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INVESTIGATION OF VACCINIA VIRUS ENTR PATHWAYS

THESIS

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TABLE OF CONTENTS

ABBREVIATIONS

1. Reagents and Terms

AB……..antibodie

AKT or PKB……..Protein Kinase B

AraC……...Cytosine β-D-arabinofuranoside

BFLA…….Bafilomycin A1

Bleb……blebbistatin

BSA……bovine serum albumin

$Ca2^{+}$.....Calcium ion

CAV1…….caveolin-1

CD98……..type II cell membrane protein

CHX……...cycloheximide

Cy5…….Cyanine dye

CypB……..Cyclophilin B

DAPI……..4'-6'-diamidino-2-phenylindole

DMEM……...Dulbecco's Modified Eagle Medium

DMSO……dimethyl sulfoxide

DRM.....detergent-resistant domain

DTT……dithiothreitol

EDTA…….Ethylene diamine tetraacetic acid

EGTA…….ethylene glycol tetraacetic acid

EM…….Electron micrograph

ERK……extracellular signal-regulated kinase

EV……..Extracellular virus

EZR……ezrin

FACS……..fluorescence-activated cell sorting

FAK……....Focal adhesion kinase

FBS…….....Fetal bovine serum

FITC……....fluorescein isothiocyanate

FN……...fibronectin

GAGs……..glycosaminoglycans

Gpt……..E. coli xanthine guanine phosphoribosyltransferase gene

IPTG……...isopropyl-β-D-thiogalacto-pyranoside

ITGβ1…….....Integrin β1

IV……....Immature virus

J2R…….viral protein, virus thymidine kinase

LacI……....E. coli lac repressor gene

LacO……...E. coli lac operator;

LN……..Laminin-1

LY294002……...PI3K inhibitor

MAB……..monoclonal antibodie

mβCD…….m - β - cyclodextran

MEF……mouse embryonic fibroblast

MV……..Mature virions

NP-40……..Nonidet P-40

p7.5 and p11……....viral promoters

PAK1……...p21-activated kinase 1

PFA…….paraformaldehyde

PFU……..Particle Forming Unit

PI3K…….Phosphatidylinositol 3-kinase

PKA…….Protein Kinase A

PKC…….Protein kinase C

PLL……..Poly-L-lysine

siRNA……..Small interfering Ribonucleic acid

SDS buffer……...Sodium dodecyl sulphate buffer

SDS PAGE……..dodecyl sulfate polyacrylamide gel electrophoresis

TK……thymidine kinase

T7 Pol……..T7 RNA polymerase

TfR……transferrin receptor

TLN1……talin-1

VV……Vaccinia virus

WV……Wrapped virus

X-Gal……(5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

2. Viruses and Cells

WR-VV or VACV……Western Reserve strain of Vaccinia Virus

VT7LacOI…….WR-VV, which encodes a continuously expressed *E. coli lac* repressor and an IPTG-inducible bacteriophage T7 RNA polymerase

vi53.5L…… Vaccinia virus contains an inducible WR53.5L/E. coli gpt marker gene cassette inserted into its endogenous WR53.5L locus

viG3L……Vaccinia virus contains an inducible WRG3L/E. coli gpt marker gene cassette inserted into its endogenous locus

WRΔ53.5L……..A deletion mutant virus deleting the endogenous WR53.5L ORF

IHD-J……..Vaccinia virus strain International Health Department-J

CPXV……..Cowpox virus (Brighton Red strain or GRI 90)

VARV……Variola virus (INDIA-1967/isolate IND3)

MYXV…….Myxoma virus

LSDV……..Lumpy skin disease virus

YMTV……Yaba-like monkey tumor virus

FWPV…….....Fowlpox virus

AMV……...Amsacta moorei entomopoxvirus

MSV……Melanoplus sanguinipes entomopoxvirus

Cop…….Copenhagen strain of vaccinia

MVA……...Modified virus Ankara (strain MVA-1721 or Acambis 3000)

CMLV…….....Camelpox virus (strain M96)

ECTV……..Ectromelia virus (strains Moscow and Naval)

LC16m8 and LC16mO……..VACV Lister strain

RPXV-UTR……Rabbitpox virus (strain Utrechht)

MPXV……Monkeypox virus (strains Walter Reed and Zaire)

VACV……Vaccinia virus

A28, A21, A16, A4, B5, G1, G2, G3, L5, L1, H2, H3, D8, J2, F3, C2, A55F13, F14, F14.5,

F15……Vaccinia viral proteins

GD25……Integrin β1 knockout embryonic stem cells

GD25 β1A …….....GD25 expressing wild-type human integrin β1

BSC1……African green monkey (kidney) cells

BSC40……...Continuous cell line of African green monkey derived from BSC 1

HeLa……..Human immortal cell line

GENERAL DESCRIPTION OF THE WORK

RELEVANCE OF THE WORK

Vaccinia virus is the prototype of the orthopoxvirus genus in the family of Poxviridae. Poxviruses differ from other animal viruses in their large size and complexity. The *Poxviridae* are large enveloped DNA virus that replicate in cytoplasm of the host cells (33, 49). The best-known member of this family is variola virus, the causative agent of smallpox disease in humans (15). Being specifically human infectious disease virus took millions of human lives over the millenniums, until was eradicated by global vacciniation. Although, on 1980, the World Health Organization (WHO) declared that smallpox eradicated from natural environment, the original smallpox virus stocks are still kept by various laboratories in Russian Federation and the United States and remains as a bioterrorism agent. In addition to the concern of variola, other poxviruses can be transmitted from animals to human and cause fatal diseases in human population. Example is monkeypox virus, which can transmit to people from a variety of wild animals, spread in human population and produce a smallpox-like disease. Suggesting that monkeypox might replace smallpox as a serious epidemic threat. Therefore the development of new antiviral agents, the full information about virus entry and replication is a great concern for the researchers working on the poxvirus field.

Vaccinia virus is a large ~200 Kb double-stranded enveloped DNA virus that encodes more than 200 open reading frames (ORFs), information about Vaccinia virus complete genomes can be searched from Poxvirus Bioinformatics Resource Center (PBR) (http://www.poxvirus.org/). Has a broad range of infectivity in vitro as well as in vivo (49). Vaccinia virus contains early, intermediate

and late classes of viral genes that encode viral transcription factors to activate viral genes expression in a cascade-regulated manner by stage-specific transcription factors that recognize distinct early, intermediate, and late promoter sequences (128, 155). Virion morphogenesis occurs exclusively in the cytoplasm of the host cells and produces multiple infectious forms of virus particles, e.g., (Figure 2) mature virus (MV), is the most abundant virion with a single membrane in cells; however, the origin of the membrane is unknown (64, 65). Some MVs are wrapped with Golgi-derived membranes to form wrapped virus (WV) and are transported through microtubules to the cell periphery to become extracellular virus (EV) (33).

Previous work in our laboratory showed that cell-bound Vaccinia MV particles were clustered at the plasma membrane lipid rafts prior to virus entry and that the interruption of lipid raft integrity with m-β-cyclodextran significantly reduced vaccinia MV entry into HeLa cells. Since lipid rafts on the plasma membrane are known to act as platforms for receptor clustering, endocytosis, and signal transduction also for other viruses (122) we hypothesized that cellular proteins within plasma membrane lipid rafts mediate vaccinia MV entry into HeLa cells. Therefore, we isolated detergent-resistant domains from HeLa cells upon vaccinia MV infections and extracted proteins for quantitative proteomic analyses.

PURPOSE AND OBJECTIVES OF THE RESEARCH

The main goal of this study was to reveal the specific molecular and cellular mechanisms, pathways, cellular receptors and proteins involved in Vaccinia virus attachment and penetration processes.

To achieve this goal, the following tasks were accomplished:

- Identification of the cellular receptors and proteins within lipid raft microdomains involved in the Vaccinia MV entry process by the investigation of their association with Vaccinia MV on the surface of infected HeLa cells;
- Investigation of the role of these receptors and proteins in the Vaccinia virus life cycle and Vaccinia MV entry process into HeLa and mouse cells;
- Investigation of the pathways and mechanisms through which these receptors and proteins mediate Vaccinia MV cell penetration and entry process.
- Identification and investigation of the functions of two Vaccinia MV proteins that previously were unknown.

SCIENTIFIC NOVELTY AND SIGNIFICANCE OF THE WORK

As such I worked in several aspects of Vaccinia virus biology. Vaccinia virus has a broad range of infectivity infects many cell lines and animals. Although it is known that the Vaccinia mature virus binds to cell surface glycosaminoglycans and extracellular matrix proteins, whether additional cellular receptors are required for virus entry remains unclear. Our previous study showed, that Vaccinia mature virus enters through lipid rafts, suggesting the involvement of raft-associated cellular proteins. Here we investigated one lipid raft-associated protein, integrin β1, and

showed that it is important for Vaccinia mature virus entry into HeLa and mouse MEF cells. More importantly Vaccinia mature virus infection triggers the activation of phosphatidylinositol 3-kinase (PI3K)/Akt signaling. And we showed, that virus induced P13K activation is occur in integrin β1-dependent manner. In addition, we showed that outside-in activation process of integrin function also facilitate Vaccinia virus entry into HeLa cells, since the disruption of focal adhesions also reduced Vaccinia MV entry. Thus, for the first time we show cellular receptor that mediates Vaccinia virus endocytosis.

As mentioned above recently proteomic analyses studied in our laboratory and by others reveled that Vaccinia MV are produced in large quantity in the infected cells and contain ~80 viral proteins in viral particles. Investigation revealed several proteins with unknown function. We also interested to explore their role and the function on Vaccinia virus life cycle. One such protein is WR53.5L/F14.5L to further investigate the WR53.5 we generated a recombinant Vaccinia virus, vi53.5L that expressed WR53.5 protein under isopropyl-β-D-thiogalactopyranoside (IPTG) regulation and found that the Vaccinia virus life cycle proceeded normally with or without IPTG when WR53.5 is not expressed, suggesting that WR53.5 protein is not essential for Vaccinia virus growth in cell cultures. Interestingly, we showed that C-terminal region of WR53.5 protein was exposed on the cell surface of infected cells and mediated calcium-independent cell adhesion. Finally, when we use viruses lacking WR53.5L gene, virulence of the mice was dramatically reduced in intra-nasal inoculation, thus we identified a new Vaccinia MV envelope protein, WR53.5 that mediates cell adhesion and is important for virus virulence in vivo.

Another Vaccinia MV protein we investigated is G3. Alignment of amino acid sequence of vaccinia G3 the 12.8 KD protein with its orthologues in the orthopoxvirus genus revealed high level of homology between the proteins, ~46% conserved residues. Hydropathy analyses predicted that G3 has two hydrophobic domains at the N and C terminus. All this suggested, that G3 would play an important role for Vaccinia virus life cycle. Therefore in order to investigate the function of G3, here again we have constructed a recombinant Vaccinia virus, viG3, expressing G3 protein under

IPTG (isopropyl-β-D-thiogalactopyranoside) regulation. And we showed that under permissive condition when G3 protein was expressed, the Vaccinia virus life cycle proceeded normally, resulting in plaque formation in BSC40 cells. In contrast, under non-permissive conditions when G3 protein expression was repressed, no plaques were formed, showing that G3 protein is essential for Vaccinia virus growth in cell cultures. In fusion assay, where cells were infected with viG3 in the presence or absence of IPTG and briefly treated with acidic buffer only cells infected in the presence of IPTG developed into gigantic fused cells. In the absence of IPTG when G3 protein is not expressed no cell fusion was observed. Thus, G3 protein is an essential component of entry fusion complex, in addition to the A28, A21, H2, J5, L5, F9, G3, G9, L1, O3, I2, A16.

APPROBATION OF THE WORK

The main results of this dissertation have been extensively discussed with the experts in the field and presented in the seminars organized by the Dr. Wen Chang's Laboratory, Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan R.O.C. (Taipei, Taiwan, 2006-2011), http://www.imb.sinica.edu.tw/%7Embwen/index.htmlфтв. and by the Laboratory of Cell Biology and Virology (head, D.Sc. Zaven Karalyan) of the Institute of Molecular Biology NAS RA, at the meetings of the scientific council of the Armenian institution (Yerevan, RA, 2012-2016), as well as at 12 international scientific conferences, symposiums and meetings, such as: International FASEB Summer Research Conference, Poxviruses (Indian Wells, California, USA, 2006). International meeting of the Molecular Biology in the XXI Century: Interface, Integration, and Perspectives, (Academia Sinica Taipei, Taiwan, R.O.C., 2006). XVII International Poxvirus and Iridovirus Conference, (Grainau-Bavaria, Germany, 2008). Institute of Molecular Biology retreat meeting: (Kaoshong, Taiwan, R.O.C., 2008). International conference, American Society for Virology: XVIII Annual Meeting, (University of British Columbia Vancouver, BC, Canada, 2009). Institute of Molecular Biology retreat meeting: (Taoyuan, Taiwan, R.O.C., 2009). XII SCBA International Symposium Science for a Healthier and Better Life (Taipei,Taiwan, R.O.C. 2009). XVIII

International Poxvirus, Asfivirus and Iridovirus symposium: (Arizona Sate University, Sedona, USA, 2010). Institute of Molecular Biology retreat meeting: (Taoyuan, Taiwan, R.O.C., 2011). International meeting Gordon Research Conference (GRC), Viruses and Cells: (Lucca-Barga, Italy, 2011). International conference, American Society for Virology: 30th Annual Meeting, (University of Minnesota, Minneapolis, USA, 2011). XIX International Poxvirus and Iridovirus Conference, (Salamanca, Spain, 2012).

PUBLICATIONS

The main results of this study are involved in 10 scientific publications, including 4 articles in high-impact factor peer-reviewed journals, 1 article in Armenian journal, and 5 reports in abstract books of international conferences.

THE VOLUME AND THE STRUCTUR OF THE THESIS

The dissertation comprises of 114 pages, includes 1 table and 30 figures, and consists of the following sections: List of Abbreviations and Symbols, Introduction, Literature Review, Materials and Methods, Results and Discussion, Conclusions, Inferences and References. The list of references includes 160 cited literature sources.

CHAPTER 1

LITERATURE REVIEW

1.1. POXVIRUSES AS HUMAN DISEASE

The *Poxviridae* are large enveloped DNA virus that replicate in cytoplasm of the host cells (33, 49). The best-known member of this family is Variola virus, the causative agent of smallpox disease in humans (15). Smallpox has been a part of human history for thousands of years, was specifically human infectious disease that took millions of human lives over the millenniums. Earliest occurrence of smallpox as a human disease can be only guessed. According for some evidences virus appeared first time in northeastern Africa, circa 10,000 BC (66). Another evidences of smallpox, comes from ancient Egypt. Scientific knowledge based on investigation of the skin of 3 mummies from Egypt mentions mummy to be covered with lesions that looked like those of a smallpox rash and speculates that smallpox may have occurred among the inhabitants of Egypt more than 3000 years ago (50). Or examination of the mummified head of Pharaoh Ramses **V** of Egypt, circa who died at 1157 BC showed the pustules that may have been due to smallpox (50). The virus most likely made its way from Africa to India by way of Egypt. International traders spread smallpox throughout the Old World during the 4th-15th centuries of Christ age. Smallpox was introduced to Europe sometime between the fifth and seventh centuries and was frequently epidemic during the middle Ages. The disease greatly affected the development of Western civilization (124).

It has claimed hundreds of millions of lives between its first recorded outbreak (Ancient Egypt) and its eradication in 1980 (99). (Table 1)

To eliminate variola virus around the globe, in 1967, the World Health Organization (WHO) organized the Smallpox Eradication Program, which relied on mass vaccination. On 1980th, the WHO declared that smallpox eradication in the natural environment had been achieved worldwide and that there was no indication that smallpox would return as an endemic disease (50).

Table 1. Milestones in smallpox virology (Mahalingam S. et al. 2004)

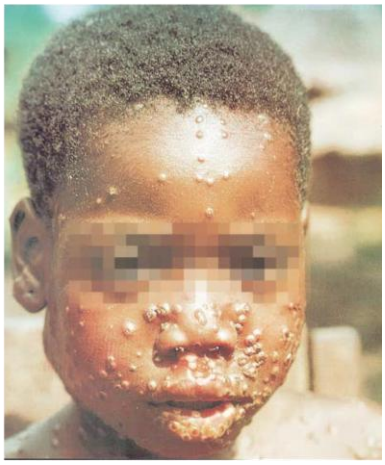
Year(s)	Milestones in smallpox virology
8000–1000 B.C.E.	Smallpox virus (*Variola*) transmitted to human species from an unknown host
1157 B.C.E	Pharaoh Ramses V died, possibly due to smallpox
430 B.C.E.	The Plague of Athens might have been smallpox
340 C.E.	Description of smallpox in China by a medical doctor Ho Kung
300–400 C.E.	Smallpox might have resulted in the decline in the population of Italy, weakening the Roman empire
910 C.E.	Persian doctor al-Razi (Rhazes) reports of smallpox in Baghdad
1000 C.E.	Smallpox: endemic in Japan
1520	Smallpox spread through Mexico, central America and south America killing half of the native American population of those areas.
1763	Deliberate infection of Ottawa tribes with smallpox by British commander. First known deliberate use of smallpox as a biological weapon
1796	Edward Jenner demonstrates his smallpox vaccine
1958	Soviet physician calls for the eradication of smallpox at the World Health Assembly
1974	Last major outbreak of *Variola major* on earth (Bangladesh)
1977	The last natural case of *Variola minor* on earth reported in Somalia
December 9, 1979	World Health Organisation certification of the global eradication of smallpox
1991	Centers for Disease Control scientists sequence the entire DNA of *Variola major*

However, although eradicated from natural environment the original smallpox virus stocks are still kept by various laboratories in Russian Federation and the United States and being highly infectious virus it remains as a bioterrorism agent. If smallpox virus released as an act of terrorism, the results could be catastrophic as nova days a large proportion of the population has no immunity and there is little available vaccine and no effective treatment.

In addition to the concern of variola, other poxviruses can be transmitted from animals to human and cause fatal diseases in human population. For example, monkeypox virus produces a smallpox-like disease a picture depicted in Figure 1 obtained from the 'The New England Journal of Medicine' shows how monkeypox resembles that of smallpox, suggesting that monkeypox might replace smallpox as a serious epidemic threat (16, 111) and Molluscum contagiosum virus causes relatively benign wart-like lesions (38).

In Africa, monkeypox infection has been found in many wild animal species: rope squirrels, tree squirrels, Gambian rats, striped mice, dormice and primates and can be transmitted to people

and spread in human population through human-to-human transmission. In early 2003 monkeypox was reported among several people in the USA (58), the virus thought to have been transmitted from African animals to a number of susceptible non-African species (like prairie dogs) with which they were co-housed. CDC fact sheet N°161 February 2011 www.cdc.gov/ncidod/monkeypox. Molluscum contagiosum (Water Warts) virus causes relatively benign wart-like lesions. It is most common in children 1 to 10 years of age. People with weakened immune systems like HIV-infected or persons being treated for cancer are at higher risk for getting molluscum (http://www.cdc.gov/ncidod/dvrd/molluscum/faq/everyone.htm#whogets.



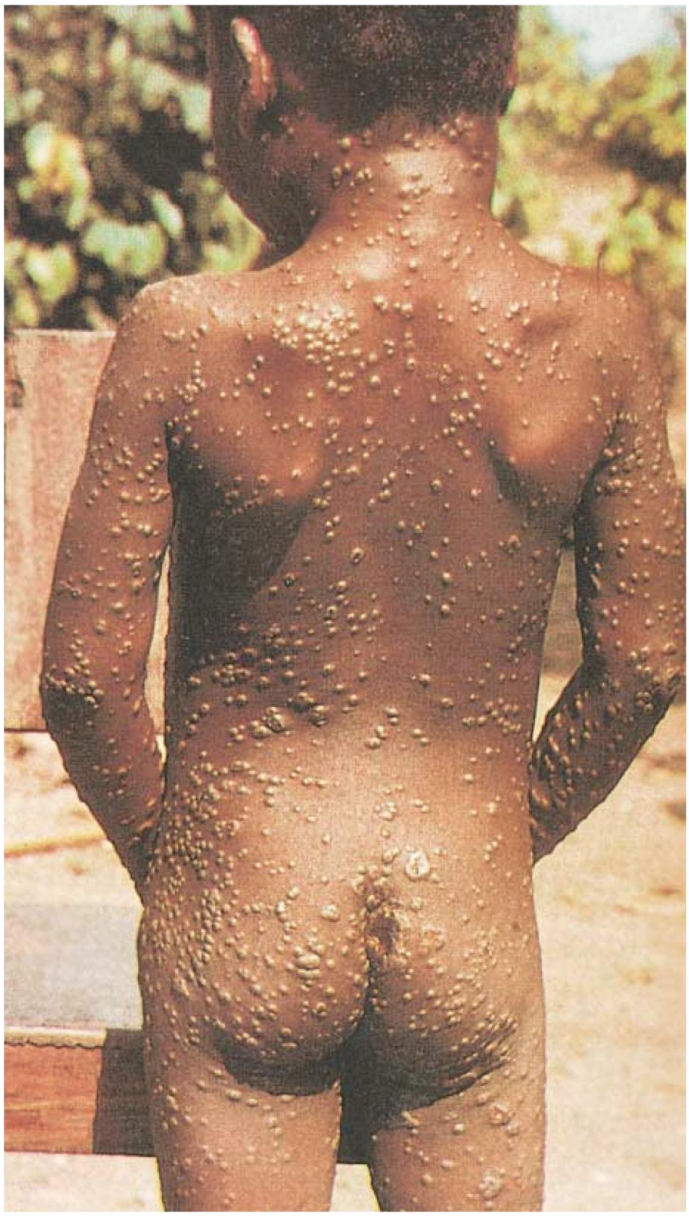


Figure 1. The clinical picture of monkeypox resembles that of human smallpox (Mark Szczeniowski.). Human Monkeypox in a Six-Year-Old Girl in Zaire, Now the Democratic Republic of the Congo. Adapted from 'The New England Journal of Medicine'

An interesting work was published by Brazilian scientists how Vaccinia virus involve to infect dairy cattle and humans, and that during the outbreaks local economies and public health in Brazil was impacted (147).

Recently poxviruses are also in great interest in viral therapies for cancer. For Vaccinia virus agents have been developed that are harmless to normal tissues but selectively able to kill cancer cells (45, 63). Therefore complete understanding of poxvirus biology, the development of new antiviral agents, the full information about virus entry and replication is a great concern for the researchers in the field working on Vaccinia and other poxviruses.

Many of the poxvirus genomes, including those of different strains of VV, have been sequenced and information can be found in the web: (http://www.poxvirus.org/viruses.asp).

1.2 VACCINIA VIRUS

Vaccinia virus (VV) is a prototype of the orthopoxvirus genus, a member of poxvirus family. VV was used as a vaccine against smallpox with which, have approximately 90% sequence identity. On May 14, 1776, an English physician named Edward Jenner inoculated 8-year-old boy with cowpox virus isolated from the infected hand of milkmaid and demonstrated that the boy was resistant to smallpox and the new procedure became known as vaccination ("vacca" from Latin cow) (81). The discovery of vaccination enabled the eradication of smallpox.

In addition to serving as a vaccine against smallpox and other poxviruses, VV has been for the study of poxviruses, developed as a recombinant expression vector (18) used in immunological studies, genetically engineered poxviruses are a great tools for veterinary and human vaccines (86, 101, 108, 121, 156). Can be use as vectors for infectious diseases and tumors (126, 150). Several

genetically engineered vectors are generated for cancer therapy based on VV strains such as Wyeth, Lister and Copenhagen that demonstrated oncolytic potency, while the Western Reserve strain seems to have the strongest oncolytic effect. VV can be used for cancer therapy by different approaches: gene delivery vectors can be used to express and transfer therapeutic genes or oncolytic vaccinia can be use to directly lyse tumor cells and as a cancer vaccine to induce anticancer immunity (60).

VV is a large, linear, double-stranded DNA (dsDNA) virus has a broad range of infectivity infects many cell lines and animal (49, 102), as other member of poxvirus family has a unique characteristic, that replicates exclusively in the cytoplasm of the host cell ref. (33). VV DNA genome is 200Kb in length, encodes approximately 200 open reading frames (ORFs) (33, 109). VV has a complex virion structure, EM examination reveled MV virions are barrel shaped, with dimensions of 360-270-250 nm (33) with a mass of approximately 5-10 femtogram (fg) (83).

To transmit virus infections in host cells Vaccinia virus uses its complex multiple forms of infectious particles, including mature virus (MV), intracellular wrapped virus (WV), and extracellular enveloped virus (EV) particles (Figure 2).

To insert virus core and transport their genetic material into the host cytoplasm Vaccinia virus should first attached to the cell membrane, several viral attachment proteins contribute the process (19, 30, 70, 96), followed by viral membrane fusion with host cell plasma membrane (110). Once in the cytoplasm viral transcriptional program is activated (4, 19), with the recognition of distinct early, intermediate and late promoter sequences (8, 39)

The virion core particles contain numerous virus encoded enzymes which are required for synthesis and modification of early mRNA, including a nine subunit RNA polymerase, a virus early transcription factor (VETF), a capping enzyme, and a poly (A) polymerase (33).

The most abundant and complex particle is MV, which is exclusively in the cytoplasm and can be moved out as cell lyses. MV responsible for virus production and to spread the virus between an organism small portion of MV will move through golgi, wrapped with additional

membrane became Wrapped Virus (WV), move through microtubio exocytose from the cells by fusing with cell membrane. The types of viruses are responsible for virus spreading within an organism.

According to the earlier studies several distinct approaches were used to analyze the composition of MV. First, virion proteins have been cataloged by electrophoretic resolution of whole virions or virion subfractions (44, 76, 82, 127, 154). Second, numerous proteins have been purified from MV and followed behavior of their enzymatic activities and subsequently characterized (106, 113), Finally, antibodies have been generated for individual gene products in a targeted fashion, allowing proteins to be subsequently localized to virions by immunoblot analysis. More recently, the combination of genetics and protein sequencing techniques has made possible to study and understand genes and investigate individual virion proteins more closely.

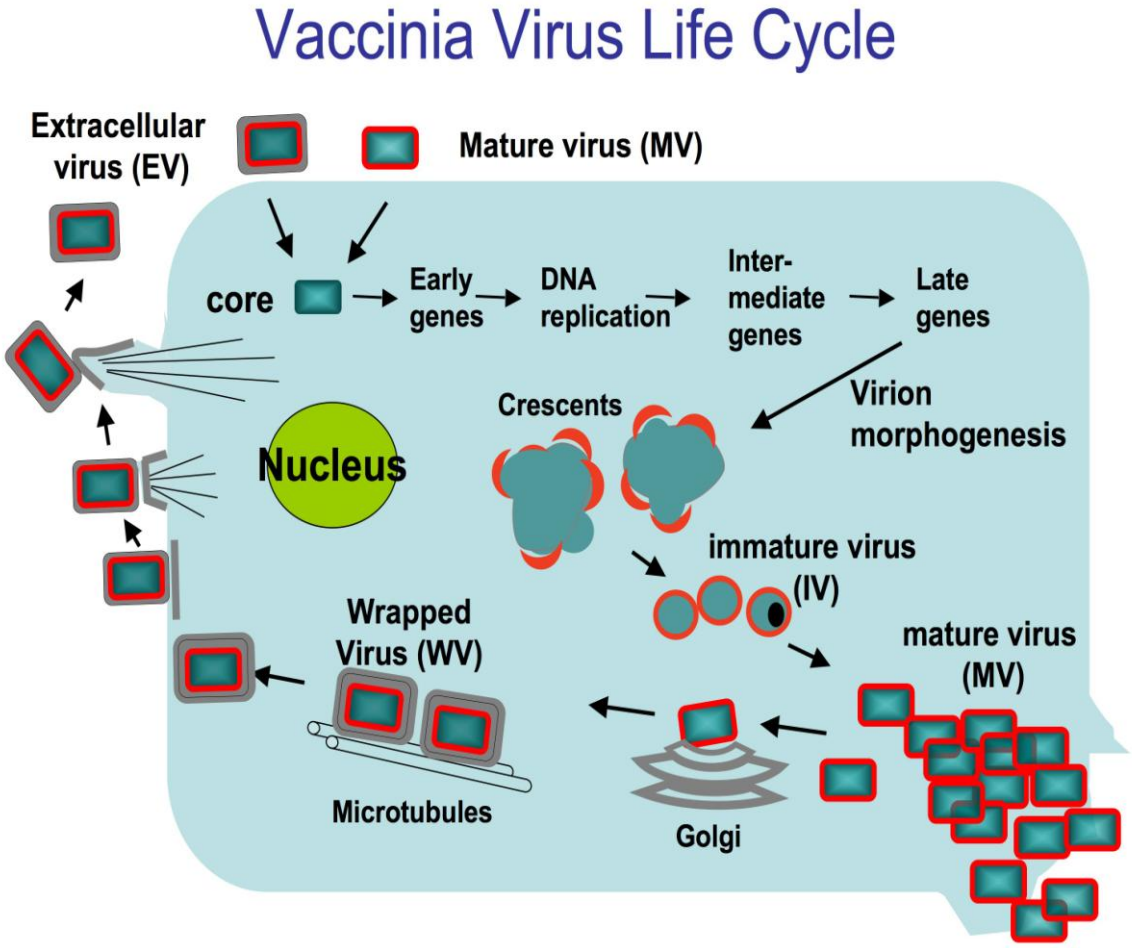


Figure 2. Vaccinia virus life cycle

Adapted from Wen Chang's laboratoty, Institute of Molecular Biology, Academia Sinica, Taipei Taiwan.

Professor Wen Chang's laboratory working on two major directions and has a great contribution on exploring and understanding Vaccinia virus biology.

I. Virus Entry and Host Cell Signaling

II. Host Restriction Mechanism Antagonized By a VV Host Range Protein CP77

In the restrictive CHO cells Vaccinia virus growth but often leads to apoptosis and no virion production. In these cells, expression of a viral host range (hr) protein, CP77, is essential to rescue virus growth. This part of research is mainly focusing on structural and functional analyses of CP77 proteins and its cellular targets to understand how cells trigger antiviral signaling and how viral hr

proteins overcome host restriction (22, 67, 71).

For the investigations we use high level of modern biological technology and experimental conditions, antibodies and reagents. We perform cloning to obtain recombinant viruses lacking or expressing the gene of interest. Usually to investigate Vaccinia MV or to identify a now components in MV composition, in our laboratory we use purified particles of MV which we isolate and purify from virus infected cell lysates, ether by sucrose gradient centrifugation or through a CsCl gradient (21, 79, 85). We performed proteomic analyses of purified MV (29, 80) and presented MV protein composition, novel envelope proteins are identified, and we are also interested to investigate their roles in virus entry. Two such viral proteins with unknown functions were WR53.5 and G3 (79, 80). We have shown that Vaccinia virus penetrates into cells through plasma membrane lipid rafts which act as platforms for signaling regulation. We have identified a cellular molecule that is specifically recruited to lipid rafts during virus entry and is required for virus penetration into cells.

The large number of viral proteins in MV composition contributes to its complex viral entry processes. Purified VV particles of Western Reserve (WR) strain was subjected to proteomic analyses in our laboratory and by others which reveled that Vaccinia MV are produced in large quantity in the infected cells and contains ~ 80 viral proteins in viral particles (29, 157).

Below is current knowledge regarding vaccinia virus entry pathways.

1. Vaccinia MV binds to multiple cell surface components such as glycosaminoglycans (GAGs), via H3, A27 and D8 viral proteins (19, 30, 70, 96). Beside that A27L protein mediates MV binding to cell surface GAGs (30) and it's N-terminal region contains a cluster of positively charged amino acids that are important for binding to HS (69). In addition A27L protein N-terminal region is essential for fusion of virus-infected cells, suggesting that conformational changes triggered after HS-A27L protein interaction initiate membrane fusion. Furthermore, A27 associates with the viral membrane protein A17 to anchor to the viral membrane (151) and has a critical function and complex formation with A26 protein (23).

D8L protein was previously reported to bind to cells (89, 97). However, it was not clear what cell surface molecules it interacts with. Direct evidence of D8L protein interaction with cell surface CS was provided by Hsiao, et al 1999, authors use cell lines expressing different GAGs such as parental mouse L cells express both HS and CS on cell surfaces, or a mutant cell line selected from L cells, gro2C, expressing only CS (57) or sog9 cells, that was selected from gro2C cells and expresses neither HS nor CS (70). The function of Vaccinia MV protein H3 in vaccinia virus life cycle was studied by Lin and colleagues experiments showed that Soluble H3 protein binds to cell surface HS and blocks MV adsorption to cells (96). Further Investigation demonstrated that Vaccinia H3 has a consensus two putative GAG-binding sites that may be responsible for HS binding. Thus, vaccinia virus MV includes three envelope proteins that recognize cell surface GAGs: A27 and H3 proteins bind to HS, and D8 protein binds to CS.

2. Vaccinia MV binds to extracellular protein laminin (26, 28) and sulfatides (117). Soluble laminin blocked MV entry in the Vaccinia virus expressing A26 protein but not in virus defective in A26. Indicating interaction of extracellular matrix Laminin is through viral A26 protein. In addition A26L binding to laminin during virus entry may affect cellular stress responses. It is well documented that cell adhesion via the ECM is important for cell survival and that disruption of the cell surface receptor-ECM interaction results in anoikis, a term describing apoptosis induced by cell detachment (52, 53). Immediately after infection, cells infected with virus expressing A26 protein exhibits a slower cell rounding-up phenotype than those infected with virus lacking A26 protein expression, suggesting that cell-associated A26 helps maintain cell adhesion. Whether any specific cellular signaling is regulated by A26 during virus entry is an open question to be study.

3. Vaccinia MV contains an entry fusion complex (EFC) of 12 viral envelope proteins that are evolutionarily conserved and essential for membrane fusion (13, 17, 32, 79, 132, 145). One

member of EFC called Vaccinia virus G3 protein was studied in our laboratory and is a part of my dissertation as well detailed information about the subject is described in the section 3.4.

4. Several proteins on Vaccinia genome, A55R and C2L, were reported to affect calcium-independent cell adhesion and the formation of long projections (10, 118). Both proteins belong to the kelch protein family with a POZ/BTB domain at the N terminus and multiple kelch repeats at the C terminus. Both proteins are intracellular proteins that were nonessential for virus growth in cell cultures and were not packaged into virions; a loss of either protein had no effect on virus virulence in an intranasal infection mouse model (10, 118). However, deletion of the C2 or A55 protein caused a lesion in mice that took longer to heal in an intra dermal infection mouse model. Investigation demonstrated that this, proteins are expressed abundantly on the cell surface of virus infected cells and mediated calcium-independent cell adhesion. The third kelch protein in vaccinia virus is F3 protein, deletion of this protein behave differently from C2 and A55, had no effect on adhesion behavior of the virus-infected cells, suggesting that F3 protein function is distinct from others (54). Interestingly Wild-type WR53.5 protein was also identified by our MV proteomic that was not reported by others. We demonstrated that the WR53.5 protein is incorporated into Vaccinia MV expressed abundantly on the cell surface and mediated calcium-independent cell adhesion. WR53.5 is not important for virus growth in cell culture but deletion of the gene attenuated virus virulence in vivo, suggesting that the WR53.5 protein may interfere with host antiviral pathways in animals. Compare to the C2, A55, F3 proteins WR53.5 does not contain any kelch homology sequences, suggesting a different mode of regulation of cell adhesion from that of viral kelch proteins. Whether the WR53.5 protein interacts with other viral or cellular proteins to mediate cell adhesion is still unknown. Previous studies mentioned calcium-independent adhesion could be mediated by integrins (90) and recent observation that integrins involved in Vaccinia entry leaves an open question to know if there is any direct connection between this viral and cellular molecules.

An independent work done by Zhang et al., also reveled the importance of WR53.5 protein for Vaccinia virus virulence. They constructed a recombinant Vaccinia virus GLV-1h68, by the insertion of three marker genes into the WR53.5/F14.5 loci of the viral genome into wild type LIVP strain (159). And the recombinant virus was used for cancer therapy studies. Then they injected GLV-1h68, intravenously into tumor bearing nude mice, which exhibited enhanced tumor targeting specificity, reduced toxicity and was a better oncolytic viral therapeutic agent than its parental LIVP strain containing WR53.5 gene (159). Although our experimental designs were different from their study, our results that WR53.5/F14.5 contributes to virus virulence in BALB/c mice are consistent with their observations.

5. An Interesting observation was presented by Huang, et al who showed that a novel cellular protein, Vaccinia virus penetration factor (VPEF), is important for Vaccinia MV penetration into HeLa cells (73), authors demonstrated, that Vaccinia MV entry into HeLa cells has several interesting features. First, vaccinia MV required actin polymerization prior to cell entry. That virus "surfing" requires normal actin dynamics has been reported for some retroviruses and vesicular stomatitis virus (92), authors demonstrated that it is also true for Vaccinia virus, making it as a common transport mechanism for many viruses. In their study plasma membrane ruffling and actin protrusions regulated by actin dynamics were also involved in Vaccinia MV recruitment. In addition, MV particles were shown to be endocytosed into HeLa cells in a manner independent of clathrin and caveola-mediated pathways but dependent on dynamin, suggesting a virus entry pathway through fluid phase endocytosis. Conclusion of the study was MV entry is dynamin-dependent and vaccinia virus penetration factor (VPEF)-dependent fluid-phase endocytosis (73).

6. In WR VV mutations viral envelope proteins A25 and A26 resulted different entry of MV to the cells (21). It has been repeatedly shown that, WR□VV can trigger Fusion From Without (FFWO) in HeLa cells only in the case when virus infected cells are treated with acidic buffer (pH

4.7), (56, 143, 146), interestingly Chang, et al demonstrated that MV made from recombinant WR VV viruses lacking A25 and A26 proteins can trigger FFWO in neutral pH (pH 7) on HeLa, CHO-K1 and L Cells whereas wild type WR VV did not, authors further demonstrated that this two proteins are low pH sensitive fusion suppressors. Examples of CHO-K1 cells are shown bellow in Figure 3.

7. Another interesting feature of VV MV entry is involvement of lipid rafts, which was demonstrated by Chung, et al (31). Lipid rafts on the plasma membrane have been shown to be a platform for the entry of many pathogens, viruses and bacteria (9, 25, 43, 135).

In addition, lipid rafts are also involved in cell surface receptor-mediated activation, which requires the aggregation of membrane receptor and adaptor molecules to transmit membrane-proximal signaling into cells (40, 100, 112, 119). Since lipid rafts are rich in sphingolipids and cholesterol`, they are more resistant to detergent extraction`, which is usually the method of choice to physically separate lipid rafts from other membrane components (31).

Studies in our laboratory by Chung, et al demonstrated that VV infection is defective when cell surface cholesterol was depleted by mβCD (Figure 4A), in co patching experiment GM1 which is a marker for raft can be patched with WR VV on the surface of infected HeLa cells. (Figure 4B). This interesting observation was further investigated to understand whether raft associated cell surface proteins or receptors are involved in VV entry rout (78, 131). Intracellular Transport of Vaccinia Virus in HeLa cells was studied and showed that virus transport in cells requires WASH-VPEF/FAM21-retromer complexes as well as recycling molecules of Rab11 and Rab22 (68).

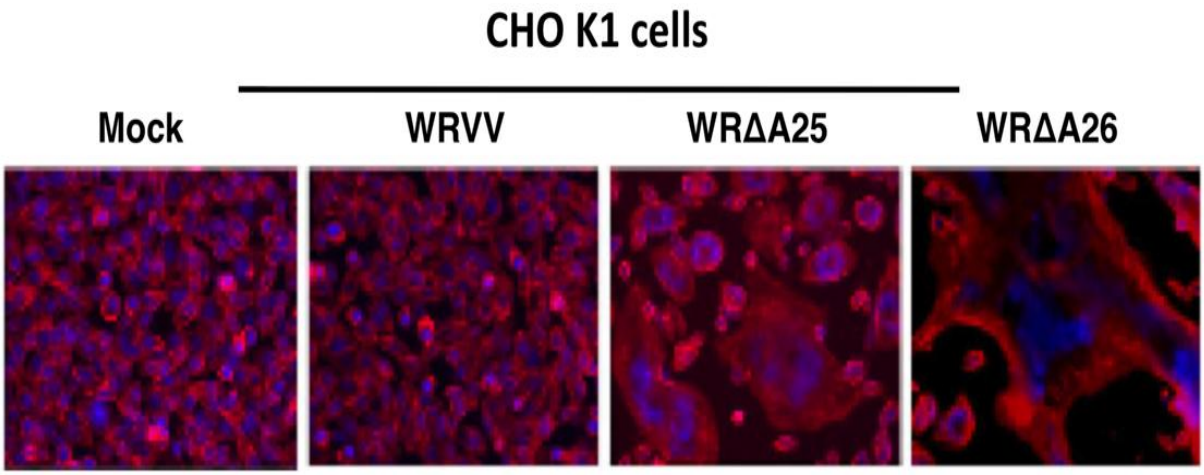


Figure 3. Fusion from without at neutral pH on CHO-K1. (Chang, et al 2010)

CHO-K1 cells were infected with WR VV, WRΔA25, and WRΔA26 viruses at an MOI of 100 PFU per cell at 37°C for 120 min at a neutral pH. Cells were fixed, nuclei were stained with DAPI and plasma membrane is stained with a fluorescent dye, PKH26, to visualize cell shapes.

8. Taking advantage, that rafts are important for Vaccinia MV entry study was extended using proteomic analyses. Detergent-resistant domains (DRM) from HeLa cells before and after Vaccinia MV infections were isolated and proteins were extracted for quantitative proteomic analyses (131) using the methods described earlier (11, 31, 120). A total of 717 cellular proteins were identified, and among those, 570 proteins (79%) were quantified, (131). A type II membrane glycoprotein, CD98, was enriched in lipid rafts upon vaccinia MV infection compared to mock-infected HeLa cells. Using siRNA knockdown approach on HeLa cells or CD98 knockout (KO) mouse embryonic fibroblasts (MEFs) authors demonstrated that in both cell types without CD98 expression virus entry significantly reduced. Infections of wild-type and CD98 KO MEF cells with different strains of vaccinia MV provided further evidence that CD98 plays a specific role in MV endocytosis, but not in plasma membrane fusion.

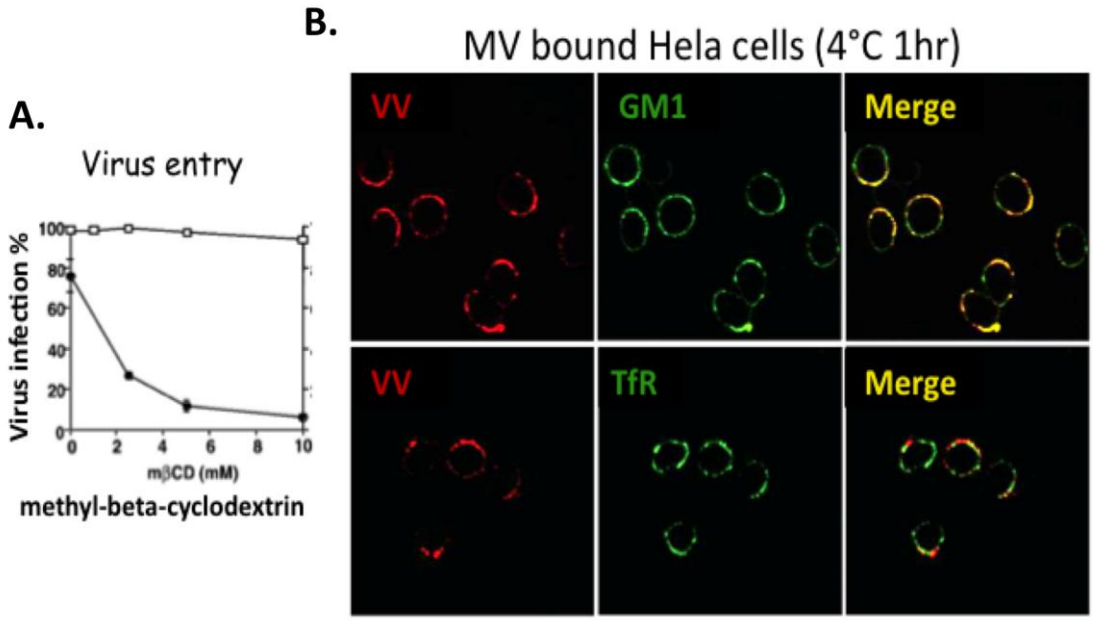


Figure 4. (A) Vaccinia virus MV infection is blocked by mβCD. (Chung, et al) Cells were pretreated with 0, 2.5, 5, or 10 mM of mβCD and infected with WT WR VV expressing lacZ from a viral early promoter, the beta-galactosidase activity was then determined. **(B)** WR VV protein co localized with GM1 but not TfR. HeLa cells were infected with WT VV at an MOI of 50 PFU per cell and prepared for copatching analyses as described in Materials and Methods. VV stained (red), cell surface GM1 or TfR (green), co localization detected by yellow Merged.

9. To extend studies above, additional biological network analyses were performed (Figure 6), which revealed the presence of integrin β1 and its associated proteins, such as CD9, CD47, CD59, CD98, talin, ezrin, and Fyn/yes/lyn, suggesting a possibility that integrin β1-mediated biological signaling may participate in vaccinia MV entry.

We further study and show for the first time that cell receptor integrin β1 (78) mediats VV endocytoses and that VV induced intracellular signaling are dependent from integrin β1. Results studied in the section 3.1, focused primarily on integrin β1, however the significance of the involvement of integrin in Vaccinia virus entry goes beyond this molecule. Blebbistatin, an

inhibitor of myosin II light-chain ATPase (95), was recently shown to interrupt integrin adhesome formation (129) and in our experiments, blebbistatin-treated HeLa cells lost the outside-in activation of integrin and reduced Vaccinia virus endocytosis. This finding implies that other components of the integrin adhesome may be involved in vaccinia virus endocytosis. Indeed, a recent study conducted in our laboratory by Schroeder et al., demonstrated that CD98, a type II membrane protein found in lipid rafts and a component of the integrin adhesome (129) is required for vaccinia MV endocytosis (131). The KD of CD98 does not affect vaccinia MV binding to cells but reduces virus endocytosis and core uncoating (131), suggesting that it participates in Vaccinia virus entry at a step subsequent to integrin β1. It is worth noting that CD98 was reported previously to promote integrin-dependent signaling, leading to the activation of FAK, PI3K, Akt, and Rac (48, 51, 158). VV MV entry pathways also was shown to vary among different cell lines (24) and multiple kinases such as extracellular signal-regulated kinase (ERK), protein kinase A (PKA), protein kinase C (PKC) and p21-activated kinase 1 (PAK1), have been shown to be activated upon virus entry (3, 87, 104). However the mechanism how signaling are activated was unknown our current model proposes that vaccinia MV binds to integrin β1, recruits CD98 in rafts, activates kinases, and induces cytoskeleton rearrangements to trigger MV endocytosis in cells section 3.1 (Figure 13).

1.3. INTEGRINS

Integrins are a large family of cell surface receptors composed of 18 different α and 8β subunits (84). In the surface of cell membrane Integrins can switch between active and inactive conformations. In the inactive state, integrins have a low affinity for ligands. Intracellular signalling events such as protein-kinase-C stimulation can prime the integrins, which results in a conformational change that exposes the ligand-binding site Ligand binding activates signalling cascades that lead to the assembly of a multiprotein complex at the site of cell adhesion to the ECM.

These events have two important impacts on the cell: they key connection between the ECM and the actin cytoskeleton, and they alter the fluxes of many intracellular signalling pathways. (91),

An important member of integrin family is Integrin β1, that is known to associate with multiple α subunits including α1-11 and αV and is widely distributed virtually in all mammalian cell types (84, 91, 93, 142). Through interaction with the extracellular matrixes, integrin β1 regulates multiple aspects of cell biology, involves in many intracellular transduction activation pathways (91), They regulate both outside-in and inside-out signaling to maintain important cell functions related to cell adhesion, migration and survival (1, 46) all of which are important processes for actin cytoskeleton organization (91, 153).

The linkage of integrin β1 to the ECM and intracellular proteins results in various intracellular signal transduction. Schematic depiction on in Figure 5 shows how various it could be.

The unique properties of integrins, may explain why they are so frequently targeted for pathogen invasions. This are the studies which described that integrins are involved in the invasion of many pathogens, viruses (2, 12, 20, 42, 47, 59, 98, 136, 141) and bacteria (36, 61, 94). The pathogen-host interaction may promote the clustering of integrins and focal adhesion formation but subvert the downstream signaling network to modify membrane traffic and cytoskeletal dynamics to meet their own needs (1). Furthermore, integrins act as global regulators of endocytosis, affecting the intracellular trafficking of growth factor receptors and endosome localization in cells (63). A genomewide screen of endocytosis regulators identified adhesion mediated molecules that support cross talk between cell adhesion regulation and endocytic activity. The fate of endocytosed vaccinia MV is not well understood. Recently, it was shown that integrin internalization is mediated through macropinocytosis. Internalized integrins were found to transit through early endosomes to recycling endosomes and then back to the cell surface to form new adhesions (93). Currently, we are investigating whether integrin β1 regulates the intracellular trafficking of vaccinia virions through the same route prior to virus-cell membrane fusion. More studies will be needed in the future to dissect the structure-function relationship of integrins in vaccinia virus entry.

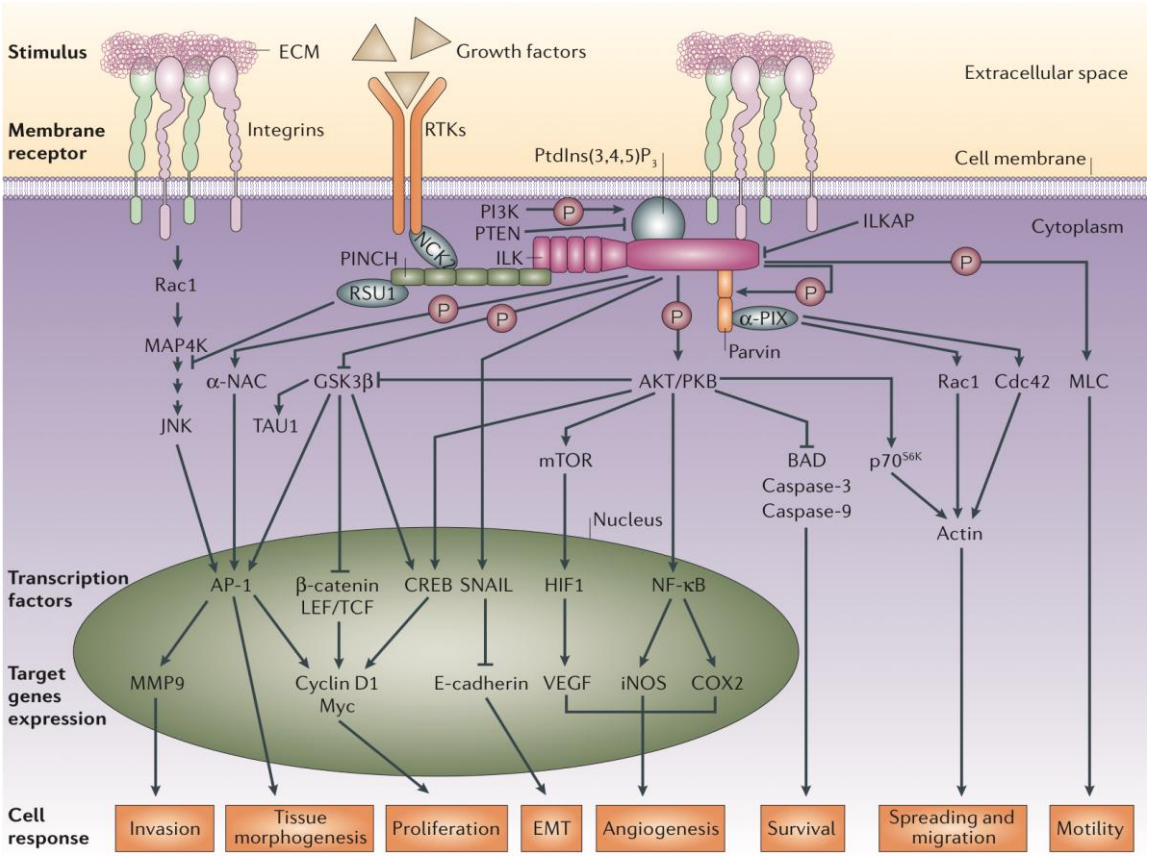


Figure 5. Signalling through integrins and intracellular proteins. (Legate, et al 2006) The integrin-linked kinase (ILK), particularly interesting Cys-His-rich protein (PINCH), parvin (IPP) complex has been implicated in the control of signalling pathways through both phosphorylation of downstream targets (most notably AKT/protein kinase B (PKB) and glycogen-synthase kinase-3β (GSK3β) and binding to upstream effectors of the Jun N-terminal kinase (JNK) signalling pathway and regulators of small- molecular-weight GTPases. The activity of the complex is upregulated by phosphatidylinositol 3-kinase (PI3K) and downregulated by the phosphatases ILK-associated protein (ILKAP) and phosphatase and tensin homologue deleted in chromosome 10 (PTEN). Growth-factor-mediated signalling through receptor tyrosine kinases (RTKs) might be transduced to the IPP complex through the receptor-tyrosine-kinase adaptor protein NCK2. The signalling pathways that are shown are limited to those that have been experimentally described to be influenced by the IPP complex. AP-1, activator protein-1; BAD, BCL2-antagonist of cell death; COX2, cyclooxygenase-2; CREB, cAMP-response-element-binding protein; ECM, extracellular

matrix; HIF1, hypoxia-inducible factor-1; iNOS, inducible nitric-oxide synthase; LEF/TCF, lymphoid enhancer factor/T-cell factor; MAP4K, mitogen-activated-protein-kinase-kinase-kinase kinase; MLC, myosin light chain; MMP9, matrix metalloprotease-9; mTOR, mammalian target of rapamycin; α-NAC, nascent polypeptide- associated complex and co-activator-α; NF-kB, nuclear factor-kB; p70S6K, p70 ribosomal S6 kinase; α-PIX, activating PAK-interactive exchange factor-α; PtdIns(3,4,5)P3, phosphatidylinositol-3,4,5-trisphosphate; RSU1, Ras suppressor-1; VEGF, vascular endothelial growth factor.

CHAPTER 2

2.1. EXPERIMENTAL MATERIALS AND METHODES

2.1.1. Cells and viruses

Two mouse cell lines, GD25 and GD25β1A, were kindly provided by Dr. Reinhard Fässler (Max Planck Institute of Biochemistry, Germany). The GD25 cell line was derived from integrin β1 knockout (KO) embryonic stem cells (46). The stably transformed cell line GD25β1A resulted from GD25 electroporation with wild type human integrin β1 cDNA (115). HeLa, BSC40, GD25 and GD25β1A cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 2% penicillin/streptomycin (Gibco) in a 5% CO_2 incubator at 37°C. The Western Reserve strain of vaccinia MV (WR-VV) was purified through 25-40% sucrose gradients as previously described (80, 85). A recombinant WR-VV was also used in this study. It was constructed previously to express a dual gene cassette inserted in the tk locus containing the luciferase (*luc*) gene driven by a viral early promoter and the *lacZ* gene driven by a viral late promoter.

2.1.2. Antibodies and reagents

The anti-integrin β1 mAbs Ts2/16 and 12G10 were purchased from Santa Cruz Biotechnology and Abcam, respectively, and 9EG7 and Mab13, rat mAbs, were acquired from BD Pharmingen. Transferrin receptor (TfR) Anti-TfR (CD71) was from AbD Serotec. Anti-paxillin antibody was purchased from BD Transduction Laboratories. Alexa Fluor 647 phalloidin was purchased from Invitrogen. Anti-phospho-Akt (Ser473) and anti-Akt antibodies were purchased from Cell Signaling Technology. Anti-phospho-FAK (pY397) antibody was purchased from Invitrogen. Anti-FAK antibody was purchased from BD Biosciences. Anti-cyclophilin B (CypB) antibody was obtained from Santa Cruz Biotechnology. Anti-β actin antibody was purchased from Sigma-Aldrich. Anti-A4 and anti-vaccinia MV (anti-

VV) rabbit antibodies were previously described (73). The mouse mAb clone 2D5 against vaccinia viral L1 protein was obtained from Y. Ichihashi (75). Bafilomycin (BFLA), cycloheximide (CHX) and blebbistatin (Bleb) were purchased from Sigma-Aldrich. PI3K inhibitor (LY 294002) and Akt inhibitor (Akt IV) were purchased from Calbiochem. Laminin-1 (LN), fibronectin (FN) andpoly-L-Lysine (PLL) were purchased from Sigma-Aldrich. The cyclophilin B (CypB) small interfering RNA (siRNA) duplex and the integrin β1 siRNA duplex (AAUGUAACCAACCGUAGCAUU) were purchased from Dharmacon Inc..

2.1.3. Biological network analysis

Cellular proteins identified in lipid rafts isolated from HeLa cells (131) were subjected to subcellular localization analyses with NCBI Gene Ontology. The "integrin β1 (ITGβ1) signaling network" contains cellular proteins that are known to physically interact with integrin β1 and was constructed using ARIADNE Pathway Studio 7.0 software, which uses automated text-mining engines to extract information from the website http://www.ariadnegenomics.com/products/pathway-studio/. Plasma membrane proteins identified in lipid rafts were compared with those in the "integrin β1 signaling network" and displayed in a graphical network using the open source software Cytoscape (134).

2.1.4. Virus entry assays

Several cell-based biological assays were used to quantify vaccinia MV entry into host cells based on previously established methods (56, 146, 148). MV particles bound to cells were quantified by vaccinia MV virion binding assays at 4ºC for 60 min with anti-L1 antibody (2D5) (148). Viral core numbers present in the cytoplasm after membrane fusion were quantified by viral core uncoating assays using an antibody against A4 (138). Luciferase assays driven by a viral early promoter were performed with cell lysates harvested at 2 h p.i.

as described previously (146). Acid-bypass treatment that forced cell-bound MV to fuse with plasma membrane was performed as previously described (56). In brief, HeLa cells were pretreated with 25 nM bafilomycin A1 or 50 μM PI3K inhibitor at 37ºC for 30 min, cooled at 4ºC for 20 min and subsequently infected with vaccinia MV at an MOI of 20 PFU per cell for 1 h. After washing, the infected cells were treated with neutral (pH 7.2) or acidic (pH 5) buffer for 5 min, incubated in growth medium and fixed at 2 h p.i.. These infected cells were permeabilized and stained with anti-core A4 antibody for confocal microscopy analyses as described (138).

2.1.5. Plaque formation on GD25β1A and GD25 cells

Freshly confluent cells were infected with WR-VV (approximately 300 PFU per well in a 6-well plate) at 37°C for 1h, washed and cultured in growth medium containing 1% agarose, fixed at 2 days p.i. and stained with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) as previously described(7). Alternatively, cells were pretreated with DMSO or LY-294002 (25 or 50 μM) in serum-free DMEM prior to infection and the inhibitors remained in cultures after infection until cell fixation and X-Gal staining as described above.

2.1.6. Integrin β1 siRNA

HeLa cells were either mock-transfected (si-control) or transfected with siRNA duplexes (20 nM) targeting either cyclophilin B (si-CypB) or integrin β1 (si-ITGβ1) using the Lipofectamine 2000 reagent (Invitrogen) as described previously (73).

2.1.7. Confocal immunofluorescence microscopy

(i) Copatching experiments. The experiments of copatching integrin β1 and vaccinia MV were performed as previously described (73, 139). In brief, HeLa cells seeded on glass cover slips were infected with MV at an MOI of 50 PFU per cell for 1 h at 4°C, washed and transferred to 12°C where the cells were incubated with anti-VV antibody (1:500) and anti-integrin β1 mAb (12G10) (1:1,000) for 1 h. Tetramethylrhodamine-conjugated goat anti-rabbit IgG (1:1,000) and FITC-conjugated goat anti-mouse IgG (1:1,000) were subsequently added for another 1 h incubation prior to cell fixation for confocal microscopy. Cells were mounted in Vectashield medium (Vector Laboratories, Burlingame, CA) and images were collected. (ii) Surface staining of integrin β1 with virus in GD25β1A cells. GD25β1A cells were seeded ($1.2x10^5$) on glass cover slips in 12-well plates. The next day cells were cooled at 4°C for 20 min and subsequently infected with vaccinia MV at an MOI of 60 PFU per cell for 1 h, washed 3 times in PBS, fixed and incubated with primary antibodies of anti-integrin β1 mAb (12G10) (1:1,000) and anti-VV rabbit antibody (1:500) for 1 h. Tetramethylrhodamine-conjugated goat anti-rabbit IgG (1:1,000) and FITC-conjugated goat anti-mouse IgG (1:1,000) were subsequently added for 30 min and analyzed by confocal microscopy.

2.1.8. Outside-in integrin signaling activation assay

(i) Glass cover slips in 24-well plates were coated with FN (10 μg/ml), LN (20 μg/ml) or PLL (100 μg/ml) in PBS at 4°C. After 24 h the dishes were blocked with 1% BSA in PBS at 37°C for 1 h. Serum-starved HeLa cells were seeded at a density of $8x10^4$ to each well in serum-free DMEM, incubated at 37°C for 20, 30, 45 and 90 min, and lysed in cold lysis buffer (10 mM Tris pH 7.4, 150 mM NaCl, 0.5% NP-40, 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 1 mM EGTA, 1 mM EDTA with 1x protease inhibitor cocktail (tablets purchased from Roche Applied Science)). Samples were then prepared for immunoblot analyses with anti-phospho-Akt (1:1,000), anti-phospho-FAK (1:1,000), anti-Akt (1:1,000) and anti-FAK (1:1,000)

antibodies. Alternatively, cells were fixed after 90 min plating, permeabilized and stained with anti-paxillin antibody (1:1,000). **(ii)** HeLa cells seeded for 90 min as described above were subsequently infected with vaccinia MV at an MOI of 5 PFU per cell at 37°C for 1.5 h and harvested for luciferase assays. **(iii)** HeLa cells were seeded as described in (**i**) for 15 min at 37°C. DMSO or blebbistatin (25 and 50 μM) was added to cells and incubated for another 45 min. The cells were either fixed, permeabilized and stained with anti-paxillin antibody (1:1,000) or were infected with vaccinia MV at an MOI of 5 PFU per cell for 1.5 h and harvested for luciferase assays.

2.1.9. Flow cytometry

Cells were detached from the dishes with 20 mM EDTA, washed and stained with anti-integrin β1 mAb (9EG7) (1:1,000) in PBS containing 150 mM NaCl and 1% BSA for 1 h at 4°C. Samples were then washed 3 times, followed by incubation with FITC-conjugated goat anti-rat secondary antibody for 1 h at 4°C. Following a final wash the cells were analyzed by fluorescence-activated cell sorting (FACS).

2.1.10. Vaccinia MV infections activated Akt phosphorylation

HeLa ($7.5x10^4$/well) or GD25β1A ($1x10^5$/well) cells were cultured in 12-well plates for 24 h. Cells were serum-starved at 37°C for 2 h (for HeLa cells) or 18 h (for GD25β1A cells) and then stimulated with medium containing 20% FBS or purified vaccinia MV (at an MOI of 40 PFU per cell for HeLa and 60 PFU per cell for GD25β1A) at 37°C for various times. Cell lysates were prepared with cold lysis buffer (10 mM Tris pH 7.4, 150 mM NaCl, 0.5% NP-40, 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 1 mM EGTA, 1 mM EDTA with 1x protease inhibitor cocktail (tablets purchased from Roche Applied Science)), for immunoblot analysis.

2.1.11. Cell spreading and adhesion assays

Spreading and adhesion assays were performed as described previously (5, 46). In brief, non-tissue culture grade 96-well plates (FALCON) were coated with fibronectin (FN) or laminin-1 (LN-1) at 20µg/ml in 50µl volume overnight at 4°C, the next day coated wells were washed once with PBS and blocked with PBS containing 1% BSA at 37°C for 1 to 2hrs. While blocking the coated substrates cell suspension were prepared. HeLa KD cells (si-control and si-ITGβ1) that were grown to confluency and serum starved for 2hrs were detached from the culture dish by trypsin/EDTA spin, washed once, suspended in serum free DMEM $2x10^4$ cells per well was added to the substrates and incubated at 37°C for various time. Non adhered cells were washed out and attached cells were fixed with 95% ethanol for 10 min at RT stained with crystal violet for 30 min, after being washed with water images were collected with Nikon inverted microscope, or for colorimetric analyses 0.2% Triton X-100 was added to the stained cells for 5 minutes at RT then OD in 550nm was measured in an ELISA reader.

2.1.12. Wound healing assay

Cells were seeded in tissue culture dish and grown for ~100% confluency then cell monolayer were wounded using pipette tip, cells were washed once with PBS to remove wounded cell debris and incubated with complete medium at 37°C. Images were collected with Nikon inverted microscope at 0 hrs (right after wounding) or 24 hrs of incubation.

2.2. EXPERIMENTAL MATERIALS AND METHODES

2.2.1.Reagents, cells and viruses

Mycophenolic acid, hypoxanthine, and xanthine (Sigma) were dissolved at a concentration of 10 mg/ml in 0.1 N NaOH and stored at –20°C. Cytosine β-D-arabinofuranoside (araC) was purchased from Sigma. BSC40, BSC1 and RK13 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 2% penicillin and streptomycin (PS) from GIBCO. Alexa Flour 647-phalloidin (200U/ml) was obtained from Molecular probes Inc. and used at a dilution of 1:125. Two wild-type WR strain vaccinia viruses were used in this study: the original WR strain obtained from Dr. S. Pennathur (designated WR-1) and another wild-type WR strain obtained from Dr. G. L. Smith (WR-2). VT7LacOI was obtained from Dr. B. Moss (152). MV virions were purified on a 36% sucrose cushion, followed by 25-40% sucrose gradient centrifugation as described previously (85) and stored at -70°C. Since sucrose gradient centrifugation resulted in mostly MV particles with little IEV/EEV contamination (37), further purification using a CsCl gradient to separate MV preparation from IEV or EEV was not performed. Rabbit Abs recognizing the vaccinia virus proteins H3L and D8L were described previously (70, 96). An anti-cores rabbit Ab was provided by Dr. J. Krijnse Locker and was described (82, 116). The anti-B5R MAb 206C5 is a mouse MAb from a hybridoma generated by immunization of mice with a recombinant B5 protein expressed as a glutathione S-transferase fusion protein in Escherichia coli. Rabbit anti-53.5 Ab was generated using a synthetic peptide, EENNEEDARIKEEQELLLLY, derived from the WR53.5 peptide sequences (QCB Inc.) and Rabbit anti-G3L antibodies were generated by using a synthetic peptide, NGKKHTFN LYDDNDIRTL, derived from the G3L sequence. They were used as 1:1,000 dilution for all experiments.

2.2.2. Generation of vi53.5L virus

(i) Plasmid construction. To construct pMITEO-53.5 containing an inducible copy of the WR53.5L gene, the full length of WR53.5L ORF was generated by PCR using the primers

5'-AAACCATGGTCATCGGTCTAGTCATA-3' and

5'-CCCGGATCCTCAATATAGCAACAGTAGTTC-3' (*Nco*I and *Bam*HI restriction sites underlined) and the genomic DNA of vT7LacOI as the template (72). The PCR product was digested with *Nco*I and *Bam*HI and cloned into pMITEOlac.20/3 to produce pMITEO-i53.5L. Three DNA fragments were used to replace the endogenous WR53.5L gene with a xanthine-guanine phosphoribosyltransferase ORF expression cassette (*gpt*). The 560 bp 5' flanking fragment containing the WR54L promoter and coding sequences was generated by PCR using the primers 5'-TATAGACTAAAAAAGAAACGT-3' and 5'-GCGGCCGC*G*TCTCTAGCTTTCACTTAA-3' (*Not*I site underlined) and vT7lacOI genomic DNA as the template. The 580 bp 3' flanking fragment containing the WR53L promoter and the coding sequences was generated by PCR using the primers 5'-GCGGCCGCGTACATAATTGAAAATCTA-3' and 5'-CTCGAGGACTTTGTAGCTCTCCCA-3' (*Not*I and *Xho*I sites underlined) and vT7lacOI genomic DNA as the template. The 5' and 3' flanking DNA fragments were cloned into the pCRII-Topo vector (Invitrogen) to create pCRII-Topo-54/53-52. The 1.95 kb i53.5L expression cassette was purified from *Not*I-digested pMITEO-i53.5 and cloned into pCRII-Topo-54/53 to obtain pCRII-Topo-54/i53.5/53. The sequences of the PCR fragments were confirmed by DNA sequencing. (ii) Construction of the recombinant vi53.5L **virus.** The recombinant vi53.5L virus was constructed following previously established protocols (79). In brief, $3x10^5$ BSC1 cells were infected with vT7LacOI at a MOI of 1 PFU per cell and subsequently transfected with 1 μg of pCRII-54/i53.5L/53 with lipofectamine (Invitrogen). After removal of the transfection mixture, DMEM containing 10% FBS and 100 μM IPTG was added to cells and lysates were prepared at 30 hours post infection (h p.i.) and used to select for plaques formed by vi53.5L, which expresses xanthine-guanine phosphoribosyltransferase (Gpt) and WR53.5 protein. Pure recombinant vi53.5L viruses were obtained after three rounds of plaque purification. The insertion of Gpt and the inducible WR53.5L gene into the endogenous WR53.5L locus was confirmed by PCR.

2.2.3. Generation of viG3L virus

(i) Plasmid construction**.** To construct pMITEO- G3L containing an inducible copy of the G3L gene, the full-length G3L open reading frame (ORF) was generated by PCR using the primers 5'-AAACCATGGCATCTTTATTATATCTT-3' and 5'-CCCGGATCCTCATTTACTAAGGAGTAAAAT-3' (the NcoI and BamHI restriction sites are underlined) and the genomic DNA of vT7LacOI as the template (72). The PCR product was digested with NcoI and BamHI and cloned into pMITEOlac.20/3 to produce pMITEO-iG3L. Three DNA fragments were used to replace the endogenous G3L gene with a gpt expression cassette. The 531-bp 5' flanking fragment containing the G2R promoter and coding sequences was generated by PCR with the primers 5'-GCGGCCGCGGATAATATGTAAAATAA-3' (the NotI site is underlined) and 5'-TAAATGTAACTTGAGAAA-3' and vT7lacOI genomic DNA as the template. The 558-bp 3' flanking fragment containing the G1L promoter and the coding sequences was generated by PCR using the primers 5'-GCGGCCGCG TAAAATTATAATGTCAC-3' (the NotI site is underlined) and 5'-CTCGAGTATTAAGATTATCTATCA-3' (XhoI site underlined) and vT7lacOI genomic DNA as the template. The 5' and 3' flanking DNA fragments were cloned into the pCRII-Topo vector (Invitrogen) to create pCRII-Topo-G2/G1. The 2.2-kb G3L expression cassette was purified from NotI-digested pMITEO-iG3L and cloned into pCRII-Topo-G2/G1 to obtain pCRII-G2/iG3L/G1. The sequences of the PCR fragments were confirmed by DNA sequencing. (ii) Construction of the recombinant viG3L virus. The recombinant viG3L virus was constructed based on previously described established protocols (105). In brief, 3 X 10^5 BSC40 cells were seeded in a 60-mm dish, incubated for 1 day, and infected for 1 h at 37°C with vT7LacOI at a multiplicity of infection (MOI) of 1 PFU per cell. The cells were then washed three times with DMEM and transfected with 4 μg of pCRII-G2/iG3L/G1 in 40 μl of Arrest-In transfection reagent (Open Biosystems, Inc.). After 5 h, the transfection mixture was removed and replaced with DMEM containing 10% FBS and 50 μM IPTG (isopropyl-β-D-thiogalactopyranoside). Lysates were prepared at 2 days post infection (p.i.) and used to infect BSC40 monolayer cells in the

presence of 25 μg of mycophenolic acid/ml, 250 μg of xanthine/ml, 15 μg of hypoxanthine/ml, and 50 μM IPTG to select for plaques formed by viG3L, which expresses xanthine- guanine phosphoribosyltransferase (Gpt) and G3L. Pure recombinant viG3L viruses were obtained after three rounds of plaque purification. The insertion of Gpt and the inducible G3L gene into the endogenous G3L locus was confirmed by PCR.

2.2.4. Construction of the recombinant WR Δ53.5 virus

A deletion mutant virus deleting the endogenous WR53.5L ORF was constructed as below. The pCRII-Topo-54/53-52 containing the 5' and 3' flanking DNA fragments was created as described above, and a *gpt* cassette was inserted. The resulting plasmid, pCRII-Topo-54/gpt/53, was transfected into the cells infected with wild-type WR vaccinia virus (WR-2) and the Gpt-positive clones were purified clonally as described above.

2.2.5. Virus growth curves

In brief, BSC40 cells were infected with vi53.5L, viG3 or parental virus vT7LacOI at a MOI of 5 PFU per cell for 1 h at 37°C, cells were washed, incubated in complete DMEM containing 10% FBS with or without 100 μM IPTG, then harvested at various time after infection for virus titer determination on BSC40 cells in the presence of 100 μM IPTG. The experiments were repeated three times and the averages are presented.

2.2.6. Membrane protein extraction from MV

Vaccinia MVs were extracted with 1%NP40 with or without 50mM DTT and separated into membrane and core fractions, essentially as described previously (27). Proteins in the pellet and supernatant were separated on SDS-PAGE using 12.5 or 15% polyacrylamide gels and transferred to nitrocellulose membrane and subjected to immunoblot analyses.

2.2.7. Protease treatment of MV

Purified MV virions were treated with trypsin as described previously (77). In brief, $1x10^8$ particles/sample were incubated with buffer containing 1 mM $CaCl_2$ (pH 7.6) / 50 mM Tris-HCL (pH 7.8) in the presence or absence of 25, 50 and 100μg/ml trypsin (Promega). After 1 hr at 37°C, virus was centrifuged and the samples were subject to immunoblot analyses.

2.2.8. Extracellular Ca^{2+} depletion assay

Extracellular Ca^{2+} depletion assays were performed as previously described (10, 118). BSC40 cells were either mock-infected or infected with vi53.5 virus at a MOI of 5 PFU per cell, cultured in medium with or without 100 μM IPTG for 2 days, and washed three times with PBS. Cell morphology was recorded under microscopy from 7 random areas immediately before and after treatment with 1 mM EGTA at 37°C for 20 min.

2.2.9. Cell surface and total protein staining assays for WR53.5

(i) Confocal immunofluorescence microscopy, BSC40 cells were seeded on coverslips in 12 well plates and infected with WR-2 or vi53.5L virus at a MOI of 5 PFU per cell at 37°C for 60 min. Cells were cultured in medium with or without 100μM IPTG for 1 and 2 days, washed three times with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. These infected cells were stained with anti-53.5 Ab (1:1,000) without permeabilization (non-permeabilized, NP), followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit Abs (Sigma) (1:1,000) in PBS/0.2% BSA to visualize cell surface staining of WR53.5. These surface-stained cells were subsequently permeabilized (P) in PBS/0.2% saponin and stained with anti-53.5 Ab (1:1,000) again, followed by tetramethyl-rhodamine goat anti-rabbit IgG (1:1,000) (Molecular probes) to visualize total WR53.5 protein in cells. Intracellular actin cytoskeleton was stained with Alexa Flour 647-phalloidin (1:150) (Molecular probes). Intracellular DNA was visualized by staining with 0.5μg /ml

of diamidino-2-phenylindole dihydrochloride (DAPI; Molecular Probes) Cell images were collected with a LSM510 META confocal laser scanning microscope (Carl Zeiss, Germany) using a 63X objective lens and confocal microscopy software Release 2.8 (Carl Zeiss). (ii) For flow cytometry, BSC40 cells ($4.5x10^5$) in a 35 mm dish were either mock-infected or infected with WR-2 or vi53.5 virus at MOI of 5 PFU/cell at 37°C cells for 60 min, washed twice with PBS and incubated in appropriate medium (WR-2 in normal medium, vi53.5 in medium with (vi53.5L+IPTG) or without (vi53.5L-IPTG) 100μM IPTG for another 24 h. Cells were subsequently detached by addition of 5 mM EDTA, stained with rabbit anti-53.5 Ab (1:1, 000) at 4°C for 1 hr and followed by FITC-conjugated goat anti–rabbit Ab for fluorescence-activated cell sorter analyses. Besides mock-infected cells that were included as background calibration in FACS, two additional controls were included in the experiments: (i). Anti-53.5 Ab staining of cells infected with vi53.5L and grown in the absence of IPTG (vi53.5L-IPTG) and (ii). Secondary FITC-conjugated goat anti–rabbit Ab staining of cells infected with vi53.5L and grown in the presence of IPTG ((vi53.5L+IPTG) –anti-53.5 Ab)).

2.2.10. Measurement of WRΔ53.5 virus virulence in mice

Groups of 5 male BALB/c mice between 7 to 8 weeks old were anesthetized and inoculated intranasally with either PBS (mock-infected control), $1x10^5$, $1x10^6$ or $1x10^7$ PFU /mouse of sucrose-purified MV of wild-type vaccinia virus WR (WR-2) or WRΔ053.5 in 10μl volume. A portion of the virus inoculums we titrated on BSC40 cells again to ensure that the inoculated titers were accurate. Mice were weighed daily and recorded as described previously (96). All the mice were housed and treated in accordance with Academia Sinica animal care guidelines.

2.2.11. Immunoblot analysis

Viral proteins from purified MV virions or extracts from virus infected cells were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto

nitrocellulose membranes, which were then blocked by incubation in I-block (2 μg/ml; Tropix) in phosphate-buffered saline (PBS) containing 0.5% Tween 20, and then incubated with primary antibodies to viral proteins, followed by alkaline phosphatase-conjugated secondary antibody. Bound antibody was then detected by using a chemiluminescence method according to the manufacturer's protocol (Tropix).

2.2.12. Electron microscopy of virion morphogenesis and purified MV particles

The experiments to monitor virion morphogenesis in cells were done as previously described (27). In brief, BSC40 cells were infected at an MOI of 5 PFU per cell, cultured in medium with or without IPTG, fixed at 12 or 24 h p.i., treated with 1% OsO4, dehydrated, and embedded as described previously (140). After embedding, the cells were stained with uranyl acetate and lead citrate and analyzed under a Zeiss 902 transmission electron microscope (123). For negative staining of MV, 1μl portions of serial dilutions of purified vaccinia MV virions were spotted onto 300 mesh Parlodion-coated grids and stained with 2% phospho-tungstic acid for 15s, and then the virion particles were photographed with a Zeiss 902 transmission electron microscope as described previously (96).

2.2.13. Confocal immunofluorescence microscopy for viG3 study

Virion entry assays, the amounts of cell surface-attached virions and uncoated cores within the cells were measured by confocal microscopy as described previously (148). In brief, HeLa cells (10^5 per well) were seeded on coverslips in 12-well plates. The cells were then infected for 1 h at 4°C with G3L$^+$□ virus at an MOI of 40 PFU per cell. Alternatively, the cells were infected with equal amounts of G3L$^-$□ virus, as measured by determining the optical density at 260 nm. These cells were then subsequently washed three times with PBS and either fixed immediately or first incubated for 2 h at 37°C in the presence of cycloheximide (30 μg/ml) and then fixed. Cells were fixed by incubation with 4% paraformaldehyde for 5 min at 4°C and then for 15 min at room

temperature. The cells were then permeabilized in PBS–0.2% saponin and stained with rabbit anti-A4L antibody or mouse anti-L1R MAb, followed by fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (IgG) or Cy5-conjugated goat anti-mouse IgG antibody, respectively. DNA was visualized by staining with 0.5 μg of DAPI (4',6-diamidino-2-phenylindole dihydrochloride; Molecular Probes) in mounting solution. Cell images were collected with the LSM510 META confocal laser-scanning microscope (Carl Zeiss, Germany) using a □63 X objective lens and confocal microscopy software (release 2.8; Carl Zeiss). The number of fluorescent-staining particles was counted from multiple photos, and the averaged numbers of surface-bound virions and uncoated cores per cell were determined.

To visualize actin tails on CEV, HeLa cells (7×10^4) were seeded on coverslips in 12-well plates and infected with viG3L at an MOI of 5 PFU per cell. The infected cells were cultured in medium with or without 50 μM IPTG for 17 h p.i., fixed for 20 min at room temperature with 4% paraformaldehyde in PBS–0.5 μM Taxol (Molecular Probes), washed three times, permeabilized with 0.2% saponin-PBS, and incubated for 1 h at room temperature with anti-B5R MAb (1: 2,500) and Alexa Fluor 647-phalloidin (Molecular Probes) and then for 30 min with Alexa Fluor 488-conjugated goat anti-mouse IgG antibody (1:1,000; Molecular Probes). After three washes with PBS, the cells were stained for 5 min with 0.5 μg of DAPI (Molecular Probes)/ml and washed, images were collected on a LSM510 META confocal laser scanning microscope as described above.

2.2.14. Cell fusion assay (fusion-from-within) induced by low-pH treatment

Freshly confluent BSC-1 cells were infected with viG3L virus at an MOI of 5 PFU per cell and incubated at 37°C for 21 h in medium with or without IPTG. Cells were washed three times with PBS (pH 7.2), treated with PBS (pH 7.2) or PBS (pH 4.7) for 3 min at room temperature, washed again, and replaced with normal medium. These cells were incubated for another 3 h and photographed with a Nikon inverted microscope.

Statistical analysis

Statistical analyses were performed using Student's t-test in Prism software (GraphPad). The P-value is shown **, P < 0.001., ***, P < 0.0001.

Immunofluorescence data

All fluorescence images were collected with an LSM510 Meta Confocal Laser Scanning Microscope (Carl Zeiss, Germany) using a 63x objective lens.

CHAPTER 3

RESULTS

3.1. INTEGRIN β1 MEDIATES VACCINIA VIRUS ENTRY THROUGH ACTIVATION OF PI3K/AKT SIGNALING

3.1.1. Integrin β1 associates with vaccinia MV on HeLa cells

In order to identify cellular proteins within lipid raft microdomains, we previously performed stable isotope labeling for quantitative proteomic analyses and identified 570 cellular proteins. Those proteins with altered levels after vaccinia virus infection constitute about 3% of the total candidates and are described elsewhere (131). To complement the above study, here we analyzed the remaining 97% of "constitutive" raft-associated proteins whose quantification fell within the range of the ratio of 1.0±0.5, i.e., a ratio that was considered not significantly changed. These proteins were analyzed through biological network analyses in order to identify specific signaling complexes resident in the rafts. As described in the Materials and Methods, biological network analyses (Figure 6) revealed the presence of integrin β1 and its associated proteins such as CD9, CD47, CD59, CD98, talin, ezrin and Fyn/yes/lyn, suggesting a possibility that integrin β1-mediated biological signaling may participate in vaccinia MV entry.

We thus investigated whether integrin β1 associates with vaccinia MV on the surface of infected HeLa cells. HeLa cells were infected with the vaccinia WR strain MV at an MOI of 50 PFU per cell for 60 min at 4°C and then washed and moved to 12°C. Anti-integrin β1 (ITGβ1) and anti-vaccinia MV (VV) antibodies were subsequently added to cells for copatching (Figure 7), as previously described (73, 139). An anti-transferrin receptor (TfR) antibody was also included as a negative control. The results show that vaccinia MV on HeLa cells copatched with cell surface

integrin β1 but not with the transferrin receptor on HeLa cells (Figure 7), suggesting a role of integrin β 1 in vaccinia MV entry.

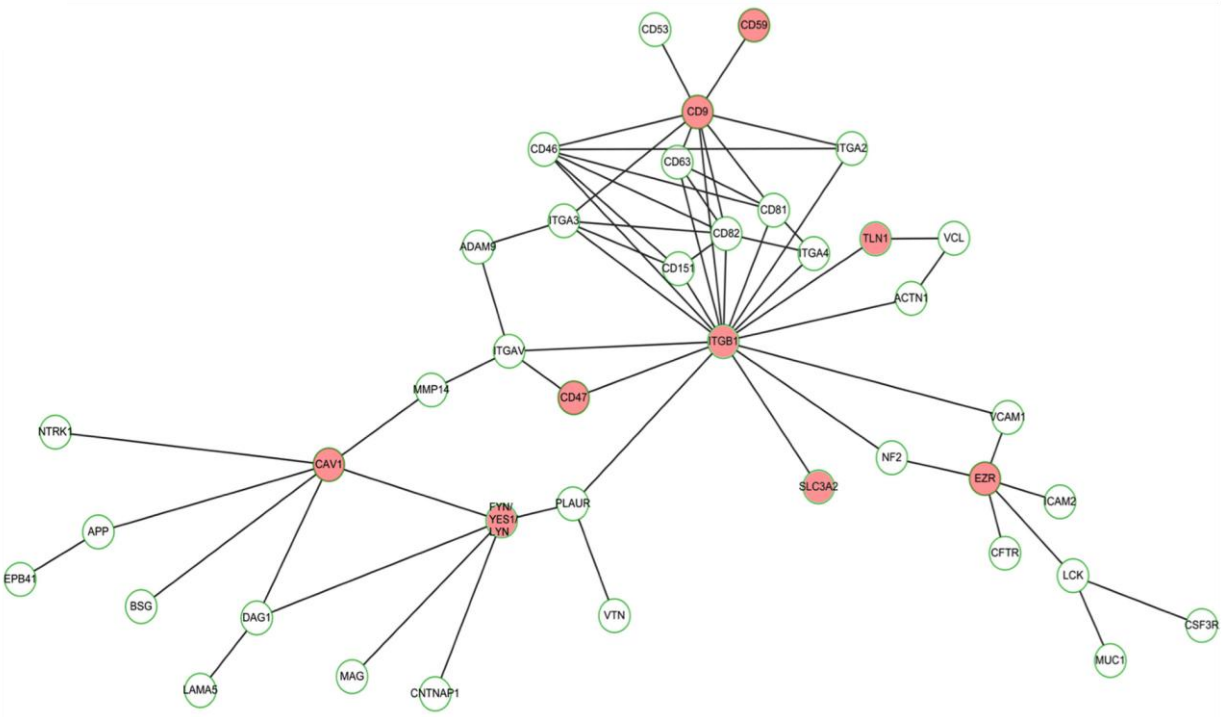


Figure 6. Hierarchical ITGβ1 network. Constructed by ARIADNE Pathway Studio 7.0 software (see Material and Methods), which shows integrin β1 and its direct and indirect interacting membrane proteins. The nodes are labeled with gene symbols and the red nodes represent the cellular proteins identified in the lipid raft fractions upon vaccinia MV infections (131). ITGβ1 (integrin β1), TLN1 (talin-1), EZR (ezrin), CAV1 (caveolin-1), SLC3A2 (CD98).

3.1.2. Vaccinia virus entry is reduced upon ITGβ1 siRNA knockdown in HeLa cells.

To test whether integrin β1 mediates vaccinia MV entry into HeLa cells, control siRNA (si-cont) or siRNA targeting integrin β1 (si-ITGβ1) or cyclophilin B (si-CypB) were transfected into HeLa cells followed by harvesting for immunoblots (Figure 8A) and FACS analyses (Figure 8B). The si-ITGβ1 and si-CypB constructs specifically knocked down (KD) the total amounts of integrin β1 and cyclophilin B proteins, respectively (Figure 8A). In addition, integrin β1 expression

on the cell surface was effectively reduced in si-ITGβ1 KD HeLa cells but not si-control nor si-CypB KD cells (Figure 8B).

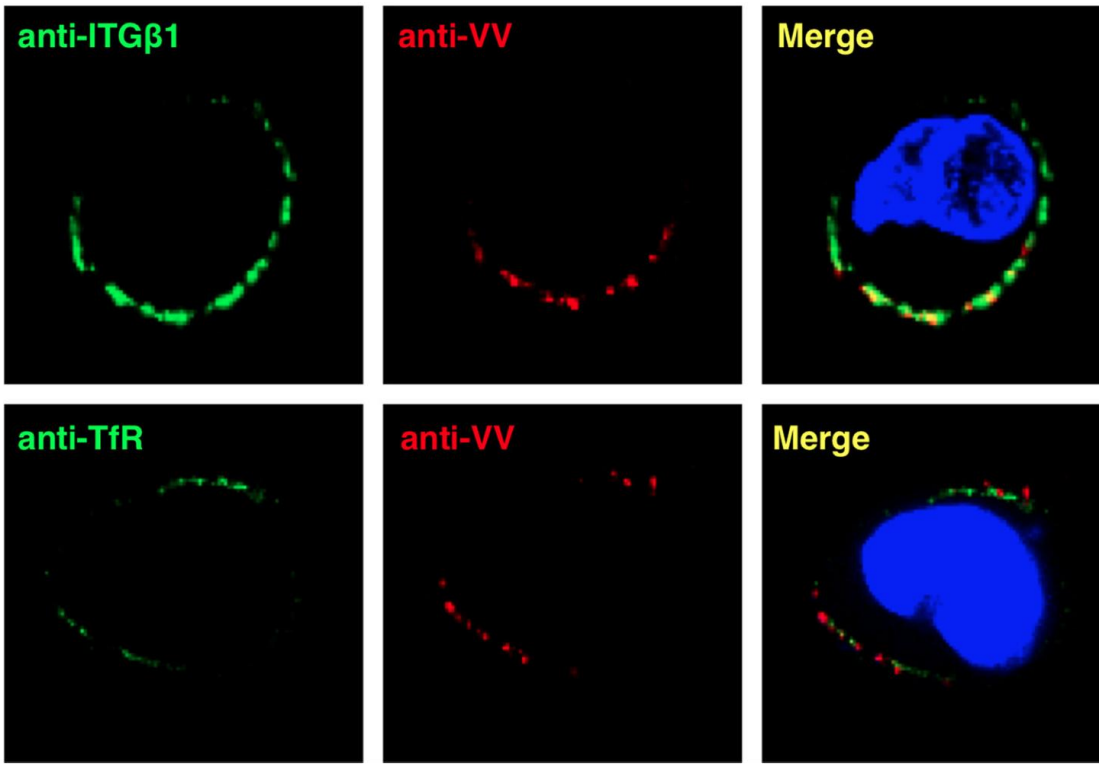


Figure 7. Co-patching experiment. Vaccinia MV co localizes with ITGβ1 at the surface of HeLa cells at 1 hr binding stage. Rabbit anti-VV antibody (red) and anti-integrin β1 mAb (12G10) (green). As a negative control, alternatively, anti-VV antibody was incubated with anti-transferrin receptor (TfR) mAb (green). DNA was visualized by DAPI staining (blue). Image was processed with confocal microscopy.

The si-ITGβ1 also affected integrin β1-mediated cell adhesion resulting in alterations in cell morphology and disorganization of focal adhesions, as determined by actin and paxillin staining in si-ITGβ1 KD HeLa cells (Figure 8C), confirming the specificity of si-ITGβ1. We then infected these HeLa KD cells with vaccinia MV at an MOI of 5 PFU per cell and harvested at 2 or 4 h p.i. for viral early luciferase activity assays. The results showed that vaccinia virus infection of si-

ITGb1 KD HeLa cells was reduced to 40% (at 2h p.i.) and 38% (at 4h p.i.) of the infection rates seen in the si-control and si-CypB KD HeLa cells (Figure 8D), suggesting that the reduction was not due to a delayed kinetics**.**

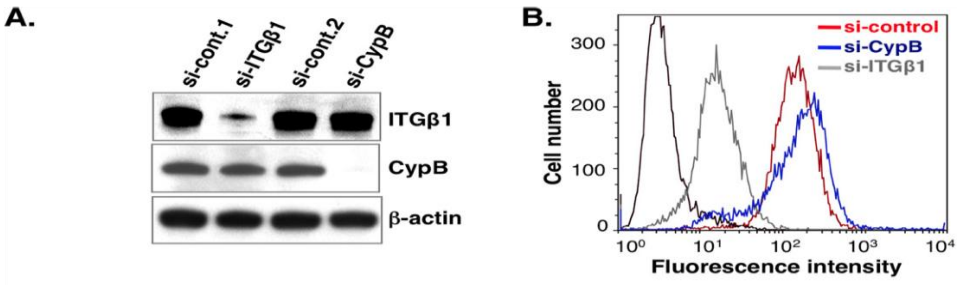


Figure 8. (**A) Total protein expression.** Immunoblots of lysates prepared from mock-KD (si-Cont.1 and 2), si-ITGβ1 KD and si-CypB KD HeLa cells using anti-ITGβ1 (Mab13), anti-CypB or anti-β-actin antibodies. **(B) Cell surface expression of ITGβ1 by flow cytometry**. The KD HeLa cells described in (A) were stained with anti-ITGβ1 mAb (9EG7) followed by FITC-conjugated goat anti-rat secondary antibody and analyzed by FACS. si-Control is shown in red, si-CypB in blue and si-ITGβ1 in gray. The background staining (shown in black) represents the si-control cells that were stained with the secondary antibody only.

Finally, we performed vaccinia MV binding assays and virus core uncoating assays by Immunofluorescence analyses as previously described (148). The KD HeLa cells as described in (Figure 8A) were infected with purified vaccinia MV particles at an MOI of 20 PFU per cell for 60 min at 4°C, fixed and stained with anti-ITGβ1 Mab13 (green) and anti-VV-L1 mAb 2D5 (red). The cell-bound virions were quantified as described previously (148). The results showed that MV attachment was reduced to 52% in si-ITGβ1 KD HeLa cells (Figure 8E). For core uncoating the KD HeLa cells described in (A) were infected as above and cultured in the presence of cycloheximide (10□mg/ml) for an additional 2 h at 37°C, fixed and stained with anti-ITGβ1 Mab13 mAb as above. These cells were subsequently permeabilized, stained with anti-A4 antibody and internalized viral

cores were quantified as described previously (138). Experiment showed that MV penetration was reduced to 32% (Figure 8F), indicating that integrin β1 is important for vaccinia virus entry at both the attachment and penetration steps.

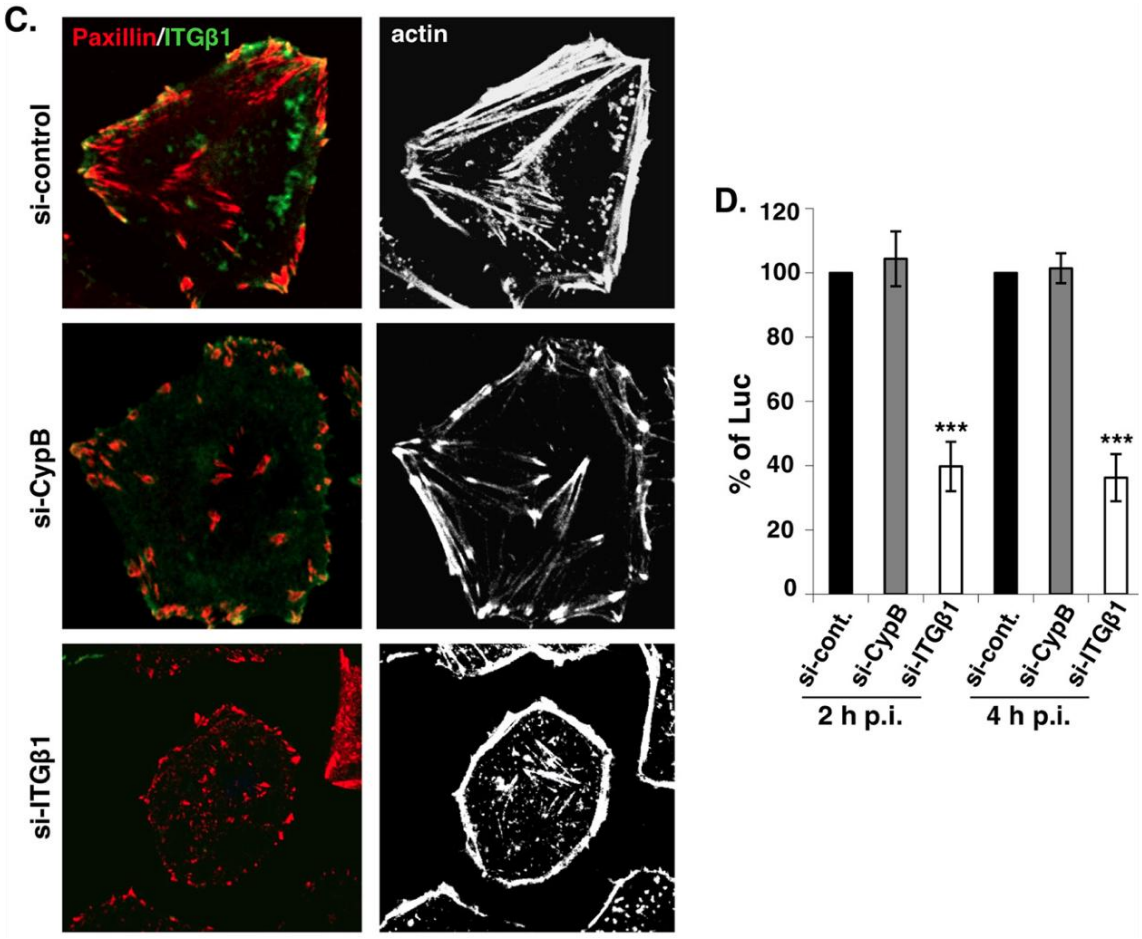


Figure 8. (C) Immunofluorescence analysis of ITGβ1, paxillin and actin proteins in KD HeLa cells. The KD HeLa cells described in (A) were fixed and stained with anti-ITGβ1 mAb (Mab13) followed by FITC-conjugated goat anti-rat secondary antibody (green). These cells were subsequently permeabilized and stained with Alexa Fluor 647-phalloidin (white) and anti-paxillin antibodies followed by tetramethylrhodamine-conjugated goat anti-mouse IgG (red). **(D) Viral early luciferase assays.** The KD HeLa cells described in (A) were infected with VV-WR at an MOI of 5 PFU per cell, harvested at 2 and 4 h p.i. for luciferase assays. The luciferase activity in

the si-control cells was defined as 100%. The bars represent the standard deviations from three independent experiments.

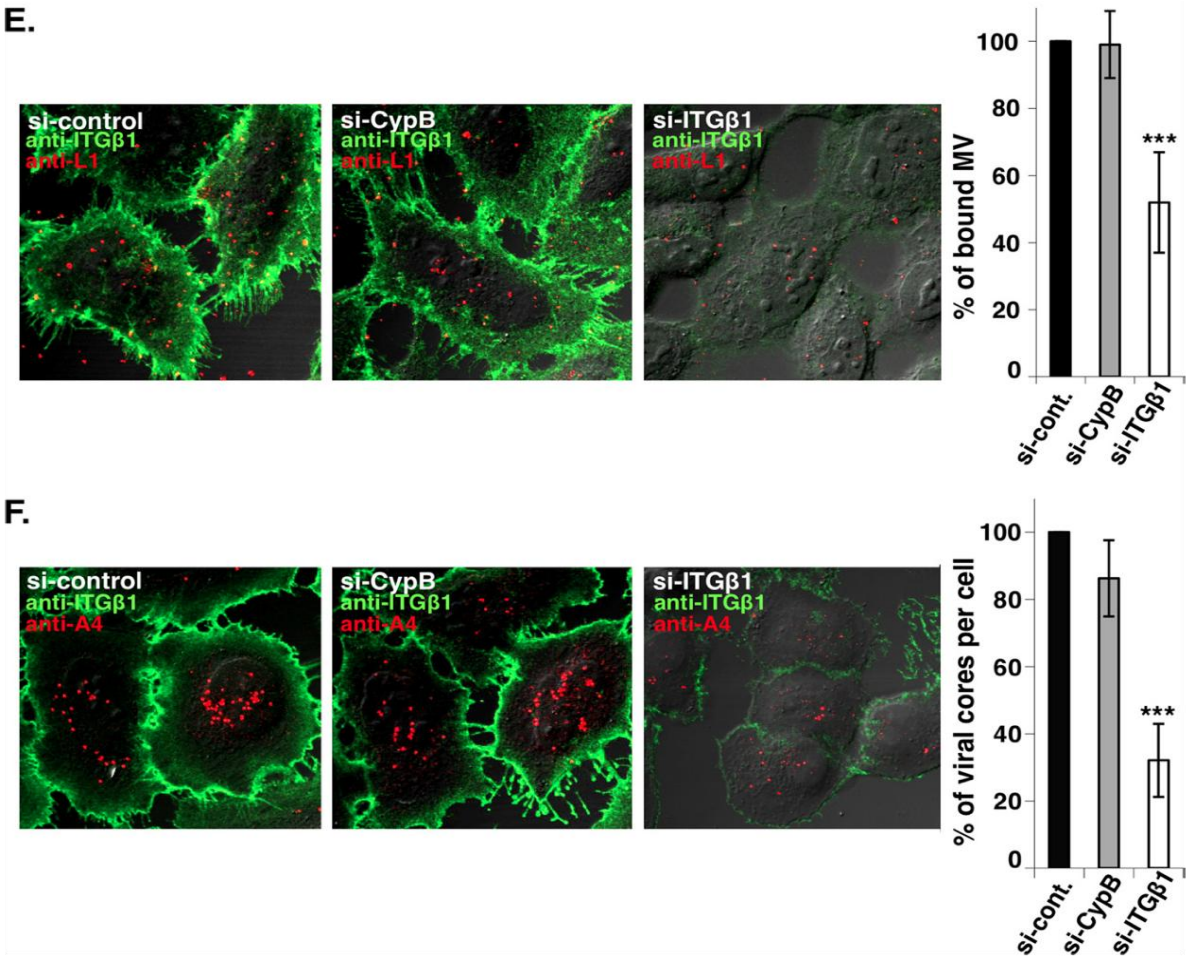


Figure 8. Integrin β1 is important for vaccinia virus entry at both the (E), attachment and (F) penetration steps. Anti-ITGβ1 (green) and anti-VV-L1 mAb 2D5 (red). The bars represent the standard deviations from five independent experiments. Statistical analyses in D, E and F were performed using Student's t-test in Prism software (GraphPad). The P-value is shown ***, $P < 0.0001$.

3.1.3. Vaccinia virus entry is reduced in mouse cells lacking integrin β1 expression

In addition to HeLa cells, we obtained a mouse cell line GD25β1A, derived from an integrin β1 KO cell line GD25 (46), that expresses only human integrin β1 (115). Indeed, integrin β1 was detected on the surface of GD25β1A, but not on GD25 cells in FACS analyses (Figure 9A).

GD25β1A and GD25 cells were infected with vaccinia MV at a MOI of 60 PFU per cell at 4°C for 60 min, washed and fixed for immunofluorescence analyses.

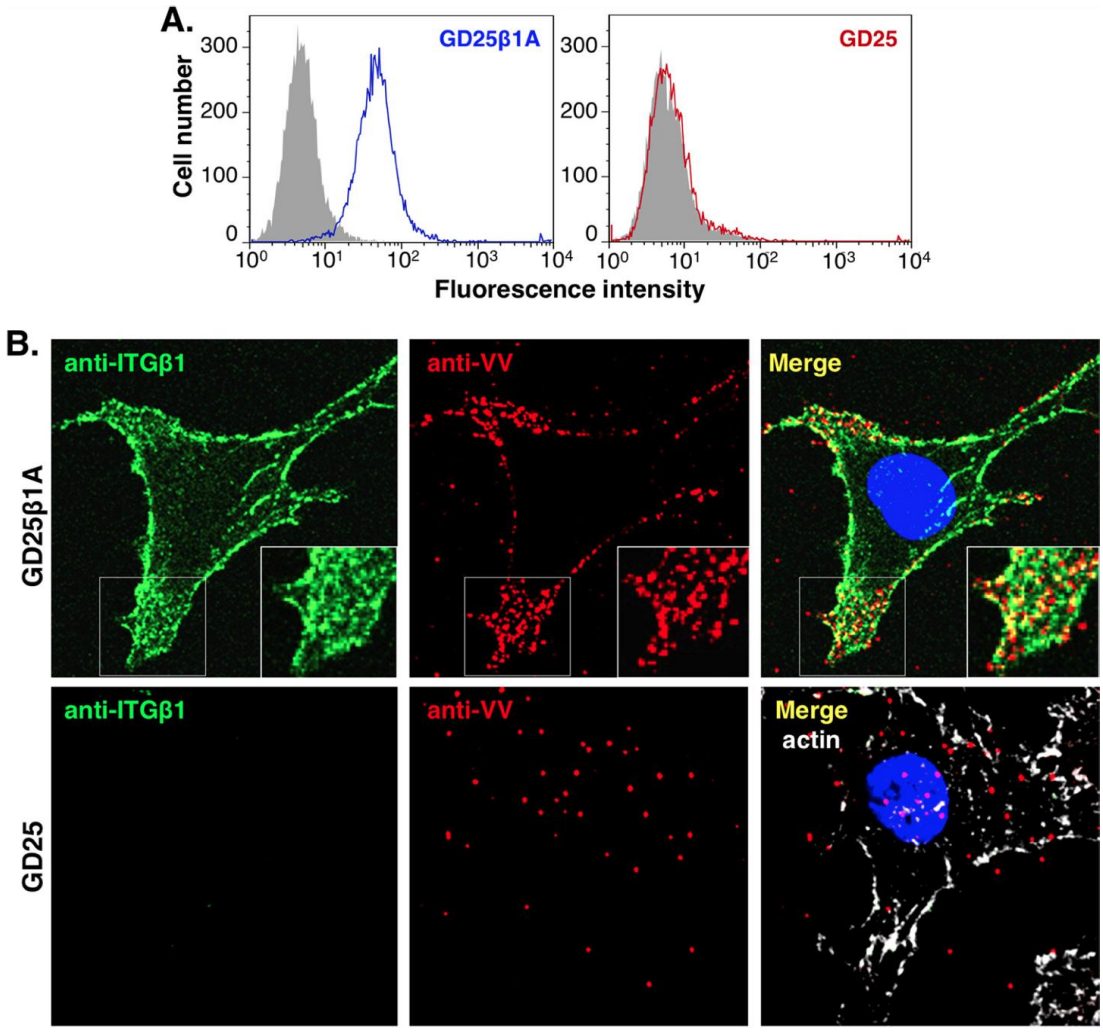


Figure 9. **Vaccinia virus entry is reduced in mouse cells lacking integrin β1 expression.** **(A) Flow cytometry analyses of cell surface expression of ITGβ1.** GD25β1A (blue) and GD25 (red) cells were stained with anti-ITGβ1 mAb (9EG7) followed by FITC-conjugated goat anti-rat secondary antibody and analyzed by FACS. The background staining (in gray) represents the cell staining with the secondary antibody only. **(B) Confocal immunofluorescence of vaccinia MV particles colocalized with integrin β1 in GD25β1A cells.** GD25β1A and GD25 cells were infected with purified vaccinia MV particles at an MOI of 60 PFU per cell at 4°C for 1 h, washed and stained with anti-ITGβ1 (12G10) (green) and anti-VV (red) antibodies, followed by FITC-

conjugated goat anti-mouse IgG and tetramethylrhodamine-conjugated goat anti-rabbit IgG. GD25 cells are negative to ITGβ1 staining and were subsequently permeabilized and stained with Alexa Fluor 647-phalloidin (white) to mark the cell body.

Abundant MV particles bound to GD25β1A and colocalized with surface integrin β1 concentrated at cellular protrusions, whereas less MV particles (~34%) bound to GD25 cells (Figure 9B). In addition, both GD25β1A and GD25 cells were infected with vaccinia WR-VV at different MOI from 10 to 40 PFU per cell and harvested to measure the early luciferase activity at 2 h p.i. or the late β-Galactosidase (β-Gal) activity at 8 h p.i. (Figure 9C). Both enzymatic activity assays together with core uncoating assay revealed that vaccinia MV entry into GD25 cells was less efficient as compared to GD25β1A cells. We also performed plaque assays on GD25β1A and GD25 cells and stained the plaques with X-gal, Consistently, vaccinia MV produced fewer plaques (~30%) on GD25 than in GD25β1A cells (Figure 9D). Interestingly, plaques formed in GD25 cells appeared also smaller in size than GD25β1A cells implying a role of integrin β1 in vaccinia virus spreading among cells although we did not pursue it further. Taken together, the results show that cell surface integrin β1 mediates vaccinia MV infections.

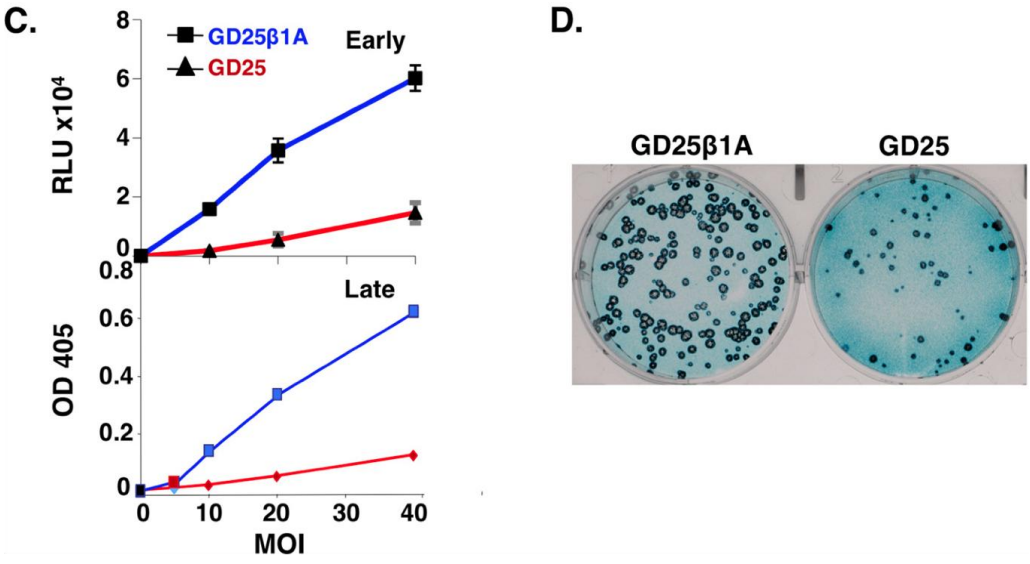


Figure 9. (C) GD25β1A and GD25 cells were infected with WR-VV at an MOI of 10, 20 and 40 PFU per cell and harvested at 2 h p.i. for luciferase assays (Early) and 8 h p.i. for β-Gal activity assays (Late). **(D)** Vaccinia MV formed more plaques in GD25β1A when compared with GD25 cells. Both cells were infected with ~300 PFU of WR-VV, fixed and stained for X-Gal at 2 days p.i. to visualize the blue plaques.

3.1.4. Vaccinia mature virus particle binds to integrin β1 to induce PI3K/Akt activation that leads to virus endocytosis into HeLa cells.

Integrin β1 was shown to mediate multiple inside-out and outside-in signaling pathways that play crucial roles in cell-to-cell and cell-to-matrix communications (62, 91, 107). Clustering of integrin at the cell membrane leads to kinase activation, which is followed by alterations to the cytoskeleton and receptor endocytosis. Since it has been shown that PI3K and Akt are critical regulators downstream of integrin β1 on GD25β1A cells (6, 34, 115), we wanted to test whether vaccinia MV binding to integrin β1 activates PI3K/Akt signaling in GD25β1A cells and whether such kinase activation is critical for vaccinia MV entry. GD25β1A cells were serum-starved and subsequently infected with vaccinia MV at an MOI of 60 PFU per cell at 37°C for 5, 10 and 20 min. and then harvested for immunoblot analysis with anti-phospho-Akt (Ser473) antibody. As shown in (Figure 10A), vaccinia MV stimulated robust phosphorylation of Akt in GD25β1A cells as early as 5 minutes after virus addition as compared to medium alone. Pre-treatment of GD25β1A cells with the PI3K inhibitor LY 294002 completely abolished MV-induced Akt phosphorylation, showing that vaccinia MV infection triggered activation of Akt through PI3K. To determine whether PI3K/Akt activation was required for vaccinia virus entry we pre-treated both GD25β1A and HeLa cells with inhibitors that blocked PI3K (LY 294002) and Akt (Akt IV) activities, subsequently infected with vaccinia MV and measured viral early promoter luciferase activity at 2 h p.i. As shown in (Figure 10B), PI3K as well as Akt inhibitors readily reduced vaccinia MV infections in

both GD25β1A and HeLa cells in a dose-dependant manner, showing that PI3K/Akt signaling is crucial for vaccinia MV entry into both cell lines. It is worth noting that GD25 cells were not suited for kinase signaling studies because it has been previously shown that this cell line contains altered kinase regulation that compensates for the loss of integrin β1 and that multiple tyrosine kinases are activated without integrin β1(5).

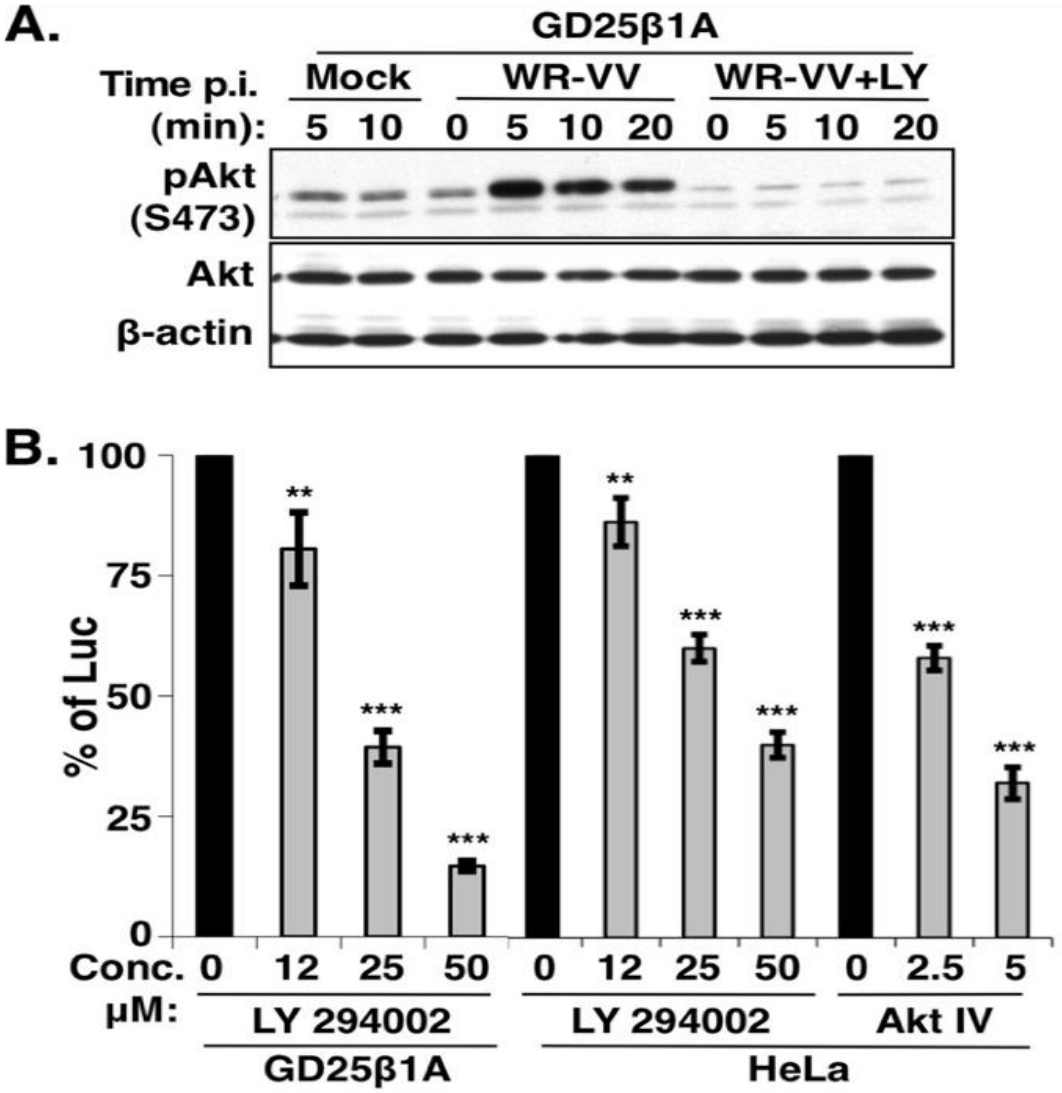


Figure 10. PI3K/Akt activation induced by WR-VV is required for virus entry. (A) WR-VV induced phosphorylation of Akt in GD25β1A cells. GD25β1A cells were serum-starved, pretreated with DMSO or PI3K inhibitor (LY 294002) and were either mock-infected or

infected with medium containing purified MV particles (WR-VV) at an MOI of 60 PFU per cell at 37°C. Cells were harvested at indicated times after addition of virus for immunoblot analyses. **(B) Viral early luciferase activity in GD25β1A and HeLa cells was blocked by PI3K/Akt inhibitors.** Cells were pretreated with DMSO, PI3K inhibitor (LY 294002) at concentrations of 12, 25 or 50 μM or Akt inhibitor (Akt IV) at concentrations of 2.5 or 5 μM and subsequently infected with WR-VV at an MOI of 10 (for GD25β1A) and 5 (for HeLa cells) PFU per cell and harvested at 2 h p.i. for luciferase assays as described above.

Thus, in order to demonstrate that MV-induced PI3K activation requires integrin β1 we infected si-control and si-ITGβ1 KD HeLa cells with vaccinia MV and harvested at 0, 60 and 90 min for phospho-Akt immunoblot analysis. When these cells were infected with vaccinia MV, phosphorylation of Akt was also induced in si-control HeLa cells, although with slower kinetics than GD25β1A cells (Figure 10C). Most importantly, phosphorylation of Akt was significantly reduced in si-ITGβ1 KD HeLa cells. Finally, GD25β1A cells were pretreated with 25 and 50 mM LY 294002 and infected with vaccinia MV for plaque formation assays which showed a dosage-dependent reduction of plaque number with LY294002 and not DMSO control (Figure 10D), consistent with the luciferase assays in Figure 11B. Taken together, these results demonstrate that vaccinia MV induces PI3K/Akt activation that is mediated through integrin β1 and that such activation is required for vaccinia virus entry and plaque formation.

3.1.5. PI3K/Akt activation is required for vaccinia MV endocytosis

Vaccinia virus MV enters cell through eather fluid-phase endocytosis/macropinocytosis or plasma membrane fusion. (21). To determine whether the PI3K inhibitor specifically blocks viral endocytic entry, we performed acid bypass experiments in which the cell-bound viruses were briefly treated with acidic buffer to force viral entry through plasma membrane fusion as described previously (56). HeLa cells were pre-treated with DMSO, bafilomycin A (a drug known to inhibit endosomal acidification) or the PI3K inhibitor LY 294002 and infected with vaccinia MV at 4°C

for 1 h. The unbound virions were washed and the infected cells were briefly exposed to either neutral (pH 7.4) or acidic (pH 5) buffer for 5 min at 37°C. The cultures were then maintained for another 2 h in growth medium prior to fixation for viral core uncoating assays. As shown in Figure 11, control HeLa cells treated with DMSO (Figure 11A, a), were successfully infected and abundant viral cores were detected in the cytoplasm. BFLA pre-treatment significantly reduced viral core number in cells, confirming virus entry through a low-pH dependent endocytic process (Figure 11A, b). Pre-treatment of HeLa cells with LY 294002 also reduced viral cores in cells, suggesting that PI3K/Akt is important for vaccinia virus uncoating (Figure 11A, c). Most importantly, exposure of these cells to a low pH buffer converted MV entry through plasma membrane fusion resistant to inhibition by BFLA (Figure 11A, d) and LY 294002 (Figure 11A, e). Quantification of viral uncoated cores in each samples are shown in Figure 11B. Taken together, these results demonstrate that PI3K/Akt activation induced by vaccinia MV is important for virus endocytosis into HeLa cells.

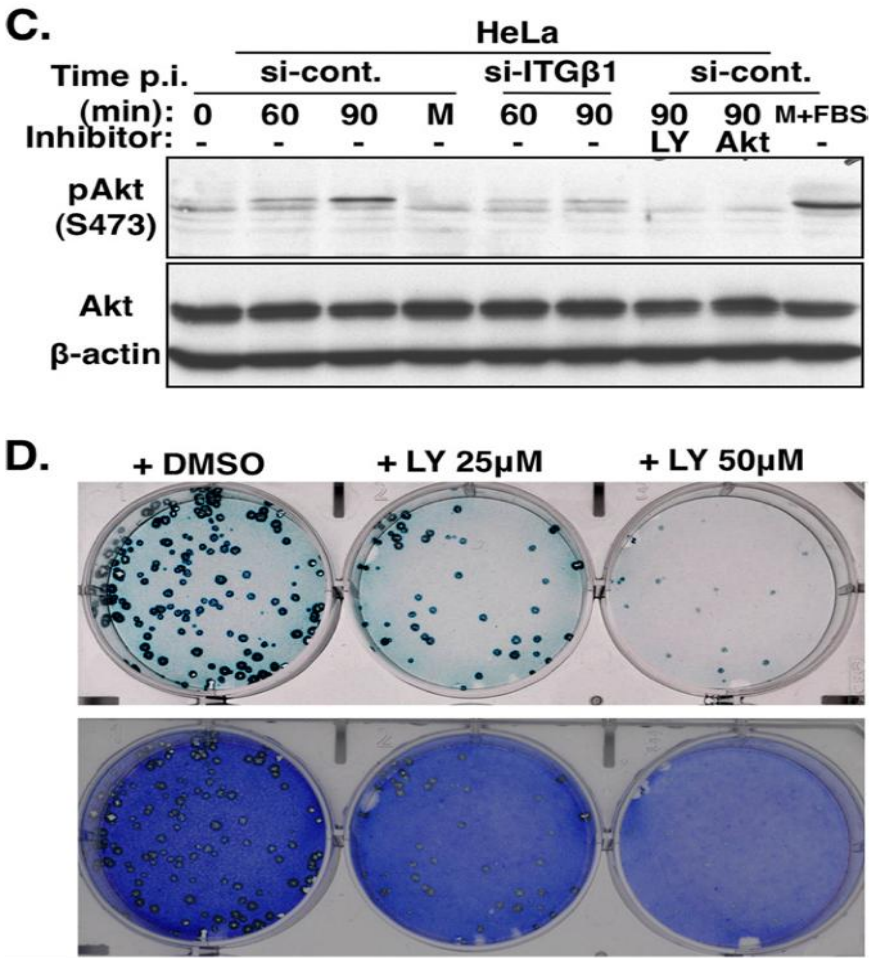


Figure 10. (C) WR-VV induced phosphorylation of Akt required integrin β1 in HeLa cells. The si-control and si-ITGβ1 KD HeLa cells, as described in figure 9A, were serum-starved, pretreated with or without inhibitors of PI3K (LY 294002) or Akt (Akt IV) and subsequently infected with purified MV particles. **(D) Vaccinia virus plaque formation was reduced with PI3K inhibitor LY-294002 in GD25β1A cells.**

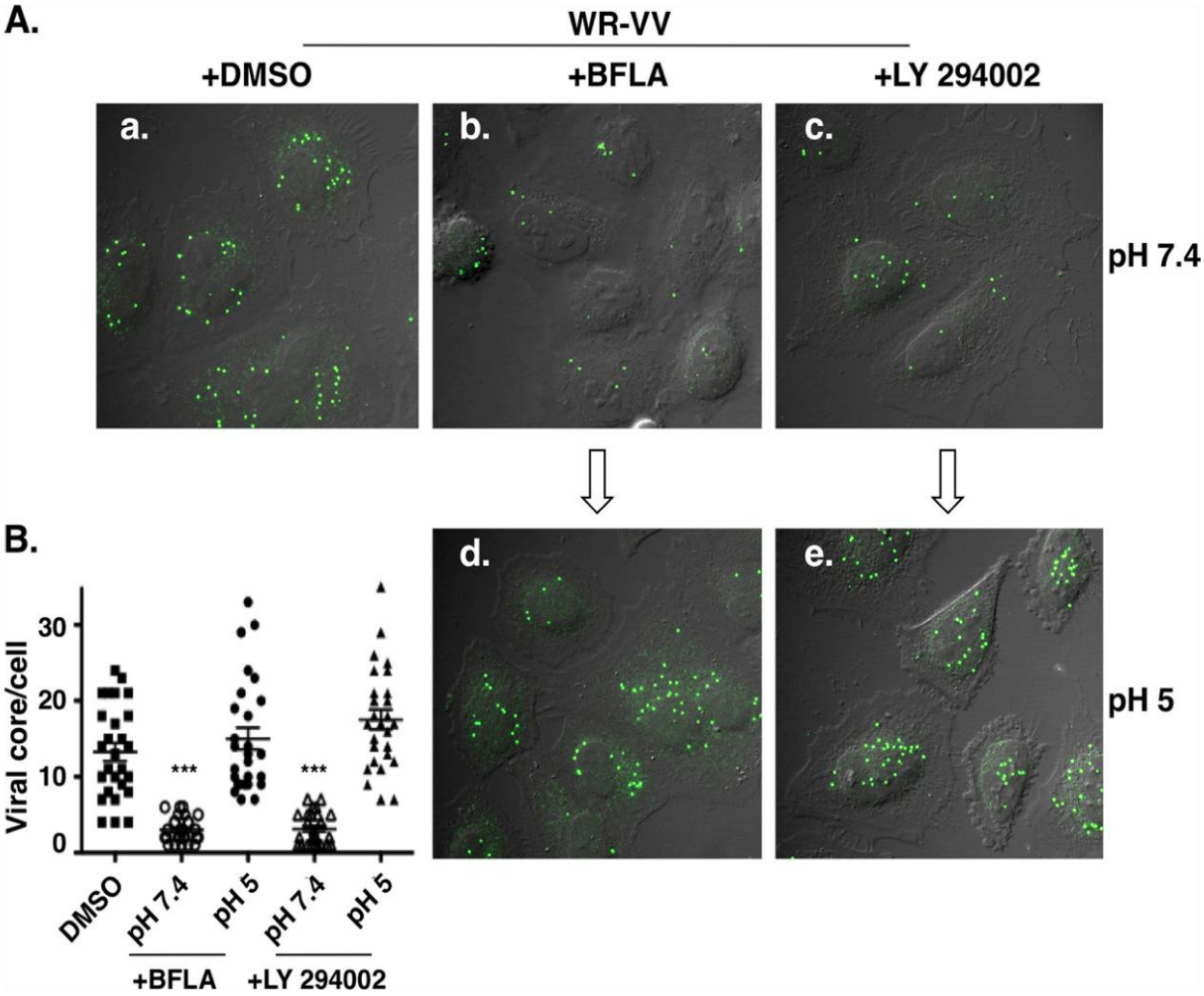


Figure 11. PI3K/Akt activation is required for vaccinia MV endocytosis. (A) Immunofluorescence analyses of virus uncoating assays in HeLa cells. HeLa cells were pretreated with DMSO (a), 25 nM BFLA (b and d) or 50 μM PI3K inhibitor (LY 294002) (c and e) and subsequently infected with purified MV particles at an MOI of 20 PFU per cell for 60 min at 4°C. Cells were then treated with neutral (pH 7.4) (a-c) or acidic (pH 5) (d and e) buffer for 5 min, washed and cultured at 37°C in medium containing 10 μM cycloheximide for an additional 2 h and analyzed by virus core uncoating assays using anti-A4 antibody as described previously **(B) Quantification of viral core numbers per cell from each group (30 cells / group) described in (A).** Statistical analyses were performed using Student's t-test in Prism software (GraphPad). The P-value is shown ***, $P < 0.0001$.

3.1.6. Outside-in activation of integrin β1 facilitates vaccinia MV entry.

The fact that the integrin network is essential for cell viability, migration and growth may suggest that vaccinia MV exploits the integrin/PI3K/Akt signaling pathway to modulate cellular environments preferable for viral entry and growth. We thus performed outside-in activation experiments to turn on the integrin-dependent signaling pathway in HeLa cells. HeLa cells were plated on dishes pre-coated with the extracellular matrix proteins fibronectin (FN) and laminin (LN) only for a short time so little extracellular matrix proteins were secreted from cells to induce integrin-mediated cell adhesion. As a negative control, HeLa cells were plated onto dishes pre-coated with polylysine (PLL), which mediates cell attachment through electrostatic interactions and is independent of integrins (129, 130). Cells were allowed to adhere and spread for 20, 30, 45 and 90 min before harvesting for immunoblot analysis. As shown in Figure 12A, integrin-mediated phosphorylation of FAK and Akt were detected as early as 20 min after cell plating on FN and LN but not on PLL, showing specific activation of integrin/PI3K/Akt through cell-matrix interactions. As expected, immunofluorescence staining with anti-paxillin antibody revealed formation of focal adhesions in cells plated on FN and LN but not on PLL (Figure 12B). When these cells were infected with vaccinia MV, early luciferase activity was higher in HeLa cells plated on FN and LN than those on PLL (Figure 12C). Altogether, these results demonstrate that outside-in activation of integrin β1-mediated-PI3K signaling is important for vaccinia MV entry into HeLa cells. Integrin adhesome formation induced by outside-in activation, has been shown to be disrupted upon blebbistatin treatment (129). When the above outside-in activation procedures were performed in HeLa cells in the presence of 25 and 50 mM of blebbistatin, formation of focal adhesions was completely dispersed (Figure 12D). These drug-treated HeLa cells were subsequently infected with vaccinia MV and a dosage-dependent reduction of viral entry was observed, when compared with the mock-treated cells (Figure 12E). These results suggested that recruitment of cellular proteins to form integrin adhesomes, which was blocked by blebbistatin treatment, is important for vaccinia MV entry.

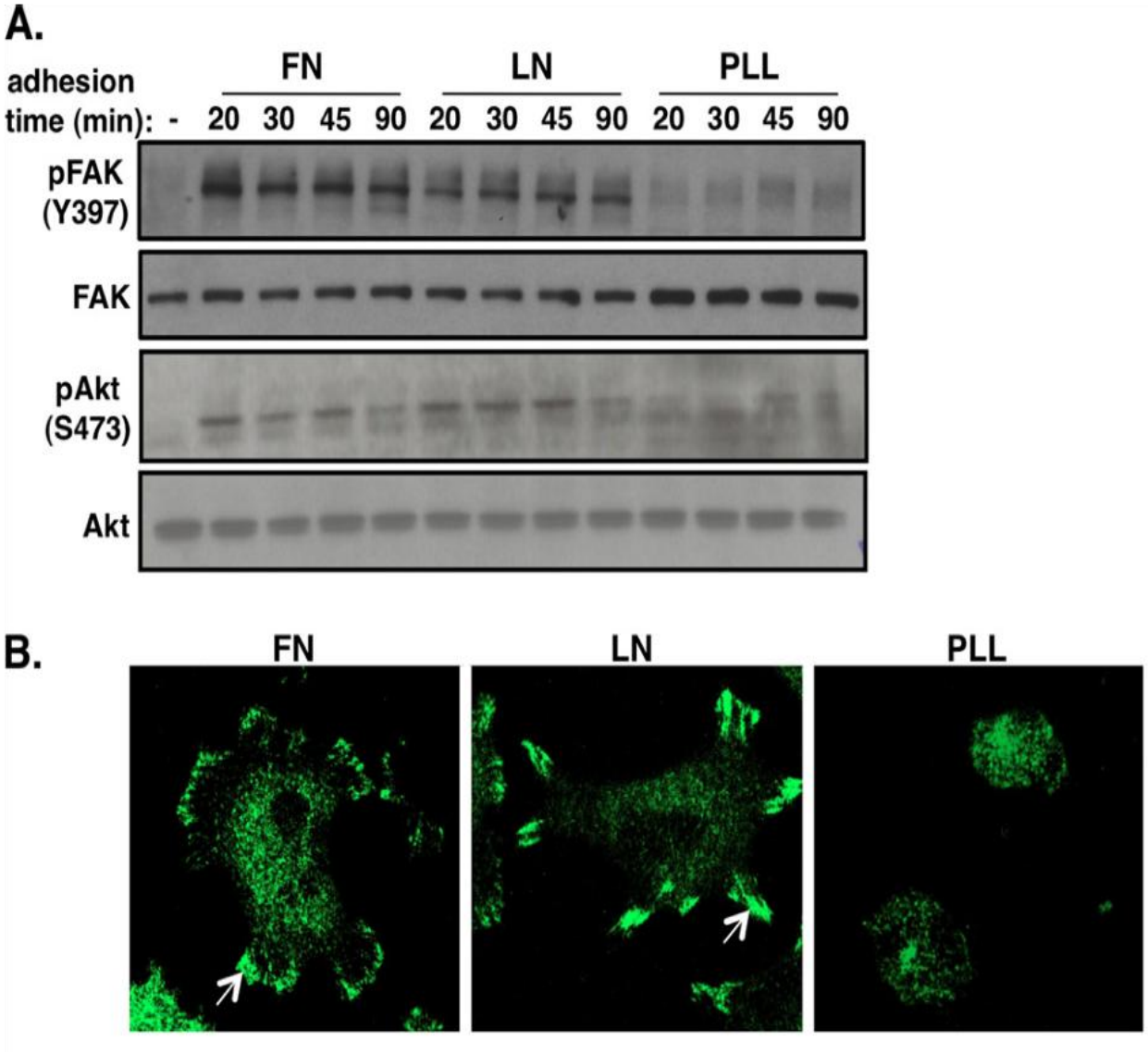


Figure 12. **Outside-in integrin activation enhances vaccinia MV entry. (A)** Cell adhesion to ECM activated FAK and Akt phosphorylation. Serum-starved HeLa cells in suspension (-) were seeded on fibronectin (FN), laminin-1 (LN) and poly-L-lysine (PLL) at 37°C and harvested at 20, 30, 45 and 90 min for immunoblot analysis with anti-phospho-FAK (pFAK, Y397), anti-phospho-Akt (pAkt, S473), anti-FAK and anti-Akt antibodies. **(B)** Immunofluorescence staining of paxillin in HeLa cells seeded on different ECM. HeLa cells were seeded onto FN, LN and PLL as described above for 90 min, fixed, permeabilized and stained with anti-paxillin antibody followed by FITC-conjugated goat anti-mouse IgG.

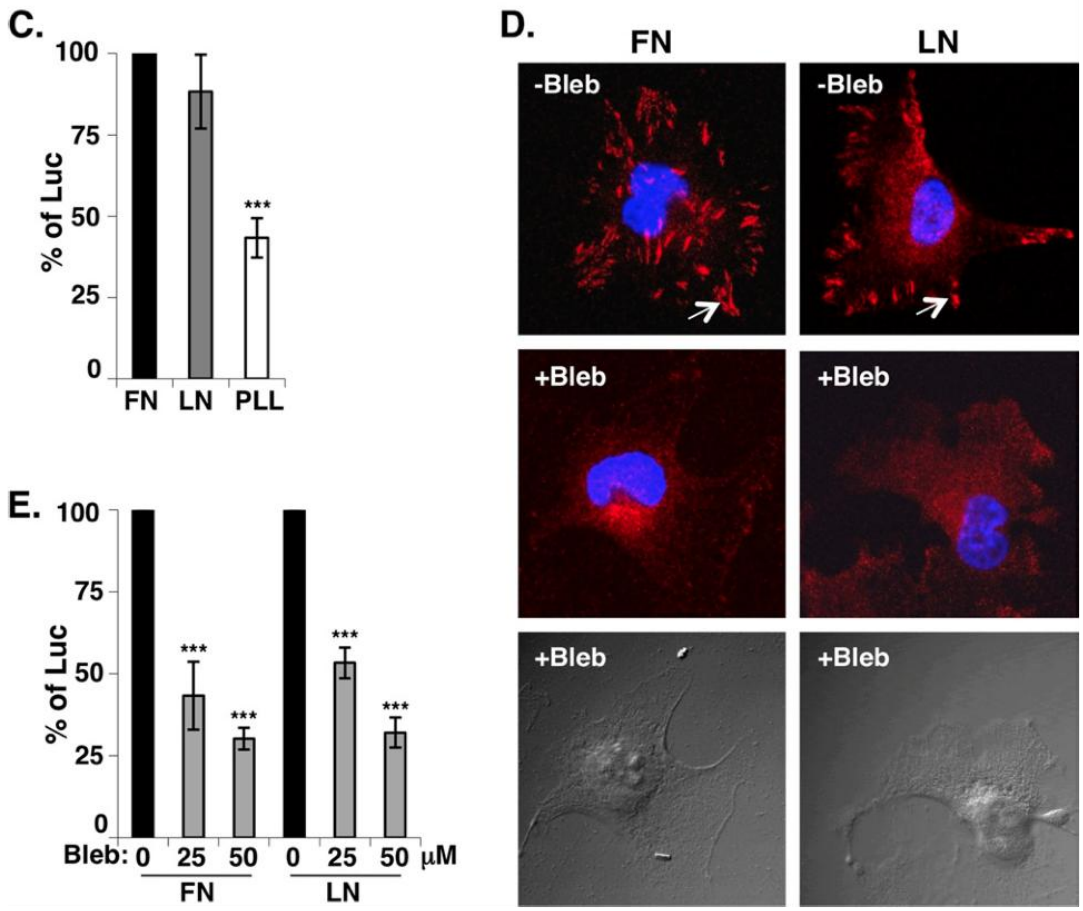


Figure 12. (C) Viral early luciferase assays. HeLa cells were seeded as described in B and infected with WR-VV at an MOI of 5 PFU per cell and harvested at 1.5 h p.i. for luciferase assays as described in Materials and Methods. The luciferase activity in HeLa cells seeded on FN was defined as 100%. **(D)** Immunofluorescence staining of paxillin in HeLa cells treated with blebbistatin. Hela cells were seeded on FN and LN as described in (A) and incubated with 50 µM blebbistatin (+Bleb) or DMSO (-Bleb) (see Materials and Methods) for 60 min. Cells were fixed, permeabilized and the focal adhesions were stained with anti-paxillin antibody (red). Cell nuclei were stained with DAPI (blue). In B and D white arrow identifies focal adhesion. **(E)** HeLa cells seeded as described above were treated with DMSO or blebbistatin (25 and 50 µM) and subsequently infected with WR-VV at an MOI of 5 PFU per cell and harvested at 1.5 h p.i. for luciferase assays. All bars represent the standard deviations from three independent experiments. Statistical analyses in B, C and E were performed using Student's t-test in Prism software (GraphPad). The P-value is shown ***, $P < 0.0001$.

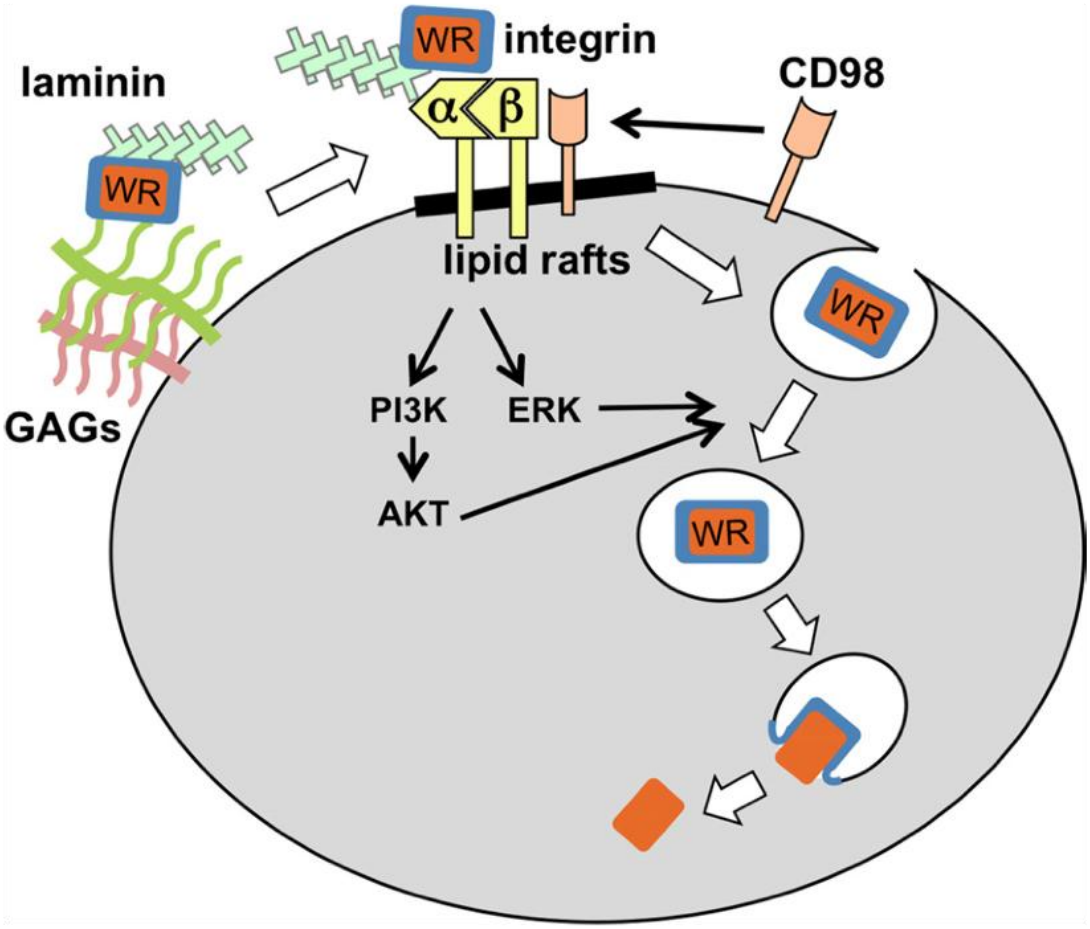


Figure 13. Model for MV binding and endocytosis: The vaccinia MV WR strain binds to cell surface glycosaminoglycans (GAGs) and laminin, which induce further interactions between MV and cellular surface receptor integrin in lipid rafts. The subsequent recruitment of the cellular membrane protein CD98 and the activation of downstream kinases such as PI3K/Akt and ERK lead to the endocytosis of vaccinia MV.

RESULTS

3.2. INTEGRIN β1 REQUIRES FOR ADHESION, SPREADING AND MIGRATION OF HeLa CELLS

Results above on outside-in activation experiments to turn-on the integrin dependent signaling pathway in an inactive HeLa cells demonstrated, that upon activation of the cells on fibronactin and Laminin several integrin β1 downstream signaling like FAK, Akt and Erk are phosphorylated (Figure 12 and 13). To address if integrin β1 is essential to mediating HeLa cell activation on extracellular matrix proteins we performed adhesion and spreading experiments from HeLa ITGβ1 KD cells. HeLa si-control and si-ITGβ1 KD cells were seeded overnight in the complete medium. The next day cells were serum starved detached from culture dish with trypsin/EDTA and plated onto substrates coated with fibronactin (FN) and Laminin (LN) for 60 minutes.

Although both, si-control and si-ITGβ1 KD cells adhere and spread to FN (Figure 14A-ab), however si-ITGβ1 KD cells have smaller shape and disorganized cytoskeleton, consistent with results in (Figure 8C). In contrast, si-ITGβ1 KD cells attached to Laminin poorly and those attached cells did not spread at all (Figure 14A-cd).

This difference was still obvious when cells were incubated even longer 120 minutes. Indicating integrin β1 binding to laminin is crucial for HeLa cells attachment and spreading. As a negative control cells were plated onto dishes coated with PBS/BSA (Fig. 14A-ef). Consistent result was obtained when adhesion assay was repeated using GD25 cells, MEF lacking integrin β1 (46, 78) (Izmailyan unpublished results). Earlier studies with GD25 cells showed that without integrin β1 these cells attachment to fibronactin is through αvβ3 receptors whether it is also true for HeLa cells remain to identify.

Quantification of attached cells with colorimetric assay is shown in (Figure 14B). Percentage of si-ITGβ1 KD cells spread on fibronactin and laminin was also counted from 7 different fields, 40 cells in each field. (Figure 14C). Thus, integrin β1 mediates cell shape adhesion and spreading in HeLa cells.

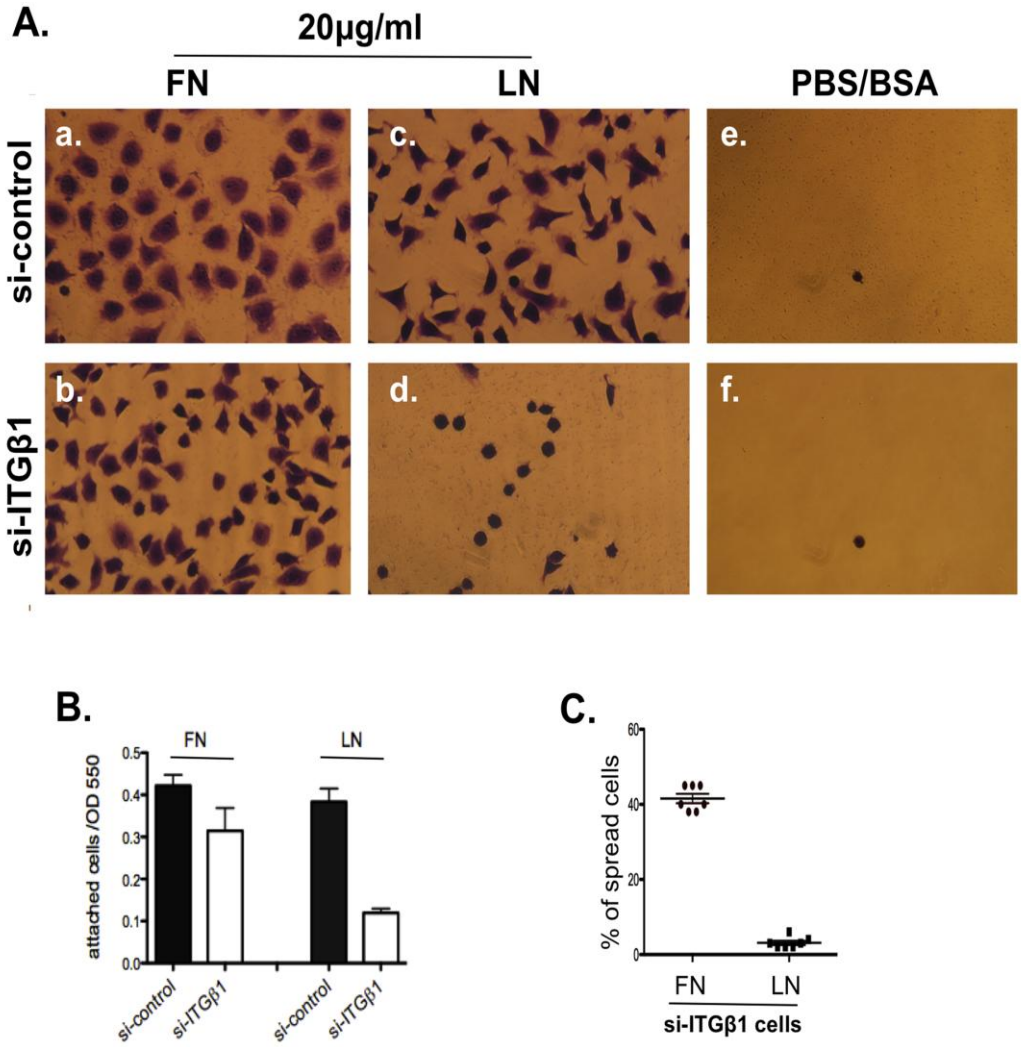


Figure 14. Adhesion and spreading of the si-control and si-ITGβ1 KD HeLa cells seeded on substrate coated with fibronactin or Laminin-1 (A) Morphology of the cells. Non tissue culture plates were coated with 20µg/ml fibronactin (FN) or laminin-1 (LN) blocked with 1% BSA cells were incubated at 37°C for 60 minutes, fixed stained and photographed. **(B)** Quantification of adhered cells. **(C)** Quantification of si-ITGβ1 KD HeLa cells spread on LN. Graph analyses were performed using Prism software (GraphPad).

Adhesion is a complex mechanism and involves variety processes like cell migration, invasion, wound healing and tissue remodeling. Effect of integrin β1 was examined in migration assay. Confluent monolayer of HeLa si-control and si-ITGβ1 KD cells were wounded and incubated for indicated time shown in (Figure 15) only in the presence of integrin β1 cells were able to migrate and repair the wound (Figure 15ab) in contrast to si-ITGβ1 cells (Figure 15cd). Resent years HeLa cells are commonly used and important cell line for the researchers in molecular and cellular biology field which makes an interest to know more about these cell biology. More works can be done to investigate and dissect which downstream signaling and receptors are directly involved for integrin β1 function in HeLa cells.

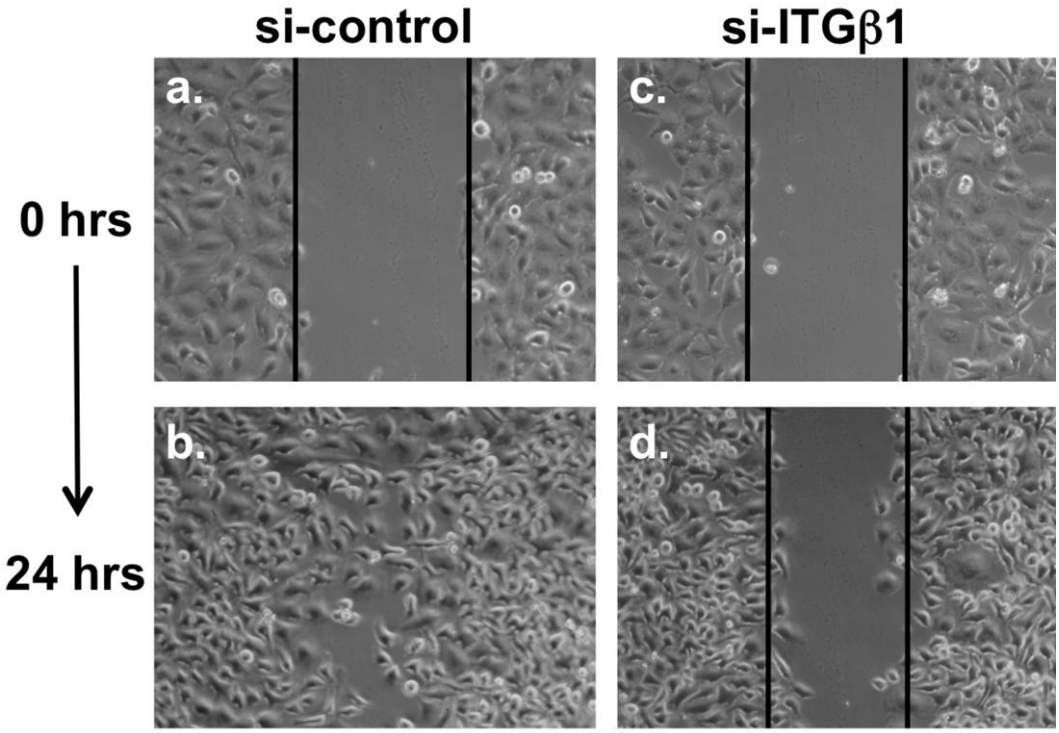


Figure 15. Migration of si-control and si-ITGβ1 KD HeLa cells. Confluent cell monolayer was wounded and photographed ether right after wounding 0 or 24 hrs post incubation. (see Material and Methods)

RESULTS

3.3. VACCINIA VIRUS WR53.5/F14.5 PROTEIN IS A NEW COMPONENT OF INTRACELLULAR MATURE VIRUS AND IS IMPORANT FOR CALCIUM-INDEPENDENT CELL ADHESION AND VACCINIA VIRUS VIRULENCE IN MICE

3.3.1. Conserved vaccinia WR53.5/F14.5 ORF encodes a late viral envelope protein in MV particles

Our recent MASS analysis of newly prepared vT7lacOI MV virions revealed two tryptic peptides that had not been detected before. These two peptides, YVEENNEEDAR and IKEEQELLLLY, matched a small ORF WR53.5 protein in vaccinia virus WR strain genome (Figure 16A), encoding a conserved polypeptide of 49 amino acids with a predicted molecular weight of 5.5 kDa (http://www.poxvirus.org/). Hydropathy analysis predicted that the WR53.5 protein has two hydrophobic domains at the N-and C-termini (Figure 16B). Alignment of the amino acid sequences of vaccinia WR53.5L and its orthologues present in the *Orthopoxvirus* genus revealed a high level of homology among these proteins (~98% conserved residues) including the F14.5L ORF in vaccinia Copenhagen strain (55). Rabbits were immunized with a synthetic peptide derived from the C-terminal region of WR53.5 protein amino acid sequences (see Materials and Methods) and the antiserum, anti-53.5, was tested in immunoblots of lysates prepared from virus-infected cells (Figure 16C). The anti-53.5 antiserum recognized a small 3.5 kDa viral protein in cells infected with our original WR strain vaccinia virus (WR-1) and a 5.5 kDa protein in cells infected with other vaccinia viruses including two WR strains (vT7lacO/I and WR-2), Copenhagen (Cop) and IHD-J strains. Although WR and Cop strains have been sequenced before we re-

sequenced the WR53.5L gene locus in the above virus genomes and found that the WR53.5L ORF in WR-1 specifies a K at residue 44, encoded by AAA, whereas an E encoded by GAA was found in other vaccinia virus genomes such as vT7LacO/I, WR-2, Cop and IHD-J strains (Figure 16D). This suggested to us that a non-conserved G-to-A mutation occurred in the WR-1 genome, resulting in a glutamic acid-to-lysine change at position 44 and a faster electrophoretic mobility on SDS-PAGE gels. Since E44 was more frequently found than K44 in the different WR53.5 orthologues (Figure 16B), we tentatively named that the WR53.5^{E44} in WR-2 as the wild-type WR53.5 protein. Wild-type WR53.5 protein was detected in MV particles prepared from vT7lacO/I and viG3L virus (79), a recombinant virus derived from vT7LacO/I; however, the WR53.5^{K44} protein was barely detected in purified WR-1 MV (Figure 16E). Expression of the wild-type WR53.5 protein was monitored in cells infected with vaccinia virus WR-2, and a 5.5 kDa protein was detected at 4 h p.i, which increased in abundance until 24 h p.i. (Figure 16F). Expression of the 5.5 kDa protein was blocked by araC, which inhibits viral DNA replication, suggesting that WR53.5L is a late gene. Indeed, there is a canonical later promoter TAAATG (125) overlapping the initiation codon of WR53.5 ORF in vaccinia virus genome.

To determine whether WR53.5 protein is present in the membrane fraction of MV, purified wild type WR-2 MV were extracted with 1% NP-40 with or without 50 mM DTT and the virion membrane proteins (supernatant) were separated from the insoluble core components (pellet) by centrifugation. As shown in Figure 17A, WR53.5 protein was not extracted from vaccinia MV by buffer containing 1% NP-40; inclusion of 50 mM DTT during extraction did, however, result in partial release of WR53.5 protein into the supernatant, suggesting an association of WR53.5 protein with membranes. Another MV membrane protein, H3, which served as a control, was readily extracted into the supernatant fraction. In contrast, the viral core proteins 4a/4b were resistant to detergent extraction.

It was previously shown that viral membrane proteins exposed on the surface of MV are sensitive to protease digestion (37, 77, 114). We then tested the protease sensitivity of WR53.5 protein in MV

particles (Figure 17B). If the C-terminus of WR53.5 protein is exposed on the MV, trypsin treatment would result in a loss of immunoreactivity to anti-53.5 Ab. Similar to H3 (37) and D8 proteins (114) that are sensitive to trypsin digestion, WR53.5 protein was also sensitive to trypsin treatment, in contrast to viral core proteins that were resistant to trypsin treatment. Together, these results show that WR53.5 is a late protein that is present in the membrane of vaccinia MV with the C-terminus of the protein exposed on the virions.

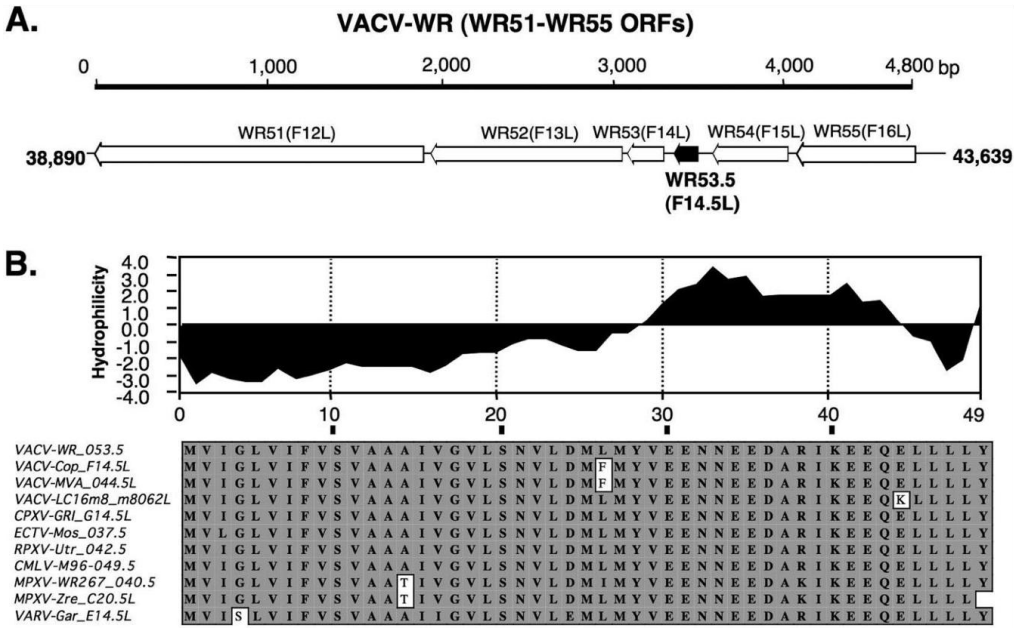


Figure 16. (A) Schematic drawing of the genomic locus of WR53.5 ORF and its neighboring ORFs (from WR51-WR55) on vaccinia virus WR strain genome. The length of the genomic region shown here is 4.8kb long. The numbers 38,890 and 43,639 are the ORF start/stop sites of the protein in the virus genome. The arrows represent each ORFs and are pointed toward the direction of gene transcription. WR51/F12L is expressed early and late and encodes an IEV protein required for virus egress (160). WR52/F13L is a late gene and encodes a palmitylated EEV protein important for cell-to-cell spread (14, 74). WR53/F14L, WR54/F15L and WR55/F16L are putative ORFs with unknown functions. **(B) Hydropathy plot of WR53.5 protein and its orthologues in the *Orthopoxvirus* genus.** The numbers at the bottom of the plot indicate the amino acid residues.

Alignment of the deduced amino acid sequences for vaccinia WR53.5 and its orthologues in other poxviruses is also shown. VACV, vaccinia virus; WR, Western Reserve strain; Cop, Copenhagen strain; MVA, modified virus Ankara (strain MVA-1721); CMLV, camelpox virus (strain M96); ECTV, ectromelia virus (strains Moscow, Naval); CPXV, cowpox virus (strains Brighton Red, GRI 90); Acam, Acambis 3000 MVA; VACV-Lister (LC16m8; LC16mO); RPXV-UTR, rabbitpox virus (strain Utrechht); VARV, variola virus (strains INDIA-1967/ isolate IND3, Garcia, Bangladesh); MPXV, monkeypox virus (Strain Walter Reed, Zaire). The orthologue sequences are obtained from http://www.poxvirus.org. The boxed sequences in gray are conserved amino acid sequences.

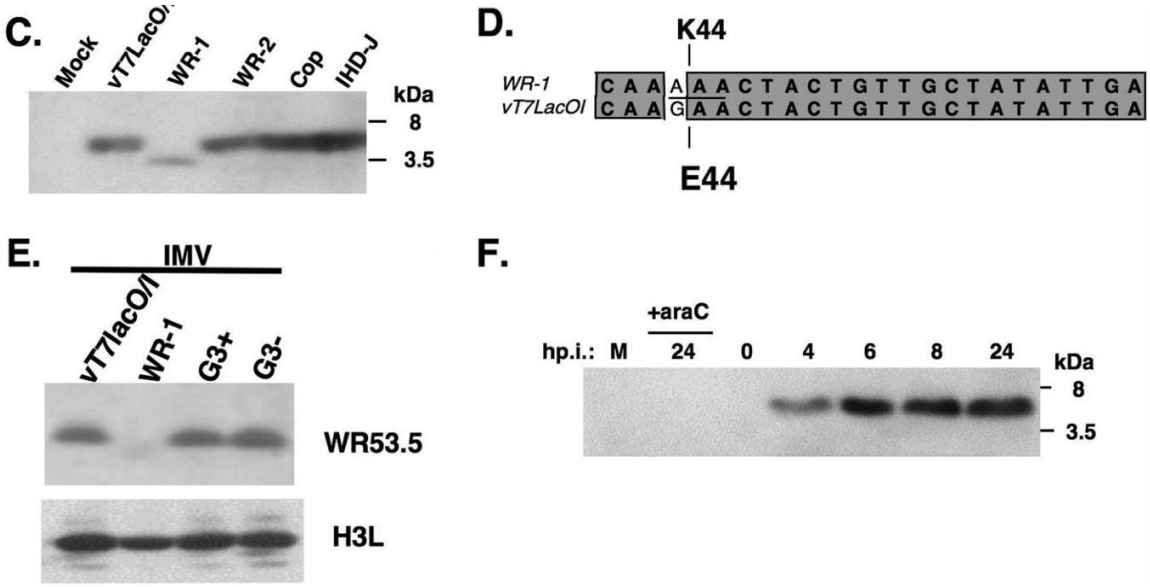


Figure 16. (C) Expression of WR53.5 protein in cell lysates infected with different vaccinia virus strains. BSC40 cells were infected with indicated viruses at a MOI of 5 PFU per cell and harvested at 24 h p.i. for immunoblot analyses with anti-53.5 Abs (1:1,000). **(D) Alignment of partial sequences of WR53.5 ORF of wild type WR-1 with vT7LacOI. (E)** Detection of WR53.3 protein in MV particles of vT7LacOI, WR-1, viG3L virus (79) grown in the presence (G3+) or absence of IPTG (G3-). **(F) WR53.5 protein is a viral late protein.** BSC40 cells were infected with vT7LacOI at a MOI of 5 PFU per cell and harvested at the indicated time p.i. for immunoblot analyses using anti-53.5 Abs (1:1,000). M: mock-infected cells. AraC (40 mg/ml) was added to cells immediately after infection.

It was previously shown that viral membrane proteins exposed on the surface of MV are sensitive to protease digestion (37, 77, 114). We then tested the protease sensitivity of WR53.5 protein in MV particles (Figure 17B). If the C-terminus of WR53.5 protein is exposed on the MV, trypsin treatment would result in a loss of immunoreactivity to anti-53.5 Ab. Similar to H3 (37) and D8 proteins (114) that are sensitive to trypsin digestion, WR53.5 protein was also sensitive to trypsin treatment, in contrast to viral core proteins that were resistant to trypsin treatment. Together, these results show that WR53.5 is a late protein that is present in the membrane of vaccinia MV with the C-terminus of the protein exposed on the virions.

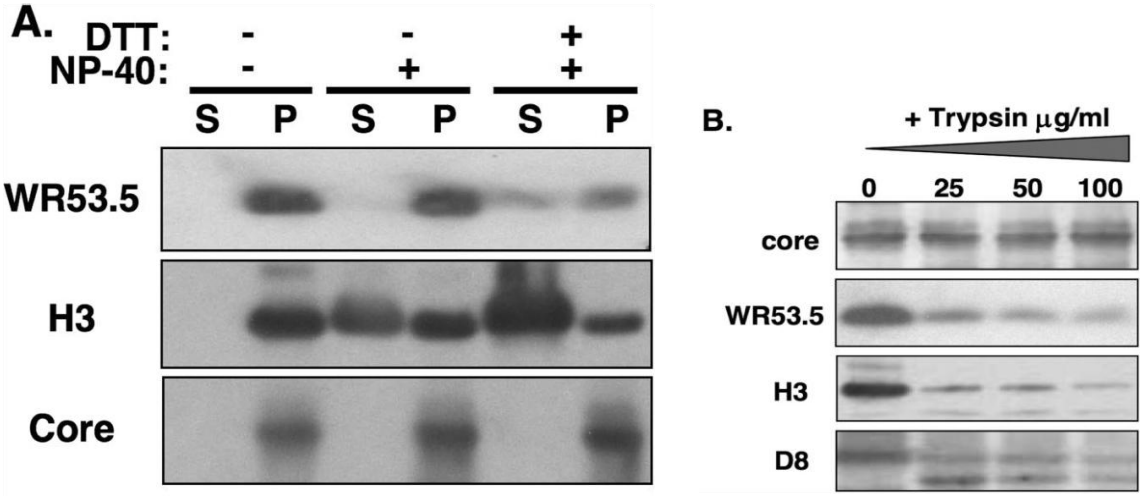


Figure 17. (A) Membrane extraction of vaccinia MV WR53.5 protein. Equivalent amount of purified wild-type (WR-2) MV were extracted with buffer containing 1% NP40 with or without 50 mM DTT, centrifuged to separate the supernatant (S) and pellet (P) for immunoblots using anti-53.5 (1:1,000), anti-H3 (1:2,000) and anti-core (1:2,000) Abs. **(B) Trypsin digestion of MV envelope proteins.** Purified wild type WR-2 MV particles were either mock-treated (50 mM Tris) or treated with an indicated concentration of trypsin at 37°C for 60 min, sedimented by centrifugation and immunoblotted with antibodies against vaccinia proteins.

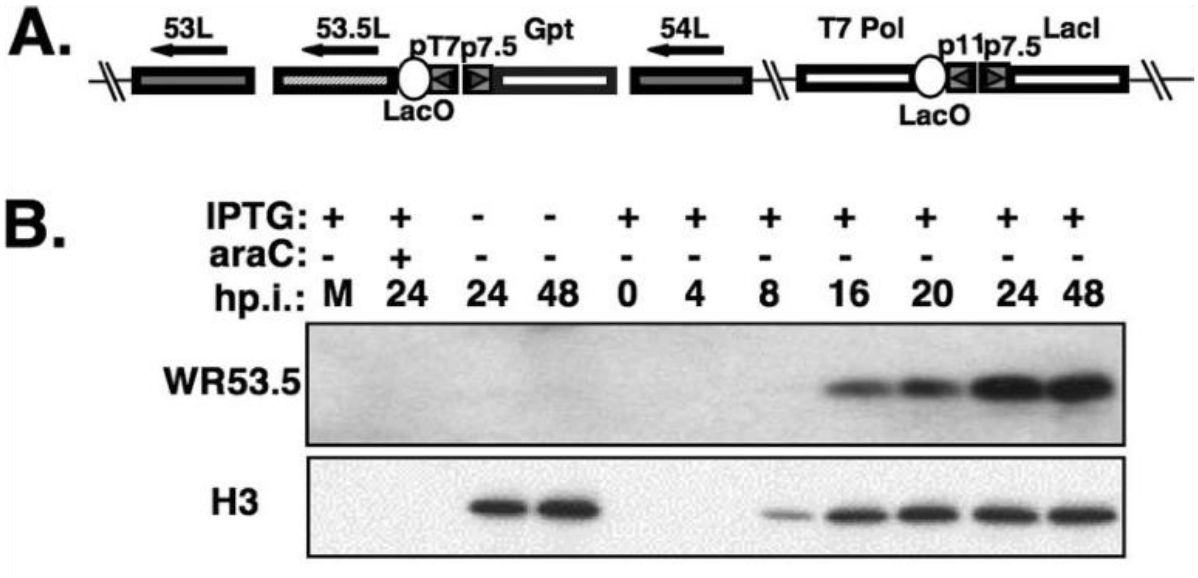


Figure 18. (A) Schematic diagram of vi53.5L virus. The WR53.5L and J2R (tk) loci in the vi53.5L recombinant virus are indicated. The J2R locus contains T7 RNA polymerase and the lacI repressor gene as described previously (152). The inducible WR53.5L is shown as a shaded box and the flanking WR53L/F14L and WR54L/F15L genes as gray boxes. The arrows indicate the transcription direction. Abbreviations used are: *T7 Pol,* T7 RNA polymerase; *LacO, E. coli* lac operator; *lacI*, *E. coli* lac repressor gene; *Gpt*, *E. coli* xanthine guanine phophoribosyltransferase gene; p7.5 and p11, viral promoters; pT7, promoter for T7 RNA polymerase. **(B) Expression of WR53.5 protein in cells infected with vi53.5L virus.** BSC40 cells were infected with vi53.5L at a MOI of 5 PFU per cell and incubated with or without 100μM IPTG, harvested at the indicated time for immunoblot analyses with the anti-53.5 (1:1,000)) or with anti-H3 Abs (1:2,000). M, mock-infected cells.

3.3.2. WR53.5 protein is not required for plaque formation and MV/EEV production in cell culture

The role of WR53.5L during the vaccinia virus life cycle in cell culture was explored using a recombinant vaccinia virus, vi53.5L that was generated from the vT7LacOI parental virus (Figure 18A). vi53.5L contains an inducible WR53.5L/ *E. coli gpt* marker gene cassette inserted into its

endogenous WR53.5L locus and was isolated in the presence of mycophenolic acid and purified after 3 rounds of plaque purification. Abundant WR53.5 protein was only detected late in the infected cells in the presence of 100μM IPTG, and its production was blocked by araC (Figure 18B), demonstrating that expression of the WR53.5L gene was tightly regulated at the late phase by IPTG.

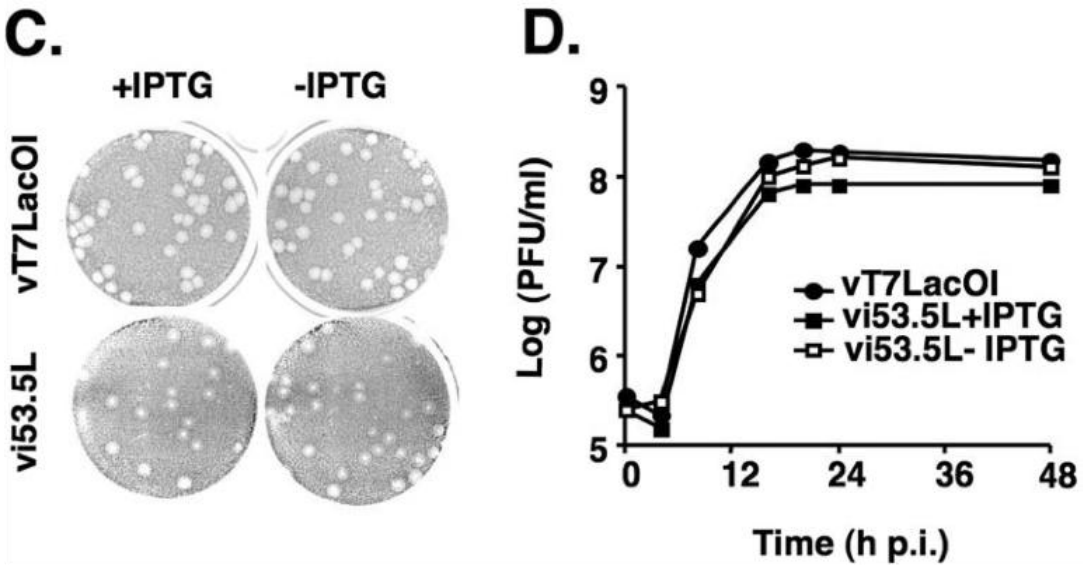


Figure 18. **(C) Plaque formation of vi53.5L and parental vT7LacOI virus on BSC40 cells in the presence (+IPTG) or absence (-IPTG) of IPTG. (D) One-step growth curve analysis of vi53.5L.** BSC40 cells were infected with vT7LacOI or vi53.5L at a MOI of 5 PFU per cell, incubated in the presence (vi53.5L+IPTG) or absence (vT7LacO/I and vi53.5L-IPTG) of 100 μM IPTG and harvested for plaque assays.

The role of WR53.5L in vaccinia virus life cycle was examined in BSC40 cells infected with vi53.5L in the presence or absence of IPTG. At 3 days p.i., both the control vT7LacOI and vi53.5L virus formed similar plaques on BSC40 cells in the presence or absence of IPTG, showing that WR53.5 protein is not required for plaque formation (Figure 18C). One-step growth analysis was performed with BSC40 cells infected with vi53.5L at a MOI of 5 PFU per cell and cultured in the

presence or absence of IPTG, after which cell lysates were collected and the MV titers determined. As shown in Figure 18D, removal of IPTG did not affect growth of vi53.5L, which grew to titers similar to the vT7lacOI parental virus. Multiple round virus growth analyses were performed with a low MOI of 0.1 infection and the yields of both MV in cells and EEV in culture media were not affected by addition of IPTG. We thus concluded that WR53.5 protein is not required for vaccinia virus life cycle in cell cultures.

3.3.3. WR53.5 protein regulates cell morphology and adhesion of virus-infected BSC40 cells

When BSC40 cells were infected with vi53.5L and maintained in medium containing 100μM IPTG the morphology of virus-infected cells gradually changed into elongated shape at 1-2 days p.i. (Figure 19A). In the absence of IPTG, the infected cells rounded up and became loosely attached to dishes at 2 days p.i., suggesting that expression of WR53.5 protein induced by IPTG affected cell morphology. However, the elongated morphology was not obvious in BSC40 cells infected with the parental virus vT7LacOI that expresses WR53.5 protein from its endogenous promoter and is not regulated by IPTG (Figure 19A). IPTG alone did not induce such elongated phenotype when tested on BSC40 cells infected with viG3L, a recombinant virus in which expression of viral G3 protein is induced by IPTG. When immunoblot analyses were performed with the above-mentioned cells, a higher level of WR53.5 protein was present in cells infected with vi53.5L+IPTG than with vT7LacOI (Figure 19B). Moreover, in BSC40 cells infected with vi53.5L, increased concentrations of IPTG from 12.5 μM to 25 μM and 50 μM in the medium induced a corresponding increase of WR53.5 protein expression and the elongated morphology in the infected cells (Figure 19C), demonstrating that a high level of WR53.5 protein expression allowed detection of its function in cell adhesion. When tested on other cell lines, WR53.5 protein also mediated cell adhesion in RK13 and BSC1 cells, though not as obvious as in BSC40 cells, not shown.

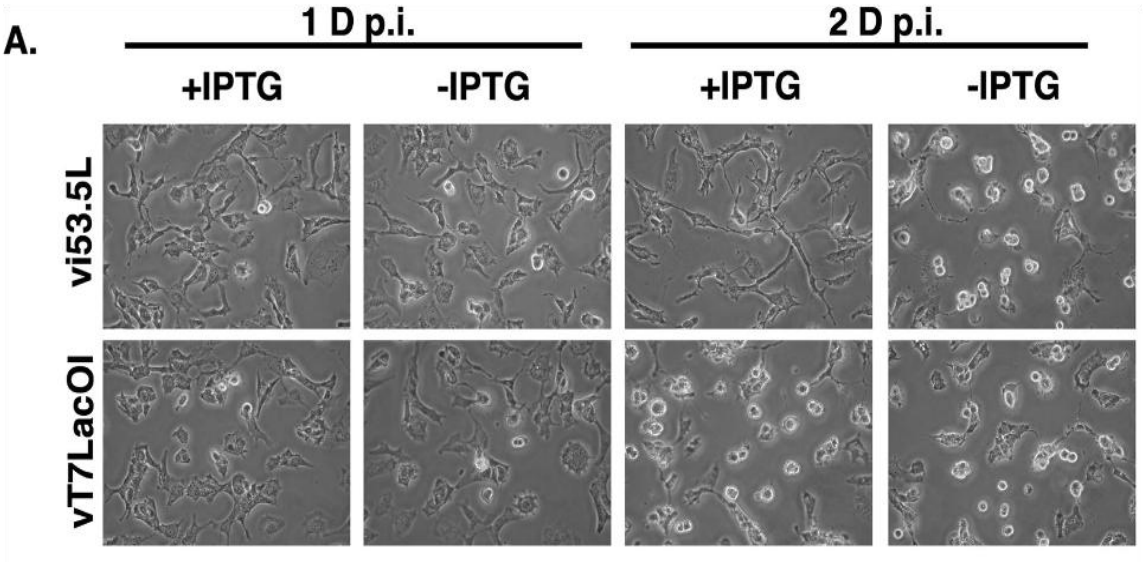


Figure 19. (A) WR53.5 protein regulates cell morphology and adhesion. Elongated morphology of BSC40 cells infected with vi53.5L virus in the presence of 100μM IPTG at the late phase of infection. BSC40 cells were infected with vi53.5L or vT7LacOI at a MOI of 5 PFU per cell and cultured in medium with (+IPTG) or without (-IPTG) 100μM IPTG for 1 and 2 days.

3.3.4. WR53.5 protein mediates Ca^{2+}-independent cell adhesion in BSC40 cells infected with vi53.5L virus

To test whether WR53.5-mediated cell adhesion was dependent on calcium, we infected BSC40 cells with vi53.5L and treated the infected cells with EGTA (Figure 19D). Mock-infected BSC40 cells adhered well to plates but rounded up after EGTA treatment for 20 min. On the other hand, cells infected with vi53.5L, which adhered well in the presence of IPTG, remained adherent even after 20 min EGTA treatment, showing that WR53.5 protein was required for calcium-independent cell adhesion of the infected cells.

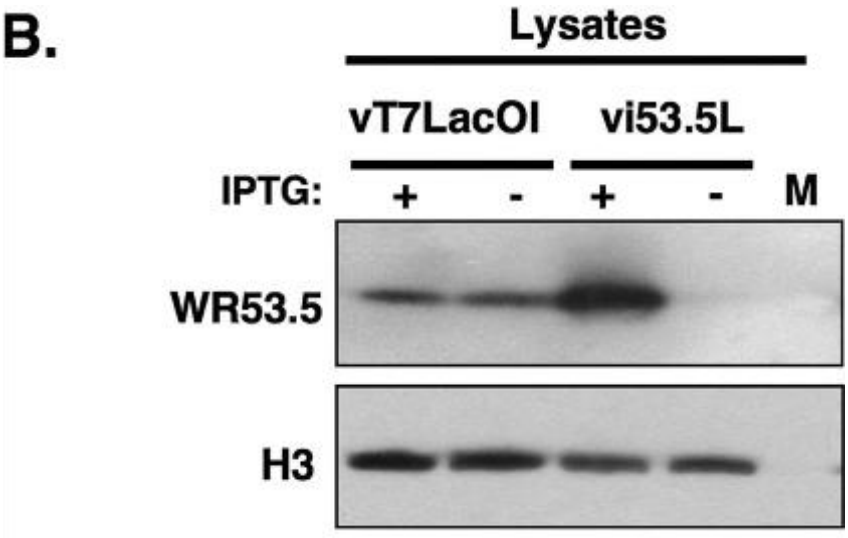


Figure 19. (B) Immnoblots of WR53.5 protein expressed in the infected cells at 2 days p.i. in the presence (+) or absence (-) of 100μM IPTG. M: mock-infected lysates.

3.3.5. Cell surface and intracellular distribution of WR53.5 protein in the infected cells

The fact that WR53.5 mediates cell adhesion suggests a possibility that it is expressed on the surface of virus-infected cells. To test this hypothesis, BSC40 cells were infected with vi53.5L, cultured in media with or without IPTG for 1 day and harvested for FACS analyses using anti-53.5 antibodies. Wild type WR-2 virus was also included to measure WR53.5 protein expression from its endogenous promoter. As shown in Figure 20A, strong fluorescent staining of WR53.5 protein was detected on BSC40 cells infected with vi53.5L in the presence of IPTG. Less strong expression of WR53.5 protein was detected on cells infected with wild type WR-2. In the absence of IPTG, anti-53.5 Ab detected a low level of fluorescence staining that was comparable to the background staining with the secondary Ab only.

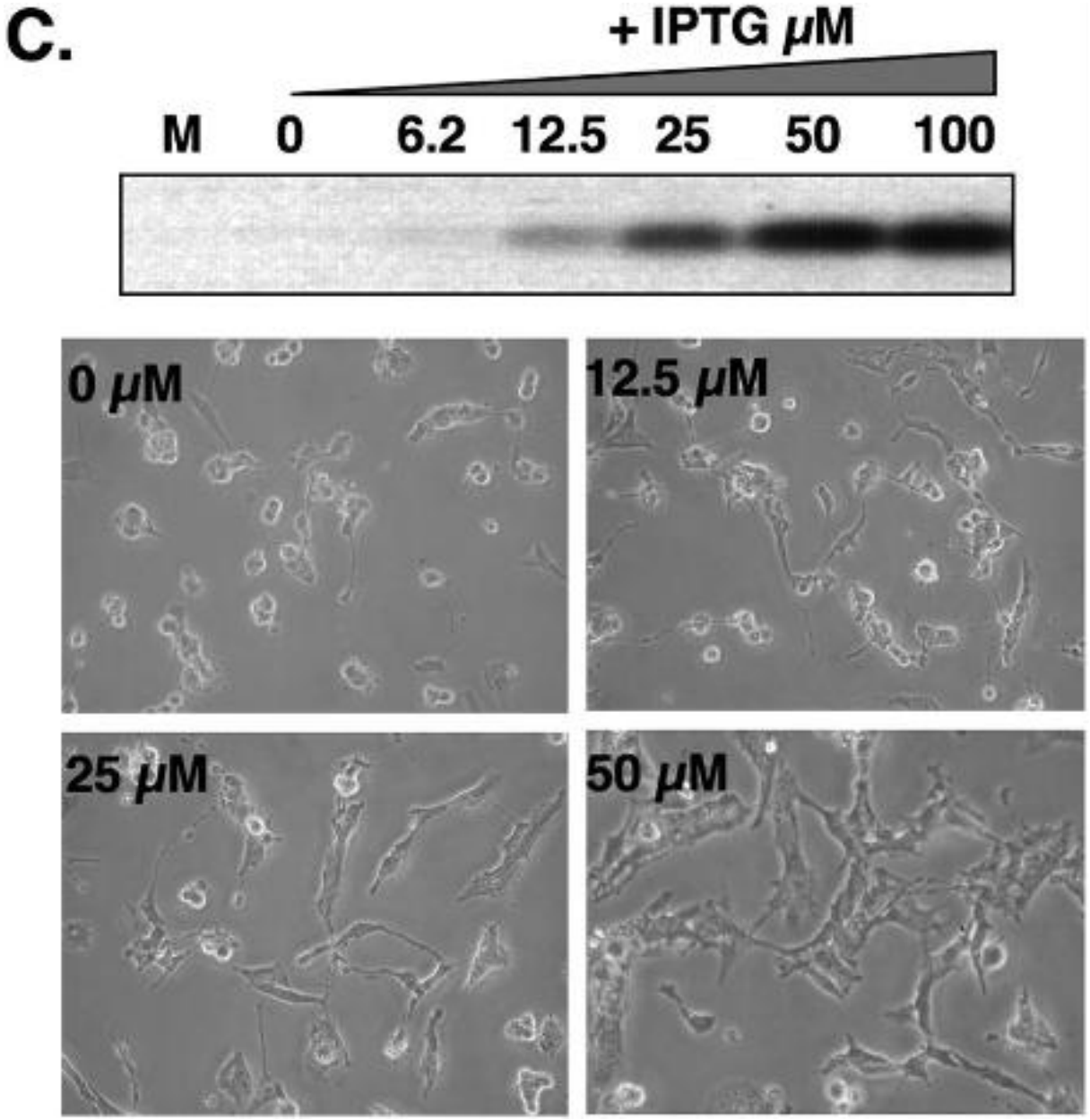


Figure 19. (C) WR53.5 protein mediates cell adhesion of virus-infected cells. (Top panel), Immunoblot analyses of WR53.5 protein induced by different IPTG concentration at 2 days p.i.. M, mock-infected cells. (Bottom panel), BSC40 cells infected with vi53.5L, cultured with (12.5, 25 and 50μM) or without (0 μM) IPTG for 2 days and photographed with a Nikon inverted microscope.

D.

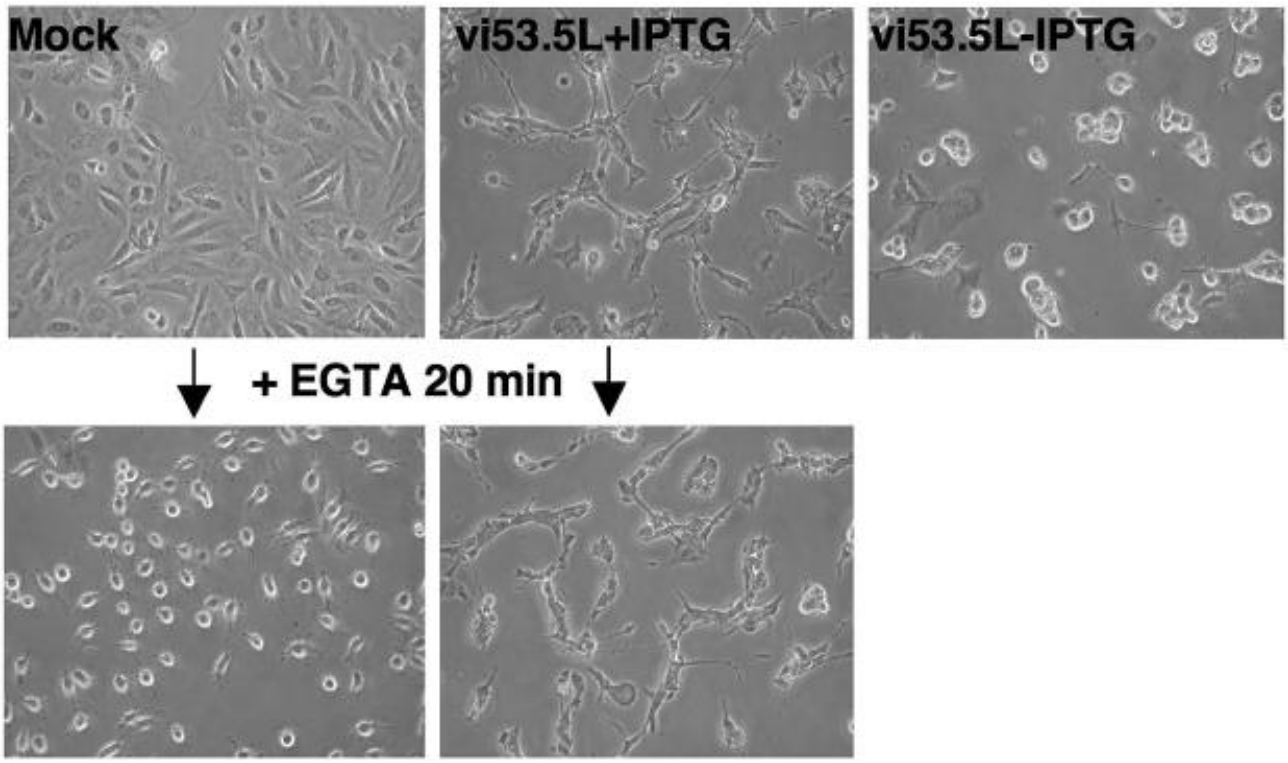


Figure 19. (D) WR53.5 protein mediates Ca^{2+}-independent cell adhesion in BSC40 cells infected with vi53.5L virus. Experiments were conducted as described before. In brief, confluent monolayers of BSC40 cells were either mock-infected (Mock) or infected with vi53.5L virus at a MOI of 5 PFU per cell and incubated at 37°C in the presence (vi53.5L+IPTG) or in the absence (vi53.5L-IPTG) of 100 μM IPTG. At 2 days p.i., cells were washed 3 times with PBS (pH 7.2) and photographed before and after 20 min EGTA treatment as described previously (118).

To investigate the intracellular distribution of WR53.5 protein in cells, we performed immunofluorescence analyses using anti-53.5 Ab on mock- and virus-infected cells at 1 day p.i. (Figure 20B). In non-permeabilized (NP) conditions, anti-53.5 did not stain mock-infected cells nor cells infected with vi53.5L and cultured without IPTG (vi53.5L-IPTG). With IPTG, anti-53.5 Ab stained WR53.5 protein on cells infected with vi53.5 (vi53.5L+IPTG) as small patches on cell

edges and at cell tips. The patch-like surface staining of WR53.5 protein was also observed in cells infected with wild type WR-2. After permeabilization (P), abundant intracellular staining of WR53.5 protein was specifically detected in cytoplasm of cells infected with vi53.5L in the presence of IPTG (vi53.5L+IPTG) and with wild type WR-2. The intracellular staining of WR53.5 looked like small dots and we believe some of these dots may represent intracellular virion particles in cytoplasm.

When cells infected with vi53.5L were allowed to grow to 2 days p.i. in the presence of IPTG (Figure 20C), elongated cells with extremely long cell protrusions were prevalent in cultures. Cell surface staining of WR53.5 protein decorated specific patches along these cell protrusions, consistent with a role in mediating cell adherence.

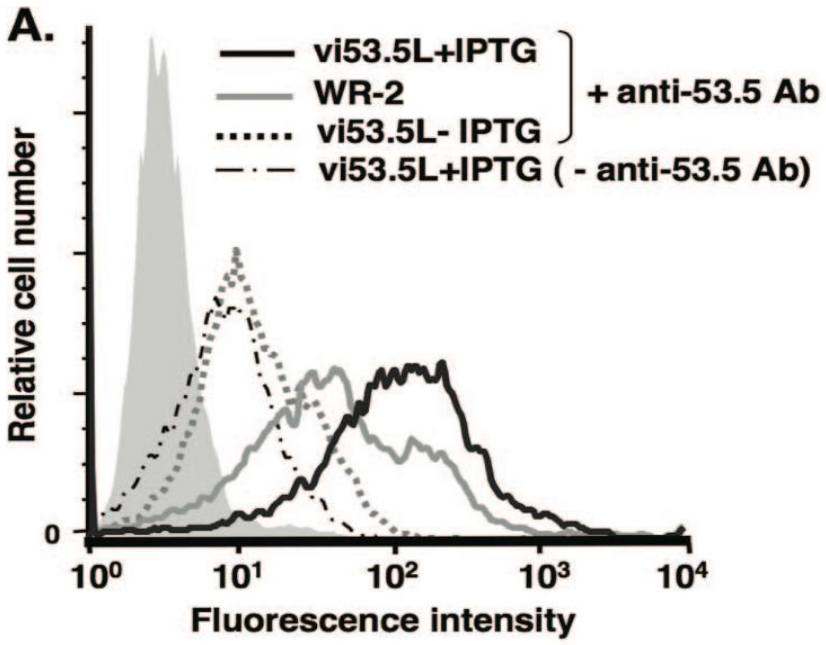


Figure 20. (A) Flow cytometry of WR53.5 protein expression on virus-infected cells. BSC40 cells were either mock-infected or infected with WR-2 or vi53.5L, cultured in the medium with (vi53.5+IPTG) or without IPTG (vi53.5-IPTG, WR-2) for 24 h, detached by 5mM EDTA and stained with anti-53.5 Ab and FITC-conjugated goat anti–rabbit Ab and analyzed by fluorescence-activated cell sorter.

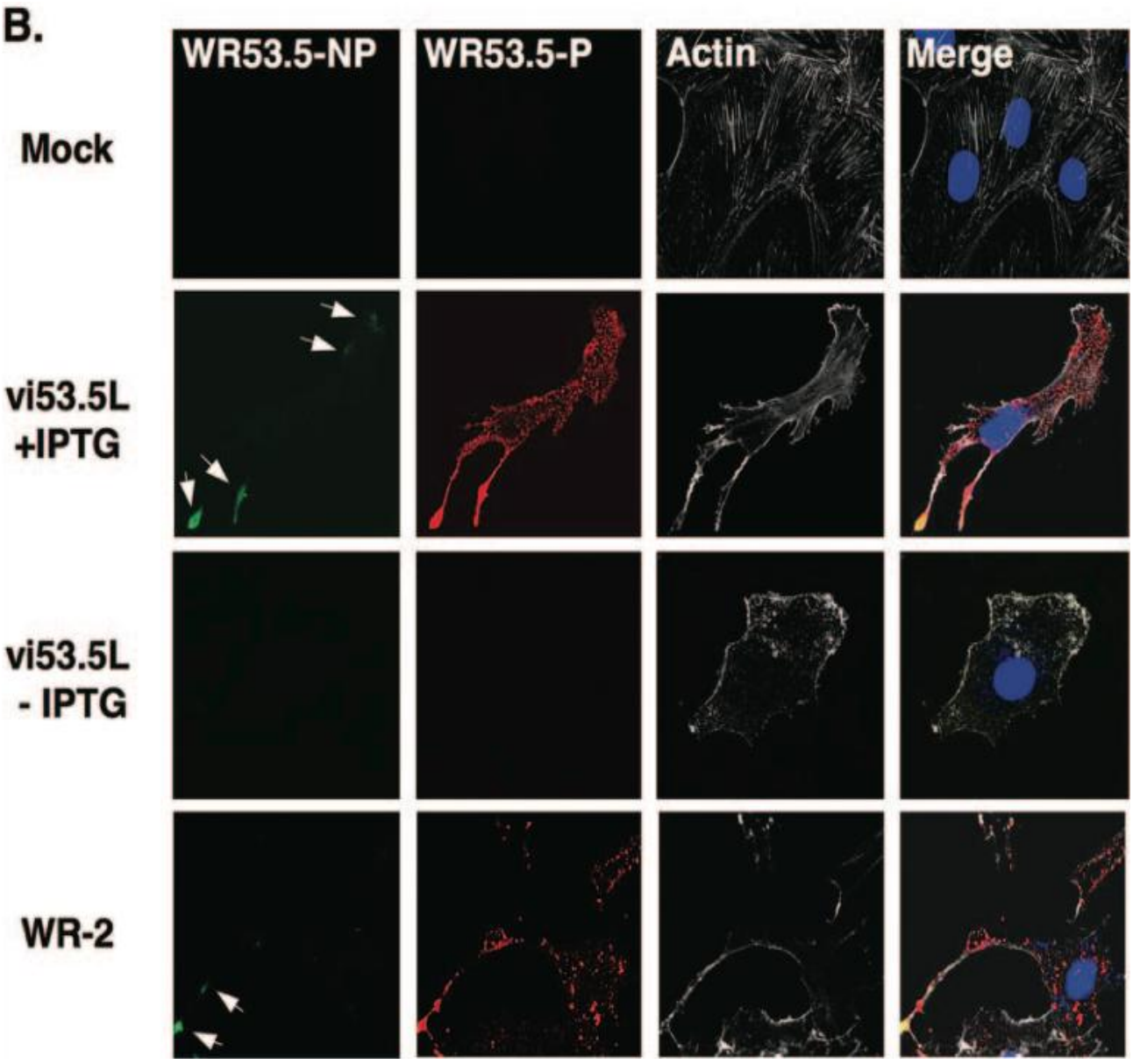


Figure 20. (B) Immunofluorescence analyses of WR53.5 protein in the infected cells at 1 day p.i.. BSC40 cells were mock-infected or infected with WR-2 or vi53.5L and cultured in medium with (+IPTG) or without (-IPTG) for 24h, fixed and stained with anti-53.5 Ab at non-permeable (NP) or permeable (P) condition. Cell surface WR53.5 protein is shown in green (pointed by white arrows) and the total WR53.5 protein in cells is shown in red. Actin stained with Alexa Flour 647-phalloidin is shown in white and DNA stained with DAPI is shown in blue.

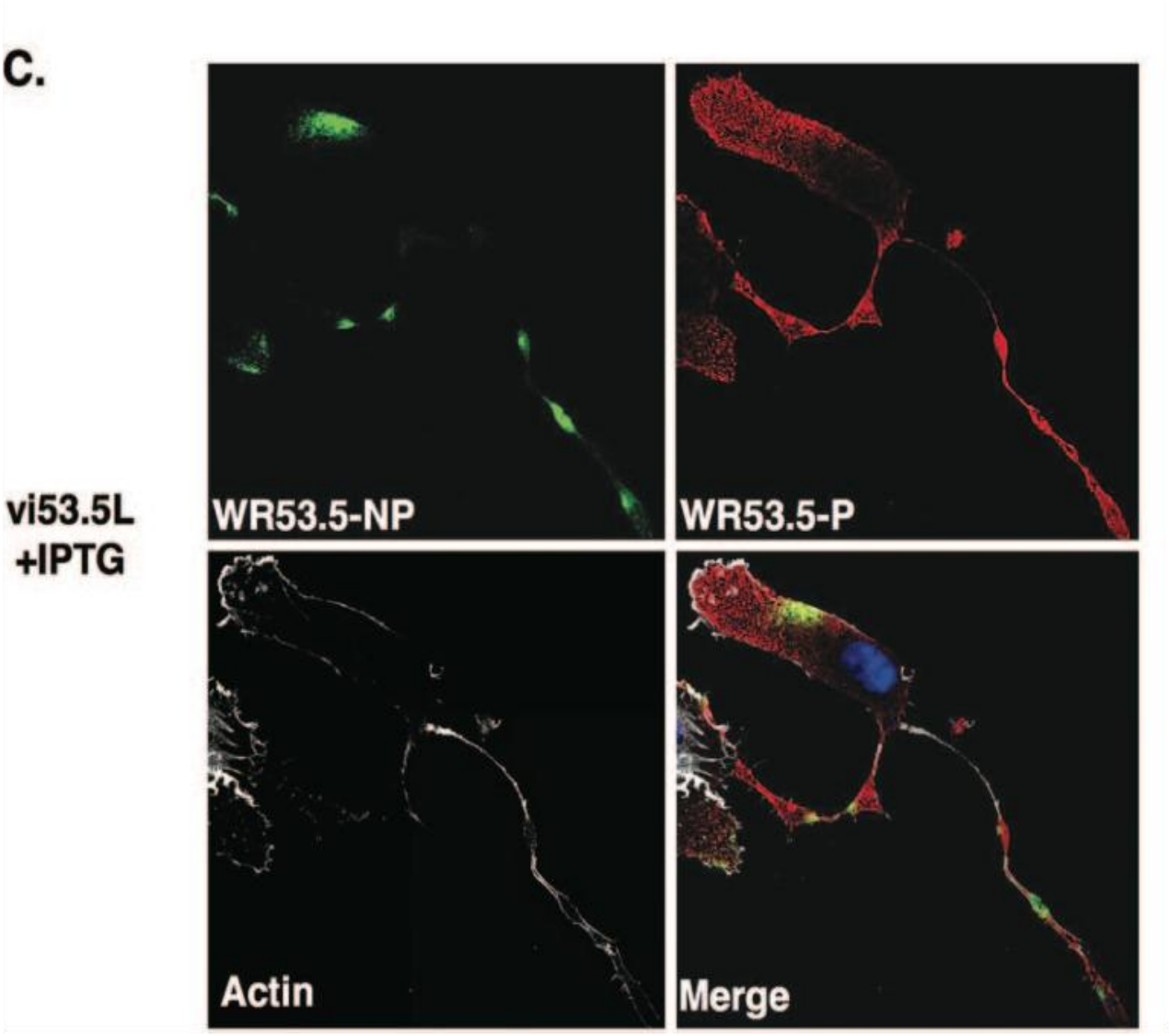


Figure 20. (C) Immunofluorescence analyses of WR53.5 protein in virus-infected cells at 2 day p.i.. The infected cells described in B were fixed at 2 days p.i. and processed to visualize cell surface WR53.5 protein (green) and total WR53.5 protein (red) in cells. Note that the cells shown in (C) were composed from two overlapping photos due to the presence of the extra long protrusions extended from cell bodies. Cell images were collected with a LSM510 META confocal laser scanning microscope (Carl Zeiss, Germany) using a 63X objective lens and confocal microscopy software Release 2.8 (Carl Zeiss).

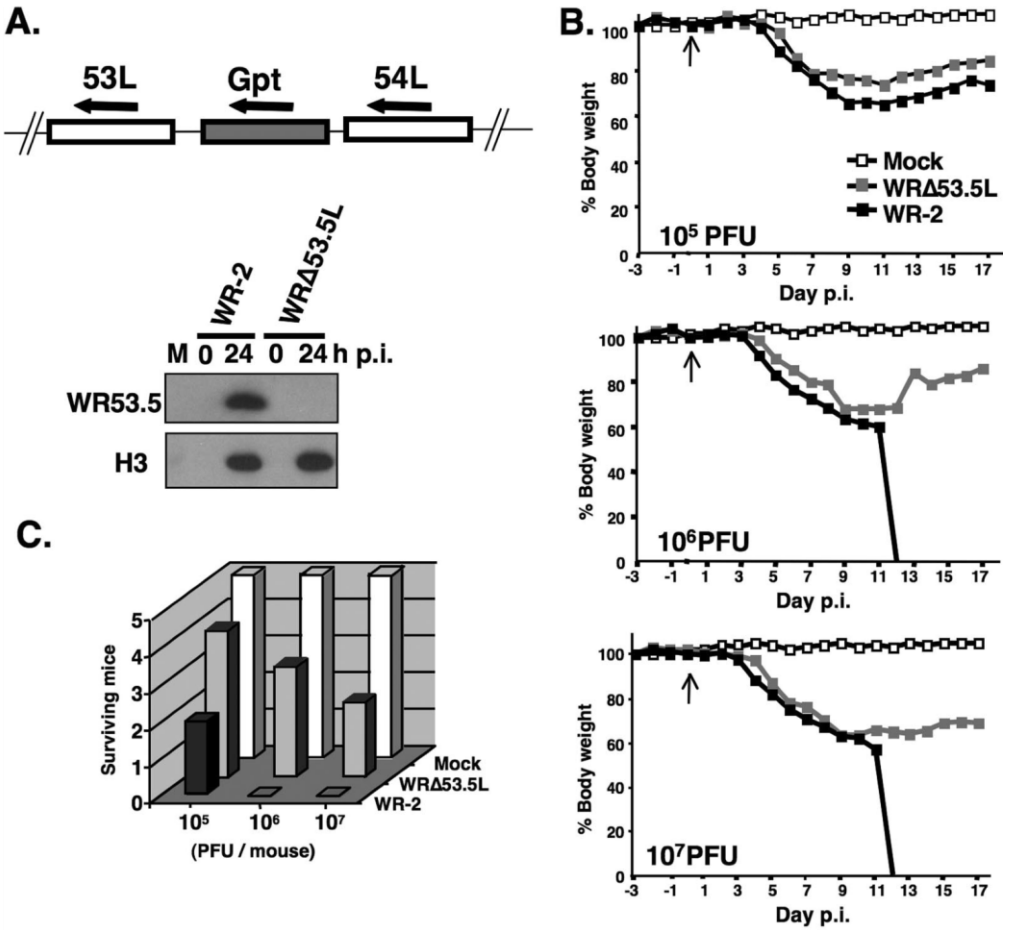


Figure 21. WR53.5 protein contributes to vaccinia virus virulence in mice. (A) Generation of WRΔ53.5L mutant virus from wild type vaccinia virus WR-2. (Top panel): Schematic representation of the WR53.5 locus that is interrupted in WRΔ53.5L by Gpt substitution. Gpt, *E. coli* xanthine guanine phophoribosyltransferase gene. (Bottom panel): Immunoblots of cell lysates from BSC40 cells infected with wild type WR-2 or WRΔ53.5L using anti-53.5 (1:1,000) or anti-H3 (1:2,000) Abs. M: Mock-infected cells. **(B) Body weight changes of mice infected with wild type WR-2 and WRΔ53.5L viruses.** Groups of 7-8 week old, male BALB/c mice (*n* = 5 per group) were either mock-infected or infected with WR-2 or WRΔ53.5L virus intranasally at a dosage of 10^5, 10^6 or 10^7 PFU per mouse. The arrows indicate the time of virus inoculation. These mice were weighed daily for a period of 17 days. **(C) Death of mice inoculated with wild type WR-2 and WRΔ53.5L virus in each group.**

3.3.6. WR53.5 protein is important for vaccinia virus virulence in mice

Although WR53.5 expression was tightly regulated by IPTG in vi53.5L virus, it is not suited for *in vivo* studies because its tk locus was inactivated by inserting a T7 RNA pol cassette. To address whether WR53.5 protein is important for virus virulence *in vivo* we generated a deletion virus, WRΔ53.5L, that inactivated WR53.5L ORF from wild type WR-2 vaccinia virus (Figure 21A). The WRΔ53.5L virus grew well in cell cultures and no difference in titers and plaque morphology was observed when compared with that of wild type WR-2 virus. Balb/C mice were inoculated intranasally with 10^5, 10^6 and 10^7 PFU of wild type WR-2 or WRΔ53.5L mutant virus per mice and the body weights of individual mice were measured every day for a period of 18 days. As shown in (Figure 21B), all the mice infected by wild type WR-2 virus or WRΔ53.5L virus started losing weight at 4 days p.i., continued weight loss for another 5-7 days. Some infected mice were severely ill and died at day 11 whereas others slowly recovered. The difference of weight loss between mice infected by wild type WR-2 virus or WRΔ53.5 virus was small in all three dosages (Figure 21B). However, all the mice infected with 10^6 and 10^7 PFU wild type WR-2 virus died by 12 days p.i. whereas 60% and 40% of mice infected by WRΔ53.5L mutant virus survived respectively (Figure 21C). We thus concluded that WR53.5 protein contributes to vaccinia virus virulence in vivo.

RESULTS

3.4. THE ENVELOPE G3L PROTEIN IS ESSENTIAL FOR ENTRY OF VACCINIA VIRUS INTO HOST CELLS

3.4.1. The conserved vaccinia G3L gene encodes a late viral envelope protein associated with MV particles

The vaccinia G3L gene encodes a conserved polypeptide of 111 amino acids with a predicted molecular mass of 12.8 kDa (55). The G3L transcript was previously detected at the late phase of virus infection, indicating that G3L belongs to the viral late gene family (103). Hydropathy analysis (88) predicted that G3L has two hydrophobic domains at the N and C termini (Figure. 22A). Alignment of the amino acid sequences of vaccinia G3L and its orthologues in the poxvirus family (Figure. 22B) revealed a high level of homology between these proteins (~46% conserved residues), suggesting that G3L might play an important function in the poxvirus life cycle. The internal region of G3L was less conserved than the N- and C-terminal regions. Anti-G3L antibody was used to study the expression of G3L during vaccinia virus infection. Rabbits were immunized with a synthetic peptide derived from the G3L amino acid sequence (see Materials and Methods), and the antiserum produced was tested on immunoblots of lysates prepared from virus-infected cells. The antiserum did not recognize any protein in mock-infected cells but recognized a 12.8-kDa protein in virus infected cells that was detected at 8 h p.i. and increased in abundance until 24 h p.i. (Figure. 23C). The 12.8-kDa protein was expressed at late phase in virus-infected cells, since araC, which inhibits viral DNA replication, blocked G3L expression. The antiserum also recognized a 12.8-kDa protein in purified MV, demonstrating that G3L protein is present in MV particles, a finding consistent with our proteomic analysis of VV MV.

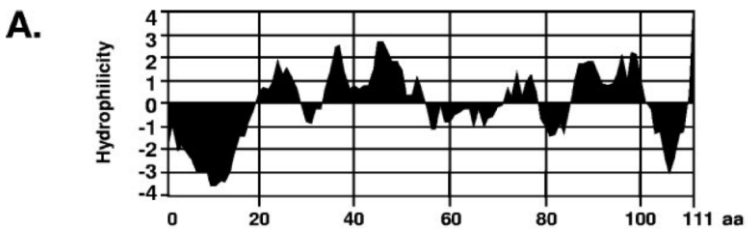


Figure 22. (A) Hydropathy plot of G3L protein. The numbers at the bottom are the aa residues.

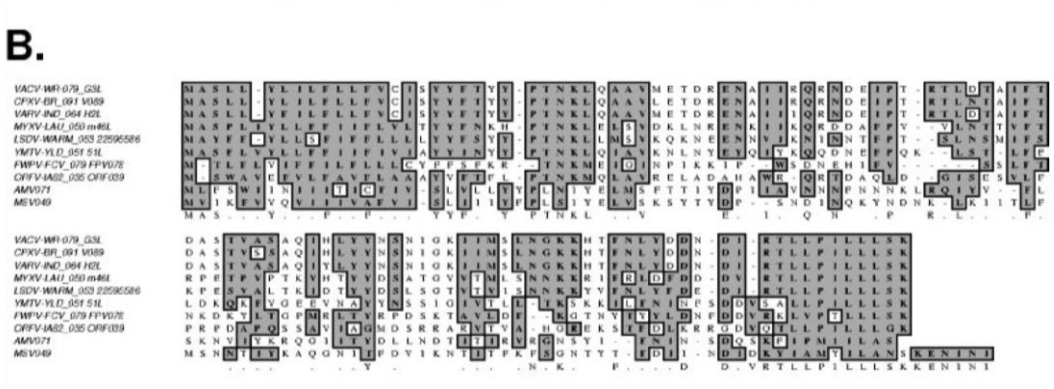


Figure 22. (B) Alignment of the deduced amino acid sequences for vaccinia G3L and G3L orthologues in other poxviruses. VACV, vaccinia virus (WR strain); CPXV, cowpox virus (Brighton Red strain); VARV, variola virus (INDIA-1967/isolate IND3); MYXV, myxoma virus; LSDV, lumpy skin disease virus; YMTV, Yaba-like monkey tumor virus; FWPV, fowlpox virus; AMV, Amsacta moorei entomopoxvirus; MSV, Melanoplus sanguinipes entomopoxvirus. The boxed sequences in gray are conserved amino acid sequences.

3.4.2. Vaccinia G3 protein is MV membrane component.

To determine whether G3L is present in the membrane fraction of MV, purified MVs were extracted with 1% NP-40 with or without 50 mM dithiothreitol (DTT), and the virion membrane proteins (supernatant) were separated from the insoluble core components (pellet) by centrifugation. As shown in Figure 23, G3L was extracted from purified vaccinia MV by buffer containing 1% NP-40; inclusion of 50 mM DTT during extraction did not result in greater release of G3L into the supernatant. These results showed that G3L is associated with membranes. Another MV membrane protein, H3L, served as a control and was similarly extracted into the supernatant fraction (Figure

23). In contrast, the viral core proteins 4a/4b were resistant to detergent extraction. Our results showed that G3L is a late protein that is present in the membrane of vaccinia MV.

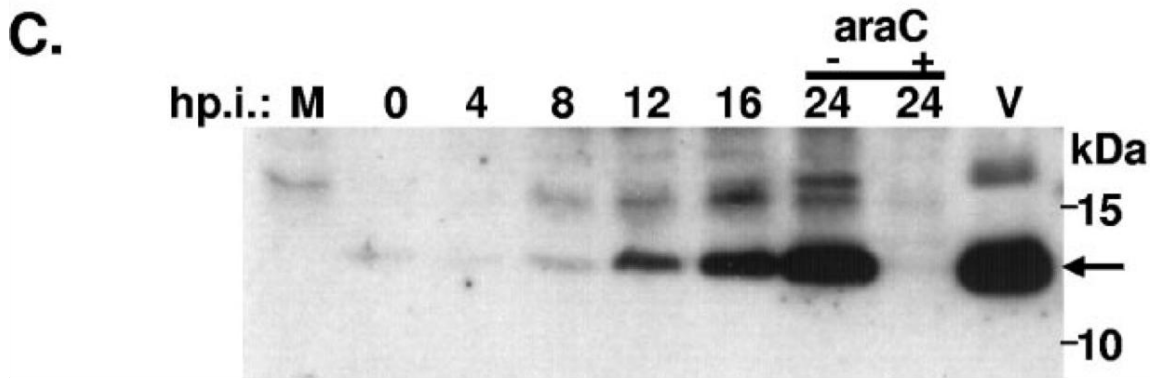


Figure 22. (C) Expression of G3L protein in infected cells and purified MV virions.

BSC40 cells were infected with parental vT7LacOI at an MOI of 5 PFU per cell and harvested at the indicated times post infection; the lysates were then separated by SDS–12.5% PAGE and transferred to nitrocellulose for immunoblotting with the anti-G3L antiserum. M, mock-infected cells; V, purified MV particles. When araC treatment was required, the drug (40 μg/ml) was added to the cells immediately after infection. The arrow shows the position of G3L protein.

3.4.3. Construction of a recombinant vaccinia virus expressing the inducible G3L gene under the control of the Lac operator

The role of G3L during the vaccinia virus life cycle in cell culture was explored using a recombinant vaccinia virus, viG3L, that expressed G3L conditionally regulated by IPTG (Figure. 24). ViG3L was generated from the vT7LacOI parental virus (152), with an inducible G3L cassette containing the E. coli gpt marker gene inserted into its endogenous locus (Figure 24 A). Recombinant virus, viG3L, was isolated in the presence of mycophenolic acid and purified after three rounds of plaque purification. BSC40 cells were infected with viG3L and cultured in medium with or without IPTG for 24 h and harvested for immunoblot analysis. Abundant G3L was only

detected in the infected cells from 8 to 24 h p.i. in the presence of IPTG, and its production was blocked by araC (Figure 24B), demonstrating that expression of the G3L gene was tightly regulated at the late phase by IPTG.

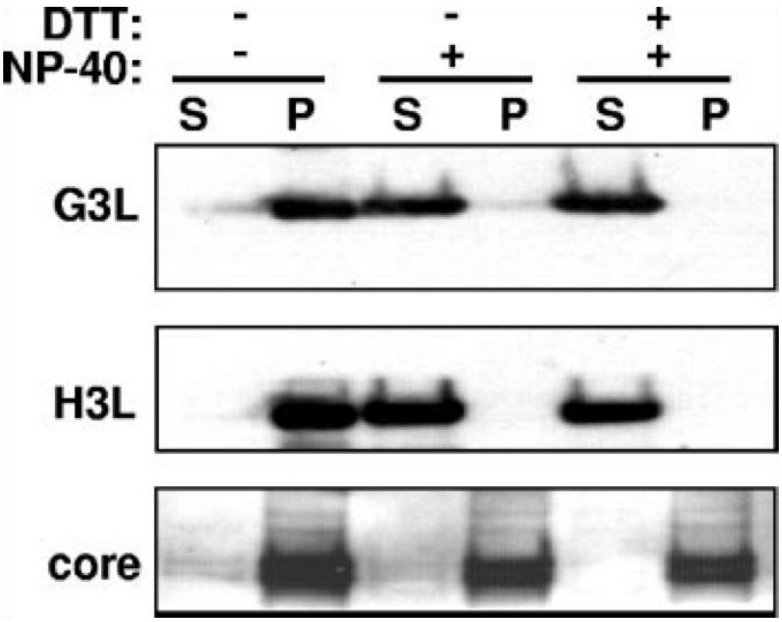


Figure 23. Membrane and core proteins after NP-40–DTT extraction of vaccinia MV. Sucrose-purified vaccinia MVs were incubated with buffer containing 1% NP-40 with or without 50 mM DTT. After centrifugation, the supernatant (S) and pellet (P) were analyzed on immunoblots with antibodies to G3L, H3L as knowen membrane protein control, or core proteins as indicated in Materials and Methods.

3.4.4. G3L is required for plaque formation and MV production in cell culture.

The role of G3L during vaccinia viral infection was examined in BSC40 cells infected with viG3L in the presence or absence of IPTG. As shown in Figure 25A, at 3 days p.i. the control parental virus, vT7LacOI, formed similar plaques on BSC40 cells in the presence or absence of IPTG, whereas viG3L only formed plaques in the presence of IPTG, thus showing that G3L expression is required for plaque formation.

The viG3L titer was measured in cell culture by using one- step growth analysis. BSC40 cells were infected with viG3L at an MOI of 5 PFU per cell and cultured in the presence or absence of IPTG, and then cell lysates were collected and the virus titers were determined. As shown in Figure 25B, viG3L showed a 2-log increase in titer at 24 and 48 h p.i., similar to the vT7lacOI parental virus, whereas viG3L grew poorly in the absence of IPTG, with no increase in titer at 24 or 48 h p.i., showing that G3L is required for vaccinia virus growth in cell culture.

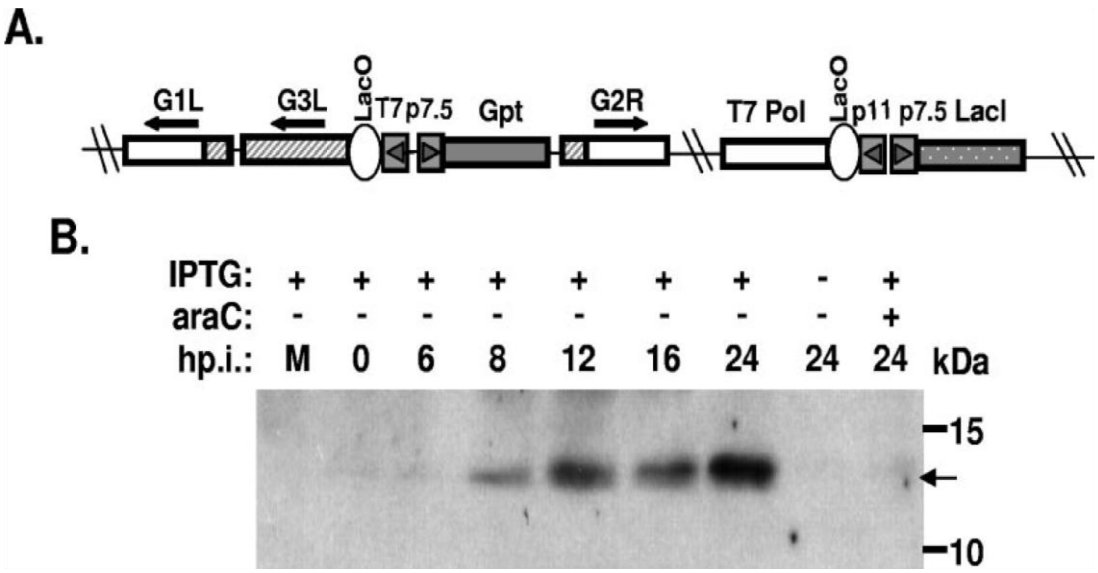


Figure 24. (A) Schematic diagram of viG3L virus. The G3L and J2R (tk) loci in the viG3L recombinant virus are indicated. The J2R locus contains T7 RNA polymerase and the lacI repressor gene as described previously (152). The inducible G3L (shaded box)/Gpt selectable marker (black box) gene cassette is also shown. The arrows indicate the transcription direction. Abbreviations: T7 Pol, bacteriophage T7 RNA polymerase; LacO, E. coli lac operator; LacI, E. coli lac repressor gene; Gpt, E. coli xanthine guanine phosphoribosyltransferase gene; p7.5 and p11, viral promoters; T7, promoter for T7 RNA polymerase. **(B) Expression of G3L in viG3L-infected cells.** BSC40 cells were infected with viG3L at an MOI of 5 PFU per cell, incubated in culture medium with or without 50 μM IPTG, then harvested at the indicated times, and subjected to SDS-PAGE and immunoblot analyses with the anti-G3L antiserum.

3.4.5. G3L is not required for virion morphogenesis of MV and EV

To demonstrate that mature virions were produced in cells, BSC40 cells infected with viG3L in the presence or absence of IPTG were analyzed at 12 and 24 h p.i. by electron microscopy. At 12 h p.i., viral crescents, immature virions, and other intermediate membrane structures were detected in cells infected with viG3L in the presence or absence of IPTG (Figure 26 A). At 24 h p.i., a large number of dense mature MV particles were detected in the cytoplasm of cells infected with viG3L in the presence or absence of IPTG (Figure 26B). These MV particles in both cells appeared to be indistinguishable from each other. We therefore concluded that G3L is not required for the formation of MV

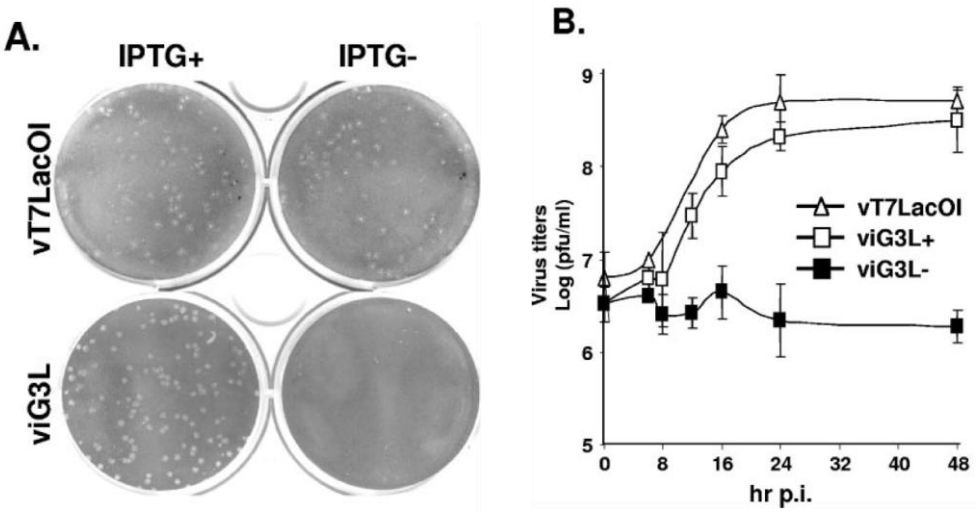


Figure 25. (A) ViG3L virus does not form plaques in BSC40 cells when the expression of G3L protein is repressed. BSC40 cells were infected with viG3L and incubated in medium with or without IPTG for 3 days, fixed, stained with crystal violet, and photographed. VT7LacoI parental virus serfes as control. **(B) One-step growth curve analysis of viG3L.** BSC40 cells were infected with control vT7LacOI or viG3L at an MOI of 5 PFU per cell and then incubated in the presence (viG3L+) or absence (viG3L-□of 50 μM IPTG for 0, 8, 12, 16, 24, or 48 h p.i. Virus titers in the lysates were determined by plaque formation on BSC40 cells. These experiments were performed three times.

3.4.6. Actin tail formation in association with EV in Vaccinia infected cells

A fraction of MV in cells is transported to the Golgi, where they are wrapped in additional membranes to form EV, which, upon fusion with the plasma membrane, form CEV, with localized actin polymerization that can be readily detected as an "actin tail" by confocal microscopy (35, 137). HeLa cells were infected with viG3L in the presence or absence of IPTG, fixed at 17 p.i., and stained with phalloidin to examine actin tail formation. As shown in Figure 27, formation of actin tails with CEV (anti-B5R MAb) at the tips was seen in the infected cells in the presence or absence of IPTG. The kinetics and the extent of actin tail formation appeared comparable in these cells, showing that G3L is not required for CEV formation.

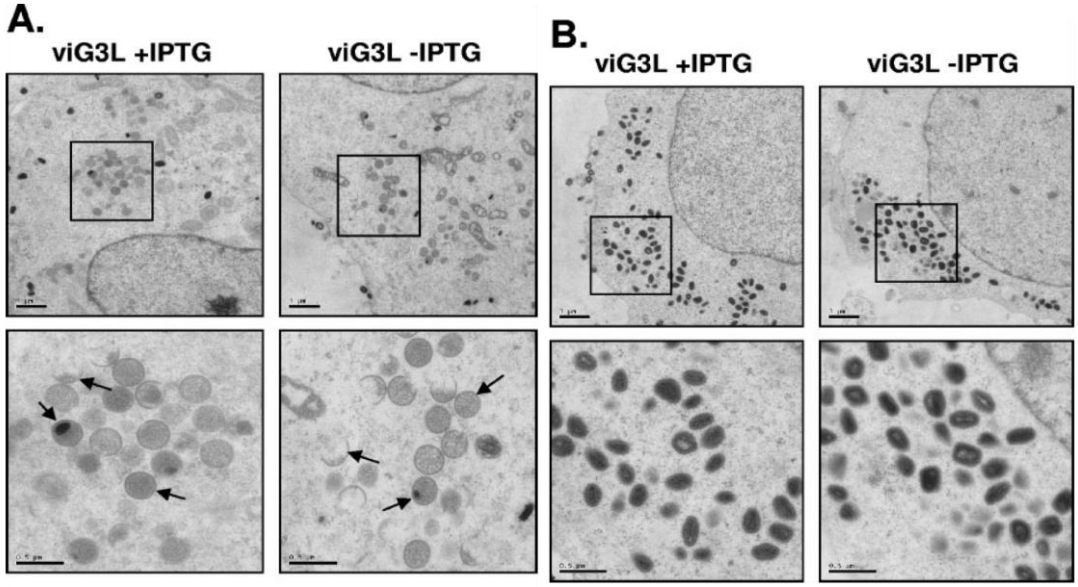


Figure 26. Electron micrographs of vaccinia virion morphogenesis in cells infected by viG3L. BSC40 cells were infected with viG3L in the presence or absence of IPTG and then fixed at **12 h (A)** or **24 h (B)** p.i. for electron microscopy. Photographs were taken at magnifications of □3,000 (top rows in panels A and B) and □9,300 (lower rows in panels A and B). The arrows in panel A represent typical viral intermediate structures such as crescents and IV.

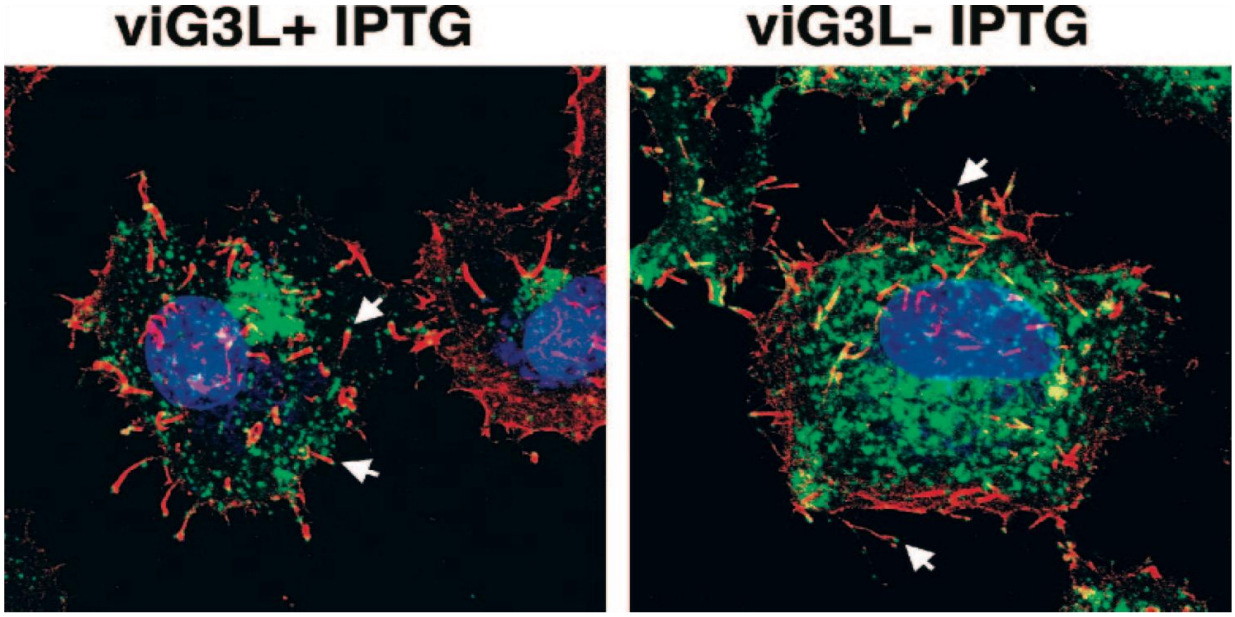


Figure 27. Actin tail formation in association with EV-CEV in Vaccinia infected HeLa cells. HeLa cells were infected with viG3L in the medium with (+IPTG) or without (-IPTG) 50 μM IPTG for 17 h p.i., fixed with 4% paraformaldehyde, permeabilized, and stained for 1 h at room temperature with VV anti-B5R MAb (1:2,500) (green) and Alexa Fluor 647-phalloidin (red). DNA was visualized by staining with DAPI (blue). The white arrows indicate representative EV-CEV with actin tails on their tips. See Materials and Methods 2.2.13.

3.4.7. MV particles devoid of G3L have a normal morphology and major protein content

MV particles containing G3L ($G3L^+$) or lacking G3L ($G3L^-$) were purified, respectively, from cells infected with viG3L cultured in the presence or absence of IPTG. The two types of MV particles ($G3L^+$ and $G3L^-$) showed the same sedimentation profile on sucrose gradient centrifugation (Figure 28A). When both types of MV virions were analyzed by electron microscopy, no detectable differences in morphology or size were detected (Figure 28B). SDS-PAGE analysis of these MV showed an identical major protein pattern (Figure 28C). Although G3L was not incorporated into purified MV, the abundance of the envelope proteins, L5R, A28L, H2R, and A21L, all of which known to interact with G3L, was not affected (Figure 28D).

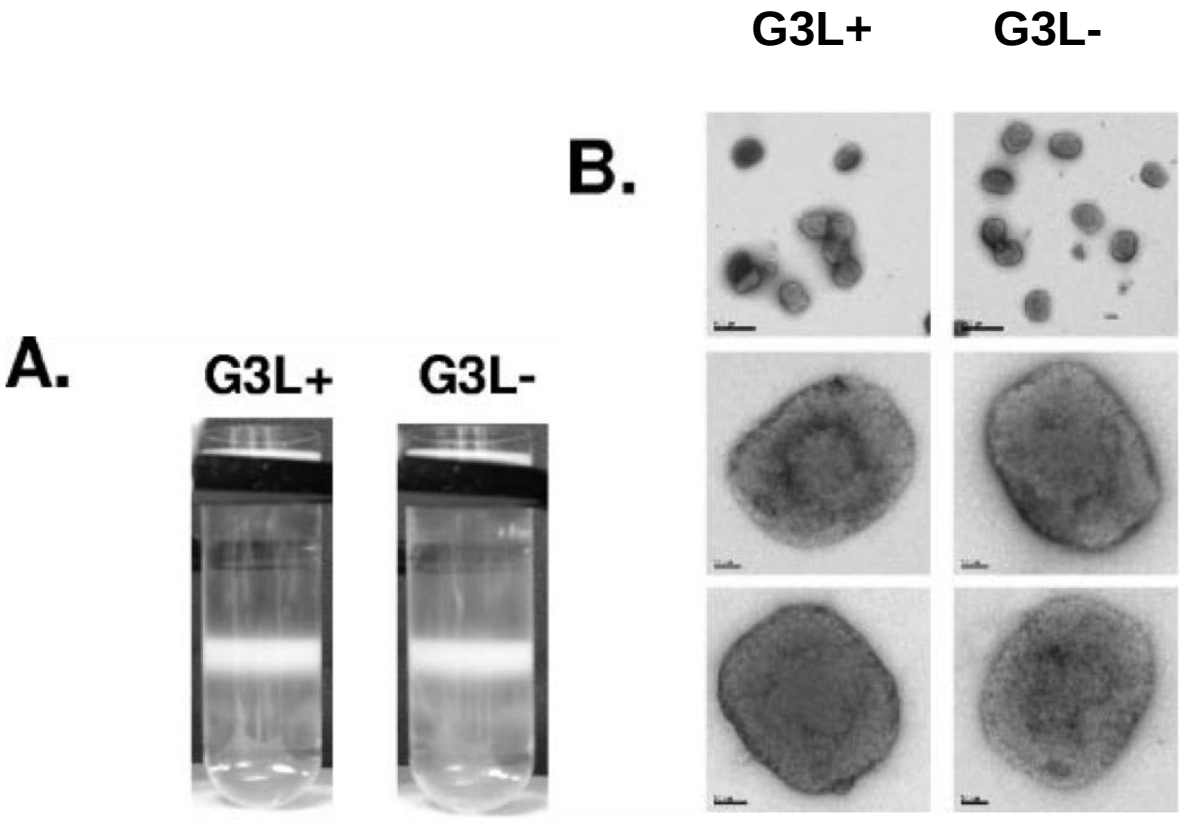


Figure 28. (A) Purified G3L$^+$ and G3L$^-$ MV on 25 to 40% sucrose gradients. (B) Electron micrographs of negatively stained G3L$^+$ and G3L$^-$ MV. Virions were deposited on grids, washed with water, and stained with 7% uranyl acetate in 50% ethanol for 30 s.

3.4.8. G3L is essential for MV penetration into cells

To investigate the role of G3L in the MV entry process, we used a previously described virus entry assay (149). HeLa cells were infected for 1 h at 4°C with purified G3L$^+$ and G3L$^-$ MVs and then examined by confocal microscopy to determine the number of MV particles bound to cells (Figure 29A). Alternatively, HeLa cells were infected as described above and then, after being washed, were cultured at 37°C for 2 h to allow MV penetration into cells before harvesting (Figure 29B). The infected cells were stained with anti-L1R MAb to detect bound virions on the cell surface or with anti-A4L antiserum to detect un- coated cores of penetrated virus in the cytoplasm. As

shown in Figure 29A, similar numbers of G3L^{+} and G3L^{-} MV particles bound to cells, indicating that G3L is not required for MV binding to cells. In contrast, as shown in Figure 29B, significant A4L staining was only seen in cells infected with G3L^{+} virus and not with G3L^{-} virus indicating that G3L viruses penetrated into the cells and successfully uncoated inside the cells. These results showed that G3L plays an essential role in MV penetration into cells.

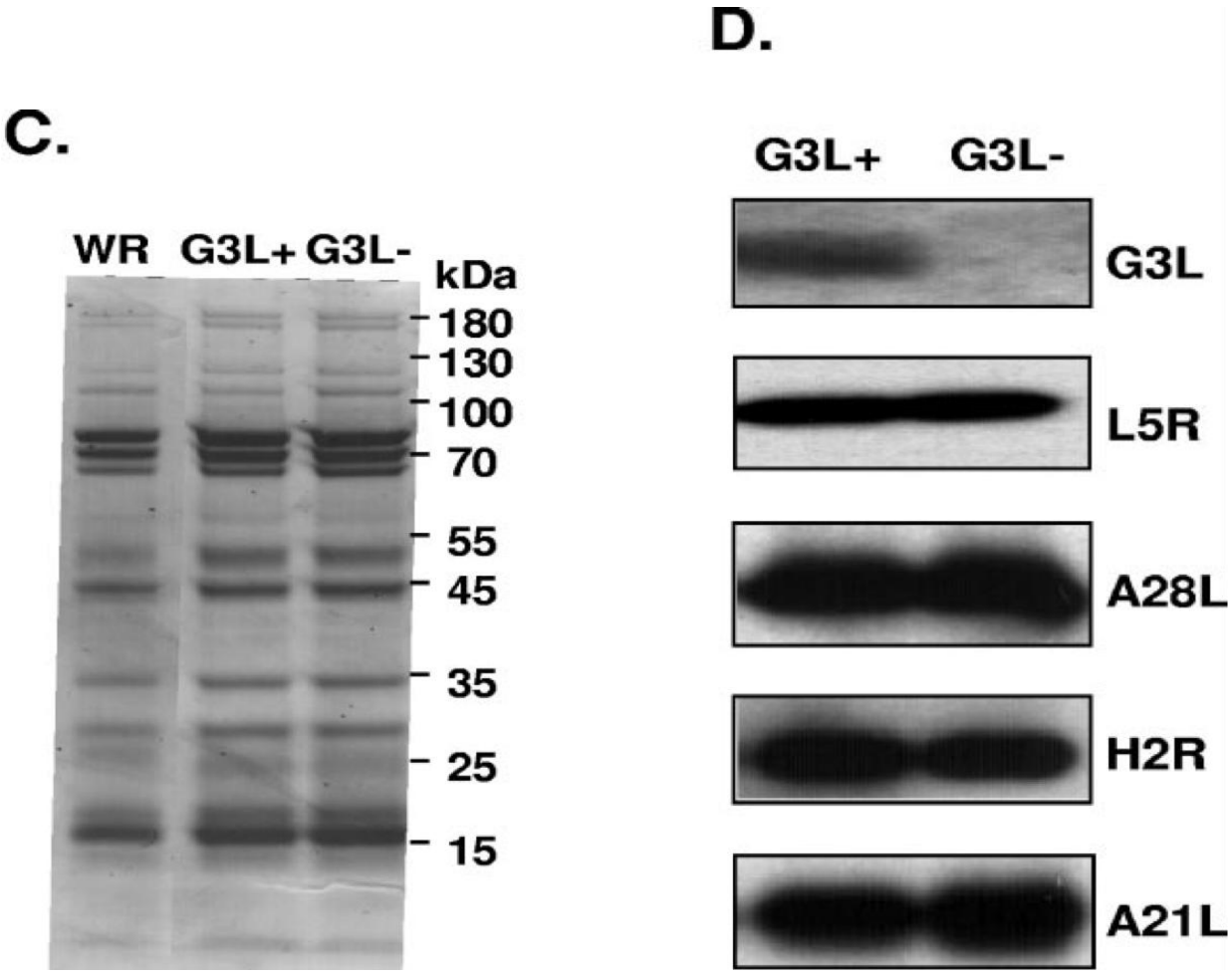


Figure 28. (C) SDS-PAGE of sucrose gradient-purified wild-type (WR) vaccinia, G3L^{+} or G3L^{-}□MV. The numbers of particles were determined from the optical density at 260 nm, and equal amounts of the three types of virions were analyzed by SDS-PAGE with Coomassie blue staining. **(D) Immunoblotts of G3L^{+}□and G3L^{-}□MV.** Equivalent amounts of G3L^{+}□and G3L^{-}□MV were separated on SDS-PAGE and analyzed by immunoblots with antibodies recognizing the indicated envelope proteins.

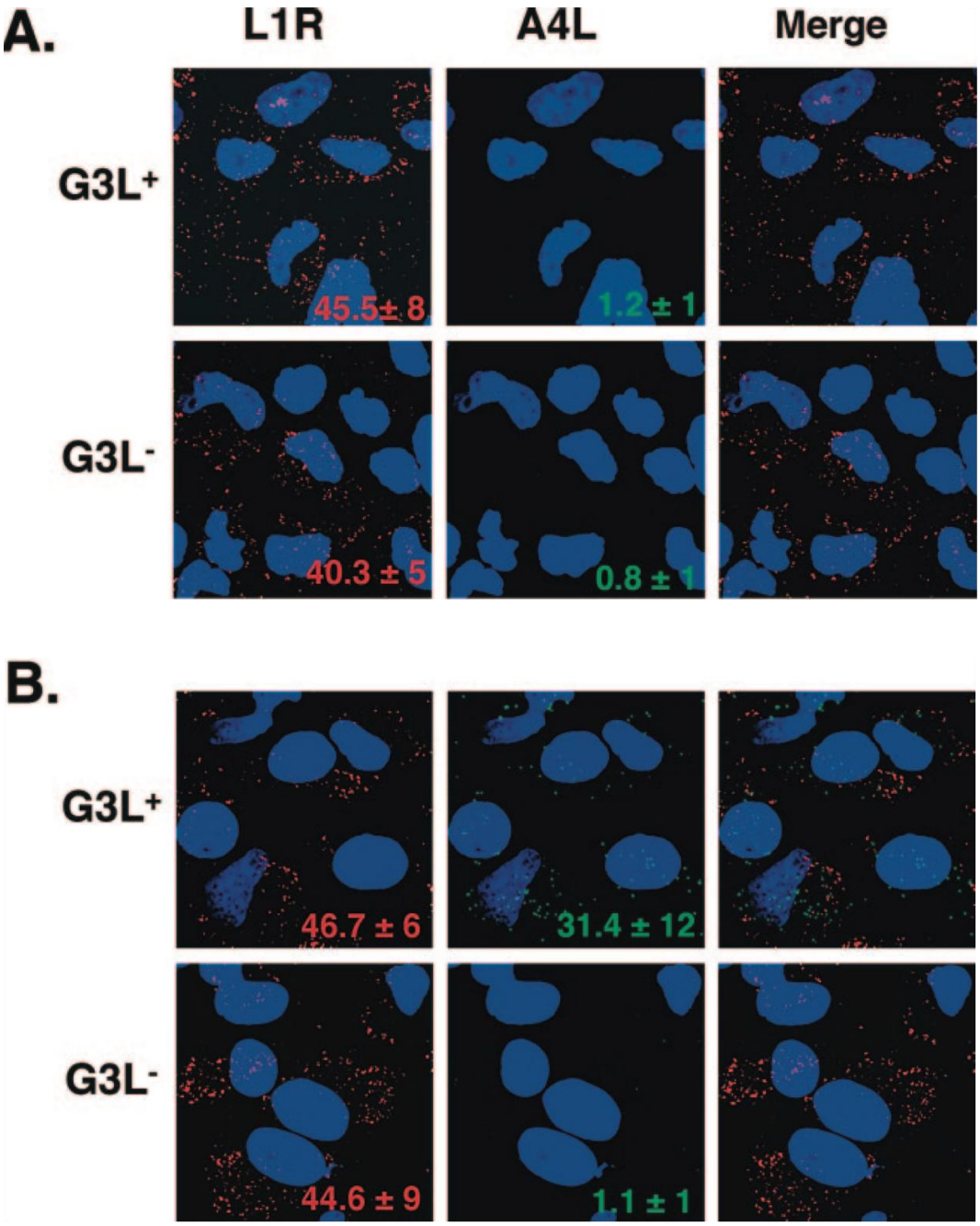


Figure 29. G3L is required for virion penetration to release cores into the cytosol. HeLa cells were infected for 1 h at 4°C with equivalent amounts of G3L^{+} and G3L^{-} virions c**ells were either immediately fixed (A)** or incubated for a further 2 h at 37°C in the presence of cycloheximide **before fixation (B).** Cells were stained with anti-L1R MAb, followed by Cy5-conjugated goat anti-mouse IgG antibody (red) or with rabbit anti-A4L antiserum, followed by FITC-conjugated goat anti-rabbit IgG antibody (green). DNA was visualized by staining with DAPI.

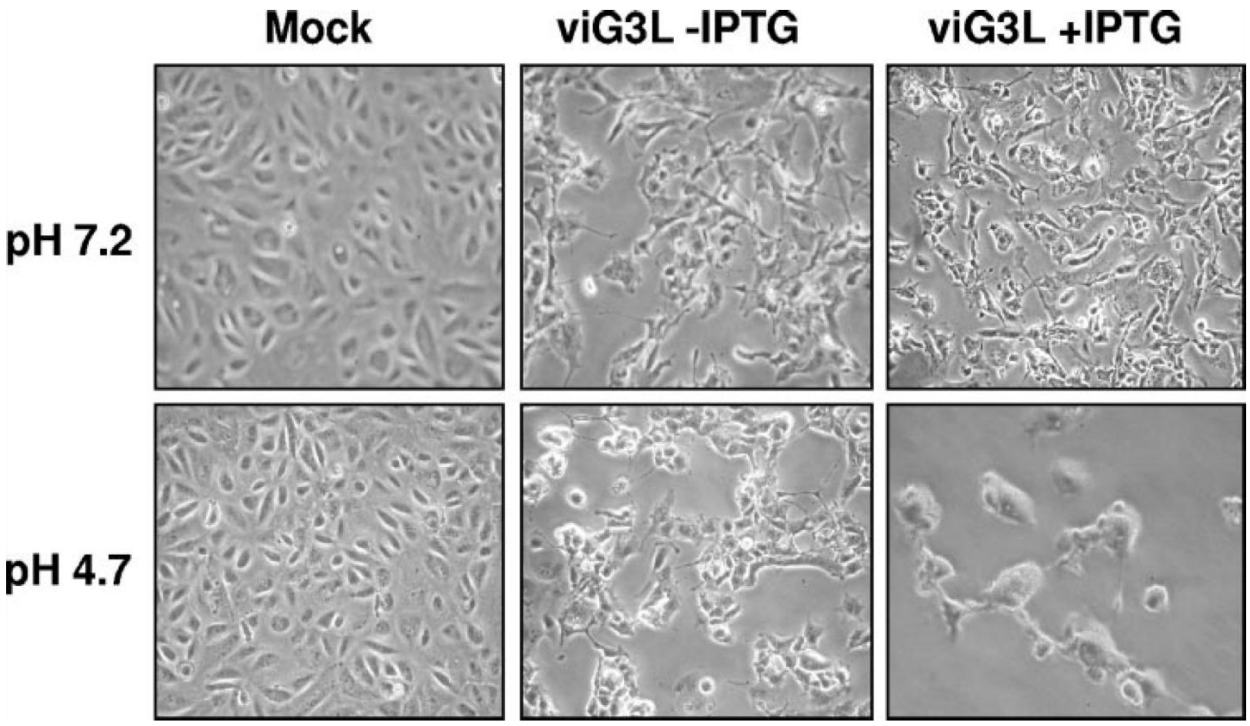


Figure 30. G3L is required for cell fusion. Freshly confluent BSC-1 cells were infected with viG3L virus at an MOI of 5 PFU per cell and incubated at 37°C for 21 h in medium with or without IPTG. Cells were washed three times with PBS (pH 7.2) and treated with PBS (pH 7.2) or PBS (pH 4.7) for 3 min at room temperature, and washed again, and the PBS was then replaced with normal medium. These cells were incubated for another 3 h and photographed with a Nikon inverted microscope.

3.4.9. G3L is essential for cell-cell fusion induced by low-pH treatment

Cells infected by vaccinia virus undergo cell fusion when they are briefly incubated in acidic buffer with a pH of 6 (41, 56). Although the parameters mediating virus and cell fusion (fusion from without) are not necessarily identical to those for the fusion of infected cells (fusion from within), both cell fusion assays have been widely used to investigate virus-mediated cell fusion. Moreover, several components of viral entry-fusion proteins-A28L, H2R, L5R, and A21L were

shown to be required for low pH-induced cell fusion from within or fusion from without (132, 133, 144), Since G3L protein is essential for MV penetration, its role in cell fusion from within was examined (Figure 30). BSC-1 cells infected with viG3L virus and maintained in medium containing IPTG did not develop cell fusion at neutral pH at 24 h p.i. However, when these cells were briefly treated with acidic buffer they developed into gigantic fused cells. In the absence of IPTG when G3L protein is not expressed no cell fusion was observed at either neutral or acidic buffer. We thus conclude that G3L protein is required for low-pH-triggered cell fusion.

CONCLUSIONS

1. It is shown for the first time that lipid raft protein integrin β1 is important for Vaccinia virus entry. It associates with Vaccinia virus mature particles in lipid raft microdomain and is mediates both Vaccinia virus attachment and penetration steps.
2. Intracellular integrin β1 downstream signaling PI3K/Akt is crucial for Vaccinia virus entry into cells and is dependant from integrin β1. Vaccinia virus mature particles bind to the cells and activate PI3K/Akt phosphorilation. Moreover, pharmocological inhibitors of PI3K (LY294002) and/or Akt (Akt IV) reduce Vaccinia virus infection in both MEF and HeLa cells.
3. Outside-in activation of integrin β1 facilitates Vaccinia MV entry. Induced virus infection was observed in HeLa cells that were plated to extracellular matrix proteins fibronectin and laminin, known to be integrin ligands, but not to the HeLa cells that were plated onto control dishes pre-coated with poly-L-lysine (PLL), as cell attachment to the poly-L-lysine is independent of integrins.
4. First time investigated, small, 49aa Vaccinia *WR-53.5* protein is expressed abundantly on the surface of virus infected cells and regulates cell morphology and adhesion. Attachment phenotype of *WR53.5* protein is Ca2+-independent.
5. WR53.5 protein is not required for plaque formation and MV/EEV production in cell culture.
6. *WR53.5* contributes to Vaccinia virus virulence in vivo.
7. Vaccinia *WR-G3L* gene is conserved encodes a late viral envelope protein and present in the membrane fraction of Vaccinia mature virion.
8. First time was shown Vaccinia virus *WR-G3* protein is essential for virus entry, plaque formation, mature virus production and virus penetration into cells.
9. *WR-G3* protein mediates low-pH-triggered cell-cell fusion and is an essential component of entry fusion complex.

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