by *env*- retrovirus was blocked when cells were treated with the anti-β_1 antibody (Figure 3.3a) but not when they were treated with the isotype control (R26.4C, Figure 3.3c). Rac1 was not activated when HeLa cells were incubated with DMEM and anti-β_1 antibody and, consistent with our previous results, *env*- retrovirus, but not conditioned medium, activated Rac1 in HeLa cells that were not treated with the anti-β_1 antibody (Figure 3.3a and c).

Since some retroviruses have been shown to contain integrins in their lipid bilayers (9), we wondered if the anti-β_1 antibody had blocked retrovirus activation of Rac1 by interacting with integrins on the surfaces of the retroviruses rather than with integrins on the surfaces of the HeLa cells. To examine this possibility, complexes of *env*- retrovirus, CSC, and PB were pelleted and resuspended in PBS that contained 0 or 10 μg/ml of anti-β_1 antibody, incubated for 1 hr at 37°C, pelleted and resuspended in DMEM, incubated with HeLa cells for 1 hr at 37°C, and then lysates of the cells probed for activated Rac1. As a control, we repeated the experiment using complexes formed with conditioned medium. We found that pretreatment of the *env*- retrovirus with the anti-β_1 antibody did not block their ability to activate Rac1 in HeLa cells (Figure 3.3b). Consistent with our previous results, *env*- retrovirus, but not conditioned medium, activated Rac1 in HeLa cells (Figure 3.3b). Taken together, these results suggest that retroviruses activate Rac1 by interacting, directly or indirectly, with β_1 integrins on the surfaces of HeLa cells, and that this interaction does not involve retroviral envelope proteins.

Retrovirus treated with heparinase III do not activate Rac1. We wondered which molecules on the surfaces of retroviruses were required to activate Rac1. Retroviruses bud from lipid rafts and incorporate a number of lipid raft proteins into their lipid bilayers (3, 25). Lipid rafts are highly enriched in GPI-anchored molecules, which suggests that GPI-anchored proteins might be incorporated into the lipid bilayers of retroviruses and play a role in Rac1 activation. To investigate this possibility, we generated *env*- retrovirus that was deficient in GPI-

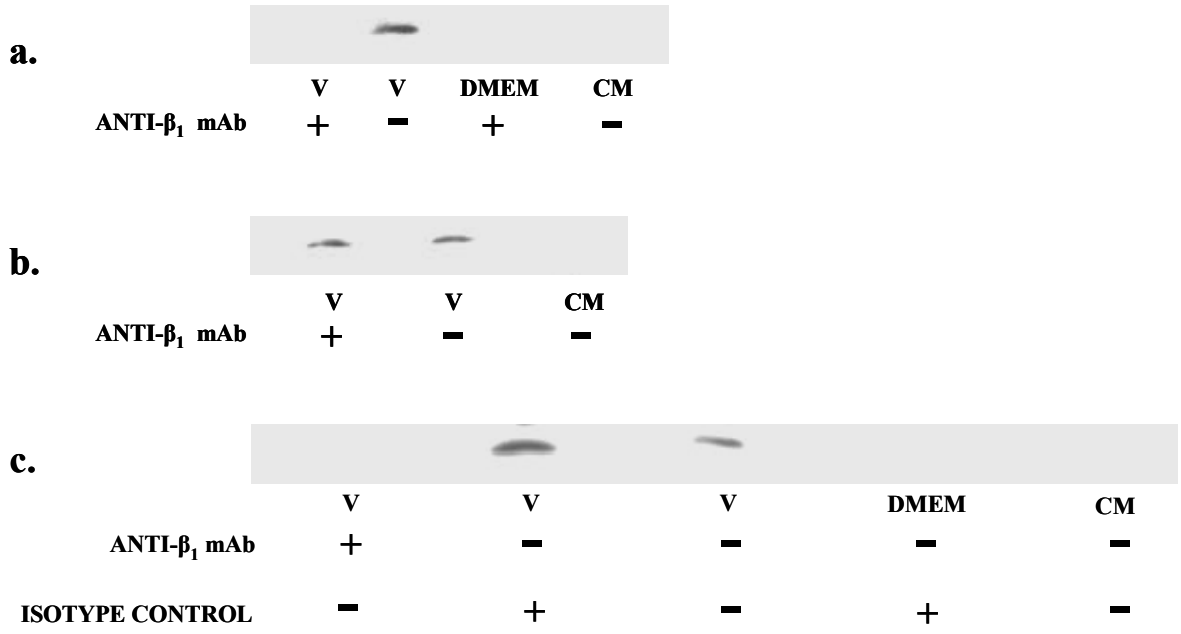

Figure 3.3 Retrovirus activation of Rac1 is blocked by an anti-β_1 integrin antibody. (a). HeLa cells (3 wells of a 6 well dish plated at 260,000 cells/well the previous day) were plated on glass coverslips (5 per well). Next day, the cells were exposed to 25 µl per cover slip (10 µg/ml in cell culture media) of function blocking anti-β_1 integrin antibody for 1 hr at 37^{o}C and then incubated with *env*- retroviruses (18 ml) concentrated 3-fold using PB and CSC containing 10 µg/ml anti-β_1 integrin antibody. As controls, HeLa cells were mock treated with cell culture media for 1 hr at 37^{o}C and exposed to *env*- retroviruses (18 ml) or CM (18 ml) concentrated 3-fold using PB and CSC. As an additional control, HeLa cells were treated with anti-β_1 integrin antibody for 1 hr at 37^{o}C and exposed to fresh DMEM. Rac1-GTP was isolated as described above. Lanes V- and CM- depict mock treated HeLa cells exposed to *env*- retroviruses and CM respectively. Lanes V+ and DMEM+ depict HeLa cells pretreated with the anti-β_1 antibody and exposed to *env*- retroviruses and DMEM respectively. (b). *Env*- retroviruses (18 ml) were pelleted using PB and CSC, the pelleted material resuspended in 500 µl of 1X PBS containing anti-β_1 integrin antibody (10 µg/ml) and incubated for 1 hr at 37^{o}C with constant agitation. After antibody exposure, *env*- retroviruses were pelleted by centrifugation at 10,000 x g for 5 min, the supernatant discarded, the pellets washed with 500 µl of 1X PBS, resuspended in 6 ml fresh DMEM and added at 2 ml per well to 3 wells of HeLa cells (260,000 cells/well in a 6 well dish plated the previous day) that had not been previously exposed to anti-β_1 integrin antibody. As controls, we exposed HeLa cells to *env*- retroviruses (18 ml) or CM (18 ml) that were pelleted using PB and CSC, mock treated with 500 µl of 1X PBS for 1 hr at 37^{o}C and processed exactly as *env*- retroviruses treated with anti-β_1 integrin antibody. Rac1-GTP was isolated as described above. Lanes V- and CM- depict HeLa cells that were exposed to mock treated *env*- retroviruses or CM respectively. Lane V+ depicts HeLa cells exposed to *env*- retroviruses pre treated with anti-β_1 integrin antibody. (c). The experiment was performed as described in (a) except, the isotype control antibody was used where indicated with a "+".

anchored proteins by incubating complexes of *env*- retrovirus, CSC, and PB with phosphatidylinositol-specific phospholipase C (PIPLC; 2 U/ml in phosphate buffered saline (PBS)) for 2 hr at 37°C. Next, we pelleted the PIPLC-treated *env*- retrovirus, discarded the supernatant, washed the pellets once with PBS, resuspended them in fresh DMEM, incubated them with HeLa cells for 1 hour at 37°C, and then probed lysates of the cells for activated Rac1. As controls, we incubated HeLa cells with *env*- retrovirus and conditioned medium that had been processed in the same way, with the exception that they were mock digested with PBS that did not contain any PIPLC. We found that Rac1 activation was not blocked by PIPLC digestion of the retrovirus particles. As expected, mock treated *env*- retrovirus, but not conditioned medium, activated Rac1 in HeLa cells (Figure 3.4a). To verify that our PIPLC treatment was sufficient to completely digest GPI anchored molecules on the surfaces of the retrovirus particles, we generated stocks of viruses that contained TAC-CD16 (6), a GPI anchored protein, in their lipid bilayers, incubated the viruses for 2 hr at 37°C with (2 U/mL) or without (0 U/mL) PIPLC, and then probed lysates of the viruses for TAC-CD16 by western blot. As expected, TAC-CD16 was readily detected in virus that had not been treated with PIPLC, but absent in PIPLC-treated virus (Figure 3.4b). These observations suggest that GPI anchored molecules on *env*- retrovirus particles are not involved in mediating Rac1 activation in HeLa cells.

Based on recent studies that show retrovirus-associated heparin sulfate proteoglycan (HSPG) can tether retrovirus to fibronectin (12), which in turn can bind to cell-surface integrins, we decided to investigate the role of retrovirus-associated HSPG in Rac1 activation. *Env*- retrovirus was pelleted by centrifugation, resuspended in enzyme buffer (PBS, 0.1 mg/ml BSA) and heparinase III (0.08 IU/ml) for 5 hr at 37°C. Next, we used CSC and PB to form complexes with the viruses, pelleted and resuspended them in fresh DMEM, incubated the virus-polymer complexes with HeLa cells for 1 hr at 37°C, and then probed lysates of the cells for activated

Rac1. As controls, we incubated HeLa cells with *env*- retrovirus and conditioned medium that had been processed in the same way, with the exception that they were mock digested with enzyme buffer (5 hr at 37^{o}C) that did not contain heparinase III. Interestingly, we found that *env*- retrovirus that had been digested with heparinase III did not activate Rac1 (Figure 3.4c). As expected, mock treated *env*- retrovirus, but not conditioned medium, activated Rac1 in HeLa cells (Figure 3.4c). Treatment of *env*- retrovirus with heparinase III led to 90% digestion of HSPG on their surface as quantified by a retronectin ELISA (Figure 3.5a). Using a p30 ELISA, we confirmed that virus binding to HeLa cells was not affected by heparinase III digestion (Figure 3.5b). Taken together, these results suggest that retrovirus particles require HSPG to activate Rac1 in HeLa cells.

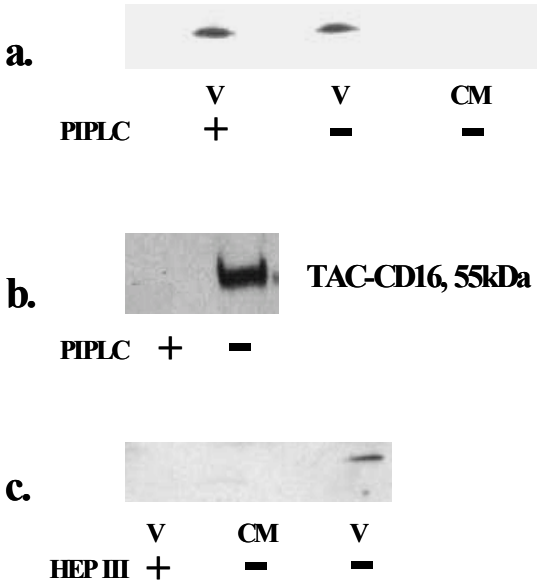

Figure 3.4 Retrovirus treated with heparinase III do not activate Rac1. (a). *Env*- retroviruses (18 ml) were pelleted using PB and CSC, the pelleted material resuspended in 100 µl of 1X PBS containing PIPLC (2 U/ml) and incubated for 2 hr at 37^{o}C. After PIPLC exposure, the *env*- retroviruses were pelleted by centrifugation at 10,000 x g for 5 min, the supernatant discarded, the pellets washed once with 500 µl of 1X PBS, resuspended in 6 ml DMEM and added to HeLa cells (3 wells of a 6 well dish plated at 260,000 cells/well the previous day) at 2 ml per well. As controls, we exposed HeLa cells to *env*- retroviruses (18 ml) or CM (18 ml) that were pelleted using PB and CSC, mock treated with 100 µl of 1X PBS for 2 hr at 37^{o}C and processed exactly as PIPLC treated *env*- retroviruses. Rac1-GTP was isolated as described above. Lanes V- and CM- depict HeLa cells that have been exposed to mock treated *env*- retroviruses and CM respectively. Lane V+ depicts HeLa cells that have been exposed to *env*- retroviruses treated with PIPLC. (b). Lentivirus (7 ml) derived from packaging cells expressing TAC CD16 (55kDa) was pelleted using PB and CSC, the pelleted material resuspended in 100 µl of 1X PBS containing PIPLC (0 or 2 U/ml), digested for 2 hr at 37^{o}C, pelleted again, the pellets washed with 500 µl of 1X PBS, resuspended in denaturing conditions and tested in an immunoblot with anti-TAC antibodies. Lane "+" depicts virus digested with PIPLC and Lane "-" depicts undigested virus. (c). *Env*- retroviruses (18 ml) were concentrated by centrifugation (6000 x g, 4^{o}C, 16 hr), the pelleted material resuspended in 600 µl of PBS/BSA (0.1 mg/ml BSA) and digested with heparinase III (0.08 IU/ml) for 5 hr at 37^{o}C. After digestion, the reaction mix was diluted with 9 ml DMEM, the *env*- retroviruses pelleted using PB and CSC, the pelleted material resuspended in 6 ml fresh DMEM and added to HeLa cells (3 wells of a 6 well dish plated at 260,000 cells/well the previous day) at 2 ml per well. As controls, we exposed HeLa cells to *env*- retroviruses (18 ml) or CM (18 ml) concentrated by centrifugation (6000 x g, 4^{o}C, 16 hr), mock treated with 600 µl of PBS/BSA for 5 hr at 37^{o}C and processed exactly as *env*- retroviruses treated with heparinase III. Rac1-GTP was isolated as described above. Lanes CM- and V- depict HeLa cells exposed to mock treated CM and *env*- retroviruses respectively. Lane V+ depicts HeLa cells that have been exposed to *env*- retroviruses treated with heparinase III.

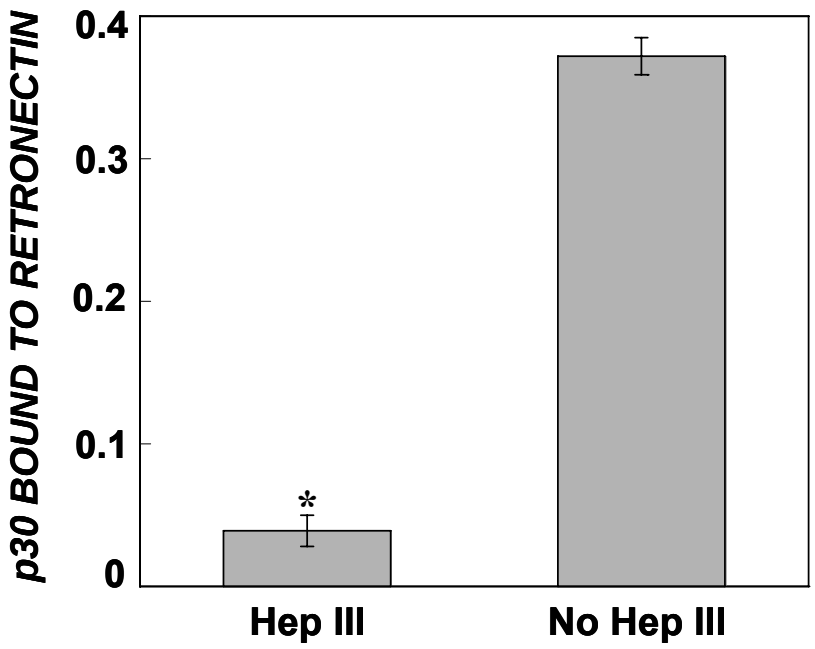

Figure 3.5a Treatment of *env*- retrovirus with heparinase III digests 90% HSPG on their surface. 96 well ELISA plates were coated with 20 μg/ml (in 1 x PBS) retronectin at 60μl per well for 2 hrs at room temperature. Next, the wells were blocked with PBS/BSA (2% BSA) at 100 μl per well for 30 min at room temperature and washed once with 1 X PBS. *Env*- retroviruses (3 ml) were concentrated by centrifugation (6000 x g, 4°C, 16 hr), the pelleted material resuspended in 100 μl of PBS/BSA (0.1 mg/ml BSA) and digested with heparinase III (0 or 0.08 IU/ml) for 5 hr at 37°C. Following digestion the samples were diluted 17.5-fold in 1X PBS and transferred at 100 μl per well to 96 well ELISA plates coated with retronectin for 4 hr at 37°C. Following virus binding, the wells were washed three times with 1 X PBS (100 μl per well) to remove any unbound virus, and the bound virus was lysed to expose virus capsid p30, with 125 μl per well of lysis buffer (1% Triton X-100/150mM NaCl/0.02% sodium azide) for 1 hr at 37°C. The lysate was then transferred to an ELISA to quantify p30. The amount of p30 corresponds to the amount of virus bound on retronectin, which is proportional to the number of HSPG on the virus surface. (*) denotes statistically significant differences ($p < 0.05$).

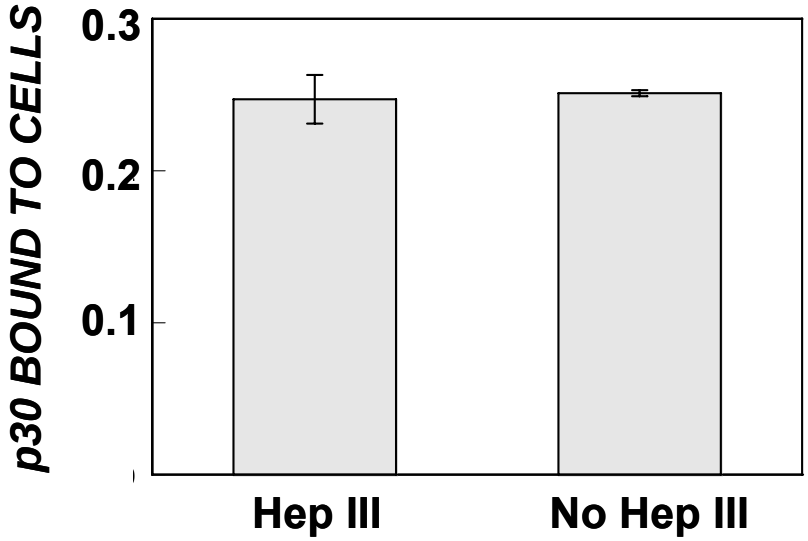

Figure 3.5b Heparinase III treatment does not affect virus binding to HeLa cells. HeLa cells were plated at 40,000 cells/well is a 96 well tissue culture dish. Next day, *env*- retrovirus that had been previously treated with heparinase III (0 or 0.08 IU/ml) was centrifuged onto HeLa cells for 30 min at 37^{o}C and 2100 x g to maximize virus binding and incubated for an additional 30 min at 37^{o}C. Following virus exposure, the cells were lysed with 125 μl per well lysis buffer (6/7 part (150mM NaCl, 1% Igepal, 50mM Tris Base), 1/7 part (complete mini protease inhibitor cocktail tablet, Roche)) for 30 min at 4^{o}C, the cell lysates centrifuged at 15000 x g for 10 min at 4^{o}C to pellet the nuclei and the supernatant analyzed for p30 in an ELISA.

3.5 Discussion

Our study shows that recombinant murine leukemia viruses activate Rac1 in HeLa cells. We found that retrovirus-mediated Rac1 activation requires cell-surface β_1 integrin subunits, cholesterol in the plasma membranes of the cells, and virus-associated heparin sulfate proteoglycans, but does not involve interactions between the envelope proteins of the virus and their cellular receptors.

Rac1 appears to regulate the entry of adeno-associated virus type 2, adenovirus, and the intracellular mature form of vaccinia virus, (18, 19, 30) and may play a role in retrovirus infection (27). Pontow et al showed that gp120, when expressed in cells, can activate Rac1 in cells that express its co-receptors, CD4 and CCR5. It is not known, however, if HIV-1 particles themselves activate Rac1 when they infect cells. Our findings show that retrovirus particles activate Rac1 without the need for envelope-receptor interactions. Previous work has demonstrated that envelope-independent interactions affect retrovirus binding and infection (26, 34), although a role for Rac1 activation in these processes has not, to our knowledge, been previously identified. For example, fibronectin appears to increase transduction in an envelope-independent manner by binding, at the same time, to virus-associated heparin sulfate proteoglycans (HSPG) and cellular integrins. Retroviruses can also bind directly to integrins via ligands, such as the cellular adhesion molecule ICAM-1, that are incorporated into their lipid bilayers during virus budding (34). HIV-1 particles, when they incorporate ICAM-1, interact with their cognate receptor (LFA-1), and complete early steps of infection more rapidly, boosting the efficiency of infection more than 10-fold (8). It is hypothesized that infection is enhanced because the virus-associated ICAM-1 bind to and activate cell-surface LFA-1 proteins, which promotes the active transport of the virus particles to lipid rafts (34). Presumably, since lipid rafts are enriched for the cellular receptors for HIV-1 (i.e., CD4 and CCR5), viruses that are

localized to these sites are more likely to interact with them and fuse with the plasma membrane of the cell (34).

Although our results show that Rac1 activation requires virus-associated HSPG and cell surface β_1 integrins, we do not know the precise mechanism by which retroviruses activate Rac1, or the role of Rac1 activation in retrovirus infection. We speculate that virus-associated HSPG bind to integrin ligands in the extracellular matrix (ECM), such as fibronectin or collagen, which in turn bind to and induce cell-surface β_1 integrins to cluster and activate Rac1 (Figure 3.6). Previous studies have shown that integrin clustering by multivalent ligands such as fibronectin is sufficient to activate Rac1 (2, 23, 24, 29). Although we can only speculate, we suspect that activated Rac1 helps to stimulate and regulate the organization and rearrangement of the actin cytoskeleton at the point of retrovirus binding and entry, which is consistent with previous work that showed the actin network plays a critical role in an early step of retrovirus infection (13). Actin rearrangement may help to destabilize the plasma membrane and induce lipid-mixing, processes that facilitate the formation of a fusion pore during virus fusion (7, 22, 27). Alternatively, actin rearrangement may help viruses navigate through the cortical cytoskeleton, a barrier that is known to slow or prevent the inward movement of internalized viruses (32).

Most efforts to understand, interfere with, or otherwise control early events in retrovirus infection have focused on manipulating the interactions between the virus-encoded envelope proteins and their cell-surface receptors, since these interactions are absolutely required for retrovirus fusion and infection (4). Nevertheless, our findings and other recent work suggest that proteins that are not encoded by the virus, but which are incorporated into their lipid bilayers from the plasma membranes of the cells that produce them, may also have a significant influence on the outcome of infection (34). An improved understanding of how these molecules interact with host cells and influence infection is likely to prove useful for the design of more efficient or targeted gene transfer vectors, or for the development of novel antiviral therapies.

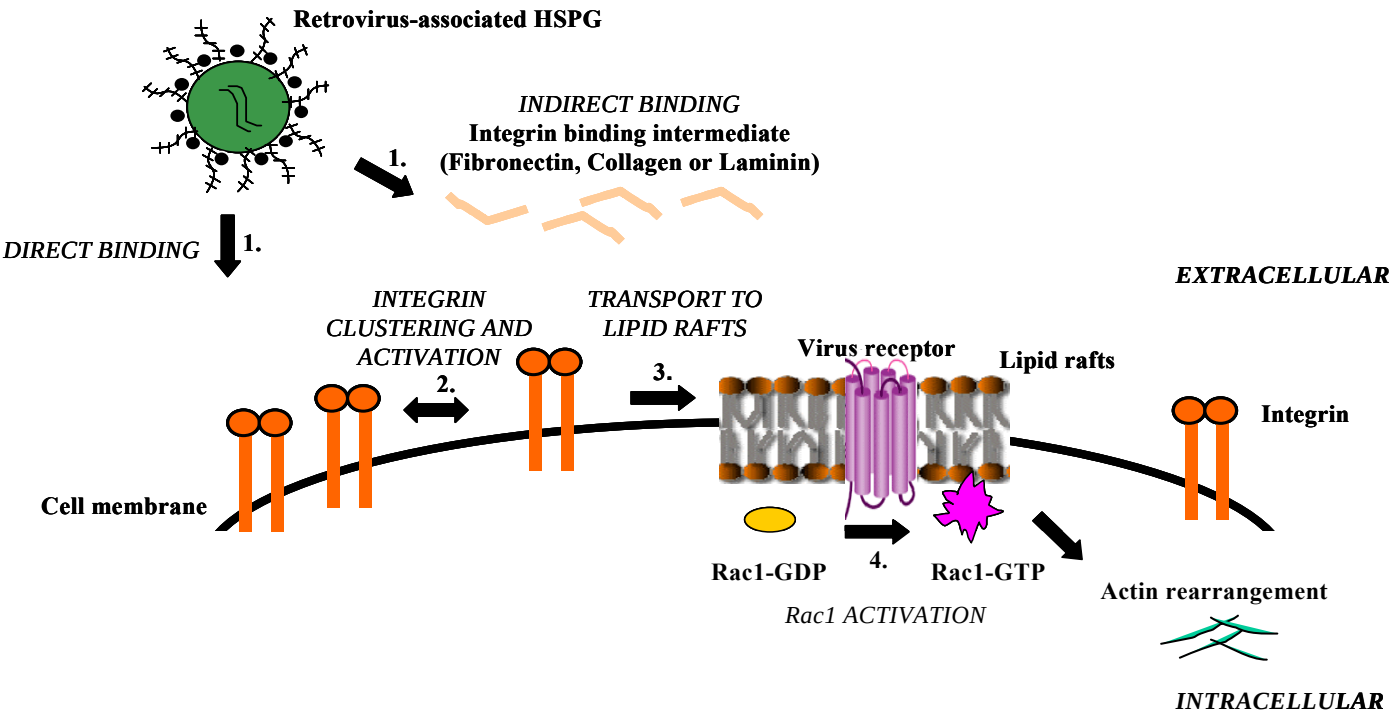

Figure 3.6 Proposed model for the mechanism of retrovirus mediated Rac1 activation in HeLa cells. (1). Retrovirus-associated HSPG interact with cell surface integrins either directly or indirectly by binding to ECM proteins such as fibronectin or collagen. (2). Binding to integrins promotes integrin clustering and activation. (3). The bound integrins are activated and are actively transported to lipid rafts, where retrovirus receptors are localized. (4). Rac1 is activated following clustering of integrins and initiates a signaling cascade which, among other effects, can promote actin rearrangement and subsequent virus entry and fusion.

For example, it may be possible to genetically engineer retroviruses to induce intracellular signaling that improves the efficiency of virus entry and infection.

In addition, further study of these interactions may reveal important rate-limiting steps of infection in cells that have proven difficult to transduce, such as human hematopoietic stem cells. For example, it is interesting to consider whether or not the state of the host cell, in terms of its ability to interact with non-envelope virus-associated proteins, affects the efficiency of retrovirus transduction. One recent study showed that when the cells are placed in suspension, lipid rafts and Rac1, which preferentially localizes to lipid rafts, are rapidly internalized, which significantly downregulates the ability of the cells to activate Rac1 in response to extracellular signals (5). We found that retroviruses do not activate Rac1 in HeLa cells that are cultured in suspension. Furthermore, a number of previous studies suggest that retroviruses, in general, bind to and transduce suspension cells less efficiently than adherent cells (11, 15, 17). Perhaps suspension cells are difficult to transduce, at least in part, because they do not efficiently engage with non-envelope proteins on the surface of retroviruses.

Retrovirus mediated Rac1 activation was observed in HeLa cells in our experimental system. Retrovirus entry mechanism into target cells is cell line dependent. For instance, ecotropic retroviruses fuse at the plasma membrane in rat XC sarcoma cells but enter via endocytosis in mouse NIH3T3 fibroblasts (13). However it has been shown that Adenovirus type 2 entry using the clathrin coated pit endocytic pathway requires Rac1 activation (32). HIV-1 on the other hand, fuses at the plasma membrane and the interaction of HIV-1 envelope with coreceptor CCR5 activates Rac1 (27). Since Rac1 in expressed ubiquitously in human cells, our results taken together with the above studies suggest that retroviruses may trigger Rac1 signaling events in other cell types besides HeLa cells, although further experiments are required to examine this possibility.

In summary, we found that recombinant retroviruses activate Rac1 in HeLa cells. Rac1 activation is independent of viral envelope proteins but requires the presence of virus-

associated HSPG, and β_1 integrins and cholesterol in the plasma membranes of the host cells. In the future, it will be important to investigate the mechanism by which virus-associated HSPG molecules and cell-surface β_1 integrins activate Rac1, and the functional role of activated Rac1 in retrovirus infection.

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CHAPTER 4

β_1- MEDIATED RAC1 ACTIVATION IN HELA CELLS BY MURINE LEUKEMIA VIRUS PARTICLES FACILITATES EARLY STEPS OF VIRUS TRANSDUCTION

4.1 Abstract

Our previous results indicate that retrovirus particles engage β_1 integrins to activate Rac1 in HeLa cells. In order to determine the functional significance of this phenomenon, we exposed recombinant retrovirus particles to HeLa cells transfected with mutants that perturb endogenous levels of active Rac1 or HeLa cells where β_1 integrins had been blocked. We found that over-expression of active Rac1 led to increased virus uptake but directed the virus towards non-productive intracellular pathways. Suppression of Rac1 activity decreased the rate of virus entry 3-fold by affecting a post-binding step of virus transduction. Blocking β_1 integrins in HeLa cells decreased the rate of virus internalization by 30% and the efficiency of gene transfer by 50%. The implications of these findings with respect to retrovirus-cell interactions are discussed.

4.2 Introduction

Recombinant retroviruses are frequently used as gene delivery vectors because they permanently integrate the therapeutic gene into the chromosomal DNA of the target cell (13, 19). Successful gene transfer with retroviruses begins with the binding of the virus to the cell, transport of the bound virus to a location where its cellular receptors are expressed, followed by an interaction between the envelope proteins of the virus and their cellular receptors that leads to fusion of the virus with the cell and its entry into the cytoplasm (7). It is important that we understand the mechanism by which retroviruses enter cells because such knowledge could lead to the development of more efficient and

selective strategies for genetically modifying cells in human gene therapy protocols, or for blocking infection by wild-type, pathogenic retroviruses.

Retroviruses encounter several barriers towards successful entry in target cells. Retroviral receptors are sequestered in specific regions of the cell surface called lipid rafts that occupy 1-10% of the cell surface (2, 9, 16). Depending on the region of cell surface where the initial binding event occurs, successful entry depends on the ability of the virus to find its receptor before losing its infectious activity. Following fusion with the cellular membrane, retroviruses encounter the barrier presented by the cortical actin network against the inward movement of viral capsids (4, 21).

Recent studies suggest that retroviruses utilize target cell machinery to find their receptors and to overcome the actin barrier (6, 15, 21, 22). HIV-1 interaction with target cell integrin LFA-1 enhances virus attachment and actively transports the virus to lipid rafts thereby allowing sufficient number of interactions between the HIV-1 envelope gp120 and its cellular receptor CD4 (22). The fusion of HIV-1 envelope with its cellular coreceptor CCR5 activates Rac1 and it has been suggested that Rac1 activation enables actin rearrangement required during virus fusion (15). In our previous study, we found that retroviruses engage β_1 integrins to activate Rac1 in HeLa cells. Since interaction with integrins and Rac1 activation in target cells, assist processes that are involved in HIV-1 entry, we hypothesized that β_1-mediated Rac1 activation in HeLa cells by murine leukemia virus particles facilitates early steps of virus transduction.

4.3 Materials and methods

Reagents, antibodies and plasmids. Polyfect was purchased from Qiagen (Valencia, CA), Exgen was purchased from Fermentas (Hanover, MD). Dimethyl amiloride, Igepal and polybrene were purchased from Sigma Chemical Company (St, Louis, MO). Mouse anti-EE monoclonal antibody, mouse anti-LAMP and plasmid DNA

encoding Rac1, RacQ61L and RacT17N were a kind gift of Harish Radhakrishna (School of Biology, Georgia Tech) (17). Cy3-conjugated donkey anti-mouse, AMCA-conjugated donkey anti-mouse, and goat anti-rabbit and donkey sera were purchased from Jackson Immunoresearch Laboratories (WestGrove, PA). Function blocking anti-β_1 integrin monoclonal antibody clone P5D2 and rabbit anti-EE primary antibody were purchased from Covance Research Products (Denver, PA). Hydrogen peroxide 30%, and Polyoxyethylene 20-Sorbitan Monolaurate (Tween 20) were from Fisher Scientific (Fair Lawn, NJ). Non-fat dry milk (blotting grade) was from Bio-Rad Laboratories (Hercules, CA). o-Phenylenediamine Dihydrochloride (OPD) was from Sigma(St. Louis, MO). Mouse anti-p30 antibodies were purified from the supernatant of the CRL-1219 hybridoma cell line (ATCC, Rockville, MD) following standard procedures. The goat polyclonal anti-p30 antibody (78S221) was from Quality Biotech (Camden, NJ). The horseradish peroxidase conjugated rabbit anti-goat immunoglobulin G polyclonal antibody was from Zymed Laboratories (South San Francisco, CA). Complete mini protease inhibitor cocktail tablets were purchased from Roche Diagnostics (Indianapolis, IN). Chlorophenol-red- β-D-galactopyranoside (CPRG) was purchased from EMD Biosciences (San Diego, CA).

Cell culture. HeLa (human cervical cancer) cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone Labs Inc., Logan, UT) with 10% fetal bovine serum (FBS; Hyclone Labs Inc.), 100 U/mL of penicillin, 100 μg/mL of streptomycin (Hyclone Labs Inc.), and 110 μg/mL of sodium pyruvate (Hyclone Labs Inc.) (DMEM/FBS). TELCeB6 cells (a kind gift from F.L. Cosset) expressing Moloney MLV Gag and Pol, and the retroviral vector MFGnlsLacZ, and 293T/17 (human embryonic kidney epithelial) cells were cultured in DMEM/FBS.

Virus production. An amphotropic packaging cell line (TELCeB6-A) was generated by stable transfection of TELCeB6 cells. Five micrograms of the plasmid FB4070ASALF, an expression plasmid that encodes for the amphotropic envelope glycoprotein (a kind gift of Stephen Russell), was dissolved in 400 μL of 0.25M $CaCl_2$, mixed with 400 μL of 2X HEPES buffered saline (274 mM NaCl, 42 mM Hepes acid, 10 mM KCl, 1.4 mM Na_2HPO_4 and 12 mM dextrose), incubated at room temperature for 20 minutes, then added to a 50% confluent T75 flask of TELCeB6 cells. Twelve hours after transfection the cells were washed with PBS, and then the medium replaced with fresh DMEM/FBS. Two days later the cells were trypsinized, pelleted, and resuspended. Two hundred microliters of the resuspended cells were diluted in 10 mL of selective medium (DMEM, 10% FBS, 50 μg/mL of phleomycin, and 7 μg/mL of blasticidin), and plated in a T75 flask. Fourteen days later pooled clones of stably transfected cells were frozen for later use. To generate retrovirus stocks, virus-producing cells were grown to confluence in T175 tissue culture flasks, and then incubated for 24 h with 35 mL of cell culture medium. The virus-laden tissue culture medium was harvested, filter sterilized (0.45-μm), then frozen (-80°C) for later use. GFP-labeled lentivirus was produced by transient transfection of 293T/17 cells. The cells were plated in a 10 cm dish (1.7×10^7 cells/dish) and co-transfected with Lipofectamine 2000 and 6 μg each of plasmid DNA encoding the lentiviral packaging construct pCMVΔR8.91 (kind gift of Scott S. Case), lentivirus vector pTY-EfnlacZ and plasmid FB4070ASALF expressing amphotropic murine leukemia virus envelope protein and plasmid encoding GFP-Vpr (kind gift of Thomas J. Hope). Virus laden tissue culture medium was harvested 36, 48, and 60 h after transfection, filtered (0.45 μm), and frozen (-80°C) for later use. Envelope-deficient GFP-labeled lentivirus was produced by the transient transfection of 293T/17 cells as described above except the addition of FB4070ASALF to the transfection mix.

Immunofluorescence microscopy. All cells were immunostained by plating them on coverslips (#1.5, 12 mm, Fisher Scientific; Suwanee, GA). Following experimental treatment, the cells were fixed with 2% paraformaldehyde (1 mL/well) for 10 min, and then blocked with PBS/sera (1 mL/well) (5% donkey sera in PBS, PBS/sera) for 15 min on a shaker. Next, the cells were incubated with primary antibody in PBS/sera/0.2% saponin for 1 h at room temperature, washed 3 times with PBS, incubated with the secondary antibody in PBS/sera/0.2% saponin for 1 h at room temperature and washed 3 times with PBS. The cells were subsequently washed with double distilled water (1 mL/well) and the coverslips mounted on glass slides with gelvatol. The following dilutions were used for immunofluorescence staining. Mouse anti-EE antibody (1:200), Rabbit anti-EE (1:50), Mouse anti-LAMP (1:5000), Cy3-conjugated donkey anti-mouse (1:800), AMCA-conjugated donkey anti-mouse (1:400), AMCA-conjugated goat anti-rabbit (1:100), Alexa-594-conjugated Concanavalin A (1:500). All cells were visualized by confocal microscopy (Zeiss LSM 510, 40X oil objective). For each experimental condition tested, pinhole, objective magnification, zoom, optical slice thickness, scan averaging, and pixel resolution were kept constant among the red, green and the blue channels and eight cells were randomly chosen and analyzed for the extent of colocalization of fluorescent probes using Metamorph Imaging System Software (Universal Imaging Corp, WestChester, PA).

ELISA for p30. We used an enzyme-linked immunosorbent assay (ELISA) to determine the concentration of virus capsid protein (p30) in cell lysates. ELISA plates (Nunc immuno Maxisorp 96-well plates, Nalge Nunc International, Rochester, NY) were coated overnight at 4°C with 10 μg/mL of mouse anti-p30 antibody (100 μL/well) in PBS. The next day, the antibody solution was removed and blocking buffer (PBS, 0.05% Tween-20, 5% non-fat milk) added (200 μL/well) for 2 h at 37°C to block non-specific

binding sites. Samples were brought to 0.5% Triton-X to expose the p30 antigen, then added to the ELISA plate (100 μL/well) and incubated for 1 h at 37°C. Bound p30 was sandwiched by the addition of the goat polyclonal anti-p30 antibody diluted 1:1000 in blocking buffer, and incubated for 1 h at 37°C. The horseradish peroxidase conjugated polyclonal rabbit anti-goat immunoglobulin G was diluted 1:5000 in blocking buffer then added to the ELISA plate (100 μL/well) for 1 hour at 37°C to enable detection and quantitation of the sandwiched p30 antigen. The plates were developed for 5 min using hydrogen peroxide (H_2O_2) and OPD (100 μg/well) from a solution of 10 mg of OPD and 10 μl H_2O_2 in 25 mL of substrate buffer (24 mM citric acid-monohydrate, 51 mM Na_2HPO_4-$7H_20$, pH 5.0). 8N sulfuric acid (50 μL/well) was used to stop the reaction and the optical density at 490 nm (OD_{490}) measured using an absorbance plate reader and the non-specific background at 650nm subtracted. Values for replicate wells without virus were subtracted as background. Values for each point are the average of at least triplicate wells.

Beta-galactosidase (β-gal) assay (CPRG assay). HeLa cells were plated at 7000 cells/well in a 96 well dish and 24 hours later transduced with *lac Z* encoding virus under the experimental conditions tested. Two days after transduction, the medium was removed and the cells washed once with 100 μL of phosphate-buffered saline (PBS) containing 1mM $MgCl_2$. After removal of the wash solution, 50 μL of lysis buffer (PBS with 1mM $MgCl_2$ and 0.5% Igepal) were added to each well, and the plate incubated at 37°C. After 30 min, 50 μl of lysis buffer with 2.4 mg/ml CPRG warmed to 37°C were added to each well, and the plate incubated at 37°C for 5 to 60 min until a visible red color was obtained. The reactions were halted by the addition of 20 μL per well of stop buffer (1M Na_2CO_3). The optical density at 570 nm (OD_{570}) was measured using an absorbance plate reader (Molecular Devices, Menlo Park, CA) and the non-specific

background at 650 nm subtracted. Values for replicate wells without virus were subtracted as background. Values for each point are the averages of at least triplicate wells.

4.4 Results

Over-expression of Rac1 and Rac1-GTP increases virus uptake in HeLa cells. Our hypothesis is that β_1-mediated Rac1 activation in HeLa cells by retroviruses affects early steps of virus transduction. As a first step towards testing our hypothesis, we decided to examine the effect of over-expression of wild-type Rac1 or constitutively active Rac1, Rac1-GTP, in HeLa cells on virus entry. HeLa cells were plated on coverslips in a 12 well dish at 10,000 cells/well and 24 hours later transfected with Polyfect complexed plasmid DNA encoding for Rac1 (EE *(Glutamine-Glutamine)* tagged for visualization) or a constitutively active mutant of Rac1, RacQ61L (EE tagged for visualization). Transfection with plasmid DNA encoding Rac1 produces Rac1 protein above endogenous cellular levels and transfection with RacQ61L leads to over-expression of activated Rac1, Rac1-GTP, in HeLa cells. Twenty-four hours later, we exposed the transfected cells to GFP-labeled lentivirus pseudotyped with the amphotropic murine leukemia virus (MLV) envelope protein. Lentivirus brought to 20µg/ml polybrene (PB) was centrifuged onto HeLa cells for 30min at 2100 x g and 37^{o}C to maximize virus binding. Following centrifugation, the virus was removed and the cells exposed to medium prewarmed to 37^{o}C for 60 min. The cells were subsequently fixed, permeabalized, stained for Rac1 or RacQ61L expression using mouse anti-EE primary antibody and Cy3 conjugated donkey anti-mouse secondary antibody and visualized using confocal microscopy. Virus entry in cells was quantified using Metamorph Imaging System Software. We found that virus uptake in cells over-expressing Rac1 was 2.3-fold higher than non-transfected cells and virus uptake in cells expressing RacQ61L was 3-4

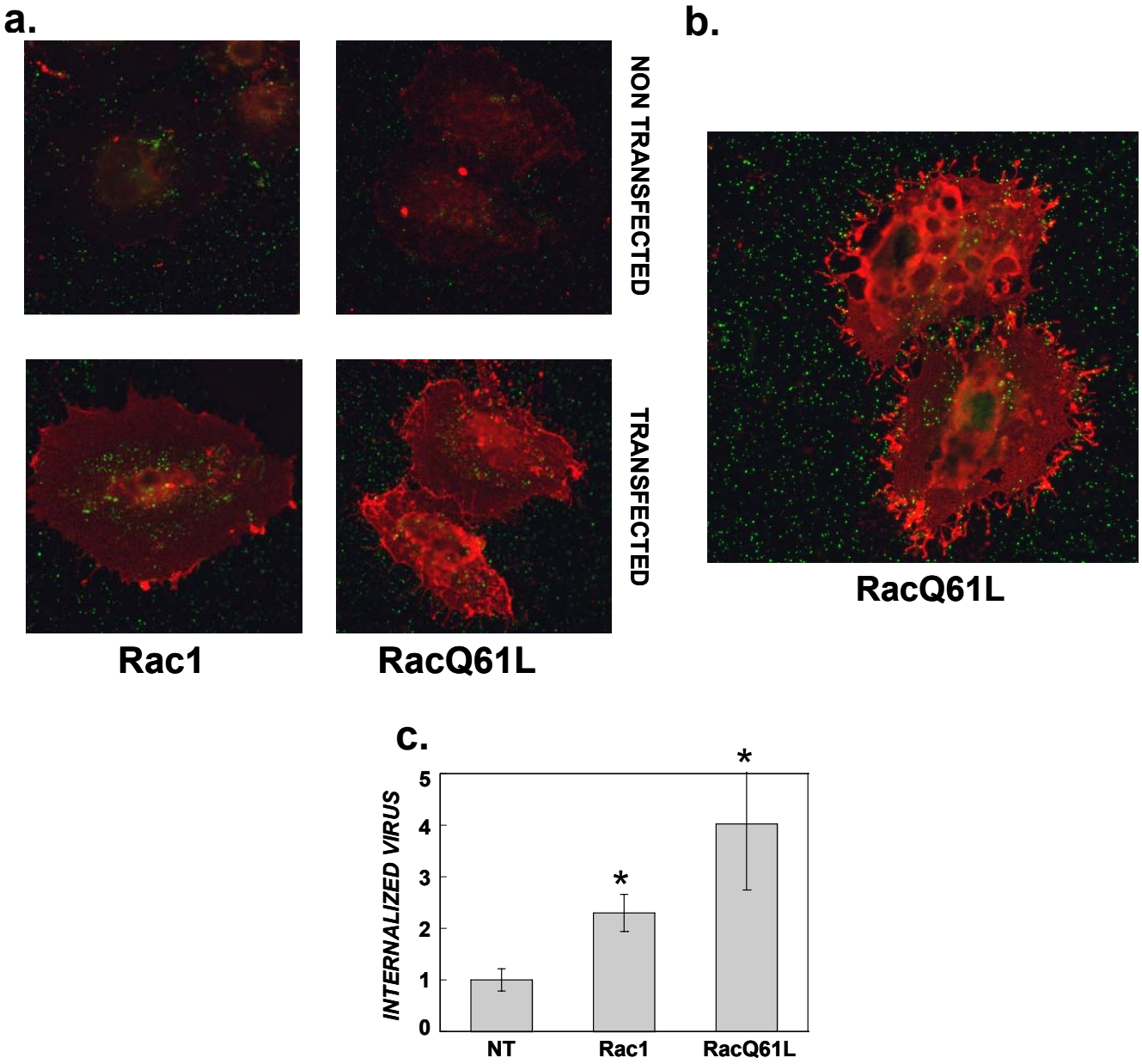


Figure 4.1 Over-expression of Rac1 and Rac1-GTP increases virus uptake in HeLa cells. HeLa cells were plated on coverslips in a 12 well dish at 10,000 cells/well and 24 hours later transfected with Polyfect complexed plasmid DNA encoding for Rac1 or RacQ61L. Twenty-four hours later, the transfected cells were exposed to GFP-labeled lentivirus pseudotyped with the amphotropic MLV envelope protein brought to 20µg/ml PB. Virus was centrifuged onto HeLa cells for 30min at 2100 x g and 37^{o}C, supernatant removed and the cells exposed to medium prewarmed to 37^{o}C for 60 min. (a and b). The cells were subsequently fixed, permeabalized, stained for Rac1 or RacQ61L expression using mouse anti-EE primary antibody and Cy3 conjugated donkey anti-mouse secondary antibody and visualized using confocal microscopy. (c). Virus entry in cells was quantified using Metamorph. Values are normalized to virus uptake in non-transfected cells. Eight cells were analyzed for each experimental condition. (*) denotes statistically significant ($p < 0.05$) differences from NT condition. Transfected cell-red, Virus-green; Non-transfected cell denoted as-NT

-fold higher than non-transfected cells (Figure 4.1a and c). On close examination of the plasma membrane in transfected cells we found that virus was engulfed in membrane extensions on the cell surface that resembled lamellapodia (Figure 4.1b). Cells transfected with RacQ61L displayed a greater number of lamellapodia than cells transfected with Rac1 (Figure 4.1a).

Formation of lamellapodia in transfected cells increases the rate of virus entry. In order to determine the effect of lamellapodia formation on virus entry we decided to quantify the amount of virus entry per unit time in transfected cells. HeLa cells were plated on coverslips in a 12 well dish at 10,000 cells/well and 24 hours later transfected with plasmid DNA encoding for Rac1 or RacQ61L. Twenty-four hours later, we exposed the transfected cells to GFP-labeled lentivirus pseudotyped with the amphotropic MLV envelope protein. Lentivirus brought to 20μg/ml PB was centrifuged onto HeLa cells for 30min at 2100 x g and 37^{o}C to maximize virus binding. Following centrifugation, the virus was removed and the cells exposed to medium prewarmed to 37^{o}C for 0, 10, 20, 30, 45 and 60 min. The cells were subsequently fixed, permeabalized, stained for Rac1 or RacQ61L expression, visualized using confocal microscopy and virus entry in cells quantified using Metamorph. We found that lamellapodia formation increases the rate of virus entry 2-fold in Rac1 transfected cells and 3-4-fold in RacQ61L transfected cells (Figure 4.2 a and b).

Increased virus uptake is not envelope-receptor mediated. Since the interaction of the virus envelope protein with its cellular receptor is central to a productive gene transfer event, we wanted to determine if the increase in virus entry due to the formation of lamellapodia was envelope-receptor mediated. HeLa cells were plated on coverslips in a 12 well dish at 15,000 cells/well and 24 hours later transfected

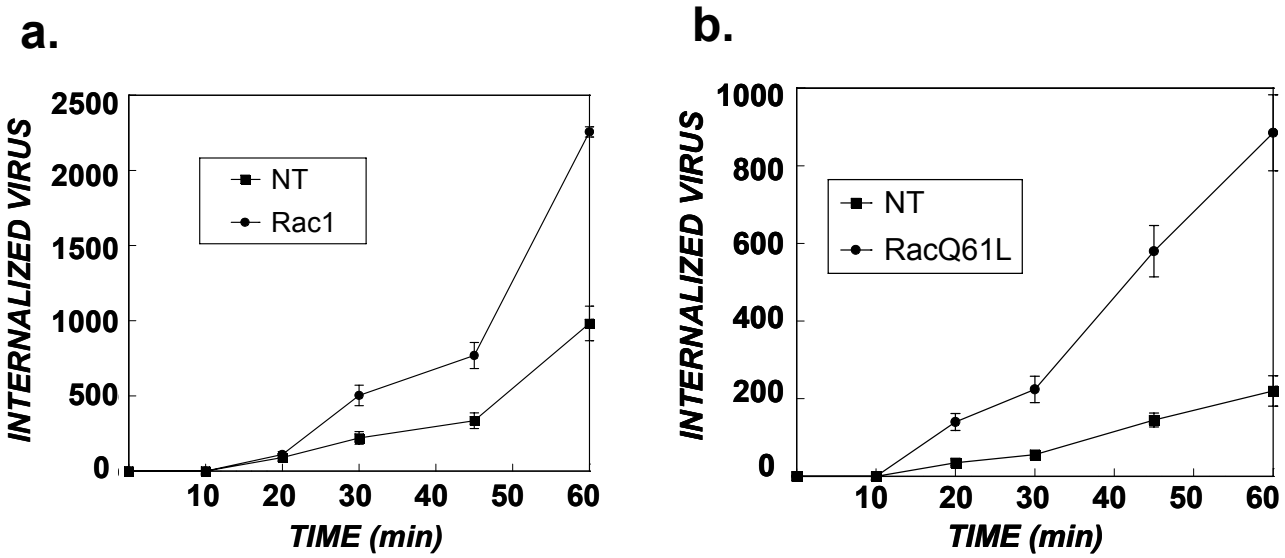


Figure 4.2 Formation of lamellapodia in transfected cells increases the rate of virus entry. HeLa cells were plated on coverslips in a 12 well dish at 10,000 cells/well and 24 hours later transfected with plasmid DNA encoding for (a) Rac1 or (b) RacQ61L. Twenty-four hours later, we exposed the transfected cells to GFP-labeled lentivirus pseudotyped with the amphotropic MLV envelope protein. Lentivirus brought to 20µg/ml PB was centrifuged onto HeLa cells for 30min at 2100 x g and 37^{o}C to maximize virus binding. Following centrifugation, the virus was removed and the cells exposed to medium prewarmed to 37^{o}C for 0, 10, 20, 30, 45 and 60 min. The cells were subsequently fixed, permeabalized, stained for Rac1 or RacQ61L expression, visualized using confocal microscopy and virus entry in cells quantified using Metamorph. Eight cells were analyzed for each experimental condition. Non-transfected cell denoted as-NT

with Exgen complexed plasmid DNA encoding RacQ61L. Twenty-four hours later, we exposed the transfected cells to envelope-deficient GFP-labeled lentivirus brought to 20µg/ml PB. Envelope-deficient lentivirus was centrifuged onto HeLa cells for 30min at 2100 x g and 37^{o}C to maximize virus binding. Following centrifugation, the virus was removed and the cells exposed to medium prewarmed to 37^{o}C for 60 min. The cells were subsequently fixed, permeabalized, stained for RacQ61L expression and visualized using confocal microscopy. Virus entry in cells was quantified using Metamorph. We found that expression of RacQ61lL in HeLa cells led to a 3.5-fold increase in envelope-deficient lentivirus uptake as compared to non-transfected cells (Figure 4.3 a and b). Taken together our results suggest that the rate of virus entry is increased in cells over expressing Rac1 or constitutively active RacQ61L. Increased virus uptake is due to the formation of lamellapodia that engulf virus in the cell vicinity in a non envelope-receptor mediated mechanism.

Increased virus uptake is due to macropinocytosis. Since cells over expressing RacQ61L have been shown previously to increase macropinocytic activity (3), we decided to investigate the effect of macropinocytosis inhibitor Dimethyl Amiloride (DMA) on virus uptake in RacQ61L transfected HeLa cells. HeLa cells were plated at 10,000 cells/ well on a coverslip in a 12 well tissue culture dish and transfected the next day with RacQ61L. Twenty-four hours later, the cells were preincubated with 0 or 100 µM DMA for 1 hr at 37^{o}C and then exposed to GFP-labeled lentivirus brought to 20 µg/ml PB. Virus was centrifuged onto the cells for 30min at 37^{o}C to maximize binding, the supernatant removed and the cells incubated for an additional 60 min with prewarmed medium. The cells were fixed, premeabalized, stained for RacQ61L, visualized by confocal microscopy and intracellular virus quantified by Metamorph. We found that virus entry was 4-fold lower in DMA-treated transfected cells as compared

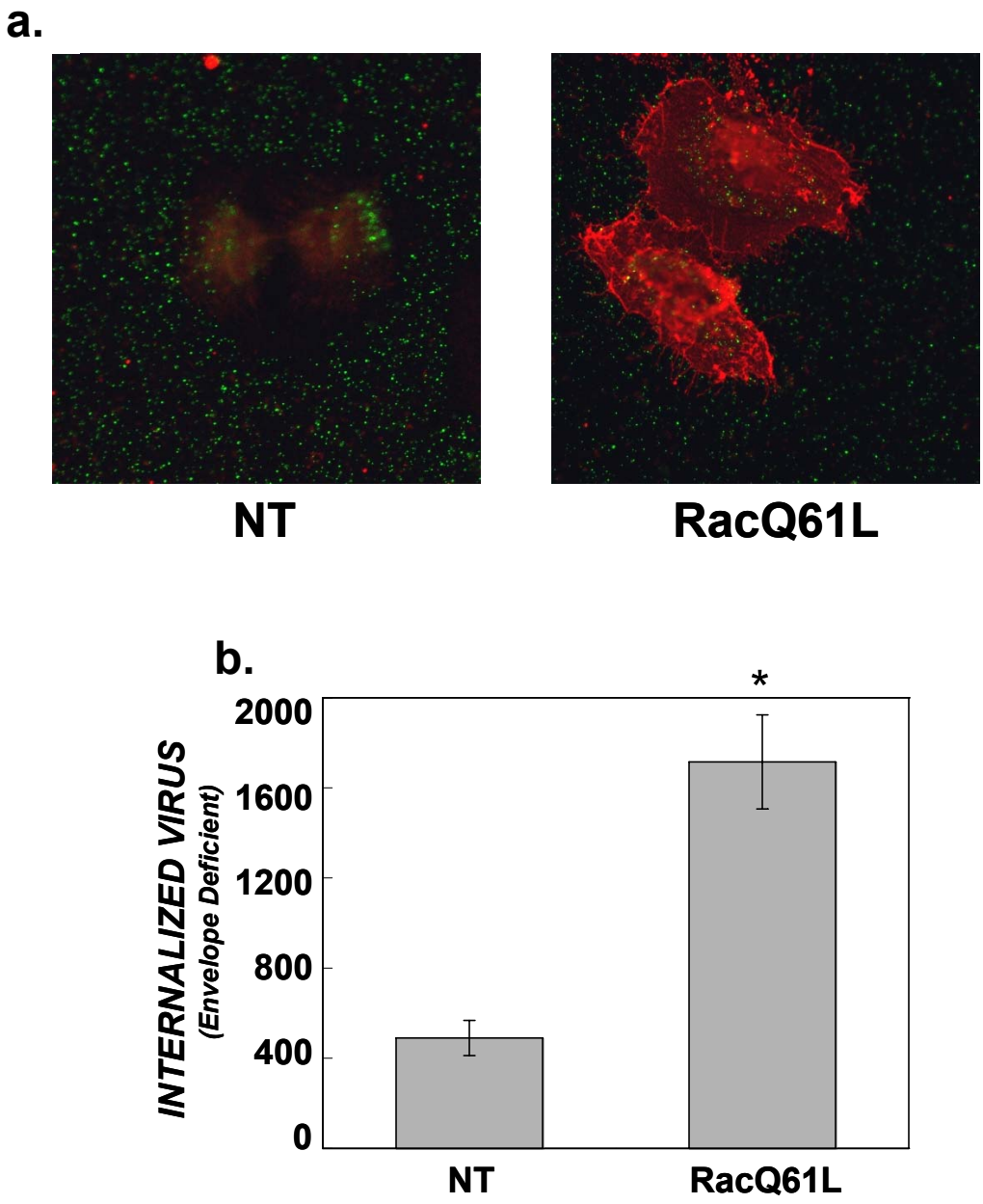


Figure 4.3 Increased virus uptake is not envelope-receptor mediated. (a).HeLa cells were plated on coverslips in a 12 well dish at 15,000 cells/well and 24 hours later transfected with Exgen complexed plasmid DNA encoding RacQ61L. Twenty-four hours later, we exposed the transfected cells to envelope-deficient GFP-labeled lentivirus brought to 20µg/ml PB. Envelope-deficient lentivirus was centrifuged onto HeLa cells for 30min at 2100 x g and 37^{o}C to maximize virus binding. Following centrifugation, the virus was removed and the cells exposed to medium prewarmed to 37^{o}C for 60 min. The cells were subsequently fixed, permeabalized, stained for RacQ61L expression and visualized using confocal microscopy. (b). Virus entry in cells was quantified using Metamorph. Eight cells were analyzed for each experimental condition. (*) denotes statistically significant ($p < 0.05$) differences from NT condition. Transfected cell-red, Virus-green; Non-transfected cell denoted as-NT

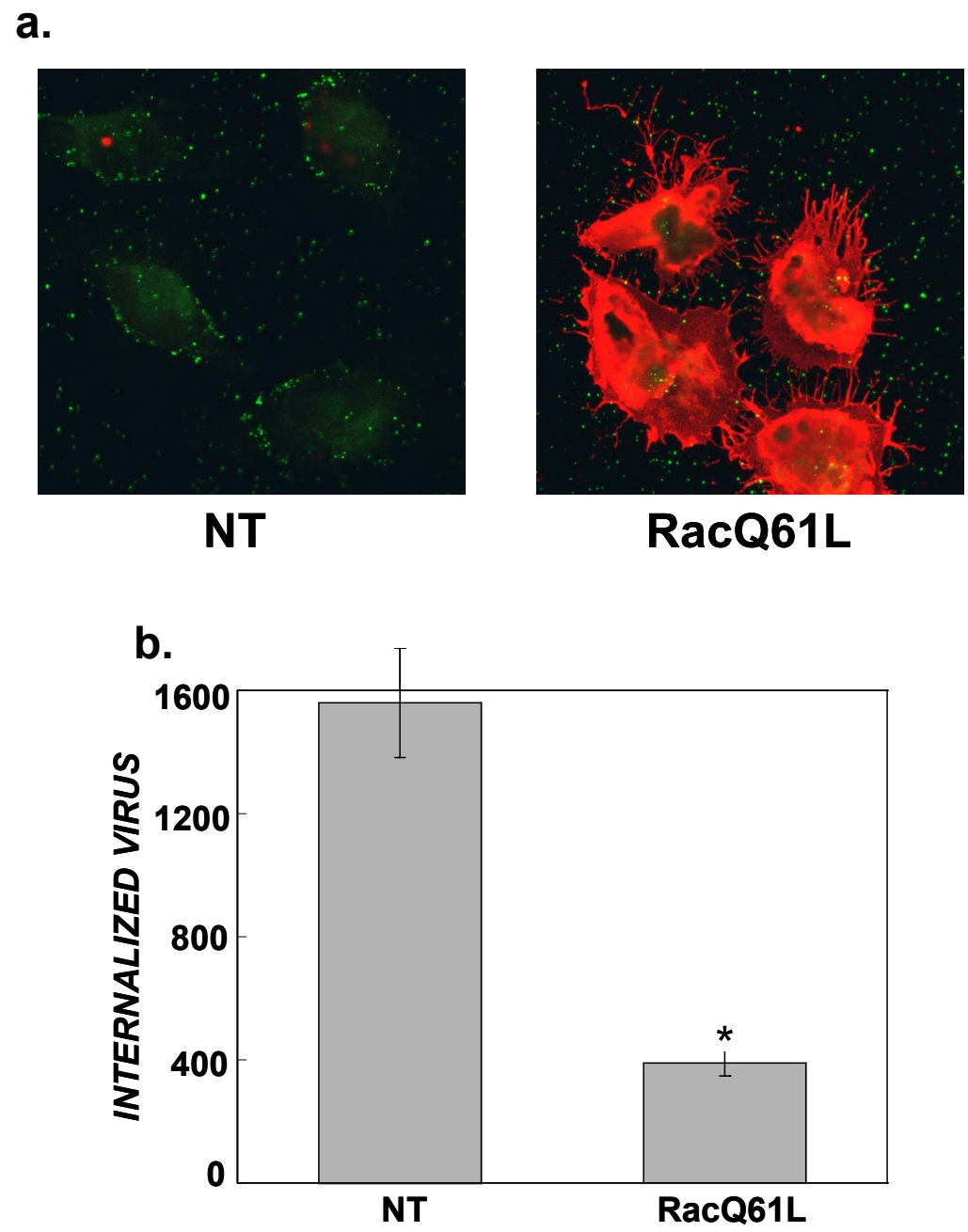


Figure 4.4 Increased virus uptake is due to macropinocytosis. (a). HeLa cells were plated at 10,000 cells/ well on a coverslip in a 12 well tissue culture dish and transfected the next day with RacQ61L. Twenty-four hours later, the cells were preincubated with 0 or 100 μM DMA for 1 hr at 37^{o}C and then exposed to GFP-labeled lentivirus brought to 20 μg/ml PB. Virus was centrifuged onto the cells for 30min at 37^{o}C to maximize binding, the supernatant removed and the cells incubated for an additional 60 min with medium prewarmed to 37^{o}C. The cells were fixed, premeabalized, stained for RacQ61L, visualized by confocal microscopy and (b). intracellular virus quantified by Metamorph. Eight cells were analyzed for each experimental condition. (*) denotes statistically significant ($p < 0.05$) differences from NT condition. Transfected cell-red, Virus-green; Non-transfected cell denoted as-NT

with DMA-treated non-transfected cells (Figure 4.4 a and b). Presence of DMA did not affect virus entry in non-transfected cells (Figure 4.4 c and d). Taken together our results suggest that increased virus uptake in cells transfected with RacQ61L is due to macropinocytosis and independent of envelope-receptor interaction.

Viruses are colocalized in lysosomes after macropinocytic uptake. It has been previously shown that retrovirus entry via macropinocytosis is non-productive and leads to intracellular degradation of the virus (8). To examine this possibility, we decided to investigate the localization of virus in HeLa cells transfected with RacQ61L. HeLa cells were plated, transfected with RacQ61L and exposed to GFP-labeled lentivirus for 90 min as previously described. Following virus exposure the cells were fixed, permeabalized and stained for lysosomes using mouse anti-LAMP1 primary antibody and Cy3 conjugated donkey antimouse secondary antibody. After lysosomal staining, the cells were stained for RacQ61L with rabbit anti-EE primary antibody and AMCA-conjugated goat anti-rabbit secondary antibody, visualized by confocal microscopy and virus colocalization with lysosomes quantified using Metamorph. We found that virus colocalization in lysosomes was 3-fold higher in RacQ61L transfected cells as compared with non-transfected cells (Figure 4.5 a and b). Taken together our results suggest that expression of RacQ61L in HeLa cells increases the rate of virus entry but towards a non-productive intracellular pathway.

Expression of dominant negative Rac1 decreases the rate of virus entry in HeLa cells by affecting a post-binding step. Our results suggest that the expression of constitutively active Rac1 mutant RacQ61L, causes the formation of lamellapodia that induce macropinocytic uptake of virus particles in HeLa cells leading to a non-productive intracellular fate. Therefore, to test our hypothesis that β_1-mediated Rac1 activation is required for early steps of virus entry, we decided to investigate the effect on virus entry of a dominant negative mutant of Rac1, RacT17N, which suppresses the formation of

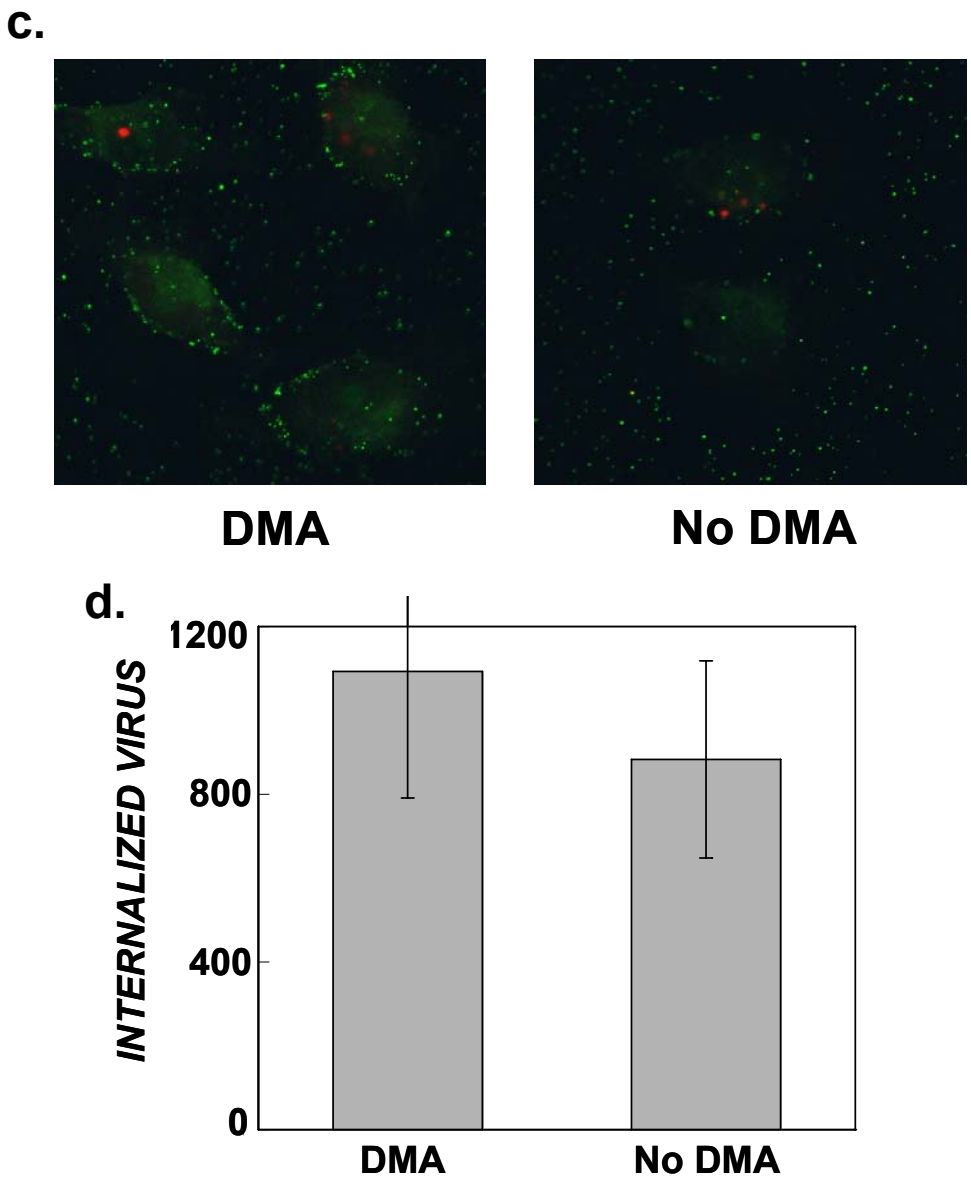


Figure 4.4 DMA does not affect virus entry in non-transfected cells (c). HeLa cells were plated at 10,000 cells/ well on a coverslip in a 12 well tissue culture dish and transfected the next day with RacQ61L. Twenty-four hours later, the cells were preincubated with 0 or 100 μM DMA for 1 hr at 37^{o}C and then exposed to GFP-labeled lentivirus brought to 20 μg/ml PB. Virus was centrifuged onto the cells for 30min at 37^{o}C to maximize binding, the supernatant removed and the cells incubated for an additional 60 min with medium prewarmed to 37^{o}C. The cells were fixed, premeabalized, stained for RacQ61L, visualized by confocal microscopy and (d). intracellular virus quantified by Metamorph. Eight cells were analyzed for each experimental condition. Shown here are non-transfected cells. Virus-green

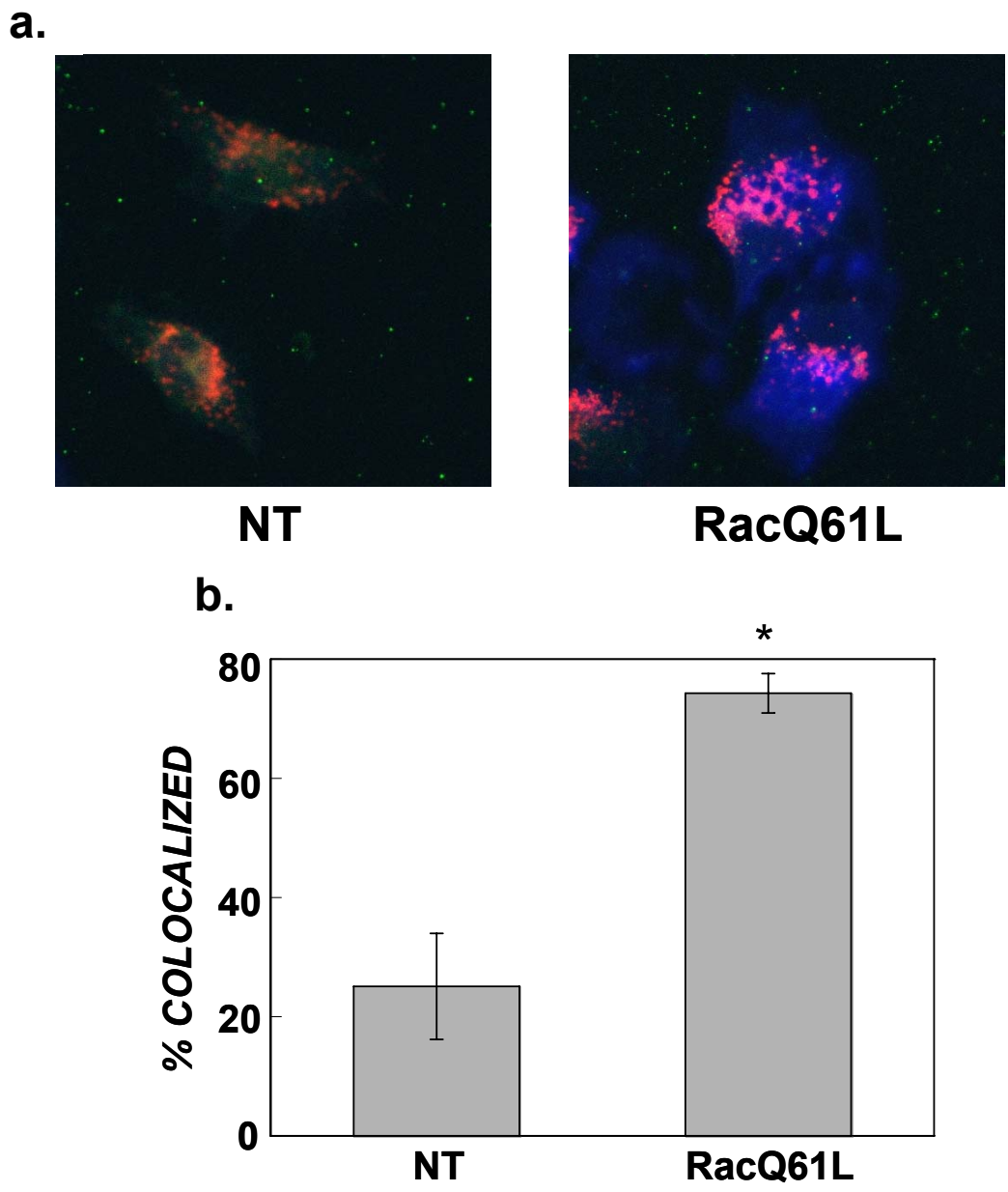


Figure 4.5 Viruses are colocalized in lysosomes after macropinocytic uptake. (a.) HeLa cells were plated at 10,000 cells per well in a 12 well dish and, transfected the next day with RacQ61L. Twenty-four hours later, GFP-labeled lentivirus brought to 20 µg/ml PB was centrifuged onto the cells at 4°C and 2100 x g for 30 min and the cells incubated for an additional 90 min at 37°C. Following virus exposure, the cells were fixed, permeabalized and stained for lysosomes using mouse anti-LAMP1 primary antibody and Cy3 conjugated donkey anti-mouse secondary antibody. After lysosomal staining, the cells were stained for RacQ61L with rabbit anti-EE primary antibody and AMCA-conjugated goat anti-rabbit secondary antibody, visualized by confocal microscopy and (b.) virus colocalization with lysosomes quantified using Metamorph. Eight cells were analyzed for each experimental condition. (*) denotes statistically significant ($p < 0.05$) differences from NT condition. Transfected cell-blue, Virus-green, lysosomes-red; Non-transfected cell denoted as-NT

active Rac1, Rac1-GTP, in HeLa cells. HeLa cells were plated, 24hrs later, transfected with RacT17N , exposed to GFP-labeled lentivirus as described above for 0, 10, 20, 30, 45 or 60 min. The cells were fixed, permeablized, stained for RacT17N (EE-tagged) with mouse anti-EE primary antibody and Cy3 conjugated donkey anti-mouse secondary antibody, visualized by confocal microscopy and the amount of intracellular virus quantified using Metamorph. We found that the rate of virus entry in RacT17N transfected cells was 3-fold lower than non-transfected cells (Figure 4.6 a and b). The lower rate of virus entry in RacT17N transfected HeLa cells as compared to non-transfected cells may be due to inefficient binding of virus to the cell surface. To examine this possibility, we quantified the amount of virus bound to surfaces of HeLa cells expressing RacT17N and compared it to virus bound to surfaces of non-transfected HeLa cells. HeLa cells were plated, transfected with RacT17N, and exposed to GFP-labeled virus brought to 20 μg/ml of PB. Virus was centrifuged onto the cells at 2100 x g and 4^{o}C for 30 min and the cells immediately chilled to block virus entry. Next the cell surface was stained with Alexa 594 concanavalin A, the cells fixed, permeabalized and stained for RacT17N with mouse anti-EE primary antibody and AMCA-conjugated donkey anti-mouse secondary antibody, visualized by confocal microscopy and the amount of virus bound to the cell surface quantified using Metamorph. We found that equal amounts of virus bound to the surfaces of RacT17N transfected cells as compared to non-transfected cells suggesting that a post binding step such as virus fusion with the cell membrane or intracellular trafficking may be impaired due to RacT17N expression (Figure 4.7 a and b).

Blocking β_1 integrins on the surface of HeLa cells affects virus internalization and efficiency of gene transfer. Our current results suggest that suppression of Rac1 activity affects virus entry in HeLa cells. Since our previous results

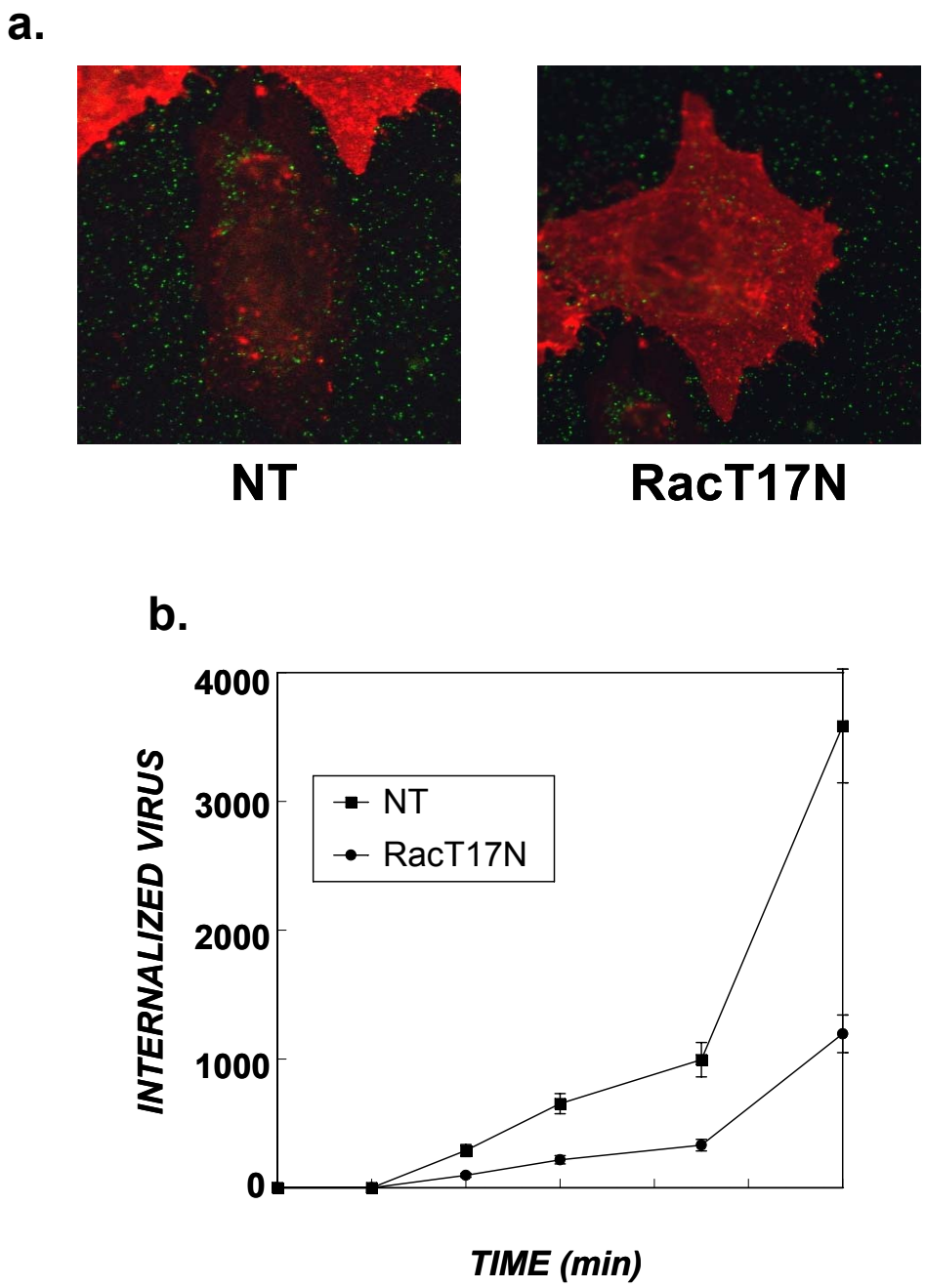
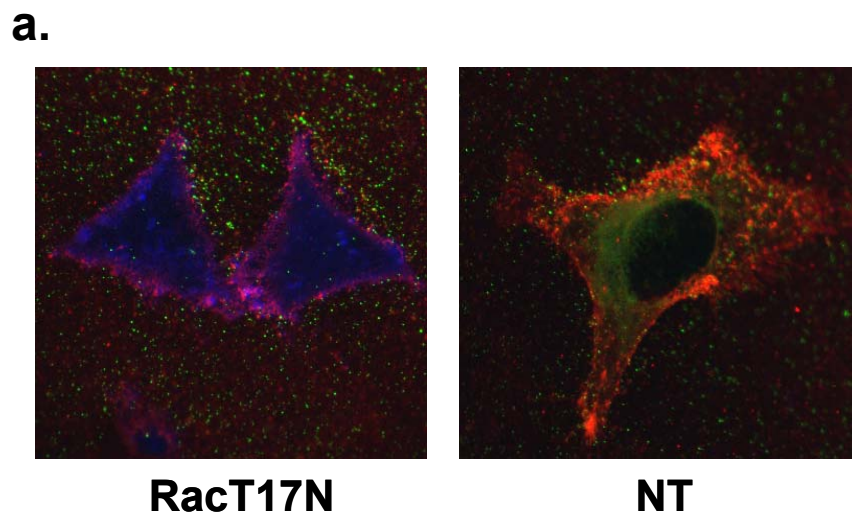


Figure 4.6 Expression of dominant negative Rac1 decreases the rate of virus entry in HeLa cells (a). HeLa cells were plated at 10,000 cells per well in a 12 well dish and, transfected the next day with RacT17N. Twenty-four hours later, the cells were exposed to GFP-labeled lentivirus brought to 20 µg/ml PB for 0, 10, 20, 30, 45 or 60 min. The cells were fixed, permeabalized, stained for RacT17N, visualized by confocal microscopy and (b). the amount of intracellular virus quantified using Metamorph. Eight cells were analyzed for each experimental condition. Transfected cell-red, Virus-green; Non-transfected cell denoted as-NT.



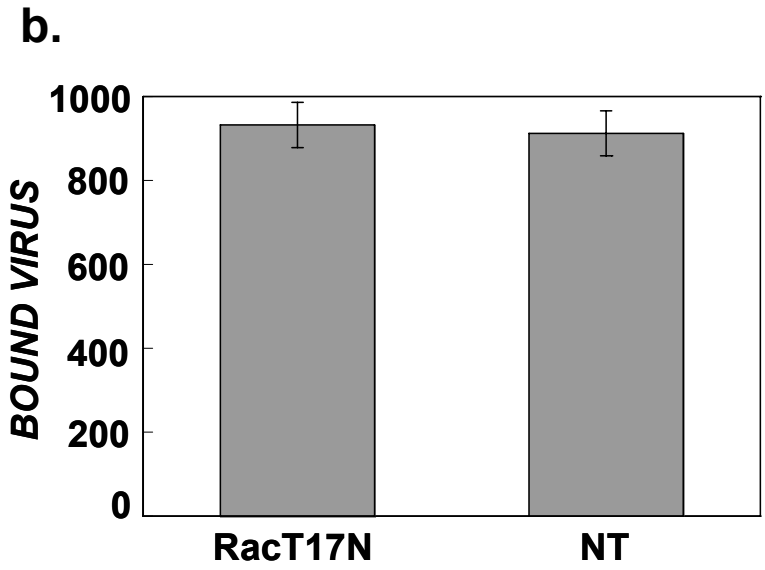


Figure 4.7 Expression of dominant negative Rac1 does not affect virus binding. (a). HeLa cells were plated, transfected with RacT17N, and the next day exposed to GFP-labeled virus brought to 20 µg/ml of PB. Virus was centrifuged onto the cells at 2100 x g and 4^{o}C for 30 min and the cells immediately chilled to block virus entry. Next, the cell surface was stained with Alexa 594 concanavalin A, the cells fixed, permeabalized and stained for RacT17N with mouse anti-EE primary antibody and AMCA-conjugated donkey anti-mouse secondary antibody, visualized by confocal microscopy and (b). the amount of virus bound to cell surface quantified using Metamorph. Eight cells were analyzed for each experimental condition. Transfected cell-blue, Virus-green, Cell surface-red; Non-transfected denoted as-NT

indicate that β_1 integrins on the surfaces of HeLa cells mediate Rac1 activation, we decided to investigate the effect of blocking β_1 integrins on virus entry. HeLa cells were plated at 300,000 cells/well in a 12 well dish and the next day chilled at 4^{o}C for 30 min in DMEM to block endocytosis. Next the cells were incubated with 42.5 µg/ml of function blocking anti-β_1 integrin antibody in DMEM for 60 min at 4^{o}C. Next, virus (with 0 or 10 µg/ml anti-β_1 integrin antibody) previously chilled at 4^{o}C and diluted to 40% by volume with fresh medium was exposed to HeLa cells for 4 hr at 4^{o}C. Following virus exposure, the cells were lysed and the amount of virus bound to cells was quantified in a p30 ELISA. In parallel, cells were exposed to medium prewarmed at 37 ^{o}C (with 0 or 30 µg/ml anti-β_1 integrin antibody) for 0, 157, 217, 277 or 337 min. At each time point the cells were washed with 1 X PBS to remove any unbound virus, trypsinized to remove any extracellular virus, and lysed to expose the internalized virus that was quantified in a p30 ELISA. We found that equivalent amounts of virus bound to HeLa cells in the presence or absence of the function blocking anti-β_1 integrin antibody. However, virus internalization in the presence of the antibody was 30% less than virus internalization in the absence of the antibody (Figure 4.8 a and b). Next, we decided to investigate the effect of blocking β_1 integrins on the efficiency of gene transfer of the virus. HeLa cells were plated in a 96 well dish at 7000 cells/well and the next day chilled at 4^{o}C for 30 min in DMEM to block endocytosis. Next the cells were incubated with 42.5 µg/ml of function blocking anti-β_1 integrin antibody in DMEM for 60 min at 4^{o}C. Next, virus encoding the *lac Z* gene (with 0 or 10 µg/ml anti-β_1 integrin antibody) previously chilled at 4^{o}C and diluted to 40% by volume with fresh medium was exposed to HeLa cells for 4 hr at 4^{o}C. Following virus exposure, the supernatant was removed and the cells exposed to medium (with 0 or 30 µg/ml anti-β_1 integrin antibody) prewarmed to 37^{o}C to internalize the virus. At 0, 2, 4, 6, 8, 24 and 48 hours after the addition of warm media, the cells were exposed to 8 µM AZT to block virus reverse transcription. This allowed us to

quantify the amount of virus that had successfully passed the reverse transcription step at each time point. Forty-eight hours later, the cells were analyzed for β-galactosidase activity using the CPRG assay. We found that the efficiency of gene transfer in the presence of anti-β_1 integrin antibody was 40-50% lower than the efficiency of gene transfer in the absence of the antibody. At each time point, 40-50% less virus had reverse transcribed in the presence of the antibody as compared to its absence (Figure 4.9).

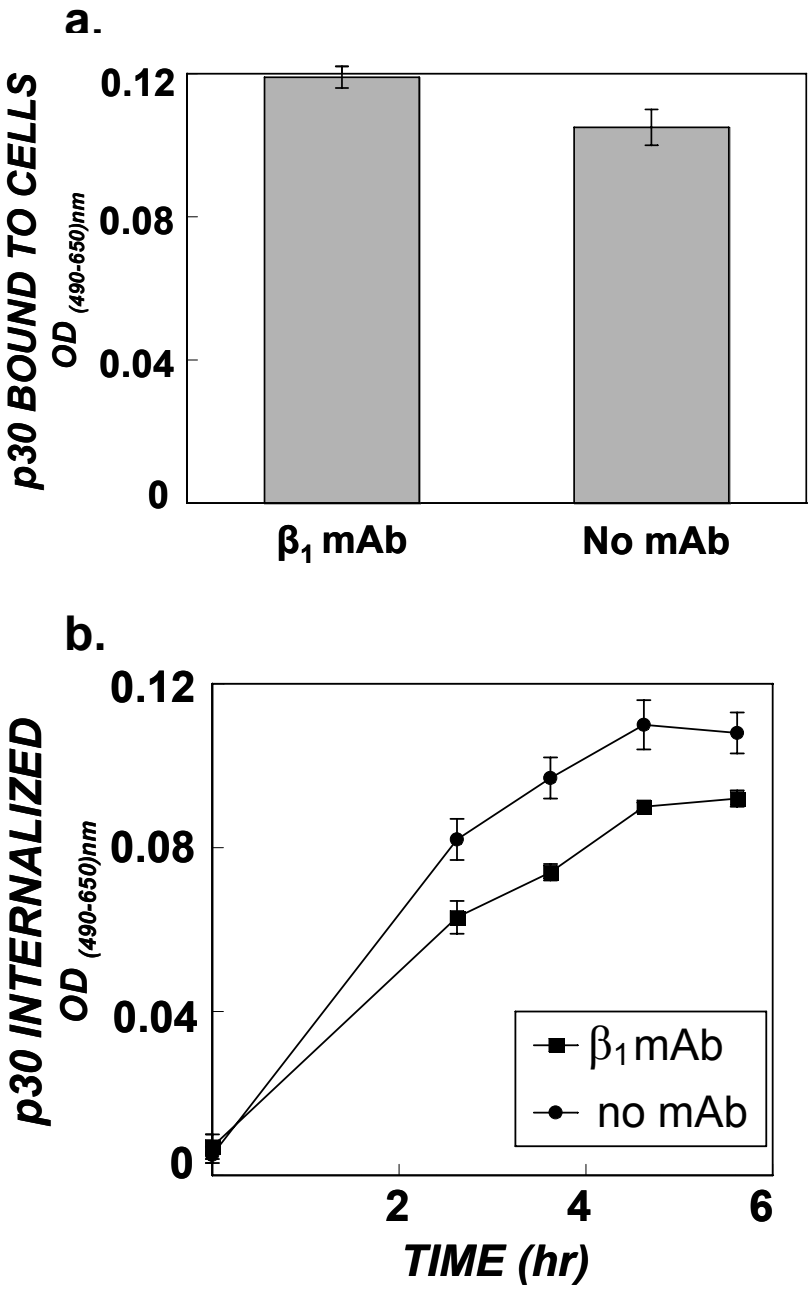


Figure 4.8 Blocking β_1 integrins on the surface of HeLa cells affects virus internalization. HeLa cells were plated at 300,000 cells/well in a 12 well dish and the next day chilled at 4°C for 30 min in DMEM to block endocytosis. Next the cells were incubated with 42.5 µg/ml of function blocking anti-β_1 integrin antibody in DMEM for 60 min at 4°C. Next, virus (with 0 or 10 µg/ml anti-β_1 integrin antibody) previously chilled at 4°C and diluted to 40% by volume with fresh medium was exposed to HeLa cells for 4 hr at 4°C. (a). Following virus exposure the cells were lysed and the amount of virus bound to cells was quantified in a p30 ELISA. (b). In parallel, cells were exposed to medium prewarmed to 37 °C (with 0 or 30 µg/ml anti-β_1 integrin antibody) for 0, 2.62, 3.62, 4.62 or 5.62 hr. At each time point the cells were washed with 1 X PBS to remove any unbound virus, trypsinized to remove any extracellular virus, and lysed to expose the internalized virus that was quantified in a p30 ELISA. Each value represents a mean of triplicate wells.

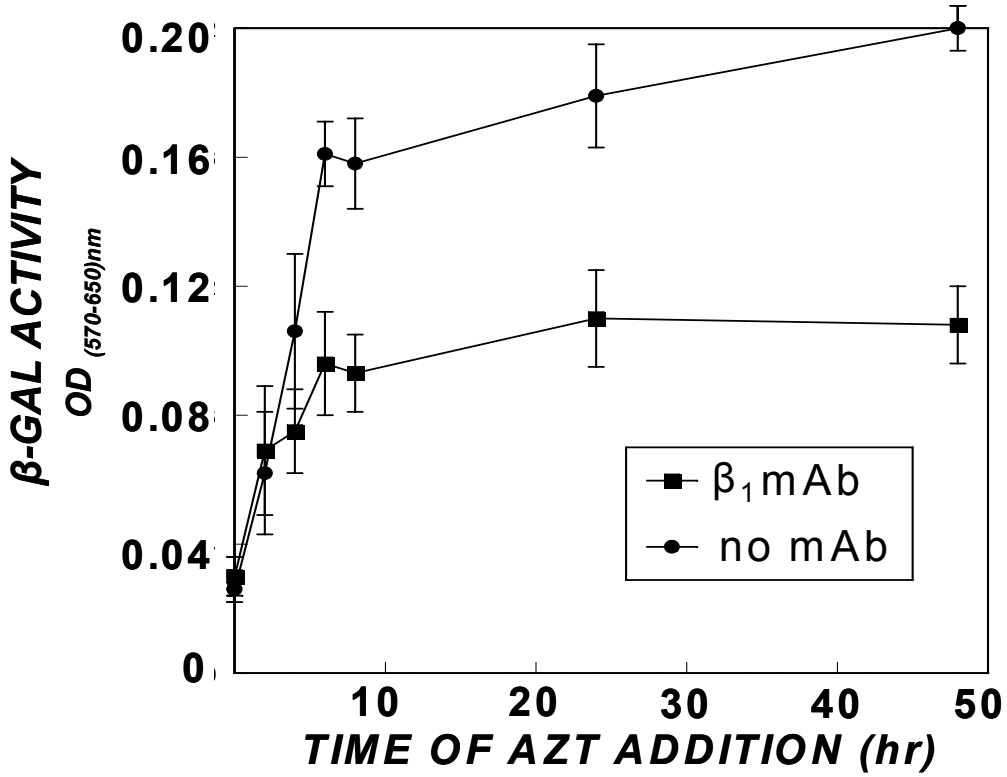


Figure 4.9 Blocking β_1 integrins on the surface of HeLa cells affects the efficiency of virus gene transfer. HeLa cells were plated in a 96 well dish at 7000 cells/well and the next day chilled at 4°C for 30 min in DMEM to block endocytosis. Next the cells were incubated with 42.5 µg/ml of function blocking anti-β_1 integrin antibody in DMEM for 60 min at 4°C. Next, virus encoding the *lac Z* gene (with 0 or 10 µg/ml anti-β_1 integrin antibody) previously chilled at 4°C and diluted to 40% by volume with fresh medium was exposed to HeLa cells for 4 hr at 4°C. Following virus exposure, the supernatant was removed and the cells exposed to medium (with 0 or 30 µg/ml anti-β_1 integrin antibody) prewarmed to 37°C to internalize the virus. At 0, 2, 4, 6, 8, 24 and 48 hours after the addition of warm media, the cells were exposed to 8 µM AZT to block virus reverse transcription. Forty-eight hours later, the cells were analyzed for β-galactosidase activity using the CPRG assay. Each value represents a mean of triplicate wells.

4.5 Discussion

Our hypothesis is that β_1-mediated Rac1 activation in HeLa cells by murine leukemia virus particles facilitates early steps of virus transduction. We found that perturbation of the level of active Rac1 using Rac1 mutants or blocking β_1 integrins using a monoclonal function blocking anti-β_1 antibody in HeLa cells affects a post-binding step of virus entry.

We found that HeLa cells over expressing Rac1-GTP developed numerous membrane protrusions that closely resembled lamellapodia and that engulfed virus particles in the cell vicinity leading to an increased rate of virus entry, albeit towards a non-productive intracellular pathway. Rac1 is a member of the Rho family of GTPases that play a central role in regulating the actin cytoskeleton (3). Rac1 cycles between an active GTP-bound conformation and an inactive GDP-bound conformation and in the GTP-bound form interacts with downstream target proteins to, among other things, induce actin polymerization (18). The expression of activated Rac1 in cells induces the formation of lamellapodia and causes cells to accumulate large vesicles containing material from the extracellular environment, behavior described as macropinocytosis in mammalian cells (3). Macropinocytosis is considered to be a non-specific mechanism for internalization not reliant on ligand binding to a specific receptor (20). HIV-1 entry into macrophages is primarily mediated by macropinocytosis and a large part of macropinocytosed virions is degraded, because macropinocytosis intersects the endosome/lysosome pathway in these cells (10). HIV-1 entry into brain microvascular endothelial cells occurs through macropinocytosis and most viruses in these cells as well are degraged in lysosomes (8). In our study we found that the uptake of virus in HeLa cells over expressing Rac1-GTP was envelope-receptor independent and 75% of the internalized enveloped virus colocalized in the lysosomes. Our previous results indicate however that viruses activate Rac1 when incubated with HeLa cells. Taken together

these results suggest that perhaps retroviruses activate Rac1 locally at points of cell entry, and that global activation of Rac1 through mutants or other means (e.g. pharmacological reagents) may not facilitate productive virus entry.

We found that the rate of virus entry in HeLa cells transfected with the dominant negative mutant RacT17N was 3-fold lower than non-transfected cells and the expression of RacT17N affected a post-binding step of virus entry. It has been previously suggested that Rac1 activity is required for productive fusion of HIV-1 envelope gp120 with its cellular coreceptor CCR5 (15). However from our data, several possibilities emerge about the effect of suppression of Rac1 activity on virus entry. It is possible that suppression of Rac1 activity inhibits fusion of the virus membrane to the cellular membrane or intracellular trafficking of the virus or both. If only virus fusion was completely inhibited, most viruses in the vicinity of the transfected cell would collect at the plasma membrane. Alternatively if only intracellular trafficking was completely inhibited, most viruses would internalize and collect in the cytoplasm close to the plasma membrane. We compared virus localization in HeLa cells transfected with RacT17N with neighboring non-transfected cells. In a non-transfected cell, viruses had internalized and collected primarily in the cytoplasm close to the cell nucleus. However in a neighboring cell expressing RacT17N, some virions had collected outside the cell around the plasma membrane, 3-fold less virus had internalized, and of the internalized virus, some had collected in the cytoplasm close to the plasma membrane and some had collected in the cytoplasm close to the cell nucleus. Since RacT17N expression does not affect virus binding to cells, our observations suggest that suppression of Rac1 activity through RacT17N expression affects both the efficiency of virus fusion *and* intracellular trafficking, but does not completely inhibit either steps of virus entry. Moreover, other actin regulating GTPases may also play a role in virus entry since RacT17N expression in our experimental system did not completely abolish virus uptake.

Since our previous results indicate that β_1 integrins are involved in retrovirus mediated Rac1 activation, we examined the effect of blocking β_1 integrins on early steps of virus transduction. Blocking β_1 integrins did not affect binding of the virus to surfaces of HeLa cells. Since the first step of virus gene transfer to target cells is binding of the virus to the cell surface, our results suggest that either β_1 integrins do not participate in facilitating this step or are not rate controlling. Cell surface glycosaminoglycans have been shown previously to partially facilitate the initial binding of virus to the cell surface (7, 14). Blocking β_1 integrins reduced virus internalization to 70% of control (virus internalized in non-blocked cells) but viral mediated gene transfer was reduced to 50% of control (gene transfer in non-blocked cells) in our experimental system. This suggests that β_1 integrins facilitate a post-binding event, possibly fusion of the virus to the cellular membrane, but since a fraction of the virus that is internalized cannot successfully transfer genes, interaction of the virus with β_1 integrins may also facilitate intracellular transport of the virus. Blocking integrins (α_2 and α_5) has been previously shown to decrease the efficiency of retroviral gene transfer in keratinocytes by 50-60% and it was suggested that integrins facilitate binding and internalization of virus (1). However, integrin engagement with ligands stimulates the activity of numerous cytoskeletal signaling molecules, and therefore blocking integrins may also affect intracellular transport of the virus (5, 11, 12). Our previous data indicates that when β_1 integrins are blocked, viruses cannot activate Rac1 in HeLa cells, but our current data indicates that viruses can still enter cells and transfer genes albeit at a lower rate. This suggests that retrovirus mediated Rac1 activation may be only one of the many mechanisms by which viruses induce cell signaling to facilitate entry and gene transfer. A better understanding of signaling pathways involved in facilitating retroviral entry and gene transfer will aid in the development of more efficient gene transfer vectors or anti-viral drugs.

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CHAPTER 5

CONCLUSIONS AND FUTURE RESEARCH

5.1 Conclusions

In chapter 2 we developed an experimental system to study the effect of pathway of virus entry and the intracellular trafficking itinerary of the targeted receptor, on the efficiency of gene transfer of targeted retroviruses. Our results indicate that interaction with a targeted receptor affects the efficiency of gene transfer of a targeted retrovirus by altering the residence time of the virus on the cell surface, by changing the region of the cell surface that the virus is exposed to, with respect to its natural receptor or by changing the pH that the virus is exposed to during intracellular transport.

In chapter 3 we investigated if recombinant retroviruses are capable of inducing signaling events while entering target cells. We found that retroviruses activate actin regulating GTPase Rac1 in HeLa cells during the first hour of transduction. Virus envelope proteins are not required for Rac1 activation. Retroviruses cannot activate Rac1 in HeLa cells that are cultured in suspension. Rac1 activation requires functional lipid rafts and β_1 integrins on the HeLa cell surface and heparan sulfate proteoglycans on the virus surface.

In chapter 4 we investigated the role of active Rac1 and β_1 integrins on retrovirus-cell interactions. We found that over-expression of active Rac1 causes virus to enter the cell in an envelope independent manner and get degraded in lysosomes. Suppression of Rac1 activity decreases the rate of virus entry 3-fold by affecting a post-binding step of transduction. Blocking β_1 integrins decreases the rate of virus entry by 30% and the efficiency of gene transfer by 50%.

5.2 Suggestions for future research

TAC system

We have developed an experimental system that consists of cell lines that express targeted receptors with the same ligand binding site but different post ligand-binding properties and a recombinant amphotropic retrovirus that binds to these receptors via envelope proteins that contain binding motifs inserted at the N-terminus. The virus that we constructed has 1000-fold lower titer on HeLa cells as compared to the titer of amphotropic virus on HeLa cells. Based on our results we make the following conclusions.

1. N-terminus modification of retroviral envelope proteins leads to inefficient processing of viral envelope proteins in packaging cells. Modified envelope proteins may not be cleaved into surface and transmembrane units effectively or may not be able to efficiently undergo conformational changes upon interaction with the natural viral receptors leading to poor exposure of fusion peptide on the envelope proteins that results in inefficient fusion of the viral and cellular membranes and low titers on target cells.
2. We also conclude that non-viral receptors (such as TAC in our system) are incapable of functioning as natural viral receptors and are unable to trigger fusion and productive entry of a targeted virus. In addition, non-viral receptors may sequester the virus away from the natural viral receptor resulting in adverse effects on efficiency of gene transfer.
3. Our results therefore suggest that in order to successfully construct a targeted retrovirus, it is undesirable to modify the envelope protein of the virus and to choose targeted receptors on cell types of interest that are incapable of facilitating the interaction of the virus with its natural receptor.

For the purpose of illustration, based on our experimental results, we recommend the following guidelines for designing recombinant retroviruses targeted to metastasized cancer cells.

1. Since the first interaction of retroviruses with target cells is the non-specific adsorption of virus on the cell surface enabled by the interaction of packaging cell derived molecules on the virus surface with extracellular matrix or cell surface proteoglycans, we recommend coating retrovirus particles with polymers such as poly ethylene glycol (PEG), that can potentially block non-specific interactions between the virus and the non-target cells as well as non-specific adsorption of virus at the target cell surface.
2. In order to concentrate virus particles on target cancer cells, we recommend engineering PEG coated viruses to express ligands that bind with high affinity to receptors enriched on the cancer cell surface. The choice of the receptor should be such that the receptor cell surface expression levels, distribution and kinetics of entry are favorable such that attachment to the receptor facilitates interaction with the natural virus receptor that is capable of supporting fusion and productive entry of the virus into the cancer cell.

Nevertheless, we have constructed an experimental system that can be used to systematically examine the effect of properties of a targeted receptor on the efficiency of gene transfer of a retrovirus targeted to the receptor by insertion of cell-specific binding motifs on the envelope protein. Future research with the experimental system in the following areas will provide information that will be useful in the design and development of targeted retroviruses with high efficiency of gene transfer.

1. Envelope protein design: Changes in envelope protein design affect the ability of a targeted retrovirus to successfully bind with a target cell receptor of interest and fuse with the natural viral receptor The affinity of virus binding to a target cell

receptor of interest may be varied by inserting ligands with different binding affinities to the targeted receptor. The presence of different ligands will also alter the fusogenicity of the envelope protein. Studies with viruses expressing modified envelope proteins with different binding and fusion characteristics will help develop a rational criterion for the design of envelope proteins with cell specific binding motifs.

2. Properties of the targeted receptor: Our results suggest that the properties of the targeted receptor affect the gene transfer of the targeted virus. By evaluating the kinetics of targeted virus binding, fusion, internalization and reverse transcription, in HeLa cells expressing TAC receptors, the step(s) of virus life cycle that are dependent on the properties of a targeted receptor can be determined. This information can be used to determine the targeted receptor-viral vector pair that would be most suitable for a particular cell type.

Rac1 system

We have discovered that amphotropic retroviruses are capable of inducing Rac1 activation to facilitate their entry into HeLa cells. Future work should examine the following ideas.

1. The mechanism of retrovirus mediated Rac1 activation: Although we have determined that HSPG molecules on the virus surface and cell surface β_1 integrins mediate Rac1 activation, we do not know the mechanism of interaction of HSPG with β_1 integrins. The possibility that virus-associated HSPG molecules may cluster β_1 integrins by interaction through extracellular matrix molecules such as fibronectin should be investigated. It should also be investigated if viruses activate integrins during the process and initiate downstream signaling events such as activation of FAK and Src kinases that phosphorylate proteins

(guanine nucleotide exchange factors) that facilitate the formation of Rac1-GTP. Moreover, since activated integrins are transported to lipid rafts, it should be investigated if viruses localize to their natural receptors in lipid rafts by attaching to integrins.

2. The function of retrovirus mediated Rac1 activation: Although Rac1 activation initiates signaling events in a cell that cause actin rearrangement, we do not know the significance of this phenomenon in the retrovirus life cycle. Perhaps retrovirus particles overcome the cortical actin barrier through Rac1 activation. This possibility should be examined and in addition, it should be examined if retroviruses are capable of activating other actin-regulating GTPases namely Rho and Cdc42. Since HSPG molecules on the virus surface enable Rac1 activation in HeLa cells, the significance of Rac1 activation can be determined by examining the ability of fluorescent retrovirus particles deficient in HSPG, to bind, fuse and traffic within target cells.
3. Applicability of results to other experimental systems: Our experiments were performed in HeLa cells with retroviruses derived from TELCeB6 packaging cells. The gene transfer efficiency of retrovirus particles depends on the target cell type. For instance, the titer of retroviruses derived from TELCeB6 cells is 10-fold higher in NIH3T3 cells as compared to HeLa cells (unpublished results). Perhaps the differences in gene transfer reflect the efficiency with which viruses induce Rac1 signaling in these cell types. This possibility should be examined, especially in clinically relevant cell types such as hematopoetic stem cells. The nature of molecules expressed on the virus lipid bilayer depends on the identity of the virus packaging cell type. Viruses from different packaging cells have different abilities to transfer genes. For instance, amphotropic viruses derived from ψ-CRIP packaging cells have 100-fold lower titer on NIH3T3 cells as

compared to amphotropic viruses derived from TELCeB6 packaging cells (unpublished results). It should be investigated if differences in titer of viruses derived from different packaging cells, correlates with the ability of the viruses to induce signaling events in target cells to facilitate attachment and entry. In addition since non-specific adsorption of virus to the target cell surface is the first interaction between a virus and a target cell, it is necessary to characterize molecules on the virus surface instrumental in this process. This information will be useful in increasing the specificity of viruses for targeted virus applications or development of anti-viral therapies or in designing viruses to transfer genes to cells that are refractory to viral gene delivery.

APPENDIX A1

PROPOSED MODEL TO DETERMINE THE EFFECT OF ACTIVE TRANSPORT ON VIRUS ENTRY

Virus entry into target cells begins with the transport of the virus to the cell surface by diffusion or convection. Since the receptor for MLV-A is primarily found in lipid rafts that typically occupy 1-10% of the cell surface, to fuse successfully, the virus must "find" its receptors on the cell surface (2, 4, 8). We propose that virus interaction with its cognate receptor is facilitated by active transport of the virus to sites where receptors are located, enabled through interaction with molecules such as integrins, or, by the active transport of receptors to sites on the plasma membrane where virus initially binds. To illustrate the advantages of active transport, we will consider the case of active transport of the virus to its cognate receptor through interaction with proteins (such as integrins) that diffuse on the plasma membrane.

Each virus particle is multivalent and interaction with several fusogenic receptors is required for a successful fusion event (6). Let us assume that fusogenic receptor aggregates are evenly distributed on the cell surface and refer to them as "virus fusion sites". We assume that the cell is circular with radius "a", each fusion site is surrounded by a circular capture area of radius "b", and that the total number of fusion sites is "N". Let us also assume that following initial contact with the plasma membrane; a virus particle interacts with a laterally diffusing membrane protein that transports the virus to a fusion site. We can then calculate the average capture time of the virus by a fusion site by using the formalism derived by Berg and Purcell (3, 4).

$$tc := \frac{b^4}{2 \cdot D \cdot \left(b^2 - s^2\right)} \cdot \ln\left(\frac{b}{s}\right) - \frac{3 \cdot b^2 - s^2}{8 \cdot D}$$

where

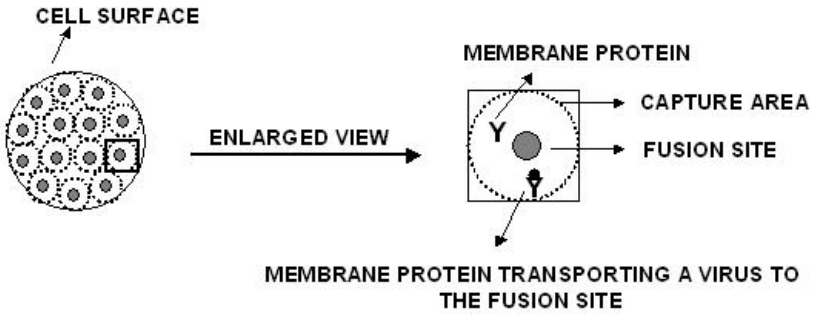
t_c = average capture time of the virus by a fusion site

b = radius of the capture area occupied by a fusion site, $N \pi b^2 = \pi a^2$

s = radius of the fusion site

D = lateral diffusivity of a membrane protein that transports the virus

= lateral diffusivity of the virus-membrane protein complex



Shown above is a schematic view of the cell surface. Assuming a = 10 μm, s = 20 nm, N = 10 – 35000 and D = 10^{-8}cm^2/s – 10^{-19}cm^2/s, we plot in Figure A1.1 and A1.2, the average capture time of the virus by a fusion site using diffusional transport, versus the number of fusions sites on the cell surface. For comparison, virus half-life for losing infectious activity and half-life for constitutive cell endocytosis are also shown. For successful fusion, a virus particle at the cell surface has to find a fusion site before it loses infectious activity or before it is internalized by the cell through constitutive endocytosis. The Berg and Purcell formalism therefore predicts that for a constant number of fusion sites, as the diffusivity of the virus on the cell surface decreases, the time to capture by a fusion site increases, and, for a constant diffusivity, as the number of fusion sites increase, the time to capture decreases. Since non-envelope protein -

non-receptor interactions precede virus interaction with fusogenic receptors at the cell surface (5), it is possible that such interactions may tether and immobilize the virus on the cell surface, resulting in virus diffusivities less than the diffusivities of individual membrane proteins. Since membrane proteins have lateral diffusivities in the range of $10^{-11}cm^2/s$ – $10^{-9}cm^2/s$ (4), the model predicts that transport of a virus particle enabled by attachment to a membrane protein capable of localizing virus to sites of fusion would facilitate virus entry.

Next, assuming active transport, of the initial concentration of virus particles on the cell surface, we calculated the fraction of virus that will find a fusogenic site. Shown below is a schematic of the virus particle on the cell surface. We define the following terms.

V = concentration of virus at the cell surface at any given time

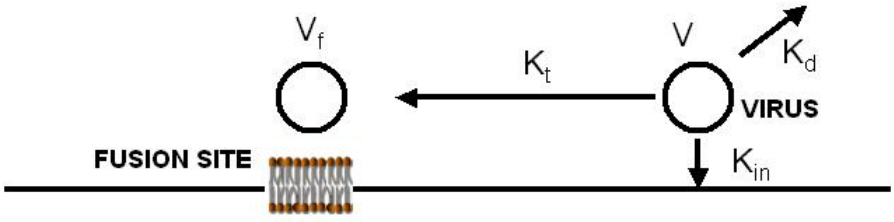
V_o =initial concentration of virus at the cell surface

V_f = concentration of virus that finds a fusion site

K_t = rate constant for active transport

K_d = rate constant for virus losing infectious activity

K_{in} = rate constant for constitutive cell endocytosis



Assuming active transport, virus decay and cell endocytosis to be first order reactions, we can write

$dV/dt = - (K_t + K_d + K_{in}) V$

$dV_f/dt = K_t V$

which yields,

$V = V_o e^{-(Kt + Kd + Kin) t}$

$dV_f/dt = K_t V_o e^{-(Kt + Kd + Kin) t}$

Integrating the above equation from (0 - ∞) with respect to t, we arrive at the following expression for fraction of virus that will find a fusion site.

$V_f/V_o = K_t / (K_t + K_d + K_{in})$

$K_t = \ln 2 / t_c$

$K_d = \ln 2 /$ (half-life for virus losing infectious activity)

$K_{in} = \ln 2 /$ (half-life for constitutive cell endocytosis)

The half-life for MLV losing infectious activity is approximately 6 hr, and half-life for constitutive cell endocytosis is approximately 0.5 hr (1, 7). Figures A1.3 and A1.4 below depict the fraction of virus that will find a fusion site as a function of number of virus fusion sites. The data below indicates that for a constant number of fusion sites, a higher fraction of virus will find fusion sites on the cell surface if membrane proteins with high diffusivities actively transport them, and, with increasing number of fusion sites, an increasing fraction of viruses will find fusions sites at lower diffusivities. The model proposed above thus demonstrates that active transport of the virus on the cell surface facilitates virus entry in target cells. Techniques such as Total Internal Reflection

Fluorescence can be used to study the motion of virus particles on the cell surface and will prove useful in further understanding virus entry in target cells.

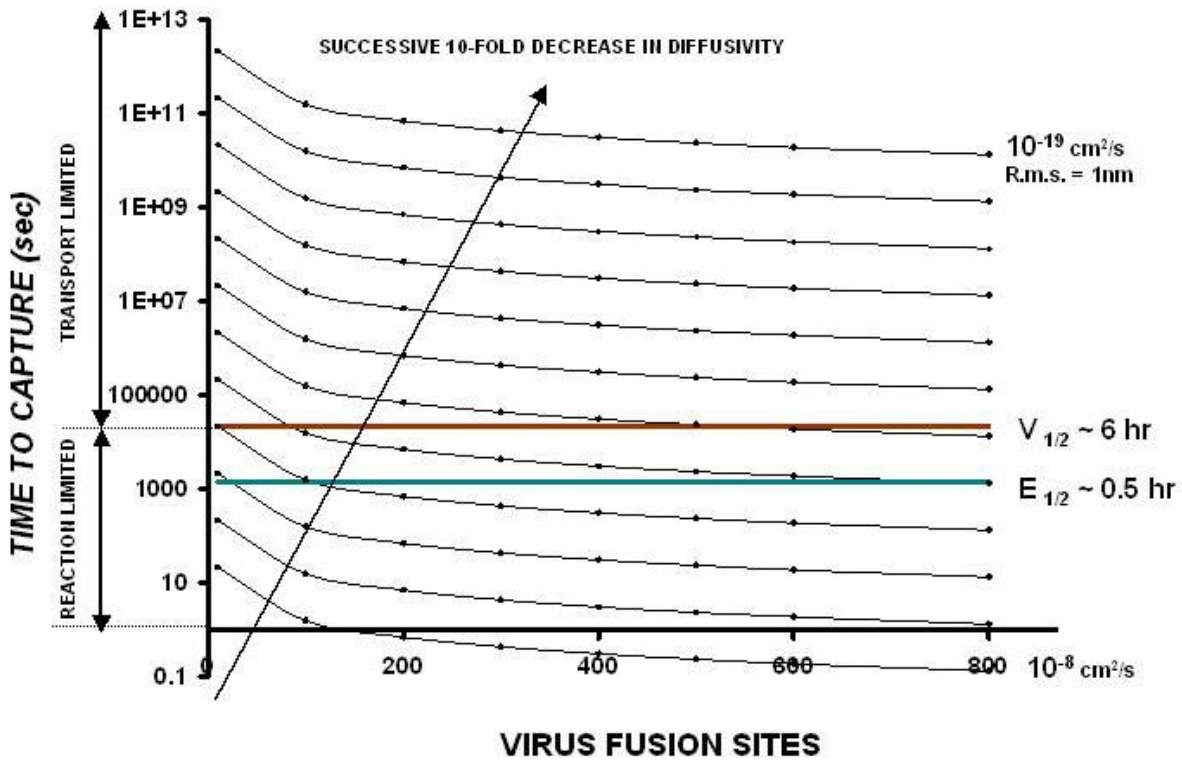


Figure A1.1 Average capture time of the virus by a fusion site versus the total number of fusion sites (10-800) on the cell surface. Y-axis is plotted on logarithmic scale. $V_{1/2}$ is defined as virus half-life for losing infectious activity and $E_{1/2}$ is defined as half-life for constitutive cell endocytosis. R.m.s is defined as the root mean square distance traveled by a virus in one virus half-life using 2-D diffusional transport. R.m.s = $\sqrt{}$ (4 D $t_{1/2}$)

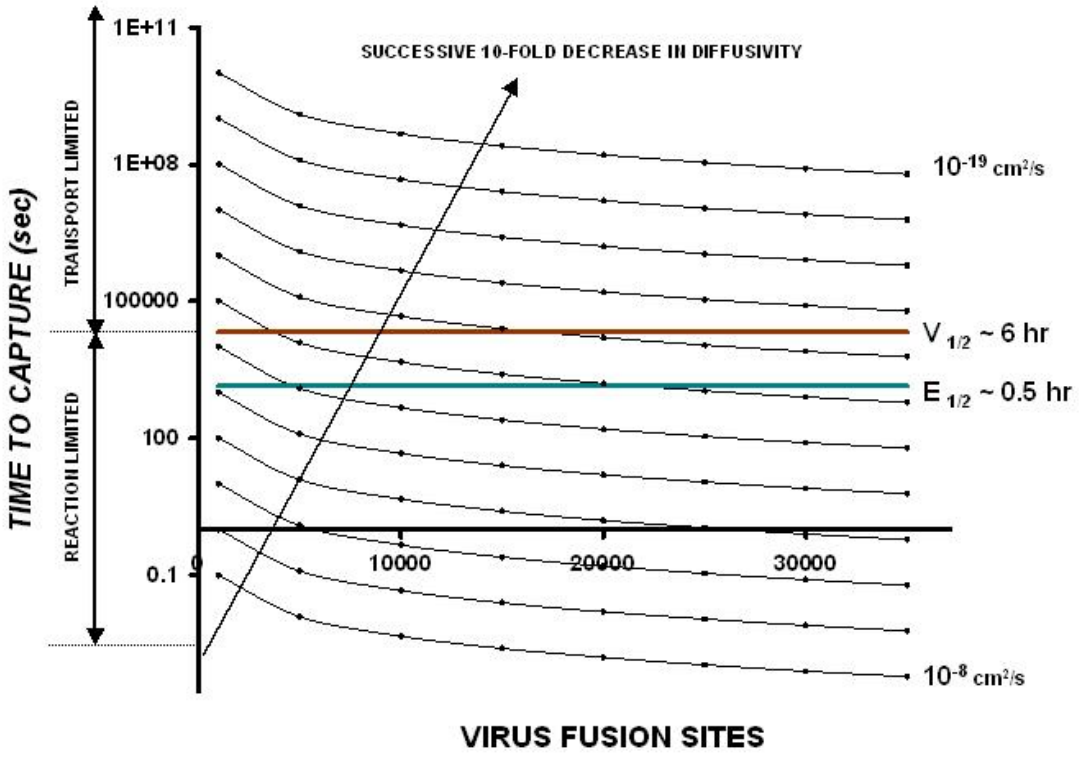


Figure A1.2 Average capture time of the virus by a fusion site versus the total number of fusion sites (1000-35000) on the cell surface. Y-axis is plotted on logarithmic scale. $V_{1/2}$ is defined as virus half-life for losing infectious activity and $E_{1/2}$ is defined as half-life for constitutive cell endocytosis.

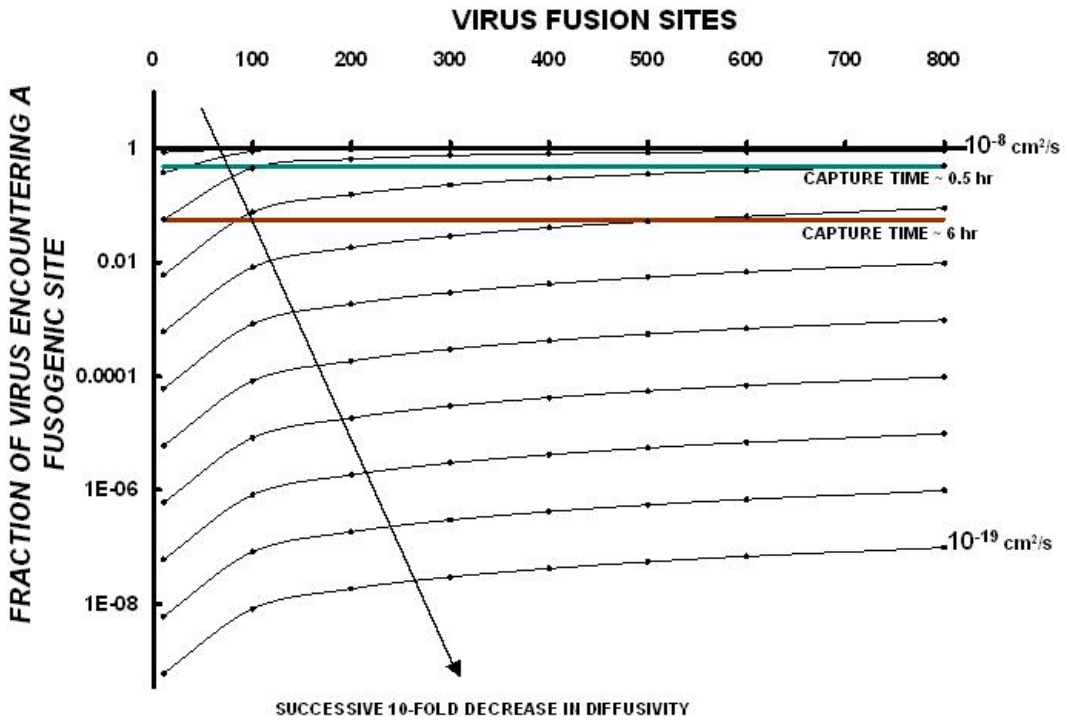


Figure A1.3 Fraction of virus particles encountering a fusion site versus the total number of virus fusion sites (10-800) on the cell surface. Y-axis is plotted on logarithmic scale. Also shown are fractions of virus that will find a fusion site if the capture time is equal to virus half-life of ~ 6hr or equal to half-life of constitutive cellular endocytosis of ~ 0.5 hr.

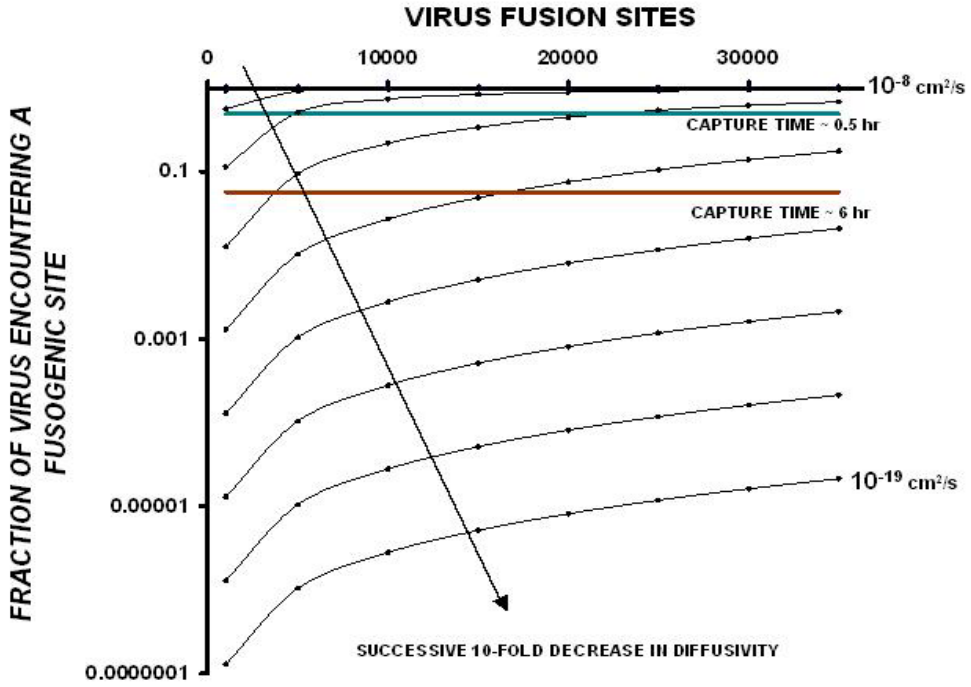


Figure A1.4 Fraction of virus particles encountering a fusion site versus the total number of virus fusion sites (1000-35000) on the cell surface. Y-axis is plotted on logarithmic scale. Also shown are fractions of virus that will find a fusion site if the capture time is equal to virus half-life of ~ 6hr or equal to half-life of constitutive cellular endocytosis of ~ 0.5 hr.

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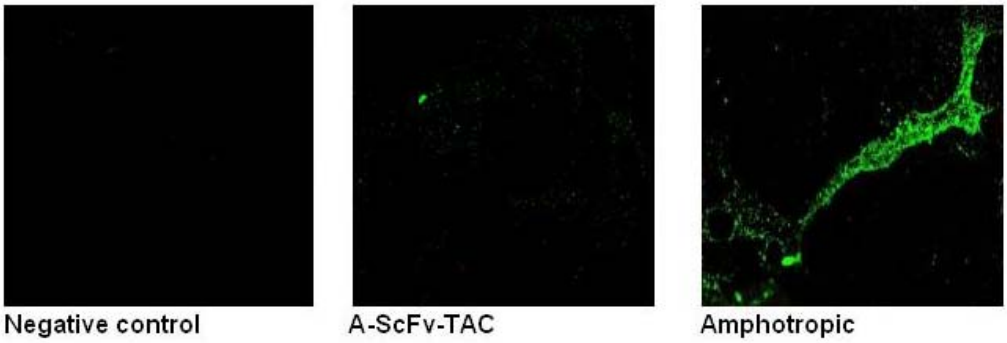
APPENDIX A2

THE EFFECT OF ENVELOPE EXPRESSION PLASMID ON TAC-BINDING ENVELOPE PROTEINS

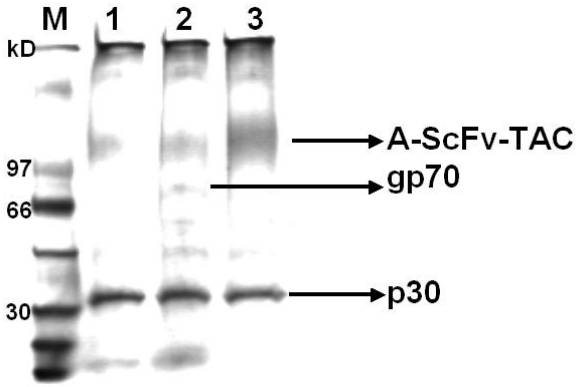
As discussed in chapter 2 we constructed a pcDNA3.1+/Neo derived expression plasmid (pAScFvTAC) that encoded a TAC-binding amphotropic envelope protein with the structure 5' – ecotropic envelope signal peptide – *Nhe*I site – ScFv TAC (single chain antibody against TAC) – *Not*I site – *Fse*I site – amphotropic envelope protein – 3'. To produce retrovirus that bind to TAC, pAScFvTAC was transfected into TELCeB6 cells, which express MLV Gag-Pol core particles and an nlslacZ retroviral vector. Transfected cells were plated at clonal density 48 hours later and cultured with neomycin (500 μg/mL) to eliminate cells that were not stably transfected. Stably transfected clonal cell lines that produced virus with the highest titer were selected and used to make TAC-binding viruses. Envelope expression by these cells was examined by fixing and staining them with an antibody (83A25) against the amphotropic envelope protein (Figure A2.1a). As controls, cells that expressed no envelope protein and cells that expressed the wild-type envelope protein were also examined. Cell surface expression of the modified envelope proteins was visible in pAScFvTAC transfected cells, although they were present at significantly lower levels as compared to cells that expressed the wild-type amphotropic envelope protein and compared to cells transfected with pCAAGS-AScFvTAC (Figure 2.6a).

To determine if the modified envelope proteins produced from the transfection of TELCeB6 with pAScFvTAC were incorporated into retrovirus particles, virus supernatants were harvested from packaging cells metabolically labeled with S-35, concentrated, separated by size by gel electrophoresis, and then the Gag (p30; CA) and envelope protein content (Figure A2.1b) quantified by autoradiography. As controls,

supernatant from cells that produced viruses pseudotyped with the wild-type amphotropic envelope or with no envelope protein were also examined. Envelope proteins were detected in the wild-type (70 kD) retrovirus stocks, but not, as expected, in the virus stocks produced by cells that did not express any envelope protein. Envelope proteins were also detected in TAC-binding virus stocks (113 kD) but at a significantly lower level as compared to the amphotropic envelope expressing virus stocks and as compared to envelope proteins detected in virus made from transfection of TELCeB6 cells with pCAAGS-AScFvTAC (Figure 2.6b). TAC-binding virus produced from pAScFvTAC had 10-fold lower titer as compared to TAC-binding virus produced from pCAAGS-AScFvTAC. We therefore conclude that pcDNA3.1+/Neo is not a desirable vector for expressing retroviral envelope proteins.



a.



b.

Figure A2.1 TAC-binding amphotropic envelope proteins are expressed on the surface of virus producer cells and incorporated into virus particles at significantly lower levels. (a) Envelope protein expression on the surface of virus producer cells. TELCeB6 cells were transfected to express the TAC-binding amphotropic envelope protein (A-ScFv-TAC), the wild-type amphotropic envelope protein (amphotropic), or no envelope protein (negative control), and then fixed but not permeabilized, immunostained with a rat monoclonal antibody (83A25) against the gp70 envelope protein and donkey anti-rat Cy2 conjugated secondary antibody (green), and visualized by confocal microscopy. (b) TAC-binding envelope proteins are incorporated into virus particles. Supernatants from metabolically labeled virus producer cells were concentrated 18-fold, resuspended in IP buffer, separated by size by SDS-PAGE electrophoresis, and then the gel dried and visualized by autoradiography. Supernatants from TELCeB6 cells which produce retroviruses with no envelope proteins (lane1), TELCeB6-A cells which produce retroviruses with amphotropic envelope proteins (lane 2),and TELCeB6-AScFvTAC cells which produce retroviruses with TAC-binding amphotropic envelope proteins (lane 3) are shown. Molecular weight markers are in lane M.