THE ROLE OF CELL SIGNALING IN POXVIRUS TROPISM: THE CASE OF THE M-T5 HOST-RANGE PROTEIN OF MYXOMA VIRUS

By

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2009
To my family
ACKNOWLEDGMENTS

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<tr>
<td>4E-BP</td>
<td>eukaryotic initiation factor 4E binding protein</td>
</tr>
<tr>
<td>Akt</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>ANK</td>
<td>ankyrin</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell leukemia/lymphoma 2</td>
</tr>
<tr>
<td>BHK</td>
<td>baby hamster kidney</td>
</tr>
<tr>
<td>BTB</td>
<td>Broad-complex, Tramtrack and Bric-a-Brac</td>
</tr>
<tr>
<td>C-</td>
<td>carboxy-</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin dependent kinase</td>
</tr>
<tr>
<td>CEF</td>
<td>chicken embryo fibroblast</td>
</tr>
<tr>
<td>CEV</td>
<td>cell associated enveloped virus</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>CHOhr</td>
<td>cowpox virus host-range protein of 77 kDa, also called CP77</td>
</tr>
<tr>
<td>CPXV</td>
<td>cowpox virus</td>
</tr>
<tr>
<td>COP</td>
<td>Copenhagen</td>
</tr>
<tr>
<td>CUL1</td>
<td>cullin 1</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dsDNA</td>
<td>double-stranded DNA</td>
</tr>
<tr>
<td>ECTV</td>
<td>ectromelia virus</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EEV</td>
<td>extracellular enveloped virus</td>
</tr>
<tr>
<td>eGFP</td>
<td>enhanced green fluorescent protein</td>
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eIF2α  α of subunit of eukaryotic initiation factor 2
FBS  fetal bovine serum
g  gravitational acceleration
GAGs  glycosaminoglycans
GFP  green fluorescent protein
GST  Glutathione-S-Transferase
H  α-helix
h  hours
HA  hemagglutinin
HCMV  human cytomegalovirus
HECT  homologous to E6-associated protein C-terminus
HMG20  high mobility group 20
hpi  hours post infection
HPV  human papillomavirus
HRP  horseradish peroxidase
IEV  intracellular enveloped virus
IFN  interferon
IκBβ  inhibitor of kappa B beta
IL  interleukin
IMV intracellular mature virus
IP  immunoprecipitation
IRF3  interferon regulatory factor-3
IRS-1  insulin receptor substrate 1
kbp  kilobase pairs
l  liter
LB  Luria-Bertani
LRR  leucine-rich repeats
MARCH  membrane-associated RING-CH
MCV  molluscum contagiosum virus
mg  milligram
μg  microgram
MGF  myxoma growth factor
min  minute
ml  milliliter
μl  microliter
mM  millimolar
μM  micromolar
μm  micrometer
MOI  multiplicity of infection
MPXV  monkeypox virus
mTOR  mammalian target of rapamycin
MVA  modified vaccinia virus Ankara
MYXV  myxoma virus
N-  amino-
NEB  New England BioLabs
NF-κB  nuclear factor kappa b
NLS  nuclear localization sequence
nm  nanometers
ORF  open reading frame
p27/Kip1  CDK inhibitor of 27 kDa
PAGE  polyacrylamide gel electrophoresis
PBS  phosphate buffered saline
PCR  polymerase chain reaction
PDK  phosphoinositide dependent kinase
PH  pleckstrin homology
PHLPP  pleckstrin homology domain leucine-rich repeat protein phosphatase
PI3K  phosphoinositide 3-kinase
PIKE-A  phosphatidylinositol 3-kinase enhancer-activating Akt
PKB  protein kinase B, also called Akt
PKR  double-stranded RNA-activating protein
pMEF  primary mouse embryo fibroblast
PP2A  protein phosphatase 2A
PRANC  pox proteins repeat of ankyrin C-terminal
PYD  pyrin domain
RING  really interesting new gene
RK13  rabbit kidney fibroblasts
RL-5  rabbit CD4+ T-lymphocyte cell line
rpm  rotations per minute
RT  room temperature
S6K  S6 kinase
SCF  Skp-cullin-F-box
SDS  sodium dodecyl sulphate
Serpin  serine protease inhibitor
Skp  S-phase kinase-associate protein
Src  v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog
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<tr>
<td>STAT</td>
<td>signal transducers and activators of transcription</td>
</tr>
<tr>
<td>TBS</td>
<td>tris buffered saline</td>
</tr>
<tr>
<td>TIR</td>
<td>terminal inverted repeats</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>TNT</td>
<td><em>in vitro</em> transcription/translation</td>
</tr>
<tr>
<td>TSC</td>
<td>tuberous sclerosis heterodimer</td>
</tr>
<tr>
<td>VACV</td>
<td>vaccinia virus</td>
</tr>
<tr>
<td>VARV</td>
<td>variola virus</td>
</tr>
<tr>
<td>VSV</td>
<td>vesicular stomatitis virus</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>WR</td>
<td>Western Reserve</td>
</tr>
<tr>
<td>U</td>
<td>unit</td>
</tr>
<tr>
<td>UV</td>
<td>ultra violate</td>
</tr>
<tr>
<td>Y2H</td>
<td>yeast two-hybrid</td>
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A productive poxvirus infection is heavily dependent upon the synthesis of a vast array of host modulatory products that specifically target and manipulate both extracellular immune response pathways of the host as well as intracellular signal transduction pathways of the individually infected cells. The unique pathogenesis and host tropism of specific poxviruses can be attributed to the broad diversity of host modulatory proteins they express. Most poxviruses express multiple proteins containing ankyrin (ANK)-repeats accounting for a large superfamily of related but unique determinants of poxviral tropism. Recently, select members of this novel family of poxvirus proteins have drawn considerable attention for their potential roles in modulating intracellular signaling networks during viral infection. M-T5 is an ANK-repeat protein encoded by the rabbit-specific poxvirus, myxoma virus (MYXV) which functions to regulate tropism of MYXV for rabbit lymphocytes and some human cancer cells. At the molecular level, M-T5 binds and alters at least two distinct cellular proteins: Akt and cullin 1. Furthermore, the direct interaction between M-T5 and Akt was shown to upregulate the kinase activity of Akt and enhance viral replication in a spectrum human cancer cells. The aim of this study was to examine the molecular mechanism by which
the M-T5 protein interacts with and manipulates host proteins to establish an
intracellular environment leading to a productive viral replication.

To understand the significance of these viral-host protein interactions, the various
binding domains of M-T5 were mapped. The N-terminal ANK-repeats I and II were
identified as being important for interaction with Akt, whereas the C-terminal PRANC/F-
box like domain was essential for binding to Skp1. We also report that M-T5 binds Akt
and the host Skp-cullin-F-Box (SCF) complex (via Skp1) simultaneously in MYXV-
infected cells. Furthermore, M-T5 specifically mediates the re-localization of Akt from
the nucleus to the cytoplasm during infection with the wild-type MYXV, but not the M-T5
knockout version of the virus. When an array of Akt inhibitor compounds that selectively
manipulate the Akt signaling network were screened, certain inhibitors significantly
blocked MYXV replication in previously permissive human cancer cells. In contrast
PP2A specific phosphatase inhibitors, such as okadaic acid, promoted an increased Akt
kinase activation and rescued MYXV replication in human cancer cells that did not
previously support viral replication. It was also demonstrated that hemi-phosphorylation
of Akt at residue Thr308 dictates physical interaction between Akt and M-T5, which
ultimately leads to productive MYXV replication in Type II cancer cells. Finally, we
conclude that M-T5 is functionally interchangeable with the host PIKE-A protein, and
that the activation of host Akt by either M-T5 or PIKE-A is critical for the permissiveness
of human cancer cells by MYXV.

In summary, this study further examines the intricate relationship between M-T5
and components of the host cell signaling networks and how these elaborate
interactions can profoundly impact poxvirus tropism. Currently, little is known regarding
the factors that regulate poxvirus tropism and the mechanisms by which poxviral encoded ANK-repeat proteins micromanipulate the signaling pathways of the infected cell to establish an environment that favors virus replication. Understanding the function of this unique family of viral host range genes will prove to be incredibly useful in our efforts to decipher the mechanisms that regulate poxvirus tropism. More importantly, this knowledge will further develop poxviruses as potential oncolytic candidates, as selectively replicating vaccine platforms, and for other diverse biotherapeutic applications.
CHAPTER 1
INTRODUCTION

The Poxviruses

Poxviruses are a highly successful collection of pathogens, which includes variola virus (VARV), the causative agent of smallpox in humans. Believed to have emerged in the human population about 10,000 BC, smallpox has plagued mankind for centuries and was responsible for an estimated 300-500 million deaths during the 20th century (17). Following the most successful vaccination campaigns in history, VARV was officially declared eradicated by the World Health Organization (WHO) in 1980, a tremendous achievement for public health. However, the recent outbreak of human monkeypox virus (MPXV) in the United States suggests the potential for poxviruses as emerging pathogens (65). MPXV is presently the most important orthopoxvirus infection in man since the eradication of VARV and the recent outbreak emphasizes the importance of understanding mechanisms by which poxviruses can sporadically leap from an evolutionary host species to cause zoonotic infections in humans (213). Furthermore, heightened concerns over the potential use of VARV as a biological weapon has increased the interest of this unique family of viruses.

Poxvirus Taxonomy

Members of the Poxviridae family are divided into two subfamilies according to their capacity to infect either vertebrate (Chordopoxviridae) or insect (Entomopoxviridae) hosts (Table 1-1). To date, more than 25 entomopoxviruses have been identified and are divided into three genera, however very little is known about the biology of these viruses and therefore they will not be further discussed. In contrast, chordopoxviruses have been extensively studied and are subdivided into eight genera.
<table>
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<th>Genus</th>
<th>Type species</th>
<th>Host(s)</th>
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<td><strong>Alphaentomopoxvirus</strong></td>
<td><strong>Melolontha entomopoxvirus</strong></td>
<td>Beetles</td>
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<tr>
<td></td>
<td></td>
<td><strong>Melolontha</strong></td>
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<td></td>
<td><strong>Betaentomopoxvirus</strong></td>
<td><strong>Amsacta moorei entomopoxvirus</strong></td>
<td>Butterflies, locusts grasshoppers and moths</td>
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<td></td>
<td><strong>Gammaentomopoxvirus</strong></td>
<td><strong>Chironomus luridus entomopoxvirus</strong></td>
<td>Flies and mosquitoes</td>
</tr>
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<td><strong>Avipoxvirus</strong></td>
<td><strong>Canarypox virus</strong></td>
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<td><strong>Fowlpox virus</strong></td>
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<td><strong>Capripoxvirus</strong></td>
<td><strong>Goatpox virus</strong></td>
<td>Goats</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Lumpy skin disease virus</strong></td>
<td>Cattle</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Sheepox virus</strong></td>
<td>Sheep</td>
</tr>
<tr>
<td></td>
<td><strong>Leporipoxvirus</strong></td>
<td><strong>Hare fibroma virus</strong></td>
<td>Lepus capensis</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Myxoma virus</strong></td>
<td>Sylvilagus brasiliensis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sylvilagus bachmani</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sylvilagus floridanus</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Rabbit fibroma virus</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Squirrel fibroma virus</strong></td>
<td>Sciurus carolinensis</td>
</tr>
<tr>
<td><strong>Molluscipoxvirus</strong></td>
<td></td>
<td><strong>Molluscum contagiosum</strong></td>
<td>Humans</td>
</tr>
<tr>
<td><strong>Orthopoxvirus</strong></td>
<td></td>
<td><strong>Camelpox virus</strong></td>
<td>Camels</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Cowpox virus</strong></td>
<td>Rodents (?)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Ectromelia virus</strong></td>
<td>Rodents</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Horsepox virus</strong></td>
<td>Horse</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Monkeypox virus</strong></td>
<td>Monkeys</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Rabbitpox virus</strong></td>
<td>Rabbits</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Racconpox virus</strong></td>
<td>Gerbils</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Taterapox virus</strong></td>
<td>Rodents (?)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Vaccinia virus</strong></td>
<td>Humans</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Variola virus</strong></td>
<td>Humans</td>
</tr>
<tr>
<td><strong>Parapoxvirus</strong></td>
<td></td>
<td><strong>Bovine popular stomatitis virus</strong></td>
<td>Cattle</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Orf virus</strong></td>
<td>Sheep</td>
</tr>
<tr>
<td><strong>Suipoxvirus</strong></td>
<td></td>
<td><strong>Swinepox virus</strong></td>
<td>Swine</td>
</tr>
<tr>
<td><strong>Yatapoxvirus</strong></td>
<td></td>
<td><strong>Tanapox virus</strong></td>
<td>Humans/Rodents</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Yaba monkey tumor virus</strong></td>
<td>Monkeys</td>
</tr>
<tr>
<td><strong>Unclassified</strong></td>
<td></td>
<td><strong>Melanoplus sanguinipes entomopoxvirus ‘O’</strong></td>
<td>Insects</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Mule deer poxvirus</strong></td>
<td>Deer</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Nile crocodile poxvirus</strong></td>
<td>Crocodiles</td>
</tr>
</tbody>
</table>
Poxviruses within the same genus share a variety of characteristics including; similar particle morphology, similar host-ranges and the members are genetically and antigenetically related (97, 182).

**Characteristics of the Poxviruses**

Amongst the largest known viruses, the poxvirus genome is encapsulated within a brick-shaped virion having dimensions in the order of several hundred nanometers (nm) (82). The outer membrane surface of the virus particle is extremely complex, containing many proteins (more than 100) and lipids that encase a walled, bioconcave core (57). Numerous viral enzymes involved in virus uncoating and early RNA transcription are packaged within the core, which is positioned between two “lateral bodies” of unknown function (Fig. 1-1). The poxvirus genome, also located within the core, is a double-
stranded (ds) DNA molecule with covalently closed hairpin termini, ranging from 130 kilobase pairs (kbp) in parapoxviruses to about 300 kbp in avipoxviruses (185). Flanked by terminal inverted repeat (TIR) regions, these identical DNA sequences are oppositely oriented at each end of the linear genome and vary in size amongst poxviruses, even within members from the same genus. More interestingly, poxviruses encode a collection of gene products that enable the virus to replicate exclusively in the cytoplasm of the infected cell. Many of the genes important for viral replication and virion assembly are centrally located in the viral genome and highly conserved among the various poxviruses (97, 281) (Fig. 1-2). In contrast, genes located towards the ends of the genome, either within the TIR or near-terminal regions, encode proteins with greater diversity among the poxvirus family and are believed to encode many of the specific determinants critical for immunomodulatory properties and host-range functions of the individual poxvirus member (174, 229, 277).

Figure 1-2. General organization of the poxvirus genome. Schematic representation of the poxvirus genome with the central core region containing highly conserved genes and the terminal regions, which encode genes predominantly involved in host modulation. TIR – terminal inverted repeat.
Poxvirus Replication Cycle

Poxvirus replication is represented by a complex sequence of events in which at least two distinct virus particle types, the intracellular mature virus (IMV) and the extracellular enveloped virus (EEV), can productively initiate the infectious cycle (243) (Fig. 1-3). These two particles differ in their membrane components and are thought to enter cells by different mechanisms (242, 283, 284). It is generally believed that most, if not all, poxviruses can efficiently bind and enter a wide range of mammalian cells and all of the known host anti-viral pathways that block viral replication in nonpremissive cells operate downstream of virus entry (171). While multiple viral proteins have been shown to be essential for virus entry/fusion into cells (184), the cellular determinant for poxvirus binding are largely unknown and believed to be ubiquitously expressed surface glycosaminoglycans (GAGs) or extracellular matrix proteins (49, 55). Once the virion has bound to the cell membrane, fusion takes place and the virion core is released into the cytoplasm of the cell (184). At this stage, poxvirus RNA polymerase and encapsulated transcription factors initiate the first wave of early viral gene transcription, synthesizing viral mRNA under the control of early viral promoters (41, 182). Interestingly, many of these gene products are immunomodulatory and host-range factors that function to prepare the intracellular environment for genome replication and transcription of intermediate viral genes. Next, viral DNA is released into the cytoplasm following core (or second stage) uncoating, where it can serve as a template for viral DNA replication and the subsequent intermediate and late transcription of poxviral genes (132). Transcription of intermediate genes only begins after replication of the viral genome has been initiated (288). A subset of intermediate gene products function to drive transcription of late genes, which encode structural products involved in virion
Figure 1-3. The single cell replication cycle of poxviruses. Poxvirus replication occurs almost exclusively in the cytoplasm of the infected cell. Shown is the entry of the intracellular mature virus (IMV) and the extracellular enveloped virus (EEV), both of which bind the surface of the target cell to initiate the replicate cycle. See text for a detailed description of the replication process.
assembly (100, 302). Many of the other late gene products are packaged directly into the progeny virion and play a pivotal role in early transcription during the next round of infection.

As late viral gene products accumulate, the virus undergoes morphogenesis and assembly of infectious virus particles at cytoplasmic sites called viral factories. The first infectious progeny that forms are the IMV, which travel along microtubules and then become enwrapped in Golgi-derived membranes to form intracellular enveloped virus (IEV) (104, 114, 275). As the IEV fuse with the cellular membrane, it loses a membrane wrapping to form the cell associated enveloped virus (CEV), an important component of cell-to-cell infection (243). CEV is either projected into the neighboring cells via actin-tail formation, or is released from the cell as EEV (241). CEV and EEV are important in cell-to-cell spread in vivo, while IEV infection becomes important in virus spread after the membrane has been ruptured following cell death (241, 294).

**Myxoma Virus**

The leporipoxvirus genus includes four species of which myxoma virus (MYXV) is the most studied. MYXV provides an excellent model to examine the numerous mechanisms by which poxviruses exquisitely evade and micromanage the immunological and anti-viral responses of the host. A natural pathogen in South and Central American tapeti rabbit (*Sylvilagus brasiliensis*), and the North American brush rabbit (*Sylvilagus bachmani*), MYXV infection is characterized by a benign cutaneous fibroma restricted to the site of inoculation (77). However, when the virus infects European rabbits (*Oryctolagus cuniculus*), MYXV causes a rapid systemic and highly lethal infection known as myxomatosis (78).
Historical Relevance of Myxoma Virus

Much of the initial characterization of MYXV was reported by Aragão in 1927 and based on the high lethality and specificity of the virus, he formally suggested using MYXV for the purpose of combating the rabbit plague that was devastating Australia (9). After comprehensive investigation the Australian Government introduced MYXV in 1950, which significantly diminished the rabbit population. Shortly following MYXV release, naturally attenuated strains with reduced pathogenicity emerged and rapidly replaced the virulent strain (79-81). Moreover, the selective pressure of myxomatosis favored rabbits with greater resistance to the viral disease. The increasingly resistant population of rabbits present in Australia and the selection of dramatically attenuated field variants of MYXV, has been well documented and provides an excellent insight into the coevolution of both host and virus (80).

Since the initial discovery of the virus, MYXV has spread across the planet and is currently endemic on four continents: South America, North America, Europe and Australia. In much of South America, MYXV is endemic in rabbits of the genus *Sylvilagus*, particularly *S. brasiliensis*. Yet in countries such as Chile, the principal reservoir of MYXV is the wild European rabbit (80). Whereas, in the Western United States the Californian strain of MYXV is endemic primarily in California brush rabbit (*S. bachmani*), which serves as a reservoir of infection for domestic rabbits (166, 214). MYXV has been endemic in the wild European rabbit population of Australia since its introduction in 1950. Similarly, MYXV was intentionally released in France in 1952 and is now endemic in many European countries, with *O. cuniculus* being the predominant host species (64). However, other rabbit species such as the European hare (*Lepus*
eropaeus), the mountain hare (Lepus timidus) and other Sylvilagus species can also serve as hosts (163).

**Pathogenesis of Myxoma Virus**

Among the natural and pathogenic rabbit hosts, the clinical signs of myxomatosis are considerably different. Rabbits of the genus Sylvilagus are relatively resistant to MYXV, resulting in the development of localized skin tumors (fibromas) 4-8 days following exposure and may persist up to 40 days (134). While young rabbits may succumb to a generalized disease, most Silvilagus rabbits fully recover from MYXV infection (77). In stark contrast, MYXV infection in European rabbits is far more severe and results in a high mortality rate. The symptoms and high mortality rates associated with myxomatosis are believed to be a result of multi-organ dysfunction as well as uncontrolled secondary bacterial infections of the respiratory tract that result from the progressive impairment of the host immune response (77, 80, 82). Early clinical signs of typical myxomatosis include pronounced edema at the base of ears, muzzle and anogenital region, as well as blepharoconjunctivitis (80, 82). By day 10 following infection, hard convex lumps on the body, head and ears are often observed. Rabbits survive for 1 to 2 weeks following infection and death is often preceded by terminal convulsions (134). Furthermore, the MYXV strain used can significantly impact the severity of the clinical disease observed. For example, rabbits infected with the original South American isolate of Moses have a mean survival time of 11 days (64). MYXV strain Lausanne, which is the predominant strain in Europe, causes severe disease symptoms with mortality close to 100% (64). Whereas, naturally attenuated MYXV isolates have emerged, in both Australia and Europe, which have decreased morbidity and significantly lower mortality rates in susceptible rabbits (33). Likewise, the
laboratory-attenuated neuroMYXV has little or no mortality and produces only mild symptoms (163).

In European rabbits, MYXV infection and pathogenesis is heavily dependent upon the ability of the virus to disseminate from the primary site of infection and establish secondary sites of viral infection in distal tissues (134). Typically, MYXV replicates in the skin at the site of infection and spreads from there into the draining lymph nodes. Spread of MYXV is achieved by means of infected migratory leukocytes, which function as viral transporters and disseminate virus infection to the distal tissues (32, 88). The ability of MYXV to infect lymphocytes in particular is a critical determinant to the success of MYXV and essential to the progression of myxomatosis in susceptible European rabbits. To date, a number of MYXV encoded proteins have been identified as tissue-specific host-range factors, which function to promote MYXV replication and block the induction of anti-viral responses, particularly apoptosis, in rabbit lymphocytes (297).

**Myxomatosis Prevention, Control and Vaccination**

Control of virus spread among the feral rabbit population is critical to prevent outbreaks from occurring, especially in regions of the world where MYXV has become endemic. Fortunately, proper screening to exclude arthropod vectors such as mosquitoes and fleas is the single most effective method of disease prevention (64). If a sick rabbit appears, the animal must immediately be quarantined to prevent new outbreaks from occurring within the colony. A live attenuated form of MYXV designated MSD strain has been used as a vaccine strain and provides immunity for approximately 9 months (216). Serial passage of this virus in rabbit kidney cultures has further attenuated the virus without sacrificing its immunity and has been designated MSD/B.
Furthermore, Shope Fibroma virus, which is closely related to MYXV, has also been used as a vaccine strain with variable results, yet is generally effective (80).

A collection of recombinant MYXV strains have been characterized and several recombinants from this collection have been shown to have the potential to be excellent vaccine candidates based on their attenuated virulence. Many of these genes have been shown to have immunomodulatory roles and selective deletion of such genes does not reduce in vitro replication levels (Table 1.2). For example, rabbits infected with MYXV deficient in Serp-1 (vMyxSerp1KO) suffered moderate clinical signs of myxomatosis but the disease was unable to progress to later stages. The majority of infected rabbits showed significant recovery within 14 days post infection and when re-challenged with the parental MYXV strain, were resistant to myxomatosis (162). Such attenuated viruses lacking host immunomodulatory genes would make great vaccine candidates, yet comprehensive studies into their effectiveness as vaccines have yet to be completed.

**Poxvirus Immunoregulatory Genes Dictate Tropism**

Despite considerable advances in the understanding of poxvirus replication, the fundamental mechanisms that mediate the basis for host-range and poxvirus tropism remains poorly understood (171). As a family of viruses, albeit with a few exceptions like molluscum contagiosum virus (MCV), poxviruses have the capacity to infect a relatively wide spectrum of eukaryotic hosts in vitro, whereas individual virus members within the family are commonly restricted to either an exclusive host, or a relatively small number of potential host species (171). At the cellular level, poxvirus tropism is dependent not upon specific cell surface receptors, but rather upon the ability of the cell to provide intracellular complementing factors needed for productive virus replication,
and the ability of the specific virus to successfully manipulate intracellular signaling networks that regulate cellular anti-viral processes downstream of virus entry (124, 171). Consequently, the ability of poxviruses to manipulate the signal transduction networks of infected cells has a major impact on the outcome of a specific viral infection.

Table 1-2. Selected immunomodulatory proteins of myxoma virus

<table>
<thead>
<tr>
<th>ORF(s)</th>
<th>Gene names</th>
<th>Protein function and/or properties</th>
<th>Required for infection</th>
<th>References(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>In vitro</strong></td>
<td><strong>In vivo</strong></td>
</tr>
<tr>
<td>M001L/R</td>
<td>M-T1</td>
<td>Chemokine binding protein, inhibits chemokine chemoattractant properties</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>M002L/R</td>
<td>M-T2</td>
<td>TNF receptor homolog, binds rabbit TNF-α</td>
<td>Only RL-5</td>
<td>Yes</td>
</tr>
<tr>
<td>M004L/R</td>
<td>M-T4</td>
<td>ER-localized apoptosis regulator</td>
<td>Only RL-5</td>
<td>Yes</td>
</tr>
<tr>
<td>M005L/R</td>
<td>M-T5</td>
<td>ANK/PRANC, host-range, cell cycle/apoptosis inhibitor</td>
<td>Only RL-5</td>
<td>Yes</td>
</tr>
<tr>
<td>M007L/R</td>
<td>M-T7</td>
<td>IFN-γ receptor homolog, binds C-C and C-X-C chemokines in vitro</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>M008.1L/R</td>
<td>SERP-1</td>
<td>Secreted serpin</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>M010L</td>
<td>MGF</td>
<td>EGF-like growth factor</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>M011L</td>
<td>M11L</td>
<td>Integral membrane protein, apoptosis regulator</td>
<td>Only RL-5</td>
<td>Yes</td>
</tr>
<tr>
<td>M013L</td>
<td>M13L</td>
<td>Pyrin-containing inhibitor of the inflammasome</td>
<td>Only RL-5</td>
<td>Yes</td>
</tr>
<tr>
<td>M063R</td>
<td>-</td>
<td>Unknown</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>M128L</td>
<td>vCD47</td>
<td>Integrin-associated protein, CD47 homolog</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>M130R</td>
<td>-</td>
<td>Unknown</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>M135R</td>
<td>-</td>
<td>Cell surface α/β IFN receptor homolog</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>M141R</td>
<td>vOX-2 / vCD200</td>
<td>Immunoglobulin domain / OX-2 homolog</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>M148R</td>
<td>-</td>
<td>ANK/PRANC</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>M149R</td>
<td>-</td>
<td>ANK/PRANC</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>M150R</td>
<td>MNF</td>
<td>ANK/PRANC, sequesters NF-κB in the cytoplasm</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>M151R</td>
<td>SERP-2</td>
<td>Serpin</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>M152R</td>
<td>Serp-3</td>
<td>Serpin</td>
<td>No</td>
<td>Yes</td>
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</table>
The distinct tropism of each individual poxvirus is thus strictly dependent upon the unique repertoire of host-range genes expressed by that virus (171). Some of the proteins encoded by poxvirus host-range genes, called host-range factors, have been demonstrated to specifically target host intracellular pathways, which are often configured to prevent viral infections, to establish a cellular microenvironment more favorable to viral replication (229). In fact, the outcome of the dynamic struggle between viral host-range factors and cellular anti-viral pathways can determine whether a specific infection will be permissive or not. Before one can gain a better appreciation of the sophisticated interplay between viral and cellular proteins, the operational roles that host-range factors play in the manipulation of the targeted host intracellular signaling pathways must be better understood.

Generally speaking, the discovery of specific poxvirus host-range genes has usually been the result of targeted gene-knockout analysis in which the mutant virus isolate is subsequently shown to be replication deficient in a subset of cultured cells for which the parental virus is permissive (125). All poxviruses are predicted to encode a unique collection of host-range factors, however their specific identification as host-range genes has been largely fortuitous. Thus, our current understanding of the host target pathways with which the protein products of these poxvirus host-range genes interact is incomplete. To date, a relatively small number of poxvirus host-range gene have been identified and studied, and only a handful of these have been functionally characterized (Table 1-3). Interestingly, the majority of the host-range genes that have been discovered and analyzed are from members of only two of the eight chordopoxvirus genera; namely, the orthopoxviruses and leporipoxviruses.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein type</th>
<th>Function</th>
<th>Cultured cells with defects in virus host-range</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPI-1</td>
<td>Serpin</td>
<td>May inhibit caspase-independent pathway of apoptosis</td>
<td>PK15, A549</td>
<td>(40)</td>
</tr>
<tr>
<td>K1L</td>
<td>ANK-repeat</td>
<td>Inhibit NF-κB activation</td>
<td>RK13</td>
<td>(238)</td>
</tr>
<tr>
<td>C7L</td>
<td>Cytoplasmic</td>
<td>Inhibition of apoptosis</td>
<td>RK13, hamster Dede cells</td>
<td>(192)</td>
</tr>
<tr>
<td>C7L/K1L</td>
<td>-</td>
<td>-</td>
<td>PK13, RK13 and most human cells</td>
<td>(203)</td>
</tr>
<tr>
<td>CHOhr</td>
<td>ANK/PRANC</td>
<td>Prevent protein synthesis shutoff</td>
<td>CHO</td>
<td>(248)</td>
</tr>
<tr>
<td>P28/N1R</td>
<td>E3-ubiquitin ligase</td>
<td>Degradation of proteins, inhibit apoptosis</td>
<td>Murine macrophages</td>
<td>(38, 194)</td>
</tr>
<tr>
<td>B5R</td>
<td>Membrane glycoprotein</td>
<td>Activate Src</td>
<td>Vero, CEF, PK-15 and quail (QT-6)</td>
<td>(257)</td>
</tr>
<tr>
<td>E3L</td>
<td>PKR inhibition</td>
<td>Inhibit IFN responses</td>
<td>HeLa, Vero, murine dendritic cell line, CEF (MVA-E3L)</td>
<td>(27, 147)</td>
</tr>
<tr>
<td>K3L</td>
<td>dsRNA-bind protein</td>
<td>Pseudosubstrate inhibitor of PKR</td>
<td>BHK, mouse L929</td>
<td>(147)</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Myxoma virus</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>M-T2</td>
<td>TNF receptor</td>
<td>Inhibit rabbit TNF-α</td>
<td>Rabbit T-cells</td>
<td>(161)</td>
</tr>
<tr>
<td>M-T4</td>
<td>ER-localized</td>
<td>Inhibit apoptosis</td>
<td>Rabbit T-cells</td>
<td>(23)</td>
</tr>
<tr>
<td>M-T5</td>
<td>ANK/PRANC</td>
<td>Cell cycle progression, phosphorylation of AKT</td>
<td>Rabbit T-cells, human tumour cells</td>
<td>(186, 267, 292)</td>
</tr>
<tr>
<td>M11L</td>
<td>Mitochondrial</td>
<td>Inhibit apoptosis</td>
<td>Rabbit T-cells</td>
<td>(71, 161)</td>
</tr>
<tr>
<td>M13L</td>
<td>PYD domain</td>
<td>Inhibit inflammasome</td>
<td>Rabbit T-cells</td>
<td>(123)</td>
</tr>
<tr>
<td>M063</td>
<td>C7L-like</td>
<td>Unknown</td>
<td>Rabbit cells</td>
<td>(20)</td>
</tr>
</tbody>
</table>

**Orthopoxivirus Host-Range Genes**

The family of orthopoxviruses collectively exhibits the broadest host-range of the chordopoxviruses and most of the individual orthopoxvirus members are capable of replicating in a wide array of cell types from various species, at least in vitro. This unique cellular tropism can in part be explained by the large coding capacity of the canonical orthopoxviral genome, which encodes a spectrum of specific viral proteins that enable the virus to cross species barriers (6). The interaction between host cell...
proteins and poxvirus host-range proteins is so diverse that no single viral host-range ortholog common to all poxvirus genomes has yet been identified (229). However, many of the better characterized host-range genes in orthopoxviruses are sometimes fairly well conserved among the family members and suggests the importance of these genes in poxvirus biology by counteracting host cell responses and manipulation of the host cellular microenvironment. For example, some of the known poxvirus host-range factors have been associated with manipulation of a diverse array of cellular targets, which includes cellular kinases and phosphatases, apoptosis and various antiviral pathways. Vaccinia virus (VACV) is the most extensively studied orthopoxvirus and for that reason has provided the bulk of our knowledge in regard to the discovery and functional understanding of orthopoxvirus host-range genes.

**Myxoma Virus Host-Range Genes**

Like all poxviruses, MYXV expresses a distinct repertoire of immunoregulatory factors that have been experimentally shown to subvert the host immune and anti-viral responses to virus infection and mediate MYXV tropism (254). Included among these viral encoded proteins are homologs of cellular cytokine receptors (viroreceptors), secreted mimics of host ligands or regulators (virokines) (172) and inhibitors of the apoptotic pathway (24, 73). In the absence of these immunomodulatory factors, for example in MYXV constructs in which individual viral genes have been deleted, the myxomatosis disease progression in susceptible rabbits is often considerably attenuated, thus demonstrating their importance in mediating the ability of MYXV to evade the host immune system and establish a successful infection.

In general, MYXV proteins that affect viral pathogenesis or dissemination are termed virulence factors, and those that specifically mediate the tropism of MYXV in
specific cell types or tissues are referred to as host-range factors. Several of the host-
range gene products of MYXV have orthologs in other poxviruses, however MYXV is
the only poxvirus for which there has been a systematic study of the comparative
biological roles of viral host-range gene products both *in vitro* and *in vivo* (254). A
collection of MYXV host-range genes have been identified (Table 1.3) and examined to
determine the mechanism(s) by which they mediate MYXV tropism and regulate virus
virulence.

**M-T5 is a Host-Range Gene Encoded by Myxoma Virus**

Of the known poxvirus host-range genes described to date, the MYXV M-T5 gene
was first discovered over a decade ago to be critical for MYXV replication within rabbit
T-lymphocytes (186). M-T5 possesses no extensive sequence similarity to non-viral
proteins but does share some sequence similarity with VACV gene B4R and B6R
encoded by VARV. Although little is currently known about the function of these
particular gene products, similarity between the ANK-repeats of M-T5 and the well-
studied host-range gene CHOhr has been previously noted (106). The cowpox virus
(CPXV) CHOhr gene encodes a 77 kDa protein that was initially identified for its ability
to rescue VACV replication in normally nonpermissive Chinese hamster ovary (CHO)
cells (140, 211, 232, 248).

VACV also possesses two additional host-range genes, named K1L and C7L,
which have been demonstrated to be required for productive virus growth in rabbit
kidney fibroblasts (RK13) and HeLa cells, respectively (90, 203, 262). Viral replication of
VACV deficient in either or both C7L and K1L can be rescued in restrictive cells by the
expression of the CHOhr gene product (203). Additionally, the CHOhr gene was shown
to functionally replace K1L and permit VACV replication in RK13 cells, suggesting that
expression of CHOhr also possesses rabbit cell host-range properties (211). It is relevant to note that when M-T5 is compared to CHOhr, sequence similarity between the two extends across the entire length of the protein with the exception of a 106 amino acid deletion in the central region of M-T5. The functional significance of this apparent internal deletion remains yet to be determined (186).

**Myxoma Virus Replication in Rabbit Lymphocytes is Dependent Upon M-T5**

Two copies of the M-T5 open reading frame (ORF) are present in the MYXV genome, one within each copy of the virus TIR (Fig. 1-4) (44). The M-T5 gene is 1452 nucleotides in length and encodes a protein of 483 amino acids, which is expressed rapidly following infection and remains as an abundant and stable 49 kDa cell-associated protein throughout the course of viral infection (127, 186). To begin to understand the functional role of M-T5 during virus infection, a recombinant MYXV was constructed in which both copies of the M-T5 gene were disrupted by the insertion of a selectable marker, namely β-galactosidase (186). The replication kinetics of the M-T5 deficient MYXV (vMyxT5KO) in cultured rabbit kidney fibroblasts was indistinguishable from cells infected by wild-type MYXV.

![Figure 1-4. Structural features of the M-T5 protein. The myxoma virus genome encodes two copies of the M-T5 open reading frame, one within each copy of the virus terminal inverted repeats (TIR). Structurally M-T5 contains seven ankyrin (ANK)-repeat domains within the N-terminus and central regions of the protein and a conserved C-terminal PRANC motif, which closely resembles a cellular protein motif called the F-box domain.](image-url)
However, rabbit T-lymphocytes infected with vMyxT5KO resulted in an abortive infection characterized by rapid inhibition of both viral and host gene synthesis accompanied by extensive cellular apoptosis. (186). Thus, it would appear that M-T5 specifically promotes MYXV replication in lymphocytes by preventing the nonspecific shutdown of protein synthesis, which is probably the stimulus leading to the induction of apoptosis that aborts the infection of rabbit lymphocytes with the vMyxT5KO virus. Similarly, host-range studies of VACV demonstrated early and extensive inhibition of viral and host-range protein synthesis during infection of CHO cells resulting in an abortive infection, unless the CHOhr gene is inserted into the VACV genome (210).

**M-T5 Inhibits the Induction of Apoptosis in Rabbit Lymphocytes**

The initiation of a rapid apoptotic response following virus infection provides an effective cellular mechanism to abort viral infections (73). Consequently, many viruses, including poxviruses, have been shown to encode modulatory proteins that function to block various components of the apoptotic pathway (268). These anti-apoptotic effects have been shown to manipulate the cell death pathways within the infected cell in a variety of ways, including the inhibition of caspases and the disruption of key mitochondrial checkpoints to prevent apoptosis (73-75). For example, CPXV encodes the protein CrmA, an intracellular serpin that functions to specifically inhibit the cellular proteins Caspase-1, Caspase-8 (212) and granzyme B (208). The Serp-2 protein encoded by MYXV is a viral serpin related to CrmA and is critical for the pathogenesis of MYXV by preventing apoptosis in virus-infected lymphocytes (178, 204).

Another MYXV anti-apoptotic gene, M11L is targeted to the cytoplasmic surface of the outer mitochondrial membrane where it is able to inhibit the release of cytochrome $c$ and block apoptotic signals at the mitochondrial checkpoint (71, 72). In addition, M11L
was shown to block apoptosis by sequestering Bak and Bax, pro-apoptotic multi-domain Bcl-2 family members (260, 291). Induction of the apoptotic pathway serves as an effective anti-viral response at the level of individually infected cells and it is no surprise that poxviruses have an extensive repertoire of strategies targeted at inhibiting the apoptotic cascade. Viral counteractive proteins, such as M-T5, have proven beneficial in elucidating the control points of the cellular apoptotic pathway and have provided further understanding into the mechanisms by which viruses can micromanipulate the intracellular environment of the infected cell.

**M-T5 is a Potent Virulence Factor in European Rabbits**

Pathogenesis studies of European rabbits infected with vMyxT5KO further demonstrated the importance of M-T5 as a potent virulence factor and its critical role during MYXV infection *in vivo*. For example, four days following infection with wild-type MYXV, large lesions were observed at the primary site of inoculation and by one week rabbits had developed all the classical signs of myxomatosis, including multiple sites of secondary infection. In contrast, rabbits infected with vMyxT5KO developed only small primary lesions at the site of inoculation and remained basically asymptomatic, with no evidence of viral spread to sites of secondary infection. While all rabbits infected with wild-type MYXV had to be scarified by day 10 post-infection, complete regression of all primary lesions was observed in rabbits infected with vMyxT5KO by 14 days post-infection. Following a full recovery, rabbits initially inoculated with vMyxT5KO were challenged by the wild-type strain of MYXV but were found to be completely immune to developing myxomatosis (186).

Histological analysis of rabbits infected with vMyxT5KO demonstrated a rapid and effective inflammatory response at the primary lesion shortly following infection. In
contrast, rabbits infected with wild-type MYXV were unsuccessful at mounting an inflammatory response capable of controlling virus infection. In the absence of the M-T5 gene, MYXV was unable to spread from the primary site of infection to distal tissues via infected lymphocytes due to a host-induced anti-viral response (186). The dramatic attenuation of vMyxT5KO appears to reflect an increased ability of the host to rapidly control the infection at the primary site of inoculation. M-T5 therefore functions as a critical virulence factor, probably in part by permitting infection of rabbit lymphocytes and thus allowing the replication and dissemination of the virus throughout the lymphoreticular system.

**Ankyrin-Repeat Protein Superfamily**

Detailed analyses of the M-T5 sequence predicted the presence of seven ANK-repeat domains within the N-terminus and central regions of the protein (Fig. 1-4). The ANK-repeat, a 33-residue sequence domain, is one of the most common protein-protein interaction motifs found in nature (181). Proteins containing ANK-repeats have been demonstrated to mediate diverse protein-protein interactions between cellular proteins having a broad spectrum of functional roles (181, 228). Solved crystal structures have revealed a conserved fold structure of the ANK-repeat unit, by which each repeat forms a characteristic helix-loop-helix structure with a beta-hairpin/loop region projecting out from the helices at a 90° angle (26, 92, 119, 158). However, the ANK fold appears to be defined by its structure rather than any conserved biological function since there is no specific conserved substrate or binding partner structure that is universally recognized by members of the superfamily. The ANK motif has been extensively identified in eukaryotes, bacteria, and archaebacteria but are relatively rare in viruses, with the exception of poxviruses (37).
Poxvirus Encoded Ankyrin-Repeat Proteins

Generally members of this unique family of poxvirus-encoded proteins are 400 to 600 amino acids in size and contain 5-10 ANK-repeats, which are normally clustered towards the N-terminus. Almost all poxviruses express multiple proteins containing ANK-repeats, thus accounting for a large family of related but unique poxviral gene products (176). Canarypox virus, for example, encodes approximately 50 ANK-repeat proteins, which represents over 20 percent of the entire genome (276). Aside from M-T5, MYXV encodes three additional ANK-repeat containing proteins, termed M148, M149 and M150, all of which are expressed by single copy genes and share some sequence similarity to each other (44). However, it is believed that each one performs unique biological functions, and to date only M-T5 has been directly shown to possess host-range properties.

Although the number of poxviral encoded ANK-repeat genes is large, the functions of only a few proteins have been clearly identified. Based on the limited amount of data regarding poxvirus ANK-repeat proteins, it would suggest they function to interact with host cellular signaling networks during virus infection. The exact functional significance for the ANK-repeats in M-T5 has yet to be identified, however it is reasonable to predict that they facilitate protein-protein interactions with host targets. Therefore, a further understanding of the mechanism by which this large family of ANK-repeat protein interact with host partners may provide additional clues to how viruses manipulate the intracellular signaling environment and promote a productive virus replication.
Most ANK-repeat Poxviral Proteins Contain a Conserved F-Box Domain

The majority of poxvirus ANK-repeat proteins, including M-T5, contain a highly conserved F-box motif located at their C-terminus (Fig. 1-4) (176). In contrast, F-box domain-containing proteins identified from yeast and humans most commonly contain other protein-protein interaction domains such as leucine-rich repeats (LRR) (137) or WD repeats (244) and the F-box motif is normally located at the N-terminus (121). The consensus sequence for the F-box domain is approximately 50 amino acids and has relatively few invariant residues (136). The F-box consensus from poxviruses is comparably shorter than the established F-box consensus in other eukaryotes but still contains a significant number of conserved residues. (176).

To date, hundreds of cellular proteins containing an F-box motifs have been identified, however the domain is very rare among viral proteins (176). It would therefore appear that poxviruses acquired the F-box domain early in evolutionary history from ancestrally infected host(s). The acquisition of this unique domain likely provides poxviruses additional strategies to regulate key cellular pathway that mediate pathogenesis and tropism.

F-box Proteins are Specific Factors of SCF Ligases

Operationally, the F-box motif mediates protein-protein interactions and was first described as a recognition subunit of the E3 ubiquitin ligase complex, known as SCF (Skp1, cullin-1, F-box protein) (76, 239). The SCF complex is a multi-protein complex that facilitates the ubiquitination of substrates destined for degradation by the 26S proteasome (103). Targeted proteins are recognized by a variety of F-box proteins, such as Skp2, and are delivered to the E3 ligase complex via an adaptor protein (62). Interaction between the adaptor protein, Skp1, and the F-box protein occurs by way of
the F-box motif. The SCF complex plays a critical role in the selective degradation of regulatory proteins that mediate various cellular functions, such as signal transduction and regulation of the cell cycle (62). For example, p27/Kip1 is a key mediator of G1 arrest, and is phosphorylated at Thr187, which provides a recognition site for the binding of Skp2 and subsequent targeting to the SCF complex (62). Therefore, the degradation of p27/Kip1, via the ubiquitin-proteasome system provides a targeted mechanism to regulate the cell cycle.

**M-T5 Binds to Cullin 1**

The importance of the ubiquitin-proteasome pathway in the regulation of cellular processes and the presence of an F-box domain at the C-terminus of M-T5 suggested that the M-T5 protein functions to target host proteins to the SCF complex. As predicted, M-T5 was identified as a cellular binding partner of a cellular complex that includes cullin 1 (CUL1), suggesting a functional link to Skp2 (Fig. 1-5). Consistent with this interaction, M-T5 was shown to promote cell cycle progression beyond the G₀/G₁ checkpoint during virus infection, whereas cells infected with vMyxT5KO entered cell cycle arrest and accumulated at G₀/G₁. Furthermore, enhanced ubiquitination of p27/Kip1 and subsequent degradation through the proteasome pathway was observed through the interaction of M-T5 and CUL1, although whether this interaction was direct or through adaptor protein(s) was not defined (127). Progression of cells through the cell cycle is a tightly regulated process and viral infection often leads to cell cycle arrest and the induction of apoptosis. Therefore, strategically manipulating the host cell cycle is a key factor to promote productive viral replication and for that reason is the target for a plethora of viral proteins. Besides the obvious importance of M-T5 during MYXV pathogenesis, the protein is also critical in protecting virus infected cells from the stress
of cell cycle arrest induced by the host innate antiviral response (127). Thus the ability to overcome cell cycle arrest following infection is a key functional determinant of the cell tropism of MYXV.

Figure 1-5. M-T5 blocks cell cycle arrest through its interaction with cullin 1. Protein interaction between virus-encoded M-T5 and cullin-1 of the SCF complex mediate the proteasomal degradation of p27/Kip, however additional substrates of M-T5 may be regulated in a similar manner.

**Expanding Myxoma Virus Tropism**

Despite recent advances in understanding poxvirus replication (183), the molecular basis underlying the strict species barrier for poxviruses remains relatively mysterious. For many eukaryotic viruses, the binding and entry stage is a critical discrimination step and is highly dependent upon the presence of specific cell surface receptors (165, 240). However, and in stark contrast to most other eukaryotic viruses, no specific host-cell receptor has been identified for poxviruses, which are relatively promiscuous at binding and entering a wide variety of mammalian cells. Instead, poxvirus binding and entry is an efficient process in most mammalian cells, and all of
the known anti-viral pathways that block replication in nonpremissive cells operate downstream of virus entry (171). It is therefore believed that a productive poxvirus infection depends heavily on the ability of the virus to micromanipulate the cellular signaling machinery of the host cell (95). Although MYXV has a narrow host-range in nature, and is pathogenic only to rabbits, *in vitro* MYXV can productively infect many nonrabbit cells as well, including immortalized baby green monkey kidney (BGMK) cells and some primary human dermal fibroblasts (126). A further understanding into the mechanisms governing the MYXV species barrier was uncovered when it was shown that manipulation of the ERK-IFN-STAT1 signaling pathway could eliminate the replicative block to MYXV in mouse cells, rendering them susceptible to a productive MYXV infection. It was demonstrated that MYXV infection of primary mouse embryo fibroblasts (pMEF) induced the rapid activation of the interferon (IFN) regulatory factor-3 (IRF3) via ERK activation, thereby promoting the induction of type I IFN leading to an abortive infection (290).

In comparison, the negative-stranded RNA virus vesicular stomatitis virus (VSV) is extremely sensitive to IFN and in human cells that respond normally to IFN the VSV does not propagate (29). However, in a variety of human cancer cells with defective IFN signaling pathways, VSV propagates efficiently (258). Since many human cancer cells exhibit defective IFN responses, a broad spectrum of human cancer cell lines were screened for their ability to support MYXV replication. Productive MYXV replication was observed in the majority (15/21) of the cell lines tested providing compelling evidence for the potential of MYXV as an oncolytic candidate (267).
**Myxoma Virus as a Candidate for Oncolytic Virotherapy**

Oncolytic viruses are quickly emerging as promising new experimental therapeutics, however the use of viruses to specifically infect and kill cancerous tissues is hardly a new concept (133). To date, a wide range of viruses that demonstrate an increased replicative potential in transformed cells have been explored for their use as therapeutic agents (30, 54). Although VACV does not exhibit increased binding or replicative specificity for tumor cells, efforts to engineer the virus as an oncolytic vector are ongoing (274). Deletion of both the thymidine kinase and VACV growth factor genes severely attenuated VACV virulence but the capacity to infect rapidly growing tumor cells was maintained (170). The large coding capacity of poxviruses make them attractive oncolytic vector platforms because multiple therapeutic genes may be inserted into the viral genome to increase both anti-cancer potential and replicative specificity to tumor cells. The nonpathogenic nature of MYXV in humans, in conjunction with its ability to infect and kill a wide spectrum of human cancer cells, thus makes MYXV an excellent oncolytic candidate.

When tested in cancer models *in vivo*, MYXV was shown to have significant anti-tumoral activity against human glioblastoma in a murine xenograft model (159). Furthermore, MYXV infection in tumor tissues persisted long after the initial viral inoculation and virus replication was not detected in non-cancerous neighboring cells (159). The inherent host tropism of MYXV provides an ideal platform for the development of next generation viruses with increased specificity and cytotoxicity for human cancer cells. Future oncolytic viral vectors will rely on the ability of the virus to exploit signaling differences often observed in tumor tissues, promoting the efficient targeting and killing of cancer cells. Therefore, the cellular anti-viral restriction
mechanisms which apparently differ between human cancer cells and normal untransformed somatic cells must be better understood before MYXV could be used in a clinical setting to treat cancer in patients.

**Intracellular Signaling Manipulation: The Role of M-T5**

The ability of MYXV to replicate in human cancer cells has generated considerable interest into characterizing the viral and cellular host-range factors responsible for this unique tropism. Thus far, M-T5 is the only MYXV encoded protein shown to influence MYXV tropism in human cancer cells (267), although other such examples are likely still to be discovered. In the absence of M-T5, the mutant MYXV was nonpermissive in a particular subset of human cancer cells (referred to as Type II cells), which supported replication of wild-type MYXV. To further investigate the tropism of MYXV in human cancer cells, a number of cellular pathways frequently dysregulated in cancer cells were examined for their potential correlation with MYXV permissiveness. Results of this work identified the Akt signaling pathway as a key restriction determinant for the permissiveness of human cancer cells by MYXV (Fig. 1-6) (292). The serine/threonine protein kinase Akt/protein kinase B (PKB) is a cellular homolog of the viral oncogene v-Akt and plays a critical role in a variety of biological processes, which include regulating the balance between cell survival and apoptosis. Akt contains three functionally distinct domains that include: an N-terminal pleckstrin homology (PH) domain, a central catalytic domain, and a hydrophobic motif at the C-terminal. The binding of PIP3 to the PH domain anchors Akt to the plasma membrane and stimulates Akt phosphorylation by several kinases including phosphoinositide dependent kinase 1 (PDK1) (7) and mTORC2 (118, 219). Fully active Akt is regulated through the phosphorylation of two residues, Thr308 on the kinase domain and Ser473 on the hydrophobic motif.
Figure 1-6. Akt activation correlates with permissiveness for myxoma virus. Binding of M-T5 to cellular Akt induces phosphorylation of Akt and is a key restriction determinant for myxoma virus permissiveness in human cancer cells. Human cancer cells were categorized into three distinct groups based on their level of endogenous Akt activation and ability to support myxoma virus replication. M-T5 is uniquely required for permissiveness of Type II cells.

In addition to its role in survival, Akt is also involved in cell cycle regulation by preventing GSK-3β mediated phosphorylation and degradation of cyclin D1 (63) and by negatively regulating the cyclin-dependent kinase inhibitors p27/Kip1 (89) and p21 Waf1/Cip1 (311) (Fig. 1-7). In the majority of human cancer cells, the Akt pathway is either mutated or constitutively activated, contributing to cancer progression through both the stimulation of cellular proliferation and inhibition of apoptosis (197, 271). A wide spectrum of human cancer cells were screened for MYXV permissiveness and divided into three distinct categories, designated Type I, II and III based on their ability to
Figure 1-7. The Akt signaling pathway. Akt a serine/threonine protein kinase and a key regulator of several cellular pathways which mediate either cell growth and proliferation or the inhibition of apoptosis. Once correctly localized to the cell membrane via binding of PIP₃ to the PH domain, Akt can be phosphorylated by its activating kinases. Fully active Akt is regulated through the phosphorylation of two residues, Thr308 on the kinase domain and Ser473 on the hydrophobic region of the protein. The upstream molecules PI3K and PTEN provide an additional level of control to regulate Akt activation. The role of M-T5 protein is to bind Akt and promote its phosphorylation and activation.

support replication of either wild-type MYXV or vMyxT5KO (292). Importantly, it was noted that the level of endogenous phosphorylated Akt (Ser473 and Thr308) directly corresponded to MYXV permissiveness in all the human cancer cells tested (292). Cells support replication of either virus (Type III). A unique subset of cells (Type II) was identified that were permissive to wild-type MYXV but did not support replication of vMyxT5KO. Low levels of Akt phosphorylation were initially identified in these Type II cells, however following infection with the wild-type MYXV, phosphorylation increased to
levels comparable to Type I cells. No increase in Akt phophyloration was detected when cells were infected by vMyxT5KO (292). It was further demonstrated that in MYXV infected cells, M-T5 forms a complex with cellular Akt and upregulates its kinase activity. Thus, if Akt was pre-activated or could be activated by MYXV infection via M-T5, the cancer cells were permissive but if Akt remained inactivated the cells were non-permissive for MYXV infection (292). Productive virus infection in permissive cells often occurs only after successful manipulation of signaling pathways that regulate various cellular anti-viral processes. Consequently, the intricate relationship between viral encoded proteins and components of the host cell signaling networks can have profound impact on virus tropism.

**Dissertation Objectives**

It is generally believed that successful poxvirus replication is dependent upon the production of a diverse array of host modulatory products that specifically target and manipulate both extracellular immune response pathways as well as intracellular signal transduction pathways. Therefore, the ability of an individual poxvirus to micromanipulate signaling pathways of the infected cell directly dictates the outcome of a viral infection. The overall objective of this dissertation is to determine the molecular mechanism by which the M-T5 protein encoded by MYXV interacts with and manipulates host proteins to establish an intracellular environment leading to a productive viral replication. Knowledge acquired from this study will provide further insights into the factors that regulate poxvirus tropism and the mechanisms by which poxviruses specifically target signal transduction pathways to block the host antiviral response during viral infection.
CHAPTER 2
MATERIALS AND METHODS

DNA Methods

Reagents

- **Luria-Bertani (LB) broth**: 1% tryptone, 0.5% yeast extract, 1% NaCl
- **2YT agar plates**: 1.6% tryptone, 1% yeast extract, 0.5% NaCl, 2% agar
- **2YT agar plates + ampicillin**: 1.6% tryptone, 1% yeast extract, 0.5% NaCl, 2% agar, 40 μg/ml ampicillin
- **2YT agar plates + kanamycin**: 1.6% tryptone, 1% yeast extract, 0.5% NaCl, 2% agar, 50 μg/ml kanamycin
- **0.5 M EDTA (ethylenediamine tetraacetic acid)**: Na₂EDTA-2H₂O, 10 M NaOH
- **TAE (Tris-acetate-EDTA) DNA electrophoresis buffer**: 40 mM Tris base, 0.114% glacial acetic acid, 1 mM EDTA
- **6X DNA loading sample buffer**: 40% sucrose, 0.02% bromophenol blue

Polymerase Chain Reaction

Cellular and viral genes were amplified by polymerase chain reaction (PCR) from both plasmids and cDNA libraries. PlatinumTaq DNA polymerase (Invitrogen) was used according to the manufacturer’s instructions. Reaction mixtures of 50 μl total volume contained; 100-500 ng of template DNA, 1 unit (U) of PlatinumTaq polymerase, 1X PCR buffer (Invitrogen), 10 mM dNTP mixture, 0.2 μM of each primer and 1.5 mM MgCl₂. An Eppendorf Mastercycler thermal cycler was used for the amplification at the following settings; 95°C for 10 min, 30 cycles of: 95°C for 30 s, 55°C for 30 s and 72°C for 1 min per kilobase, followed by 72°C for 10 min. PCR products were analyzed by agarose gel electrophoresis using 1% gels which contained SYBR Safe DNA gel stain (Invitrogen). DNA was visualized with UV light.
Purification of PCR Products

Reaction samples were resolved on a 1% agarose gel containing SYBR Safe DNA gel stain and bands were visualized with UV irradiation. PCR products were excised from the gel and DNA was purified using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer’s recommendations.

Restriction Enzyme Digestion of DNA

DNA was digested according to the restriction enzyme manufacturer’s instruction (New England BioLabs, NEB) using 1 U of enzyme per 1 μg DNA. DNA was digested at 37°C for 1 h. The digested DNA was resolved on a 1% agarose gel containing SYBR Safe DNA gel stain, visualized with UV irradiation and purified with the QIAquick Gel Extraction Kit (Qiagen), according to the manufacturer’s recommendations. Where digestion with two enzymes in different buffers was required, the DNA was digested with one restriction enzyme first, purified using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer’s recommendations, then digested with the second restriction enzyme, and resolved on a 1% agarose gel containing SYBR Safe DNA gel stain, visualized with UV irradiation. The PCR product was excised from the gel and DNA was purified using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer’s protocol.

DNA Ligation

Approximately 200 ng of inset DNA was incubated with 500 ng of linerized plasmid DNA in 50 μl ligation mix containing 1 U of T4 DNA ligase (NEB) and 1X ligation buffer (NEB). Ligations were carried out at 4°C overnight. The enzyme was heat inactivated according to the manufacturer’s instructions for 15 min at 65°C.
Transformation of *E. coli*

Competent cells were thawed quickly and 20 μl of cell suspension was added to 100-500 ng plasmid or 100-500 ng ligated DNA mix. After 30 min on ice the cells were heat shocked for 90 s at 42°C. 100 μl of LB broth was added and the cells were incubated at 37°C for 1 h with shaking at 200 rpm. Transformed cells were plated on 2YT agar plates containing the appropriate antibiotic (ampicillin or kanamycin) and incubated overnight at 37°C. The following day 5-10 colonies were selected and screened by PCR, using insert specific primers, to determine if the plasmid contained the correct insert. Once a positive was identified the colony was grown in 5 ml of LB broth containing the appropriate antibiotic overnight at 37°C with shaking at 200 rpm. The next day 750 μl of the bacterial culture was added to 250 μl of glycerol and stored at -80°C as a stock sample.

**Plasmid Production and Purification**

Qiagen MiniPrep and MaxiPrep kits were used according to the manufacturer's recommendations to produce concentrated plasmid preparations. *E. coli* transformed with the plasmid of interest were streaked on 2YT agar plates containing the appropriate antibiotics (ampicillin and kanamycin) and a single colony was picked. The *E. coli* from this colony were either grown in 10 ml LB broth (MiniPrep) or 250 ml LB broth (MaxiPrep) overnight at 37°C with shaking at 200 rpm. *E. coli* were then centrifuged, lysed and plasmid DNA was purified according to the manufacture’s suggestions resulting in 30 μl of concentrated DNA preparation of 200-400 ng/μl (MiniPrep) and 500 μl of concentrated DNA preparation of 500-1000 ng/μl (MaxiPrep). DNA concentrations were determined using an Ultraspec 3100 pro spectrophotometer from BioChrom.
**DNA Sequencing**

DNA templates, purified as described above and sequencing primers that were complementary to the plasmid backbones outside of the insert regions (custom made by Invitrogen) were supplied to the Interdisciplinary Center of Biotechnology Research (ICBR, http://langsat.biotch.ufl.edu) at the University of Florida, as per ICBR instructions.

**Plasmids**

Vectors encoding MYXV genes M-T5, M148, M149 and M150 were PCR amplified from viral genomic DNA and subcloned into pDONR221 (Invitrogen). Using Gateway recombination (Invitrogen) the viral ORFs were transferred to the expression plasmid pANT7_nHA, which was kindly provided by Harvard Institute of Proteomics (HIP). The plasmids encoding human CUL1, Skp1 and Akt all fused to C-terminal GST (pANT7_cGST) were also received from HIP, CUL1 tagged with hemagglutinin (HA) (HA-CUL1) or Flag (Flag-CUL1) epitopes were gifts provided by Y. Xiong (University of North Carolina, Chapel Hill) and Z. Q. Pan (Mount Sinai School of Medicine, New York), respectively. Furthermore, the plasmid MT5-GST was constructed using the vector pANT7_cGST.

M-T5 ORF fragment constructs were amplified from viral genomic DNA by PCR. N-terminal M-T5 deletions were generated using the forward primers listed in Table 2-1 and the reverse primer MT5rev (5’-AAACTCGAGCGGTATCTTTAC-3’) (XhoI underlined). Alternatively, internal M-T5 deletions were generated by amplifying the right and left flanks of the deletion region using the primer sets listed in Table 2-1. Afterwards, a second round of PCR was performed using the forward primer MT5for (5’-AAAGGATCCATGGATCTACGGG-3’) (BamHI underlined) and the reverse primer
Table 2-1. List of primers used to PCR amplify M-T5 and Akt constructs

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
</tr>
</thead>
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<td>MT5for</td>
<td>AAAGGATCCATGGATCTATACGGG</td>
</tr>
<tr>
<td>MT5rev</td>
<td>AAACTCGAGCCGCTGTATCTTTAC</td>
</tr>
<tr>
<td>ΔNfor</td>
<td>AAAGGATCCATGGGATACCCCTTTCGC</td>
</tr>
<tr>
<td>ΔN-Ifor</td>
<td>AAAGGATCCATGGTTCGTGGTTCTCGTACG</td>
</tr>
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<td>ΔN-Illfor</td>
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<td>ΔN-Vfor</td>
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<td>ΔN-VIIfor</td>
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<tr>
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<tr>
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</tr>
<tr>
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<tr>
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</tbody>
</table>

MT5rev. PCR products were digested with BamHI and Xho1, gel purified (Qiagen) and cloned directly into the vector pcDNA3.1(+)myc-His A (Invitrogen). The vector contains a human cytomegalovirus immediate-early promoter for high-level expression in a wide range of mammalian cells and a T7 promoter/priming site, which allows for in vitro transcription.

The plasmid 1036 pcDNA3 Myr HA Akt1 (HA-Akt), which has a N-terminal myristoylation sequence followed by the HA epitope was acquired from Addgene and
previously documented (209). Substitution of lysine 179 for alanine destroys the ATP-binding site, resulting in a kinase-deficient mutant of Akt. Another plasmid, 1014 pcDNA3 T7 Akt1 K179M T308A S473A (209) was also purchased from Addgene, and was subsequently subcloned into the vector pcDNA3/myr-HA (HA-AktΔ3). Alternatively, HA-AktT308A, HA-AktK179M and HA-AktΔ2 were generated by amplifying the right and left flanks at the point of mutation using the primer sets listed in Table 2-1. Afterwards, a second round of PCR was performed using the forward primer Akt.for (5’-GGATCCCATGAGCGACGTGGCTATTGT GAAGGA -3’) (BamHI underlined) and the reverse primer Akt.rev (5’-CTCGAGGGCCGCTGCTGGCCGAGT A-3’) or Akt.S473A.rev (5’-CTCGAGGGCCGCTGCTGGCCGAGTAGGCGAACTGGGGGAAGT-3’) (EcoR1 underlined). Whereas, HA-AktS473A was cloned using the primer set Akt.for and Akt.S473A.rev. PCR products were digested with BamHI and EcoR1, gel purified (Qiagen) and cloned directly into the vector pcDNA3/myr-HA.

Myc-PIKE-A was a generous gift from K. Ye (Emory University School of Medicine, Atlanta) and has previously been described (2, 3). Lastly, pcDNA3 (Invitrogen) was used as a control vector for experiments in which Myc-PIKE-A was used.

The identity of all clones was confirmed by sequence analysis and expression of fusion proteins was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.

**Cell Culture**

**Reagents**

- **FBS (fetal bovine serum):** heat inactivated at 56°C for 30 min.
- **DMEM (Dulbecco's modified Eagle medium):** (Gibco), 2g/L NaHCO₃, 4.77 g/l HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]), 10% FBS, 1.5 μl β-mercaptoethanol, 50 μg/ml gentamycin, 100 units penicillin/ml and 100 μg/ml streptomycin.

- **PBS (phosphate buffered saline):** 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄-7H₂O, 1.4 mM KH₂PO₄

- **Neutral buffered formalin:** 3.7% formaldehyde in PBS

- **X-Gal stain:** 100 μg/ml X-Gal [5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside], 500 mM potassium ferricyanide, 500 mM potassium ferrocyanide, 100 nM MgCl₂ in PBS

**Cell Culture**

Established cell lines used include; baby green monkey kidney (BGMK), human embryonic kidney (HEK)293, and the human tumor cell lines; HeLa, HOS, Caki, 786-0, SK-MEL-5, MD-MBA435 and MCF-7. All cells were propagated in DMEM at 37°C in 5% CO₂. MYXV permissivity, endogenous phospho-Akt level and cell origin for each of the human tumor cells is listed in Table 2-2. During cell splitting and seeding, cells were treated with 1% trypsin for 5 min.

**Table 2-2. Human cancer cell lines used in this study**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cell origin</th>
<th>Endogenous p-Akt status*</th>
<th>Permissive Type</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOS</td>
<td>Osteosarcoma</td>
<td>High</td>
<td>YES vMyx</td>
<td>YES</td>
</tr>
<tr>
<td>Caki</td>
<td>Renal</td>
<td>High</td>
<td>YES vMyxT5KO</td>
<td>YES</td>
</tr>
<tr>
<td>786-0</td>
<td>Renal</td>
<td>Low</td>
<td>YES NO</td>
<td>NO</td>
</tr>
<tr>
<td>SK-MEL-5</td>
<td>Melanoma</td>
<td>None</td>
<td>NO NO</td>
<td>NO</td>
</tr>
<tr>
<td>MD-MBA435</td>
<td>Breast</td>
<td>None</td>
<td>NO NO</td>
<td>NO</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Breast</td>
<td>None</td>
<td>NO NO</td>
<td>NO</td>
</tr>
</tbody>
</table>

*endogenous phospho-Akt status was detected by immunoblotting using Akt phospho-specific antibodies (pAkt-Ser473 and pAkt-Thr308). Table adapted from (292)

**Viruses and Infection**

The recombinant MYXVs (strain Lausanne) used in this study have been described previous and include; vMyx (200), vMyx-gfp (127), vMyxT5KO (186) and
vMyxT5KO-gfp (295). All recombinant viruses contain a LacZ marker, which has been inserted between M11L and M12L, whereas vMyx-gfp and vMyxT5KO-gfp also have an eGFP cassette, located between M135R and M136R. Furthermore, both vMyxT5KO and vMyxT5KO-gfp fail to express the M-T5 gene product due to targeted disruption of both copies of the M-T5 ORF (M005R/L). It should also be noted that both LacZ and the eGFP cassette are both under the control of the poxviral synthetic early/late poxviral promoter. All viruses were propagated and titrated by focus formation on BGMK cells as described previously (200).

For infection studies, cells were incubated with the indicated multiplicity of infection (MOI) of either virus for 1 h at 37°C, infected cells were then washed three times with serum-free medium to remove excess virus and cultured in normal medium until used in subsequent experiments. Viral replication was analyzed by single-step growth curve analysis as outlined previously (267). Briefly, cells (5 X 10^5) were either mock infected or infected with MYXV at the indicated MOI for 1 h. Unabsorbed virus was removed by washing the cells with serum-free medium three times, and cells were grown in DMEM. Cells were harvested following infection at the indicated time points and virus titers were determined by serial dilution and infection of BGMK. For β-galactosidase expression studies, cells infected with vMyx or vMyxT5KO were washed with PBS at various time points postinfection, fixed for 5 min in neutral buffered formalin and stained with X-Gal for 4 to 8 h at room temperature (RT). Blue foci, indicating virus replication and spread, were counted, and virus production was determined. Whereas, viral replication of vMyx-gfp or vMyxT5KO-gfp was determined by the formation of fluorescent green-expressing foci as viewed by fluorescence microscopy. Titration of each time point was done in
triplicate and data was expressed as FFU per $10^6$ cells. Results were graphed using Microsoft Excel, with corresponding error bars.

**Transfection of Mammalian Cells**

Cells were seeded in six-well plates at a density of $5 \times 10^5$ cells per well in DMEM prior to transient transfection. Transfections were performed with LipofectAMINE 2000 (Invitrogen) or Effectene (Qiagen) in accordance with the manufacturer’s instructions. The cells were harvested at various time points following transfection and cell lysates were stored at -80°C until used for subsequent studies.

**siRNA Transfections**

Cellular monolayers were transfected with the indicated siRNA as per manufacturer’s protocol (Dharmacon) and two days later cells were infected with either vMyx-gfp or vMyxT5KO-gfp at an MOI of 0.1. Innoculum was allowed to adsorb for 1 h before being removed. Cells were washed, supplemented with DMEM and incubated at 37°C. Focus formation was examined at 48 hours post infection (hpi), under a fluorescence microscope to determine viral replication.

**Drug Inhibition Studies**

For each inhibitor, confluent cultures of cells in six-well plates were pre-incubated for 4 h at 37°C with the working concentration of the drug. The inhibitor solution was removed, and the cells were washed and infected with MYXV at an MOI of 3. After 1 h, excess virus was removed, and cells were cultured for 48 h in media containing the specific inhibitor. Cultures infected in the absence of inhibitor served as controls. The number of GFP-expressing cells or foci present in wells infected with vMyx-gfp or vMyxT5KO-gfp was assayed by fluorescent microscopy and efficiency of infection was determined by comparing the GFP levels present in untreated, infected controls vs.
treated, infected cells. All inhibitors were purchased from Calbiochem and initially reconstituted according to the manufacture’s directions in either dimethyl sulfoxide (DMSO) or water. The working concentrations of each drug were prepared by dilution in DMEM and used as follows unless stated otherwise: Akt inhibitor I, 5 μM; Akt inhibitor V, Triciribine (API-2), 10 μM; Akt inhibitor VIII, isozyme-selective, Akti-1/2, 2 μM; Akt inhibitor X, 3 μM; rapamycin, 20 nM; endothall, 90 nM; Fenvalerate, 4 nM; DARPP-32, phospho-, rat, recombinant, E. coli, 1 μM; α-naphthyl acid phosphate, monosodium salt, 1 mM; okadaic acid, Prorocentrum sp. 0.1 nM. FTY720 was purchased from Clayman Chemicals and the cytotoxic effect of the drug on HOS, 786-0 and SK-MEL-5 cell lines was determined by the CellTiter 96 Non-Radioactive Cell Proliferation Assay (MTT) from Promega. Three independent experiments were performed to determine an IC<sub>10</sub> dose of 6 μM, which was used as the working concentration.

Protein Methods

Reagents

- **TBS (Tris buffered saline):** 150 mM NaCl, 10 mM Tris pH 8.0
- **PBS:** 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O, 1.4 mM KH<sub>2</sub>PO<sub>4</sub>
- **GST pull-down wash buffer:** 50 mM Tris pH 7.5, 1M NaCl, 1% NP-40
- **Protease inhibitor cocktail solution:** 1X Complete Mini, EDTA-free (Roche) (phenylmethanesulphonyl fluoride (PMSF), Pefabloc SC, Pefabloc SC Plus, Aprotinin, Leupeptin, α<sub>2</sub>-Macroglobulin, E-64) in water
- **Cell lysis buffer:** 20mM Tris pH 7.5, 137mM NaCl, 15% glycerol, 2 mM NaF, 1% NP-40, 10mM Na pyrophosphate, 1mM NaV<sub>3</sub>O<sub>4</sub>, 2mM PMSF, 25mM β-glycerolphosphate, protease inhibitor cocktail solution
- **5X SDS-loading dye:** 0.5 M Tris-HCl pH 6.8, 10% SDS, 30% glycerol, 5% β-mercaptoethanol, 0.02% bromophenol blue
- **SDS-PAGE electrophoresis running buffer:** 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3
Table 2-3. SDS-PAGE gels

<table>
<thead>
<tr>
<th>Component</th>
<th>3% Stacking gel (Upper)</th>
<th>10% Resolving gel (lower)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>6.8 ml</td>
<td>4 ml</td>
</tr>
<tr>
<td>30% Acrylamide mix&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.7 ml</td>
<td>3.3 ml</td>
</tr>
<tr>
<td>1.0 M Tris</td>
<td>1.25 ml (pH 6.8)</td>
<td>2.5 ml (pH 8.8)</td>
</tr>
<tr>
<td>10% SDS</td>
<td>100 μl</td>
<td>100 μl</td>
</tr>
<tr>
<td>10% ammonium persulphate</td>
<td>100 μl</td>
<td>100 μl</td>
</tr>
<tr>
<td>TEMED&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10 μl</td>
<td>4 μl</td>
</tr>
</tbody>
</table>

<sup>a</sup> 29.2% acrylamide and 0.8% N,N’-methylene-bis-acrylamide
<sup>b</sup> N,N,N’,N-Tetramethylethylenediamine

- **Transfer buffer**: 192 mM glycine, 25 mM Tris pH 8.3, 20% methanol
- **Western blot blocking solution**: 5% non-fat milk in PBS
- **Western blot wash buffer**: 0.1% Tween-20 in PBS

Table 2-4. Antibodies used in Western blotting

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit-anti-Akt</td>
<td>Cell Signaling</td>
<td>1/1000&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Rabbit-anti-pAkt(Ser473)</td>
<td>Cell Signaling</td>
<td>1/1000&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Rabbit-anti-pAkt(Thr308)</td>
<td>Cell Signaling</td>
<td>1/1000&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Rabbit-anti-Skp1</td>
<td>Cell Signaling</td>
<td>1/1000&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Rabbit-anti-Cleaved Caspase-3</td>
<td>Cell Signaling</td>
<td>1/1000&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mouse-anti-Serp1</td>
<td>Viron Biotherapeutics</td>
<td>1/1000&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mouse-anti-HA (12CA5)</td>
<td>Roche</td>
<td>1/3000&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mouse-anti-Myc (9E10)</td>
<td>Santa Cruz Biotechnology</td>
<td>1/3000&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Rabbit-anti-GST</td>
<td>NeoMarkers</td>
<td>1/500&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Goat-anti-rabbit-HRP</td>
<td>Jackson ImmunoResearch</td>
<td>1/5000&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Goat-anti-mouse-HRP</td>
<td>Jackson ImmunoResearch</td>
<td>1/5000&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> diluted in PBS, 5% non-fat milk and incubated for 16 h at 4°C
<sup>b</sup> diluted in PBS, 5% non-fat milk and incubated 1 h at room temperature

**Cell Lysis**

Cultured cells were harvested and lysed in cell lysis buffer, sonicated to solubilize proteins and cleared by centrifugation at 15,000 x g for 30 min at 4°C. Thereafter, protein levels were quantified using the Quick Start Bradford Dye Reagent 1X from BioRad and samples were used in subsequent experiments.
**Immunoprecipitation Assay**

Samples were incubated with protein A/G beads (Pierce) at 4°C for 1 h in a preclear phase. After centrifugation, supernatants were removed and incubated with the specified antibody at 4°C overnight with shaking. The next day protein A/G beads (Pierce) were added and further incubated at 4°C for 2 h. The beads were washed three times with cell lysis buffer, resuspended in SDS-loading buffer and boiled for 5 min to elute the proteins. The eluted supernatants were resolved by SDS-PAGE and analyzed by Western blotting.

**GST Pull-Down Assay**

For GST fusion proteins, plasmids were expressed by *in vitro* transcription/translation (TNT) according to manufacture’s protocol (Promega) and were incubated with GST-coated agarose beads for 2 h. Beads were pelleted by centrifugation and washed 5 times with GST pull-down wash buffer. Thereafter, samples were resuspended in SDS-loading dye and boiled for 5 min to elute the proteins. The eluted supernatants were resolved by SDS-PAGE and analyzed by Western blotting.

**SDS-PAGE**

Immediately before loading onto the SDS-PAGE gel, the samples were sedimented in a bench top centrifuge at 12,000 rpm. Samples were then loaded onto a 10% SDS-PAGE gel and proteins were separated for 1.5 h at 150 volts using a Mini Protean 3 Cell system (BioRad). The marker SeeBlue Plus2 Pre-stained standard (Invitrogen) was used alongside the samples.
**Immunoblot Analysis**

After gel electrophoresis, separated proteins were transferred to nitrocellulose membrane (Hybond-C Extra, Amersham Bioscience) using the XCell II Blot Module (Inviviteogen) for 1.5 h at 25 volts. The membranes were blocked with Western blot blocking solution for 1 h at RT. Primary antibodies were diluted in 5% skim milk–PBS at the concentrations listed above and incubated with membranes overnight at 4°C. Membranes were then washed three times with Western blot wash buffer and incubated for 1 h at RT with horseradish peroxidase-conjugated secondary antibodies diluted 1:5000 in 5% milk-PBS. Once again the membranes were washed three times with Western blot wash buffer and incubated with Western Lightening chemiluminescence (PerkinElmer) for 2 min to detect immunoreactive proteins. Loading of equal amounts of protein from each sample was confirmed by detection of the gene coding for the housekeeping protein, actin. Densitometric levels of Akt were detected by Molecular Imaging software (Kodak).

**Yeast Two-Hybrid Screening**

Yeast two-hybrid (Y2H) screening was carried out at Myriad Genetics using the M-T5 protein as bait with a mating based method. The details of the human cDNA libraries and the Y2H screening methods have been reported elsewhere (180).

**AlphaScreen Binding Protocol**

Cells were seeded in six-well plates at a density of 5 x 10^5 cells per well in DMEM. Transfections were performed with Effectene (Qiagen) in accordance with the manufacturer’s instructions. Cultured cells were collected and cell lysis was prepared as previously described. All AlphaScreen assays described were performed in triplicates in 384-well white opaque plates (PerkinElmer) using PBS containing 0.1% FBS as buffer.
For the detection of HA fusion proteins, the HA detection kit containing anti-HA–coated Acceptor beads (PerkinElmer) was used. Equal concentrations of Acceptor beads and streptavidin Donor beads were used at a final concentration of 20 μg/ml in a final volume of 25 μl per well. First, 5 μl of cell lysate, followed by biotinylated-anti-HIS (10 nM) or biotinylated-anti-Flag antibody (25 nM) and Acceptor beads in buffer were added to each well and incubated for 2 h at room temperature. A total of 5 μl of a 1:50 dilution of the Donor beads was then added to give a final volume of 25 μl, and the mixture was incubated at room temperature for 2 h. All additions and incubations were made in subdued lighting conditions due to photosensitivity of the beads and finally, the assay plates were read in an EnVision plate reader (PerkinElmer). Likewise, AlphaScreen SureFire phospho-Akt (Thr308) and phospho-Akt (Ser473) assay kits were used according to the manufacture’s recommendations to detect Akt phosphorylation.

**Nuclear Extraction**

Nuclear and cytoplasmic extractions were performed according to manufacturer’s recommendations (Thermo Scientific). Briefly, ice-cold CER I (Thermo Scientific) was used to resuspend the cell pellet, which was then placed on ice for 10 min. Afterwards, ice-cold CER II (Thermo Scientific) was added to the sample, vortexed and incubated on ice for 1 min. The sample was centrifuged for 5 min at 16,000 x g and the supernatant (cytoplasmic extract) was removed and stored at -80°C for later use. The insoluble (pellet) fraction was resuspended in ice-cold NER (Thermo Scientific) and placed on ice. Every 10 min the sample was vortexed for 15 s, for a total of 40 min. Lastly, the sample was centrifuged at 16,000 x g for 10 min and the supernatant (nuclear faction) was removed and stored at -80°C until needed.
Microscopy - Cellular Localization

Reagents

- **PBS**: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na$_2$HPO$_4$·7H$_2$O, 1.4 mM KH$_2$PO$_4$
- **Fixative**: 4% paraformaldehyde in PBS, 3 min at RT
- **Permeabilization solution**: 0.3% Triton X 100 in PBS, 2 min at RT
- **Blocking solution**: 1% FBS in PBS, 1 h at RT
- **Wash buffer**: 0.1% Tween-20 in PBS

Table 2-5. Antibodies used in immunostaining

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit-anti-Akt</td>
<td>Cell Signaling</td>
<td>1/100&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mouse-anti-HA (12CA5)</td>
<td>Roche</td>
<td>1/100&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Goat-anti-mouse- TexasRed</td>
<td>Invitrogen</td>
<td>1/1000&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Goat-anti-rabbit-FITC</td>
<td>Invitrogen</td>
<td>1/1000&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>diluted in PBS, 1% FBS and incubated for 1 h at RT

Fixation, Permeabilization and Blocking

HeLa cells were grown on glass cover slips to 70% confluence and were transiently transfected with HA-CUL1 using Effectene (Qiagen) according to the manufacturer’s recommendations. Two days later, cells were mock infected or infected with vMyx or vMyxT5KO at an MOI of 5. At 4 hpi, cells were fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X 100 and blocked with 1% FBS for 1 h. Cells were washed three times using PBS with gentle agitation between each preparation step.

Immunostaining

After cells were fixed and prepared as described above, cover slips were blocked with blocking solution and then incubated with the appropriate antibody at the concentrations listed above in a volume of 100 μl. Antibody solutions were placed on parafilm and the cover slip was inverted so that cells were exposed to the antibody. HA-CUL1 expression was detected by indirect immunofluorescence using monoclonal
antibody specific for the HA epitope and a TexasRed-conjugated goat-anti-mouse secondary antibody. Detection of endogenous Akt was determined by polyclonal Akt antibody and the fluorescein isothiocyanate-conjugated (FITC) goat-anti-rabbit secondary antibody. Prior to addition of the secondary antibody and before cover slips were mounted, cells were washed three times with wash buffer. Furthermore, incubations using the secondary antibodies were performed in the dark. Cover slips were washed three times with wash buffer and then mounted onto microscope slides using Vectashield (Vector Laboratories) containing the fluorescent marker DAPI (4,6-diamino-2-phenylindole) to assay nuclear localization. Protein localization was examined using the 40X objective of an Olympus DSU-IX81 Spinning Disc Confocal/Deconvolution fluorescent microscope.
CHAPTER 3
M-T5 IS A NOVEL ADAPTOR THAT COORDINATELY LINKS THE CELLULAR SIGNALING PATHWAYS MEDIATED BY AKT AND SKP1 IN VIRUS-INFECTED CELLS

Introduction

MYXV has proven to be a useful model system for studying the mechanism by which virus-encoded immunoregulatory proteins function to manipulate the various host immune responses during the course of viral infection (254). The success of MYXV as a pathogen can be attributed to the ability of the virus to effectively avoid recognition and clearance by the immune system of susceptible rabbit hosts. At the level of individual virus-infected cells, poxviruses like MYXV are particularly adept at binding and entering most mammalian cells where they attempt to establish a favorable intracellular environment. Thus, the ability of poxviruses to re-configure or disable the various host antiviral responses of the infected cell directly dictates the outcome of a viral infection at the cellular level (171). To this end, poxviruses possess a large genomic capacity, and all encode a unique repertoire of immunoregulatory and host-interactive proteins that have evolved to specifically mediate a broad range of cellular process critical for successful viral replication. To date, a large collection of poxvirus-encoded immunoregulatory proteins have been identified and characterized, including a novel category of poxvirus ANK-repeat proteins that have recently drawn considerable attention for their potential roles in modulating intracellular signaling networks during viral infection (247, 249, 282).

With the exception of poxviruses, the ANK motif is not commonly reported in viruses, although numerous examples have been identified in eukaryotic, bacterial and archaeal proteins (37). Almost all chordopoxviruses encode multiple ANK-repeat
containing proteins, the majority of which include a conserved C-terminal PRANC (pox proteins repeats of ankyrin C-terminal) motif that closely resembles a cellular protein motif called the F-box domain (176). MYXV encodes four unique ANK-repeat proteins, termed M-T5, M148, M149 and M150, all of which have all been described as virulence factors for myxomatosis in European rabbits (34, 47, 186). The MYXV host-range factor M-T5 was first characterized for its ability to regulate viral tropism within rabbit lymphocytes and later some classes of human cancer cell lines (186, 267). In human cancer cells, the direct physical interaction between M-T5 and the host cell Akt was shown to upregulate the kinase activity of Akt and enhance MYXV replication in human cancer cells (292). M-T5 was also demonstrated to protect MYXV-infected cells from virus-induced cell cycle arrest, a property which was linked to its ability to interact with CUL1, a member of the host cell SCF complex (127). Unlike M-T5, no specific host binding partners or target substrates have yet been identified for M148, M149 or M150. However, in TNF-α stimulated cells, M150 was shown to co-localize in the nucleus with NF-κB p65, suggesting this MYXV protein may modulate the NF-κB pathway (47).

In this study, we demonstrate that M-T5, M148, M149 and M150 all have functional C-terminal PRANC/F-box like domains and each one can interact directly with the Skp1 component of the host SCF complex. We further examined the various binding domains of M-T5 and identified ANK-repeats I and II as being important for interaction with Akt, whereas the PRANC/F-box like domain was essential for binding to Skp1. We also show that the previously reported interaction of M-T5 to CUL1 was in fact, indirect linking of M-T5 to the host SCF complex via Skp1. More specifically, we investigated the ability of M-T5 to function as a molecular scaffold to link disparate cellular binding
partners together within a single complex and report that the viral protein binds Akt and
the SCF complex (via Skp1) simultaneously in MYXV-infected cells. Finally, we
demonstrate that M-T5 specifically mediates the relocalization of Akt from the nucleus to
the cytoplasm during MYXV infection. These results suggest that ANK/PRANC proteins,
such as M-T5, play a critical role in reprogramming disparate cellular signaling
cascades to establish a new cellular environment more favorable for viral replication.

Results

MYXV ANK-Repeat Proteins All Contain a Functional Carboxy-Terminal PRANC/F-
Box Like Domain and Bind Skp1

With the exception of MCV, all sequenced members from each of the poxvirus
genera encode multiple ANK-repeat containing proteins, the majority of which include a
PRANC/F-box like domain located at the C-terminus (176). MYXV, a member of the
leporipoxvirus genus, encodes four ANK/PRANC containing proteins; M-T5, M148,
M149 and M150 (44, 296). Similar to other poxviral ANK/PRANC proteins, the four
MYXV proteins range between 400 to 700 amino acids in length, possess 5 to 10 copies
of the ANK motif clustered towards the N-terminus of each protein and include a single
C-terminal PRANC/F-box like domain (Fig. 3-1A). However, amino acid sequence
alignment between M-T5, M148, M149 and M150 demonstrate the proteins otherwise
exhibit little sequence similarity to one another (33.4 - 43.4% similarity; 18.8 - 23.3% 
identity, Table 3-1). M148 was the least similar ORF amongst the four MYXV ORFs and
appears to contain an additional 179 resides at the N-terminus, accounting for an extra
2 to 4 ANK-repeats.

Notably, the PRANC/F-box like domains for each of the MYXV proteins share a
high degree of sequence similarity to one another (Fig. 3-1B). The PRANC motif is
| Identity/similarity matrix of myxoma virus ANK-repeat containing proteins | Similarity scores (%) |
|---|---|---|---|---|
| | M-T5 | M148 | M149 | M150 |
| M-T5 | ----- | 33.6 | 43.3 | 44.2 |
| M148 | 18.8 | ----- | 38.0 | 33.4 |
| M149 | 23.2 | 20.1 | ----- | 36.3 |
| M150 | 23.3 | 19.1 | 20.8 | ----- |

comparably shorter than the established F-box consensus sequence common to eukaryotes but still contains a few invariant positions; these include positions 1 (leucine), 2 (proline), 9 (isoleucine or valine), and 13 (leucine or valine) (176). Based on co-crystal structures, the cellular F-box is usually composed of three α-helices, however the poxviral version of the motif is frequently truncated and lacks the third helix (246). Many of the invariant residues within helices H1 and H2 are well conserved in the MYXV PRANC/F-box like domains, but little sequence similarity was maintained within α-helix H3 (Fig. 3-1B). Despite the lack of H3, the 2-α-helix PRANC/F-box like domain found in poxviral proteins can still be capable of interacting faithfully with the cellular SCF complex (246).

The presence of a C-terminal PRANC/F-box like domain suggests the four ANK-repeat proteins encoded by MYXV likely interact with the SCF complex by means of binding to an adaptor protein such as Skp1. To determine if these viral proteins have a functional PRANC/F-box like domain, we tested their ability to bind host Skp1 protein using a GST pull-down assay. A TNT protocol was used to co-express Skp1-GST, tagged at the C-terminus with GST, plus one of the MYXV ANK/PRANC proteins fused to a common N-terminal HA tag (HA-MT5, HA-M148, HA-M149 and HA-M150). Samples were then incubated with GST-coated beads to pull-down Skp1-GST fusions, and complexes were resolved by SDS-PAGE and immunoblotted with anti-HA antibody
Figure 3-1. MYXV ANK-repeat proteins include a functional C-terminal PRANC/F-box like domain that binds Skp1. (A) Schematic representation of the four ANK-repeat containing proteins encoded by MYXV. Predicted ANK-repeats and putative C-terminal PRANC/F-box domains are represented by the white and gray boxes respectively (151, 226). The first amino acid residue of each predicted ANK-repeat and the amino acid length for the corresponding protein are indicated. (B) Alignment of the amino acid sequences of the C-terminal region of each MYXV ANK-repeat protein. A consensus PRANC/F-box like sequence adapted from Mercer et al. (176) is shown with lower case letters representing variant positions and X indicates nonconserved positions. Residues identical with the poxviral consensus motif are dark shaded, while conserved substitutions are denoted with light shading. Non-conserved differences contain no shading. The F-box α-helical secondary structures are represented by H1, H2, and H3 (202, 303). Known residues of Skp2 that contact the linker protein Skp1 are underlined (225). (C) TNT was used to express the indicated (+) combination of plasmids which include; Skp1-GST and HA-tagged MYXV ANK-repeat proteins M-T5, M148, M149 and M150. Samples were subjected to a GST pull-down assay. Precipitates and total lysates were resolved by SDS-PAGE and probed with anti-HA antibody to detect co-precipitated viral proteins (lower panels). Expression of MYXV ANK-repeat proteins (upper panels) and Skp1 (middle panels) was confirmed by immunoblotting with antibody against HA (anti-HA) and GST (anti-GST) epitopes, respectively. Bands of interest are represented by asterisks.
to analyze binding of HA-tagged MYXV proteins. As predicted, all four of the MYXV encoded ANK/PRANC proteins were detectable in the GST pull-down only when co-expressed in the presence of Skp1-GST (Fig. 3-1C, lanes 1, 5, 9 and 13). However, no MYXV ANK/PRANC proteins were detected following GST pull-down in the absence of Skp1-GST expression (Fig. 3-1C, lanes 2, 6, 10 and 14). It should be noted the binding of HA-M148 to Skp1-GST is difficult to appreciate, however when this interaction was further examined by the AlphaScreen assay, the signal produced by the interaction between M148 and Skp1 was comparable to M-T5, M149 and M150 (data not shown). Our findings suggest that M-T5, M-148, M149 and M-150 each contain a functional PRANC/F-box like domain, which can interact with the host Skp1 as previously reported with other poxviral ANK-repeat proteins (247, 249, 282).

**Yeast Two-Hybrid Screen for Potential Cellular Binding Partners of M-T5**

Although PRANC proteins are predicted to manipulate the cellular ubiquitination machinery, surprisingly little is known about the molecular mechanism by which this largest family of poxvirus proteins function during viral infection. Aside from Skp1, identification of additional binding partners, either cellular or viral, has resulted in little success and coincidentally has hindered the study of this superfamily of proteins. The Y2H system has become an increasingly important tool to identify and map novel protein-protein interactions. For example, systematic Y2H analysis of the unique VARV genes with human cDNA libraries revealed the presence of a novel viral inhibitor of NFκB1/p105 (180). A similar Y2H screen was performed using various human cDNA libraries to identify potential human cellular binding partners of M-T5, the best characterized of the four MXYV ANK-repeat proteins. Multiple independent human cDNA libraries were analyzed for interactions and the results from this screen identified
a total of 13 potential human binding partners for M-T5 (Table 3-2). Notably, neither of
the two previously identified binding partners for M-T5, namely CUL1 or Akt, were
identified in this Y2H screen but the Skp1 component of the host SCF complex (that
includes CUL1) was picked up in this screen.

Table 3-2. Potential cellular binding partners of myxoma virus encoded M-T5 identified
by a yeast two-hybrid screen of human cDNA libraries

<table>
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<th>Symbol</th>
<th>Name</th>
<th>NCBI RefSeq</th>
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</tr>
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<td>Chromobox homolog 3, isoform 1</td>
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<td>Epsin</td>
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</tr>
<tr>
<td>KIN13A(1749)</td>
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<td>NM_022113</td>
</tr>
<tr>
<td>NEB</td>
<td>Nebulin</td>
<td>NM_004543</td>
</tr>
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<td>6-phosphogluconate dehydrogenase</td>
<td>NM_002631</td>
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<td>NM_002654</td>
</tr>
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<td>DNA repair and recombination protein Rad50</td>
<td>NM_005732</td>
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<td>NM_152281</td>
</tr>
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<td>NM_006930</td>
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**Creation of M-T5 Deletion Constructs for Protein Partner Binding Studies**

The intricate relationship between viral encoded proteins and components of the
host cell signaling networks can have profound impact on poxvirus tropism. To
investigate the significance of these viral-host protein interactions in controlling virus
tropism, a collection of myc-His (C-terminus) tagged M-T5 fragment constructs were
created and used to map the binding domain(s) critical for interaction with the cellular
proteins (Fig. 3-2A). Briefly, ANK-repeats of M-T5 were systematically deleted from the
N-termini of the M-T5 ORF and fused to a C-terminal myc-His tag. To complement, an
additional set of plasmids were constructed which include; internal ANK-repeat
deletions, PRANC/F-box like only (MT5\N -VII) and M-T5 without the PRANC/F-box like
domain (MT5\PRANC) (Fig. 3-2A). Upon completion, each plasmid was sequence
verified and protein expression was confirmed by western blot analysis with anti-Myc antibody (Fig. 3-2B). It is interesting to note that some plasmids produced multiple

Figure 3-2. Summary of constructs employed in this study. (A) Schematic representation of the M-T5 constructs used during this study, indicated is the first residue for each of the seven predicted ANK-repeats and the C-terminal PRANC/F-box like domain. All plasmids contain a C-terminal myc-His tag. (B) Immunoblot analysis of M-T5 constructs expressed by TNT, detected by a Myc specific antibody. Bands of interest are represented by asterisks.
bands, which we predict are shorter M-T5 protein fragments that were translated from an alternative start codon by the polymerase during the TNT reaction. Furthermore, no prominent bands were observed when an empty vector was used (Fig. 3-2B, lane 16), suggesting these particular bands are not the result of non-specific binding from the anti-Myc antibody.

**M-T5 Domains Needed to Bind the Host Cell SCF Complex**

To determine whether the PRANC/F-box like domain of M-T5 was responsible for the observed binding between Skp1 and M-T5 (Fig. 3-1C), GST pull-downs were performed. Skp1 fused to GST (Skp1-GST) and one of the various M-T5 myc-His tagged constructs (MT5, MT5ΔPRANC or MT5ΔN -VII) were co-expressed by TNT and protein complexes isolated using GST-coated beads. After washing the beads, samples were resolved by SDS-PAGE and probed with an anti-Myc antibody. Co-precipitation of Skp1-GST and M-T5 was observed only when full length M-T5 (MT5/myc-His) was expressed (Fig. 3-3A, lane 1). The truncated M-T5 constructs (MT5ΔPRANC/myc-His or MT5ΔN-VII/myc-His) failed to interact with Skp1 (Fig. 3-3A, lanes 2 and 3). Another essential component of the SCF complex, CUL1, has been previously reported to form a complex with M-T5 (127). When the interaction between CUL1 and M-T5 were examined by GST pull-down the results were equivalent to Skp1 binding. In other words, CUL1-GST co-precipitated with full length M-T5 (MT5/myc-His) but not with the truncated M-T5 constructs (Fig. 3-3B).

To further verify the GST pull-down experiments by an independent protein-protein interaction method, the bead-based assay, AlphaScreen, was used to examine binding interactions. Shown schematically in Fig. 3-3A, activation of the Donor beads at 680 nm results in the generation of excited singlet oxygen radicals, which migrate to
Figure 3-3. Skp1 and CUL1 members of the SCF complex bind M-T5 via the PRANC/F-box like domain. The specified (+) combination of plasmids that include; HA-Skp1 (A) or HA-CUL1 (B) and a variety of myc-His tagged fragments of M-T5 were transiently transfected in HEK293 cells or expressed by TNT. Samples were subject to GST pull-down assay, resolved by SDS-PAGE and probed with anti-Myc antibody to detect co-precipitated proteins. Protein expression of HA-CUL1, HA-Skp1 and M-T5 fragments was detected using anti-HA and anti-Myc antibodies, respectively. (C) Principle of AlphaScreen detection for the interaction between an HA-fusion host protein (CUL1 or Akt) and the myc-His tagged M-T5 fragment constructs. Excitation of Donor bead causes the conversion of ambient oxygen to the singlet-state. If a biomolecular interaction brings the Acceptor bead within close proximity (>200 nm) to the Donor bead, a cascade of chemiluminescence occurs, resulting in the emission of a characteristic fluorescent signal between 520–620 nm. Conversely, if binding between bead bound proteins does not occur, the distance between Donor and Acceptor beads is too great for the oxygen singlet to migrate and no emission signal is produced. Biotin-conjugated anti-Myc antibody is used to indirectly link streptavidin coated Acceptor beads to myc-His tagged protein. (D) Cell lysates were harvested after 48 h and incubated with biotin conjugated anti-Myc antibody and Donor/Acceptor beads before an AlphaScreen assay was performed. Each sample was performed in triplicate and standard deviations are represented by the error bars.
react with chemiluminescers on the Acceptor bead. Subsequently, triggering a cascade of chemical reactions that induce the release of light (520-620 nm) from activated fluorophores. For this reaction to occur, the two beads must be within close proximity (<200 nm) via specific interactions of the proteins–protein complexes coupled to them (Fig. 3-3A, upper). However, if the bound test proteins do not interact, no light is emitted by the unstimulated chemiluminescers on the Acceptor bead (Fig. 3-3A, lower). To investigate the interaction between M-T5 and the SCF complex, cells were co-transfected with HA-CUL1 and one of the various M-T5 constructs (MT5, MT5∆PRANC or MT5∆N-VII). The day following transfection, cell lysates were collected and prepared for protein-protein interaction analysis. AlphaScreen Acceptor beads, coupled anti-Myc antibody. Two hours later, AlphaScreen Donor beads coated with streptavidin were added, again the sample was allowed to incubate for a couple of hours before the luminescent signal is measured by an EnVision plate reader. The presence of a distinct signal was only detectable when the PRANC/F-box like domain of M-T5 was present for binding CUL1 (Fig. 3-3D, lane 1 and 3). In contrast, the signal was dramatically reduced when the PRANC/F-box like domain of M-T5 was deleted (Fig. 3-3D, lane 2). Interestingly, interaction between the M-T5 domain containing only the PRANC/F-box domain like (MT5∆N-VII/myc-His) and CUL1-GST was observed by AlphaScreen, however when this same domain was previously examined by GST pull-down, this M-T5 PRANC-only domain was unable to bind either Skp1 or CUL1 (Fig. 3-3A and B, lane 3). Furthermore, MT5∆N-VII/myc-His dissociates more rapidly from Skp1-GST compared to MT5/myc-His when binding was examined by Biacore (data not shown). Thus, we propose that the half-life of the PRANC-only M-T5 complex with CUL1 might be shorter...
than that of the full length M-T5 protein with CUL1, and thus be detectable only by AlphaScreen and not by GST pull-down. Together, these results demonstrate that Skp1 and CUL1 are both potential cellular binding partners of M-T5 and that deletion of the C-terminal PRANC/F-box like domain from M-T5 clearly abolishes protein-protein interactions with both partners. However, since reticulocyte lysates have endogenous untagged SCF complex proteins, we wanted to determine whether the CUL1 binding to M-T5 might be indirect, via interactions to the Skp1 component of the SCF complex.

**Skp1 Serves as the Direct Adaptor Protein that Binds M-T5 to the SCF Complex**

In the SCF complexes, Skp1 generally serves as a common adaptor that directly links the various F-box proteins to CUL1. M-T5 has been shown by immunoprecipitation and pull-down assays to co-precipitate with both Skp1 and CUL1, suggesting that M-T5 might bind indirectly to CUL1 through the direct interaction with Skp1 within the SCF complex. Unfortunately, binding assays that utilize tagged proteins expressed in transfected cells *in vivo* or in reticulocyte translation assays *in vitro* are unable to discriminate between direct and indirect interactions of the cellular proteins and M-T5, because of the presence of endogenous pools of these proteins. Thus, HA-MT5 co-precipitated with CUL1-GST following GST pull-down when co-expressed by TNT (Fig. 3-4, lane 1). However, when endogenous Skp1 was cleared first by immunoprecipitation with anti-Skp1 antibody from the TNT lysate prior to the co-expression of HA-MT5 and CUL1-GST, the binding of M-T5 to CUL1 was not detected following GST pull-down (Fig. 3-4, lane 2). This result clearly indicates that CUL1 binds to M-T5 only when Skp1 (either tagged or untagged) is present.

To further confirm the role of Skp1 as the direct adaptor and its ability to coordinate the binding of M-T5 and CUL1/SCF, siRNAs was employed to downregulate
Figure 3-4. Binding of M-T5 to the SCF complex is dependent upon Skp1. (A) The plasmids HA-MT5 and CUL1-GST were expressed by TNT and subjected to GST pull-down assay. Precipitates and total lysate were immunoblotted and probed with anti-HA antibody to detect co-precipitated proteins. Prior to the addition of plasmids, Skp1 was pre-cleared by immunoprecipitation from the TNT lysate using anti-Skp1 antibody (lane 2). Expression levels of the endogenous Skp1, HA-MT5 and CUL1-GST were confirmed using the designated antibodies. Bands of interest are represented by an arrowhead.

the expression of Skp1 in HEK293 cells. Cells were co-transfected with Skp1 specific siRNA, plus plasmids that express HA-CUL1 and one of the various M-T5 constructs (MT5, MT5∆PRANC or MT5∆N-VII), cells were harvested two days later and assayed by AlphaScreen for protein-protein binding. Knockdown of Skp1 expression (Fig 3-5A insert) significantly decreased the binding interaction between the PRANC/F-box like motif of M-T5 and CUL1, as demonstrated by a reduction in AlphaScreen signal (Fig. 3-5A, lanes 2 and 4). In contrast, transfection of control siRNA did not interrupt the binding interaction between the viral and cellular proteins (Fig. 3-5A, lanes 1 and 3).

Collectively, these results demonstrate the interaction between CUL1 and M-T5 is dependent upon the adaptor protein Skp1, which functionally bridges M-T5 with CUL1 and the SCF complex. Interestingly, siRNA-mediated knockdown of either Skp1 or
Figure 3-5. Knockdown of Skp1 with siRNA disrupts binding of M-T5 to the SCF complex. (A) HEK293 cells were transfected with either control (dark bars) or Skp1 specific (light bars) siRNA and the indicated (+) combination of plasmids. Harvested after 48 h, cell lysates were assayed for binding by AlphaScreen as described in Fig. 3-3. Knockdown of Skp1 protein expression by the siRNA treatment in HEK293 cells was determined by Western blot analysis (inset). (B) The Type II human cancer cell line 786-0 (292) was mock transfected or transfected with either, control, Skp1 or CUL1 specific siRNA and 24 h later were infected with vMyx-gfp or vMyxT5KO-gfp at a MOI of 0.1. Viral foci formation was measured at 48 hpi by fluorescence microscopy.

CUL1 expression in the human Type II cancer cell line 786-0 considerably blocked replication of vMyx-gfp at 48 hpi compared to cells transfected with control siRNA (Fig. 3-5B). No replication of vMyxT5KO-gfp was observed (Fig. 3-5B) since these particular Type II cancer cells express low levels of endogenous phosphorylated Akt and do not
normally support MYXV replication in the absence of M-T5 (292). These findings demonstrate how the interaction between M-T5 and the SCF complex significantly contribute to the permissiveness of MYXV replication in these cells. Furthermore, these results provide further insights into the molecular mechanism by which M-T5 functions via the host cell SCF complex during viral infection.

**Identification of the M-T5 ANK-Repeats Essential for Binding to Cellular Akt**

Almost all poxviruses express multiple proteins containing ANK-repeats, thus accounting for a large superfamily of related but unique poxviral gene products (176). Detailed analyses of the M-T5 sequence predicted the presence of approximately seven ANK-repeat domains within the N-terminus and central regions of the protein (Fig 3-1A). The functional significance for any of the specific ANK domains in M-T5 has yet to be identified, however it is reasonable to predict they facilitate protein-protein interactions with host targets critical for viral replication. Since the ANK-repeat is one of the most common protein-protein interaction motifs found in nature (181), it can be hypothesized that one or more of these ANK domains function as the docking site for binding viral or cellular substrate proteins.

The host protein Akt, was previously demonstrated to bind M-T5 by co-immunoprecipitation assays, however the details of this interaction were not fully investigated at the molecular level (292). To investigate this protein-protein interaction in greater detail, HEK293 cells were co-transfected with one of the various M-T5 fragment constructs fused to myc/His (Figure 3-2A) together with HA-Akt and cell lysates were assayed for binding by AlphaScreen. The presence of a distinct emission signal was detectable when full length M-T5 (MT5/myc-His) was available for binding HA-Akt (Fig. 3-6A, lane 1) likewise, deletion of the first 31 amino acids at the N-terminal of M-T5
Figure 3-6. ANK-repeats I and II of M-T5 are critical for binding Akt. The plasmids HA-Akt and a variety of myc-His tagged M-T5 fragments (Fig 3-2A) were transiently transfected into HEK293 cells or expressed by TNT in the indicated (+) combinations. (A) Cell lysates were collected 48 h following plasmid transfection and assayed for binding by AlphaScreen as described in Fig. 3-3. (B) GST recombinant proteins were pulled-down with GTS-coated beads, and the associated proteins were resolved by SDS-PAGE and analyzed by immunoblotting with anti-Myc antibody. (C) Endogenous untagged Akt protein was immunoprecipitated with anti-Akt antibody and samples were analyzed by SDS-PAGE and probed with anti-Myc antibody to detect co-precipitated proteins. The specified antibodies were used to detect the protein expression of HA-Akt, Akt-GST, endogenous Akt and myc-His M-T5 tagged fragments.
(MT5ΔN/myc-His) did not affect binding (Fig. 3-6A, lane 2). However, deletion of the first ANK-repeat (MT5ΔN-I/myc-His and MT5ΔI/myc-His) reduced the binding affinity between the two proteins by approximately half (Fig. 3-6A, lane 3 and 10). Furthermore, deletion of both ANK I plus II completely abolished the interaction between M-T5 and Akt, as demonstrated by the significant decrease in the emission signal for all the constructs tested (Fig. 3-6A, lanes 4-9 and 12). Interestingly, internal deletion of ANK II only (MT5ΔII/myc-His) was also able to ablate binding (Fig. 3-6A, lane 11), however whenever both ANK I and II were both present, the interaction between M-T5 and Akt appeared to be maintained (Fig. 3-6A, lanes 1, 2 and 13-15).

Next, GST pull-downs were performed to confirm the interaction between Akt and the ANK I and II domains of M-T5. Individual M-T5-myc/His fragment constructs were co-expressed with Akt-GST by TNT and incubated with GST-coated beads overnight. Precipitated complexes were resolved by SDS-PAGE and only those M-T5 constructs which contained both ANK I and II were detected when immunoblotted with an anti-Myc antibody (Fig. 3-6B, lanes 1, 5 and 6). Similarly, co-immunoprecipitation of M-T5 fragment constructs containing both ANK I and II were detected when immunoblotted with an anti-Myc antibody (Fig. 3-6B, lanes 1, 5 and 6). Similarly, co-immunoprecipitation of M-T5 fragment constructs containing both ANK I and II was observed when endogenous untagged Akt was immunoprecipitated using an Akt specific antibody (Fig. 3-6C, lanes 1, 5 and 6). In both experiments, interaction between Akt and M-T5 fragments lacking ANK I and/or II was not detected (Figs. 3-6B and C, lanes 2, 3 and 4). Taken in aggregate, these data clearly demonstrate that the composite ANK I-II domain of M-T5 is critical for binding to cellular Akt.
M-T5 Functions as a Molecular Scaffold that Binds Akt and the SCF Complex Simultaneously

Functional diversity among ANK-repeat proteins suggests the motif is far more important structurally, as scaffolding modules, rather than as an enzymatic role (228). Thus, the potential of viral ANK-repeat proteins to operate as a molecular scaffold may provide poxviruses a mechanism to generate novel protein-protein interactions fundamental to successful viral replication. Results from this study and previous reports have shown M-T5 can independently bind CUL1/Skp1/SCF (Figs. 3-2 and 3) (127) and Akt (Fig. 3-5) (292), however the potential ability of M-T5 to bind both cellular partner complexes simultaneously, had not been examined. To determine if M-T5 functions as a molecular scaffold that can link Akt directly with the SCF complex, HEK293 cells were co-transfected with HA-Akt, Flag-CUL1 and one of the various myc-His tagged M-T5 constructs (MT5, MT5∆PRANC or MT5∆N-VII), the following day cell lysates were collected and assayed for binding by AlphaScreen. In Figure 3-7A, we show that HA-Akt and Flag-CUL1 do not directly interact (Fig. 3-7A, lane 6), however the addition of M-T5 (MT5/myc-His) elevated the emission signal dramatically (Fig. 3-7A, lane 1). In contrast, deletion of either the PRANC/F-box like domain (Fig. 2-7A lane 2) or the ANK I and II domain (Fig 3-7A, lane 3) from M-T5, significantly reduced the capacity of the viral M-T5 protein to bridge HA-Akt with Flag-CUL1. As a control, cells co-transfected with MT5/myc-His and either HA-Akt or Flag-CUL1 alone did not produce a significant emission signal when binding was analyzed (Fig. 3-7A, lanes 5 and 6). Although, Flag-CUL1 will indirectly bind to the Acceptor bead via the biotin conjugated anti-Flag antibody, the Donor bead which is coated with a HA specific antibody will not recognize the myc-His epitope expressed by M-T5. As a result, the interaction between M-T5 and
Figure 3-7. M-T5 binds Akt and SCF components simultaneously. The designated (+) combinations of plasmids were used to transiently transfect HEK293 cells or were expressed by TNT. (A) Harvested 48 h after transfection, cell lysates were subjected to AlphaScreen binding assay as described by Fig. 3-3. (B) Cells were infected with vMyx (lanes 1-4), vMyxT5KO (lanes 4-8) or mock infected (lanes 9-12) the day following transfection. At 48 hpi cell lysates were collected and assayed for protein-protein binding by AlphaScreen. (C) Antibody specific for Akt (anti-Akt) was used to immunoprecipitate Akt bound protein complexes in mock infected cells or cells infected with vMyxT5KO or vMyx. Immunoblot analysis in SDS-PAGE detected the resulting protein complexes with anti-Skp1 antibody. (D) Endogenous Skp1 protein was immunoprecipitated with anti-Skp1 antibody in samples that expressed HA-MT5 by TNT. Detection of Akt co-immunoprecipitation was determined by Western blot analysis using Akt specific antibody (anti-Akt). Protein expression of HA-CUL1, HA-Skp1 and M-T5 fragments were detected using the designated antibodies.
CUL1 was not detected based on the design of this experiment. The same principle applies to HA-Akt, which binds directly to the Donor beads.

To confirm the ability of M-T5 to simultaneously bind the cellular proteins in virus-infected cells, HEK293 cells were first co-transfected with HA-Akt plus Flag-CUL1 and then infected with either vMyx or vMyxT5KO the following day. Specific binding interaction between Akt and CUL1, as indicated by the elevated emission signal, was only observed when cells were infected with wild-type MYXV (Fig. 3-7B, lane 4). In contrast, cells that were mock infected (Fig. 3-7B, lane 12) or infected with MYXV that does not express the M-T5 gene product (Fig. 3-7B, lane 8) produced only a low emission signal, indicating that the M-T5 protein bridges Akt with components of the SCF complex in MYXV-infected cells. These results are congruent with the M-T5 binding data (Figs. 3-3 and 3-5) and suggest the viral M-T5 protein has the capacity to bind Akt and CUL1-containing SCF simultaneously during viral infection.

Next, to verify that Skp1 is also within the SCF complex that binds Akt in the presence of M-T5, HEK293 cells were either mock infected or infected with vMyx (that expresses M-T5) or vMyxT5KO and at 48 hpi untagged cellular Akt was immunoprecipitated from the cellular lysate with an anti-Akt antibody. Co-immunoprecipitation of Skp1 was only observed in cells infected with the wild-type virus that expresses M-T5, and not in mock or vMyxT5KO infected cells (Fig. 3-7C). Similarly, co-immunoprecipitation of endogenous Akt was detected only when full length M-T5 (MT5/myc-His) was co-expressed by TNT (Fig 3-7D, lane 1). In contrast, Akt co-immunoprecipitation with Skp1 was not identified in TNT lysates that expressed either MT5ΔI-II or MT5ΔPRANC (Fig. 3-7D, lanes 2 and 3). Collectively, the results clearly
demonstrate that M-T5 operates as a scaffolding protein to bridge two distinct cellular binding partners; Akt and the SCF complex, containing both CUL1 and Skp1, during viral infection.

**Cellular Localization of Akt is Influenced by M–T5 During Virus Infection**

The potential capacity of poxviral ANK-repeat proteins to operate as molecular scaffolds to link disparate cellular partners together may result in novel cross-communication between distinct host signaling pathways that are normally independent of one another. Such modifications of the host cellular signaling networks, induced by poxvirus-encoded host-range genes, would thus be critical in establishing an environment within the host crucial for successful virus replication. Confocal immunofluorescence microscopy was used to examine the ability of M-T5 to alter the cellular localization of Akt and/or members of the SCF complex in the context of a viral infection. Briefly, HeLa cells were transfected with HA-CUL1 and two days later mock infected or infected with vMyx or vMyxT5KO for 4 h (M-T5 is expressed early during infection). Cells were fixed with 4% paraformaldehyde, permeabilized with 1% Triton-X 100, and then rabbit polyclonal anti-Akt antibody (to monitor untagged cellular Akt) and a mouse monoclonal antibody against HA (to monitor the HA-tagged CUL1) were applied to the samples and the protein localizations were assessed by counterstaining with fluorescently labeled secondary antibodies. In both the mock control and vMyxT5KO-infected cells, Akt was abundantly detected in the nucleus, colocalizing with the DNA stain DAPI. Smaller amounts of Akt also appeared to be located at the plasma membrane, where the protein is thought to reside when phosphorylated and activated. In contrast, within cells infected with wild-type MYXV that expresses M-T5, significant relocalization of Akt, predominantly from the nucleus to the cytoplasm, was observed. In
Figure 3-8. Relocalization of Akt in MYXV-infected cells requires M-T5. (A) The cellular localization of endogenous Akt (FITC, Green) and transiently transfected HA-CUL1 (TexasRed, Red) was assayed in HeLa cells mock infected (A-C), or infected with either vMyx-lac (D-F) or vMyxT5KO (G-I). Nuclei (Blue) were detected using DAPI reagent as shown in merged panels C, F and I. (B) HeLa cells were infected with either vMyx-lac (dark bar) or vMyxT5KO (open bar) and collected at 0, 1, 4, 8, 12 and 24 hpi. Nuclear and cytoplasmic extracts were isolated, separated by SDS-PAGE electrophoresis and blots were probed with an Akt specific antibody. Cellular distribution of Akt was determined by quantifying the level of Akt present in both the nucleus and cytoplasm by densitometry (Molecular Imaging software, Kodak).
contrast, we did not observe any obvious cellular relocalization of CUL1 in either the presence or absence of virus. Thus, in MYXV-infected cells, there was no obvious relocalization of CUL1-containing SCF complex (which remained in the cytoplasm) but a fraction of the nuclear pool of Akt was specifically relocalized from the nucleus to the cytoplasm only when M-T5 was present. Thus, the results support our previous data and exhibit the ability of M-T5 to simultaneously interact with both Akt and the SCF complex.

To further examine the relocalization of Akt following vMyx infection the cellular distribution of Akt was examined. HeLa cells were infected with either vMyx or vMyxT5KO and cells were collected at various time points after infection. Nuclear and cytoplasmic extracts were isolated, separated by SDS-PAGE electrophoresis and blots were probed with an Akt specific antibody. Cellular distribution of Akt was determined by quantifying the level of Akt present in both the nucleus and cytoplasm by densitometry (Fig. 3-8B). Initially Akt resided predominantly within the nucleus, however 4 h following vMyx-lac infection the cytoplasmic levels of Akt increased and remained elevated for up to 24 h. Whereas, during the same time period the level of Akt present in the nucleus decreased. In stark contrast, relatively no change in the level of Akt localized to the nucleus was observed in cells infected with vMyxT5KO. Furthermore, lower Akt levels were observed in the cytoplasmic samples after vMyxT5KO infection. The data suggest that expression of M-T5 by MYXV appears to induce at least some of the nuclear Akt to migrate to the cytoplasm with the CUL1/SCF complexes.

**Discussion**

Generally speaking, most poxviruses exhibit a narrow or restricted host-range in which the distinct tropism is linked with the unique repertoire of host-range genes
expressed by each individual virus (171). During poxviral infection, these host-range genes contribute significantly to modulating the intracellular environment by specifically targeting a diverse array of cellular factors and pathways to establish optimal conditions for permissive viral replication (173, 229). Many of the known poxviral host-range genes are not only functionally diverse but also demonstrate a wide spectrum of biochemical and structural characteristics (297). The ANK-repeat has been noted within many poxvirus host-range factors from different genera, such as; K1L from VACV, CHOhr from CPXV, both of which are orthopoxviruses, and M-T5 of MYXV, which is a leporipoxvirus. Generally, this unique family of poxvirus-encoded proteins are 400 to 600 amino acids in size, contain from 5-10 ANK-repeats, and a C-terminal PRANC/F-box like domain (176). The cellular versions of the F-box domain commonly exist in concert with additional protein-protein interaction motifs such as WD repeats or LRR, which are though to mediate substrate specificity (137, 244). The presence of the ANK-repeat motifs within F-box proteins is exclusive to poxviral proteins, suggesting the collaborative role of these distinct binding domains likely provides poxviruses novel strategies to regulate key cellular pathways that mediate pathogenesis and tropism.

Like many previously characterized cellular F-box proteins, all four MYXV encoded ANK-repeat proteins were demonstrated to interact directly with the common core SCF adaptor component, Skp1 (Fig. 3-1C). These four MYXV proteins add to a growing list of ANK-repeat containing poxviral proteins, which bind to the Skp1 component of the SCF complex via a C-terminal PRANC/F-box like domain (247, 249, 282). However, the identification of other cellular substrates for the poxviral ANK/PRANC protein family members, either cellular or viral, has proven to be a major obstacle. In this study,
multiple independent human cDNA libraries were screened by Y2H for potential binding partners of M-T5 and a total of 13 unique potential host protein interactions were identified (Table 3-2). Aside from Skp1, all proteins were novel potential M-T5 binding partners and have not been previously reported, but their verification as true binding partners in virus-infected cells remains to be confirmed.

Using a combination of experimental approaches, we successfully demonstrate that M-T5 binds to the SCF complex via the adaptor protein Skp1 by means of the C-terminal PRANC/F-box like domain (Figs. 3-3 and 4). Previous studies report that cellular F-box proteins without WD or LRR motifs can also bind to SCF components in vivo (50). Likewise, the ORF008 protein encoded by ORF virus was able to directly interact with Skp1, even following the deletion of all the ANK-repeats (247). Conversely, binding between components of the SCF complex and the ectromelia virus (ECTV) ANK-repeat protein ECT005 was abrogated when the ANK-repeat domains of the viral protein were removed (282). In the case of M-T5, CUL1 interacted with both M-T5 and a mutant of M-T5 lacking all the ANK-repeats, when analyzed by AlphaScreen (Fig. 3-3D). However, the GST pull-down protocol could only detect M-T5 binding with CUL1 when the ANK-repeats of M-T5 were present (Fig. 3-3A and B), suggesting the ANK-repeat domains may contribute to the stability of the interaction between M-T5 and components of the SCF complex. We propose that stability of the complex formed between the solitary PRANC/F-box like domain of M-T5 and SCF is compromised in the absence of the ANK domains, making the more transient interactions difficult to identify when using methods that rely upon the complex having a longer half-life, such as GST pull-downs or co-immunoprecipitations to assay the complexes. AlphaScreen provides
greater sensitivity and is much more rapid than the above assays, therefore may be a more sensitive method to measure more transient interactions between potential binding partners that have shorter half-lives.

When the protein binding domains of M-T5 were examined, the PRANC/F-box like domain was shown to be essential for binding to Skp1 (Fig. 3-3), whereas ANK-repeats I and II were identified as being important for interaction with Akt (Fig. 3-6). Furthermore the ability of M-T5 to link disparate cellular binding partners together within a single complex was investigated and we report that the viral protein binds Akt and the SCF complex (via Skp1) simultaneously in MYXV-infected cells. The ability of M-T5 to function as a molecular bifunctional adaptor defines a novel mechanism by which a viral host-range protein can influence two distinct signaling pathways, consequently altering the intracellular environment to promote viral replication. However, more details are necessary to further characterize the functional role of M-T5 and its coordinated interaction with the cellular SCF complex and various substrates such as Akt during viral infection. The capacity of poxviral ANK/PRANC proteins to function as molecular scaffolds may coordinate the cross-communication between host signaling pathways that normally function independently. At the molecular level, such viral proteins have the potential to re-configure intracellular signaling networks into downstream effects that may previously not exist due to spatial or temporal constraints, but which may be crucial for viral replication.

In summary, viruses such as poxviruses encode a myriad of proteins that have the capacity to hijack the ubiquitin-proteasomal pathway, for example to target and eliminate unwanted cellular proteins such as inhibitors of the cell cycle or various anti-
viral factors that would otherwise function to block viral replication. Understanding the mechanisms by which viral host-range proteins interact with host factors and signaling networks should continue to provide invaluable insights into how vital cellular networks can be re-programmed by viral factors to increase virus survival at the cellular level.
CHAPTER 4
PHARMACOLOGICAL MANIPULATION OF THE AKT SIGNALING PATHWAY REGULATES MYXOMA VIRUS REPLICATION AND TROPISM IN HUMAN CANCER CELLS

Introduction

Following viral infection, substantial alterations in cellular physiology often lead to the modification of various cellular pathways critical to the success of viral replication. The demand for energy, nutrients and macromolecular synthesis that accompany viral replication can be substantial; thus, many viruses have evolved elaborate strategies to hijack key cellular signaling networks necessary to support their demands (43). By the same token, anti-viral pathways activated by the virus infection may also need to be blocked or subverted to ensure successful virus replication. Poxviruses possess large dsDNA genomes that encode multiple gene products that specifically modify or debilitate the various host signaling responses of the infected cell (171).

The serine/threonine kinase Akt was initially discovered as the cellular homolog of the viral oncogene (v-Akt) encoded by the AKT8 retrovirus isolated from a murine T cell lymphoma (31, 128, 251). There are three isoforms found in mammals (Akt1, -2 and -3) encoded by separate genes, but sharing over 80% amino acid sequence identity. Akt is a key regulator of many important cellular functions that include; cell survival, proliferation, glucose metabolism and protein synthesis. The critical role of Akt in the regulation of multiple biological processes makes it a central regulator of cellular signaling and therefore it is not surprising that many viruses have developed sophisticated strategies to manipulate the activation of Akt (43, 58). Activation of Akt is predominantly dependent upon phosphoinositide 3-kinase (PI3K), which phosphorylates phosphoinositides at the D3-position of the inositol ring to generate PI(3,4,5)P₃ (PIP₃).
Akt possesses an N-terminal PH domain that binds PIP₃ to promote its translocation of the plasma membrane. Once localized at the membrane, Akt becomes phosphorylated at residue Thr308 in the activation loop by PDK1 and also within the carboxy-terminus at residue Ser473 by mTORC2 (7, 256, 259). Phosphorylation of both sites is necessary for full induction of Akt kinase activity. In the majority of human cancer cells, the Akt pathway is either mutated or constitutively activated, contributing to cancer progression through both the stimulation of cellular proliferation and inhibition of apoptosis (197, 271).

Although MYXV has a narrow host-range in nature, and is pathogenic only to European rabbits, the tropism of MYXV has recently been extended to include human cancer cells in vitro (18, 252, 267, 292, 301) and in xenografted mice in vivo (159, 160, 304). The mechanisms that mediate MYXV tropism in human cancer cells are still being investigated but one signalling requirement has been linked to the state of cellular Akt kinase activity (292). Human cancer cells (called Type I) that exhibit high levels of endogenous phosphorylated Akt (Ser473 and Thr308) supported permissive MYXV replication while cells with no detectable endogenous phosphorylated Akt, that was unaffected by the virus infection, were non-permissive (Type III). A unique subset of cancer cells (Type II) were found to be permissive to wild-type MYXV, but did not support MYXV replication following the deletion of the viral host-range factor M-T5 (vMyxT5KO). These Type II cells constitutively expressed only low levels of endogenous phosphorylated Akt (mostly at Thr308), but following infection with permissive MYXV a significant increase in Akt phosphorylation (particularly at Ser473) was observed. In stark contrast, the endogenous levels of phosphorylated Akt remain
essentially unchanged when Type II cells were infected with the nonpermissive M-T5 knockout MYXV (vMyxT5KO) (292).

In this study, we screened an array of Akt inhibitor compounds that selectively manipulate the Akt signaling network at some level and report that certain Akt inhibitors significantly blocked MYXV replication in previously permissive Type I and II human cancer cells. An additional set of inhibitors selectively inhibited only the replication of MYXV deleted for M-T5 and did not modify the replicative ability of the parental wild-type virus. Furthermore, the decrease in viral replication efficiency was correlated with lower levels of phosphorylated Akt at residues Ser473 and Thr308. In contrast certain PP2A specific phosphatase inhibitors, such as okadaic acid, promoted an increased Akt kinase activation and rescued MYXV replication in Type III human cancer cells that did not previously support viral replication. Finally, we demonstrate that the hemi-phosphorylation of Akt at residue Thr308 dictates physical interaction between Akt and M-T5, which ultimately leads to productive MYXV replication in Type II cancer cells. These studies show that activation of the Akt signaling cascade is essential for efficient MYXV replication in human cancer cells and further demonstrate the dynamic role by which M-T5 manipulates Akt signaling to establish a cellular environment more favorable for viral replication.

Results

Certain Akt Inhibitors Block Myxoma Virus Replication in Permissive Type I and II Human Cancer Cells

An ever-increasing number of compounds and drugs have been characterized for their ability to target various components of the PI3K-Akt signaling pathway and provide novel mechanisms to selectively manipulate the Akt network (70). To further examine
the mechanism by which the phosphorylation status of Akt affects MYXV replication in human cancer cells, a collection of small molecular compounds that specifically target the Akt signaling pathway at diverse stages were examined for their ability to regulate MYXV replication in permissive and nonpermissive human cell lines. Akt inhibitor I is a D3-modified PI ester analog, a competitive inhibitor of PI3K with respect to PI (110), whereas Akt inhibitor V is a synthetic triacyclic nucleoside which selectively blocks phosphorylation and activation of Akt but does not inhibit kinase activity nor upstream Akt activators such as PI3K and PDK1 (305). Interaction of Akt inhibitor VIII with the PH domain of Akt prevents conformational change required for phosphorylation by upstream kinases, thus blocking the phosphorylation of Akt (16, 154). In contrast, Akt inhibitor X is a N-substituted phenoxazine that inhibits the activity of Akt even in the absence of its PH domain and it has been suggested that it may bind in the ATP binding site (272). Type I, II and III human cancer cells were treated with various inhibitors (Akt Inhibitor I, V, VIII and X) prior to infection with either vMyx-gfp or vMyxT5KO-gfp at an MOI of 3 and viral replication was determined by the formation of fluorescent green-expressing foci as viewed by fluorescence microscopy at 48 hpi. In HOS (Type I) cells, Akt inhibitors VIII and X were able to successfully reduce viral replication of both vMyx-gfp and vMyxT5KO-gfp, while Akt inhibitors I and V were only able to reduce replication of vMyxT5KO-gfp (Fig. 4-1A, upper two panels). As demonstrated previously, 786-0 (Type II) cells do not support replication of vMyxT5KO, whereas the wild-type virus grows permissively (292). In the presence of Akt inhibitors VIII and X, these cells became less permissive to vMyx-gfp replication as well, however Akt inhibitors I and V did not effect MYXV replication in these Type II cells (Fig. 4-1A, middle two panels). SK-
Figure 4-1. Effects of Akt inhibitors on MYXV replication in human cancer cells. (A) HOS, 786-0 and SK-MEL-5 cells were infected with either vMyx-gfp or vMyxT5KO-gfp in the absence (mock) or presence of various Akt inhibitors, and viral foci were detected by fluorescence microscopy at 48 hpi. Inhibitor concentrations are as stated in Materials and Methods section. (B) Permissive HOS cells were infected with either vMyx-gfp (white bars) or vMyxT5KO-gfp (gray bars) at an MOI of 1 in the absence (mock) or presence of various Akt inhibitors, and virus was collected at 48 hpi and tittered on BGMK cells. Titers are expressed as FFU/10^6 cells and represent the mean standard deviation of triplicate wells. (C-F) Representative Western blots showing detection of Akt in cell lysates from HOS cells at 24 hpi following mock infection (lanes 1 and 2), or infected with either vMyx (lanes 3 and 4) or vMyxT5KO (lanes 5 and 6) at MOI 3 in the presence (+) or absence (-) of various Akt inhibitors. Kinase activation of Akt is demonstrated by detection of specific phosphorylated forms pAkt-Ser473 and pAkt-Thr308. Equal sample loading was confirmed by detection of total Akt and the housekeeping protein actin. (G) Cell lysates prepared from (B) were assayed for Akt phospho-Ser473 (left) and Akt phospho-Thr308 (right) using the standard AlphaScreen SureFire protocol. Each sample was performed in triplicate and standard deviations are represented by the error bars. Mock treated cells are represented by white bars, whereas cells treated with FTY720 are represented by gray bars.
MEL-5 (Type III) cells do not support MYXV replication and none of the Akt inhibitors rescued MYXV replication in these cells (Fig. 4-1A, lower two panels).

To quantitatively assess the generation of infectious progeny MYXV, HOS cells were individually treated with these Akt inhibitors and infected with either vMyx-gfp or vMyxT5KO-gfp. Samples were harvested for infectious virus at 48 hpi, and titrated on BGMK cells by serial dilution. Viral titer of vMyx-gfp was considerably reduced when cells were treated with Akt inhibitors VIII and X (Fig. 4-1B, lanes 7 and 9 vs. 1), whereas cells treated with the Akt inhibitors I and V produced viral progeny levels comparable to control treated cells (Fig. 4-1B, lanes 3 and 5 vs. 1). In cells infected with vMyxT5KO-gfp, reduced viral titers were observed in samples treated with all four Akt inhibitors (Fig. 4-1B, lanes 4, 6, 8 and 10 vs. lane 2).

Successful MYXV replication in human cancer cells is dependent upon activation of endogenous Akt (292), suggesting that in the presence of these Akt inhibitors, a reduction in MYXV replication efficiency can be attributed to a decrease in the level of phosphorylated Akt. Thus, cell lysates of virus-infected HOS (Type I) cells treated with each of these Akt inhibitors were collected and phosphorylation of Akt at residues Ser473 and Thr308 was monitored by western blot using Akt phospho-specific antibodies. As predicted, the level of Akt phosphorylation at both phospho-residues was dramatically reduced in the presence of Akt inhibitors VIII or X in cells infected with vMyxT5KO (Figs. 4-1E and F, lane 5 vs. 6). Furthermore, a drop in Akt phosphorylation at residue Ser473 and to a lesser extent at Thr308 was observed in cells treated with either Akt inhibitor VIII or X and infected with vMyx (Figs. 4-1E and F, lane 3 vs. 4). No significant decrease in Akt phosphorylation, at either Ser473 or Thr308, was observed
in cells treated with Akt inhibitors I or V and infected with vMyx (Figs. 4-1C and D, lane 3 vs. 4). In contrast, cells treated with Akt inhibitors I or V and infected with the M-T5 knockout virus (vMyxT5KO) exhibited levels of Akt phosphorylation that were considerably reduced at residue Ser473 but not Thr308 (Figs. 4-1C and D, lane 5 vs. 6). Elevated levels of endogenous Akt phosphorylation at both Ser473 and Thr308 resides was observed in uninfected cells in the absence of any Akt inhibitor (Figs. 4-1C-F, lanes 1). Conversely, all Akt inhibitors were successful at reducing the level of phosphorylated Akt at residue Ser473 in uninfected cells, but only Akt inhibitor VIII and X also blocked phosphorylation of Thr308 (Figs. 4-1C-F, lanes 1 vs. 2).

To complement the western blot results, the levels of phosphorylated Akt were also be assayed by the SureFire modification of Alpha Screen. SureFire is a homogenous bead-based platform that uses AlphaScreen technology to provide an extremely sensitive and rapid assay to quantitate the site and extent of Akt phosphorylation. Like all proximity-based assays, AlphaScreen depends on bringing together two distinct types of beads whose proximity causes the production of an emission fluorescence signal. The SureFire assay employs phospho- and non-phospho antibodies, specific to Akt, which coat the surface of the beads. Only phosphorylated Akt interacts with both antibodies and brings the two sets of beads together, thus producing an emission signal that is detected. As demonstrated in Fig. 4-1G, the relative level of endogenous phosphorylated Akt at residues Ser473 and Thr308 was decreased in cells treated with Akt inhibitors VIII or X and then infected with either virus (Fig. 4-1G, lanes 7-10). Alternatively, Akt phosphorylation levels in cells treated with Akt inhibitors I or V and infected with vMyx remain high and are comparable to mock treated
cells (Fig. 4-1G, lanes 3 and 5). Furthermore, decreased levels of phosphorylated Akt at residue Ser473, but not Thr308, was observed in vMyxT5KO infected cells treated with Akt inhibitors I or V (Fig. 4-1G, lanes 4 and 6). Lastly, drug treatment or virus infection did not affect the total amount of Akt protein present in the cell (Fig. 4-1C-F, third panels). Taken together, these data demonstrate that manipulation of the Akt signaling network by small molecular inhibitors can have profound effects on MVXY tropism in human cancer cells. Specifically, two of these inhibitors (I and V) can distinguish between infections with wild-type MYXV vs vMyxT5KO in Type I and II cells, and their inhibitory effects are suppressed in the presence of M-T5.

**Dephosphorylation of Akt by FTY720 Blocks Myxoma Virus Replication**

The immunosuppressant FTY720 is a derivative of ISP-1 (myriocin), and was originally demonstrated to prolong allograft transplant survival in numerous models by inhibiting lymphocyte emigration from lymphoid organs (190, 265). FTY720 induces G0/G1 arrest in Jurkat and HL-60RG cells via dephosphorylation of retinoblastoma protein (191) and promotes apoptosis in the human prostate cells line DU145 (234, 293). Furthermore, FTY720 was shown to activate PP2A-like phosphatases and dephosphorylate Akt, resulting in the enhancement of apoptosis via the mitochondrial pathway (149, 168). To examine if FTY720 could inhibit MYXV replication via Akt dephosphorylation, and whether M-T5 could circumvent this block, Type I, II and III cells were treated with or without FTY720 prior to infection with either vMyx-gfp or vMyxT5KO-gfp. Viral replication was determined by the formation of fluorescent green foci as viewed by fluorescence microscopy. Replication of the wild-type virus in the presence of FTY720 was not affected in permissive cell lines (Type I HOS and Type II 786-0), however treatment of HOS cells with FTY720 considerably reduced replication
of vMyxT5KO-gfp (Fig. 4-2A). This phenotype was not observed in the Type II cells because they already do not support replication of vMyxT5KO.

To further examine viral replication in the presence of FTY720, viral one-step growth curves were performed in Type I and II human cancer cell lines. In HOS cells, both viruses replicated efficiently and to similar levels in the absence of FTY720, however virus titer of vMyxT5KO was significantly and uniquely lower in the presence of drug (Fig. 4-2B, left panel). In contrast, FTY720 had little or no inhibitory effect on viral titers of HOS cells infected with vMyx (Fig. 4-2B, left panel). Likewise, replication kinetics of wild-type virus in the 786-0 cells was relatively similar in the presence or absence of drug (Fig. 4-2B, right panel). As previously demonstrated, 786-0 cells do not support replication of vMyxT5KO (Fig. 4-2B, right panel).

Based on the reported ability of FTY720 to dephosphorylate Akt, cell lysates of MYXV-infected Type I and II cells were collected and Akt phosphorylation was analyzed by both Western blot and AlphaScreen SureFire. The addition of FTY720 had little or no effect on the phosphorylation status of Akt at residue Thr308 in the two cell lines in the presence or absence of either vMyx or vMyxT5KO (Fig. 4-2C and 2D, right panel). Conversely, phosphorylation of Akt at residue Ser473 was significantly reduced in HOS cells following treatment with FTY720 (Fig. 4-2C, lane 1 vs. 2). However, in HOS cells treated with FTY720 and infected with wild-type MYXV, but not vMyxT5KO, phosphorylation of Akt at residue Ser473 remained relatively unchanged (Fig. 4-2C, lane 3 vs. 4 and Fig. 4-2D, lane 1 vs. 2). Elevated levels of Akt phosphorylation (Ser473) were observed in 786-0 cells in the presence or absence of FTY720 only when cells were infected with vMyx (Fig. 4-2C, lanes 9 and 10, Fig 4-2D, right panel lanes 5.
Figure 4-2. Inhibition of vMyxT5KO replication by FTY720. (A) The human cancer cells HOS, 786-0 and SK-MEL-5 were infected with either vMyx-gfp or vMyxT5KO-gfp in the absence (-) or presence (+) of FTY720, and at 48 hpi viral foci formation was determined by florescence microscopy. (B) HOS (left) and 786-0 (right) cells were infected with either vMyx-gfp (square and triangle) or vMyxT5KO-gfp (circle and diamond) at an MOI of 1 in the absence (square and circle) or presence (triangle and diamond) of FTY720, and virus collected at various hpi was tittered on BGMK cells. Titers are expressed as FFU/10^6 cells and represent the mean standard deviation of triplicate wells. (C) Representative Western blots showing detection of Akt in cell lysates from HOS (left) and 786-0 (right) cells at 24 hpi following mock infection (lanes 1 and 2), or infected with either vMyx (lanes 3 and 4) or vMyxT5KO (lanes 5 and 6) at MOI 3 in the presence (+) or absence (-) of FTY720. Kinase activation of Akt is demonstrated by detection of specific phosphorylated forms pAkt-Ser473 and pAkt-Thr308. Equal sample loading was confirmed by detection of actin. (D) Cell lysates of HOS cells prepared from (C) were assayed for Akt phospho-Ser473 (left) and Akt phospho-Thr308 (right) using the standard AlphaScreen SureFire protocol. Each sample was performed in triplicate and standard deviations are represented by the error bars. Mock treated cells are represented by white bars, whereas cells treated with FTY720 are represented by gray bars.
and 6). The data indicates that FTY720 dephosphorylates Akt at residue Ser473, which coincidently blocks vMyxT5KO replication in previously permissive HOS (Type I) cells but not vMyx, which can exploit M-T5 to keep this site phosphorylated.

**Phosphatase Inhibitors Promote Myxoma Virus Replication in Previously Non-Permissive Human Cancer Cells**

Activation of Akt is highly regulated by the intricate role of kinases and phosphatases that function to control the addition or removal of phosphates at the residues Ser473 and Thr308. The two phosphatases, protein phosphatase 2A (PP2A) and the recently identified pleckstrin homology domain leucine-rich repeat protein phosphatase (PHLPP) inactive Akt via the dephosphorylation of residues Thr308 and Ser473, respectively. In non-permissive Type III human cancer cells the level of endogenous phosphorylated Akt remains extremely low even in the presence of virus that expresses M-T5, suggesting the possibility these phosphatases may be overexpressed or excessively active. To test this hypothesis, a collection of protein phosphatase inhibitors were selected and tested for their ability to rescue MYXV replication in non-permissive human cancer cells. HOS, 786-0 and SK-MEL-5 cells were treated with one of the phosphatase inhibitors (DARPP-32 [PP1], Endothall [PP2A], Fenvalerate [PP2B], α-napthyl acid phosphate [broad spectrum] and okadaic acid [PP2A]) prior to infection with either vMyx-gfp or vMyxT5KO-gfp and viral replication was determined by the formation of fluorescent green foci as viewed by fluorescence microscopy (Fig. 4-3). In the HOS cells, none of the phosphatase inhibitors appeared to significantly alter replication of either virus, as based on the production of fluorescent green foci. The phosphatase inhibitors were unsuccessful at restoring replication of vMyxT5KO in the 786-0 cells and did not change the replicative ability of vMyx. In stark
Figure 4-3. PP2A-specific protein phosphatases rescue MYXV replication in non-permissive Type III cancer cells. MYXV foci formation was detected at 48 hpi by florescence microscopy in HOS, 786-0 and SK-MEL-5 cells infected with either vMyx-gfp or vMyxT5KO-gfp in the absence (mock) or presence of various protein phosphatase inhibitors. A-NAP, α-naphthyl acid phosphate, monosodium salt.

contrast, the PP2A inhibitor, okadaic acid, was capable of releasing the replicative block in SK-MEL-5 cells to restore vMyx permissivity. Endothall, another PP2A inhibitor, had a similar effect as okadaic acid but to a lesser effect. However neither okadaic acid nor endothall was able to rescue replication of vMyxT5KO in the SK-MEL-5 cells. These findings suggest that inhibition of the PP2A phosphatase can restore vMyx permissivity in Type III human cancer cells that previously did not support viral replication.
The Combination of Okadaic Acid and Rapamycin Induces Full Akt Phosphorylation to Rescue vMyxT5KO Replication

To confirm the ability of okadaic acid to rescue MYXV replication in Type III cells, viral titers of infected cells was determined. The viral titer of both vMyx-gfp and vMyxT5KO-gfp was indistinguishable in HOS and 786-0 cells treated with or without okadaic acid (Fig. 4-4A, lanes 1-8). In contrast, SK-MEL-5 cells treated with okadaic acid prior to infection with vMyx resulted in a significantly higher viral titer compared to cells not treated with okadaic acid (Fig. 4-4A, lane 9 vs. 10). Whereas, only a small increase in viral titer was observed in Type III cells treated with okadaic acid and infected with vMyxT5KO-gfp (Fig. 4-4A, lane 11 vs. 12). Previous work in our lab demonstrated the drug rapamycin was able to dramatically increase vMyxT5KO replication and spread in Type II but not Type III human cancer cells. Furthermore, rapamycin treatment was shown to induce Akt phosphorylation via the mTOR (mammalian target of rapamycin) signaling network (252). The combination of okadaic acid and rapamycin was used to treat SK-MEL-5 cells prior to infection with either vMyx-gfp or vMyxT5KO-gfp and viral titers were determined at 48 hpi. In the presence of okadaic acid and rapamycin the titer of vMyxT5KO was dramatically higher compared to either drug alone or no drug (Fig. 4-4B, lane 8 vs. 4 and 6). Interestingly, the combination of okadaic acid and rapamycin did not significantly increase vMyx replication when compared to okadaic acid only treatment (Fig. 4-4B, lane 3 vs 7).

The endogenous level of phosphorylated Akt was measured in the SK-MEL-5 cells and treatment with okadaic acid and rapamycin considerably increased the phosphorylation level at both Ser473 and Thr308 residues (Fig. 4-4C, lane 4). This pattern of Akt phosphorylation was also observed in vMyxT5KO infected cells (Fig. 4-4C
Figure 4-4. Combination of okadaic acid plus rapamycin enhances vMyxT5KO replication in Type III cancer cells. The effect of okadaic acid on MYXV replication in HOS (lanes 1 to 4), 786-0 (lanes 5 to 8) and SK-MEL-5 (lanes 9 to 12) cells. All cells were either mock treated (white bars) or pre-incubated (gray bars) with okadaic acid for 4 h prior to infection with vMyx-gfp (WT) or vMyxT5KO-gfp (T5KO) and at 48 hpi foci formation was determined by fluorescence microscopy. (B) Single-step growth analysis of vMyx-gfp (open bars) and vMyxT5KO-gfp (gray bars) at 48 hpi in Type III SK-MEL-5 cells. Prior to viral infection, cells were mock treated (lanes 1 and 2) treated with either okadaic acid (lanes 3 and 4), rapamycin (lanes 5 and 6) or both okadaic acid and rapamycin (lanes 7 and 8). Titers are expressed as FFU/10^6 cells and represent the mean +/- standard deviation of triplicate wells. (C) Cell lysates were prepared from Type III SK-MEL-5 cells mock infected (lanes 1 to 4), or infected with either vMyx (lanes 4 to 8) or vMyxT5KO (lanes 9 to 12) at MOI 3 in the absence (-) or presence of okadaic acid and/or rapamycin (+).
Cells infected with vMyx and treated with either okadaic acid or rapamycin expressed higher levels of Akt phosphorylation, however the level of Akt phosphorylation was appreciably increased following the combination of drugs (Fig. 4-4C, lane 6 and 7 vs. lane 8). We currently do not understand why increased Akt phosphorylation was observed only in SK-MEL-5 cells treated with okadaic acid or rapamycin alone and infected with wild-type vMyx (Fig. 4-4C, lanes 6 and 7) but not in the absence of virus (Fig. 4C, lanes 2 and 3) or vMyxT5KO-infected cells (Fig. 4-4C, lanes 10 and 11). However, the results clearly demonstrate that M-T5 influences the ability of both these drugs to upregulate the Akt activation in Type III cancer cells. Pharmacological manipulation of the Akt signaling network may provide mechanism by which cellular tropism can be altered, thus offering clues into the cellular blocks that inhibit MYXV replication in non-permissive human cancer cells. The results indicate that okadaic acid and rapamycin facilitate MYXV replication in Type III cancer cells by increasing Akt phosphorylation at both Thr308 and Ser473.

**M-T5 Binding is Dependent Upon Prior Akt Phosphorylation**

Although MYXV is a rabbit-specific poxvirus pathogen, a broad collection of human cancer cells can support productive viral replication (267). Permissive Type I cancer cells were found to possess high levels of endogenously activated Akt, whereas in non-permissive Type III human cancer cells the level of endogenous phosphorylated Akt remained essentially non-detectable, even following wild-type MYXV infection (292). To investigate whether full or partial phosphorylation of Akt might be critical for binding M-T5, HEK293 cells were co-transfected with MT5/myc-His and either HA-Akt or dominant negative Akt (HA-AktΔ2), in which the two major sites of ligand-induced phosphorylation (Thr308 and Ser473) are both replaced by alanine. Cells transfected
with M-T5 and wild-type Akt produced a strong binding signal when lysates were tested for M-T5/Akt binding by AlphaScreen the following day (Fig. 4-5A, lane 1). Alternatively, transfection of HA-AktΔ2 dramatically reduced binding affinity between the two proteins (Fig. 4-5B, lane 2), suggesting that at least some phosphorylation of Akt is critical for M-T5 binding.

Subsequently, the importance of Akt phosphorylation for binding M-T5 was examined in human cancer cells. Type I (Caki-1), II (786-0) and III (MCF-7) human cancer cells were co-transfected with MT5/myc-His and either HA-Akt, a constitutively active mutant of Akt (HA-Myr-Akt) or HA-AktΔ2. Fusion of the myristoylation signal from the human Src protein to the amino terminus of Akt (Myr-Akt), leads to enhanced association of the protein kinase with the plasma membrane and constitutive activation (1). A distinct M-T5/Akt binding signal was observed in both Type I and II cells co-transfected with HA-Akt and MT5/myc-His (Fig. 4-5B, lanes 1 and 2), however in Type III cells only a relatively low binding signal was detected (Fig. 4-5B, lane 3), suggesting M-T5 was essentially unable to bind Akt in these MYXV-nonpermissive cells. Strikingly, transfection of HA-Myr-Akt promoted binding to MT5/myc-His in Type III human cancer cells as demonstrated by the increase in binding signal (Fig. 4-5B, lane 6). In contrast, binding between Akt and M-T5 was completely lost in Type I, II and III human cancer cells when MT5/myc-His was co-transfected with HA-AktΔ2 (Fig. 4-5B, lanes 7-9). Our findings suggest that at least some phosphorylation of Akt is critical for binding M-T5 in both HEK293 and human cancer cells and that manipulation of the Akt phosphorylation status can perturb the interaction between these two binding partners.
Figure 4-5. The cellular environment regulates the interaction between Akt and M-T5. Cells were transiently transfected with the specified (+) combination of plasmids that include; MT5/myc-His, HA-Akt, HA-AktΔ2 and HA-Myr-Akt, in (A) HEK293 or (B and C) Caki, 786-0 and MCF-7 cell lines. Cell lysates were harvested after 48 h and pre-incubated with biotin conjugated anti-Myc antibody and Donor/Acceptor beads before an AlphaScreen assay was performed to determine binding. Each sample was performed in triplicate and the error bars represent standard deviation. (C) Cells were either mock treated or treated with the Akt inhibitors VIII or X prior to collection of cell lysis and AlphaScreen analysis of M-T5 binding to Akt.
Since some phosphorylation of Akt is critical for physical binding to M-T5, we next investigated the ability of Akt inhibitors VIII and X to block the interaction of these binding partners. Type I, II and III human cancer cells were co-transfected with MT5/myc-His and HA-Akt and were treated with either Akt inhibitor VIII or X the following day. Cell lysates were collected two days after transfection and were assayed for M-T5/Akt binding by AlphaScreen. In the presence of either inhibitor, the binding affinity between M-T5 and Akt, as demonstrated by the signal strength, was significantly reduced in Type I (Fig. 4-5C, compare lanes 1 to 4 or 7) and Type II (Fig. 4-5C, compare lanes 2 to 5 or 8) human cancer cells. No interaction between M-T5 and Akt in the Type III cells was observed in the presence or absence of inhibitors (Fig. 4-5C, lanes 3, 6 and 9), in agreement with the results in Fig. 4-5B. Taken together, these data provide evidence into the molecular interaction between M-T5 and Akt and the importance of at least some Akt phosphorylation prior to M-T5 binding.

**Phosphorylation of Akt Residue Thr308 Promotes M-T5 Binding**

To further examine how Akt phosphorylation dictates M-T5 binding, a collection of Akt mutants were constructed (Fig. 4-6A) and assayed for interactions using a GST pull-down assay. Each plasmid was sequence verified and Akt protein expression was confirmed by Western blot analysis with anti-HA antibody (Fig. 4-6B, upper lanes). A TNT protocol was used to co-express MT5-GST, tagged at the C-terminus with GST (Fig. 4-6B, middle lanes), plus one of the Akt proteins fused to a common N-terminal hemagglutinin (HA) tag (HA-Akt, HA-AktT308A, HA-AktS473A, HA-AktΔ2, HA-AktK179M, HA-AktΔ3). Samples were then incubated with GST-coated beads to pull-down MT5-GST fusions, and complexes were resolved by SDS-PAGE and immunoblotted with anti-HA antibody to analyze binding of HA-tagged Akt proteins (Fig.
Figure 4-6. Phospho-status of Akt residue Thr308 mediates M-T5 binding. (A) Schematic representation of the Akt constructs used during this study, amino acid substitutions are indicated. All Akt constructs contain an N-terminal HA tag. (B) The plasmids MT5-GST and the various HA-Akt constructs were co-expressed by TNT and subjected to a GST pull-down assay. Precipitates and total lysates were resolved by SDS-PAGE and probed with anti-HA antibody to detect co-precipitated viral proteins (lower panels). Expression of Akt proteins (upper panels) and M-T5 (middle panels) was confirmed by immunoblotting with antibody against HA (anti-HA) and GST (anti-GST) epitopes, respectively. Bands of interest are represented by arrows. 4-6B, lower lanes). Co-precipitation of MT5-GST and HA-Akt was observed only when threonine was present at Akt residue 308, whereas binding was abolished following substitution of alanine at this position (Fig. 4-6B, lower lanes 2, 4 and 5). In contrast, mutations at either residue 473 (S→A) or 179 (K→M) did not significantly affect the ability of M-T5 to bind Akt (Fig. 4-6B, lower lanes 3 and 6). These findings demonstrate that Akt phosphorylation of Thr308, but not residue Ser473, is critical for binding M-T5.
Discussion

An emerging body of evidence clearly demonstrates the remarkable ability of poxviruses to specifically manipulate a wide spectrum of cellular signaling networks to establish a cellular environment more favorable to viral replication (171). The PI3K/Akt/mTOR pathway has been reported to influence a diverse range of biological functions (43) and therefore provides an ideal target for which poxviruses to reconfigure the intracellular signaling according to their replicative requirements. Previously, we have shown that MYXV replication in human cancer cells is dependent upon the phosphorylation status of Akt and cells that failed to support productive viral replication (Type III) exhibited very low levels of phosphorylated Akt, which was not altered by virus infection (292). Furthermore, the MYXV host-range protein M-T5, promotes Akt activation through direct interaction with Akt to achieve optimal viral propagation in Type II cancer cells where the level of Akt phosphorylation is increased in an M-T5-dependent fashion following infection with MYXV. However, the mechanism by which M-T5 recognizes and interacts with Akt in some cancer cells (ie Type I and II) but not others (ie Type III) is poorly understood. Further examination of the binding requirements and the signaling consequences of this interaction may provide clues into the downstream events that regulate MYXV tropism in the human cancer cells.

The replicative strategies of many viruses is highly influenced by the PI3K/Akt/mTOR signaling cascade, accordingly viruses have evolved elaborate mechanism to modulate various aspects of this integral pathway (43, 58). As an example, a growing number of viral proteins have been characterized for their ability to activate PI3K through binding of its p85 adaptor or p110 catalytic subunit. For example, the hepatitis B X protein (HBx) associates with the catalytic subunit of PI3K to promote
phosphorylation of the p85 adaptor subunit, which subsequently elevates the phosphorylation levels of both Akt and the pro-apoptotic protein, BAD (150). Furthermore, activation of PI3K by HBx prevents activation of Caspase-3 and blocks transforming growth factor (TGF)-β-induced apoptosis in hepatoma cells (236).

Likewise, middle T (MT) antigen encoded by murine polyomavirus was reported to interact with the SH2 domain of the PI3K p85 regulatory subunit and block apoptosis independently of p53 (60, 261, 298). Lastly, the orthopoxviruses, VACV and CPXV have been shown to rapidly activate the PI3K/Akt pathway following infection and blockage of PI3K signaling with the inhibitor, LY294002, significantly decreased viral titer and induced an apoptotic response (245). Similarly, MYXV tropism is dependent upon activation of Akt, however in contrast to the orthopoxviruses like VACV and CPXV, inhibition of PI3K by LY294002 did not block MYXV replication (292), suggesting that MYXV possesses a unique ability to modulate Akt activation directly at the level of Akt itself. On the other hand, direct inhibition of Akt phosphorylation by the transfection of a dominant negative form of Akt significantly reduced the replicative potential of MYXV in permissive human cancer cell lines (292). In this current study, four inhibitors that function of block Akt signaling, each with a unique mode of action, were exploited to investigate the details of M-T5 interaction with Akt.

Many commercially available Akt inhibitors act by either preventing the generation of PIP3 by PI3K or by blocking the binding of PIP3 to Akt. This inhibitor mode is utilized by PI analogs, such as Akt inhibitor I, which bind to the PH domain of Akt to occupy the binding site used by PIP3 (110). Another mode of inhibition is by preventing the activation of Akt via inhibition of upstream effectors. For example, the triacyclic
nucleoside Akt inhibitor V targets an Akt effector molecule other than PI3K or PDK1 to selectively inhibit phosphorylation and activation of Akt (131, 305). Furthermore, this inhibitor exhibits little effect towards cellular signaling pathways mediated by PKC, PKA, SGK, Stat3, p38, ERK or JNK and has been shown to preferentially induce apoptosis and growth arrest in cancer cells with aberrant Akt activity both \textit{in vitro} and \textit{in vivo} (131, 305). Alternatively, other drugs like Akt inhibitor VIII specifically bind the PH domain of Akt to promote formation of an inactive conformation, which does not allow phosphorylation by upstream kinases (16, 61, 154, 310). Interestingly, Akt inhibitor VIII does not exhibit any inhibitory effects against PH-domain lacking Akt variants or other closely related AGC family kinases, PKA, PKC and SGK even at concentrations as high as 50 μM (16). Whereas, Akt inhibitor X binds in the ATP binding site of Akt and the mode of inhibition is not PH domain-dependent. Subsequently, the drug inhibits IGF-I-stimulated nuclear translocation of Akt and blocks phosphorylation of the downstream Akt targets, mTOR and p70S6 kinase (272).

We report here that only Akt inhibitor VIII and X were able to reduce viral replication when cells were infected with MYXV (with or without M-T5), however in the absence of M-T5, replication of the knockout virus (vMyxT5KO) was selectively lower in Type I cancer cells treated with the Akt inhibitors I and V (Fig. 4-1). Interestingly, neither Akt inhibitor I or V directly target Akt, but rather function by targeting elements immediately upstream of Akt itself, which may explain the inability of these inhibitors to efficiently reduce replication when M-T5 is expressed by MYXV. In other words, M-T5 can specifically circumvent the effects of these two Akt inhibitors by binding Akt directly. In contrast, the Akt inhibitors VIII and X appear to block activation of Akt in a manner by
which M-T5 is unable to overcome and this suggests that these two drugs block required signaling elements that are exploited when M-T5 activates Akt.

The immunomodulator FTY720, is a synthetic sphingosine analogue of myriocine and has been extensively studied in experimental allotransplantation models and autoimmune disease models (39, 53, 169, 263-265). The combination of FTY720 with cyclosporin (CsA) was reported to further reduce kidney rejection in humans, however in phase III clinical trials, FTY720 alone was found to be no better than the existing standard of care (130). Furthermore, clinical trials in patients with relapsing-remitting multiple sclerosis are currently taking place and preliminary results show benefit for patients receiving FTY720 (198). An increasing number of studies have examined the anti-tumoral properties of FTY720 and in bladder cancer, breast cancer and leukemia FTY720 was found to be effective at inducing apoptosis (11, 12, 168). At the molecular level, FTY720 was shown to activate PP2A-like phosphatases and dephosphorylate Akt resulting in the enhancement of apoptosis through mitochondria by inhibition of Bcl-2 (168). Additionally, dephosphorylation of Akt at residue Ser473 was more pronounced compared to the level of phosphorylation of Thr308 both \textit{in vitro} and \textit{in vivo} models (149) and this specificity prompted us to examine the effects of this drug on MYXV replication. As we report here, FTY720 had little if any effect on wild-type MYXV replication, however in the absence of M-T5, viral titers were specifically and dramatically reduced in Type I human cancer cells compared to mock treated cells. A correlation between lower viral titers of vMyxT5KO and decreased phosphorylation of Akt at residue Ser473 was observed in cells treated with FTY720. In stark contrast, no decrease in phosphorylated Akt was observed in wild-type MYXV-infect cells treated
with FTY720, suggesting M-T5 is able to counteract the mechanism by which FTY720 induces dephosphorylation of Akt in these cells. Similarly, reduced vMyxT5KO replication was observed in cells treated with either Akt inhibitor I or V (Fig. 4-1), however the mechanism by which these compounds block vMyxT5KO may or may not be identical. In either case, FT720 provides an alternative reagent for studying how Akt phosphorylation at specific sites influences MYXV infection in human cancer cells.

Reversible phosphorylation is an important intracellular regulatory mechanism for many diverse cellular processes. Protein phosphatases are a diverse group of proteins that are classified into three distinct categories according to their substrate specificity: serine/threonine-specific, tyrosine-specific, and dual-specificity phosphatases. The serine/threonine protein phosphates are further divided in two subclasses, PPP and PPM, based on biochemical parameters, substrate specificity, and sensitivity to various inhibitors. The PPP class consisting of PP1, PP2A, and calcineurin (also known as PP2B), have high sequence similarity in their catalytic domains but differ in their substrate specificities and interactions with regulatory proteins (120). PP2C shares no sequence similarity with the PPP family of enzymes and is a representative member of the PPM class of phosphatases.

The two phosphatases, PP2A and PHLPP, tightly regulate the cellular activity of Akt via the dephosphorylation of residues Thr308 and Ser473 respectively. Interestingly, when a collection of phosphatase inhibitors was screened for their ability to rescue MYXV replication in nonpermissive Type III human cancer cells, only okadaic acid and endothall were effective at rescuing virus replication. Both okadaic acid and endothall are specific inhibitors of PP2A, suggesting that inhibition of the protein phosphatase
PP2A can relieve the MYXV replicative block in these cells. Furthermore, increased MYXV replication was correlated with elevated levels of Akt phosphorylation at both Ser473 and Thr308. However, in the absence of M-T5, neither okadaic acid nor endothall could successfully restore MYXV permissivity even though increased levels of Akt phosphorylation of Akt at residue Thr308 but not Ser473 were observed. This would imply that M-T5 functions to pro-actively promote the phosphorylation of Akt at residue Ser473, and that this is necessary for productive MYXV replication to occur. Thus, the failure of MYXV to replicate in these nonpermissive Type III cancer cells may be attributed to over expression of PP2A, however this has not been examined in further detail. In this study, the concentration of inhibitors used was low to reduce toxicity, and so some of the phosphatase inhibitors that did not significant alter MYXV replication may have been insufficiently active in these cells at the chosen concentrations.

Previously our lab reported that rapamycin increased the levels of constitutively activated Akt in human giloma cells and enhanced the oncolytic potential of MYXV in an orthotopic human medulloblastoma xenograft mouse model (160). Based on the ability of rapamycin to promote Akt phosphorylation and rescue replication of vMyxT5KO in Type II human cancer cells (252), we were prompted to investigate whether the combination of rapamycin plus okadaic acid could successfully restore vMyxT5KO replication in Type III SK-MEL-5 cells (Fig. 4-4). Even in the absence of MYXV infection, increased Akt phosphorylation of Akt at both Ser473 and Thr308 residues was observed following the dual drug treatment of these cells, but not with either drug alone. The results suggest that following hemi-phosphorylation of Akt at residue Thr308, M-T5 is able to promote the subsequent phosphorylation of residue Ser473 and thus fully
activate Akt. Moreover, these results show that MYXV replication in Type III human cancer cells can be achieved through manipulation of the PI3K/Akt/mTOR signaling cascade, which ultimately lead to the full activation of Akt.

Although many virus-encoded proteins have been reported to target the PI3K/Akt/mTOR signaling cascade, M-T5 is the only viral protein shown to date that directly binds and activates Akt. Interestingly, no physical interaction between M-T5 and Akt was observed in Type III cells that exhibit very low levels of constitutively phosphorylated Akt, which suggests that the phospho-status of Akt may dictate the initial binding of M-T5. In support of this, when a constitutively active variant of Akt was tested, binding of M-T5 and Akt could be readily detected in each of the three classes of cancer cells. On the contrary, a non-phosphorylatable Akt variant (with alanine at the two phosphorylation sites) was unable to bind M-T5 in all three cell lines examined (Fig. 4-5). These results are congruent with previous studies in which expression of constitutively active Akt was able to rescue MYXV infectivity in Type III human cancer cells, however even transfection of M-T5 could not rescue MYXV replication in these cells (292), which we now believe is because the M-T5 cannot bind completely unphosphorylated Akt. The rate-limiting step to Akt activation is the binding of PIP₃ to the PH domain of Akt, which promotes Akt localization to the plasma membrane. Once correctly positioned at the plasma membrane, Akt can then be phosphorylated by its activating kinases, leading to full activation. Furthermore, phosphorylation of both residues is required for full Akt activity, and phosphorylation of Thr308 is not dependent on phosphorylation of Ser473 or vice versa, as measured by in vitro kinase assays (7). Analysis of Akt mutants in which the Thr308 or Ser473 sites were individually converted
to alanine showed that phosphorylation of Thr308, but not residue Ser473, was critical for binding M-T5 (Fig. 4-6). These results suggest that MYXV tropism in human cancer is largely dependent upon the endogenous phospho-status of Akt residue Thr308. As one model, phosphorylation of Akt at residue Thr308 may induce a conformational change allowing M-T5 access to a previously blocked binding site. Once M-T5 is bound to hemi-phosphorylated Akt, it is not yet known whether M-T5 alters availability of Akt to kinases or phosphatases that regulate the Ser473 site, however the subsequent phosphorylation of Ser473 is particularly dramatic. Future studies will examine the possible mechanism(s) by which M-T5 regulates Ser473 phosphorylation, for example by increasing access to the kinase, mTORC2, or decreasing access to the protein phosphatase, PHLPP.

In summary, poxviruses encode a myriad of proteins that coordinate remarkable intracellular signaling modifications to establish an environment, which will support a productive virus replication. Although M-T5 has been shown to enhance MYXV replication through interaction with Akt, many of the fundamental questions regarding the mechanism of this interaction remain poorly understood. The results in this study suggest that binding of M-T5 is dependent upon the endogenous phospho-status of Akt residue Thr308 and pharmacological manipulation of the Akt signaling pathway can significantly influence the outcome of MYXV replication in human cancer cells. Thus, this knowledge may have significant implications for the identification of novel compounds to use in conjunction with MYXV virotherapy to increase the oncolytic potential of this virus. Furthermore, understanding the interaction between viral host-range factors like M-T5 and cellular targets such as Akt will provide invaluable insights
into how vital cellular networks can be re-programmed by viral factors to increase virus survival at the cellular level.
CHAPTER 5
M-T5 CAN BE FUNCTIONALLY REPLACED BY CELLULAR PIKE-A

Introduction

Functional and sequence similarities between many virus-encoded host-range and immunomodulator genes suggest they have been originally hijacked directly from an ancestral host. However, the evolutionary origins of certain virus-encoded immunomodulatory proteins are often complicated to identify. For example, M-T5 shares no significant sequence similarity to any known cellular genes and therefore the evolutionary origin of M-T5 has remained elusive. M-T5 was first identified as a virulence factor that is critical for productive myxomatosis in infected rabbits, based on its ability to inhibit apoptosis in rabbit T lymphocytes (186). Although MYXV is a rabbit specific pathogen, the virus can productively infect a wide variety of human cancer cells and is currently being developed as an oncolytic therapeutic because it is non-pathogenic in man (159, 266). In human cancer cells infected with MYXV, interaction between M-T5 and cellular Akt was demonstrated to upregulate the kinase activity of Akt and enhance viral replication (292). Furthermore, the susceptibility of human cancer cells to be infected and killed by MYXV is directly correlated to the basal level of endogenous phosphorylated Akt. Thus, if Akt was pre-activated or could be activated by MYXV infection, via M-T5, the cancer cells were permissive but if Akt remained unactivated the cells were unable to support

Here we demonstrate that ectopic overexpression of PIKE-A (PI3-kinase enhancer activating AKT) is able to rescue the ability of vMyxT5KO to productively infect Type II human cancer cells that were previously resistant to infection. In addition, Type III cancer cells, which did not support the replication of either wild-type vMyx or
vMyxT5KO, were rendered permissive to MYXV replication when these cells were transiently transfected to express PIKE-A before infection. Elevated levels of phosphorylated Akt was observed when PIKE-A transfected cancer cells were infected with either wild-type vMyx or vMyxT5KO. Finally, virus-induced apoptosis in infected Type II and Type III cancer cells was blocked by transfected PIKE-A prior to MYXV infection. We conclude that the MYXV M-T5 host-range protein is functionally interchangeable with the host PIKE-A protein, and that the activation of host Akt by either M-T5 or PIKE-A is critical for the permissiveness of human cancer cells by MYXV. The implications of these results for the development of MYXV as an oncolytic agent to treat human cancer will be further discussed.

Results

M-T5 Sequence Exhibits Similarity to Cellular PIKE-A

We have recently demonstrated that M-T5 forms a complex with cellular Akt and as a result upregulates its kinase activity in MYXV-infected human cancer cells (292). Given this ability to regulate Akt activation, we initiated a search for cellular proteins capable of binding and activating Akt in a fashion similar to M-T5 and one particular protein caught our attention. A physiological mediator of Akt, PIKE-A has been demonstrated to directly bind Akt in a guanine nucleotide-dependent manner, stimulating the kinase activity of Akt and promoting the invasiveness of cancer cell lines (3). Functional PIKE-A stimulates Akt kinase activation through two domains; the N-terminal GTPase domain (first 128 residues) and the ANK-repeats located at the C-terminus, both of which have been shown to independently associate with the regulatory and partial catalytic domains of Akt (4) (Fig. 5-1A). Although M-T5 shares no significant homology to any known non-viral proteins, sequence alignment identified some
Figure 5-1. M-T5 exhibits sequence similarity to PIKE-A. (A) M-T5 features including the predicted ANK-repeats (I-VII) and PRANC/F-box like domain located at the C-terminus; compared to the structure of PIKE-A. Underlined sections indicate the regions of PIKE-A sufficient to independently bind Akt, the left line matches the amino acid sequence alignment in part B and the right line matches part C. The N-terminal sequences (1-128 aa) (B) and C-terminus sequence (734-836 aa) (C) of PIKE-A were aligned with the N-terminus of MYXV M-T5. Conserved residues are boxed. Dark shading indicates identical residues and light shading indicates similar residues. The bars above the M-T5 sequence define the predicted ANK-repeats I and II.

similarity between the N-terminus of M-T5 and the N-terminal 128 residues of PIKE-A (18% identity, 33% similarity) (Fig. 5-1B). Likewise, sequence similarity was observed between the C-terminus of PIKE-A and the N-terminus of M-T5 (18% identity, 36% similarity) (Fig. 5-1C). This lead us to speculate that the sequence homology and functional similarities shared between these two proteins could be of particular importance. To further investigate whether M-T5 represented a viral molecule that had
adopted functions similar to cellular PIKE-A we performed a series of experiments to examine this functional similarity.

**Transient Expression of PIKE-A Rescues Myxoma Virus Replication in Restrictive Human Cancer Cells**

Virus permissivity was observed to be dependent upon the basal level of endogenous phosphorylated Akt, when a wide spectrum of human cancer cell lines were screened for their ability to support MYXV replication (266, 292). Since PIKE-A has been demonstrated to bind and elevate the kinase activity of Akt we wanted to determine if overexpression of PIKE-A was able to influence the permissiveness of MYXV in various cancer cell types. For this study the following human cancer cell lines were used as representatives for the three cell types; HOS (Type I), 786-0 (Type II) and MDA-435 (Type III). The cell lines, 786-0 and MDA-MB435 were transfected with control plasmid or a plasmid containing the PIKE-A gene for 8 h and then mock infected or infected with either vMyx or vMyxT5KO at an MOI of 5. Cell lysates were collected at 48 hpi and expression levels of the late MYXV gene Serp-1 were assessed by Western blot analysis. For the reason that poxvirus late genes require active virus replication to undergo expression, our lab routinely uses the MYXV late gene Serp-1 as a marker to represent successful virus replication. Based on the presence of Serp-1, 786-0 cells supported replication of vMyx (Fig. 5-2A, lane 3), however were non-permissive to vMyxT5KO because expression of Serp-1 was not detected by Western blot (Fig. 5-2A, lane 5). Similarly, MDA-MB435 cells did not support replication of either virus (Fig. 5-2A, lanes 8 and 10) as confirmed by the absence of Serp-1 expression. In stark contrast, expression of Serp-1 was detected when 786-0 (Fig. 5-2A, lanes 2 and 4) and MDA-MB435 (Fig. 5-2A, lanes 7 and 9) cells were transfected with PIKE-A 8 h prior to being
Figure 5-2. Expression of cellular PIKE-A rescues MYXV infection in restrictive cancer cells. (A) The MYXV restrictive Type II (786-0) and MYXV-nonpermissive Type III (MDA-MB435) human cancer cells were transfected with a Myc-tagged PIKE-A expressing plasmid (lanes 2, 4, 7 and 9) or a control plasmid (lanes 1, 3, 5, 6, 8 and 10) for 8 h and then mock infected (lane 1) or infected with either vMyx (lanes 2, 3, 7 and 8) or vMyxT5KO (lanes 4, 5, 9 and 10) at an MOI of 5. Cell samples were collected at 48 hpi and cell lysates were examined by immunoblotting with anti-Serp-1 (late viral gene) and anti-Myc (PIKE-A). Single-step growth analysis of (B) HOS (C) 786-0 and (D) MDA-MB435 cells were transfected with the PIKE-A containing plasmid (■) or a control plasmid (□) for 12 h and then infected with either vMyx (—) or vMyxT5KO (---) at a MOI of 5. Cells were harvested at the indicated times post infection and infectious virus titers were determined on BGMK cells. Each viral growth analysis was performed in triplicate.

infected with either vMyx or vMyxT5KO. Whereas, no Serp-1 expression was detected in either mock-infected 786-0 or MDA-MB435 cells (Fig. 5-2A, lanes 1 and 6 respectively). Samples were probed with an anti-Myc antibody to demonstrate expression of the transfected Myc-tagged PIKE-A protein and expression of the Myc epitope was only detected in cells transfected with the PIKE-A plasmid (Fig. 5-2A, lanes 2, 4, 7 and 9). Additionally, increased expression of Serp-1 did not correlate with the
level of PIKE-A expression in transfected cells, but was dependent upon infection by MYXV. Virus replication as confirmed by the expression of MYXV Serp-1 protein was observed in human cancer cells which were previously non-permissive to MYXV, when transfected with PIKE-A prior to infection. Thus suggesting that overexpression of PIKE-A preceding MYXV infection was able to rescue previously non-productive infection and allow virus replication. Furthermore, in the absence of M-T5, overexpression of PIKE-A could promote vMyxT5KO replication in cells that previously support replication of the knockout virus.

To quantitatively assess the ability of MYXV to replicate, single-step growth curves on representative Type I, II and III cells were performed. Each cell type was transfected with the PIKE-A expressing plasmid or a control plasmid and 8 h later were infected with either vMyx or vMyxT5KO at an MOI of 5. Samples were harvested for infectious virus particles at 0, 4, 8, 12, 24 and 48 hpi, and all time points were titrated on BGMK cells by serial dilutions followed by X-gal staining to visualize foci. Infection of HOS cells (Type I) with vMyx and vMyxT5KO produced growth curves characteristic of a classical poxvirus replication kinetics. A minimum virus titer was reached at approximately 4 hpi followed by a continuous increase up to 48 hpi, at which point the virus yield reached maximal levels. Identical replication curves were generated for vMyx infection of HOS cells regardless of the expression of PIKE-A. However, HOS cells infected with vMyxT5KO produced a slightly lower yield, nevertheless transfection of PIKE-A before infection restored virus titer to a level similar to that of cells infected with vMyx (Fig. 5-2B). Type II cells (786-0) completely supported vMyx infection but were non-permissive to vMyxT5KO. When 786-0 cells were transfected with PIKE-A and then infected with
vMyxT5KO, the viral titers indicated that transfection of PIKE-A before infection, was able to rescue vMyxT5KO replication to levels similar to cells infected with vMyx (Fig. 5-2C). Virus replication of either vMyx or vMyxT5KO was not supported in the Type III cells (MDA-MB435) and as a result little or no viral amplification was observed overtime. Viral titers were increased considerably when cells were transfected with PIKE-A prior to infection indicating rescue of virus replication (Fig. 5-2D). The ability of PIKE-A to rescue viral replication in previously non-premissive human cancer cell lines was further demonstrated when cells were transfected with PIKE-A and infected with viruses which express gfp (vMyx-gfp or vMyxT5KO-gfp). No foci were observed when Type II cells were infected with vMyxT5KO-gfp, however endogenous PIKE-A expression prior to infection with vMyxT5KO-gfp rescued foci formation. Virus replication of either vMyx-gfp or vMyxT5KO-gfp in Type III cells was only supported when cells were transfected with PIKE-A 8 h before infection with either virus (data not included). These data demonstrate that over-expression of exogenous PIKE-A has the ability to render previously restricted human cancer cells fully permissive to MYXV infection.

Among viruses, poxviruses are unique in that they have the ability to effectively and efficiently enter almost any cell type. However, the virus replication is often restricted because the virus is unable to complete its replicative cycle within the infected cell (184). Earlier studies have shown that in non-permissive cancer cells MYXV can successfully bind, uncoat and begin early gene expression. The block to a productive infection however lies in the inability of M-T5 to bind and activate Akt (292). In cell lines previously non-permissive to MYXV-replication, over-expression of PIKE-A is predicted to specifically upregulate the normally low kinase activity of Akt. PIKE-A therefore
directly modulates the PI3K/Akt signal pathway promoting virus permissivity by releasing the block prior to virus replication and virus late gene expression but does not alter viral entry into the cell.

**Transient Expression of PIKE-A Upregulates the Kinase Activity of Akt in Type II and Type III Human Cancer Cells.**

Amplification of PIKE-A has been observed in a variety of human glioblastoma cells which coincidently results in the upregulation of Akt kinase activity (3). A plasmid containing the PIKE-A gene was transfected into HEK293 cells, lysates were collected at various time points and resolved by Western blot. Expression of PIKE-A was detected at 8 h following transfection (Fig. 5-3A, lane 3) and expression continued to increase over time (Fig. 5-3A). To determine if overexpression of PIKE-A would have the ability to increase kinase activity of Akt, Type II cells (786-0) and Type III cells (MDA-MB435) were transfected with the PIKE-A plasmid. Cell lysates were collected at various time points following transfection of PIKE-A and Akt phosphorylation was assessed by Western blot. Low levels of endogenous Akt phosphorylation were detected at both Ser473 and Thr308 residues in the 786-0 cells at 0 h (Fig. 5-3B, lane 1). Following transfection of PIKE-A, phosphorylation of Akt at Ser473 was dramatically increased as early as 12 h (Fig. 5-3B, lane 4) and phosphorylation of Thr308 was detected at 24 h (Fig. 5-3B, lane 5). In the MBA-MB435 cells very low levels of Akt phosphorylation were detected at 0 h (Fig. 5-3C, lane 7), however at 8 h overexpression of PIKE-A considerably induced Akt phosphorylation at Ser473 and increased phosphorylation levels of Thr308 were detected at 24 h (Fig. 5-3C, lane 4). The levels of total Akt protein remained relatively constant (Fig. 5-3B and C). As predicted the
Figure 5-3. Induction of endogenous Akt phosphorylation following transfection of PIKE-A in human cancer cells. (A) HEK293 cells were transfected with the PIKE-A plasmid and expression was detected at various time point by immunoblotting with an anti-Myc antibody. PIKE-A plasmid was transfected into (B) 786-0 and (C) MDA-MB435 cells and Akt phosphorylation at Ser473 and Thr308 sites were detected in cell lysates by Western blot at various times following transfection. Levels of Akt phosphorylation at Ser473 and Thr308 were determined by Molecular Imaging software (Kodak) and compared to the protein level of Akt to quantify fold stimulation induced by overexpression of PIKE-A in (D) 786-0 and (E) MDA-MB435 cells. Immunoblot signal variability between films were normalized to total Akt. Overexpression of exogenous PIKE-A induces the phosphorylation of Akt at Ser473 and Thr308 sites in Type II and III cell lines. In both cell lines the phosphorylation of Ser473
occurred earlier and the band intensity was more intense in contrast to phosphorylation of Thr308 (Fig. 5-3D and E). Furthermore trivial differences in the pattern of Akt phosphorylation was observed, suggesting the response to the overexpression of PIKE-A may be unique for each cell line. Therefore, in human cancer cells, which express little or no detectable levels of endogenous phosphorylated Akt, Akt kinase activity can be induced through the overexpression of its physiological regulator, PIKE-A.

**Induction of Akt Kinase Activity Following Transfection of PIKE-A in vMyxT5KO Virus Infected Type II Human Cancer Cells**

Susceptibility of human cancer cells to MYXV infection is dependent upon the basal level of endogenous phosphorylated Akt (292). In the 786-0 cells (Type II) the level of endogenous phosphorylated Akt was shown to be very low, however infection with vMyx dramatically induced Akt phosphorylation. The viral protein M-T5 was a critical determinant of MYXV tropism in human cancer cells and in its absence endogenous levels of phosphorylated Akt remained relatively unchanged in vMyxT5KO infected 786-0 cells (292). As overexpression of PIKE-A increases Akt kinase activity we wanted to examine levels of phosphorylated Akt following vMyx or vMyxT5KO infection in the presence or absence of exogenous PIKE-A. Cells were transfected with the PIKE-A plasmid or a control plasmid for 8 h and then infected with either vMyx or vMyxT5KO. Cell lysates were collected at various time points and phosphorylation of Akt was assessed by Western blot analysis. Increased levels of endogenous phosphorylated Akt at both Ser473 and Thr308 residues were detected at 8 hpi in 786-0 cells infected with vMyx either in the presence or absence of endogenous expression of PIKE-A (Fig. 5-4A and B, lanes 1-6). In contrast, 786-0 cells infected with vMyxT5KO exhibited very little Akt phosphorylation (Fig. 5-4A, lanes 7-12) yet transfection of PIKE-A prior to infection
Figure 5-4. PIKE-A upregulates Akt phosphorylation in Type II cells infected with vMyxT5KO. Human 786-0 (Type II) cancer cells were either transfected with (A) control plasmid or (B) PIKE-A expressing plasmid and 8 h later were infected with either vMyx (lanes 1-6) or vMyxT5KO (lanes 7-12) at an MOI of 5. Cells were harvested at the indicated times post infection and Akt phosphorylation, at both Ser473 and Thr308 sites, was detected in cell lysates. Total Akt protein levels are shown in the bottom lines. Densitometry was used to measure induction of Akt phosphorylation at both (C) Ser473 and (D) Thr308 as described in Fig. 5-3.

by vMyxT5KO considerably induced phosphorylation of Akt at both Ser473 and Thr308 compared to levels observed in the absence of exogenous PIKE-A (Fig. 5-4B, lanes 7-12). The levels of total Akt protein remained relatively constant following vMxy and vMyxT5KO infection (Fig. 5-4A and B). Densitometry levels of Akt phosphorylation at both Ser473 (Fig. 5-4C) and Thr308 (Fig. 5-4D) were detected by Molecular Imaging software (Kodak) and compared to the protein Akt to quantitate phosphorylation induction following transfection of PIKE-A. Taken together the data in Figures 5-2 to 5-4 confirm that overexpression of PIKE-A, in Type II cells (786-0), was able to upregulate
the kinase activity of Akt, which is critical for the replication of MYXV even in the absence of M-T5.

**Transfection of PIKE-A Stimulates Akt Phosphorylation in Myxoma Virus Infected Type III Cells**

The basal level of endogenous phosphorylated Akt was undetectable in Type III cells (MDA-MB435, Fig. 5-5A, lanes 1 and 7), which do not support replication of either vMyx or vMyxT5KO. Levels of Akt phosphorylation remained undetectable following vMyx and vMyxT5KO infection (Fig. 5-5A, lanes 1-12) suggesting that M-T5 is unable to activate Akt kinase activity in Type III cells. Transfection of PIKE-A in MDA-MB435 cells prior to infection with either vMyx (Fig. 5-5B, lanes 1-6) or vMyxT5KO (Fig. 5-5B, lanes 7-12) dramatically induced phosphorylation of endogenous Akt at residues Ser473 and Thr308 at 8 hpi. Total Akt protein levels remained relatively unchanged (Fig. 5-5A and B) as observed in the 786-0 cells (Fig. 5-4A and B). Fold induction of Akt phosphorylation was determined by measuring the level of Akt phosphorylation at both Ser473 (Fig. 5-5C) and Thr308 (Fig. 5-5D) residues by Molecular Imaging software (Kodak) and was compared to the expression levels of the Akt protein. Together these data indicate that Type III cells, which do not support replication of MYXV, are unable to activate Akt kinase activity when infected with MYXV, in the presence or absence of M-T5. However, ectopic expression of PIKE-A will induce Akt phosphorylation at Ser473 and Thr308 sites and rescue replication of both vMyx and vMyxT5KO. These results help to explain the mechanism by which overexpression of PIKE-A contributes to rescuing virus replication in the Type III cells as observed previously in Figures 5-2B and D.
Figure 5-5. Overexpression of PIKE-A stimulates Akt phosphorylation in Type III cells infected with either vMyx or vMyxT5KO. MDA-MB435 (Type III) cells were either transfected with (A) control plasmid or (B) PIKE-A expressing plasmid. At 8 h following transfection cells were infected with either vMyx (lanes 1-6) or vMyxT5KO (lanes 7-12) at an MOI of 5. Cells were harvested at the indicated times post infection and Akt phosphorylation, at both Ser473 and Thr308 sites, was detected in cell lysates. Total Akt protein levels are shown in the bottom lines. Densitometry was used to measure induction of Akt phosphorylation at both (C) Ser473 and (D) Thr308 as described in Figure 5-3.

PIKE-A Inhibition of Apoptosis in Myxoma Virus Infected Human Cancer Cells

Following MYXV infection of human cancer cells a cascade of events, which induce apoptosis are initiated. When M-T5 is present and expressed it plays a critical role in preventing apoptosis through manipulation of the cell cycle (127). PIKE-A, a physiological regulator of Akt activation (109), is often amplified in human cancer cells and coincidently has been shown to promote cellular proliferation by inhibiting apoptosis through stimulation of Akt (2). Since transient expression of PIKE-A in Type II and Type
III human cancer cells stimulates phosphorylation of Akt (Fig. 5-5) we wanted to determine if overexpression of PIKE-A also functioned to inhibit apoptosis induction following vMyxT5KO infection of Type II cells and MYXV infection of Type III cells. To investigate this possibility Type II (786-0) and Type III cells (MDA-MB435) were infected with either vMyx or vMyxT5KO and cleavage of pro-Caspase-3 to its active form was assessed by Western blot. As expected from our previous studies, infection of 786-0 cells with MYXV did not induce apoptosis (127) and no cleavage of pro-Caspase-3 was observed (Fig. 5-6A, lanes 1-6). Type II cells (786-0) infected with vMyxT5KO induced activation of Caspase-3, observed as early as 8 hpi (Fig. 5-6A, lanes 7-12). However, Type II cells were transfected PIKE-A 8 h prior to infection with vMyxT5KO, cleavage of Caspase-3 was not detected, indicating that inhibition of caspase-mediated apoptosis was observed even in the absence of M-T5 (Fig. 5-6B, lanes 7-12).

![Figure 5-6](image-url)
Induction of apoptosis as indicated by Caspase-3 cleavage in Type III cells (MDA-MB435) was observed when cells were infected with either vMyx (Fig. 5-6C, lanes 1-6) or vMyxT5KO (Fig. 5-6C, lanes 7-12). Exogenous expression of PIKE-A was successful at inhibiting apoptosis following infection with either vMyx (Fig. 5-6D, lanes 1-6) or vMyxT5KO (Fig. 5-6D, lanes 7-12) by preventing the cleavage of pro-Caspase-3 into its active form. Therefore, transfection of PIKE-A in Type II cells was able to inhibit vMyxT5KO-induced apoptosis in Type II cells. Viral-induced apoptosis was also inhibited in Type III cells infected with either vMyx or vMyxT5KO when the cells had previously been transfected with PIKE-A. Stimulation of Akt kinase activity, in response to overexpression of PIKE-A, promoted the inhibition of the apoptotic signaling cascade, which would otherwise be activated in MYXV infected cells (Type III), especially in the absence of M-T5 (Type II and Type III cells).

Discussion

A number of cellular pathways, frequently mutated in cancer, were examined in MYXV permissive human cancer cells and the Akt pathway was identified to be a key restriction determinant for virus replication (292). The oncogene, Akt is a critical regulator of diverse cellular processes and has been demonstrated to contribute to cancer progression through stimulation of proliferation and inhibition of apoptosis (197, 271). The critical role of Akt in the regulation of multiple cellular functions makes it a central manipulator of cellular signaling and therefore it is not surprising a number of viruses have developed sophisticated strategies to manipulate the activation of Akt (148). For example, respiratory syncytial virus (RSV) induces activation of the PI3K/Akt signaling pathway during early viral infection (273), thereby increasing cell survival and ensuring the virus has sufficient time to complete its replicative cycle (58). MYXV also
manipulates the Akt pathway through the actions the protein M-T5 which has been shown to bind and upregulate the kinase activity of Akt during MYXV infection (292). Akt kinase activity is upregulated through direct binding to PIKE-A. Over expression of PIKE-A stimulates Akt activity promoting cellular transformation leading to the development of cancer, while knockdown of PIKE-A diminishes Akt activity and increases apoptosis. Amplified PIKE-A has been identified in a number of human glioma cancer cells which express increased levels of Akt phosphorylation and reduced activation of apoptosis (2).

Here, we demonstrate that restrictive Type II human cancer cells will switch from resistant to susceptible for vMyxT5KO-infection, following transient expression of PIKE-A. In the absence of M-T5, MYXV is unable to stimulate kinase activity of Akt, however overexpression of exogenous PIKE-A in Type II cells considerably increased levels of Akt phosphorylation at both Ser473 and Thr308 residues (Fig. 5-5). Type III human cancer cells are non-permissive to both vMyx and vMyxT5KO infection and Type III cells do not express basal levels of detectable, endogenous phosphorylated Akt (Fig. 5-5). Similar to the observation in Type II cells, expression of PIKE-A renders non-permissive Type III cells susceptible for both vMyx and vMyxT5KO infection and upregulates Akt kinase activity (Figs. 5-2, 4 and 5). The fact that exogenous PIKE-A rescues MYXV replication in previously non-premissive human cancer cells only strengthens the argument that the Akt pathway is a key restriction determinant for permissiveness of human cancer cells by MYXV. In addition to stimulating Akt kinase activity, transfection of PIKE-A was responsible for inhibiting activation of viral-induced apoptosis following MYXV infection in Type II and III human cancer cells. M-T5 also
inhibits MYXV-induced apoptosis by protecting MYXV-infected cells from cell cycle arrest which otherwise would promote the activation of the apoptotic cascade (127). Sequence similarity between MYXV M-T5 and cellular PIKE-A is limited to the previously identified region of PIKE-A necessary to bind Akt. However, both M-T5 and PIKE-A contain ANK-repeats, share the ability to upregulate the Akt pathway, block apoptosis and to interact with Akt. Functionally, M-T5 and PIKE-A represent a viral and cellular molecule evolved to control Akt activation.

A number of virus-encoded proteins, several of which are host-related immunomodulatory genes, share sequence and functional similarity with cellular proteins and are categorized as viral homologs. Many of these viral homologs are gene products which have been hijacked from their host, increasing the replicative ability of the virus (229). Although M-T5 and PIKE-A share similar functions, based on this preliminary study we would predict that M-T5 represents a viral strategy evolved to mimic the cellular activity of PIKE-A. We demonstrate that exogenous PIKE-A is able to upregulate Akt kinase activity and rescue MYXV replication in Type III human cancer cells. Interaction of Type III cells with MYXV does not produce a productive infection even though M-T5 is expressed. We suspect that M-T5 expression and localization is altered during infection of Type III cells (292). Therefore, the mechanism by which PIKE-A activates Akt may exhibit some differences from the method employed by M-T5.

PIKE-A provides an alternative model for studying the importance of Akt phosphorylation during a productive MYXV infection in human cancer cells. Understanding the mechanism by which PIKE-A rescues MYXV replication in previously non-permissive human cancer cells may provide additional clues by which M-T5
functions during MYXV infection. We predict that cells with a high level of PIKE-A expression will be naturally more susceptible to MYXV infection. Additionally, the M-T5 protein possesses seven ANK-repeat domains, which are thought to mediate specific protein–protein interactions. Therefore we speculate that M-T5 acts as a molecular scaffold bringing together proteins, which may otherwise be spatially and temporally isolated, thus stimulating signaling pathways critical for successful MYXV replication. Studying the functional role of PIKE-A may give us further insight into additional proteins, which may interact with M-T5 and counteract MYXV replication in the human cancer cells. The results in this study suggest that manipulation of the Akt pathway through the actions of PIKE-A may allow the oncolytic capacity of this virus to extend to an even broader spectrum of human cancer cells. In conclusion, this knowledge may have significant implications towards the rational design of the next generation of oncolytic viruses, as the development of new and improved cancer therapies continues.
CHAPTER 6
CONCLUSIONS

Overview of the Study

Most poxviruses express multiple proteins containing ANK-repeats, accounting for a large superfamily of related but unique determinants of poxviral tropism. Recently, select members of this novel family of poxvirus proteins have drawn considerable attention for their potential roles in modulating intracellular signaling networks during viral infection. The rabbit-specific poxvirus, MYXV, encodes four unique ANK-repeat proteins, termed M-T5, M148, M149 and M150, all of which include a C-terminal PRANC domain which closely resembles a cellular protein motif called the F-box domain. We have shown that each MYXV-encoded ANK-repeat protein, including M-T5, interacts directly with the Skp1 component of the host SCF ubiquitin ligase complex, and that the binding of M-T5 to CUL1 (127) is indirect via binding to Skp1 in the host SCF complex. To understand the significance of these viral-host protein interactions, the various binding domains of M-T5 were mapped. The N-terminal ANK-repeats I and II were identified as being important for interaction with Akt, whereas the C-terminal PRANC/F-box like domain was essential for binding to Skp1/SCF. We also report that M-T5 can bind Akt and the host SCF complex (via Skp1) simultaneously in MYXV-infected cells. Finally, we report that M-T5 specifically mediates the re-localization of Akt from the nucleus to the cytoplasm during infection with the wild-type MYXV, but not the M-T5 knockout version of the virus (vMyxT5KO). These results indicate that ANK/PRANC proteins play a critical role in reprogramming disparate cellular signaling cascades to establish a new cellular environment more favorable for virus replication.
MYXV permissiveness in at least some human cancer cells (called Type II) is dependent upon the direct interaction between M-T5 and Akt, which has been shown to induce the kinase activity of Akt. An array of compounds that selectively manipulate the Akt signaling network was screened and certain Akt inhibitors were shown to significantly decrease the ability of MYXV to replicate in previously permissive human cancer cells. Furthermore, reduced viral replication efficiency was correlated to lower levels of endogenous phosphorylated Akt. In contrast, the PP2A specific phosphatase inhibitor, okadaic acid, promoted increased Akt kinase activation and rescued MYXV replication in cancer cells that did not previously support viral replication (called Type III cells). Finally, phosphorylation of Akt at residue Thr308 was shown to dictate the physical interaction between Akt and M-T5, which ultimately leads to a productive MYXV replication in the permissive human cancer cells. The results of this study further characterize the mechanism by which M-T5 utilizes the Akt signaling cascade to enhance the replication efficiency of MYXV in human cancer cells.

In searching for a host counterpart of M-T5, we noted sequence similarity of M-T5 to a recently identified ANK-repeat cellular binding protein of Akt called PIKE-A. PIKE-A binds and activates the kinase activity of Akt in a GTP-dependent manner and promotes the invasiveness of human cancer cell lines. Here we demonstrate that transfected PIKE-A is able to rescue the ability of vMyxT5KO to productively infect Type II human cancer cells that were previously resistant to infection. As well, cancer cells that were completely non-permissive for both vMyx and vMyxT5KO infection (Type III) were rendered fully permissive following ectopic expression of PIKE-A. We conclude that the MYXV M-T5 host-range protein is functionally interchangeable with the host PIKE-A
protein, and that the activation of host Akt by either M-T5 or PIKE-A is critical for the permissiveness of human cancer cells by MYXV.

**Poxviral ANK/PRANC Proteins Influence Virus Host-Range**

As a family of viruses, poxviruses collectively exhibit a broad host-range and most of the individual members are capable of replicating in a wide array of cell types from various host species, at least in vitro (171). At the cellular level, poxvirus tropism is dependent not upon unique cell-specific surface receptors, but rather upon the ability of the cell to provide intracellular complementing factors needed for productive virus replication, and the ability of the specific virus to successfully manipulate intracellular signaling networks that regulate cellular anti-viral processes downstream of virus entry (125). The large genomic coding capacity of poxviruses enables the virus to express a unique collection of viral proteins that function as host-range factors, which specifically target and manipulate host signaling pathways to establish optimal cellular conditions for viral replication (124, 171). Functionally, the known host-range factors from poxviruses have been associated with manipulation of a diverse array of cellular targets, which includes cellular kinases and phosphatases, apoptosis and various antiviral pathways (229). To date, a relative small number of poxvirus host-range genes have been identified and fewer of these have been functionally characterized (297). For this reason, poxvirus host-range factors represent a potential gold mine for the discovery of novel pathogen-host protein interactions. Many of the known poxviral host-range genes are not only functionally diverse but also demonstrate a wide spectrum of biochemical and structural characteristics (297). Relatively little sequence similarity is shared among the family of poxvirus host-range genes, however the ANK-repeat motif is found in many of them (176).
Cowpox Virus is a Reservoir for Host-Range Genes

More than 30 poxvirus genomes have been fully sequenced to date and sequence analysis has increased our knowledge and understanding of virus-host interactions. CPXV contains one of the largest genomes known in the chordopoxvirus family and in all probability encodes more host-range factors involved in cell tropism than any other mammalian poxvirus family member. In contrast to VACV, CPXV can productively replicate in CHO cells, so the genetic basis for this difference in host-range has been of some interest. A 77-kDa CPXV protein, called either CHOhr or CP77, encoded by the V025 gene was identified for its ability to permit CPXV replication in CHO cells (248). CHOhr is a nine-ANK motif protein, which also contains a C-terminal PRANC domain and is one of 12 ANK/PRANC proteins encoded by CPXV. One functional role of CHOhr is likely to avoid the abrupt and early shutoff of protein synthesis, which is characteristic of VACV infection in CHO cells (68, 107). Furthermore, CHOhr was shown to bind and promote the dissociation of HMG20A from the viral factories, providing the first cellular target regulated by viral host-range CHOhr protein (108). The significance of this novel host protein interaction, as well as the molecular mechanism by which CHOhr functions to expand the host-range of VACV to include CHO cells, remains poorly understood and merits further study.

More interestingly, the CHOhr gene was demonstrated to provide functionally equivalent host-range functions to K1L and C7L by permitting VACV strain Copenhagen (COP) gene knockout constructs of K1L or C7L to replicate on human and porcine kidney cells (203). Similarly, the host restriction of a K1L-deleted VACV strain WR could be reversed in RK13 cells when complemented by CHOhr (56, 211). Although K1L contains multiple ANK-repeats, like CHOhr, the protein lacks a C-terminal PRANC
domain. K1L is highly conserved in orthopoxviruses and has presumably evolved a function unrelated to the SCF complex that partially overlaps with the function of CHOhr. Even more puzzling is that both motifs (ANK and PRANC) are absent from C7L, thus the mechanism by which CHOhr can functional compensate for C7L is a remarkable phenomenon. Since CHOhr gene supports orthopoxvirus replication in the broadest range of mammalian cell types tested, it would suggest this gene would be the one most conserved among members of the orthopoxviruses. On the contrary, the VACV homolog of CHOhr is deleted in strain COP (91) and relative to the CPXV CHOhr sequence the orthologous pseudogene in the VACV strain WR genome has been inactivated by multiple substitutions and frame shift mutations (140). The genomic evidence suggests that VACV evolved from an ancestral virus that contained a functional counterpart of the intact CHOhr gene and its function was lost through either mutation or deletion, possibly during the 200 years of VACV “domestication” since its first isolation in the days of Jenner.

**Modified Vaccinia Ankara Lacks Many Orthopoxvirus Host-Range Factors**

In stark contrast to CPXV, which can replicate in a wide spectrum of cells, the highly attenuated vaccinia strain modified Ankara (MVA) is severely host-range restricted and unable to efficiently propagate in mammalian cells (179). MVA was generated by over 570 passages in chicken embryo fibroblasts (CEF) and relative to the parental strain, MVA contains multiple gene mutations in addition to major deletions in both the left and right termini accounting for approximately 15 percent of its genomic information lost (179). Whereas, genes present in the central conserved region of the genome remained intact, with the exception of the major membrane proteins, F11L and O1L (8). MVA lacks many genes associated with virulence and/or regulation of virus
tropism. For example, the orthopoxvirus host-range gene K1L is partially deleted in MVA, similar to the situation in VARV and the CHOhr homolog is split into several separate ORFs. Furthermore, all ANK-repeat containing genes in MVA are either defective or deleted, with the exception of 68-kDa ANK gene (B18R), which is essential for DNA replication and complete gene expression of MVA in nonpermissive human and murine cells (250). Despite having an intact human host-range gene (100% identity to C7L of the VACV WR strain), human cells do not support the replication of MVA. The strict cellular tropism of MVA provides an ideal model for the potential discovery of novel host defense mechanisms targeted by host-range genes that enable replication in CEF cells.

**Molluscum Contagiosum: An Extreme Example of Host-Range Restriction**

Both MCV and VARV have the narrowest tropism of any poxvirus and use humans as exclusive natural hosts (85, 152). MCV replication is strictly restricted to basal keratinocytes of the human epidermis, causing a benign tumor-like lesions in children and young adults but is far more prevalent in immunodeficient individuals (93). Among the MCV genes identified in the completed DNA sequence, several potential immunomodulatory and host-range proteins that control host defenses were discovered (230). In contrast to CPXV and other orthopoxviruses, the genome of MCV is much smaller (190 kbp) and is missing more than 80 genes common to most orthopoxviruses (230). Furthermore, MCV is the only sequenced poxvirus that does not encode any ANK-repeat proteins. Many of these missing genes have been identified as having a functional role in the suppression of host response to orthopoxvirus infection (230, 231). It has been hypothesized that over evolutionary time the genetic material that provides broader tissue tropism for the orthopoxviruses has been lost from MCV (231). For
example, MCV lacks a homolog to both the E3L and K3L proteins of VACV suggesting that either the virus has evolved unique strategies to manipulate PKR and other antiviral response against viral infection, or these pathways are not operational in differentiating basal keratinocytes. Like MVA, the lack of host-range encoded by the MCV genome make this virus a perfect platform to examine the ability of host-range genes to expand the tropism of MCV.

The Unique Tropism of Myxoma Virus

In nature, MYXV has a narrow host-range and is pathogenic only to rabbits, although *in vitro* MYXV can productively infect many nonrabbit cells, including immortalized baby BGMK cells, murine cells (290), some primary human dermal fibroblasts (126) and select human cancer cells (267, 292, 301). The MYXV genome is comparably smaller than VACV, suggesting key host-range genes, not essential for MYXV replication in rabbits have been lost through evolution. Following initial analysis of the MYXV genome, no obvious K1L or CHOhr homologs were recognized, which was particularly interesting since these orthopoxvirus host-range factors were partly characterized in rabbit cells, and rabbits are the natural host for MYXV. However, three tandemly arranged ORFs, designated M062R, M063R and M064R were identified in MYXV, which share some sequence similarity with the VACV host-range gene C7L (44). Pathogenesis studies of rabbits infected with MYXV deleted of the M063R gene (vMyx63KO) failed to develop any symptoms of myxomatosis and all rabbit cell lines tested were completely non-permissive to the mutant virus (20). vMyx63KO successfully binds and enters nonpermissive rabbit cells but late stage viral gene expression and DNA replication was blocked in these cells (20). Conversely, vMyx63KO can still productively infect both human cancer cells and certain primate cell lines (such as
BGMK cells), suggesting either that these latter cells are deficient in some aspect of antiviral responses operational in rabbit cells or that M063R interacts with rabbit-specific host factors that have not yet been identified (20). Rabbit fibroblast that stably express VACV K1L were not successful at rescuing vMyx63KO replication, demonstrating that these genes are not interchangeable and likely interacts with different cellular targets (20).

It is interesting to note the sequence similarity between M063R and C7L, but C7L is not required for VACV replication in rabbit cells, suggesting M063R possesses unique rabbit host-range function. It remains unknown why MYXV would encode three related apparent orthologs of C7L, but one possibility is that each gene product interacts with a distinct cellular factor to promote virus replication (20). Among all of the other reported deletion mutants of MYXV, vMyx63KO is the only gene knockout virus reported to date that fails to replicate in all rabbit cells tested in vitro and also cannot induce any detectable primary lesion formation in infected rabbits. The molecular role of M063R is still unknown but the protein exhibits sequence similarity to the glutamate-rich domain of human DAXX, a FAS-binding death associated protein (51, 52). It is possible that M063R possess similar anti-apoptotic functions as described by other MYXV-encoded host-range proteins, but no clear link with apoptosis has yet been demonstrated (20). Although M063R is not a functional equivalent of C7L, it is obvious the gene provides a critical host-range function for MYXV in rabbit cells and most likely intersects with distinct intracellular signal cascade(s). In another study, M062R was shown to be functionally equivalent to C7L at supporting VACV replication in mammalian hosts, however M063R and M064R were unable to replace VACV lacking the host-range
genes K1L and C7L (175). It is still unclear why VACV would encode two seemingly equivalent genes but it is reasonable to speculate that C7L and K1L perform distinctive functions in some cell types, but not others. Like K1L, the molecular role of C7L still remains unknown but the gene is well conserved in all of the orthopoxvirus genomes that have been sequenced, thus indicating the importance of this gene for the replication of many poxviruses in mammalian hosts (97). Therefore, understanding the function by which the M063 family extends MYXV tropism could provide valuable insight into the narrow host-range of the virus.

Like all poxviruses, the MYXV genome includes a distinct repertoire of host-range genes, whose protein products target specific intracellular pathways to establish an environment within the infected cells that favors viral replication (19, 44, 313). Several of the host-range gene products of MYXV have orthologs in other poxviruses. However, MYXV is the only poxvirus for which there has been a systematic study of the comparative biological roles of viral host-range gene products both in vitro and in vivo (254). Functionally, the known host-range factors from poxviruses have been associated with manipulation of cellular signaling cascades and the ANK-repeat motif appears to be critical for protein-protein interactions between viral and host partners. Presently, the ANK-repeat has been the only identified motif shared by a majority of poxvirus host-range genes, however it is likely that additional protein motifs will be recognized in the future.

**Manipulation of the Akt Signaling Network by Poxviruses**

The demand for energy, nutrients and macromolecular synthesis that accompany viral replication is enormous; however upon viral infection substantial alterations in cellular physiology often lead to the shutdown of various cellular processes critical to the
success of viral replication. Akt has emerged as a central regulator of cellular signaling in a wide variety of fundamental cellular processes and subsequently many viruses have evolved elaborate mechanisms to modulate various aspects of this signaling cascade, to support their replicative requirements (13, 58). The orthopoxviruses, VACV and CPXV have been shown to rapidly induce Akt kinase activity following infection and inhibition of Akt phosphorylation by the P13K inhibitor, LY294002, was shown to reduce viral replication of both viruses (245). Similarly MYXV infection stimulates Akt phosphorylation, but in stark contrast, treatment with LY294002, did not block viral replication (292). Alternatively, inhibition of Akt phosphorylation by Akt inhibitors or a dominant negative form of Akt significantly reduced the replicative potential of MYXV in permissive human cancer cell lines (292). These findings demonstrate that induction of Akt activation, in MYXV-infected cells, occurs in a manner independent of PI3K activation and is resistant to inhibitors that target upstream of Akt.

Clearly the mechanism used by MYXV to induce activation of the Akt pathway is different compared to VACV and CPXV, which suggests individual poxviruses have evolved unique mechanisms to achieve a similar outcome. Additionally, the two orthopoxviruses examined thus far appear to use a similar if not the same mechanism to activate Akt. Therefore, it would be interesting to determine if this trend were true for all members of the orthopoxirus genus and possibly for other more distantly related poxvirus members. To date, the M-T5 protein encoded by MYXV is the only poxvirus protein reported to target the Akt signaling network by means of directly binding Akt (292). In contrast, the mechanism by which CPXV and VACV activate Akt following infection remains unknown and the poxviral protein(s) that specifically target this
pathway have not yet been identified. Interestingly, M-T5 does not share a high degree of sequence similarity with any other poxvirus proteins, suggesting the functional activity of this particular protein may be specific to the replicative requirements of MYXV in the rabbit host. Activation of the Akt pathway appears to be a prerequisite for a successful poxvirus replication; therefore understanding the mechanisms by which individual poxviruses modulate this central signaling cascade may provide valuable knowledge regarding poxvirus biology.

Many of the oncogenes and tumor suppressor genes, including those that function upstream of Akt, have the capacity to encourage cancer progression. Consequently, in the majority of human cancer cells the Akt pathways is either mutated or constitutively activated, often providing the appropriate intracellular signaling environment for a productive MYXV replication to occur. Although M-T5 has been shown to bind and activate Akt, the consequences of this interaction and how they contribute to enhance MYXV replication in the various classes of human cancer cells is poorly understood. With over 100 unique substrates covering a broad range of cellular functions, activation of Akt can concurrently impact multiple pathways, which have the potential to either promote or inhibit the replication efficiency of MYXV. Furthermore, crosstalk between the individual signaling pathways adds yet another level of complexity to the Akt pathway. Therefore, identification of the specific factors that function downstream of Akt to enhance MYXV replication, will be key to understanding how M-T5 contributes to MYXV tropism in the human cancer cells.

**Blocking Activation of the Apoptosis Pathway**

In European rabbits, MYXV infection and pathogenesis is heavily dependent upon the ability of the virus to disseminate from the primary site of infection and establish
secondary sites of viral infection in distal tissues. Spread of MYXV is achieved by means of infected migratory leukocytes, which function as viral transporters and disseminate virus infection to the distal tissues (32, 134). The ability of MYXV to infect lymphocytes in particular is a critical determinant to the success of MYXV and essential to the progression of myxomatosis in susceptible European rabbits. In rabbit T-lymphocytes infected with vMyxT5KO, an abortive infection was observed, which was characterized by rapid inhibition of both viral and host gene synthesis and extensive cellular apoptosis. (186). Interestingly, one of the principle roles of Akt is to regulate the balance between cell survival and apoptosis. Upon activation, Akt phosphorylates and inactivates several pro-apoptotic targets, including Bad (67), and the Forkhead transcription factors (42) and also functions to prevent the release of cytochrome c from the mitochondria (312). Collectively, the results suggest stimulation of Akt activity by M-T5 specifically promotes MYXV replication in lymphocytes by preventing the nonspecific shutdown of protein synthesis, which is probably the stimulus leading to the induction of apoptosis that aborts the infection of rabbit lymphocytes with vMyxT5KO. Likewise, in human cancer cells that express low levels of endogenous phosphorylated Akt (Type II) and do not support vMyxT5KO replication, activation of the apoptotic pathway was observed shortly following infection (Fig. 5-4). The initiation of a rapid apoptotic response following virus infection provides an effective cellular mechanism mechanism for limiting virus infection and replication within the host (73). Consequently, viruses with larger genomes, such as poxviruses, have evolved immunomodulatory proteins which function to block various components of the apoptotic responses during early stages of viral infection (193, 268). These anti-apoptotic effectors have been shown to manipulate the
cell death pathways within the cell in a variety of ways, including the inhibition of caspases and disruption of key mitochondrial checkpoints to prevent apoptosis (74, 75, 268). In addition to M-T5, MYXV encodes three unique proteins; M-T4, M11L and Serp-2, which individually have been shown to regulate apoptosis in response to MYXV infection. Interestingly, deletion of any individual MYXV anti-apoptotic gene interrupts the delicate balance in the intracellular signaling pathways, resulting in the initiation of apoptosis following virus infection. The multiple strategies used by these MYXV encoded proteins are reflective of the obvious complexity inherent within the apoptotic cascade and further demonstrates how manipulation of this pathway is critical to poxvirus replication. Understanding the mechanism which by M-T5 blocks the induction of apoptosis will provide valuable knowledge about strategies used by poxviruses to mediate cellular signalling networks.

**mTOR Complexes and their Activities**

The mTOR signaling pathway plays a critical role in the regulation of cell growth, proliferation, motility, survival, protein synthesis, and transcription (102). Furthermore, dysregulation of the mTOR pathway has been implicated as a contributing factor to various human diseases, especially cancer (28). mTOR, the central component, is found in two structurally and functionally distinct protein complexes, which differ in their major binding partners: Raptor in mTORC1 and Rictor in mTORC2.

Activity of mTORC1 is positively regulated by the Akt pathway via the phosphorylation and inhibition of the tuberous sclerosis heterodimer (TSC1/TSC2) (116, 270), which stimulates the intrinsic GTPase activity of Rheb, thus converting it from Rheb-GDP to Rheb-GTP. Consequently, Rheb-GTP binds directly to the mTOR catalytic domain to displace the mTORC1 inhibitor FKBP38 and stimulates mTORC1
activity (10, 157). Furthermore, in TSC1−/− and TSC2−/− cells constitutive activation of mTORC1 was observed, to suggest the negative regulatory role of these proteins (83, 143). The molecular mechanism by which TSC1/2 and Rheb function to regulate mTORC1 activity has been well established, however regulation of mTORC2 is poorly understood at the present time. It has recently been demonstrated that knockdown of TSC1/2 inhibits Akt phosphorylation, suggesting that Rheb positively regulates mTORC1 while negatively affecting mTORC2 (306). Downstream of mTORC1 are S6 kinase (S6K) and eukaryotic initiation factor 4E binding protein (4E-BP), which are both phosphorylated by mTORC1 to promote protein synthesis and cell cycle progression (69, 207). 4E-BP is a translational repressor that directly regulates the function of the eIF4F translation-initiation complex, which binds to the 5' cap of an mRNA, the first step in the initiation of cap-dependent translation.

Viral control of mTORC1 activity depends on the specific needs of the virus and those, which are reliant on cap-dependent translation during infection, must maintain mTOR kinase activity. Like all viruses, poxviruses are absolutely dependent upon cellular translational machinery for the production of viral polypeptides. Furthermore, all poxviral mRNAs are capped at their 5' end, through the action of a viral methyltransferase complex (36, 167, 285) and therefore must hijack the eIF4F to initiate cap-dependent translation. Interestingly, poxviruses have been shown to degrade the translational repressor 4E-BP and promote eIF4E assembly into an active eIF4F complex (289). Additionally, stimulation of the Mnk1 kinase, which phosphorylates and activates eIF4E, was observed following VACV-infection (289). Thus it would appear
poxviruses utilize the mTORC1 pathway to enhance viral replication and protein synthesis, however the mechanism by which this occurs is not fully understood.

mTORC2 has been shown to regulate actin cytoskeleton dynamics (135, 217) and unexpectedly also functions as the elusive "PDK2" and phosphorylates Akt at Ser473, an event which leads to full Akt activation (117, 237). The demonstration that mTORC2 has PDK2 activity places mTORC2 at the center stage of cellular signaling and multiple cellular processes. Since the replication success of many viruses is dependent upon their ability to activate the Akt pathway, one would expect that viruses have devised various strategies to activate mTORC2 (43). One particular virus, human cytomegalovirus (HCMV) alters substrate specificity of mTORC2, and has been shown to phosphorylate both 4E-BP and SK6, to maintain protein translation following infection (141). The control of mTORC2 is not well understood and to date no poxvirus encoded protein has been demonstrated to specifically target mTORC2. Although, M-T5 does not appear to directly target the mTORC2 signaling cascade, phosphorylation of Akt is observed in MYXV-infected cell. The results suggest that either M-T5 indirectly manipulates mTORC2 signaling or another MYXV protein may specifically target the mTORC2 pathway and function in conjunction with M-T5 to promote Akt phosphorylation following infection.

Rapamycin Increases Myxoma Virus Tropism in Certain Human Cancer Cells

Rapamycin, a bacterial natural product, has been shown to inhibit mTORC1 activity through association with its intracellular receptor FKBP12, whereas mTORC2 is insensitive to rapamycin under normal conditions (112, 177, 206). However, recent reports have shown prolonged inhibition of mTOR by rapamycin blocked mTORC2 assembly and subsequent activation of Akt (5, 15, 218, 308). Alternatively, inhibition of
mTOR was demonstrated to induce Akt activation via elevated expression of the receptor tyrosine kinase, insulin receptor substrate-1 (IRS1) (199, 235). In examining way to increase the tropism of MYXV to human cancer cells, a number of drugs that affect the Akt signaling pathway were examined for their ability to alter MYXV tropism in cancer cells. One particular drug, rapamycin, was shown to induce Akt phosphorylation via the mTOR signaling network and dramatically increased MYXV tropism and spread in certain cancer cells in vitro (252). Thus far, two animal studies have demonstrated enhanced MYXV oncolytic potential when used in combination with rapamycin (160, 253). Furthermore, the combination of rapamycin and okadaic acid successfully restored vMyxT5KO permissivity in cancer cells that previously did not support virus replication. As rapamycin is considered an inhibitor of the Akt-mTOR pathway and activation of Akt is vital to MYXV replication, these results appear to be counterintuitive. The molecular mechanism by which rapamycin induces Akt phosphorylation in human cancer cells remains unknown but nevertheless rapamycin provides a means to manipulate the cellular signaling environment to favor a productive MYXV replication in at least in some human cancer cells (252). Thus, rapamycin offers a novel strategy to extend MYXV tropism in human cancer cells and demonstrates that specific inhibitors can manipulate the intracellular signaling networks and influence the tropism of viruses.

As demonstrated with rapamycin, pharmacological manipulation of the Akt signaling network can significantly impact the ability of cells to support MYXV replication. For instance, inhibitors that specifically block or enhance dephosphorylation of Akt at residues Ser473 and Thr308 generally reduced MYXV replication. Whereas, increased MYXV permissivity was observed in cells treated with drugs that promote Akt
phosphorylation and impede phosphatase activity. Unfortunately, enhancement of Akt kinase activity blocks apoptosis and promotes cellular proliferation, both of which can accelerate cancer progression. Prospective studies will examine small molecular libraries for novel compounds that have the capacity to expend MYXV tropism and enhance viral replication but do not significantly influence other cellular pathways. Invaluable lessons into the tropism mechanisms, which govern host-range and pathogenesis in poxviruses, may be derived through the study of such reagents on viral tropism. Additionally, this knowledge may have significant implications for the rational design of the next generation of oncolytic viruses, as the development of new and improved synergistic cancer therapies continues.

**The Cellular Ubiquitination System**

The ubiquitin-proteasome system is an ancient eukaryotic regulatory mechanism that plays a pivotal role in the selective degradation of regulatory proteins involved in a variety of fundamental cellular processes that include progression of the cell cycle, induction of the inflammatory response and cell death (62, 139). Ubiquitination requires the coordinated action of three classes of enzymes, the ubiquitin-activating enzyme (E1) which facilitates ATP-dependent activation of ubiquitin, a ubiquitin-conjugating enzyme (E2) that transiently carries the activated ubiquitin molecule to the target protein and the ubiquitin-ligating enzyme (E3) which catalyzes the transfer of ubiquitin from the E2 onto specific lysine residues of the target protein (205). Much of the selectivity and high efficiency of the ubiquitination reaction is dependent upon E3 ligases, which utilize one of two distinct structural motifs; a HECT (homologous to E6-associated protein C-terminus) domain or a Really Interesting New Gene (RING)-finger. HECT-type E3s are modular proteins and contain two functional domains: an amino terminal that interacts
with specific target substrate(s), while the C-terminal HECT domain functions as an acceptor for the activated ubiquitin carried by the E2 enzyme (101, 142). Interestingly, one HECT domain cannot substitute for another (227), possibly because the HECT domain makes a modest contribution to the interaction with the substrate (113). Numerous potential HECT E3s exist in mammalian databases, however the vast majority of these enzymes are functionally uncharacterized. Most RING-finger type of E3 ligases contain one of seven cullin proteins and of this group, the SCF complexes is the best understood (76, 239). The SCF complex is composed of a modular E3 core containing the cullin subunit CUL1 and Rbx1 (which contains the RING domain), the variable F-box protein and the adaptor protein Skp1, that links CUL1 to the F-box protein (48). F-box proteins specifically bind Skp1 via an amino terminal 40 residue F-box motif and interacts with a target substrates through a second interaction module, such as LRR or WD-40 repeats (137, 244). The process of selective ubiquitination is an essential regulatory step for many cellular processes and the human genome encodes more than 70 F-box proteins, which collectively are thought to specifically target a broad collection of cellular substrates for delivery to the SCF complex to initiate turnover (300).

**M-T5 Protects Infected Cells from Cell Cycle Arrest**

The cellular SCF complex plays a critical role in the selective degradation of regulatory proteins that mediate the cell cycle, in particular p27/Kip1. The cyclin dependent kinase (CDK) inhibitor, p27/Kip1, is a key negative regulator of cell cycle progression, which binds to cyclin-CDK complexes in the nucleus and causes cell cycle arrest in the G1 phase. Activity of p27/Kip1 is highly regulated by its subcellular localization and phosphorylation by upstream kinases. Generally, substrates are recognized by the SCF complex after they have been phosphorylated on specific
epitopes (59). For example, phosphorylation of p27/Kip1 at residue Thr187 by active cyclin E- or cyclin A–CDK2, provides a recognition site for the binding of Skp2 and subsequent degradation via the ubiquitin-proteasome pathway (189, 233, 287). Consequently, in the absence of p27/Kip1, the cell cycle is permitted to progress beyond G1 arrest and enter S phase (287). Therefore, the degradation of p27/Kip1, via the ubiquitin-proteasome system, provides a targeted mechanism to regulate the cell cycle.

During MYXV infection, when M-T5 is expressed, enhanced phosphorylation, ubiquitination and degradation of p27/Kip1 was observed, whereas in the absence of M-T5, levels of p27/Kip1 were selectively increased, consistent with its reduced phosphorylation and ubiquitination. Moreover, cells infected with vMyxT5KO specifically entered cell cycle arrest and accumulated at G0/G1, whereas in the presence of virus that expresses M-T5, the viral protein was shown to promote cell cycle progression beyond the G0/G1 checkpoint during virus infection (127). Note that p27/Kip1 is known to be a substrate of activated Akt and the phosphorylation of p27/Kip1 at site Thr157 by Akt causes retention of p27/Kip1 in the cytoplasm and failure to induce G1 arrest (86, 286). Recent studies have shown that Akt-dependent phosphorylation leads to the cytoplasmic translocation of Skp2 and assembly of the SCF complex (87, 153). Interestingly, the interaction between M-T5 and Akt has been demonstrated to induce the activation of Akt in certain human cancer cells called Type II, which is a key determinant for permissive MYXV replication in these particular cells (292). The current results suggest that bridging of the SCF complex to Akt by M-T5, as demonstrated in Fig. 6-1, may directly enhance the activation of Akt that becomes localized to the SCF
Figure 6-1. M-T5 potentially functions as a molecular bifunctional adaptor and simultaneously binds the cellular proteins Akt and the SCF complex (including recruited substrates such as p27/Kip1). In MYXV-infected cells, the bridging of the SCF complex to Akt by M-T5 may contribute to the concurrent phosphorylation, ubiquitination and proteolytic degradation of various host proteins destined for degradation, such as p27/Kip1. The consequence of this interaction with p27/Kip1 is to prevent cell cycle arrest of infected cells and thus avert the subsequent induction of apoptosis. Significantly, Akt itself is not subject to degradation in this complex. Note that we propose that other cellular targets (denoted “X”) are also conscripted into the M-T5/Akt/SCF complex and become first substrates for Akt and then are subjected to SCF-dependent ubiquitination and degradation. Identification of these novel cellular targets will be an important future goal.

complex and contribute to the concurrent phosphorylation and proteolytic degradation of p27/Kip1. Interestingly, there is no evidence that Akt itself is subject to any excessive degradation as a consequence of its relocalization to the SCF complex, suggesting that the Akt/M-T5/SCF complex functions to increase the range of Akt targets, and not to ferry Akt into the proteasome. The ability of M-T5 to function as a molecular bifunctional adaptor defines a novel mechanism by which a viral host-range protein can influence two distinct signaling pathways, consequently altering the intracellular environment to promote viral replication. However, more details are necessary to further characterize
the functional role of M-T5 and its coordinated interaction with the cellular SCF complex and various substrates such as Akt during viral infection.

**Poxvirus ANK Proteins Contain a Functional F-Box and Assemble into SCF Complexes**

The majority of poxviral ANK-repeat containing proteins include a conserved C-terminal PRANC motif, which closely resembles a cellular protein motif called the F-box domain (176). The cellular F-box domain is usually composed of three α-helices, however the PRANC motif is comparably shorter and lacks the third helix (246). Many of the invariant residues within helices H1 and H2 are well conserved in the MYXV PRANC/F-box like domains, but little sequence similarity was maintained within α-helix H3. Despite the lack of H3, all four of the MYXV-encoded ANK-repeat proteins, including M-T5, were shown to directly interact with the Skp1 component of the host SCF ubiquitin ligase complex (Fig. 3-1). Furthermore, deletion of the PRANC domain abolished interaction between M-T5 and Skp1. These findings demonstrate each MYXV ANK protein contains a functional PRANC/F-box like domain, which can interact with the host Skp1 as previously reported with other poxviral ANK-repeat proteins (247, 249, 282). Additionally, poxviral ANK/F-box proteins did not directly inhibit the ubiquitination activity of SCF complexes, suggesting this group of viral proteins probably functions as specificity factors of the SCF complex, targeting cellular proteins to the SCF complex resulting in their ubiquitination and subsequent proteasomal degradation (247).

Alternatively, it is possible that members of this protein family function in a manner not leading to the ubiquitination of its binding partner. However, it is not clear why poxviruses would devote a protein family to targeting proteins to the SCF complex but not initiate their ubiquitination, nor is it obvious why multiple proteins would be
directed to the single task of inhibiting the SCF complex. In a recent report ubiquitination of Akt by the E3 ligase TRAF6, was shown to promote Akt translocation to the plasma membrane for subsequent activation (307). Furthermore, following treatment with growth factors in cells deficient in TRAF6; ubiquitination, membrane localization, activation, and signaling of Akt was impaired (307). Thus, the ability of M-T5 to simultaneously bind Akt and the SCF complex may promote the ubiquitination of Akt independently of TRAF6, unfortunately this hypothesis has not yet been examined in any detail. The presence of the ANK-repeat motifs within F-box proteins is exclusive to poxviral proteins, suggesting the collaborative role of these distinct binding domains likely provides poxviruses novel strategies to regulate key cellular pathways that mediate pathogenesis and tropism.

The Ubiquitin Pathway is a Popular Target of Viral Proteins

Poxviruses are known to specifically target and manipulate a variety of signaling networks that regulate critical cellular processes. Besides the ANK/PRANC subfamily of proteins, poxviruses have evolved additional strategies to subvert the host ubiquitination pathway (309). For example, poxviral proteins which contain Broad-complex, Tramtrack and Bric-a-Brac (BTB) and kelch domains have been shown to interact with cullin-3 ubiquitin ligases (138, 299). Furthermore, the p28 protein encoded by ECTV contains a single subunit RING-finger which functions as a viral E3 ubiquitin ligase (111, 194). This protein is highly conserved among orthopoxviruses, however in the VACV strains COP, WR and MVA the p28 gene products are either truncated or interrupted. Another E3 ubiquitin ligase, M153R of MYXV, is a membrane-associated RING-CH (MARCH) protein that downregulates host cell surface expression of major histocompatibility complex class I, the pro-apoptotic factor CD95 (Fas), the activated leukocyte cell
adhesion molecule (ALCAM) and CD4 (25, 98, 164). Interestingly a wide range of proteasome inhibitors were shown to dramatically affect poxvirus replication, suggesting a functional ubiquitin-proteasome system is required during poxvirus infection (269).

In summary, poxviruses encode a myriad of proteins that have the capacity to hijack the ubiquitin-proteasomal pathway, for example to target and eliminate unwanted cellular proteins such as inhibitors of the cell cycle or various anti-viral factors that would otherwise function to block viral replication. Understanding the mechanisms by which viral host-range proteins interact with host factors and signaling networks should continue to provide invaluable insights into how vital cellular networks can be reprogrammed by viral factors to increase virus survival at the cellular level.

An ever-increasing number of other viruses have been shown to also manipulate the proteosome pathway in an attempt to specifically degrade cellular proteins which function to block virus replication (14). The E6 protein from human papillomavirus was the first viral protein demonstrated to use the proteasome machinery to direct the degradation of a cellular target protein. Binding of E6 to the tumor-suppressor protein p53 was shown to promote degradation via the proteasomal pathway, which ultimately sequesters the pro-apoptotic activities of p53 and allow for cell cycle progression (221). Adenovirus also targets p53 for ubiquitination and degradation by means of the viral proteins E1B 55K and E4orf6 (255), both of which have been shown to bind to p53 near its N- and C-termini, respectively (66, 129). The use of the proteasome by a wide assortment of viral proteins emphasizes the crucial importance of manipulating this cellular pathway for the replicative success of many viruses.
Localization of ANK/F-Box Proteins

The ANK-repeat domain is known to mediate protein-protein interactions and cellular proteins containing the motif have a broad range of functions including: transcriptional regulators, cytoskeletal organizers, cell cycle regulatory proteins, and developmental regulators (181, 228). Functional diversity among ANK-repeat proteins suggests the motif is far more important structurally, as scaffolding modules, rather than as an enzymatic role (228). The capacity of poxviral ANK/PRANC proteins to operate as molecular scaffolds may coordinate the cross-communication between distant host signaling pathways that normally function independently. At the molecular level, such viral proteins have the potential to re-configure intracellular signaling networks into downstream effects that may previously not exist due to spatial or temporal constraints, but which may be crucial for viral replication.

Localization studies of the four ANK/PRANC proteins encoded by MYXV revealed that each had a unique cellular distribution pattern. For example, a punctuate pattern with a uniform distribution in the cytoplasm was observed for M149, whereas M148 also had cytoplasmic distribution but a distinct nuclear pattern was evident (34). In TNF-stimulated cells, M150 was reported to co-localize in the nucleus with NF-κB p65, however direct binding studies have not yet been performed to verify this interaction (47). Although no classical nuclear localization signal (NLS) has been identified in M148R or M150R, it has been shown that ANK-repeats can function as non-typical NLS (215). This has been verified for M150R, since deletion of the eighth ANK-repeat from M150R blocked nuclear translocation (47). Furthermore, this particular ANK-repeat exhibits significant sequence similarity to the NLS identified in IkBα and therefore M150R was named myxoma nuclear factor (47). Finally, M-T5 exhibited punctate
cytoplasmic expression in MYXV-infected human cancer cells (292) and was shown to specifically mediate the relocalization of Akt from the nucleus to the cytoplasm during MYXV infection (Fig. 3-8).

The data suggests M-T5 may perturb Akt cellular localization and thus its ability to interact with specific downstream targets. Alternatively, binding of M-T5 to Akt may promote translocation of Akt to the plasma membrane where the kinase becomes activated by way of phosphorylation. Interestingly, the SCF complex has been shown to localize throughout the cell, whereas F-box specificity factors have been identified in distinct subcellular compartments (84, 96, 115, 155, 220). Thus the unique localization patterns exhibited by the individual MYXV ANK/PRANC proteins suggests each protein has a distinctive function consistent with being an F-box specificity factor of the SCF complex. Since, cellular location potentially has a major impact on protein-protein interactions, it would be advantageous to examine the localization of many other poxvirus ANK/PRANC proteins to determine how these proteins contribute to poxvirus replication.

Identification of Substrates of ANK/PRANC Specificity Factors

Although cellular F-box proteins have been shown to manipulate the cellular ubiquitination machinery, surprisingly little is known about the molecular mechanism by which this largest family of poxvirus proteins function during viral infection. Aside from Skp1, identification of additional binding partners, either cellular or viral, has resulted in little success and coincidently has hindered the study of this superfamily of proteins. To address this issue, a small but increasing number of studies have employed proteomic methods to screen individual poxviral ANK/PRANC proteins for binding partners. For example, systematic Y2H analysis of the unique VARV genes with human cDNA
libraries revealed the presence of a novel viral inhibitor family of NFκB1/p105. Additionally, this ANK/PRANC protein, called G1R in VARV, was shown to interact with Skp1 (180). In another study, a Y2H screen was performed to identify cellular binding partners for the 68k ANK-repeat protein encoded by MVA, and interestingly 99% of the analyzed positive interactions were with cellular Skp1 (249). Likewise, multiple independent human cDNA libraries were screened by Y2H for potential binding partners of M-T5 and a total of 13 unique potential host protein interactions were identified (Table 3-2). Aside from Skp1, all proteins were novel potential M-T5 binding partners and have not been previously reported, but their verification as true binding partners in virus-infected cells remains to be confirmed.

Significantly, Akt was not identified as an M-T5 binding partner in the Y2H screen, however the protein-protein interaction between Akt and M-T5 is strictly dependent upon the phosphorylation status of Akt. Phosphorylation of Akt at residues Thr308 and Ser473 regulate the kinase activity and consequently the phospho-state of Akt may also affect the binding of M-T5. If this were the case, then proper phosphorylation of Akt would likely not occur during the Y2H screen, which may help to explain the failure of the Y2H screen with M-T5 to identify Akt as a potential binding partner. Furthermore, Skp1, but not CUL1, was identified as a direct M-T5 binding partner, to reaffirm our contention that the direct binding interaction is between M-T5 and Skp1, while CUL1 is indirect by virtue of being a component of the larger SCF complex that is ubiquitously found in cells and cellular lysates. The Y2H system has become an increasingly important tool to identify and map novel protein-protein interactions, however like any high throughput assay, all hits must be verified by other
methods to eliminate false positives that are not biologically relevant. Future studies will focus on validating these potential binding partners of M-T5 identified by the Y2H screen, using complementary methods such as AlphaScreen, co-immunoprecipitation and GST pull-down experiments. Deconstructing the intricate interactions between virus host-range proteins and their host interacting proteins will be particularly useful to decipher the targets for this class of viral ANK-repeat proteins during viral infection.

**Functional Replacement of M-T5 by Cellular PIKE-A**

Functional and sequence similarities between many virus-encoded host-range and immunomodulator genes suggest they have been originally commandeered directly from an ancestral host. However, M-T5 shares no significant sequence similarity to any known cellular genes and the evolutionary origin of M-T5 has remained elusive. Nevertheless, some clues were provided when the function of a cellular protein called PIKE-A was characterized. PIKE-A is a physiological mediator of Akt and functions to stimulate Akt activation through direct binding (2-4), in a fashion similar to M-T5. The transient expression of PIKE-A was able to rescue MYXV replication in human cancer cells that were previously restrictive to replication of vMyxT5KO (Type II) (295). Additionally, the overexpression of PIKE-A induced phosphorylation of endogenous Akt and blocked the induction of apoptosis in MYXV infected human cancer cells (295). More interestingly, transfected PIKE-A was demonstrated to rescue MYXV replication in Type III cells in the presence or absence of M-T5 (vMyxT5KO), suggesting PIKE-A provides additional properties, critical for MYXV replication, when compared to M-T5. The results demonstrate that, through the manipulation of the Akt signaling pathway, MYXV tropism may be expanded into an even broader spectrum of human cancer cells.
In a wide spectrum of human cancer cells PIKE-A is either amplified or mutated, which consequently promotes cell survival and proliferation by binding to and enhancing Akt kinase activity (2, 156). Although we have previously demonstrated that MYXV permissivity in human cancer cells is dependent upon elevated levels of endogenous phosphorylated Akt (292) the link between overexpression of PIKE-A and productive MYXV replication has yet to be established. Presumably, cells that express elevated levels of PIKE-A will support MYXV replication, whereas PIKE-A expression will be lower and coincide which decreased Akt kinase activity in non-permissive cells. However it is difficult to believe this correlation will be true for all cell lines, especially since there are so many proteins that regulate signaling through the Akt pathway, both upstream and downstream. To further validate the importance of PIKE-A overexpression, dominant-negative mutants of PIKE-A are available to examine how inhibition of PIKE-A activity affects MYXV replication in permissive cell lines. Two dominant negative PIKE-A mutants have been characterized; PIKE-A DN, which possesses mutations in the GTPase domain preventing the protein from activating its targets but does not effect substrate binding and PIKE-A KN, PH domain mutations which cause PIKE-A to lose its phosphoinositide-binding capability (3). Alternatively, siRNA can be used to specifically knockdown PIKE-A, which should generate comparable results. Lastly, construction of a recombinant MYXV that expresses PIKE-A would be a very useful biological tool to examine the biological mechanism by which PIKE-A promotes MYXV replication in greater detail. PIKE-A provides an alternative strategy for studying the importance of Akt phosphorylation during a productive MYXV infection in human cancer cells. Understanding the biological mechanism by which
expression of PIKE-A rescues MYXV replication in previously non-permissive human cancer cells potentially offers further insight into the importance of Akt phosphorylation during a productive virus infection. Studying the functional role of PIKE-A may also lead to the discovery of additional proteins that can function similarly to M-T5 and promote MYXV replication in human cancer cells.

**Concluding Remarks**

In conclusion, the ability of M-T5 to function as a molecular bifunctional adaptor defines a novel mechanism by which a viral host-range protein can simultaneously influence multiple distinct cellular signaling pathways. The intricate relationship between viral encoded ANK/PRANC proteins and components of the host cell signaling networks can have profound impact on poxvirus tropism, however the factors that regulate these protein-protein interactions are poorly understood. The lessons we continue to learn from poxvirus host-range factors like M-T5 will provide invaluable insight into the elaborate mechanism by which viral encoded proteins micro-manipulate the signal transduction pathways of the infected cells to promote an intracellular environment more conducive for optimal virus replication.
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

Steven Werden was raised in rural Southwestern Ontario where he began his education at Brooke Central School in nearby Alvinston, and upon graduation, he attended Lambton Central Colligate Vocational Secondary School in Petrolia, Ontario. He later went on to receive a Bachelor of Science from the University of Western Ontario in London, Canada, in 2005. It was during this time he joined the laboratory of Dr. Grant McFadden and started to study poxviruses. In the Fall of 2005, he enrolled in Graduate School at the University of Western Ontario and later transferred to the University of Florida to complete his Doctor of Philosophy in the Department of Molecular Genetics and Microbiology under the supervision of Dr. McFadden. His research mainly focuses on understanding the elaborate mechanisms by which poxviral encoded proteins micromanipulate the signal transduction pathways of infected cells to promote an intracellular environment for virus replication.