

2005-07-13

Mechanisms of Host-Range Function of Vaccinia Virus K1L Gene: a Dissertation

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A Dissertation Presented

By

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Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical sciences, Worcester

In partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

JULY 13, 2005

DEPARTMENT OF MOLECULAR GENETICS AND MICROBIOLOGY

Mechanisms Of Host-Range Function Of Vaccinia Virus K1L Gene.

A Dissertation Presented
By
Ritu Rakshit Bradley

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Parts of this dissertation have been presented in the following publication:

Title: Vaccinia virus K1L protein mediates host-range function in RK-13 cells via ankyrin repeat and may interact with a cellular GTPase-activating protein.

(In Press, Virus Research, Elsevier)

Authors: Ritu R. Bradley and Masanori Terajima.

ACKNOWLEDGEMENTS

I would like to thank The University of Massachusetts Medical Center, Worcester, for allowing me the opportunity to grow through this very enlightening experience.

I would like to thank the Center for Infectious Diseases and Vaccine Research.

I would like to thank my mentor Dr. Masanori Terajima for being the kindest and the most patient mentor that I could ever have. I thank him for teaching me good science and for always being ready and willing to answer all my questions. I would like to thank Dr. Francis A. Ennis for all his support in all these years through easy and difficult times. I will remember his advice and words of wisdom and will carry that wisdom with me wherever I go.

I would like to thank my mother and father for making this day possible. Without their love and affection, wisdom, and their unending support in letting me follow my heart, this day would not have been possible. I would like to thank especially my brother, Arun, for being a lover of science and sharing with me the marvels of it through all these years. I would like to thank my husband Sean, for being part of this endeavor, through good times and bad. His unending support and confidence in me, has allowed me to get over the disappointments and difficulties that I encountered during this process. I would like to thank the rest of my families, both in India and here, for all their love and affection. I would also like to thank all my friends who have supported me through all these years with their good wishes.

ABSTRACT

The K1L gene of vaccinia virus encodes for a host range protein; in the absence of which, the virus is unable to grow in certain cell lines (RK-13 and some human cell lines). K1L function can be complemented in RK-13 cells by the cowpox host range gene product CP77 despite a lack of homology between the two proteins except for ankyrin repeats. We investigated the role of ankyrin repeats of the K1L gene in the host-range restriction of growth in RK-13 cells. The growth of a recombinant vaccinia virus, with the K1L gene mutated in the most conserved ankyrin repeat, was severely impaired as evidenced by lack of plaque formation and reduction in viral titers. Infection of RK-13 cells with the mutant recombinant vaccinia virus resulted in total shutdown of both cellular and viral protein synthesis early in infection, indicating that the host restriction mediated by the ankyrin repeat is due to a translational block. A comparison of the cellular localization of the K1L wild type and mutated forms showed no difference, as both localized exclusively in the cytoplasm of RK-13 cells. We also investigated the interaction of the vaccinia virus K1L protein with cellular proteins in RK-13 cells and co-immunoprecipitated a 90 kDa protein identified as the rabbit homologue of human ACAP2, a GTPase-activating protein with ankyrin repeats. Our result suggests the importance of ankyrin repeat for host-range function of K1L in RK-13 cells and identifies ACAP2 as a cellular protein which may be interacting with K1L.

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ABBREVIATIONS

ACAP	<u>A</u> rf GAP with <u>c</u> oiled coil, <u>A</u> NK repeat and <u>P</u> H domains
ANK	Ankyrin
ARF	ADP-ribosylation factor
ASAP1	<u>A</u> rf GAP containing <u>S</u> H3, <u>A</u> NK repeats, and <u>P</u> H domain
BHK	Baby hamster kidney
53BP2	p53 binding protein 2
C3b	Complement 3b
CDK6	Cyclin dependent kinase 6
CEF	Chicken embryo fibroblasts
CEV	Cytoplasmic enveloped virion
COPI	Coat protomer I
CTA	Cholera toxin
CTL	Cytotoxic T Lymphocyte
DNA	Deoxyribonucleic acid
EEV	Extracellular enveloped virion
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
EIF-2	Eukaryotic Initiation Factor-2

ER	Endoplasmic Reticulum
GABP-α	GA-binding protein
GAP	GTPase-activating protein
GBP	Guanylate binding protein
GDP	Guanosine 5'-diphosphate
GEP	Guanine nucleotide exchange protein
GTP	Guanosine 5'-triphosphate
IEV	Intracellular enveloped virion
IκB	Inhibitors of NF- κ B
IL-1β	Interleukin -1 β
IL-1R	Interleukin-1 Receptor
IL-18	Interleukin-18
IL-18BP	Interleukin-18 Binding protein
IMV	Intracellular Mature Virion
IFN	Interferon
ITR	Inverted Terminal Repeats
JAK	Janus kinases
MALDI	Matrix assisted laser desorption ionization
mRNA	Messenger RNA
MOI	Multiplicity of infection
MVA	Modified vaccinia virus Ankara
NF-κB	Nuclear factor κ B
NLS	Nuclear localization signal

NYCBH	New York City Board Of Health strain of VV
ORF	Open Reading Frame
PA	Phosphatidic acid
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol 4, 5-bis-phosphate
PIP3	Phosphatidylinositol-3, 4,5-trisphosphate
PLD	Phospholipase D
PBMC	Peripheral blood mononuclear cells
PKR	Protein kinase R
PSD	Post source decay
STAT	Signal transducers and activators of transcription
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
VC-2	Copenhagen strain of VV
VV	Vaccinia Virus
WR	Western Reserve strain of VV

CHAPTER I

INTRODUCTION

A. Vaccinia Virus: A Member Of The *Poxviridae* Family Of Viruses

Vaccinia virus (VV) is a member of the *Poxviridae* family of double stranded DNA viruses. One of the unique features of this family is that unlike other DNA viruses, they do not replicate in the nucleus (Moss, 2001). The *Poxviridae* family consists of two subfamilies; *Entomopoxvirinae* (viruses infecting arthropods) and *Chordopoxvirinae* (viruses infecting vertebrates). Within the subfamily of vertebrate poxviruses there are eight genera, one of which is *Orthopoxvirus*. VV belongs to the *Orthopoxvirus* genus along with variola virus, cowpox virus, monkeypox virus, etc. (Table 1) (Esposito, 2001) (Fenner, 2000). The members of the *Orthopoxvirus* genus cannot be distinguished morphologically. They are related antigenically and share considerable cross reactivity between the species. Thus, a previous infection with any one of the species would offer some protection against any other member of the genus (Esposito, 2001).

Table 1: Members of the Orthopoxvirus genus

Genus and species	Reservoir host	Geographic distribution	other naturally occurring host
Camelpox virus	camels	Africa, Asia	Nil
Cowpox virus	rodents	Europe, western Asia	cats, cows, humans
Ectromelia virus	rodents	Europe	Nil
Monkeypox virus	Squirrels	Western and central Africa	monkeys, humans
Raccoonpox virus	Raccoons	Eastern USA	Nil
Skunkpox virus	Skunks	Western USA	Nil
Taterapox virus	Gerbils	Western Africa	Nil
Uashin gishu virus	Unknown	Eastern Africa	Horses
Vaccinia Virus	Unknown	Worldwide	Humans, rabbits, cows River buffaloes
Variola virus	Humans	Worldwide (eradicated)	Nil
Volepox virus	Voies	Western USA	Nil

(Esposito, 2001).

1. Human infections caused by four members of this genus are as follows:

Variola virus

Variola virus is a strictly human pathogen. It has an incubation period of 12 days. A period of high fever, head and backache, and malaise, lasting about 2-5 days is followed by maculopapular rash. The rash becomes vesicular and then pustular within 1-2 days. Death occurs either late in the first week or the second week of the illness and is associated with extensive viremia. The mortality rate during naturally occurring smallpox infections was 30% (Henderson, 1999) (Esposito, 2001).

Monkeypox virus

Monkeypox virus can cause a smallpox-like disease in humans (Esposito, 2001). Inter-human spread is very unlikely. The year 2003 saw an outbreak of monkeypox in humans in the United States. The infection started in a pet housing facility in Illinois, when infected rodents from Africa transmitted the virus to native prairie dogs housed in the same facility. Human infections resulted after close contact with their pet prairie dogs. There was a wide spectrum of clinical manifestations in the patients. Patients with a previous history of smallpox vaccination showed milder symptoms. Most of the 37 patients had a mild, febrile, rash illness. One patient had to be hospitalized for severe encephalitis (Reed et al., 2004) (Ligon, 2004).

Cowpox virus

Cowpox virus was originally regarded as a disease of European cows, causing ulcers. It was later observed that the cow is an ancillary host, being infected from a

rodent reservoir. Humans coming in contact with cows, cats, and zoo animals acquire a localized pustular skin infection (Esposito, 2001) (Baxby and Bennett, 1997).

Vaccinia virus

Strains of VV used for vaccination against smallpox cause dermal pustules and, in some cases, can cause severe side effects. The most common side effects observed were as follows:

- Progressive vaccinia usually occurs in individuals with congenital or acquired immunodeficiency. In persons suffering from agammaglobulinemia, defective cell-mediated immunity and other conditions associated with a suppressed immune system, the vaccinia lesions do not heal. One third of such patients die (Esposito, 2001) (Lane and Millar, 1969) (Lane et al., 1970).
- Eczema vaccinatum: People that previously suffered from eczema are susceptible to this kind of complication (Lane and Millar, 1969). This occurred at a rate of 66 in 14.5 million people, with no deaths. Eruptions occur at sites that are, or had been, eczematous and may eventually spread to normal skin. Persons suffering from this complication have high fever and general lymphadenopathy. Mortality in such individuals can be controlled by treating them with vaccinia immunoglobulin (Esposito, 2001).

▪ Generalized vaccinia is manifested by the breakout of rash all over the body at 6-9 days post vaccination (Brown, 1965) and occurred in 141 cases among 14.5 million people vaccinated. Lesions develop at locations other than the site of vaccination. No specific therapy is needed and patients may experience high fever and malaise (Esposito, 2001).

A subspecies of VV, buffalopox virus, mainly found in India, causes oral and skin lesions in individuals coming in contact with the animals or drinking their milk (Esposito, 2001).

2. Historical background of Variola infections and its eradication using VV

The most notable of the *Orthopoxviruses* is Variola virus, a strictly human pathogen and the causative agent of smallpox. The earliest record of smallpox comes from 1157 B.C. from the mummified remains of Ramses V (Henderson, 1999) (Behbehani, 1983). There have been major outbreaks of smallpox with high mortality all around the world, until its eradication in 1980. The name variola is derived from the Latin “*varius*” (spotted) or “*varus*” (pimple) as coined for the first time by Bishop Marius of Switzerland (Henderson, 1999) (Moore, 1815). English accounts of the disease used the word *pockes*. Variola infection was called smallpox in order to distinguish it from the Greatpox (syphilis) (Henderson, 1999) (Creighton, 1894). The history of immunization against smallpox goes back to sometime before 1000 A.D in India, where it was common to inoculate pustule fluid or scab material by either intranasal insufflations or by direct application to the skin. These practices made their way into China, western Asia, and were introduced to Europe in the early 1700s (Henderson, 1999). In 1796, Edward Jenner was able to show that inoculation using cowpox lesion material, or *variola vaccinae* (smallpox of the cow) could confer protection from smallpox in humans. He showed that material taken from the human pustular lesions caused by cowpox virus, when inoculated into the skin of another person, protected the individual from subsequent smallpox infection. However, sustaining the virus through arm-to-arm inoculation presented many

difficulties and was complicated by the transmission of syphilis among the inoculated individuals. The problem was eventually solved by growing the virus on the flank of a calf, and this provided an adequate and safer supply of vaccine material (Henderson, 1999). According to Jenner's writings, he also used lesion material from horses suffering from a disease called "grease", which is now extinct. Many passages of the virus, that he and other vaccinators had used, gave rise to what is now considered as a different virus species, known as vaccinia virus. General vaccinations came to an end after the WHO declared this disease eradicated in 1980, but vaccination of laboratory workers and some military personnel continued (Henderson, 1999).

3. Use of VV as recombinant vaccines in the post eradication era

Descriptions of recombination between VV and DNA fragments of other poxvirus gave rise to the idea of using VV as recombinant virus, carrying foreign genes that could serve as vaccines against diseases other than smallpox. The reasons for VV being a good vector are as follows:

a) VV can accommodate large amounts of foreign DNA, up to 25 kbp (Smith and Moss, 1983), so that no deletions are needed. This could lead to one recombinant virus expressing many foreign antigens and serve as a polyvalent vaccine in a single vaccination. This was exemplified by experiments where a recombinant VV

containing the hepatitis B surface antigen, herpes simplex glycoprotein D, and influenza virus hemagglutinin was injected into rabbits which then produced antibodies against all three antigens (Perkus et al., 1985).

b) The vaccine is extremely stable when freeze dried (Henderson, 1999).

c) It is cheap to make and easy to administer (Henderson, 1999).

However, as a vaccine for smallpox, the VV had an imperfect safety record and needed to be attenuated. In an attempt to attenuate the virus, researchers created deletions and this led to the discovery of many VV genes that are involved in host responses to infection. This opened up a whole realm of study involved in unraveling the details of the very complex biology of VV.

B. VV Biology

1. VV Structure VV consists of an enveloped virion containing a core, which encloses the double stranded DNA genome.

a) Vaccinia virus virions as visualized by cryoelectron microscopy appear as smooth rectangles (Moss, 2001) (Dubochet et al., 1994). The virion consists of an envelope surrounding a core, which encloses the double stranded DNA genome. Vaccinia virus can exist as four forms: 1) an envelope-less cytoplasmic infectious particle called IMV (intracellular mature virion), 2) an intracellular enveloped form called

IEV (intracellular enveloped virion), 3) a membrane bound form at the cell periphery called CEV (cytoplasmic enveloped virion), and, 4) an enveloped EEV (extra cellular enveloped virion) (Smith et al., 2002).

b) The VV core has a rectangular shape and the wall of the core is composed of two layers. The outer layer consists of a cylindrical subunit, and the inner layer is 5nm thick and smooth in texture (Easterbrook, 1966). The core is dumbbell shaped and is found associated with structures called *lateral bodies*. The core encloses the VV genome.

c) The VV genome is very large, approximately 200 kbp, encoding more than 200 gene products. The ORFs of VV are named after the 16 HindIII restriction enzyme digested fragments, by decreasing order of sizes, from A to P. The alphabet is followed by the ORF number (from left to right) within the fragment. The ORF designation also has L or R (left or right), depending on the direction of the reading frame (Moss, 2001). The genome has inverted terminal repetitions (ITRs) (Garon et al., 1978). These are identical sequences, oriented in opposite directions, occurring at the two ends of the genome. The ITRs contain an AT rich region, that is incompletely base-paired and forms a hairpin loop (Baroudy et al., 1982). The sequences of several vaccinia virus strains are now available, e.g. the Copenhagen

strain of VV(VC-2) (Goebel et al., 1990), the Western Reserve (WR) (Unpublished; Genbank Accession number AY243312) and the modified vaccinia virus Ankara (MVA) (Antoine et al., 1998) have been completely sequenced.

2. VV Life Cycle (Figure 1)

a) VV entry: The exact mechanism of VV entry into cells is not yet clear. The fact that VV can enter almost any cell makes the possibility of a ubiquitous molecule being the receptor very plausible (Moss, 2001). However, no receptor has yet been identified for the poxviruses. A report of VV binding to epidermal growth factor (EGF) receptors exists, but it does not explain which form of VV binds to the receptor (Eppstein et al., 1985) (Marsh and Eppstein, 1987). Another proposal suggested that poxviruses use chemokine receptors, but this remains to be substantiated (Lalani et al., 1999).

Four VV proteins (all IMV proteins) have been implicated in the entry of the VV into the cells. They are L1R (Ichihashi and Oie, 1996), A27L, D8R, and H3L (Lin et al., 2000). The infectivity of the IMV can be abrogated by adding antibodies to L1R and A27L. A deletion mutant of H3L, which binds to heparin sulphate, bound to cells with lower efficiency under some conditions as compared to others. The binding and entry study for EEV has not been very successful, mostly because

of low amounts of EEV, fragility of the outer envelope of EEV, and also because it is difficult to obtain pure preparations of IMV- free EEV (Moss, 2001).

b) Uncoating of the VV core: The cores come with some early proteins incorporated within them. EM images have revealed that the viral DNA is extruded out of pores in the core (Moss, 2001).

c) Early Gene expression: Almost half of the VV genes belong to the early category and are transcribed before replication (Boone and Moss, 1978) (Paoletti and Grady, 1977). The early genes are transcribed within minutes after entry of the virus into the cell (Baldick et al., 1992). The viral RNA polymerase is similar to a eukaryotic polymerase in size and subunit complexity (Baroudy and Moss, 1980). Early mRNA is synthesized within the core and then extruded out in a microtubule dependent manner. Early mRNAs extruded from the core are assembled in the cytoplasm, distinct from the core (Mallardo et al., 2001). The polyribosomes and other translational machinery are recruited to this region and the early transcripts are translated to early proteins. These early proteins are required for viral replication to progress. Some early genes are as follows: *MIL*, *K1L*, *A33R*, *A56R*, and *F12L*.

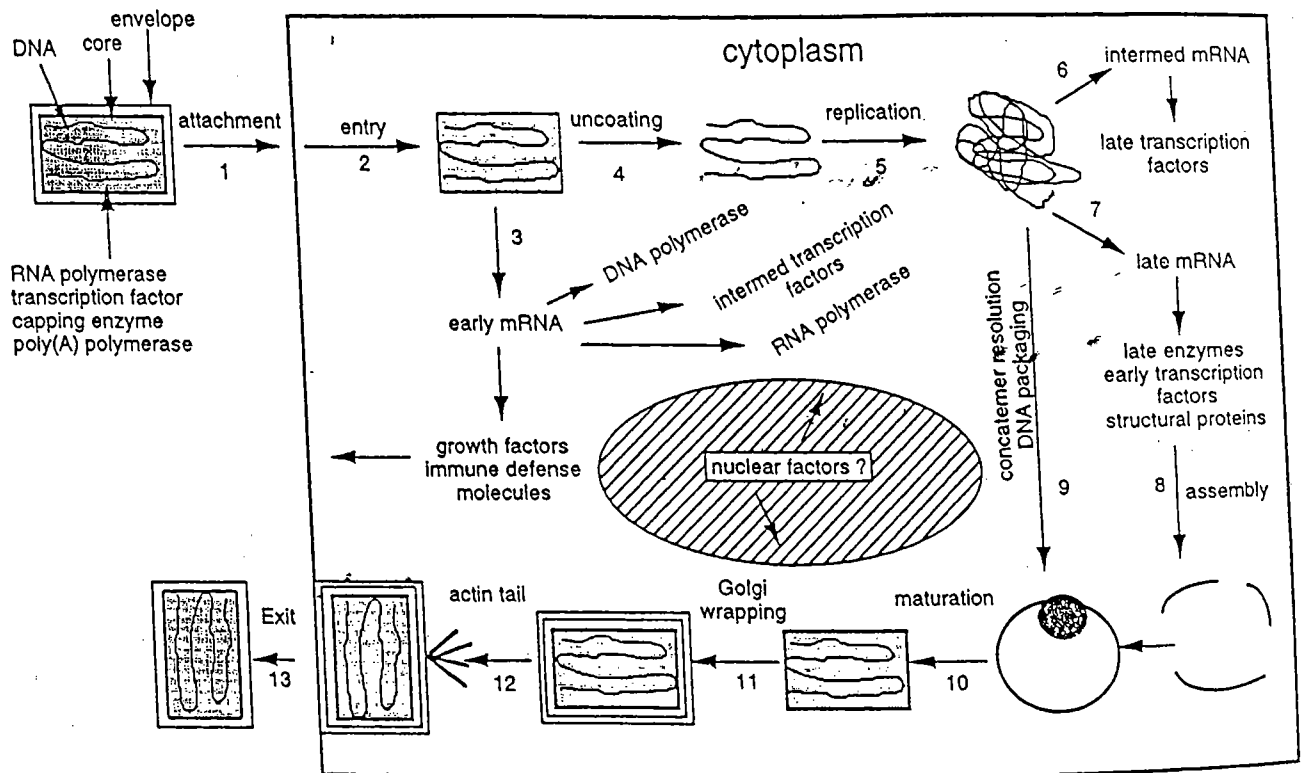
d) DNA replication: Poxviruses are distinct from other DNA viruses in that they replicate in the cytoplasm of the cell instead of the nucleus. Distinct regions (called

viroplasm, viral factory or factory areas) in the cytoplasm have been characterized by light and electron microscopy (Cairns, 1960) (Harford et al., 1966) (Moss, 2001). The time when DNA synthesis starts varies between different members of the poxvirus family. However, it usually starts 1-2 hours after infection (Joklik and Becker, 1964) (Moss, 2001). Soon after the uncoating of the core in the cytoplasm, the viral genome is extruded out of the core and the DNA associates closely with the membrane of the endoplasmic reticulum (ER). Soon after the initial DNA synthesis at 2 hrs, the replication sites are, or become, completely surrounded by the ER membranes. When virion assembly starts, around 6 hrs post-infection, the ER dissociates from the replication sites. The presence of early proteins is required for the initiation of replication. There have been some viral genes shown to be involved in viral replication e.g., the *E9L* DNA polymerase (Beaud, 1995), *B1R* protein kinase (Banham and Smith, 1992)(Beaud, 1995), *D5R* nucleic acid-independent nucleoside triphosphatase (Evans et al., 1995), *D4R* uracil DNA glycosylase (Millns et al., 1994), the *H6R* DNA topoisomerase, *I3L* single stranded-DNA binding protein, *H5R* virosome-associated protein, and the *A50R* DNA ligase (Beaud, 1995). In addition, several viral-encoded proteins regulate the level of the deoxyribonucleoside triphosphate pool: the *J2R* thymidine kinase; *A48R* thymidylate kinase; *I4L* and *F4L* subunits of ribonucleotide reductase; and *F2L* dUTPase (Beaud, 1995).

e) Intermediate and late gene expression: Intermediate gene expression starts after DNA replication and the intermediate proteins serve as transcription factors for the expression of late gene products (Moss, 2001). Many of the late proteins are packaged into the vaccinia virion and mediate early protein synthesis soon after infection (Broyles, 2003). Some intermediate genes are: *H5R*, *A1L*, *A2L*, and *G8R* (Keck et al., 1990). Some late genes are: *A34R*, *B5R*, and *F13L*.

f) Packaging and expulsion of VV from cell: The intracellular immature virion moves to the cell periphery in a microtubule-dependent manner. The IMV gets wrapped in membranes derived from Golgi/Trans-Golgi (Ichihashi et al., 1971) (Hiller and Weber, 1985), or the endosomes (Tooze et al., 1993), to form IEV. There is controversy about the origin of the membranes, and they may come from both of the above mentioned sources. The F13L (Blasco and Moss, 1991) and B5R (Engelstad and Smith, 1993) proteins have been shown to be required for efficient wrapping. The IEV is an intermediate form between the IMV and the CEV. There is evidence that the movement of IEV from within the cytoplasm to the cell periphery is assisted by microtubules (Hollinshead et al., 2001) (Rietdorf et al., 2001) (Ward and Moss, 2001). These wrapped particles fuse with the plasma membrane. Some of the fused virus particles (CEV) get released into the medium as EEV, while others remain adherent to the cell surface. The viral protein A36R is responsible for the polymerization of actin on the cell surface (Frischknecht et al., 1999). The actin tail

can grow very long and could detach from the cell with the CEV at its tip (Smith et al., 2002).



Vaccinia Virus Life Cycle

Fig 1

C. Immune Responses to VV Infection

Both humoral and cell-mediated immunity are important in protection against *Orthopoxviruses*. Genetic defects in B-cell or T-cell immunity lead to susceptibility to side effects after vaccination. Studies in mice have demonstrated that both CD4 (+) and CD8 (+) T cell-mediated immunity protect against VV infection (Xu et al., 2004). Early in infection, in a naïve individual, the cytotoxic T lymphocyte (CTL) response is responsible for clearing infection and is the first response (Ada and Blanden, 1994) (Henderson, 1999). CD8 (+) T cells can confer protection in the absence of antibody and can provide protective memory. VV specific T-cell memory can last up to 50 years post-vaccination in the absence of antigen (Demkowicz et al., 1996). Epitope specific CD8+ T cell responses were analyzed in the peripheral blood mononuclear cells (PBMCs) of HLA-A2.1-positive donors after primary immunization. The frequency of epitope-specific CD8+ T cells peaked at two weeks after immunization and declined thereafter. They were, however, still detectable 1-3 years after primary immunization (Terajima et al., 2003).

As mentioned before, VV exists in four forms; IMV, IEV, CEV and EEV. EEV is mostly responsible for the long-range dissemination of the virus and spread of the virus *in vivo* (Payne, 1980). There are contradictory reports about the inhibition of EEV infectivity by neutralizing antibody. While one report says that EEV is resistant to neutralizing antibodies (Ichihashi and Oie, 1996), another claims

that EEV infectivity can be inhibited by antibody (Law, 2001). However, many studies have shown that antibody-mediated mechanisms cannot offer protection during a primary infection. Type I CD4+ T cells also help in the induction of long lasting CTL response (Henderson, 1999). Interferons (IFNs) also play a crucial role in poxvirus infections. IFN- $\alpha/\beta/\gamma$ can effectively control viral growth (Xu et al., 2004). Inflammatory cells like PMNs and macrophages also play an important role. Inflammatory cells may limit virus spread by phagocytosis of poxvirus virions that have been either antibody coated or C3b coated (Buller and Palumbo, 1991) and by secretion of interferons and chemokines (Buller and Palumbo, 1991). However, poxviruses have developed strategies to evade the host immune system.

D. Immune Evasion by *Orthopoxviruses*

The role of viral proteins in inhibiting host responses to infection was first demonstrated by the discovery of the regulation of the complement activation pathway by poxviruses (Kotwal et al., 1990) (Kotwal and Moss, 1988b).

Subsequently, many pox-viral proteins have been identified that interfere directly with host immune responses. The viral proteins responsible for immune evasion can function in the following ways:

1. Inhibition of complement activation

In response to viral infection, the complement pathway gets activated leading to the production of C3 convertase, which can cause the inactivation of virus and destruction of the infected cells (Tomlinson, 1993). The complement pathway is regulated by cellular proteins, limiting its harmful effects on the cell. The regulators have a common structural feature, a motif called the short consensus repeat (Reid and Day, 1989). VV encodes for a protein (VCP) containing four copies of this repeat, and it is secreted from infected cells (Kotwal and Moss, 1988b). The C3 convertases are rapidly degraded by VCP, inhibiting the complement activation pathway (McKenzie et al., 1992).

2. Inhibition of TNF

Tumor necrosis factors (TNFs) are pro-inflammatory cytokines that induce death of virus-infected cells. The TNFs bind to either Type I or II TNF receptors (TNFR) (Moss and Shisler, 2001). *Leporipoxviruses* and *Orthopoxviruses* encode for TNFR homologs (Moss and Shisler, 2001). Members of the Orthopoxvirus family can encode for a varying number of TNFR homologs. Smallpox and ectromelia have one, and cowpoxvirus has three, intact TNFR Type II homologs which are called crmB, crmC and crmD (Hu et al., 1994) (Smith et al., 1996) (Loparev et al., 1998) (Chen et al., 2000). In most strains of VV, the TNFR homolog ORF is interrupted

(Howard et al., 1991). These TNFR homologs can block TNF- α mediated cytolysis and counter the anti-viral state of the cell.

3. Inhibition of IL-1 β function

Interleukin-1 β (IL-1 β) is a pro-inflammatory cytokine that binds to a high affinity cellular receptor and mediates a broad response to viral infection via signal transduction pathways (Moss and Shisler, 2001). *Orthopoxviruses* encode secreted homologs of interleukin-1 receptor (IL-1R), which specifically bind to IL-1 β . Also, in order for IL-1 β to be activated, the precursor form needs to be cleaved by caspase -1. The cowpox virus encodes for a protein called crmA, which prevents activation of IL-1 β by inhibiting caspase-1 function (Ray et al., 1992).

4. Inhibition of IFN and IL-18

Interferons bind to cellular receptors, both Type I and II, and activate the Janus kinase and signal transducers and activators of transcription (JAK/STAT) pathway, inducing an anti-viral state in cells (Darnell et al., 1994). *Orthopoxviruses* produce proteins that can bind to type I or II IFNs (Moss, 2001). The VV protein B8R has been shown to bind to IFN- γ and shares sequence similarity with the extracellular domain of the IFN- γ receptor. Interleukin 18(IL-18) is a cytokine that induces IFN- γ production in macrophages, natural killer (NK) cells, and T cells. This cytokine has potent anti-viral properties that can protect mice from infections with vaccinia

virus (Tanaka-Kataoka et al., 1999). Interleukin -18 binding protein (IL-18BP) is a soluble, secreted inhibitor of IL-18, produced by both humans and mice (Novick et al., 1999). *Orthopoxviruses* encode IL-18BP homologs that can bind IL-18 (Calderara et al., 2001).

5. Intracellular proteins with anti-viral properties

In response to IFNs, cells express products that interfere with viral replication (Samuel, 1991). VV infection leads to the production of double stranded RNA which activates protein kinase R (PKR) (Gunnery and Mathews, 1998). PKR can inhibit viral protein synthesis by phosphorylation of the sub-unit of the translation initiation factor eukaryotic initiation factor 2 (eIF-2) (Brand et al., 1997).

Orthopoxviruses encode a double stranded RNA binding protein (*E3L* gene of VV) that can compete with PKR in binding with the ds RNA and thus inhibit PKR activation (Chang and Jacobs, 1993). *E3L* is a host-range gene of VV and its ability to counteract the anti-viral responses of the cell contributes to its host-range function. VV lacking the *E3L* gene is unable to grow in HeLa cells because the endogenous level of PKR is higher in the HeLa cells compared to baby hamster kidney (BHK) cells which support the growth of the mutant VV (Langland and Jacobs, 2002). Hence, the host range restriction of VV is closely tied in with the anti-viral state of a cell and on the ability of the virus to disable the host response to infection.

E. VV Host-range Restrictions

1. Host Range genes of VV

The host-range of VV is very broad among mammalian cells. Due to the presence of a huge genome, it was thought that VV would be very likely to encode for specific genes that would allow it to cross species barriers. The first evidence of the existence of poxvirus host range mutants was the rabbitpox virus mutants (Gemmell and Fenner, 1960), which failed to grow in pig kidney cells (McClain, 1965) (McClain and Greenland, 1965). The first host range mutant (hr mutant) in VV was identified during screening of nitrous acid mutagenized stock in an attempt to isolate temperature sensitive mutants (Drillien et al., 1981). The mutant was tested on 14 different cell lines and the results showed that the growth of the mutant was restricted in most of the human cell lines tested and that the restriction was independent of the source of origin of the cell lines; i.e. whether epithelial or fibroblastic, transformed or diploid, they all restricted the growth of the mutant. The mutant was also found to be totally restricted in its growth in rabbit cells, both epithelial and kidney cell line. The mutant, however, grew much better in monkey cell lines, both in primary and a continuous cell line, and grew the best in BHK and chicken embryo fibroblasts (CEF). It was determined that the hr mutant had a deletion of 18 kbp which occurred at the left end of the genome (Drillien et al.,

1981). In order to map the exact location of the deletion, recombinant VV were created where fragments of wild type DNA were inserted into the mutant virus and the recombinants screened by their ability to grow in human cells (Gillard et al., 1986). The DNA fragment containing the M and K fragments was able to restore growth of the mutant. This fragment contained an ORF encoding for a 32.5 kDa polypeptide (Gillard et al., 1986). This ORF was eventually shown to be transcribed leftward from *HindIII* K fragment into the M fragment and was called *K1L*, following standard protocol for naming VV genes. The *K1L* ORF is disrupted in the Variola virus (Massung et al., 1993). Since the identification of *K1L* gene as the host range gene, other VV genes with host range function have been reported, namely *C7L* (Perkus et al., 1990), the function of which is not completely understood, and *E3L* (Chang et al., 1995) (Langland and Jacobs, 2002), which has been very well studied as explained above.

Even though the *K1L* gene was characterized, the exact mechanism of how it mediated its function has not been entirely elucidated. To date, there has been no characterization of any interaction between *K1L* and any host protein. *In vitro* yeast two hybrid assays have shown that *K1L* interacts with *C10L*, another VV protein, but no biological significance has yet been attributed to this interaction (McCraith et al., 2000). Experiments have been carried out in order to determine the exact stage of life cycle when the block in growth occurs in cells infected with the hr mutant.

2. RNA synthesis in cells infected with mutant VV (K1L-)

A non-permissive human transformed cell line (KB) infected with the hr mutant, shows a steady production of cytoplasmic RNA for the first 90 minutes which declines thereafter. The rate and extent of RNA synthesis during the 90 minute period was seen to be similar to that in cells infected with the wild type virus (Drillien et al., 1981). RNA synthesis was also studied in the non-permissive rabbit kidney cell line, RK-13. MVA, lacking the *K1L* ORF along with many other ORFs, shows the same host range phenotype in RK-13 cells as the hr mutant described by Drillien (Sutter et al., 1994). A study using recombinant MVA, called MVA-K1L, which expressed the *K1L* gene, was used in the study to determine the extent of RNA synthesis in RK-13 cells. Cellular RNA, collected at time points 0, 0.5, 1, 2, and 4 hours post-infection, were probed for early mRNAs (Sutter et al., 1994). In cells infected with MVA-K1L, the transcripts of these early genes were detectable at the highest levels at 2 hours. There were more transcripts detectable at 1 hr than at 4 hours. In cells infected with MVA, again, the highest levels of transcripts were detected at 2 hours post infection, but in contrast to the MVA-K1L, the levels were higher at 4 hours than at 1 hour. This indicated that there was an accumulation of early transcripts in the absence of the K1L protein. The early transcripts in infections with both viruses could be detected well beyond the shutoff of protein synthesis. Intermediate transcripts were detectable at 2 and 4 hours post infection using MVA-K1L, while none were found in cells infected with MVA (Sutter et al.,

1994). Another study (Ramsey-Ewing and Moss, 1996), using RK-13 cells infected with the wild type K1L (+) virus and a mutant K1L (-) virus, reported similar observations. RNA levels were studied using a nuclease protection assay in infected RK-13 cells. The assay indicated that the RNA levels peaked at 2 hour after infection and then went down in cells infected with K1L (+) virus. In cells infected with the mutant lacking a functional K1L gene product, RNA was detected at 2 hours but persisted till 6 hours before they decreased. Study of intermediate transcripts showed that in K1L (+) infected cells; intermediate RNA was detectable at 4 hour post infection, and in K1L (-) infected cells very little RNA was detected at 24 hours post infection. As for late transcripts, in cells infected with the wild type, they were present between 6 and 24 hours, but none detected after infection with the K1L- virus (Ramsey-Ewing and Moss, 1996). The absence of intermediate and late transcripts could contribute to the absence of DNA synthesis. There were several studies done to analyze DNA synthesis in cells infected with K1L (-) virus in comparison to that in cells infected with the K1L (+) virus and these are summarized below.

3. DNA synthesis in cells infected with VV with mutant K1L (-)

Cytoplasmic DNA synthesis was seen to be considerably lower in hr-infected cells than in wt-infected cells (Drillien et al., 1981). RK-13 cells infected with MVA did not show any DNA replication (Sutter et al., 1994), while in cells infected with MVA-K1L, there was evidence of DNA replication. This indicated that the absence of intermediate and late transcripts was due to the absence of DNA replication. In a similar study (Ramsey-Ewing and Moss, 1996), it was observed that in RK-13 cells infected with a K1L (-) virus there was no increase in the levels of DNA after the initial 0 hour time point. In contrast, in cells infected with K1L (+) virus, there was an increase in the levels of DNA by 4 hours, and it continued after that (Ramsey-Ewing and Moss, 1996).

The absence of DNA replication could be due to delay in uncoating of the template DNA or due to some defect in the replication apparatus. In order to differentiate between the two possibilities, the kinetics of the levels of DNA replication of plasmid DNA serving as template (which does not need uncoating) was studied (Ramsey-Ewing and Moss, 1996). The pattern of DNA synthesis was not any different from that of viral DNA synthesis. This indicated that the defect in DNA synthesis was due to an impairment in the replication machinery rather than being a defect of uncoating the template alone.

4. Protein synthesis in cells infected with VV with mutant K1L (-)

Since the consistent pattern in all the studies has been the accumulation of early mRNA in the absence of K1L gene product, the question that followed was whether there was a defect in the translation of these early mRNAs to early proteins. The first evidence of the lack of protein synthesis showed that in KB cells infected with the hr mutant there was a decline in protein synthesis compared to the wt virus-infected cells (Drillien et al., 1981). In order to rule out the possibility that the decline was due to the absence of DNA replication in the hr mutant infected cells, the cells infected with wt virus were treated with cytosine arbinoside (which inhibits DNA synthesis). No decline in protein synthesis was observed in these cells indicating that the absence of DNA replication was not the reason for the decline in protein synthesis (Drillien et al., 1981). Observations from a study using RK-13 cells infected with either MVA (lacking K1L) or MVA-K1L showed similar results. In cells infected with MVA there was an abrupt shutdown of protein synthesis, and no proteins, either cellular or viral, were detected between 2 and 12 hours post-infection (Sutter et al., 1994). The absence of protein synthesis in the presence of early mRNAs suggested a translational block.

5. CP77 and K1L

VO25/CP77 is the cowpox host range gene encoding for a 77-kDa polypeptide. The cowpox virus has the largest genome amongst viruses in the *Orthopoxvirus* family. The cowpox host range gene is partially deleted in the WR (Kotwal and Moss, 1988a) and the VC-2 (Goebel et al., 1990) strains of VV. In an interesting study (Ramsey-Ewing and Moss, 1996), the *CP77* gene was inserted into the genome of a mutant VV lacking a functional *K1L* gene (K1L-CP77+). In RK-13 cells, this K1L (-) CP77 (+) recombinant behaved differently than the K1L (-) mutant. Not only did the *CP77* gene product rescue viral early protein synthesis, but it also rescued DNA synthesis and late protein synthesis. In RK-13 cells infected with the recombinant virus, K1L (-) CP77 (+), the levels of early transcripts decreased initially at around 3 hours post-infection (comparable to that in cells infected with K1L (-) CP77 (-)), but started to reappear at later times. Similarly, the intermediate RNAs were detectable at 12 hours post-infection and so were the late transcripts. It was observed that in the presence of *CP77*, there was a resumption of DNA synthesis after a lag of about 8-16 hours post-infection in RK-13 cells. The *CP77* protein has been implicated in preventing apoptosis (Ink et al., 1995). More recently, it has been shown to relieve host restriction in apoptotic HeLa cells at the intermediate gene translation stage by regulating the function of eIF-2 (Hsiao et al., 2004) and has also been shown to act upstream of caspase activation. However, the role of apoptosis has been ruled out as the basis of host-restriction in RK-13 cells (Chung et al., 1997). Therefore, *CP77*

must be involved in an alternative pathway. It has been suggested that the two proteins K1L and CP77 may have multiple roles which may differ in different cell lines (Ramsey-Ewing and Moss, 1996).

As mentioned earlier, there is no homology between these two proteins except for the presence of ankyrin repeats, and this led us to analyze more deeply the ankyrin repeats.

F. Ankyrin Repeats

1. What is an ankyrin repeat?

It is a 33 amino acid repeat motif, first identified in Swi6p and Cdc10p, two yeast cell cycle regulators and in development regulators Notch (from *Drosophila melanogaster*) and LIN-12 (from *C.elegans*) (Breedon and Nasmyth, 1987). Later, the cytoskeletal protein ankyrin (ANK) was shown to contain 24 of these repeats and hence the name of the repeats (Lux et al., 1990). The range of species containing proteins with ankyrin repeats has been shown to extend from viruses to humans and at least 400 proteins are known to have this motif (Michaely and Bennett, 1992). Some proteins consist solely of ankyrin repeats while others have ankyrin repeats along with other functional domains. The number of repeats in a protein has been known to vary. For example, the ankyrin protein has 24 and NF- κ B

has 5. An interesting feature of ankyrin repeats is that these motifs usually occur as multiple copies, at least 4 copies per protein (Bork, 1993). An exception to this rule is *Orthopoxvirus* proteins containing ankyrin repeats.

A study of variola and vaccinia virus proteins revealed that many of them contain ankyrin repeats (Bork, 1993) (Shchelkunov et al., 1993). Some examples are: C19L, M1L, K1L, C17L, C15L, B4R, and B14R proteins of vaccinia virus and the O1L, B6R, B19R, B21R, and G3R of variola virus. The number of ankyrin repeats in these proteins vary from 1-3, with the G3R protein being the most typical with 5 ankyrin repeats (Shchelkunov et al., 1993). Based on the known function of ankyrin proteins, which contain 24 ankyrin repeats, it has been suggested that the viral proteins containing ankyrin repeats may have important functions in regulation of viral growth and modifications of cell structural elements.

2. The structure of an ankyrin repeat

The elucidation of the three dimensional structure of an ankyrin repeat occurred 10 years after the discovery of this motif and was resolved from the 53BP2 protein bound to p53 (Axton et al., 1994; Sedgwick and Smerdon, 1999). The structure showed that a single ankyrin repeat consists of 2 anti-parallel α -helices connected to 2 β -sheets. The β -sheets form a continuous projection away from the α -helices at an angle of almost 90° , forming an ankyrin groove (Fig.2). This groove consists of

solvent exposed residues from the α -helical bundle. A typical ankyrin repeat is considered to be a β -hairpin-helix-loop-helix ($\beta_2\alpha_2$) structure (Sedgwick and Smerdon, 1999). Conserved residues in ankyrin repeats can be divided into different categories, such as those that are involved in formation and stabilization and others in forming the interface between repeats. Most ankyrin repeats have a conserved consensus sequence of 6 amino acids (TPLHLA), of which the first four, Thr-Pro-Leu-His (TPLH) initiates the first α -helix of the repeat by forming a tight turn (Sedgwick and Smerdon, 1999). The hydrogen-bonding interaction between the Thr6 and His9 of the side chain and main chain respectively, stabilizes this turn.

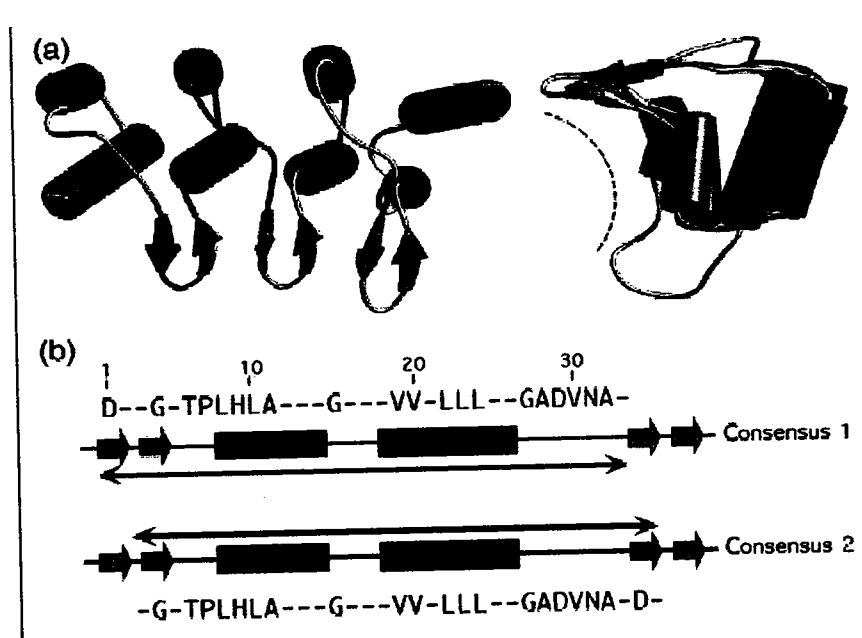


Fig 2: Structure of an ankyrin repeat.

(a) shows the arrangement of α -helices (cylinders) and β -hairpins (arrows), which is characteristic of the ankyrin (ANK)-repeat architecture viewed from the 'top' of the domain (left). A single ANK repeat is highlighted in red. The continuous β -sheet projects away from the helical stack to form the ankyrin groove, which is indicated by the dotted arc (right).

(b) The ANK-repeat consensus. ANK-repeat domains are assembled from multiple, sequential copies of a ~33 residue motif. The structure-based consensus (consensus 1) defines the ANK repeat as a $\beta 2\alpha 2$ motif highlighted in red [compare with (a)]. (consensus 2) is based on the location of exon boundaries in the ankyrin gene. In both consensus sequences, the extent of a single ANK repeat is indicated by a black arrow.

(Sedgwick and Smerdon, 1999)

3. Function of the ankyrin repeats:

The role of ankyrin repeats is to mediate protein-protein interactions and has been documented with many examples (Bork, 1993). A few examples are as follows:

p16-CDK6: Cyclin dependent kinase 6(CDK6) is a cell cycle regulatory molecule, and p16 is a CDK inhibitor. The interaction between these molecules is mediated by the ankyrin grooves (Bork, 1993).

I κ B-NF- κ B: NF- κ B is a transcription factor and I κ B is an inhibitor of NF- κ B. I κ B binds to NF- κ B so that the nuclear localization signal (NLS) of the p65 subunit is masked and the transcription factor is retained in the cytoplasm. Both proteins have ankyrin repeats, and the interaction between them is mediated by these repeats. The ankyrin groove formed by repeats 1, 2 and 3 of I- κ B makes contact with the NLS region of p65 (Bork, 1993).

GABP α -GABP- β -DNA: GA-binding protein alpha (GABP α) is a transcription factor that binds to DNA along with GABP- β . Interaction between the two proteins is mediated primarily by the β -hairpin fingers. Further contacts are mediated by residues in the ankyrin groove (Sedgwick and Smerdon, 1999).

53BP2-p53 interaction: p53 is a mediator of cellular stress response with a role in cell cycle control, DNA repair, and apoptosis. 53-BP2 interacts with p53, transactivates it, and promotes apoptosis. Although most of the interaction is mediated by the (SH3) domain of the 53BP2 protein, the β -hairpin of the ANK4 domain of the 53BP2 protein has been shown to make contact with p53 (Axton et al., 1994).

These are examples of intermolecular interactions; however, there is also evidence of ankyrin repeats being involved in intramolecular interactions. In the Swi6 protein, the region responsible for transcriptional activation interacts with the core domain, which contains five ankyrin repeats. In another example, the ankyrin repeats of the PAP protein of the ACAP (GTPase-activating protein) family of proteins interacts with the GTPase-activating (GAP) domain (Mandiyan et al., 1999).

Hence, it is clear that these repeats can be present in all kinds of proteins, in different species, with disparate biological functions including: immunomodulatory functions; signaling in cyclin dependent kinases; in developmental regulators; and toxins. The role of ankyrin repeats in viral proteins has not been well studied, especially in VV, considering the fact that so many VV proteins have ankyrin repeats.

G. OBJECTIVES OF DISSERTATION:

The function of *K1L*, a host range gene of VV, is unknown except for the fact that it is involved in the maintenance of viral protein synthesis. As mentioned earlier, the host-range restriction of a virus can also be an indication of an anti-viral state of the host cell. By studying the host-restriction of the wild type and the K1L-virus in restrictive and non-restrictive cell lines, we have tried to shed light on any novel antiviral pathway that the K1L gene product may be interfering with. The complementation of K1L function by the CP77 gene product has led to comparisons between these two proteins, and the only homology found has been the presence of ankyrin repeats. This led us to examine the importance of these repeats (known to be involved in protein- protein interactions) in mediating the host-range function. The differences in the infectivity of the mutant VV (lacking the *K1L* gene) in infection of permissive CV-1 cells and non-permissive RK-13 cells suggest that there may be a cellular protein involved. There has not been any report of cellular proteins interacting with the K1L protein of VV. In this study, we decided to address the involvement of cellular and viral proteins in the host range function of K1L. The specific aims of this dissertation are as follows:

1. To define the importance of the ankyrin repeat of K1L protein in mediating the host-range function.

a) To determine whether mutating the ankyrin repeat affects the host-range function of the K1L protein.

b) To define the intracellular localization of the wild type K1L and whether mutations in the ankyrin repeat causes the localization to change.

2. To identify cellular or viral proteins interacting with the K1L protein.

3. If interactions are found, to determine their role in mediating the host range function of the K1L protein.

CHAPTER II

MATERIALS AND METHODS

Cell Lines.

The following cell lines were used for experiments conducted for this thesis work:

The rabbit kidney cell line RK-13 (ATCC accession number, CCL-37); the African green monkey kidney cell line CV-1 (ATCC accession number, CCL-70) and the human osteosarcoma cell line lacking *TK* gene, 143B (ATCC accession number, CRL8303).

Cloning of the *KIL* gene from the New York City Board of Health (NYCBH) strain of VV.

CV-1 cells were grown to confluency in 75cm² flasks using 10% MEM (MEM containing 10% FCS). Inoculum (NYCBH strain of VV) was made up in 2.5% MEM (MEM containing 2.5% FCS). After an initial incubation of 1 hour, the innoculum was aspirated, and cells were incubated for 48 hours in 10% FCS. Cells were then washed once with sterile PBS (1X) and harvested by scraping off from the bottom of the flask and resuspended in PBS. The cells were centrifuged at 1,500

rpm for 5 minutes. The resulting cell pellet was resuspended in 600 μ l of nuclei lysis buffer (from Wizard Genomic DNA kit). Genomic DNA was extracted using the Wizard Genomic DNA purification kit (Promega, Madison, WI) according to manufacturer's instructions. *KIL* cDNA was amplified using the XL PCR amplification system (PE Applied Biosystems, Foster City CA). This PCR system amplifies DNA using the *rTth* DNA polymerase enzyme which apart from efficient DNA synthesis can also correct misincorporated bases. The PCR was performed as follows: 94°C/4 min, 1 cycle; 94°C/1 min, 52°C/1 min, 65°C/5 min for 30 cycles; 72°C/10 min, 1 cycle.

The primers were designed based on the sequence of VC-2 *KIL*.

K1Ls: 5'- CTCGAGTCAGACATGGATCTGTCACGAATTAAT -3'

K1Las: 5'- GGATCCGTGGGAGAATCTAATTAGTTTTTCTTTACAC -3'

The PCR product was first cloned into Topo TA cloning vector (Invitrogen, Carlsbad, CA). Restriction enzyme digestion using the enzymes XhoI and BamHI yielded the insert fragment which was then cloned into XhoI (CTCGAG) and BamHI (GGATCC) sites of the multiple cloning site of the mammalian expression vector pCDNA3.1 /Hygro (-) (Invitrogen, Carlsbad, CA).

GenBank Accession numbers. *KIL*: AY621082

Site directed mutagenesis.

As described before, K1Lwt was first generated in pCDNA3.1/Hygro (-). This construct was then used to generate the K1L mutated form. The 6 amino acids (96-101) comprising the consensus sequence of the most typical ankyrin repeat (amino acid 93-125) were converted to alanine using the site-directed mutagenesis kit (Quickchange, Stratagene, La Jolla, CA) as shown below.

K1L wt ACTGCATTGTATTAT 96-TALYYA-101 (amino acid sequence)
 K1Lmutank GCCGCGGCCGCTGCT AAAAAA (amino acid sequence)

Creating mutations in ankyrin repeats by converting the amino acids in the consensus sequence to alanines has been reported in other studies (Inoue et al., 1992). 10 ng of plasmid DNA template (pCDNA3.1Hygro- K1Lwt) was used in PCR amplification reaction using 125 ng of the following primers:

Anks:

5'GATGACAAAGGAAACGCCGCGGCCGCTGCTGCGGTTGATAGTGGT 3'

Ankas:

5'ACCACTATCAACCGCAGCAGCGGCCGCGGCGTTTCCTTTGTCATC 3'

The PCR cycling conditions were as follows: 95°C/30 sec, 1 cycle; 95°C/30 sec, 55°C/1min, 68°C/12 min, 12 cycles. PCR was performed using the Perkin Elmer DNA thermal cycler 480. The cDNA sequences of both K1Lwt and K1Lmutank were confirmed and did not contain any unwanted mutations.

Generation of recombinants.

K1Lwt and K1L mutank (first generated in pCDNA3.1/Hygro -) were subcloned into transfer vector pSC11ss. Recombinant VV vAbT33 (used as the parental strain) has one *lacZ* gene as does the transfer vector pSC11ss. In order to avoid recombination between the two *lacZ* genes, both K1Lwt and K1Lmutank were subcloned into transfer vector pSC11ssEE. pSC11ssEE was created by restriction enzyme digestion of pSC11ss vector with EcoRI (which deleted the *lacZ* sequence) and then religating the vector to reconstitute the Thymidine Kinase (*TK*) gene. Recombinant viruses vAbT33K1Lwt and vAbT33K1Lmutank were created following standard protocol described before (Terajima et al., 2002). In order to confirm the presence of the *KIL* gene in the recombinant viruses, genomic DNA was extracted from cells infected with the recombinants, and the *KIL* gene was amplified by PCR.

Table 2: Plasmids and Viruses used in this study:

Plasmids	Promoter
pCDNA3.1/ Hygro (-) (Invitrogen)	CMV immediate early promoter
pEGFPC-3 (BD Biosciences, Palo Alto, CA)	CMV immediate early promoter
pSC11ss (transfer vector) Kindly provided by Dr. Bernard Moss, NIH.	p7.5 early promoter
pSC11ssEE (Suguru Yamaguchi)	p7.5 early promoter
pMax-GFP (Amaya biosystems, Gaithersburg, MD 20877)	CMV immediate early promoter

Viruses.

New York City Board of Health (NYCBH) Kindly provided by Gail Mazzara and Dennis Panicali (Therion Biologics, Cambridge, MA)	Wild type VV containing a functional K1L protein
vAbT33 Kindly provided by Alicia Gomez Yafal (Therion Biologics, Cambridge, MA).	VV lacking a functional K1L protein derived from NYCBH.
vAbT33K1Lwt (In this report)	Described in text
vAbT33K1Lmutank (In this report)	Described in text

Immunoprecipitations.

RK-13 and CV-1 cells were grown (in 10% MEM) to confluency in 175 cm² flasks. Cells were metabolically labeled with [³⁵S]-methionine (0.1mCi), long-term and short-term.

Long- term labeling of cells: After aspirating the regular media (10% MEM) from the confluent cells, they were first washed with warmed long-term labeling media (methionine free media containing 10% FCS and serum free media) and then incubated in the same for 15 minutes at 37⁰ C. They were then incubated in the long-term labeling media containing 0.1mCi of [³⁵S]-methionine overnight. After the incubation, all the label was removed, and the cell monolayer was washed twice with regular media (2.5 % MEM). The cells were then infected with an MOI of 10 of the NYCBH strain (in 7 mls of 2.5 % MEM) and incubated for 1 hour at 37⁰ C. Then, the inoculum was removed and cells were incubated for an additional 2 hours in 10% MEM at 37⁰ C. Cell lysate was prepared at the end of this time point. For mock-infected cells, 2.5% MEM was added instead of inoculum and the rest of the protocol was followed as above.

Short-term labeling: Cells were first infected with an MOI of 10 of the NYCBH strain and incubated with inoculum (in 7 mls of 2.5 % MEM) for 1 hour at 37⁰ C. At

the end of this time, inoculum was removed and the cell monolayer was washed with warm short-term labeling media (100 ml methionine free media). Cells were incubated in this media for 15 minutes at 37⁰ C and then incubated for two more hours in short-term labeling media containing 0.1mCi of [³⁵S]-methionine. Cell lysate was prepared at the end of this incubation time.

Preparation of lysate: The monolayer was first washed two times with ice-cold PBS and the supernatant was discarded. Cells were lysed in cold lysis buffer (Immunoprecipitation kit, Roche, Indianapolis, IN) and harvested by scraping. Samples were transferred to a microhomogenizer (Kontes pellet pestle, VWR) and were manually homogenized with approx. 10 strokes per 1 ml of cell lysate. Lysates were then centrifuged in a table-top microfuge, at 12000 x g at 4⁰C for 10 minutes. The supernatants were then precleaned.

Precleaning of supernatants: 1 ml of sample was incubated with 50µl of protein A agarose at 4⁰C overnight on a rocking platform. Samples were then centrifuged at 12000 x g for 20s and the supernatants collected in fresh tubes.

Immunoprecipitation: Immunoprecipitation was performed as per manufacturer's instructions (Immunoprecipitation kit, using protein A agarose, Roche, Indianapolis, IN). The anti-K1L antibody (kindly provided by Dr. Robert Drillien) was used at

1:200 dilution (Gillard et al., 1989). The anti-K1L antibody was raised in rabbits against a peptide comprising 19 amino acids from the carboxy-terminal end of the K1L protein (Gillard et al., 1989). Samples were incubated with antibody for 1 hour at 4⁰C on a rocking platform. Then they were incubated with 50µl of Protein A agarose overnight at 4⁰C. Samples were centrifuged and pellet containing proteins complexed with protein A agarose were collected and were washed three times, with high and low salt buffers and denatured in SDS buffer containing DTT. Samples were run on 9% SDS-PAGE and analyzed using a phosphoimager.

Co-immunoprecipitation using anti-ACAP2 antibody: RK-13 cells were long-term and short-term labeled as described earlier with [³⁵S]-methionine.

Immunoprecipitation using anti-ACAP2 antibody (kindly provided by Dr. Paul Randazzo, NIH) (1:1000 dilution) was also performed as described earlier. The anti-ACAP2 antibody was raised in rabbits against residues 761-778 of ACAP2 protein (Jackson et al., 2000). However, the anti-ACAP2 antibody failed to immunoprecipitate the ACAP2 protein.

Silver staining and Mass spec sequencing.

RK-13 cells were grown to confluency in two 175 cm² flasks. Cells (2 x 10⁷ cell/flask) were infected with 10 MOI of virus (NYCBH strain). After an hour of incubation with inoculum, cells were washed and incubated for two more hours in

regular media (2.5% MEM). Cell lysates were prepared as per manufacturer's instructions (Immunoprecipitation kit, Roche). Proteins were immunoprecipitated using anti-K1L antibody and protein A agarose as per manufacturer's instruction (Roche). Proteins were run on 9% SDS-PAGE. The gel was stained using Silver Stain Plus (Bio-Rad, Hercules, CA). The silver staining was performed as follows: After gel electrophoresis, the gel was fixed in fixative enhancer solution (50% methanol, 10% acetic acid, 10% fixative enhancer concentrate and 30% distilled water) for 20 minutes. The gel was rinsed twice in deionized distilled water for 10 minutes with gentle agitation. It was then stained using staining solution, which was prepared immediately before use. In order to prepare 50 ml of staining solution, 5 ml of silver complex solution, 5 ml of reduction moderator solution and 5 ml of image development reagent was added to 35 ml of deionized water at room temperature. 50 ml of development accelerator solution was added to the staining solution just before staining. The gel was stained until the bands became visible. The staining reaction was stopped using 5% acetic acid solution for at least 15 minutes. The pertinent bands were excised from the gel and MALDI MS/PSD analysis (by Dr. John Leszyk, Proteomics Core Facility, UMASS Medical Center) of the silver stained band identified the protein as a rabbit homologue of Human ACAP2.

Plaque assay in RK-13 cells.

RK-13 cells were grown to confluency (1×10^6 cells/well) in 6 well plates and infected with NYCBH, vAbT33, vAbT33K1Lwt and vAbT33K1Lmutank with an MOI of 0.1. After an incubation of 2 hours, cells were washed once and incubated in 10% MEM for 48 hours at 37° C. At 48 hours post infection, cells were washed and stained with 0.2% of crystal violet in 10% ethanol.

Titration of VV by focus forming assay.

Permissive CV-1 cells were grown to confluency in 6-well plates. Cells were then infected with serial dilutions of infected cell lysates to be titrated. Infection of permissive CV-1 cells by infected cell lysates followed standard procedures (Earl et al., 1997) (Terajima and Leporati, in press). Viral titer was determined by staining foci of infection using immunohistochemistry with anti-VV antibody (Biogenesis, Brentwood, NH) and histochemical staining kit (rabbit IgG, Vecta stain, ABC kit, Vector laboratories, Burlingame, CA). The anti-VV antibody used is a polyclonal antibody raised in rabbits against Lister strain of VV (Biogenesis, Brentwood, NH).

RT-PCR of viral RNA:

Transcript levels of *KIL* in the cells infected with four viruses were analyzed. RK-13 cells (2×10^6 cells) were infected with 0.1 MOI of NYCBH, vAbT33,

vAbT33K1Lwt, vAbT33K1Lmutank. Cells were incubated with inoculum for 2 hours, after which they were washed and incubated in regular media (10% MEM) for 6 hours. Total RNA was extracted from the cells using Ultraspec RNA isolation system (Biotech laboratories Inc., Houston, TX). The extracted RNA was treated with DNase I to eliminate any contaminating DNA in the RNA preparation. The extracted RNA was incubated with 10X DNase I buffer, 1mM DTT, 20 µg/ml of DNase I, RNase inhibitor, at 37°C for 2 hours. The DNase treated RNA was again purified by Ultraspec. Reverse Transcriptase (RT) reactions were set up using 1 µg of purified RNA. A set of control reactions, without RT enzyme, was also set up. PCR was set up with templates that were treated with RT and without RT, for each sample in order to eliminate the possibility of amplification from any contaminating DNA. PCR primers for the amplification of *K1L* gene were as follows:

K1Ls: 5' - CTCGAGTCAGACATGGATCTGTCACGAATTAAT -3'

K1Las: 5' - GGATCCGTGGGAGAATCTAATTAGTTTTTCTTTACAC -3'

K1L was amplified using the XL PCR amplification system (PE Applied Biosystems, Foster City CA) using the *rTth* DNA polymerase enzyme. The PCR programme used was as follows: 94°C/4 min, 1 cycle; 94°C/1 min, 52°C/1 min, 65°C/5 min for 30 cycles; 72°C/10 min, 1 cycle.

Cloning rabbit *ACAP2*.

Total RNA was extracted from uninfected RK-13 cells. Three fragments from the middle of the open reading frame were amplified by RT-PCR, using three sets of primers that were chosen from the regions conserved between human *ACAP2* (Accession # NM 012287.3) and mouse *ACAP2* (Accession # NM 030138) sequences.

ACAP2 (1) s: 5'- TGCCCAAGTACAAAGAAACAAA -3'

ACAP2 (1) as: 5'- GGAATCTGCCTGGAGCATAAC -3'

ACAP2 (2) s: 5'- TGTATGCTCCAGGCAGATTC -3'

ACAP2 (2) as: 5'- CGCCCTATAAAGCTGAAGTCC -3'

ACAP2 (3) s: 5'- GCCTGAAGGAGAAAGGCAAGA -3'

ACAP2 (3) as: 5'- TTTCTGTGAATCTTGCTGGAAA -3'

The products were sequenced and based on these sequences the 5' end was amplified by 5' RACE (SMARTTM RACE cDNA Amplification Kit, Stratagene). Starting with the cDNA produced (RACE-ready cDNA) using the first set of primers, a 5' RACE reaction was set up using primer:

ACAP2.5.2: 5'- TACTTCTTCCAAAGCAGCCCTGAAGCGGGG -3'.

The reaction was set up as follows:

Race-ready DNA	2.5 μ l
Universal 5' primer (10X)	5 μ l (supplied by manufacturers)
Sequence specific primer (100 μ M)	1 μ l

PCR master mix 41.5 μ l (supplied by manufacturers)

The PCR cycling parameters using a Perkin Elmer DNA thermal cycler 480 were as follows: 94⁰/30 sec; 68⁰/30 sec; 72⁰/3 min; 25 cycles. The PCR products obtained were cloned into TOPO TA cloning vector and the inserts sequenced.

Sequences from at least two independent PCR products were compared to determine nucleotide sequence. The entire cDNA was then cloned using primers:-

ACAP2 s: 5'- GGCAGGATGAAGATGACGGTGGAT -3'

ACAP2as: 5'- TCAGAATTTCTGTGAGTCCTGCTGGAA -3'.

GenBank Accession numbers. Rabbit *ACAP2*: AY620244

Pulse –Chase labeling.

RK-13 cells grown in 25cm² flasks were infected with 10 MOI of virus (NYCBH, vAbT33, vAbT33K1Lwt, vAbT33K1Lmutank). Cells were incubated with inoculum for 30 minutes at 37⁰ C. After aspiration of inoculum, cells were washed once and regular media (10% MEM) was added. Cells were then labeled and analyzed at 0 hour (immediately after the 30 minute incubation) and 6 hours post infection. Labeling was done as follows: Cells were first washed in warm short-term labeling media (100 ml methionine free media). Cells were then incubated in the short term labeling media for 10 minutes. After that, cells were incubated with 3.5 μ Ci /ml [³⁵S]-methionine (diluted in short term labeling media) for 30 minutes at 37⁰ C. After the label was removed, cells were lysed

using lysis buffer [20mM Tris-HCL (pH 8.0), 10mM NaCl, 0.5% NP-40] . Cell lysates were run on 10% SDS-PAGE and analyzed by autoradiography.

Localization of K1L.

Cells were grown on cover slips to 30-40% confluency. Cells were transfected using effectene (Qiagen, Valencia, CA) with 0.4 μ g of plasmid DNA, pEGFPK1Lwt and pEGFPK1Lmutank and pEGFP. The pEGFPK1Lwt and pEGFPK1Lmutank constructs were generated by fusion of the *K1L* cDNA (both the wt and the mutated form) to the carboxy terminal of EGFP. Cells were fixed 36 hours post-transfection with 4% paraformaldehyde and mounted on slides, using 4'-6-Diamidino-2-phenylindole (DAPI) containing mounting media (Vectashield, H-1200, Vector laboratories). DAPI stains nuclei specifically, with little or no cytoplasmic labeling. Cells were then visualized using a Nikon DIAPHOT 300 Inverted Microscope with a Zeiss AxioCam digital camera using openlab version 3.0 (Improvision) image capturing software.

Study of viral factory formation in RK-13 cells infected VV:

Cells were grown to confluency on cover slips in 6-well plates. Cells were infected with 5 MOI of NYCBH and vAbT33 strains of virus. Cells were incubated for one hour at 37 °C, and then the inoculum was aspirated. Cells were washed once with media (2.5% MEM) and incubated for two hours in 2.5% MEM. Cells were then

washed once with 1X PBS and fixed with 4% paraformaldehyde at 4 °C for 20 minutes. After washing two times with 1X PBS, cells were then stained with Hoechst stain (0.25mg/ml) for 5 minutes. After removal of the stain, cells were then incubated in PBS for 5 minutes and then mounted on slide using mounting media (1% glycerol in 1% PBS).

***In vitro* transcription-translation:**

Mammalian expression vectors pcDNA3.1Hygro(-) expressing the wild type K1L and the mutated K1L were used in the *in vitro* transcription and translation assay using the TNT Quick system (Promega). pcDNA3.1Hygro(-) has a T7 promoter for *in vitro* transcription-translation. The expressed proteins (K1L wild type and K1L mutank) labeled with 10mCi/ml of [³⁵S]-methionine were incubated for 60 minutes at 30° C as per manufacturer's instruction. At the end of the incubation period, proteins were run on 10% SDS PAGE gel and the results were analyzed by autoradiography.

Designing siRNA: siRNA specific for *ACAP2* was designed using the basic siRNA design tool, available at the URL:

<http://www1.qiagen.com/Products/Genesilencing/CustomSiRNA> (Qiagen). The target DNA sequence chosen was: AAGAGGCTGAGCATTCTAAA, and was

ranked the best match with a high score of 90. The specific siRNA for *ACAP2* was of HPP scale purity, was all annealed and had DNA overhangs. A commercially available (Qiagen) negative (non-silencing) control was also used in these studies.

Determination of the efficiency of transfection of siRNA into RK-13 cells using nucleofection (amaxa biosystems). Efficiency was determined by transfecting pMax-GFP (Amaya Biosystems, Gaithersburg, MD) plasmid DNA into RK-13 cells using nucleofection protocol (Amaya Biosystems, Gaithersburg, MD). RK-13 cells were grown to confluency. Cells were trypsinised and counted. 2×10^6 cells were used for one transfection reaction. 2×10^6 cells were resuspended in 100 μ l of nucleofection solution V (supplied by manufacturers), mixed with 1.4 μ g of pMax-GFP. Cells were transfected using the nucleofection programme A-33. Cells were incubated for 24 hours, and then GFP expression was monitored by FACS analysis.

FACS analysis: RK-13 cells were transfected with either 1.4 μ g of pMAXGFP plasmid DNA or no DNA (negative control). 24 hours after transfection, cells were trypsinised and collected by centrifugation. Cells were then resuspended and washed twice in FACS buffer (PBS/1%FBS). Cells were then fixed in 1% paraformaldehyde for 10 minutes at 4 $^{\circ}$ C in the dark. They were then washed once and resuspended in 400 μ l of FACS buffer and then analyzed for GFP fluorescence.

Real-Time PCR: Transcript levels of *ACAP2* were measured quantitatively using the Taqman real-time PCR amplification system (Applied Biosystems).

Primers used were as follows:

ACAP2 Taqman forward primer: CAGGGCAGGTGTGTTTATTTCTAA

ACAP2 Taqman reverse primer: CAAAGGGTCTTTCCCTTCTTCA

Actin Taqman forward primer: CGAGATCGTGCGGGACAT

Actin Taqman reverse primer: GCCATCTCCTGCTCGAAGTC

Probes used:

ACAP2 Taqman Probe: ACGAGGTGCCAATCAACATGCCACT

Actin Taqman Probe: AAGGAGAAGCTGTGCTACGTGGCGCT

The RT reaction was set up in triplicate in a 96-well plate, starting with 3 µg of RNA for each sample. The RT reaction mix contained RT buffer, dNTPs, MgCl₂, specific reverse primer, RNase inhibitor and RT enzyme (Taqman, Reverse transcription kit, Applied Biosystems, Foster city, CA). The PCR reagents were added to the newly synthesized cDNA, and analyzed in the GeneAmp 5700 Detector (Applied Biosystems, Foster city, CA).

CHAPTER III

RESULTS

SECTION I

A. The role of ankyrin repeats in the host range function of K1L

As mentioned in the introduction, the functional complementation of K1L by CP77; despite the absence of homology (except for the presence of ankyrin repeats), was of considerable interest. The exact mechanism of how the K1L protein mediates its host range function is unknown. As mentioned earlier, there is no evidence of the presence of any other known functional protein domains in K1L. The ankyrin repeats of the K1L protein have never been analyzed previously. We decided to study these 33 amino acid residue repeats. First, we decided to study the impact that the ankyrin repeats have on the host range function of the K1L protein, both in mediating the host range phenotype and the maintenance of protein synthesis. Secondly, we decided to investigate the intracellular localization of the K1L wt protein and compare it with that of the mutated K1L protein.

1. Analysis of the ankyrin repeat of K1L in mediating its host-range function.

The presence of ankyrin repeats in the K1L protein of VV had been reported earlier in some studies; however, results differed concerning the number of these repeats. One report cited 5 ankyrin repeats (Bork, 1993) (Bork, 1993), while another cited 2 ankyrin repeats (Shchelkunov et al., 1993). A conserved domain search we performed on the K1L protein of VV Copenhagen strain (GenBank accession number AAA48005) showed one very highly conserved ankyrin repeat at amino acid positions 93-125 and a less conserved ankyrin repeat at positions 29-61. Under very low stringency conditions, we could detect two more ankyrin repeats which were much less conserved. All conserved domain searches were done using the CDD: <http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi> (Marchler-Bauer et al., 2003). After we cloned the K1L gene from the NYCBH strain of VV (as described in Materials and Methods), a conserved domain search showed the ankyrin repeat 93-125 as the most typical ankyrin repeat in the NYCBH strain, and the amino acid sequence of this repeat is completely conserved between the Copenhagen strain and the NYCBH strain. In order to study the host-range function of the K1L gene product, we initially used a transient transfection approach. RK-13 cells were first transfected with pCDNA3.1Hygro (-) K1Lwt. At 24, 48 and 72 hours post transfection, the cells were infected with vAbT33 strain of VV. It was expected that the expression of wild type *K1L* gene before infection would make the otherwise

non-permissive cells permissive to the mutant virus. This would serve as the positive control for an experiment in which RK-13 cells would be transfected, and subsequent infection with the mutant virus would indicate loss of function, if any. These transfection-infection experiments failed to yield any results, as the positive control did not work very well because of the poor transfection efficiencies of RK-13 cells. We therefore prepared recombinant VV expressing the wild type K1L and the mutant K1L with the same parental background. The vAbT33 strain of VV was chosen as the parental virus. vAbT33 is a recombinant VV, produced by deletion of the 3' end of the *K1L* gene and the adjacent M2L of the NYCBH strain, and has a β -galactosidase gene regulated by BamHI F promoter inserted at this site (Smith et al., 1993). vAbT33 has a truncated K1L ORF and hence a non-functional K1L gene product which is impaired in its ability to grow in RK-13 cells (Smith et al., 1993). We first cloned the *K1L* cDNA from the NYCBH strain of virus into the expression vector pCDNA3.1Hygro (-), as described before. In order to create a mutated form of K1L, the consensus sequence of 6 amino acids (96-101) of the ankyrin repeat 93-125 was mutated by substituting to alanine, following the protocol as described (Inoue et al., 1992). The cDNA sequences of both K1Lwt and K1Lmutank were confirmed and did not contain any unwanted mutations. The cDNA of both the K1Lwt and K1Lmutank were then cloned into the transfer vector pSC11EE. Recombinant virus was created using a standard protocol for the creation of

recombinant VV. The following aspects of the host-range restriction were studied using the recombinants produced.

a) Study of plaque forming ability of both vAbT33K1Lwt and vAbT33K1Lmutank in RK-13 cells.

RK-13 cells were infected with NYCBH, vAbT33, vAbT33K1Lwt and vAbT33K1Lmutank at an MOI of 0.1. Cells infected with NYCBH and vAbT33K1Lwt supported viral growth as evident by the presence of plaques, while cells infected with vAbT33 and vAbT33K1Lmutank failed to produce plaques after 48 hours post infection (Fig 3). Plaques produced by the vAbT33K1Lwt were smaller in size than those by NYCBH, probably due to the lower expression of K1L (described in details in the section "detection of K1L protein", page 63). The reintroduced K1L gene has a p7.5 promoter instead of its own promoter and lacks the early termination motif. These differences might explain the difference in protein expression. Another explanation of smaller plaque size could be that vAbT33K1Lwt does not have M2L reconstituted, although the function of M2L is not known.

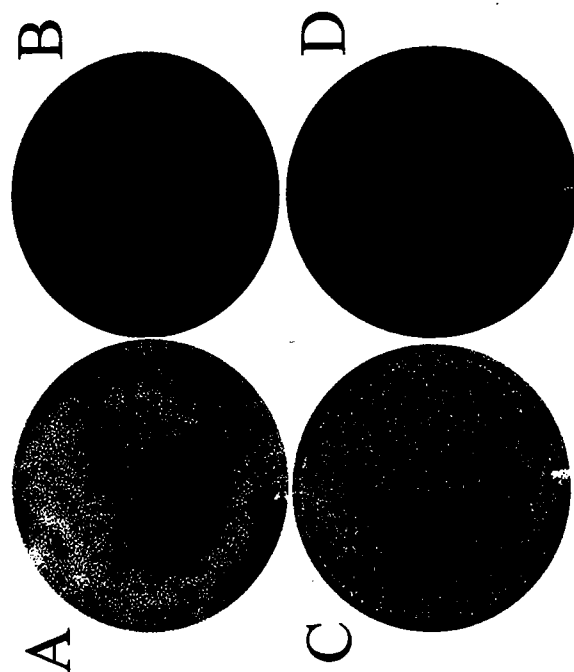


FIG. 3. Plaque Assay in RK-13 cells. Cells Infected with vAbT33K1Lmutank do not produce plaques. RK-13 cells were infected with MOI of 0.1 of NYCBH (C), vAbT33 (D), vAbT33K1Lwt (A), vAbT33K1Lmutank (B). After an initial incubation of two hours, cells were washed once and incubated for 48 hours, after which cells were stained with crystal violet. Plaques were visualized under white light.

b) Study of growth kinetics of vAbT33K1Lmutank and vAbT33K1Lwt in RK-13 cells.

We also examined the kinetics of viral growth in RK-13 cells. RK-13 cells were grown to confluency in 25 cm² flasks. Cells were infected with the four viruses; NYCBH (wild type, K1L+), vAbT33 (K1L-), vAbT33K1Lwt (K1L+) and vAbT33K1Lmutank (mutated in ankyrin repeat 93-125) at an MOI of 0.1. Cell lysates were collected at 0, 6 and 12 hours post-infection and were titrated in permissive CV-1 cells (Fig 4). vAbT33K1Lwt and NYCBH both showed very good growth in RK-13 cells. vAbT33 infected cells showed a gradual loss in growth over time. The recombinant vAbT33K1Lmutank behaved similar to the vAbT33 parent virus. The difference in the titers of vAbT33K1Lwt and vAbT33K1Lmutank was almost 3 logs (Fig 4).

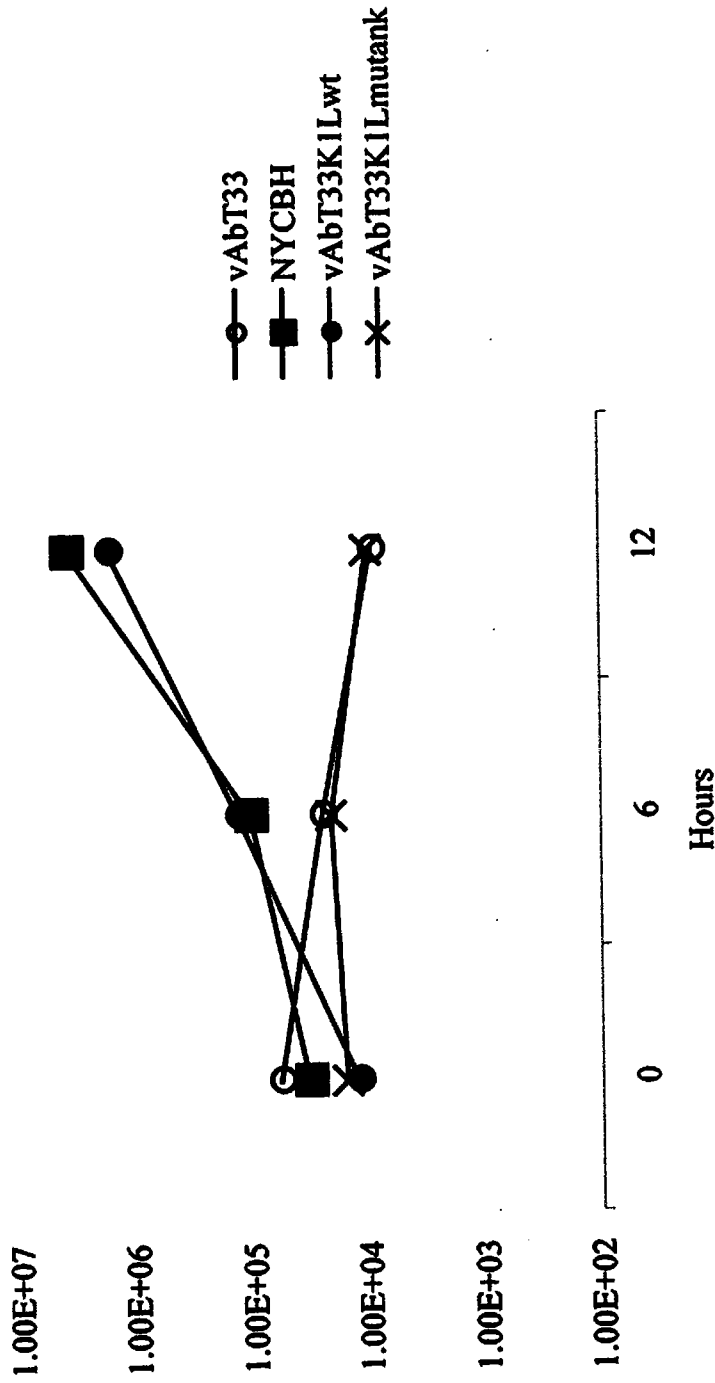


FIG. 4. Infection of RK-13 cells with vAbT33K1Lmutank results in non-productive replication. RK-13 cells were infected with 0.1 MOI of NYCBH, vAbT33, vAbT33K1Lwt, vAbT33K1Lmutank. After an initial incubation with inoculum for 1 hour, cells were washed and incubated for 0, 6, and 12 hours. Cell lysates were prepared at these time points and titrated in CV-1 cells. Titers of NYCBH (■), vAbT33 (○), vAbT33K1Lwt (●), vAbT33K1Lmutank (×) are shown as ffu/ml

c) Detection of K1L transcripts

K1L transcripts could be detected by RT-PCR using total RNA samples prepared from RK-13 cells infected with NYCBH, vAbT33K1Lwt and vAbT33K1Lmutank, (Fig 5, lanes 1, 5, 7) but not from those infected with vAbT33 (Fig 5, lane 3), uninfected RK-13 cells (Fig 5, lane 9) or in the no template control (Fig 5, lane 11). There were no transcripts detected in the absence of RT enzyme. (Fig 5, Lanes 2, 4, 6, 8, and 10).

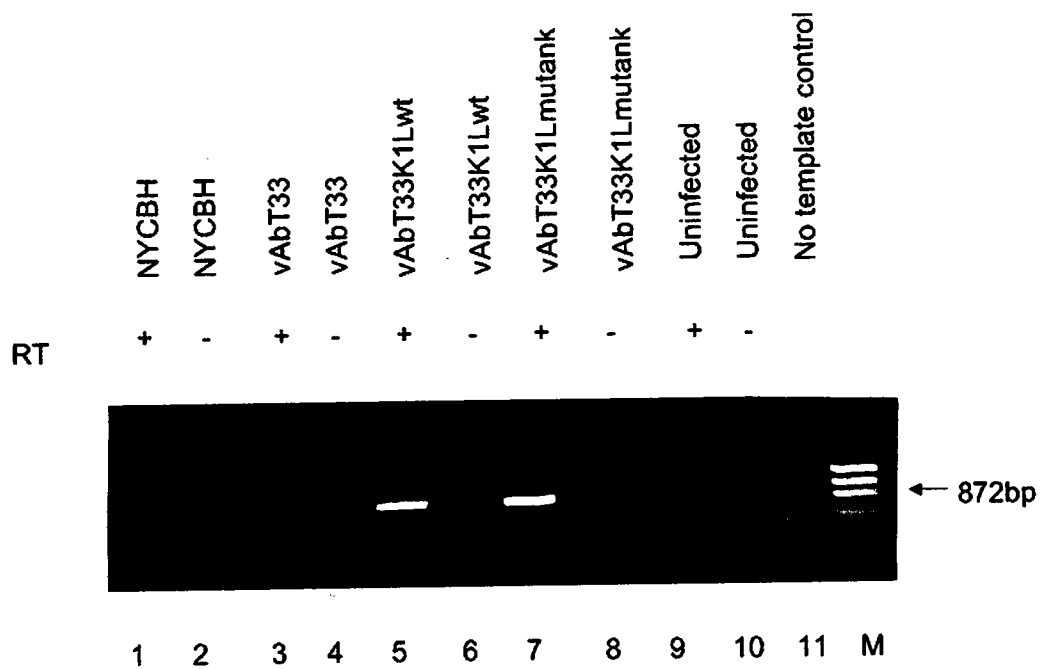


FIG. 5 Detection of K1L transcripts in VV infected RK-13 cells.

d) Detection of K1L protein.

An immunoprecipitation assay was performed using anti-K1L antibody (as described in Materials and Methods) in order to detect both the wild type and mutated K1L proteins from CV-1 cells infected with the recombinant viruses (NYCBH, vAbT33K1Lwt and vAbT33K1Lmutank). K1L protein was detectable by immunoprecipitations from CV-1 (permissive) cells infected with all three viruses (NYCBH, vAbT33K1L wt and vAbT33K1Lmutank) (Fig 6A). However, the K1Lmutank protein migrates slower than the wild type K1L protein (Fig 6A), suggesting that the mutation in the most conserved ankyrin repeat of this protein affects its secondary structure. This is expected because the consensus sequence of the ankyrin repeat that was mutated occurs at the turn of an α -helix. This was further confirmed in an *in vitro* transcription/translation assay. Mammalian expression vectors pCDNA3.1Hygro (-), expressing the wild type K1L and the mutated K1L, were used in the *in vitro* transcription and translation assay. These vectors also have a T7 promoter for *in vitro* transcription and translation. The expressed proteins (K1L wild type and K1Lmutank) labeled with 10 mCi/ml of [³⁵S]-methionine, were subjected to incubation at 30⁰C for one hour. At the end of the incubation, proteins were run on 10% SDS PAGE gel and results analyzed by autoradiography (Fig 6B). Results showed that the mutated K1L protein ran at a slightly higher size than the wild type K1L. We were unable to detect K1Lmutank protein in RK-13 cells infected with the vAbT33K1Lmutank virus. This is expected, because infection with

the vAbT33K1Lmutant is abortive in RK-13 cells and results in shutdown of protein synthesis.

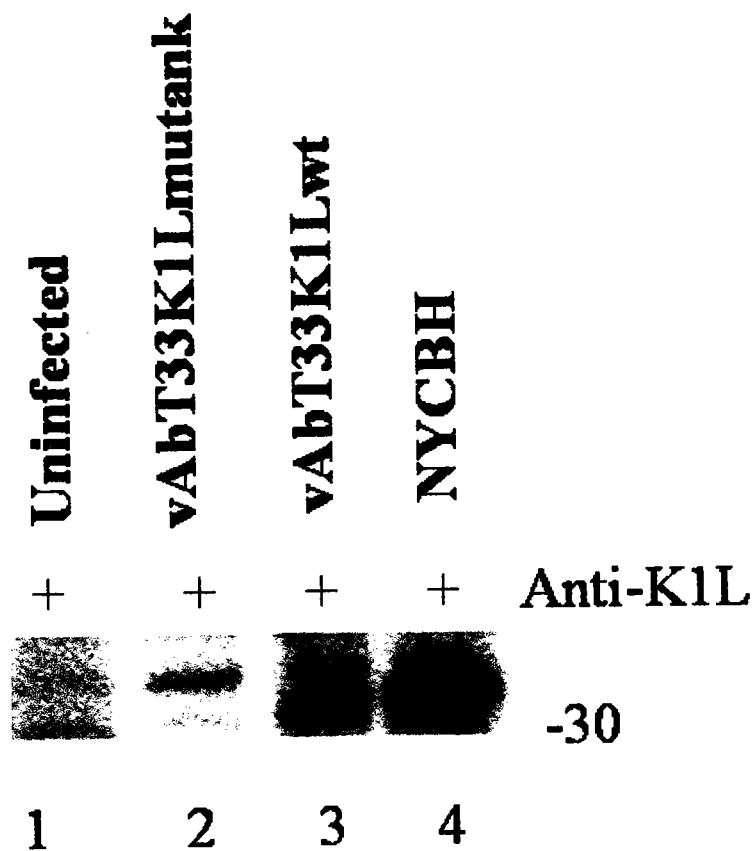


FIG. 6. A) Immunoprecipitation of K1L protein from CV-1 cells.

Cells were infected with 20 MOI of NYCBH, vAbT33K1Lwt; vAbT33K1Lmutank. The cells were incubated for 2 hours. The inoculum was aspirated and cells were incubated with labeling media containing 0.1 mCi of ^{35}S -methionine and incubated for 6 hours. Uninfected cells were also labeled with 0.1 mCi of ^{35}S -methionine. Cell lysates were prepared and incubated with protein A agarose. Proteins associated with protein A agarose were immunoprecipitated with anti-K1L antibody. Cells were analyzed by 9% SDS-PAGE.



FIG.6 B) In vitro translation of K1Lwt and K1Lmutank proteins.
35S-methionine labeled in vitro transcribed and translated K1Lwt and K1Lmutank proteins were run on a 9% SDS PAGE gel. The K1Lmutank protein (lane 1) migrated a little slower than K1Lwt protein (lane 2).

e) Study the effect of K1L mutant virus on protein synthesis.

The infection of RK-13 cells with a mutant VV lacking a functional *K1L* gene has been shown to cause the premature shutdown of synthesis of both cellular and viral proteins. We wanted to see whether vAbT33K1Lmutank would elicit the same response in protein shutdown. A pulse-chase experiment, using 3.5 μ Ci/ml of [³⁵S-] methionine to label both cellular and viral proteins, showed that in cells infected with NYCBH (K1L+), there is no effect on total protein synthesis at 6 hrs (Fig 7, lanes 1 and 2). In contrast, in cells infected with vAbT33, there is a shutdown of protein synthesis by 6 hours (Fig 7, lane 4), as compared to 0 hr (lane 3) .The recombinant virus, vAbT33K1Lwt, behaved very much like the NYCBH (Fig 7, lanes 5, 6), and, as expected, in cells infected with the recombinant vAbT33K1Lmutank, there is a total shutdown of protein synthesis at 6 hrs as compared to 0 hr (Fig 7, lanes 7 and 8).

These data show that mutating the ankyrin repeat 93-125 can bring about shutdown of protein synthesis of both cellular and viral proteins. We next decided to investigate whether differences in localization of the K1L protein in the cell, the wt and the mutated forms, may have a role to play in determining the host-restriction in RK-13 cells.

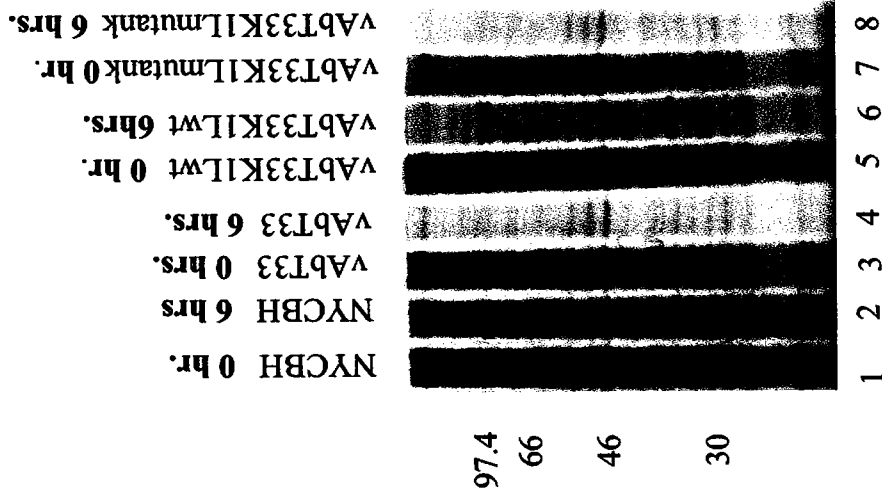


FIG. 7. vAbT33K1Lmutank caused premature shutdown of both viral and cellular protein synthesis.

RK-13 cells were infected with 10 MOI of NYCBH, vAbT33, vAbT33K1Lwt and vAbT33K1Lmutank.

Cells were incubated with inoculum for 30 minutes.

Cells were labeled with 35S-methionine at 0 hr (immediately after incubation with inoculum) or 6 hrs after incubation with inoculum. Cell lysates were analyzed by 10% SDS-PAGE.

f) Study of localization of K1Lwt and K1Lmutank RK-13 cells.

An earlier attempt to determine sub-cellular localization of K1L failed to assign the distribution of this protein into any one compartment (Gillard et al., 1989). The absence of a nuclear localization signal suggests that the protein is present in the cytoplasm. Ideally the intracellular localization of K1L protein should be determined by immunohistochemical staining using anti-K1L antibody.

Unfortunately, the anti-K1L antibody we used in this study is not suitable for immunohistochemistry, and at this point this is the only one anti-K1L antibody available to the study. As an alternative, we made a fusion protein of K1L and enhanced green fluorescent protein (EGFP), assuming fused EGFP would not change the localization of the K1L. We cloned the *K1L* gene (both wt and mutank) into the expression vector pEGFP, fused to the carboxy terminus of EGFP. EGFP is a mutant form of GFP, which upon excitation, produces more long lasting and brighter fluorescence as compared to wild type GFP. Expression of both K1Lwt (Fig 8A) and K1Lmutank (Fig 8B), in RK-13 cells, showed that both these proteins localize in the cytoplasm of the cell. The pEGFP vector alone localized all over the cell, including in the nucleus (Fig 8C). Infection of transfected cells (detected by anti-VV antibody conjugated with rhodamine associated secondary antibody) with the NYCBH strain did not show any difference between the two forms.



Fig.8 Localization of K1L in RK-13 cells: A) K1Lwt B) K1Lmutank C) pEGFP vector only.

CHAPTER IV

RESULTS

SECTION II

A. Interaction of K1L with a cellular protein:

The differences in permissibility of infection with the mutant VV (lacking the K1L gene) in different cell lines suggests the involvement of cellular factors. This has been suggested in various studies but there has been no experimental evidence indicating existence of any cellular protein interacting with the K1L protein. A yeast-two hybrid analysis of interacting VV proteins revealed that the K1L protein interacts with another VV early protein, C10L. No biological significance was, however, attributed to this interaction.

1. K1L protein interacts with rabbit ACAP2 protein in RK-13 cells.

In order to identify cellular proteins that may be interacting with the K1L protein of VV, a co-immunoprecipitation assay was performed. RK-13 cells were infected with 10 MOI of NYCBH. The cells were labeled with [³⁵S]-methionine long-term (overnight before infection, for identification of cellular proteins) and short-term (2

hours post infection, for identification of viral proteins). Cell lysates were then immunoprecipitated with anti-K1L antibody and protein A agarose, as described in Materials and Methods, and the samples were run on 9% SDS-PAGE. We were able to immunoprecipitate the K1L protein from infected cells in the presence of anti-K1L antibody, but not in the presence of rabbit control serum (Fig 9A). This antibody also co-immunoprecipitated a 90 kDa protein, representing a cellular protein being pulled down by the anti-K1L antibody, but not in the presence of rabbit control serum (Fig 9A) or in a mock infection (Fig 9B). Immunoprecipitated proteins were run on 9% SDS PAGE gel and stained with silver stain plus as described in materials and methods (Fig. 9C).

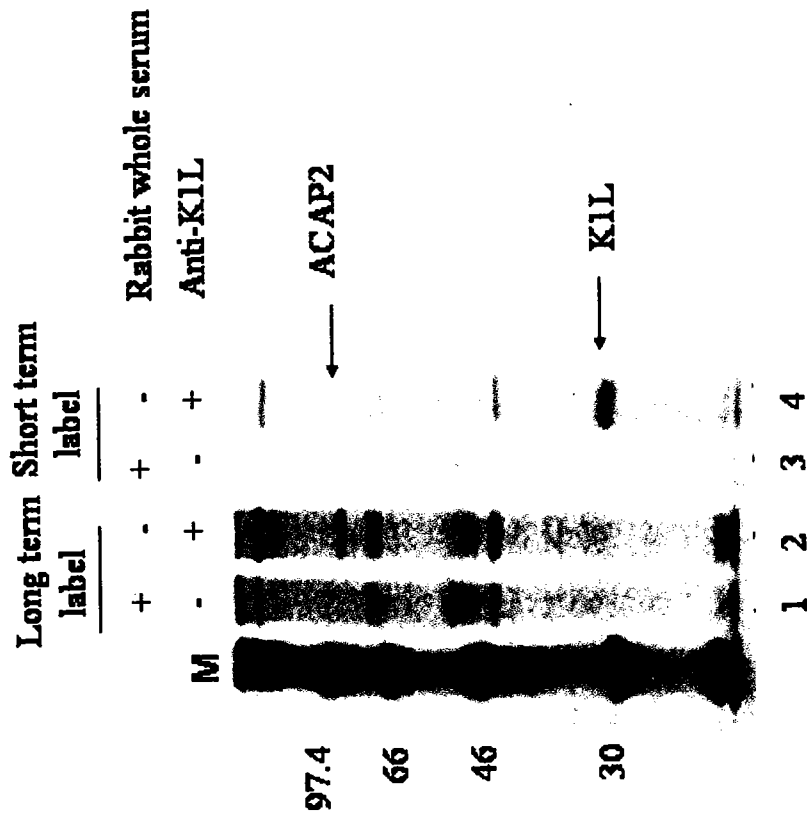


FIG. 9A. A 90 kDa band was co-immunoprecipitated with K1L protein in RK-13 cells infected with NYCBH, using anti-K1L antibody. RK-13 cells were either long-term labeled (lanes 1 and 2), or short-term labeled (lanes 3 and 4) with ³⁵S-methionine. Cell lysates were prepared and proteins were immunoprecipitated with either anti-K1L antibody (lanes 2 and 4) or control serum (lanes 1 and 3). Cells were analyzed by 9% SDS-PAGE. A 90 kDa protein was co-immunoprecipitated only in the presence of K1L antibody (lane 2).

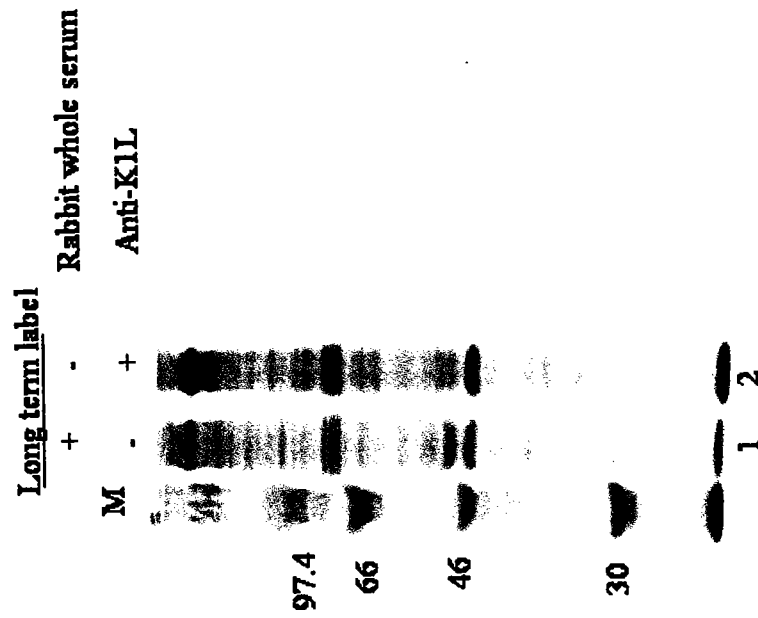


Fig.9 B. Mock infection of RK-13 cells.
 RK-13 cells were long-term labeled with ³⁵S-methionine. They were mock infected, using 2.5% MEM instead of inoculum. Cell lysates were prepared and proteins were immunoprecipitated with either anti-K1L antibody (lane2) or control serum (lane 1). Cells were analyzed by 9% SDS-PAGE.

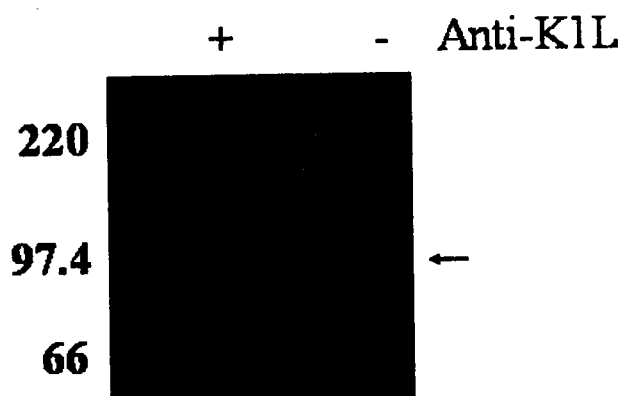


FIG. 9C. Detection of rabbit ACAP2 by silver staining.

Cell lysates were prepared from RK-13 cells infected with NYCBH and proteins were immunoprecipitated with either anti-K1L antibody (lane 1) or control serum (lane 2). Cells were analyzed by 9% SDS-PAGE. A 90 kDa protein was co-immunoprecipitated only in the presence of K1L antibody (lane 1). The band was excised and sequenced by Mass Spec.

2. MALDI MS/PSD Mass Spectrometry sequencing of the immunoprecipitated protein.

Mass spectrometric techniques are used for the identification of proteins in the database (proteomic mass spectrometry). Immunoprecipitated proteins were run on 9% SDS PAGE gel and stained with silver stain plus as described in Materials and Methods (Fig. 9C). The pertinent band was first digested with trypsin and then mass analyzed and identified by MALDI MS/PSD. Matrix assisted laser desorption ionization (MALDI) is a technique where the sample is co-crystallized with a matrix. The matrix is capable of absorbing UV, and when irradiated in vacuum, the peptide sample absorbs energy transferred from the matrix and produces gaseous ions that can be mass analyzed. After mass measurements are completed, mass analysis is done by MS/PSD. MS/PSD (Post-source-decay) analysis is based on a single peptide and the fragments derived from it; and this data is put through the database using search algorithm. The sample analyzed was identified as the rabbit homologue of human ACAP2 (Fig 10). The peptide fragments generated by the proteomics analysis and used in the blast searches are as follows:

KHSTIQQKD, RMNEEMRE, RSLGVHFSKV, RFQQDSQKF,

KAVOTSIATAYRE, KHLNPGLQLYRA (Table 3). The MALDI MS/PSD

analysis was done at the proteomics core facility at the UMASS Medical Center, Worcester, by Dr. John Leszyk.

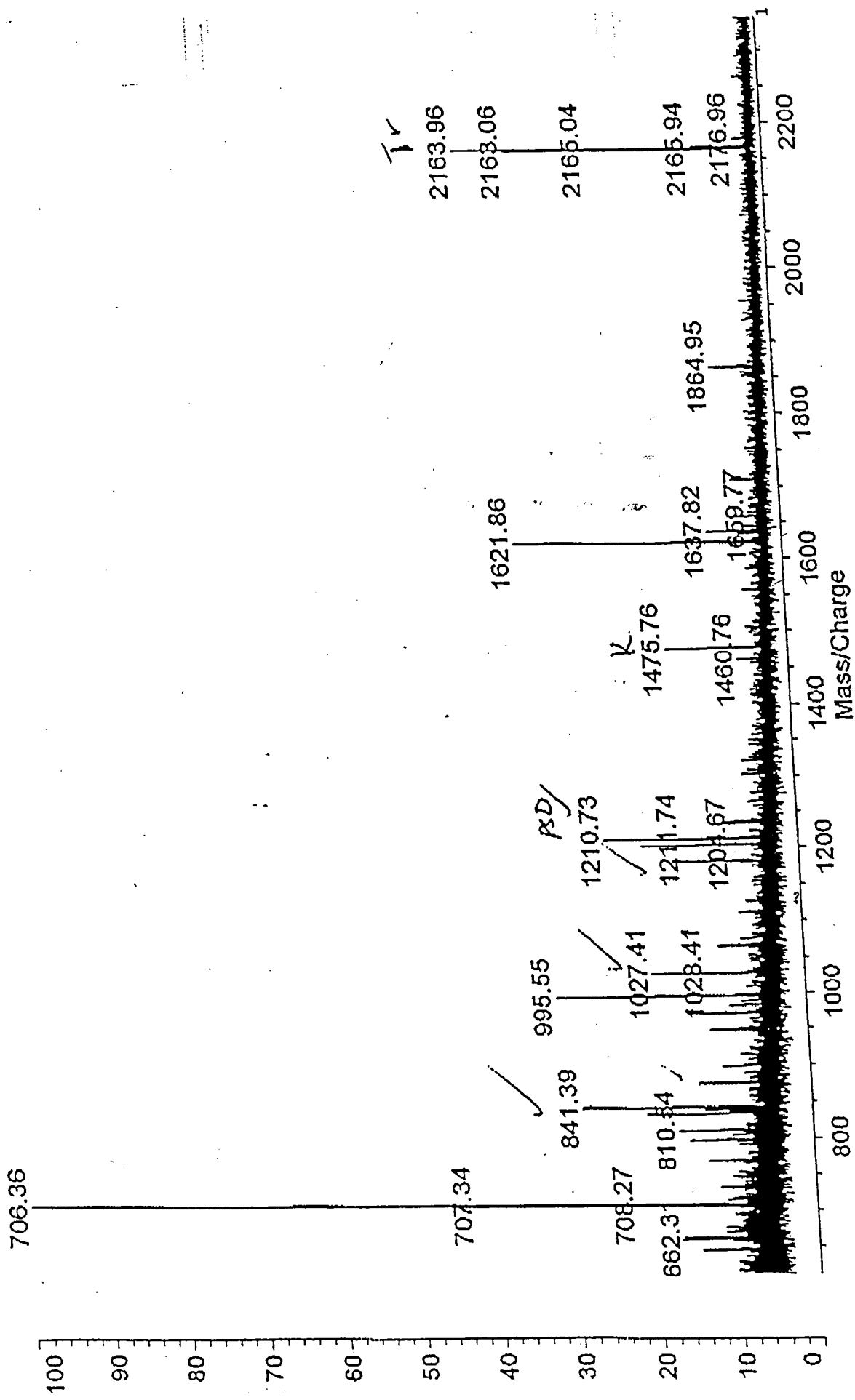


Fig 10. Post Source Decay Analysis.
Dr. John Leszyk, Proteomics core facility, UMASS medical Center

Table 3 MS-Fit Search Results

Press stop on your browser if you wish to abort this MS-Fit search prematurely.

Sample ID (comment): Magic Bullet digest
 Database searched: NCBI nr.100602
 Molecular weight search (1000 - 100000 Da) selects 1154666 entries.
 Full pI range: 1204732 entries.
 Species search (MAMMALS) selects 211101 entries.
 Combined molecular weight, pI and species searches select 199522 entries.
 MS-Fit search selects 16 entries (results displayed for top 2 matches).

Considered modifications: | Peptide N-terminal Gln to pyroGlu | Oxidation of M | Protein N-terminus Acetylated |

Min. # Peptides to Match	Peptide Mass Tolerance (+/-) ppm	Peptide Masses are monoisotopic	Digest Used Trypsin	Max. # Missed Cleavages 1	Cysteines Modified by acrylamide	Peptide N terminus Hydrogen (H)	Peptide C terminus Free Acid (O H)	Input # Peptide Masses 10
5	150.000	monoisotopic	Trypsin	1	acrylamide	Hydrogen (H)	Free Acid (O H)	10

Result Summary

Rank	MOWSE Score	#(%) Masses Matched	Protein MW (Da)/pI	Species	NCBI nr.100602 Accession #	Protein Name
1	38.7	5/10 (50%)	71243.7 / 6.60	HOMO SAPIENS	<u>3183205</u>	(D26069) Start codon is not identified
2	32.2	5/10 (50%)	87903.9 / 6.64	HOMO SAPIENS	<u>(17977656)</u>	(AJ238248) centaurin beta2

Detailed Results

1. 5/10 matches (50%). 71243.7 Da, pI = 6.60. Acc. # 3183205. HOMO SAPIENS. (D26069) Start codon is not identified.

m/z submitted	MH ⁺ matched	Delta ppm	start	end	Peptide Sequence (Click for Fragment Ions)	Modifications
841.3900	841.4532	-75.0971	96	102	(K)HSTIQQK(D)	
841.3900	841.3184	85.0890	585	590	(R)MNEEMR(E)	2Met-ox
874.4300	874.4787	-55.6642	297	304	(R)SLGVHFSK(V)	
1027.4100	1027.4849	-72.8817	625	632	(R)FOODSOKF(-)	
1180.7100	1180.6326	65.5481	209	219	(K)AVQTSIATAYR(E)	
1210.7300	1210.6697	49.8353	457	466	(K)HLNPGLQLYR(A)	

5 unmatched masses: 797.3500 832.4800 948.4800 970.4600 1475.7600

The matched peptides cover 7% (49/632 AA's) of the protein.
 Coverage Map for This Hit (MS-Digest index #): 963232

2. 5/10 matches (50%). 87903.9 Da, pI = 6.64. Acc. # 17977656. HOMO SAPIENS. (AJ238248) centaurin beta2.

3. Cloning of Rabbit *ACAP2*

To confirm the presence of the rabbit homologue of human ACAP2 protein, we cloned the rabbit *ACAP2* gene by RT-PCR and 5' RACE using total RNA from uninfected RK-13 cells as template (Fig 11), as explained in materials and methods. A Nucleotide Blast search showed 93% sequence identity between the rabbit and human *ACAP2* genes (Fig 12). A protein blast search showed 98% sequence identity between the two proteins (Fig 13). All conserved domain searches were done using the CDD: <http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>.

An experiment to co-immunoprecipitate K1L using anti-ACAP2 antibody has been performed. However, the anti-ACAP2 antibody (kindly provided by Dr. Paul Randazzo, NIH) has never been tested in an immunoprecipitation reaction and in our experiments failed to immunoprecipitate ACAP2. In order to study the interaction between ACAP2 and K1L *in vitro*, we performed the following experiment. We amplified rabbit ACAP2 and introduced a 5'T7 promoter and a 3'HA tag by PCR. We confirmed the construct by direct sequencing of the PCR product. We tried to *in vitro* translate the ACAP2 protein from the PCR product (using the commercial kit: TNT T7 Quick for PCR DNA, Promega) and were planning to perform an *in vitro* immunoprecipitation reaction using the *in vitro* translated ACAP2 and *in vitro* translated K1L proteins. However, while we were able to produce *in vitro* translated K1L protein, the ACAP2 protein was very difficult to translate.

1 MKMTVDFEELKDSPRFRAALEEVEGDVAELELKLKLVKLCIAMIDTGKAF
 2 CLANKQFMNGIRDLAQYSSNDAAVETS~~LT~~KFSDSLQEMINFHTILFDQTORSIK
 3 AQLQNFVKEDLKKFKDAKKQFEKVSSEKENALAKNAQVORNKQHEVEEATNILLT
 4 ATRKCFRHI~~ALDYVLQ~~INVLQSKRRSEILKSMLSFMYAHLAFEHQGYDLFSELG
 5 PYMKD**LGAQLDR**LVDAA**KEKREMEQKHS****TIQOKD**FSDDSKLEYNVDAANGIV
 6 MEGYLFKRA~~SN~~AFKTNRRWF~~SI~~QNNQLVYQKKFKDNPTVVVEDLRLCTVKHCE
 7 DIERRFCFEVVSPTKSCMLQADSEKLRQAWI**KAVQTSIATAYREK**GESEKLDK
 8 KSSPSTGSLDSGNEKELLLKGESALQRVQCVPGNASCCDCGLADPRWASINLG
 9 ITLCIECSGI**HRSLGVHFSK**VRS~~LT~~LDTWEPELLKLMCELGNDVINRVYEANVE
 10 KMGIKKPPQPGQROEKEAYIKAKYVERK~~FVD~~KYSVSSPPEQEKVSVSKDSEEKR
 11 LSIPKLGPGDQVRTSIQSSVKSNDGIQSSDDGRESLPSTVSANSLYEPEGER
 12 QDSSVFLDS**KHLNPG**L**LYRA**SEYK~~NLP~~KMAEALAHGADVNWANSEENKATPLI
 13 QAVLGGSLVTCEFLQNGANVNQRDVQGRPLHHATVLGHTGQVCLFLKRGANQ
 14 HATDEEGKDPLSIAVEAANADIVTLLRLA**RMNEEMRE**SEGLYGQPGDETYQDIF
 15 RDFSQMASNNPEKLN**RFOOD**SOKE 778

FIG.11. The 90 kDa protein was identified as rabbit homologue of human ACAP2. MALDI MS/PSD analysis of peptide fragments obtained from co-immunoprecipitated, silver stained protein band identified several amino acid sequences (Fig 6, underlined, bold sequences), which, when used in a blast search, showed sequence similarity to human ACAP2. Amino acid sequence of rabbit ACAP2 (nucleotide Accession number: AY620244) shows 98% identity with human ACAP2 (AJ238248). A conserved domain search of the rabbit ACAP2 showed that it has ankyrin repeats (red), Arf-GAP domain (blue), Pleckstrin homology domain (green) and a BAR domain (brown).

gi|40254841|ref|NM_012287.3| **GU** Homo sapiens centaurin, beta 2 (CENTB2), mRNA
 Length = 7102

Score = 3449 bits (1740), Expect = 0.0
 Identities = 2193/2344 (93%)
 Strand = Plus / Plus

```

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Sbjct: 171 ggcaggatgaagatgactgtggatttcgaggagtgtctgaaggactcgccccgcttcagg 230

Query: 61   gctgctttggaagaagtagaaggagatgtggctgaattggaactaaaacttgataagctt 120
          ||
Sbjct: 231   gcagctttggaagaagtagaaggatgtggcagaattggaactaaaacttgataagctt 290

Query: 121  gtgaagctctgtattgccatgattgataccggaaaagccttttgcttgcaaataaacag 180
          |||||
Sbjct: 291  gtgaaactttgtattgcaatgattgatactggaaaagccttttgcttgcaaataaacag 350

Query: 181  ttcatgaacgggatccgagacctggcccagtattctagtaatgatgctgtagttgaaaca 240
          |||
Sbjct: 351  ttcatgaatgggattcgagacctggctcagtattctagtaatgatgctgtcgttgagaca 410

Query: 241  agtttgaccaagttttctgacagctttcaagaaatgataaattttcacacaatcctgttt 300
          |||
Sbjct: 411  agtttgaccaagttttctgacagctttcaagaaatgataaattttcacacaatcctgttt 470

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Sbjct: 471  gaccaaactcagagatcaattaaggcacagcttcagaactttgttaagaagatccttaga 530

Query: 361  aaatttaagatgccaagaagcaatttgaaaaagtcagtgaagagaaagaaaacgcattg 420
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Sbjct: 531  aaattcaagatgccaagaagcaattcgaaaaagtcagtgaagagaaagaaaatgcggtta 590

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          |||
Sbjct: 651  ctgacagcaacaagaaaatgtttccgacacatatagccctcgattatgtgcttcagattaat 710
  
```

Fig 12. Nucleotide homology between human ACAP2 and rabbit ACAP2.

Query: 1201 gaaagtgcactgcagcgggtccagtggtgctccctgggaatgetagctgttgactgtggt 1260
||||||| || ||||||||||||||||| ||||||| ||||| |||||||||||||||||
Sbjct: 1371 gaaagtgcgcttcagcgggtccagtggtatccctggcaatgccagctgttgactgtggc 1430

Query: 1261 ctggcagatccacggtgggccagcatcaacttaggcatcaccttgtgcattgagtgtct 1320
||||||||||||||||||||||||||||||| | ||||||||||||||||| || |||||||||
Sbjct: 1431 ctggcagatccacggtgggccagcatcaacctgggcatcaccttgtgtatcgagtgtctc 1490

Query: 1321 gggattcacaggagtcttgggggttcatttttcaaaagtgcgatctttaactttagacacc 1380
|| ||||||| |||| ||||||||||||||||||||| |||||||||||||||||||||
Sbjct: 1491 ggaattcacggagccttgggggttcatttttcaaaagtacgatctttaactttagacacc 1550

Query: 1381 tgggagcctgaacttttaagcttatgtgtgagttggggaatgatggtataaacagagtt 1440
||||||||| ||||||||||||||||||||||||| ||||||||||||||||| |||||
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Query: 1441 tatgaagctaactggaaaaaatgggaataaagaagccacaaccaggacaaagacaggag 1500
||||||||||| ||||||||||||||||||||||||| || ||||||||||||||||| |||||
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Query: 1501 aaagaggcatatatcaaagcaaaatgtggaaggaaatttgggataaatattctgta 1560
|| ||||||||||||||||| ||||||||||||||||| ||||||||||||||||| |||||
Sbjct: 1671 aaggaggcatatatcagagcaaaatgtggaaggaaatttgggataaatattctata 1730

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||| ||||||||||||||||| ||||||||| ||| | |||||||||||||
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||||||||| ||||| ||||||||||||| ||||||||||||| ||||| ||| |||||||||
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Query: 1681 aaaagtaatgacagtggaatccagcagagttctgatgatggaagagaatctttaccctcc 1740
| ||||||||||||||||| ||| ||||||||| ||||||||||||||||| |||||||||||||
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Query: 1861 cttccaaaaatggctgaggctttggctcatgggtgcagatgtgaactgggctaattcagag 1920
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 ||||| |||||
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Query: 2341 tgaa 2344
 ||||
 Sbjct: 2511 tgaa 2514

gi|39932727|sp|Q15057|CEB2 HUMAN **G** Centaurin beta 2 (Cnt-b2)
 Length = 778

Score = 1523 bits (3943), Expect = 0.0
 Identities = 763/778 (98%), Positives = 770/778 (98%)

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 MKMTVDFEELKDSPRFRAALEEVEGDVAEELKLDKLVKLCIAMIDTGKAFCLANKQFM 60
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Query: 181 QSKRRSEILKSMLSEMYAHLAFFHQGYDLFSELGYPYMKDLGAQLDRLVVDAAKEKREMEQ 240
 QSKRRSEILKSMLSEMYAHLAFFHQGYDLFSELGYPYMKDLGAQLDRLVVDAAKEKREMEQ 240
 Sbjct: 181 QSKRRSEILKSMLSEMYAHLAFFHQGYDLFSELGYPYMKDLGAQLDRLVVDAAKEKREMEQ 240

Query: 241 KHSTIQKDFSSDDSKLEYNVDAANGIVMEGYLFKRASNAFKTWNRRWFESIQQNQLVYQK 300
 KHSTIQKDFSSDDSKLEYNVDAANGIVMEGYLFKRASNAFKTWNRRWFESIQQNQLVYQK 300
 Sbjct: 241 KHSTIQKDFSSDDSKLEYNVDAANGIVMEGYLFKRASNAFKTWNRRWFESIQQNQLVYQK 300

Query: 301 KFKDNPTVVVEDLRLCTVKHCEDIERRFCFEVVSPTKSCMLQADSEKLRQAWIKAVQTSI 360
 KFKDNPTVVVEDLRLCTVKHCEDIERRFCFEVVSPTKSCMLQADSEKLRQAWIKAVQTSI 360
 Sbjct: 301 KFKDNPTVVVEDLRLCTVKHCEDIERRFCFEVVSPTKSCMLQADSEKLRQAWIKAVQTSI 360

Query: 361 ATAYREKGDSEKLDKKSSPSTGSLDSGNESKEKLLKGESALQRVQCVPGNASCCDCGLA 420
 ATAYREKGDSEKLDKKSSPSTGSLDSGNESKEKLLKGESALQRVQC+PGNASCCDCGLA 420
 Sbjct: 361 ATAYREKGDSEKLDKKSSPSTGSLDSGNESKEKLLKGESALQRVQCIPGNASCCDCGLA 420

Query: 421 DPRWASINLGITLCIECSGIHRS LGVHFSKVRSLTLDTWEPELLKLMCELGNDVINRVYE 480
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 Sbjct: 421 DPRWASINLGITLCIECSGIHRS LGVHFSKVRSLTLDTWEPELLKLMCELGNDVINRVYE 480

Query: 481 ANVEKMGIKKPQPGQRQEKEYIKAKYVERKFDKYSVSSSPPEQEKVSKDSEEKRLS 540
 ANVEKMGIKKPQPGQRQEKEYI+AKYVERKFDKYS+S SPPEQ+KK VSK SEEKRLS 540
 Sbjct: 481 ANVEKMGIKKPQPGQRQEKEYIRAKYVERKFDKYSISLSPPEQQKFKVSKSSEEKRLS 540

Query: 541 IPKLGPGDQVRTSIQSSVKSNDSGIQSSDDGRESLPSTVSANSLYEPEGERQDSSVFLD 600
 I K GPGDQVR S QSSV+SNDSGIQSSDDGRESLPSTVSANSLYEPEGERQDSS+FLD 600
 Sbjct: 541 ISKFGPGDQVRASQSSVRSNDSGIQSSDDGRESLPSTVSANSLYEPEGERQDSSMFLD 600

Query: 601 SKHLNPGQLQYRASYEKNLPKMAEALAHGADVNWANSEENKATPLIQAVLGGSLVTCEFL 660
 SKHLNPGQLQYRASYEKNLPKMAEALAHGADVNWANSEENKATPLIQAVLGGSLVTCEFL 660
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Query: 661 LQNGANVNQRDVQGRGPLHHTVGLGHTGQVCLFLKRGANQHATDEEGKDPLSIAVEAANA 720
 LQNGANVNQRDVQGRGPLHHTVGLGHTGQVCLFLKRGANQHATDEEGKDPLSIAVEAANA 720
 Sbjct: 661 LQNGANVNQRDVQGRGPLHHTVGLGHTGQVCLFLKRGANQHATDEEGKDPLSIAVEAANA 720

Query: 721 DIVTLLRLARMNEEMRESEGLYQPGDETYQDI FRDFSQMASNNPEKLNRFQQDSQKF 778
 DIVTLLRLARMNEEMRESEGLYQPGDETYQDI FRDFSQMASNNPEKLNRFQQDSQKF 778
 Sbjct: 721 DIVTLLRLARMNEEMRESEGLYQPGDETYQDI FRDFSQMASNNPEKLNRFQQDSQKF 778

Fig 13. Amino acid sequence homology between human ACAP2 and rabbit ACAP2

4. K1L protein interacts with ACAP2 differently in RK-13 and CV-1 cells.

We were able to co-immunoprecipitate (using anti-K1L antibody) a 90 kDa band from NYCBH-infected CV-1 cells, which could be detected only after a longer exposure (Fig 14). Although it is difficult to measure a quantitative difference in cells from different species using immunoprecipitation, we detected less ACAP2 in CV-1 cells (permissive) (Fig 14) than in RK-13 cells (non-permissive) (Fig 9A). We tried to detect the constitutive expression of the ACAP2 protein in RK-13 cells as compared to that in CV-1 cells. However, western blot analysis using anti-ACAP2 antibody did not yield conclusive results because the antibody produced multiple bands at the expected size, which is 90 kDa.

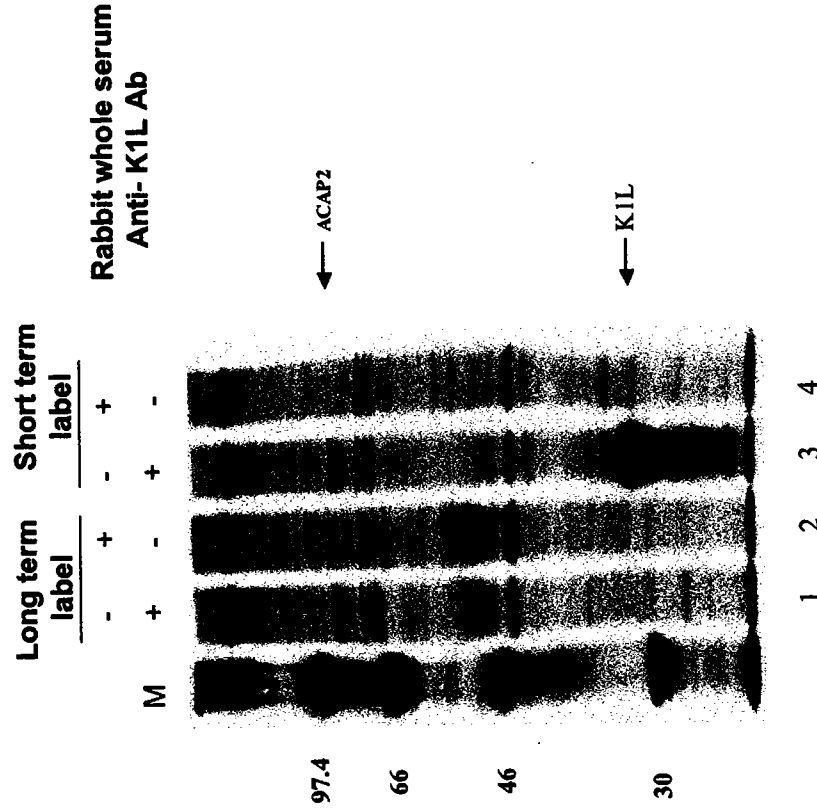


FIG. 14. ACAP2 of CV-1 cells, pulled down by K1L Antibody, was detectable after long exposure. CV-1 cells were either long-term labeled (lanes 1 and 2), or short-term labeled (lanes 3 and 4) with 35S-methionine. Cell lysates were prepared and proteins were immunoprecipitated with either anti-K1L antibody (lanes 1 and 3) or control serum (lanes 2 and 4). Cell lysates were analyzed by 9% SDS-PAGE. A weak band of 90 kDa protein was co-immunoprecipitated in the presence of K1L antibody (lane 1) but was detectable only after a long exposure.

CHAPTER V

RESULTS

SECTION III

A. Functional significance of the K1L-ACAP2 interaction

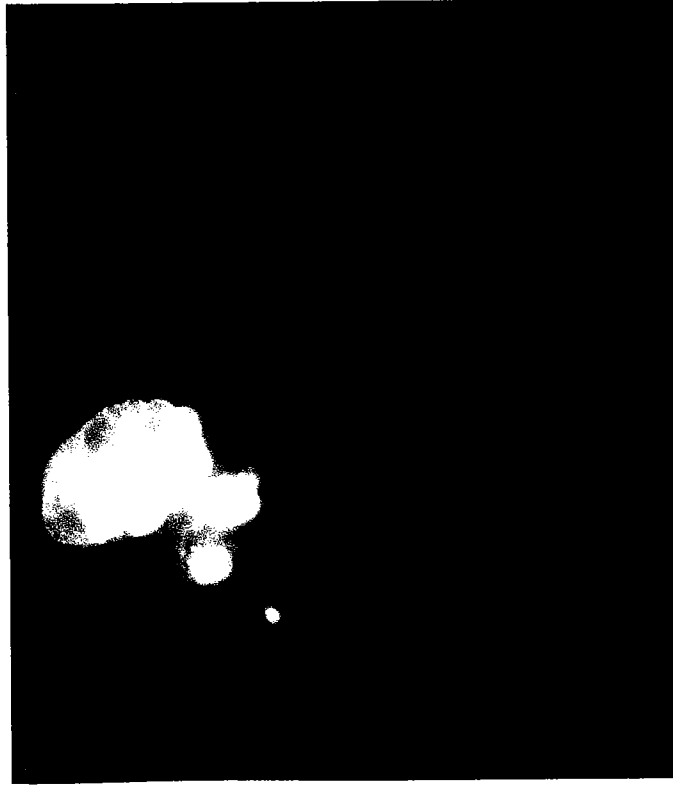
1. Study of the formation of viral factory

As mentioned above, cells infected with the mutant VV lacking the K1L gene product have a defect in early protein production along with shutdown of DNA replication and intermediate and late gene expression. Since we have been able to show that the K1L protein interacts with rabbit ACAP2, which is involved in membrane transportation, one of the questions we asked was whether the defect in DNA synthesis and intermediate and late gene expression was a result of defects in formation of the viral factory, which is the site of viral DNA synthesis.

RK-13 cells grown on cover-slips in 6-well plates were infected with MOI of 5 of the NYCBH, vAbT33K1Lwt or vAbT33 viruses. After an incubation of 3 hours at 37⁰ C, cells were fixed in 4% paraformaldehyde, washed, and then stained with Hoechst's stain that enables the visualization of DNA, both host and viral, using fluorescent microscopy. We were able to detect viral factories which appeared as blue bodies in the cytoplasm, distinct from the nucleus, in cells infected with

NYCBH (Fig 15A) and vAbT33 (Fig 15B). These bodies were, however, absent in uninfected cells (Fig 15C). The presence of these bodies in cells infected with the mutant virus vAbT33 suggested that the host-range phenotype of defective DNA replication and intermediate and late gene expression is not due to the inability of the virus to form the viral factories.

HOECHST



PHASE



FIG 15A. Detection of viral factory in RK-13 cells infected with NYCBBH.
Extra-nuclear material was visible when stained by Hoechst as well as by phase microscopy.

PHASE



HOECHST

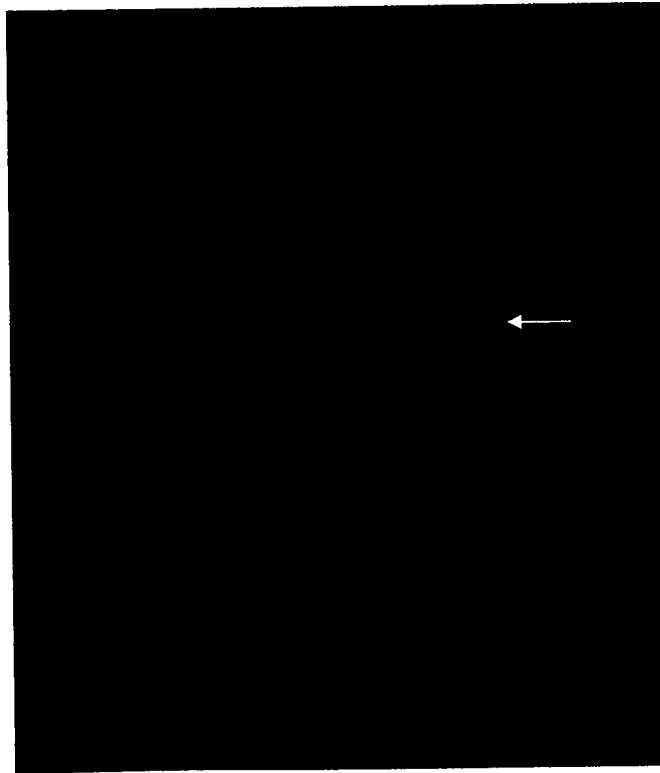


FIG 15 B. Detection of viral factory in RK-13 cells infected with vAbT33.
Extra-nuclear material was visible when stained by Hoechst as well as by phase microscopy.

PHASE



HOECHST

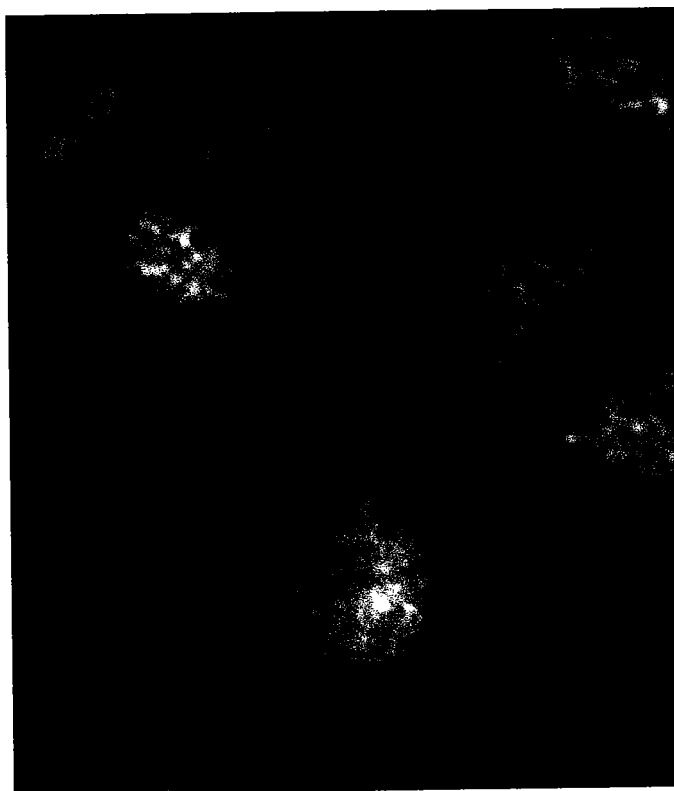


FIG. 15C. No viral factories detected in uninfected RK-13 cells.

2. Study of the effect of silencing *ACAP2* expression in RK-13 cells:

The identification of the rabbit *ACAP2* as a protein interacting with K1L is very interesting. Whether this protein is directly involved in the host range function mediated by the K1L protein was the next question to ask. The simplest way to explore the role of *ACAP2* in the K1L mediated host-range function is to silence the expression of the *ACAP2* protein in the non-permissive cell line and study the effect on viral growth. We decided to accomplish this by using the siRNA technology. siRNA (1.4 μ g) specifically targeting rabbit *ACAP2* was transfected into RK-13 cells as described in Materials and Methods. As a control, a non-silencing control siRNA (1.4 μ g) was also transfected into the RK-13 cells. The commercially available non-silencing control does not have sequence homology to any mammalian sequences. The transfection efficiency of RK-13 using conventional lipofectamine reagents has not been very good. So, the transfection efficiency was first determined using an electroporation technique as described in materials and methods. At the end of the specific time points, GFP expression was analyzed by FACS. FACS analysis showed that the transfection efficiency using the electroporation method was at least 60 % at 24 hours post- transfection (Fig 16). So, RK-13 cells were transfected with 1.4 μ g of siRNA specific to rabbit *ACAP2* and 1.4 μ g of the non- silencing control siRNA using the amaxa electroporation technology. The cells were incubated for 24 hours and then infected with MOI of 0.1 of the vAbT33 (K1L-) strain of VV. This experiment was done in duplicate. In

the first set of experiments, total RNA was extracted from the RK-13 cells, post-transcription and post-infection. After cDNA synthesis, rabbit *ACAP2* and rabbit *β -actin* messages were amplified using real time PCR (Tables 4 and 5 and Fig 17). The transcript levels of rabbit *ACAP2* were normalized against rabbit *β -actin* transcript levels. The normalized values were plotted in a graph (Fig 17). There was about a 90% reduction in *ACAP2* message in cells treated with the specific siRNA as compared to cells treated with control. In the second set of experiments cells were harvested and the virus titrated in permissive CV-1 cells. No viral growth was observed in cells treated with specific siRNA and cells treated with non-specific control siRNA. One limitation of these experiments, however, is the inability to measure the extent of infection in the transfected cells.

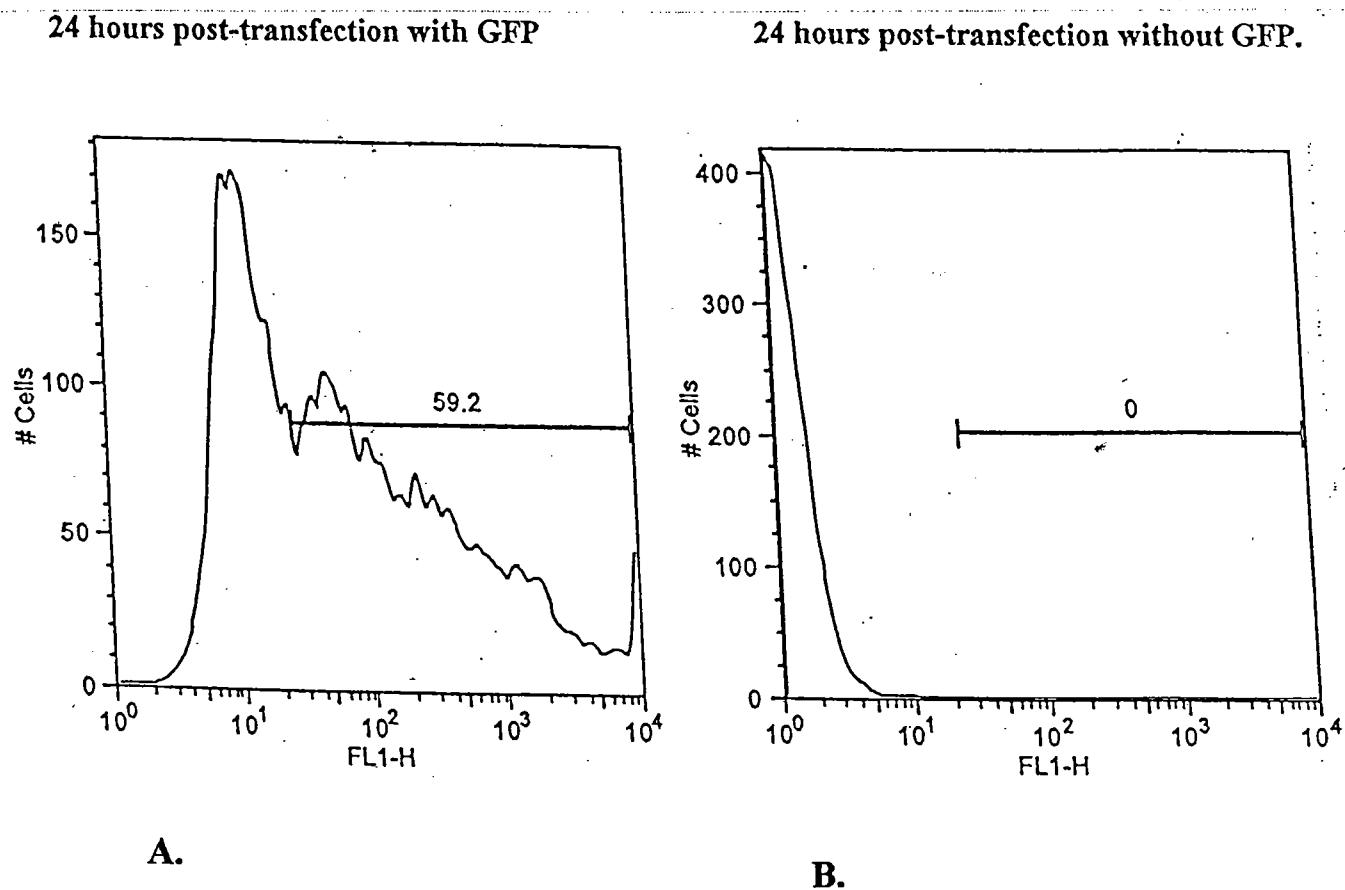


Fig.16 Transfection efficiency in RK-13 cells using amaxa nucleofection. RK-13 cells were transfected with pMax-GFP plasmid construct. 24 hours post transfection, GFP expression was monitored by FACS analysis. At least 60% cells were transfected (Fig. 16 A) as compared to the negative control (Fig. 16 B).

**Thermal Cycling
Parameters for
Two-Step PCR**

Table 4**Reverse Transcription**

Step	Incubation ^a	Reverse Transcription	Reverse Transcriptase Inactivation
	HOLD	HOLD	HOLD
Temperature	25 °C	48 °C	95 °C
Time	10 min	30 min	5 min

a. When using random hexamers or oligo d(T)16 primers for first-strand cDNA synthesis, a primer incubation step (25 °C for ten minutes) is necessary to maximize primer-RNA template binding. During incubation the hexameric or oligo d(T)16 primers are extended by reverse transcriptase. If using a sequence specific reverse primer, the incubation step is not necessary.

Table 5**PCR**

Step	UNG Incubation	AmpliTaq Gold Activation	PCR ^a	
	HOLD	HOLD	CYCLE (40 cycles)	
			Denature	Anneal/ Extend
Temperature	50 °C	95 °C	95 °C	60 °C
Time	2 min	10 min	15 sec	1 min

a. It is important to keep the reaction temperatures >55 °C to prevent amplicon degradation.

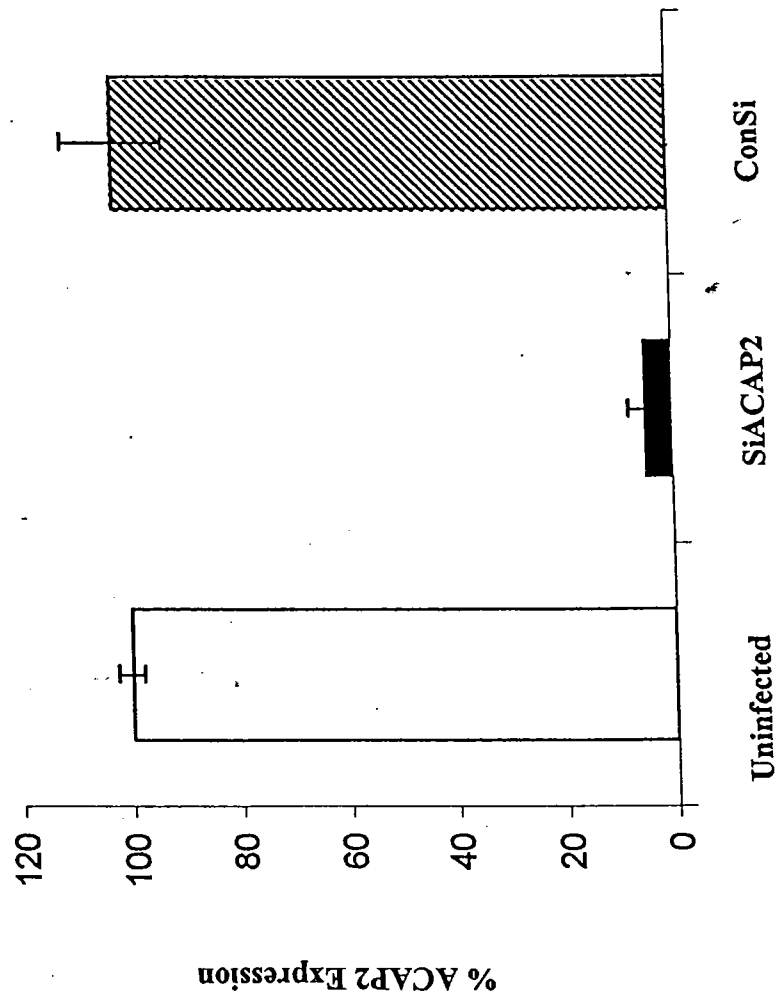


FIG.17. % ACAP2 expression in RK-13 cells: a) Uninfected b) Treated with siRNA specific to ACAP2 (SiACAP2) and infected with vAbT33 and c) Treated with control non-specific siRNA (ConSi) and infected with vAbT33

CHAPTER VI

DISCUSSION

CONCLUSIONS OF DISSERTATION

The objective of this study was to understand the role of K1L protein of VV in mediating the host restriction in the RK-13 cell line. As mentioned previously, earlier studies with this protein have shown that it is involved in the maintenance of viral protein synthesis. The reason for the abrupt shutdown of protein synthesis in the absence of K1L in RK-13 cells is not known. The cessation of cellular protein synthesis in the event of a viral infection has been well studied.

It has been reported that cellular mRNAs are transported on microtubules, which during viral infection are replaced by viral mRNA, thus bringing about a gradual cessation of cellular protein synthesis. However the shutdown of both cellular and viral protein synthesis, in the absence of K1L protein, suggests that this protein is in some way involved in the translation pathway. The absence of common functional domains in this protein has made its study very difficult. The only domains that this protein has are ankyrin repeats. We were able to show that the most conserved

ankyrin repeat is important not only for the viral growth in RK-13 cells, but also for the maintenance of viral protein synthesis. The importance of the ankyrin repeat may explain the complementation of K1L function by the CP77 protein, which also has ankyrin repeats. CP77 has two much conserved ankyrin repeats. The loss of function due to secondary effect on protein structure may be possible because the mutated K1L lost one α -helix. Crystal structural studies would be needed to determine the details of a secondary effect on the structure of the K1L protein. The definition of the mechanism by which the ankyrin repeat of K1L is involved in this host-range restriction and shut down of all protein synthesis is an important question that needs to be answered. The mutation in the ankyrin repeat, however, did not seem to make a difference in the localization of this protein, since both the wild type and mutated protein localized all over the cytoplasm.

The fact that growth of VV lacking the K1L gene product is restricted in some cell lines (rabbit kidney, human) and not in others (african green monkey, BHK, CEF) brings up the question of the differences between the two groups. Whether or not any cellular proteins are involved in the host range restriction of VV, mediated by the K1L protein, is a pertinent question. It has been suggested in earlier studies of the K1L protein, that cellular effectors or regulators may be involved. This prompted us to ask the question of whether the K1L protein mediates its host-range function via a cellular protein. In this study, we have been able to preliminarily identify a cellular protein being co-immunoprecipitated with the K1L

protein in cells infected with VV. This protein was identified as ACAP2 (GTPase-activating) protein and belongs to the ACAP family of proteins.

The identification of ACAP2 as the protein interacting with K1L, prompted us to focus on this family of proteins as well as their regulators, which are discussed in depth below. ACAP2 belongs to a GTPase-activating family, and there is evidence in the literature about many anti-viral GTPases. Hence, we also focused on these families as described below.

A. The ACAP protein family.

The ACAP family of proteins comprises GTPase-activating proteins, and is known to regulate, specifically, the Arf family of GTP-binding proteins. The ACAP2 family consists of 4 family members; ACAP1, ACAP2, PAP, and ASAP1. The family members are characterized by the presence of a GAP-activating domain, and sequences comprising PH, coiled coil, and ankyrin repeat domains (Fig 18) (Jackson et al., 2000). These proteins occur in multicellular organisms including *C. elegans*, *Drosophila*, *Arabidopsis*. No orthologs were found in *Saccharomyces*. Earlier studies have shown that the proteins are highly conserved among mammals. This is reflected in our study, where we have shown that rabbit ACAP2 is 98% identical to human ACAP2. The crystal structure of one member of the ACAP family shows that the ankyrin repeat physically associates with the GAP domain intra-

molecularly, but whether this association is involved in mediating the GAP activity is not yet known (Mandiyan et al., 1999).

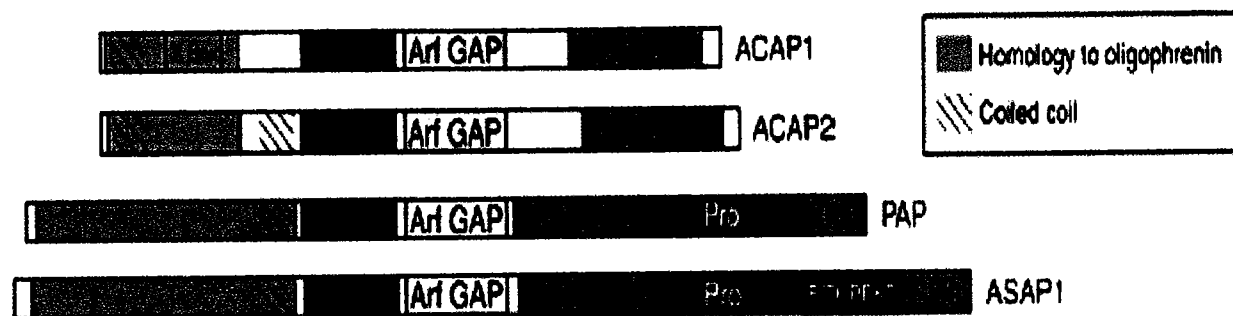


Fig 18.

Schematic of ASAP/ACAP family members.

ArfGAP: ArfGAP domain.

ANK: Ankyrin Repeat.

PH: Pleckstrin homology domain.

The "X Box" refers to the PI-PLC X-box. "Pro" refers to a proline-rich domain with SH3-binding sites. "E/DLPPKP" is a domain of tandem repeats of this consensus sequence.

(Jackson et al., 2000)

B. The Function of ACAP2

As mentioned earlier, the ACAP family of proteins have GTPase-activating (GAP activity) functions mediated by the GAP domain and have been shown to be regulators of the Arf family members (Donaldson and Jackson, 2000). The GAP proteins are stimulated by phospholipase D (PLD), phosphatidylinositol 3, 4, 5-triphosphate (PIP3) and PIP2 and phosphatidic acid (PA). These stimuli activate the GAPs to bring about GTP-binding and hydrolysis of its substrates (Randazzo et al., 2000). ACAP2 has been shown to act more specifically on Arf 6 than Arf 1. ACAP2 has been shown to be present in the cytoplasm and on tubular portions of the Arf 6 endosomal compartment (Jackson et al., 2000). As suggested in earlier reports, ACAP2 can be recruited to the membrane by activated Arf6. It has also been suggested that because of the presence of multiple domains, the ACAP family of proteins can have more than one role, and they could be acting as mediators between proteins that are involved in cytoskeletal reorganizations. In order to better understand the regulatory function of ACAP2, it is important to understand the Arf family of proteins.

C. The ARF family

The ADP-ribosylation factors (Arfs) are 20 kDa GTP-binding proteins belonging to the Ras GTPase superfamily (Donaldson and Jackson, 2000). These proteins were first identified as stimulators of the ADP-ribosyltransferase activity of the cholera

toxin (CTA) (Moss and Vaughan, 1995). The Arf proteins can be further divided into three classes: Class I (Arf 1, 2 and 3), Class II (Arf 4 and 5), and Class III (Arf 6) (Moss and Vaughan, 1998) (Moss and Vaughan, 1995). Arf 1, 3, and 6 are the most well studied of the Arf proteins. The Arf proteins are active in their GTP bound form and inactive in their GDP bound form. Both, the release of GDP and the hydrolysis of GTP bound to the Arf protein, are either very slow or undetectable under physiological conditions, respectively. The guanine nucleotide exchange protein (GEP) activates the Arf protein by converting it into its GTP bound form. Arf does not have GTPase activity and the conversion of Arf-GTP to Arf-GDP is facilitated by the GTPase activating proteins (GAPs). Thus the GEP and GAP proteins together help maintain both the GTP and GDP bound forms of Arf (Nie et al., 2003) in the cell (Fig19). In cells, the function of Arf has been related to vesicular transport in the Golgi, endosome fusion, formation of clathrin-coated vesicles, assembly of nuclear membrane, and intravesicular acidification. They have also been shown to be involved in regulating membrane traffic, affecting membrane ruffling, filopodia, and actin rich protrusions (Donaldson and Jackson, 2000) (Donaldson and Jackson, 2000).

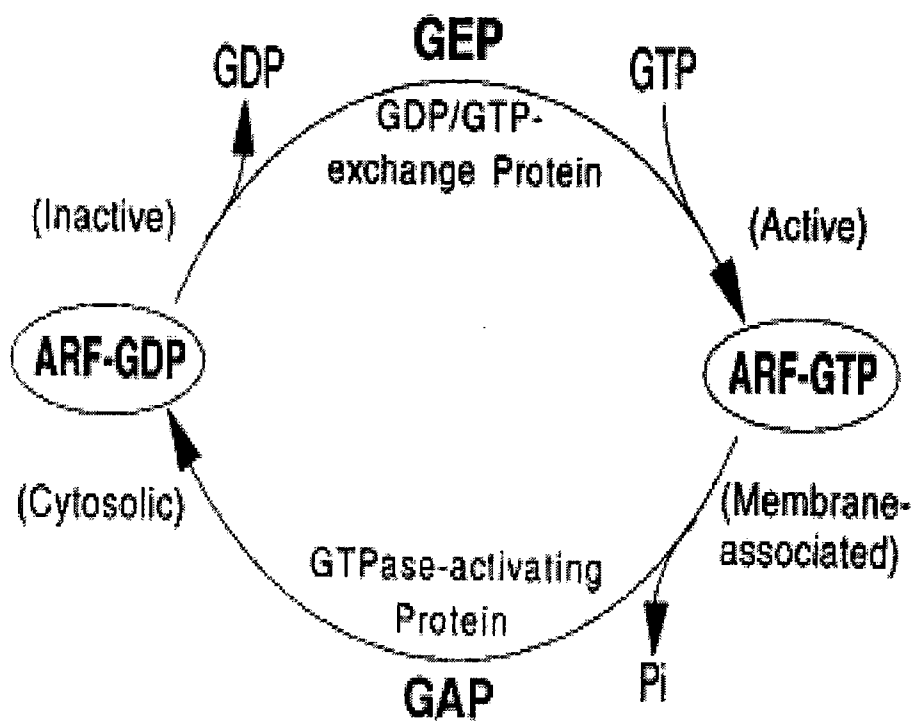


Fig 19.

Regulation of ARF activity by GEPs that accelerate GTP binding and GAPs that activate GTP hydrolysis by ARF.
(Moss and Vaughan, 1998)

D. ACAP2 may regulate other antiviral GTPases.

The involvement of GTPases in anti-viral activities is known (Taylor et al., 2004). As described before, GTPases are GTP binding proteins that can hydrolyze GTP. The GTPases have a variety of functions, and their role in anti-viral responses during an infection is still being investigated. Three families of GTPases have been shown to be induced by interferons, namely, the Mx, GBP and p47 families (Taylor et al., 2004). Being a GTPase-activating protein, the possibility of ACAP2 regulating the antiviral GTPases described below cannot be ruled out. A brief description of these GTPases and their roles in antiviral responses are as follows:

1) Mx family

The Mx proteins are interferon-induced GTPases and are members of the dynamin superfamily of large GTPases. In humans, there are two Mx proteins, MxA and MxB, encoded on chromosome 21. The MxA has been shown to have anti-viral activity against many RNA viruses, including bunyaviruses, orthomyxoviruses, and some DNA viruses like hepatitis B virus (Haller and Kochs, 2002) (Taylor et al., 2004). In infections with bunyaviruses, MxA inhibits viral assembly by interfering with transport of the viral nucleocapsid protein to the Golgi compartment (where viral assembly takes place). In the case of infection with Thogoto virus, an orthomyxovirus, MxA inhibits viral transcription and replication by interfering with the transport of incoming nucleocapsids into the nucleus. In rodents, there are two

Mx proteins, Mx1 (predominantly nuclear) and Mx2 (predominantly cytoplasmic). Most other vertebrates express only the cytoplasmic form of the protein. The subcellular localization of the rodent Mx proteins determines their anti-viral specificities, for eg. , the nuclear Mx1 protein responds against the orthomyxoviruses which replicate in the nucleus, while the cytoplasmic Mx2 protein acts against the bunyaviruses, which replicate in the cytoplasm.

2) GBP Family (guanylate-binding proteins)

The human proteins GBP1 and GBP2 and the mouse proteins Gbp1, Gbp2, Gbp3, Gbp4/Mag2 and Gbp5 are the proteins that are present most abundantly in response to IFN- γ stimulation. Apart from IFN- γ , the GBPs can be upregulated by stimulation with LPS, IL-1 β and TNF. The GBPs have high GTPase activity. The mouse Gbp2 allows targeting to intracellular vesicle like structures. HeLa cells become resistant to vesicular stomatitis virus (VSV) and encephalomyocarditis virus (EMCV) after over expression of GBP1 (Anderson et al., 1999).

3) p47 GTPase family

This group of 47-48 kDa proteins is produced in response to IFNs (Taylor et al., 2004). Present knowledge about this family shows the existence of six members in the mouse, namely, Igtg, Lrg47, Irg47, Tgtg/Mg21, Iigp and Gtpi. Homologues of these proteins exist in the humans, but have not been cloned yet. The role of these

proteins in resistance to protozoan and bacterial infections is very well studied. Their role in viral infections is not entirely understood. However, some studies have shown them to have antiviral activity *in vitro*. Overexpression of the Tgtp protein in fibroblasts decreased their susceptibility to lysis by VSV. In another study, overexpression of Igtp in HeLa cells inhibited coxsackievirus replication and apoptosis of the infected cells. The current models propose the following mechanisms of action for the p47 GTPases. P47 GTPases help in cell survival after viral infection through activation of the phosphatidylinositol-3-kinase (PI3K) survival pathway. They have also been shown to inhibit translation of viral messenger RNA.

4) Arf GTPase family

The Arf family members have been discussed above. Recent studies have shown that the HIV-1 protein Nef induces internalization of the MHC class-I molecule from the surface of the infected cell. In order to accomplish this, it activates PI3K, which results in the formation of PIP3 at the cell membrane. This in turn recruits Arf6 GEF to the PIP3 containing membrane and results in the activation of Arf6. Activated Arf6 promotes internalization of the MHC molecule from the surface. The Nef protein also blocks the recycling of the MHC molecules from the Arf6 compartment to the cell surface. The activation of Arf6, for the internalization of

MHC -I molecule, is an example of viruses hijacking cellular GTPases for immune evasion (Blagoveshchenskaya et al., 2002).

E. Early events involving cellular membrane participation

As mentioned in the introduction, the VV life cycle is completed in its entirety in the cytoplasm of the cell. This is unique among all DNA viruses, which replicate in the nucleus of the cell. Recent work elucidating early events occurring in the cells post-infection have shed light on several complicated yet interesting phenomena. Infection of cells by VV is followed by the association of intracellular viral cores with microtubules and the ER (Mallardo et al., 2002). Within minutes of the entry of the viral core containing the DNA genome into the cell about half the genome is transcribed and extruded out of the core in an ATP dependent manner. The transcripts have been shown to accumulate in regions in the cytoplasm in granular structures distinct from the cores and have been shown to be surrounded by polyribosomes (Mallardo et al., 2001). Polyribosomes are recruited to these sites, along with other host cell translational machinery, and early mRNAs are translated to early proteins (Mallardo et al., 2002). The amorphous structure containing the transcripts appeared to grow in size with infection. Both the cores and the mRNA containing structure have been shown to be associated with microtubules (Mallardo et al., 2001). It has been suggested that efficient VV early protein synthesis can be hampered if the organization of the granular site fails. It has been suggested that the

organization of these microtubules into the granular mRNA accumulation site is mediated predominantly by cellular proteins (Mallardo et al., 2002) (Mallardo et al., 2001). No cellular protein has so far been identified as mediating the formation of the mRNA accumulation site in VV-infected cells. The possibility of ACAP2 being involved in these early events is very likely. The host range restriction by K1L in non-permissive cells is associated with impairment of translation of viral proteins. An anti-viral role of ACAP2 alone or in conjunction with other proteins, in disrupting the formation of the granular structures explained above could very well account for the lack of viral protein synthesis.

F. Potential role of ACAP2 in host range function.

The difference in host-range restriction in cells of differing permissibility strongly suggests the role of a cellular protein. We were able to immunoprecipitate from infected RK-13 cells the cellular GTPase- activating protein, ACAP2. Keeping in mind the complexities of the life cycle of VV, it is possible to envision many possible ways that the ACAP2 protein may be involved in the pathway regulated by K1L.

1) An anti-viral role of ACAP2 would explain the difference in the host-range restriction phenotype observed in different cells (fig.20). The obvious question is why the host-range phenotype manifests itself differently in cells of different permissibility (CV-1 and RK-13 cells). This can be explained by a difference, either

in the expression levels of the ACAP2 protein, between the rabbit and African green monkey (Fig. 20). The simplest model would be that in non-permissive cells infected with the wild type VV, the functional K1L is capable of suppressing the anti-viral effects of ACAP2, while in those infected with the mutant (K1L-), the infection becomes non-productive (Fig 20).

2) A second possibility exists that the ACAP2 protein is recruited by the K1L protein and is essential for the growth of the virus. The question then arises as to why the host-range phenotype manifests itself differently in cells of different permissibility (CV-1 and RK-13 cells). This could be explained if there is a compensatory cellular protein, apart from ACAP2 that rescues the growth of the virus in the permissive cells and is either expressed in lower amounts or is very different in non-permissive cells.

In order to investigate whether the expression levels of the ACAP2 protein is different in the two cell lines, we performed an immunoprecipitation assay, trying to co-immunoprecipitate ACAP2 protein from infected CV-1 cells, using anti-K1L antibody. Results showed that there was less ACAP2 being pulled down in CV-1 cells as compared to that in RK-13 cells. The expected band was only detectable in lysates from CV-1 cells after longer exposure. However, it is very difficult to quantify proteins from two different species. An effort to quantify endogenous ACAP2 protein levels in the two cell lines by western blot analysis using an anti-ACAP2 antibody failed to give any conclusive results, as there were multiple bands

observed at the expected size. The role of ACAP2 in mediating host range function of K1L needs further study. The similarity between the ACAP2 sequences of human and rabbit at both the nucleotide and amino acid levels correlate with the similarity of host range restriction exhibited by the K1L (-) virus in both rabbit kidney cell lines and many human cell lines.

In order to further study ACAP2, we knocked down *ACAP2* in RK-13 cells, using siRNA technology. Although we were able to knock down rabbit *ACAP2* expression by about 90 %, we did not detect a significant increase in viral titres, suggesting that the ACAP2 protein is not the only factor (there may be other compensatory mechanisms) or may not be a direct player in regulating the host-range function of the K1L protein. However, in our siRNA studies, we were not able to determine the decrease in the ACAP2 protein levels, due to the unavailability of a good anti-ACAP2 antibody. The lack of evidence showing absence of ACAP2 protein, in RK-13 cells treated with the siRNA specific to *ACAP2*, makes it difficult to conclude that ACAP2 protein is not in any way involved in the host-range function mediated by K1L protein. Some of the possible roles that ACAP2 might have are outlined in Fig.21.

It is possible that the ACAP2 protein, is not directly anti-viral, but regulates an anti-viral target. So far, the Arf-1 and Arf-6 proteins are the known targets being regulated by ACAP2. Both Arf-1 and Arf-6 have a role in modifying the cytoskeletal elements in the cytoplasm and also have a role in the regulation of

membrane transfer and compartmentalization within the cytoplasm as well. The complexities involved in the cytoskeletal and membrane structures early in VV infection are still being resolved and more detailed studies will be necessary to answer these questions.

As has been mentioned before, the anti-viral roles of GTPases are coming to light. As a GTPase-activating protein, ACAP2 could be regulating functions of other anti-viral GTPases as well, e.g. the Mx protein family members, the GBP protein family members, and the p47 protein family members.

K1L has been shown to inhibit NF κ B activation by interfering with the degradation of I κ B (Shisler and Jin, 2004). However, it has not been shown to be responsible for the host range function.

Model of role of ACAP2 in host-range function mediated by K1L

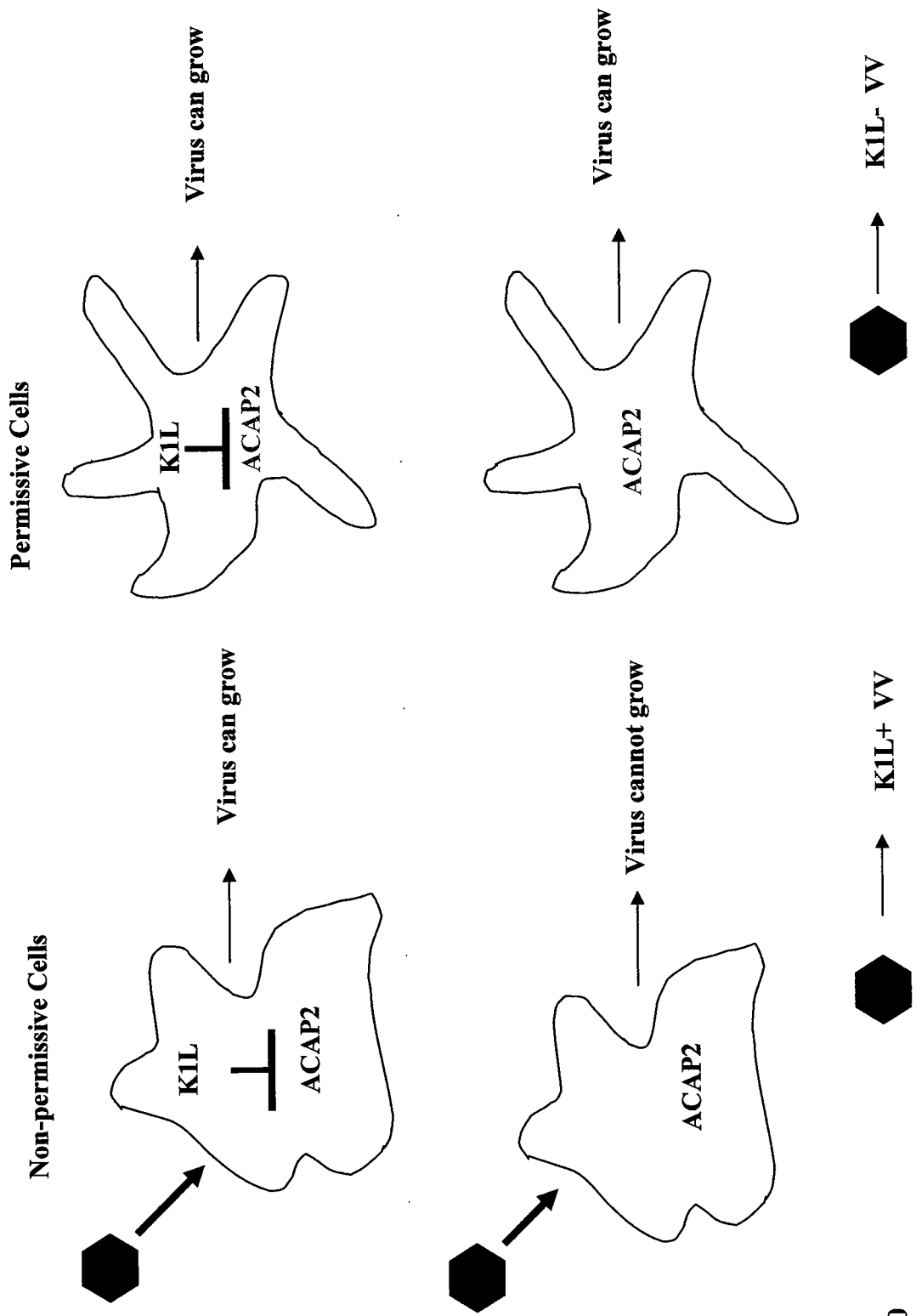


Fig. 20

Possible Roles of ACAP2

ACAP2

Not ACAP2 but Arfs are the major players. Arf expression levels or the proteins themselves may be different in permissive and non-permissive cells.

May be regulating antiviral GTPases other than Arfs.

Has a role in membrane trafficking and may interfere with VV life cycle in the early stages.

Presence of other domains indicate that it may have multiple functions.

Significance of study

The study of the host-range function of K1L over the years has shown that this protein is involved in maintaining viral protein synthesis. In the absence of this protein, there is an abrupt shutdown of viral protein translation. In these studies, it has also been suggested that the involvement of a cellular protein/proteins in mediating host range function along with the K1L protein is a distinct possibility. Interpretation of observations over the years has been difficult because of the complexity of the VV life cycle (involving over 200 viral proteins), which until very recently had not been fully realized and is still being investigated with new facts coming to light. The study of a host-range protein is significant because host restriction is also an indication of the anti-viral state of a cell and a successful infection depends on the ability of the virus to disable the host response to infection. As explained before, one very well characterized host-range gene product of VV, the E3L protein, mediates its host range function by disabling the PKR mediated antiviral mechanism. The possibility of the involvement of a similar or a novel antiviral pathway being disabled by the K1L gene product is very plausible. The preliminary identification of ACAP2 -K1L protein interaction is the first report of a cellular protein being implicated in the host range function of K1L. The mechanism by which this interaction contributes to host range function needs further studies.

Future Directions

a) Analysis of the crystal structure of both VV K1L and rabbit ACAP2 proteins.

The crystal structure resolution of the two proteins and their interacting surfaces, will allow an understanding of the domains of both proteins involved in this interaction. As has been mentioned before, the crystal structure of a member of the ACAP2 family (PAP) shows an intramolecular interaction between the ankyrin repeats and the GAP domain. It will be important to analyze whether the physical interaction between the K1L protein and the rabbit ACAP2 is an ankyrin-ankyrin or an ankyrin-GAP domain (ankyrin repeat of K1L and the GAP domain of ACAP2) interaction. Once the domains of interaction are identified, mutational analysis can be used to narrow down the interface to the few critical amino acids that are vital for this interaction. Since the K1L protein has been shown to lose its function by amino acid substitution at the most conserved ankyrin repeat, analysis of whether this mutated K1L can interact with the ACAP2 protein will be an important study. An attempt by us to answer this question was unsuccessful, as the available anti-K1L antibody failed to immunoprecipitate the mutated K1L proteins from the RK-13 cells infected with recombinant virus, vAbT33K1Lmutank.

b) Identification of other players, cellular or viral.

The possibility that the ACAP2 protein is not the only one interacting with K1L still exists. It is possible that our immunoprecipitation experiment failed to detect unstable proteins, either cellular or viral. Proteomic studies may be used to detect more proteins.

c) Analysis of the cytoplasmic amorphous site for the accumulation of the VV early mRNA.

The life cycle of VV is very complex, with new information coming to light. The existence of a specific region within the cytoplasm where the early viral transcripts accumulate and are translated is a very interesting phenomenon that has been described recently and is still being studied. It is conceivable that cellular regulators of cytoskeletal elements and membrane components are involved in the formation of these structures, and further studies using the VV recombinants we have made, may be useful to elucidate whether the K1L protein is involved in this process.

d) Study differences in Arf expression in permissive and non-permissive cells.

Using western blot analysis, the constitutive levels of Arf6, the target of ACAP2, can be studied in the permissive and non-permissive cells. Arf6 has already been shown to be involved in the anti-viral pathway in HIV-1 infection, where the viral protein Nef, mediates the down regulation of MHC class I expression on the cell surface.

e) Analysis of the interaction of mutated K1L with ACAP2 protein, using Bacterial two-hybrid system.

One limitation to the study of the interaction between the mutated K1L protein and the rabbit ACAP2 protein is that in RK-13 cells infected with the mutant virus, there is no K1L protein detectable by immunoprecipitation because of the abortive infection. Hence, an *in vitro* method needs to be devised, and may be achieved by using either a yeast two-hybrid, or a bacterial two-hybrid system.

References

- Ada, G.L. and Blanden, R.V. (1994) CTL immunity and cytokine regulation in viral infection. *Res Immunol* 145(8-9), 625-8; discussion 628-9.
- Anderson, S.L., Carton, J.M., Lou, J., Xing, L. and Rubin, B.Y. (1999) Interferon-induced guanylate binding protein-1 (GBP-1) mediates an antiviral effect against vesicular stomatitis virus and encephalomyocarditis virus. *Virology* 256(1), 8-14.
- Antoine, G., Scheiflinger, F., Dorner, F. and Falkner, F.G. (1998) The complete genomic sequence of the modified vaccinia Ankara strain: comparison with other orthopoxviruses. *Virology* 244(2), 365-96.
- Axton, J.M., Shamanski, F.L., Young, L.M., Henderson, D.S., Boyd, J.B. and Orr-Weaver, T.L. (1994) The inhibitor of DNA replication encoded by the *Drosophila* gene plutonium is a small, ankyrin repeat protein. *Embo J* 13(2), 462-70.
- Baldick, C.J., Jr., Keck, J.G. and Moss, B. (1992) Mutational analysis of the core, spacer, and initiator regions of vaccinia virus intermediate-class promoters. *J Virol* 66(8), 4710-9.

- Banham, A.H. and Smith, G.L. (1992) Vaccinia virus gene B1R encodes a 34-kDa serine/threonine protein kinase that localizes in cytoplasmic factories and is packaged into virions. *Virology* 191(2), 803-12.
- Baroudy, B.M. and Moss, B. (1980) Purification and characterization of a DNA-dependent RNA polymerase from vaccinia virions. *J Biol Chem* 255(9), 4372-80.
- Baroudy, B.M., Venkatesan, S. and Moss, B. (1982) Incompletely base-paired flip-flop terminal loops link the two DNA strands of the vaccinia virus genome into one uninterrupted polynucleotide chain. *Cell* 28(2), 315-24.
- Baxby, D. and Bennett, M. (1997) Cowpox: a re-evaluation of the risks of human cowpox based on new epidemiological information. *Arch Virol Suppl* 13, 1-12.
- Beaud, G. (1995) Vaccinia virus DNA replication: a short review. *Biochimie* 77(10), 774-9.
- Behbehani, A.M. (1983) The smallpox story: life and death of an old disease. *Microbiol Rev* 47(4), 455-509.
- Blagoveshchenskaya, A.D., Thomas, L., Feliciangeli, S.F., Hung, C.H. and Thomas, G. (2002) HIV-1 Nef downregulates MHC-I by a PACS-1- and PI3K-regulated ARF6 endocytic pathway. *Cell* 111(6), 853-66.

- Blasco, R. and Moss, B. (1991) Extracellular vaccinia virus formation and cell-to-cell virus transmission are prevented by deletion of the gene encoding the 37,000-Dalton outer envelope protein. *J Virol* 65(11), 5910-20.
- Boone, R.F. and Moss, B. (1978) Sequence complexity and relative abundance of vaccinia virus mRNA's synthesized in vivo and in vitro. *J Virol* 26(3), 554-69.
- Bork, P. (1993) Hundreds of ankyrin-like repeats in functionally diverse proteins: mobile modules that cross phyla horizontally? *Proteins* 17(4), 363-74.
- Brand, S.R., Kobayashi, R. and Mathews, M.B. (1997) The Tat protein of human immunodeficiency virus type 1 is a substrate and inhibitor of the interferon-induced, virally activated protein kinase, PKR. *J Biol Chem* 272(13), 8388-95.
- Breeden, L. and Nasmyth, K. (1987) Similarity between cell-cycle genes of budding yeast and fission yeast and the Notch gene of *Drosophila*. *Nature* 329(6140), 651-4.
- Brown, E.H. (1965) Complications of smallpox vaccination. *Postgrad Med J* 41(480), 634-5.
- Broyles, S.S. (2003) Vaccinia virus transcription. *J Gen Virol* 84(Pt 9), 2293-303.
- Buller, R.M. and Palumbo, G.J. (1991) Poxvirus pathogenesis. *Microbiol Rev* 55(1), 80-122.
- Cairns, J. (1960) The initiation of vaccinia infection. *Virology* 11, 603-23.

- Calderara, S., Xiang, Y. and Moss, B. (2001) Orthopoxvirus IL-18 binding proteins: affinities and antagonist activities. *Virology* 279(1), 22-6.
- Chang, H.W. and Jacobs, B.L. (1993) Identification of a conserved motif that is necessary for binding of the vaccinia virus E3L gene products to double-stranded RNA. *Virology* 194(2), 537-47.
- Chang, H.W., Uribe, L.H. and Jacobs, B.L. (1995) Rescue of vaccinia virus lacking the E3L gene by mutants of E3L. *J Virol* 69(10), 6605-8.
- Chen, N., Buller, R.M., Wall, E.M. and Upton, C. (2000) Analysis of host response modifier ORFs of ectromelia virus, the causative agent of mousepox. *Virus Res* 66(2), 155-73.
- Chung, C.S., Vasilevskaya, I.A., Wang, S.C., Bair, C.H. and Chang, W. (1997) Apoptosis and host restriction of vaccinia virus in RK13 cells. *Virus Res* 52(2), 121-32.
- Creighton. (1894) *History of Epidemics in Britain*.
- Darnell, J.E., Jr., Kerr, I.M. and Stark, G.R. (1994) Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science* 264(5164), 1415-21.
- Demkowicz, W.E., Jr., Littaua, R.A., Wang, J. and Ennis, F.A. (1996) Human cytotoxic T-cell memory: long-lived responses to vaccinia virus. *J Virol* 70(4), 2627-31.

- Donaldson, J.G. and Jackson, C.L. (2000) Regulators and effectors of the ARF GTPases. *Curr Opin Cell Biol* 12(4), 475-82.
- Drillien, R., Koehren, F. and Kirn, A. (1981) Host range deletion mutant of vaccinia virus defective in human cells. *Virology* 111(2), 488-99.
- Dubochet, J., Adrian, M., Richter, K., Garces, J. and Wittek, R. (1994) Structure of intracellular mature vaccinia virus observed by cryoelectron microscopy. *J Virol* 68(3), 1935-41.
- Earl, P.L., Cooper, N. and Moss, B. (1997) Preparation of Cell Cultures and Vaccinia Virus Stocks. In: F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith and K. Struhl (Eds), *Current protocols in molecular biology*, pp. 16.16.1-7. Vol. 2. 3 vols. John Wiley & Sons, New York.
- Easterbrook, K.B. (1966) Controlled degradation of vaccinia virions in vitro: an electron microscopic study. *J Ultrastruct Res* 14(5), 484-96.
- Engelstad, M. and Smith, G.L. (1993) The vaccinia virus 42-kDa envelope protein is required for the envelopment and egress of extracellular virus and for virus virulence. *Virology* 194(2), 627-37.
- Eppstein, D.A., Marsh, Y.V., Schreiber, A.B., Newman, S.R., Todaro, G.J. and Nestor, J.J., Jr. (1985) Epidermal growth factor receptor occupancy inhibits vaccinia virus infection. *Nature* 318(6047), 663-5.
- Esposito, J.J., Fenner F. (2001) Poxviruses, *Fields Virology*, 4th ed. Vol. 2.

- Evans, E., Klemperer, N., Ghosh, R. and Traktman, P. (1995) The vaccinia virus D5 protein, which is required for DNA replication, is a nucleic acid-independent nucleoside triphosphatase. *J Virol* 69(9), 5353-61.
- Fenner, F. (2000) Adventures with poxviruses of vertebrates. *FEMS Microbiol Rev* 24(2), 123-33.
- Frischknecht, F., Moreau, V., Rottger, S., Gonfloni, S., Reckmann, I., Superti-Furga, G. and Way, M. (1999) Actin-based motility of vaccinia virus mimics receptor tyrosine kinase signalling. *Nature* 401(6756), 926-9.
- Garon, C.F., Barbosa, E. and Moss, B. (1978) Visualization of an inverted terminal repetition in vaccinia virus DNA. *Proc Natl Acad Sci U S A* 75(10), 4863-7.
- Gemmell, A. and Fenner, F. (1960) Genetic studies with mammalian poxviruses. III. White (u) mutants of rabbitpox virus. *Virology* 11, 219-35.
- Gillard, S., Spehner, D., Drillien, R. and Kirn, A. (1986) Localization and sequence of a vaccinia virus gene required for multiplication in human cells. *Proc Natl Acad Sci U S A* 83(15), 5573-7.
- Gillard, S., Spehner, D., Drillien, R. and Kirn, A. (1989) Antibodies directed against a synthetic peptide enable detection of a protein encoded by a vaccinia virus host range gene that is conserved within the Orthopoxvirus genus. *J Virol* 63(4), 1814-7.

- Goebel, S.J., Johnson, G.P., Perkus, M.E., Davis, S.W., Winslow, J.P. and Paoletti, E. (1990) The complete DNA sequence of vaccinia virus. *Virology* 179(1), 247-66, 517-63.
- Gunnery, S. and Mathews, M.B. (1998) RNA binding and modulation of PKR activity. *Methods* 15(3), 189-98.
- Haller, O. and Kochs, G. (2002) Interferon-induced mx proteins: dynamin-like GTPases with antiviral activity. *Traffic* 3(10), 710-7.
- Harford, C.G., Hamlin, A. and Rieders, E. (1966) Electron microscopic autoradiography of DNA synthesis in cells infected with vaccinia virus. *Exp Cell Res* 42(1), 50-7.
- Henderson, D.S., Moss B. (1999) *Smallpox and Vaccinia, Vaccines*, 3rd ed., pp. 74-97.
- Hiller, G. and Weber, K. (1985) Golgi-derived membranes that contain an acylated viral polypeptide are used for vaccinia virus envelopment. *J Virol* 55(3), 651-9.
- Hollinshead, M., Rodger, G., Van Eijl, H., Law, M., Hollinshead, R., Vaux, D.J. and Smith, G.L. (2001) Vaccinia virus utilizes microtubules for movement to the cell surface. *J Cell Biol* 154(2), 389-402.
- Howard, S.T., Chan, Y.S. and Smith, G.L. (1991) Vaccinia virus homologues of the Shope fibroma virus inverted terminal repeat proteins and a discontinuous

- ORF related to the tumor necrosis factor receptor family. *Virology* 180(2), 633-47.
- Hsiao, J.C., Chung, C.S., Drillien, R. and Chang, W. (2004) The cowpox virus host range gene, CP77, affects phosphorylation of eIF2 alpha and vaccinia viral translation in apoptotic HeLa cells. *Virology* 329(1), 199-212.
- Hu, F.Q., Smith, C.A. and Pickup, D.J. (1994) Cowpox virus contains two copies of an early gene encoding a soluble secreted form of the type II TNF receptor. *Virology* 204(1), 343-56.
- Ichihashi, Y., Matsumoto, S. and Dales, S. (1971) Biogenesis of poxviruses: role of A-type inclusions and host cell membranes in virus dissemination. *Virology* 46(3), 507-32.
- Ichihashi, Y. and Oie, M. (1996) Neutralizing epitope on penetration protein of vaccinia virus. *Virology* 220(2), 491-4.
- Ink, B.S., Gilbert, C.S. and Evan, G.I. (1995) Delay of vaccinia virus-induced apoptosis in nonpermissive Chinese hamster ovary cells by the cowpox virus CHOhr and adenovirus E1B 19K genes. *J Virol* 69(2), 661-8.
- Inoue, J., Kerr, L.D., Rashid, D., Davis, N., Bose, H.R., Jr. and Verma, I.M. (1992) Direct association of pp40/I kappa B beta with rel/NF-kappa B transcription factors: role of ankyrin repeats in the inhibition of DNA binding activity. *Proc Natl Acad Sci U S A* 89(10), 4333-7.

- Jackson, T.R., Brown, F.D., Nie, Z., Miura, K., Foroni, L., Sun, J., Hsu, V.W., Donaldson, J.G. and Randazzo, P.A. (2000) ACAPs are arf6 GTPase-activating proteins that function in the cell periphery. *J Cell Biol* 151(3), 627-38.
- Joklik, W.K. and Becker, Y. (1964) The Replication and Coating of Vaccinia DNA. *J Mol Biol* 10, 452-74.
- Keck, J.G., Baldick, C.J., Jr. and Moss, B. (1990) Role of DNA replication in vaccinia virus gene expression: a naked template is required for transcription of three late trans-activator genes. *Cell* 61(5), 801-9.
- Kotwal, G.J., Isaacs, S.N., McKenzie, R., Frank, M.M. and Moss, B. (1990) Inhibition of the complement cascade by the major secretory protein of vaccinia virus. *Science* 250(4982), 827-30.
- Kotwal, G.J. and Moss, B. (1988a) Analysis of a large cluster of nonessential genes deleted from a vaccinia virus terminal transposition mutant. *Virology* 167(2), 524-37.
- Kotwal, G.J. and Moss, B. (1988b) Vaccinia virus encodes a secretory polypeptide structurally related to complement control proteins. *Nature* 335(6186), 176-8.
- Lalani, A.S., Masters, J., Zeng, W., Barrett, J., Pannu, R., Everett, H., Arendt, C.W. and McFadden, G. (1999) Use of chemokine receptors by poxviruses. *Science* 286(5446), 1968-71.

- Lane, J.M. and Millar, J.D. (1969) Routine childhood vaccination against smallpox reconsidered. *N Engl J Med* 281(22), 1220-4.
- Lane, J.M., Ruben, F.L., Neff, J.M. and Millar, J.D. (1970) Complications of smallpox vaccination, 1968: results of ten statewide surveys. *J Infect Dis* 122(4), 303-9.
- Langland, J.O. and Jacobs, B.L. (2002) The role of the PKR-inhibitory genes, E3L and K3L, in determining vaccinia virus host range. *Virology* 299(1), 133-41.
- Ligon, B.L. (2004) Monkeypox: a review of the history and emergence in the Western hemisphere. *Semin Pediatr Infect Dis* 15(4), 280-7.
- Lin, C.L., Chung, C.S., Heine, H.G. and Chang, W. (2000) Vaccinia virus envelope H3L protein binds to cell surface heparan sulfate and is important for intracellular mature virion morphogenesis and virus infection in vitro and in vivo. *J Virol* 74(7), 3353-65.
- Loparev, V.N., Parsons, J.M., Knight, J.C., Panus, J.F., Ray, C.A., Buller, R.M., Pickup, D.J. and Esposito, J.J. (1998) A third distinct tumor necrosis factor receptor of orthopoxviruses. *Proc Natl Acad Sci U S A* 95(7), 3786-91.
- Lux, S.E., John, K.M. and Bennett, V. (1990) Analysis of cDNA for human erythrocyte ankyrin indicates a repeated structure with homology to tissue-differentiation and cell-cycle control proteins. *Nature* 344(6261), 36-42.

- Mallardo, M., Leithe, E., Schleich, S., Roos, N., Doglio, L. and Krijnse Locker, J. (2002) Relationship between vaccinia virus intracellular cores, early mRNAs, and DNA replication sites. *J Virol* 76(10), 5167-83.
- Mallardo, M., Schleich, S. and Krijnse Locker, J. (2001) Microtubule-dependent organization of vaccinia virus core-derived early mRNAs into distinct cytoplasmic structures. *Mol Biol Cell* 12(12), 3875-91.
- Mandiyan, V., Andreev, J., Schlessinger, J. and Hubbard, S.R. (1999) Crystal structure of the ARF-GAP domain and ankyrin repeats of PYK2-associated protein beta. *Embo J* 18(24), 6890-8.
- Marchler-Bauer, A., Anderson, J.B., DeWeese-Scott, C., Fedorova, N.D., Geer, L.Y., He, S., Hurwitz, D.I., Jackson, J.D., Jacobs, A.R., Lanczycki, C.J., Liebert, C.A., Liu, C., Madej, T., Marchler, G.H., Mazumder, R., Nikolskaya, A.N., Panchenko, A.R., Rao, B.S., Shoemaker, B.A., Simonyan, V., Song, J.S., Thiessen, P.A., Vasudevan, S., Wang, Y., Yamashita, R.A., Yin, J.J. and Bryant, S.H. (2003) CDD: a curated Entrez database of conserved domain alignments. *Nucleic Acids Res* 31(1), 383-7.
- Marsh, Y.V. and Epstein, D.A. (1987) Vaccinia virus and the EGF receptor: a portal for infectivity? *J Cell Biochem* 34(4), 239-45.
- Massung, R.F., Esposito, J.J., Liu, L.I., Qi, J., Utterback, T.R., Knight, J.C., Aubin, L., Yuran, T.E., Parsons, J.M., Loparev, V.N. and et al. (1993) Potential

- virulence determinants in terminal regions of variola smallpox virus genome. *Nature* 366(6457), 748-51.
- McClain, M.E. (1965) The Host Range and Plaque Morphology of Rabbitpox Virus (Rpu+) and Its U Mutants on Chick Fibroblast, Pk-2a, and L929 Cells. *Aust J Exp Biol Med Sci* 43, 31-44.
- McClain, M.E. and Greenland, R.M. (1965) Recombination between Rabbitpox Virus Mutants in Permissive and Nonpermissive Cells. *Virology* 25, 516-22.
- McCraith, S., Holtzman, T., Moss, B. and Fields, S. (2000) Genome-wide analysis of vaccinia virus protein-protein interactions. *Proc Natl Acad Sci U S A* 97(9), 4879-84.
- McKenzie, R., Kotwal, G.J., Moss, B., Hammer, C.H. and Frank, M.M. (1992) Regulation of complement activity by vaccinia virus complement-control protein. *J Infect Dis* 166(6), 1245-50.
- Michaely, P. and Bennett, V. (1992) The ANK repeat: a ubiquitous motif involved in macromolecular recognition. *Trends Cell Biol* 2(5), 127-9.
- Millns, A.K., Carpenter, M.S. and DeLange, A.M. (1994) The vaccinia virus-encoded uracil DNA glycosylase has an essential role in viral DNA replication. *Virology* 198(2), 504-13.
- Moore. (1815) History of smallpox.
- Moss, B. (2001) Poxviridae: The viruses and their Replication, 4th ed. Vol. 2.

- Moss, B. and Shisler, J.L. (2001) Immunology 101 at poxvirus U: immune evasion genes. *Semin Immunol* 13(1), 59-66.
- Moss, J. and Vaughan, M. (1995) Structure and function of ARF proteins: activators of cholera toxin and critical components of intracellular vesicular transport processes. *J Biol Chem* 270(21), 12327-30.
- Moss, J. and Vaughan, M. (1998) Molecules in the ARF orbit. *J Biol Chem* 273(34), 21431-4.
- Nie, Z., Hirsch, D.S. and Randazzo, P.A. (2003) Arf and its many interactors. *Curr Opin Cell Biol* 15(4), 396-404.
- Novick, D., Kim, S.H., Fantuzzi, G., Reznikov, L.L., Dinarello, C.A. and Rubinstein, M. (1999) Interleukin-18 binding protein: a novel modulator of the Th1 cytokine response. *Immunity* 10(1), 127-36.
- Paoletti, E. and Grady, L.J. (1977) Transcriptional complexity of vaccinia virus in vivo and in vitro. *J Virol* 23(3), 608-15.
- Payne, L.G. (1980) Significance of extracellular enveloped virus in the in vitro and in vivo dissemination of vaccinia. *J Gen Virol* 50(1), 89-100.
- Perkus, M.E., Goebel, S.J., Davis, S.W., Johnson, G.P., Limbach, K., Norton, E.K. and Paoletti, E. (1990) Vaccinia virus host range genes. *Virology* 179(1), 276-86.

- Perkus, M.E., Piccini, A., Lipinskas, B.R. and Paoletti, E. (1985) Recombinant vaccinia virus: immunization against multiple pathogens. *Science* 229(4717), 981-4.
- Ramsey-Ewing, A.L. and Moss, B. (1996) Complementation of a vaccinia virus host-range K1L gene deletion by the nonhomologous CP77 gene. *Virology* 222(1), 75-86.
- Randazzo, P.A., Nie, Z., Miura, K. and Hsu, V.W. (2000) Molecular aspects of the cellular activities of ADP-ribosylation factors. *Sci STKE* 2000(59), RE1.
- Ray, C.A., Black, R.A., Kronheim, S.R., Greenstreet, T.A., Sleath, P.R., Salvesen, G.S. and Pickup, D.J. (1992) Viral inhibition of inflammation: cowpox virus encodes an inhibitor of the interleukin-1 beta converting enzyme. *Cell* 69(4), 597-604.
- Reed, K.D., Melski, J.W., Graham, M.B., Regnery, R.L., Sotir, M.J., Wegner, M.V., Kazmierczak, J.J., Stratman, E.J., Li, Y., Fairley, J.A., Swain, G.R., Olson, V.A., Sargent, E.K., Kehl, S.C., Frace, M.A., Kline, R., Foldy, S.L., Davis, J.P. and Damon, I.K. (2004) The detection of monkeypox in humans in the Western Hemisphere. *N Engl J Med* 350(4), 342-50.
- Reid, K.B. and Day, A.J. (1989) Structure-function relationships of the complement components. *Immunol Today* 10(6), 177-80.
- Rietdorf, J., Ploubidou, A., Reckmann, I., Holmstrom, A., Frischknecht, F., Zettl, M., Zimmermann, T. and Way, M. (2001) Kinesin-dependent movement on

- microtubules precedes actin-based motility of vaccinia virus. *Nat Cell Biol* 3(11), 992-1000.
- Samuel, C.E. (1991) Antiviral actions of interferon. Interferon-regulated cellular proteins and their surprisingly selective antiviral activities. *Virology* 183(1), 1-11.
- Sedgwick, S.G. and Smerdon, S.J. (1999) The ankyrin repeat: a diversity of interactions on a common structural framework. *Trends Biochem Sci* 24(8), 311-6.
- Shchelkunov, S.N., Blinov, V.M. and Sandakhchiev, L.S. (1993) Ankyrin-like proteins of variola and vaccinia viruses. *FEBS Lett* 319(1-2), 163-5.
- Shisler, J.L. and Jin, X.L. (2004) The vaccinia virus K1L gene product inhibits host NF-kappaB activation by preventing IkappaBalpha degradation. *J Virol* 78(7), 3553-60.
- Smith, C.A., Hu, F.Q., Smith, T.D., Richards, C.L., Smolak, P., Goodwin, R.G. and Pickup, D.J. (1996) Cowpox virus genome encodes a second soluble homologue of cellular TNF receptors, distinct from CrmB, that binds TNF but not LT alpha. *Virology* 223(1), 132-47.
- Smith, G.L. and Moss, B. (1983) Infectious poxvirus vectors have capacity for at least 25 000 base pairs of foreign DNA. *Gene* 25(1), 21-8.
- Smith, G.L., Vanderplasschen, A. and Law, M. (2002) The formation and function of extracellular enveloped vaccinia virus. *J Gen Virol* 83(Pt 12), 2915-31.

- Smith, K.A., Stallard, V., Roos, J.M., Hart, C., Cormier, N., Cohen, L.K., Roberts, B.E. and Payne, L.G. (1993) Host range selection of vaccinia recombinants containing insertions of foreign genes into non-coding sequences. *Vaccine* 11(1), 43-53.
- Sutter, G., Ramsey-Ewing, A., Rosales, R. and Moss, B. (1994) Stable expression of the vaccinia virus K1L gene in rabbit cells complements the host range defect of a vaccinia virus mutant. *J Virol* 68(7), 4109-16.
- Tanaka-Kataoka, M., Kunikata, T., Takayama, S., Iwaki, K., Ohashi, K., Ikeda, M. and Kurimoto, M. (1999) In vivo antiviral effect of interleukin 18 in a mouse model of vaccinia virus infection. *Cytokine* 11(8), 593-9.
- Taylor, G.A., Feng, C.G. and Sher, A. (2004) p47 GTPases: regulators of immunity to intracellular pathogens. *Nat Rev Immunol* 4(2), 100-9.
- Terajima, M., Cruz, J., Raines, G., Kilpatrick, E.D., Kennedy, J.S., Rothman, A.L. and Ennis, F.A. (2003) Quantitation of CD8⁺ T cell responses to newly identified HLA-A*0201-restricted T cell epitopes conserved among vaccinia and variola (smallpox) viruses. *J Exp Med* 197(7), 927-32.
- Terajima, M. and Leporati, A.M. (in press) The role of indoleamine 2,3-dioxygenase in antiviral activity of interferon- γ against vaccinia virus. *Viral Immunology*.
- Terajima, M., Van Epps, H.L., Li, D., Leporati, A.M., Juhlin, S.E., Mustonen, J., Vaheri, A. and Ennis, F.A. (2002) Generation of recombinant vaccinia

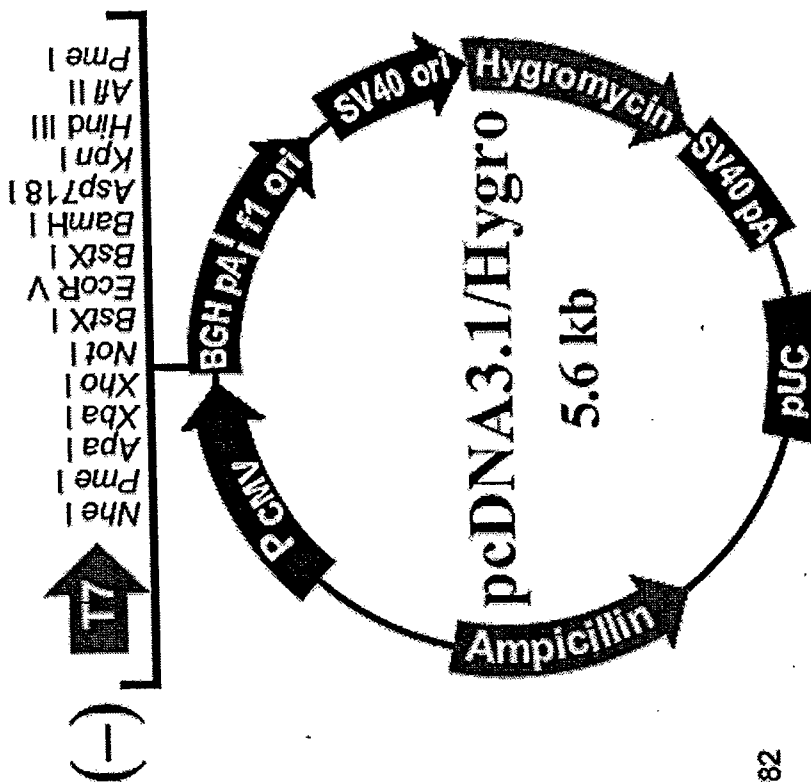
viruses expressing Puumala virus proteins and use in isolating cytotoxic T cells specific for Puumala virus. *Virus Res* 84(1-2), 67-77.

Tomlinson, S. (1993) Complement defense mechanisms. *Curr Opin Immunol* 5(1), 83-9.

Tooze, J., Hollinshead, M., Reis, B., Radsak, K. and Kern, H. (1993) Progeny vaccinia and human cytomegalovirus particles utilize early endosomal cisternae for their envelopes. *Eur J Cell Biol* 60(1), 163-78.

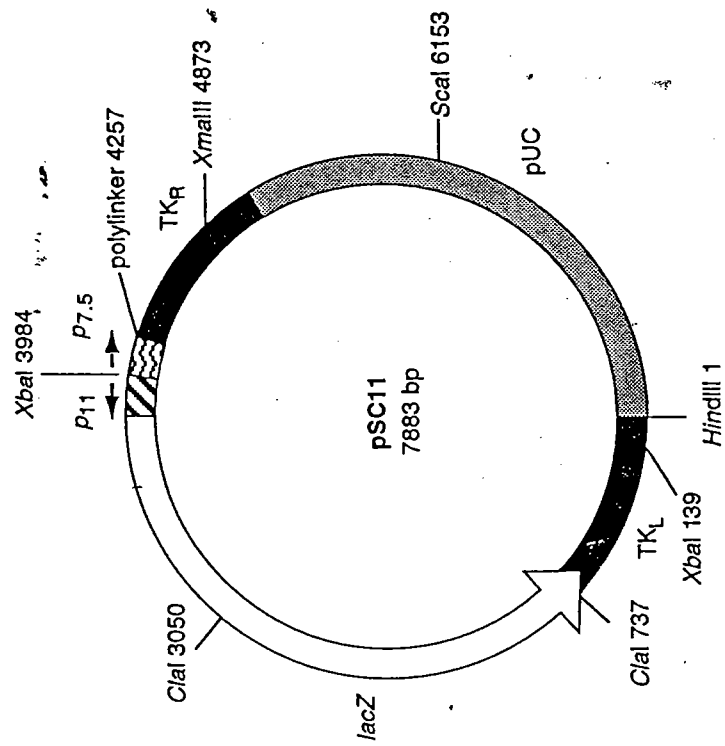
Ward, B.M. and Moss, B. (2001) Vaccinia virus intracellular movement is associated with microtubules and independent of actin tails. *J Virol* 75(23), 11651-63.

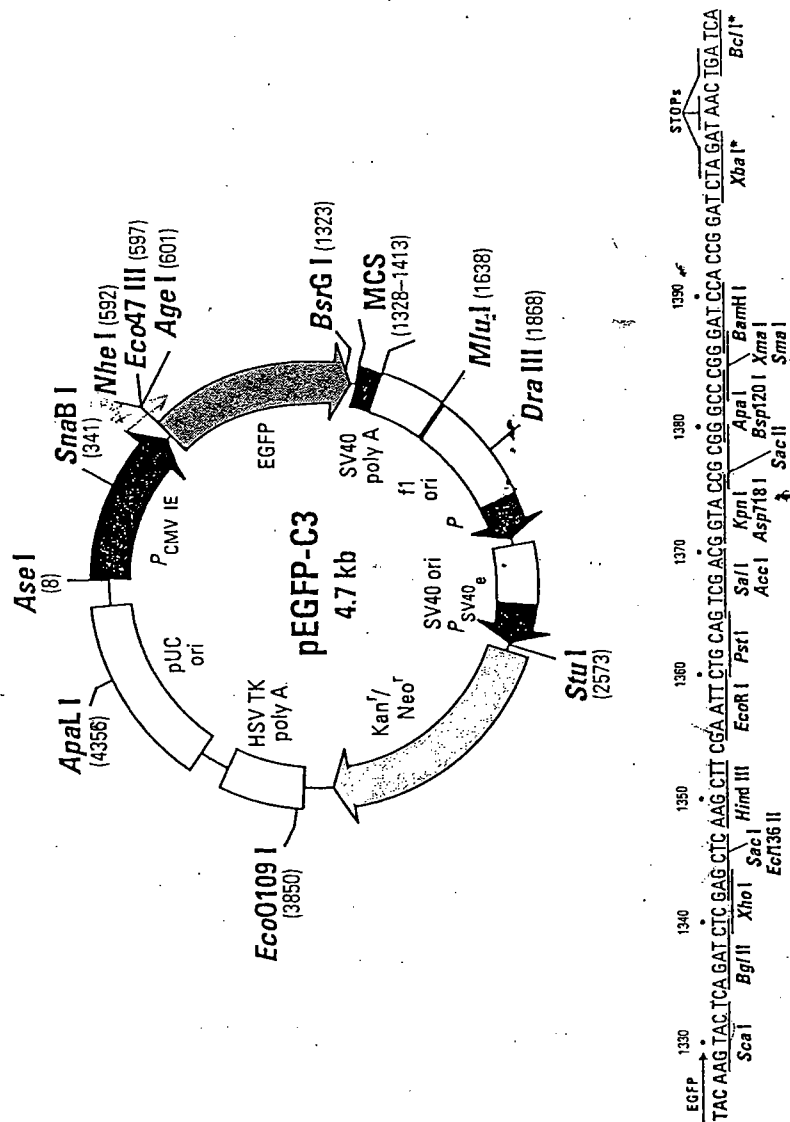
Xu, R., Johnson, A.J., Liggitt, D. and Bevan, M.J. (2004) Cellular and humoral immunity against vaccinia virus infection of mice. *J Immunol* 172(10), 6265-71.



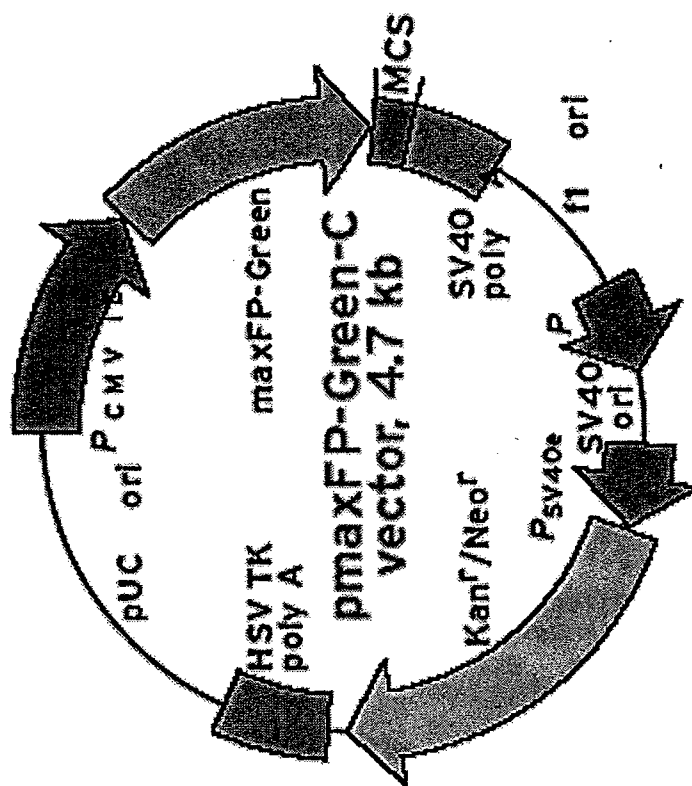
Comments for pcDNA3.1/Hygro (+):
5597 nucleotides

- CMV promoter: bases 209-863
- T7 promoter/priming site: bases 863-882
- Multiple cloning site: bases 895-1010
- BGH reverse priming site: bases 1022-1039
- BGH polyadenylation signal: bases 1021-1235
- f1 origin: bases 1298-1711
- SV40 promoter and origin: bases 1776-2100
- Hygromycin resistance gene: bases 2118-3141
- SV40 early polyadenylation signal: bases 3154-3526
- pUC origin: bases 3786-4456 (complementary strand)
- Ampicillin resistance gene: bases 4601-5461 (complementary strand)





Restriction Map and Multiple Cloning Site (MCS) of pEGFP-C3. All restriction sites shown are unique. The Bcl I site cannot be used for fusions since it contains an in-frame stop codon. The Xba I and Bcl I sites (*) are methylated in the DNA provided by BD Biosciences Clontech. If you wish to digest the vector with these enzymes, you will need to transform the vector into a dam⁻ host and make fresh DNA.



Location of features:

- P_{CMV IE}**: 1-589
- Enhancer region**: 59-465
- TATA box**: 554-560
- Transcription start point**: 583
- maxFP-Green**
- Kozak consensus translation initiation site**: 606-616
- Start codon (ATG)**: 613-615; **Stop codon**: 1378-1380
- Last amino acid in maxFP-Green**: 1306-1308