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The Partial Characterization of the 142R Protein of Tanapox Virus

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by

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Due to the complex nature of cancer, a variety of strategies are being employed to treat patients. Among these are oncolytic viruses that conditionally replicate in tumor cells with specific cellular landscapes. Several viruses including Herpesviruses, Adenoviruses, and Poxviruses, predominantly vaccinia virus (VV), have been explored for their oncolytic potential. Most of these viruses can productively infect a variety of cell types and it is one goal to create conditionally lethal viruses that can only replicate in tumor cells. Due to the prevalence of p53 inactivating mutations in cancers viruses that are restricted to p53 null cells are desirable and have been developed. We were interested if we could develop a similar mutant in the tanapox virus. Here we describe the identification of a protein from the 142R open reading frame of the tanapox virus genome that is orthologous to the B1R protein from VV which is known to down regulate p53 by phosphorylation. We determine that like B1R, TPV142R encodes a serine threonine kinase that can phosphorylate the tumor suppressor p53. However, we were not able to isolate a TPV142R knockout virus using our current system of viral mutant generation indicating that the p142R protein is essential for viral replication. This was confirmed by using shRNA to target the knockdown of 142R which resulted in a

decrease in viral replication. Due to the apparent necessary function that 142R protein provides, TPV lacking 142R may not make a suitable oncolytic virus.

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Krystal N. Seibert

TABLE OF CONTENTS

Table of Contents—continued

Table of Contents—continued

Table of Contents—continued

LIST OF TABLES

LIST OF FIGURES

List of Figures—continued

INTRODUCTION

Poxviruses

Poxviridae is a large family of DNA viruses that infect vertebrates and invertebrates (Moss, 1996). Members of the family of poxviruses are characterized as viruses that replicate in the cytoplasm of their host and are able to synthesize mRNA via the enzymes contained in the virion. These enzymes are encoded in the viral genome, which is single, linear, and dsDNA with hairpin loops at each end. These hairpins at the ends of their genomes are A + T rich, not completely base-paired and are inverted or complementary in sequence. The region directly adjacent to the hairpin loops is highly conserved amongst poxviruses (DeLange et al., 1986). At both ends of the genome are inverted terminal repeats (ITRs) that are identical to one another but oppositely oriented (Moss, 1996). These regions are highly variable and may include coding regions which puts some genes at both ends of the genome (Figure 1).

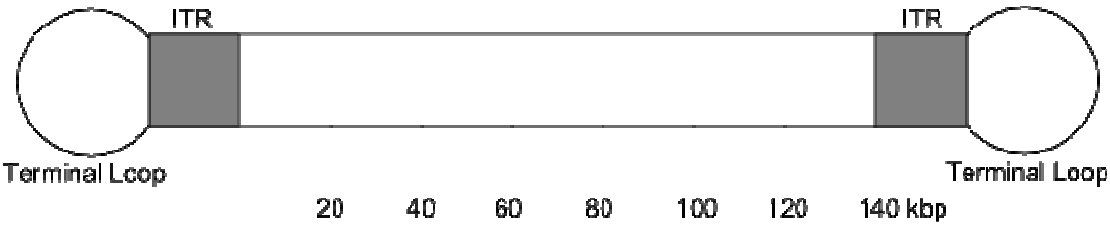

Figure 1. Inverted Terminal Repeats and Hairpin Loops of Tanapox Virus.

The family is further divided into subfamilies *Chordopoxvirinae* (vertebrate) and *Entomopoxvirinae* (insects) by host specification (Moss, 1996). Among chordopoxviruses there are eight genera based on genetic and antigenic relatedness,

similar morphology and a similar host range. Within each genus, serologic cross-reactivity for group-specific NP antigen has been demonstrated. With complete sequences being reported for each genera of *Chordopoxvirinae* and two *Entomopoxvirinae*, it has been noted that nearly 100 genes are conserved amongst *Chordopoxvirinae* and 50 genes amongst all poxviruses (Moss, 2007). These genes are largely nonoverlapping and point to the nearer end of the genome. More conserved genes are more centrally located and are essential for replication, while genes involved in host interactions, which are highly variable, are located more peripherally in the genomes.

Viral Particles

Poxviruses are larger than other animal viruses to the extent that they are visible by light microscopy (Moss, 1996). By reconstructed images of cryoprepared electrol tomography they appear to have a barrel shape measuring about 360 by 270 by 250 nm (Cyrklaff et al., 2005, Dubochet et al., 1994). There is a 30 nm membrane surface layer that surrounds a homogenous core that is studded with 20 nm spikes (Moss, 1996). The core has a dumbbell shape made up of an outer and inner layer with lateral bodies attached within the concavities (Figure 2). Within the core are four insoluble structural proteins that account for 70% of the weight of the core (Paoletti & Moss, 1974). Infectious particles contain all the enzymes and factors they need to synthesize polyadenylated, capped and methylated mRNAs (Wei & Moss,

1975). This includes early transcription factor which can begin the chain reaction of virus production (Moss, 1996).

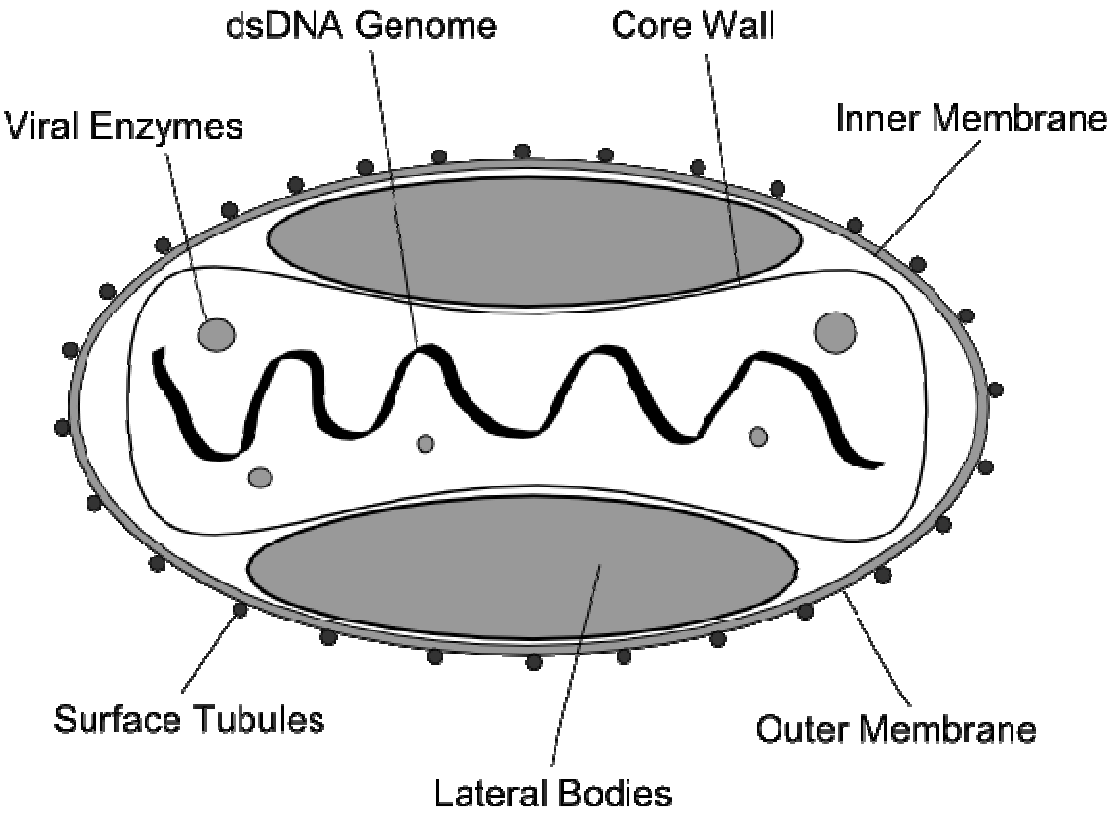

Figure 2. Schematic of a Poxvirus.

Entry and Early Transcription

Although not completely clear, mature virions (MV) are thought to fuse with the cell membrane, possibly with the help of a cellular receptor, in order to enter the cell (Chang & Metz, 1976, Twardzik et al., 1985). However, viral envelopes need a low pH environment to allow productive entry (Townsley et al., 2006), and more recently vaccinia virus has been shown to enter through apoptotic mimicry (Mercer &

Helenius, 2008). Extracellular enveloped virions (EEV) require the removal of the outer membrane prior to fusion (Moss, 2007). This disruption can occur with a neutral pH or potentially within endosomes (Ichihashi, 1996, Vanderplasschen et al., 1998). Once the virus is attached there is a signaling cascade that causes membrane rearrangements and actin formation (Locker et al., 2000). MV then enter the cell via a complex of 11-12 transmembrane proteins that are conserved in all poxviruses (Moss, 2012). Viral cores are then transported to sites of transcription via microtubules and mRNA synthesis begins (Moss, 2007). Early transcription requires both RNA polymerase and early transcription factor which are both packaged in the virion and do not need to be transcribed (Moss, 1996). The genes transcribed at this point of infection contain promoter sequences that are conserved between members with the same genera, which may explain how they can rescue one another after heat-inactivation. The mRNA from early genes can be detected as early as twenty minutes postinfection and peak at two hours (Baldick & Moss, 1993). These genes encode proteins involved in viral DNA replication, intermediate gene expression and host interactions (Moss, 1996). Serine/threonine protein kinases have been found to have early expression in addition to presence in the virions to aid in DNA replication (Kleiman & Moss, 1975). Early gene expression ends with the disruption of the virus core, called uncoating, via the nucleoprotein complex passing through breaches in the core wall (Moss, 2007). See figure 3 for an overview of poxvirus replication.

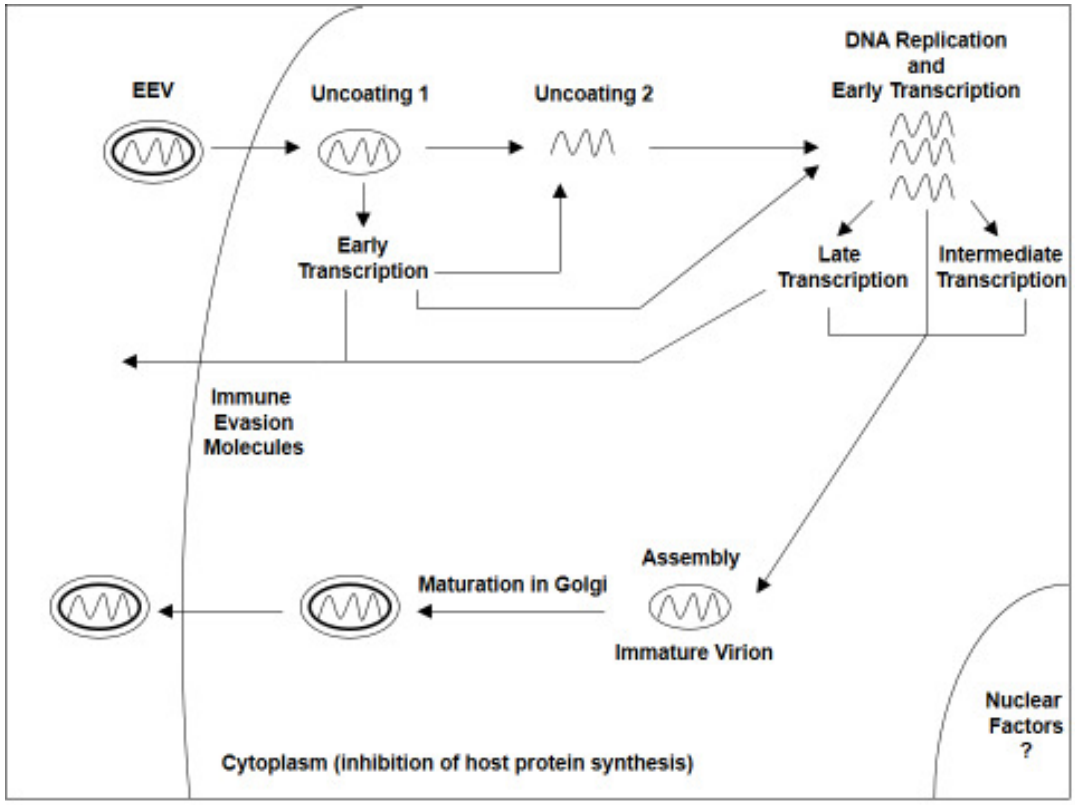

Figure 3. Hypothetical Tanapox Virus Life Cycle (Essani et al., 2011).

DNA Replication

DNA replication occurs in foci of replication sites in the cytoplasm termed factory areas, using viral counterparts of cellular replication proteins (Moss, 1996). In order to replicate DNA, deoxyribonucleotides need to be synthesized due to suboptimal precursor pools. This is accomplished through the viral enzymes thymidine kinase, thymidylate kinase, ribonucleotide reductase and dUTPase. The time it takes for DNA replication spans from one to sixteen hours depending on the poxvirus family member. It has been speculated that replication begins at both ends following terminal nicks and does not require an origin site. The proteins necessary

for replication are DNA polymerase, an ATP/GTP binding motif protein, a serine/threonine kinase packaged into virions, a protein that encodes uracil DNA glycosylates and an ATP-dependent DNA ligase. In addition, concatemer intermediates need to form and resolve into unit-length molecules. Concatemer junctions appear at the ends of the genome as copies of the hairpin loop. There is a strong connection between replication and recombination which indicates that poxviruses cannot biochemically distinguish between the two processes (Fisher et al., 1991). High rates of recombination occur within poxvirus-infected cells allowing viral genomes to rapidly eliminate direct repeats and often result in single and double crossover products (Ball, 1987).

Intermediate Transcription

Intermediate-stage genes are expressed after DNA replication but before the expression of late genes. Some early genes are required for intermediate gene transcription, just as some intermediate genes transactivate late gene expression. Intermediate-stage promoters contain two important regions: a 14-bp-core element that is 10-11 bp upstream of the critical 4-bp initiator element where initiation takes place (Baldick et al., 1992). Intermediate-stage transcription requires RNA polymerase, virus-encoded capping enzyme and intermediate transcription factor in order for production of RNA (Rosales et al., 1994, Vos et al., 1991).

Late Transcription

Late transcription follows intermediate gene expression and genes in this category are clustered in the central region of the genome (Moss, 1996). Major virion components and necessary factors for early gene production are transcribed during the late stage. Late stage mRNAs have 5' caps, are of a heterogeneous length and are polyadenylated due to RNA polymerase slippage. However, they lack a defined 3' end (Mahr & Roberts, 1984).

Viral Packaging

Soon after late transcription, intracellular immature virions (IV) are assembled in circumscribed, granular, electron dense areas of the cytoplasm (Moss, 2007). IVs begin in a crescent shape with a membrane containing a brushlike border of spicules on the convex surface and granular material on the concave side. Subsequently, IVs become circular with a dense nucleoprotein mass, which enters envelope before it is completely sealed and is embedded in the granular matrix. Major structural proteins are then processed and the IV becomes enveloped. Some poxvirus IVs then become occluded in a dense protein matrix of the cytoplasm or at sites of virus replication and assembly which help protect them from the environment. Then the IV becomes more barrel-shaped rather than spherical and is now considered to be mature.

This now mature virus then moves to the cell periphery where it becomes membrane wrapped by the Golgi or early endosomal network and is now considered a wrapped virion (WV). After being wrapped the virus is transported to the plasma

membrane on actin-containing microfilaments (Stokes, 1976) where the virus fuses and is externalized to become EEV (Moss, 1996), containing an additional lipoprotein envelope (Payne, 1978). At this time the EEV can adhere to the cell surface or are released (Moss, 1996). Adherent EEVs go on to mediate cell-to-cell spread, while released EEVs provide long-range dissemination (Blasco & Moss, 1992).

Viral Infection

Once the virus infects the cell, multiple events occur aside from DNA transcription and replication. Infection results in cytopathic effects (Bablanian et al., 1978), changes to membrane permeability, and inhibition of host DNA, RNA and protein synthesis (Moss, 1996). Upon infection, many poxviruses induce hyperplastic responses and sometimes tumor formation due to the secretion of growth factors related to both epidermal growth factor (EGF) and transforming growth factor α (Blomquist et al., 1984). These growth factors help contribute to virulence *in vivo* (Moss, 1996). In defense to poxvirus infection, the host mounts a nonspecific immune response involving interferons, complements, natural killer cells, cytotoxic T cells and antibodies (Buller & Palumbo, 1991). However, poxviruses counter and evade the immune response by several different mechanisms: including a lack of a latent phase (Moss, 1996). Poxviruses have also evolved multiple proteins that interfere with principal cytokines including interferons, interleukins and tumor necrosis factors involved in host defense. In addition, poxviruses contain short

consensus repeats to inhibit complement activation pathways to allow for maximal infection.

Yatapox

Yatapox is a genus of the subfamily *Chordopoxvirinae* that contains two viruses: yaba-monkey tumor virus (YMTV) and tanapox virus (TPV) (Nazarian et al., 2007a). Tanapox virus is considered to have two strains, the central virus called tanapox virus from equatorial Africa and the other called yaba-like disease virus (YLDV) from U.S. primate facilities (Downie & España, 1973). Both tanapox virus and YMTV have a narrow host range of primates, both human and nonhuman, and are the smallest of the chordopoxviruses (Brunetti et al., 2003). YMTV has been sequenced and has open reading frames that are highly conserved with TPV, despite their vast clinical differences. YMTV induces substantial tumor formation that is derived from histiocytes that migrate to the site of infection. Once the histiocyte forms a polyclonal tumor (though never becomes invasive), the tumor will regress when mononucleated cells move into the lesion until it is cleared and the infection is gone (Damon, 2007).

Tanapox Virus

Tanapox virus was first publicized when outbreaks occurred along the Tana River in Kenya in 1957 and 1962 (Downie et al., 1971). These were both years in which there was prolonged, extensive flooding along the river that left local tribes isolated on islands of high ground with not only their livestock, but wild animals as

well. In 1957, it was the children of the mission school in the swamp forest flood plain village of Ngau that were infected leading to speculation that it targeted children. However, in 1962 the riverine Wapakono tribe along the middle reaches of the Tana River between Garissa and Garsen were infected. In this case several hundred people were infected of both sexes and all ages, making it clear that tanapox virus is not limited to children. Due to the fact that these outbreaks were limited; despite the tribe and the children being in contact with others, implicates mosquitoes as the most likely vector candidate. The flooding in these years made ideal breeding grounds for the *Mansonia* mosquitoes that were able to feast on the stranded people at a rate of about seven hundred bites per hour in the evenings. When comparing tanapox virus with other mosquito-transmitted viruses, it was found that West Nile virus infection is quite similar (Manson-Bahr & Downie, 1973). The antibody distribution for both viruses is similar as well as the age, sex and tribe specific incidence. This adds further evidence for mosquitoes being the responsible vector of transmission.

In addition there has never been a reported case of human-to-human contraction of the disease. However, there is evidence of primate to human transmission of the disease, as in the outbreak in U.S. primate facilities in 1966 (Downie & España, 1973). The U.S. virus was eventually named yaba-like disease virus (YLDV) and is phenotypically identical to tanapox virus. However, the slight differences in their restriction digest patterns suggested YLDV and tanapox virus may be different strains of the same virus (Knight et al., 1989), which was later confirmed

when these viruses were sequenced (Lee et al., 2001, Nazarian et al., 2007a). For both of these strains it is believed that some species of primate is the reservoir due to the fact that only primates are susceptible and there are several species of primates living along the Tana River, although this has never been confirmed (Downie & España, 1973).

Endemic Virus

After these three outbreaks, it was noted that tanapox virus is actually endemic to all of equatorial Africa (Knight et al., 1989). Neutralizing and complement-fixing antibodies have been found in a wide variety of people in the area well after the outbreaks indicating their infection had been in the last two years of sampling (Manson-Bahr & Downie, 1973). Infected persons have also been found outside the Tana River Valley, as in human primates in Zaire in 1977 and 1981 and vervet monkeys in Ethiopia. Samples were taken from many of these areas and further examined for understanding of their relationship to other poxviruses.

Viral Infection

Morphologically tanapox virus resembles other poxviruses, with its dumbbell-shaped core encased in an outer membrane making an overall brick-shaped appearance (Nazarian et al., 2007a). However, tanapox virus often has an extra envelope which is rarely found in samples from orthopox virus infections (Fenner, 1996). In cell culture tanapox virus forms focal lesions that have intense granularity leading to the cells rounding up. In primates the infection symptoms manifest after an

unknown number of days in a short febrile illness (Downie et al., 1971). A lesion develops during this time that begins as a small nodule that becomes papular and increases in size until the end of week two where it can be up to fifteen millimeters in diameter (Fenner, 1996). The area around the nodule becomes an edematous zone and a large erythematous areola. The nodule then generally ulcerates and gradually heals for a few weeks, leaving behind a scar. Despite the fact that this infection can be contracted anywhere in equatorial Africa, depending in what country this occurs in can determine the number of lesions as well as the location on the body. In Kenya the lesions are usually above the waistline and singular (Downie et al., 1971), however in Zaire they are more prevalent on the lower limbs with multiple lesions occurring (Fenner, 1996).

Diagnosis

When diagnosing a patient with tanapox virus it is often difficult to differentiate from other poxviruses. Immediate, visual tests to distinguish tanapox virus from smallpox virus can be done. These are based on the fact that tanapox virus lesions are larger, firm, solid, have slower evolution while exhibiting no postulation (Downie et al., 1971). Further testing can be done on samples from patients to confirm the presence of an enveloped virion in the electron microscopy, the lack of growth on choriallantois of chick embryos, and PCR determination with tanapox virus primers (Dhar et al., 2004). Once diagnosis has been made no treatment need be administered due to its ability to heal on its own without patient risk.

Tropism

The tropism of tanapox is rather narrow, which may explain the location of lesions on the body as well as the behavior of tanapox virus. Tanapox virus is a self-limiting infection which may be due to its optimal temperature of 34ºC being below host core body temperature (Nazarian et al., 2007b). Tanapox virus uses monocytes for replication due to their rapid division and movement to sites of naturally occurring infections which may be the cause of the acute febrile illness. Tanapox virus cannot replicate in a cell in which the cell cycle is arrested, limiting its ability of infecting the body to that of proliferating cells.

Viral Genome

With both known strains of tanapox virus sequenced, many of the putative functions of the predicted 151 open reading frames have been hypothesized (Lee et al., 2001, Nazarian et al., 2007a). These sequences have been compared to both other poxviruses and tanapox virus isolates from different decades to determine homology, evolution and function of these genes. The tanapox virus genome is predicted to encode for all the necessary proteins for viral DNA replication and packaging. In addition, it also encodes for proteins involved in evading the immune response in humans, some of which have been examined in detail. Tanapox virus produces an interleukin-10 related cytokine (Bartlett et al., 2004), an inhibitor of interleukin-18 (Nazarian et al., 2008), an inhibitor of tumor necrosis factor (Brunetti et al., 2003), multiple anti-cytokines (Essani et al., 1994), and glycoproteins to inhibit interferons

(Huang et al., 2007). Furthermore, tanapox virus expresses a member of the complement control protein family (Law et al., 2004) and a chemokine receptor (Najarro et al., 2006).

Poxviruses with Oncolytic Potential

Vaccinia virus has been included in the many viruses that have been used to target cancer cells. Ever since the early 20th century, viruses have been considered promising ways of targeting and destroying cancer cells (Sinkovics & Horvath, 2000). Since that time, many viruses have been tested in both experimental settings and in humans, with most ending in failure or with side effects too harmful to warrant continuation (Vähä-Koskela et al., 2007). However, resurgence in the 1990s, with new biotechnology methods available and the beginnings of gene therapy, made virotherapy an available option for cancer treatment plans. Virotherapy can be accomplished in a variety of ways that can be either direct or indirect.

Indirect Virotherapy

Indirect methods are when the viruses induce an immune response from the host to target tumor cells. The foremost way of evoking the desired response is through tumor vaccination or cancer immunotherapy (Vähä-Koskela et al., 2007). In this way the body can maintain immunologic memory as well as evoke tumor-specific immunity to eradicate established tumors (Schuster et al., 2006). What's more is that even simple vaccination against common pathogens has indirect effect of cross-protection between viral epitopes and cancer (Kölmel et al., 2005). Viruses can also

be used as adjuvants that enhance tumor cell recognition by T cells or dendritic cells when they encode immunostimulatory cytokines (Vähä-Koskela et al., 2007). However, viral transduction into the tumor vasculature remains an ideal target as a way to prevent angiogenesis and oncotoxic prodrug production.

Direct Virotherapy

In order to target tumor cells, viruses must be able to select between normal cells and cancer cells, which can be achieved either naturally or through viral engineering. For a virus, being able to distinguish between these two cell types may determine its success in propagation. Cancer cells provide the virus with a fertile ground for replication due to the loss of intracellular defense mechanism components (Cahill et al., 1999). These cells are often easier for the viruses to infect because some malignant cells express higher levels of viral receptors than normal cells (Shafren et al., 2004). Viral tropism towards cancer can be enhanced by engineering the virus to be conditionally replicating (Vähä-Koskela et al., 2007), by taking advantage of the fact that many cancer cells have activated genetic pathways or loss of tumor suppressors (Kim et al., 2006). Adenovirus ONYX-015 is an example in which the E1B gene has been deleted, restricting its replication to cells not containing a wild-type p53, as is the case in many cancers. Viruses have also been engineered to express exogenous cytotoxic genes or modified to prevent harmful side effects due to viral infection. Other viruses inherently act by not killing the cell, but rather by preventing cellular transformation and tumorigenesis, as seen in parvoviral infection

of tumors (Iseki et al., 2005). These viruses cause oncosuppression through histone acetylation by their nonstructural proteins.

Clinical Success

Among the variety of oncolytic viruses there are three generations. The first generation are the viruses that have inherent capacity for tumor cells such as reovirus and vesicular stomatitis virus (Kirn & Thorne, 2009). Then there are the second generation viruses consisting of deletion mutants that are engineered for cancer specificity such as adenovirus and herpes simplex virus. For both of these categories clinical trials have deemed them safe and selective, but lacking in potency (Liu et al., 2008). This is why in third generation oncolytic viruses the added strategy of arming the viruses has shown more success, such as in vaccinia virus (Kim et al., 2006).

Poxviruses as Cancer Therapeutic Agents

Many viruses have been used to target cancer cells, with one of them being vaccinia virus. Several approaches have been used to adapt vaccinia virus for use as an anti-cancer agent. They are modified viruses that have been used to deliver tumor-associated antigens (Conry et al., 2000), cytokines (Mastrangelo et al., 1999) and costimulatory molecules (Hodge et al., 1999) to induce an antitumor immune response. Vaccinia virus has also been used to deliver genes for enzyme-prodrug therapy (Gnant et al., 1999b) and sensitization to systemic treatment with tumor necrosis factor (Gnant et al., 1999a). However, vaccinia virus has some drawbacks in that it infects both tumor and non-tumor cells (Guo et al., 2005), and hosts often have

a pre-existing immunity to it (Hu et al., 2001) sometimes making it a problematic system. For this reason, other poxviruses, such as YLDV, are now being examined for oncolytic potential. YLDV shows potential because it does not cross-react with vaccinia virus antibodies, replicates in human cells, and has relatively mild infection under normal conditions making it safer to work with (Paulose et al., 1998). For all of these reasons, YLDV and tanapox virus may be the future in fighting cancer through virotherapy.

p53

p53 is a classical tumor suppressor gene that is mutated in over half of all human tumors (Ryan et al., 2001). Under normal conditions p53 is kept at a low steady state level that dramatically increases under cellular stress conditions (El-Deiry, 1998). These stresses include DNA damage, telomere attrition, oncogene activation, hypoxia, and loss of normal growth and survival signals (Ryan et al., 2001). Once p53 becomes activated, many possible responses could occur which include differentiation, senescence, DNA repair, inhibition of angiogenesis, cell cycle arrest or apoptosis (Bates & Vousden, 1999) (See figure 4).

Downstream Transcription

The response depends on what p53 targets for transcriptional upregulation. Upregulation of the cyclin dependent kinase inhibitor p21 induces a cell cycle arrest response (El-Deiry, 1998). This upregulation is essential to sustain cell cycle arrest in G2 after DNA damage occurs (Bunz et al., 1998). Ribonucleotide reductase (p53R2)

can then be induced for DNA repair (Tanaka et al., 2000). If repair is not possible then p53 can stimulate apoptosis by inducing mitochondrial localized proteins Bax, NOXA (Oda et al., 2000a), PUMA (Yu et al., 2001) and p53AIP1 (Oda et al., 2000b) that trigger cytochrome c release. p53 can also trigger death receptor transcription such as Fas, Killer/DR5, and death-domain-containing protein PIDD (Lin et al., 2000).

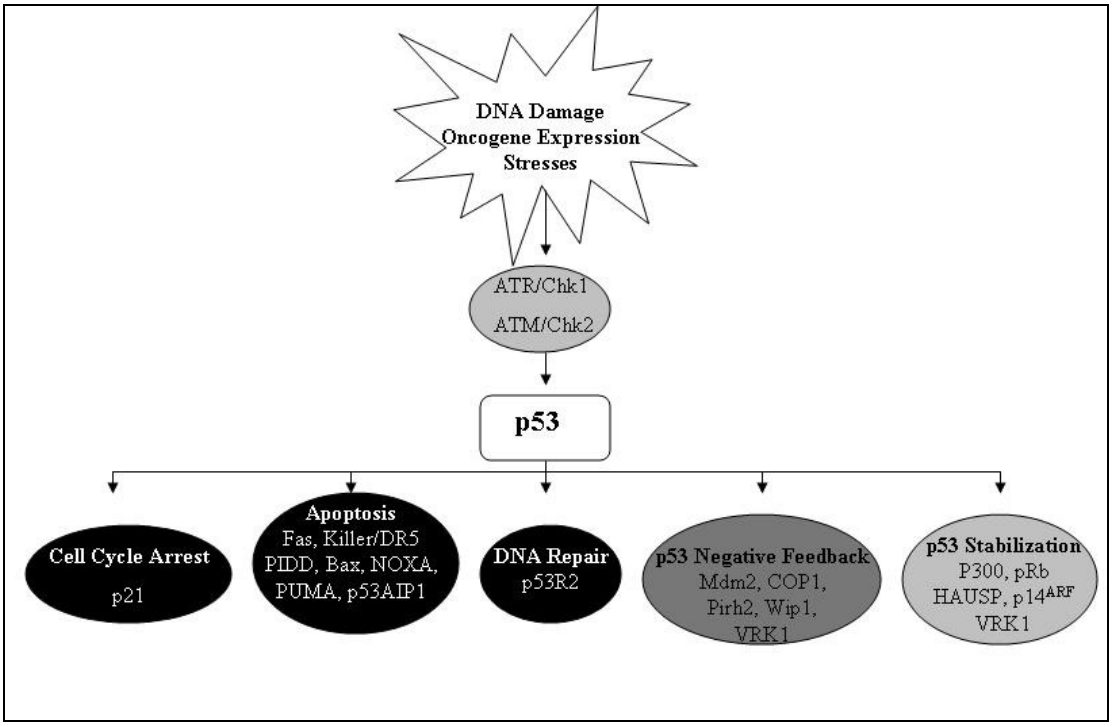

Figure 4. The p53 Pathway. Black ovals signify proteins involved in downstream transcription by p53, the dark gray oval denote proteins involved in p53 regulation and the light gray oval indicate proteins involved in p53 stabilization.

Regulation

Due to the fact that p53 can potentially cause so much harm to normal cell cycle progression, it must be under tight regulation. Amongst the multiple negative

regulators of p53, Mdm2 is considered key in protecting the cell from apoptosis. Mdm2 functions as an E3 ubiquitin-ligase that is responsible for the ubiquitination of both p53 and itself (Honda & Yasuda, 1999). Mdm2's C-terminus RING finger domain binds to p53's N-terminus to allow ubiquitination and nuclear export of the complex (Kubbutat et al., 1999). Once in the cytoplasm, the poly-ubiquitination acts as a signal for proteasome-dependent degradation (Hicke & Dunn, 2003). Mdm2 becomes upregulated when p53 becomes activated because Mdm2 is a transcriptional target of p53 putting these two cellular regulators in an autoregulatory loop (Oren et al., 2002).

Stabilization

On the other hand there are also proteins that can stabilize p53 in the cell. One of the most important ways for p53 to be stabilized is through acetylation by p300 (Yuan et al., 1999). p53 requires the acetylation of p300 in order to induce cell cycle arrest after irradiation. It is this binding that is responsible for arrest during G1, yet it does not interfere with the p53-Mdm2 complex. However, DNA-damage-induced kinase Chk1 and Chk2 do interfere with this complex by phosphorylating the N-terminus of p53, preventing the binding of Mdm2 and stabilizing p53 (Chehab et al., 2000, Shieh et al., 2000).

B1R

Since the declaration of smallpox being eradicated from the world, vaccinia virus has been the model for studying poxviruses, and its genome was the first

orthopox virus to be completely sequenced (Goebel et al., 1990). Many of the genes have since been characterized based on sequence homology as well as functional testing. The B1 gene, for example, has been thoroughly examined in respect with its homology to protein kinases. Since the discovery of B1R protein, others have explored homologs in mammalian models, specifically human and mouse (Lopez-Borges & Lazo, 2000). These homologs have since been dubbed vaccinia-related kinases (VRKs), with B1R being the prototypical model.

Protein Kinase

B1 is a 34-kDa essential gene for viral replication (Traktman et al., 1989). B1 is highly conserved in every member of the Poxvirus family except Molluscum contagiosum virus (MCV), which may be why MCV exhibits such host restriction (Boyle & Traktman, 2004). Upon sequencing B1, it was observed that B1 shows significant homology to many protein kinases, due to its predicted ATP binding domain, catalytic residues, phosphorylation receptor site, and its Gly-X-Gly-X-X-Gly motif (Traktman et al., 1989). This glycine-rich motif is a consensus sequence found in many nucleotide binding proteins, and its three-dimensional structure shows this as an ATP binding site in protein kinases (Hanks et al., 1988). The presence of an invariant lysine residue at position 149 offered evidence that supported the prediction that B1R was a serine/threonine kinase.

Activity Characteristics

B1 has been demonstrated as being expressed early in infection with temporal changes in phosphorylation as infection progresses (Traktman et al., 1989) and that it is oriented to the right (Rempel et al., 1990). It is this early expression that is required for viral DNA replication (Traktman et al., 1989). However, B1R has demonstrated a role not just in initiation of replication, but throughout the replication process with many possible substrates (Rempel & Traktman, 1992b). The activity of B1R has been further characterized in terms of optimal activity parameters. The optimal donor for phosphorylation is ATP, while GTP shows much less activity. On the other hand, VRK1 uses both ATP and GTP as phosphate donors, which makes it unique from other casein kinases (Barcia et al., 2002). This activity of VRK1 also depends on divalent cations, particularly Mn^{2+}, Mg^{2+} and Zn^{2+} to become active, as well as an intact C-terminus for correct folding. The optimal pH for B1R's kinase function is between 8 and 9 (Lin et al., 1992). B1R has been confirmed to only phosphorylate serine and threonine residues, with a preference to threonine. Within virion cores there are many protein kinases present, of which B1R makes up about 4%, which is approximately forty times the amount that is found within the virion envelope.

Targets

In addition to early expression, B1R can be found stably during late stage, as well as located in virions (Banham & Smith, 1992). Due to this fact, B1R can interact with a variety of targets (Table 1). In infected cells B1R protein localizes to

cytoplasmic factories where virus DNA synthesis occurs. In order for viral DNA synthesis to take place in the cell, there must be a switch from host DNA synthesis to viral. The 40S ribosomal proteins S13, Sa and S2 have demonstrated phosphorylation by other viral proteins that allows for a switch to viral mRNA translation with temporal association and B1R has been shown to phosphorylate both Sa and S2 (Banham et al., 1993). This is possibly the reason for B1R's presence in the virion. B1R protein has also been shown to phosphorylate a single stranded DNA-binding protein expressed by vaccinia virus (Beaud et al., 1995). This protein is the product of the H5R gene that is phosphorylated at several threonine sites and thought to be involved in virus assembly by targeting membranes and DNA-protein foci of viral replication. It is this interaction of these two viral molecules that is able to inhibit the host immune system by inhibiting CD1d1 T lymphocytes, by which the virus can survive (Webb et al., 2006). To aid in the protection of vaccinia virus from the host immune system and allow replication, B1R also targets α-2-macroglobulin, which inhibits proteases within the coagulation cascade (Rempel & Traktman, 1992b).

MAPK

In viral infections, the MAPK pathway is often activated as a way of guaranteeing the life cycle of the cell to allow the virus to replicate (Santos et al., 2006a). In MAPK pathways several kinases are working together to activate transcription factors and other kinases (Davis, 2000). In some circumstances these kinases come together in a complex that is held together by the JIP scaffold (Jaeschke

et al., 2004). This complex can then go on to activate the c-Jun transcription factor that is a component of the AP-1 complex (Weston & Davis, 2007). In vaccinia viral infections, B1R has been shown to phosphorylate c-Jun and complex with the JIP scaffold (Santos et al., 2006a). This interaction with the JIP scaffold leads to an increase in the amount of MAPK bound to JIP1 and an increase in phosphorylation of JNK. More stable interactions result in an increase in activation of transcription factors such as c-Jun. With these methods B1R is capable of stabilizing c-Jun in two different ways.

However, VRK2 interacts with the MAPK pathway in the opposite manner. Both forms of VRK2 have been seen to block the stimulation of TAK1 signaling in response to hypoxic conditions without the use of their kinase domains (Table 2) (Blanco et al., 2007). TAK1 is a mitogen activated protein kinase kinase kinase (MAPKKK) that is the first molecule to bind to the JIP1 scaffold to induce a response to cellular stress. It has been shown that VRK2A is capable of forming a stable complex with TAK1 the best, which can in turn determine the magnitude of the stress response. VRK2 has also been shown to downregulate the activation of transcription induced by the IL-1β signal by interfering with the response signal at the MAPK level (Blanco et al., 2008). VRK2 is able to bind to the JIP1 signalsome, which destabilizes the complex and displaces JNK.

Table 1. Substrates Phosphorylated by Protein Kinases.

Kinases	Substrates	References
B1R	40S Ribosomal Protein S2	(Banham et. al., 1993)
	40S Ribosomal Protein Sa	(Banham et. al., 1993)
	α-2-macroglobulin	(Rempel & Traktman, 1992)
	BAF	(Nichols et. al., 2006)
	c-Jun	(Santos et. al., 2006a)
	p53	(Santos et. al., 2004)
	Vaccinia Virus H5R	(Beaud et. al., 1995)
VRK1	ATF2	(Sevilla et. al., 2004)
	BAF	(Nichols et. al., 2006)
	Core Histones H2B, H3, H4	(Kang et. al., 2007)
	p53	(Vega et. al., 2004)
VRK2	BAF	(Nichols et. al., 2006)
	p53 (B form only)	(Blanco et. al., 2006)

Table 2. Other Interactions With VRKs.

Proteins	Substrates	References
B1R	JIP1	(Santos et al., 2006a)
VRK1	CDK2	(Santos et al., 2006b)
	SURVIVIN	(Santos et al., 2006b)
VRK2	BHRF-1	(Li et. al., 2006)
	JIP1	(Blanco et. al., 2008)
	TAK1	(Blanco et. al., 2007)
VRK3	VHR	(Kang & Kim, 2008)

BAF

B1R, VRK1 and VRK2 are all capable of phosphorylating barrier-to-autointegration factor (BAF) (Table 1) (Nichols et al., 2006). BAF is an essential, conserved, 10-kDa protein that binds to LEM domains, anchors chromatin to nuclear periphery, and inhibits the formation of the nuclear envelope (Segura-Totten et al., 2002). When BAF becomes phosphorylated on Ser4 and to a lesser extent Thr2 and Thr3, there is a decrease in interaction with the LEM domain, and a disruption of the ability to bind to DNA (Nichols et al., 2006). This is most likely due to the relocation from the normal nuclear location of the phosphorylated form to throughout the cell. The hypothesis is that BAF becomes phosphorylated during prophase allowing chromatin to condense, and then is later dephosphorylated during anaphase allowing localization at the chromosomes and the recruitment of LEM proteins. BAF has been shown to be required from the onset of anaphase until the nuclear envelope reforms in telophase, where it is needed for assembly, but not maintenance (Gorjánácz et al., 2007). During mitosis VRK remains associated with chromatin, continually phosphorylating BAF to allow mitosis to occur correctly and without VRK1 the nuclear envelope cannot form properly.

B1R's phosphorylation is a mechanism for the virus to overcome the host's defense against viral replication (Wiebe & Traktman, 2007). Normally upon infection, BAF relocates to areas of viral replication, binds to DNA, and inhibits DNA synthesis. However, the virus counteracts this through the expression of B1R protein that can phosphorylate BAF, which causes BAF to remain diffuse in the cell, unable

to bind DNA. It is able to recognize viral DNA not through the sequence, but due to its inappropriate location (Rahman & McFadden, 2007).

p53

However, B1R is not only found in the cytoplasm, but also in the nucleus of the cell (Santos et al., 2004). Here B1R is able to hyperphosphorylate p53, the tumor suppressor that is then downregulated in an Mdm2-dependent mechanism. B1R phosphorylates p53 in the same location that Mdm2 interacts with, and in fact B1R needs Mdm2 to be present in order to have effect. This is because when Mdm2 is not present, B1R phosphorylation causes an accumulation of p53. By the downregulation of p53, B1R inhibits apoptosis, allowing viral DNA synthesis to occur.

VRK1 and VRK2 also both target tumor suppressor p53 through phosphorylation of Thr-18 which stabilizes and accumulates p53, and are thought to normally help maintain a steady state of readiness (Vega et al., 2003). VRK1 does this by phosphorylating p53 on the Thr18 residue, which stabilizes p53 by interfering with the degradation of p53 by the proteasome, as well as recruiting other stabilizing proteins p300 and Mdm4 (Vega et al., 2004). VRK2B can phosphorylate p53 to induce accumulation and activate its transcriptional activity as well (Table 2), in a mechanism very similar to the nature of VRK1 despite the differences in the last ninety amino acids of these kinases (Blanco et al., 2006). It has been hypothesized that this redundant function of VRK2B may be utilized when VRK1 is cytosolic.

VRK1 Rescues B1R

VRK1 has even been shown to rescue a B1R mutant in regards to viral DNA synthesis, without the phosphorylation of H5R protein (Boyle & Traktman, 2004). hVRK1 is able to fully restore intermediate and late expression genes, while mVRK1 can only restore some genes. However, in both cases viral production is only partially restored, which may be due to the fact that cellular VRK localizes to the nucleus while vaccinia virus replicates in the cytoplasm.

142R

142R is a putative early serine/threonine kinase of tanapox virus (Nazarian et al., 2007a). It has been found to exhibit homology with vaccinia viral protein B1R in both sequence and genome location. This has thus led to the speculation that both proteins will have similar transcriptional timing and function in the host cell. The predicted kinase function is based on the residues found in 142R that are highly conserved in most protein kinases.

Protein Kinases

Protein kinases make up a large family of enzymes that are responsible for cellular responses to internal and external stimuli (Hanks et al., 1988). These enzymes have a well conserved catalytic domain that most often lies near the C-terminus and is 250 to 300 amino acids in length. Within the catalytic domain there are eleven major conserved subdomains (Table 3), which are separated by regions of

lower conservation in the form of gaps or inserts. This alternating arrangement is a common feature of homologous globular proteins (Chothia & Lesk, 1986), where the conserved regions are active and the nonconserved make up the loops that bring the active sites together (Hanks et al., 1988).

Table 3. Conserved Regions of Serine/threonine Kinases With Respect to Adenosine 3', 5'-Monophosphate (cAMP)-dependent Protein Kinase Catalytic Subunit, α Form (cAPK).

Subdomain	Motif	Function	Found in 142R
I	Gly-X-Gly-X-Gly	Nucleotide Binding	Yes
II	Lys^{72}	Phosphotransfer and Proton Transfer	Yes
III	Glu^{91}	ATP Binding	
VI	Val^{157}	ATP Binding	
	Asp-Leu-Lys-Pro-Glu-Asn	ATP Binding	Yes
VII	Asp^{184}, Phe^{185}, Gly^{186}	ATP Binding	Yes
VIII	Ala-Pro-Glu	Catalytic Domain Indicator	Yes
IX	Asp^{220}, Gly^{225}	ATP Binding	Yes
XI	Arg^{280}	ATP Binding	

Serine/threonine Kinases

There are three major types of kinases: those that phosphorylate serine/threonine, those that phosphorylate tyrosine and those that phosphorylate all three amino acids (Hanks et al., 1988). Serine/threonine kinases then go on to be further categorized based on the mode of regulation, whether they are retroviral transforming proteins, their oncogenic potential, relatedness to yeast cell cycle control relatives, or from herpes.

Conserved Domains

Based on the prototypical protein kinase, bovine adenosine 3', 5'-monophosphate (cAMP)-dependent protein kinase catalytic subunit, α form (cAPK) sequence there are nine identical amino acid residues: Gly^{52}, Lys^{72}, Glu^{91}, Asp^{166}, Asn^{171}, Asp^{184}, Gly^{186}, Glu^{208}, and Arg^{280} (Hanks et al., 1988). There are also five slightly less conserved amino acid residues present as Gly^{50}, Val^{157}, Phe^{185}, Asp^{220}, and Gly^{225}. Many of these conserved residues are responsible for binding with ATP for phosphotransfer.

Within subdomain I there is a conserved consensus sequence of Gly-X-Gly-X-X-Gly very near the N-terminus of the catalytic domain. This sequence is responsible for binding nucleotides with the first Gly contacting the ribose and the second near the terminal pyrophosphate (Sternberg & Taylor, 1984). Working with this sequence are two hydrophobic residues lying upstream in positions one and seven, in addition to an invariant valine two positions downstream, that may position these glycines (Hanks et al., 1988). In subdomain II there is an invariant lysine at position 72 that is directly involved in the phosphotransfer reaction and possibly proton transfer as well (Kamps & Sefton, 1986). In 142R this lysine is located at residue 57.

The central core region, which includes subdomains VI through IX, is usually the most highly conserved (Hanks et al., 1988). The invariant residues of subdomains VI and VII have been implicated in the binding of ATP. Subdomain VIII contains a key protein kinase catalytic domain indicator of the consensus triplet Ala-Pro-Glu which is required for activity (Bryant & Parsons, 1983). However, in both B1R and

142R, this consensus triplet has been changed to Thr-Leu/Ile-Glu. This alteration is still considered to be conserved because the two changes are to other non-polar residues. This sequence has been shown to lie very near the catalytic site of the enzyme (Hanks et al., 1988). Both subdomains VI and VIII contain consensus sequences that are indicative of serine/threonine kinase activity (Hanks, 1987). Implicated in ATP binding is the Asp-Leu-Lys-Pro-Glu-Asn consensus sequence of subdomain VI (Hanks et al., 1988). While B1R and 142R do contain most of this sequence, both have alterations for the Pro-Glu residues which have been changed to Ala-Ser. Both changes retain polarity properties, however the Glu to Ser substitution changes from an uncharged polar molecule to an acidic polar molecule. Once again these changes are not considered to alter the conserved region's function. Subdomain VIII contains a sequence immediately on the N-terminus side of the Ala-Pro-Glu sequence of Gly-Thr/Ser-X-X-Tyr/Phe-X-Ala-Pro-Glu.

Phylogeny

Upon phylogenetic analysis of known kinase catalytic domains, it was determined that the evolution of these domains stem from gene duplication events and/or speciation events (Hanks et al., 1988). Kinases that were found clustered were at least 35% similar within the cluster, and they were found 20-25% similar when not from the same cluster, although tyrosine kinases show higher clustering ability. We propose to add 142R to this family and determine the relatedness to the plethora of known kinases.

MATERIALS AND METHODS

Cell Lines and Virus

All cell lines were obtained from American Type Culture Collection. Cos-7 were cultured in DMEM maintenance medium consisting of Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (Dot Scientific) and 1x antibiotic-antimycotic (Invitrogen). Owl Monkey Kidney (OMK) cells were cultured in EMEM maintenance medium consisting of Eagle's minimum essential medium (EMEM) (Sigma) and supplemented with 10% newborn calf serum (NBCS) (Life Technologies), 1.5 g/L sodium bicarbonate, 2 mM L-glutamine and antibiotics (penicillin G sodium 100 units/ml and streptomycin sulfate 100 µg/ml) (Invitrogen).

TPV-Kenya was obtained from the Center for Disease Control. Inoculated cell monolayers were maintained in EMEM supplemented with 2% NBCS, 1.5 g/L sodium bicarbonate, 2 mM L-glutamine and antibiotics.

Plasmid Constructs

pGEX-142R

TPV-Kenya DNA was isolated by phenol/chloroform extraction and ethanol precipitation (Sambrook et al., 1989). The 142R ORF was then amplified by polymerase chain reaction (PCR) using DyNAzyme polymerase (Fisher) and oligonucleotides 5'- GGGGAATTCATGTCAAAAAACCAAG and 5'-

CTCGAGTTAAAAATGGTT as primers. The product was then purified using the QIAquick PCR purification kit (Qiagen), digested with EcoRI and XhoI endonucleases and repurified by gel electrophoresis and the QIAquick gel extraction kit (Qiagen). This fragment was inserted into the multiple cloning site (MCS) of the pGEX-5X-1 vector (gift from Dr. PE Hoppe) to create pGEX-142R for expression in bacteria.

pcDNA-142R

The pcDNA-142R plasmid was created in a similar fashion as the pGEX-142R plasmid using oligonucleotides 5'- GGGCTCGAGATCTCAAAAAAC and 5'-GGGGAATTCAAAATGGTTTAAAAATA as primers and pcDNA3.1myc/his (Invitrogen).

p2KOΔ142R

TPV-Kenya DNA was used to amplify a left flanking region, which spans from the start of 141R and continues 210bp into 142R, using oligonucleotides 5' - GGGGGGGAGCTCAATTATGAACTACTGCTAT and 5'-GGGGGGGCGGCCGCTTGATAAAATACTTGTTCTA as primers. A right flanking region, which includes the last 230bp of 142R and the first 438bp of 143R was amplified by PCR using oligonucleotides 5'-GGGGGGGAATTCTAAAAGTGGATGAGTGGTAA and 5'-CCCCCCCTCGAGTTTTCCTTTTTTGTGTATAG as primers. The PCR products were then purified, digested with SacI and NotI endonucleases for the left flank and

EcoRI and XhoI endonucleases for the right flank, and gel purified. The two fragments were then inserted into the p2KO plasmid (created by S. Conrad and D. Jeng). This plasmid was created by inserting two pox viral promoters and a mCherry tag into the MCS of pBluescript (Invitrogen). The resulting plasmid was designated p2KOΔ142R.

pcDNA-142R-GFP

The GFP region from the SV40 origin site to the SV40pA site of the pTracer-CMV plasmid (Invitrogen) was amplified by PCR using oligonucleotides 5'-GTTAACCCCAGGCTCCCCAGG and 5'- ATCGATGCAGTGAAAAAAATG as primers. The PCR product was purified, digested with HpaI and ClaI endonucleases and gel purified. This fragment was ligated into the pcDNA-142R plasmid after it was digested with HpaI and ClaI endonucleases and gel purified to create the pcDNA-142R-GFP plasmid.

pET21-142R and pPICZA-142R

The pcDNA-142R plasmid was digested with EcoRI and XhoI endonucleases to isolate the fragment containing 142R. This fragment was then gel purified and ligated into the MCS of pPICZ A vector to create pPICZA-142R plasmid for expression in yeast and pET-21a(+) vector (gift from Dr. S Rossbach) to create pET21-142R plasmid for expression in bacteria.

pET15-142R

The pGEX-142R plasmid was digested with BamHI and XhoI endonucleases to isolate the 142R fragment. This fragment was gel purified, ligated to the linker oligonucleotide consisting of the two strands 5'- GATCCGGTACCCCA and 5'- TATGGGGTACCG and inserted into the MCS of pET-15b vector (gift from Dr. B Tripp) to create pET15-142R plasmid for expression in bacteria.

pET45-142R

The pGEX-142R plasmid was digested with EcoRI and XhoI endonucleases to isolate the fragment containing 142R. This fragment was then gel purified and ligated into the MCS of pET-45b(+) vector (Novagen), which was digested with the HindIII endonuclease, blunted with DNA polymerase I and gel purified, to create pET45-142R plasmid for expression in bacteria.

pET21-GA

142R was codon optimized for expression in *E.coli* using Geneart (Invitrogen). The plasmid that was produced was digested with EcoRI and XhoI endonucleases, gel purified and inserted into the MCS of pET-21a(+) vector to create pET21-GA plasmid for expression in bacteria.

shRNA Plasmids

Short hairpin RNA (shRNA) plasmids were created using the pSUPERIOR.neo expression vector (OligoEngine). The BLOCK-iT RNAi program (Invitrogen) was used to predict shRNA targeting 142R. The oligonucleotide 5'-GATCCCCGCAGGTACGTACATAGCGATATAACGAATTATATCGCTATGTACGTACCTGCTTTTTGGAAA and 5'-AGCTTTTCCAAAAAGCAGGTACGTACATAGCGATATAATTCGTTATATCGCTATGTACGTACCTGCGGG were annealed and ligated into the pSUPERIOR.neo vector according to manufacturer's instruction to produce shRNA 464 plasmid, which targets 142R starting at the 464 base pair from the beginning. Likewise oligonucleotide 5'-GATCCCCGCAGGAGCATATCCTTCTAGAAGACGAATCTTCTAGAAGGATATGCTCCTGCTTTTTGGAAA and 5'-AGCTTTTCCAAAAAGCAGGAGCATATCCTTCTAGAAGATTCGTCTTCTAGAAGGATATGCTCCTGCGGG were annealed and inserted into pSUPERIOR.neo vector to create shRNA 652 plasmid. The control plasmids c-Jun and Fra were created by Denise Smith as described (Smith & Bejcek, 2011).

Protein Expression and Purification

Bacterial Protein Expression

All plasmids for bacterial expression, including plasmids obtained from Addgene p3113 GST-p53 (Huibregtse et al., 1991) and pGEX-4T MDM2 WT (Zhou

et al., 2001), were expressed in *Escherichia coli* strains. Among those used were BL21-A1 (gift from Dr. PE Hoppe), BL21-Star (gift from Dr. B Tripp), Rosetta (gift from Dr. Huffman), Top10, DH5α and BL21-plysS. Transformed bacteria were grown to an OD_{600} of 0.4 at 37°C, before the addition of 1 mM isopropyl-β-$_D$-thiogalactopyranoside (IPTG) (US Biologicals) and 0.2% L-arabinose (for BL21-A1) for induction of protein expression. Bacteria was then grown for 4 hours at either 21°C, 30°C or 37°C. Soluble protein was extracted by centrifugation and sonication. It was then subjected to affinity chromatography on a glutathione-agarose column (Novagen) for pGEX-142R, p3113 GST-p53 and pGEX-4T MDM2 WT. All other bacterial produced protein were isolated using either a nickel or a TALONspin cobalt column (Clontech) as all these proteins have His tags. Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay (Pierce) with a BSA standard.

Cos-7 Cell Protein Expression

For expression in Cos-7 cells, the 142R protein was produced by transfection of the pcDNA-142R plasmid with FuGENE 6 transfection reagent (Roche), according to the manufacturer's instruction. As determined by the NanoDrop 2000 spectrophotometer, 10 µg of pcDNA-142R plasmid was transfected into Cos-7 cells for 48 hours. Cells were then harvested in 1x cell lysis buffer (Promega) with 1 mM phenylmethanesulfonylfluoride (PMSF), sonicated and centrifuged. The whole cell lysate was then purified by TALONspin columns

(Clontech). Elutions were pooled and concentrated by Centricon10 filter devices (Millipore) at 4000rpm. Protein concentrations were determined with a BCA protein assay (Pierce). Protein expressed from the pcDNA-142R-GFP and empty vector pcDNA3.1 myc/his plasmids were treated in identical manner.

Yeast Protein Expression

For yeast expression pPICZA-142R was transformed into *Pichia pastoris* GS115/pPICZ/*lac*Z Mut$^+$ β-galactosidase from the Easy Select *Pichia* Expression Kit (Invitrogen). Protein isolation was performed according to the manufacturer's recommendation.

Protein Kinase Assays

Protein kinase assays were performed in a solution containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM dithiothreitol, 100 μM vanadate, 10 μM [γ-32P] ATP, and one of the following protein samples: 0.2 μg to 1 μg of 142R protein, 0.5 μg 142R-GFP protein or 0.6 μg pcDNA protein. Reactions were incubated at 30^{o}C for 1 hour, before termination by the addition of sodium dodecyl sulfate (SDS) gel sample buffer. Proteins were separated on 12% SDS-PAGE and exposed to Kodak X-Omat LS film or a Molecular Dynamics storage phosphor screen overnight. Screens were then imaged using the Storm860 scanner. Where indicated 85 μg dephosphorylated α-casein (Sigma), 100 μg BSA (New England Biolabs), 7.6 μg MDM2 protein or 37.5 μg p53 protein were included in the reaction mixture.

Recombinant Virus

To create the TPVΔ142R virus, 80-90% confluent OMK cells were inoculated with a 1X-lysate of TPV-Kenya for one hour. The media was then replaced with DMEM maintenance media and placed at 37ºC with 5% CO_2 until the pH returned to a normal level. Then the cells were transfected with either 2 µg pcDNA-142R-GFP or p2KOΔ142R plasmid (see Figure 5) with jetPRIME DNA transfection reagent (Polyplus-transfection) according to manufacturer's recommendation. Media was replaced four hours post transfection. Four to seven days later the cells were harvested, subjected to freeze/thawing on dry ice and reinoculated in new plates with 25 µl, 50 µl, 100 µl or 200 µl. Individual red plaques were picked from these plates, freeze/thawed, diluted 1:200 and reinoculated in new plates. This process was repeated at least 3 times or until all plaques were fluorescent. DNA was then harvested via phenol/chloroform extraction and subjected to PCR to verify that 142R had been successfully removed from the virus.

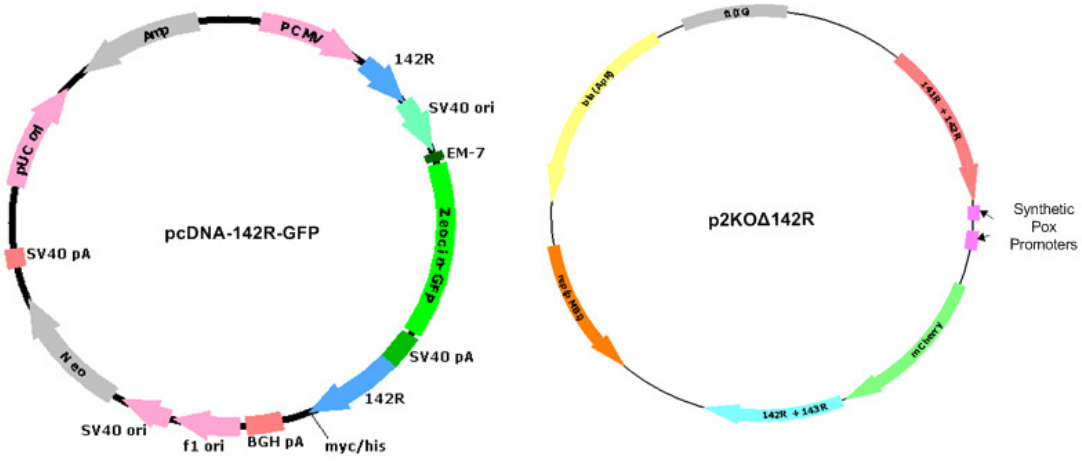

Figure 5. Plasmid Diagrams for Recombinant Virus Creation. The pcDNA-142R-GFP plasmid contains a GFP gene inserted into the middle of 142R. The p2KOΔ142R plasmid contains a mCherry gene between the two ends of 142R, with the middle of the gene removed. Both plasmids should integrate their fluorescence genes into the TPV-Kenya genome at the 142R locus through recombination generating knockout viruses.

shRNA

One microgram of pSUPERIOR.neo, pSUP-cJun, pSUP-Fra, shRNA 464 and shRNA 652 plasmids were all transfected into OMK cells in a 6-well plate with jetPRIME DNA transfection reagent (Polyplus-transfection) according to manufacturer's recommendation. Media was replaced 4 hours post transfection. Twenty-four hours post transfection, the cells were inoculated with TPV-GFP with an MOI of 0.1 pfu/cell. One hour post inoculation the media was replaced with EMEM maintenance media containing 0.4% methyl cellulose. In the sixth well there was no plasmid transfection introduced. Four days later, cells were scraped, freeze/thawed on dry ice, sonicated and diluted for viral quantitation.

Viral Quantitation

To determine viral concentration, TPV-GFP was serially diluted from 10^{-2} to 10^{-5} and inoculated in 6-well plates with 400 µl per dilution (3 wells per dilution). One hour post inoculation the media was replaced with EMEM maintenance media containing 0.4% methyl cellulose. These plates were then incubated for 10 days at 37ºC with 5% CO_2. The media was then removed and cells were stained with 0.1% crystal violet in 10% formaldehyde for 1 hour. Cells were then rinsed in deionized water and air-dried. The average number of plaques were counted and used to determine the total yield of virus.

Student's t-Test

P values were calculated using Graphpad Prism software by unpaired Student's t-Test comparing each treatment to the control that contained no plasmid. Differences with a $P < 0.05$ were considered as statistically significant.

RESULTS

Protein Expression

pGEX Plasmids

In order to express protein for use in kinase assays, pGEX-5X-1 plasmid (without an insert) was induced for protein expression in 2 different cell lines to determine which cell line would express protein more efficiently. Samples were separated on a 12% SDS-PAGE and stained with Coomassie blue (see Figure 6). Since BL21-A1 cells appeared to express the most protein this cell line was utilized in further expression experiments.

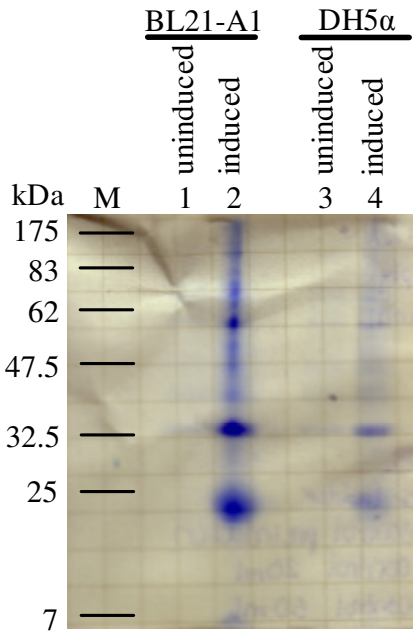

Figure 6. Comparison of Cell Lines for Expression of the GST Protein From the pGEX-5X-1 Plasmid. Lane M is the marker lane containing protein standards. Lane 2 shows that BL21-A1 has a higher level of protein expression upon the induction of the plasmid, than that of lane 4 of DH5α.

pGEX-142R, p3113 GST-p53 and pGEX-4T MDM2 WT plasmids were all induced for expression of protein, which was then isolated using a glutathione column or with a GST bead slurry and visualized on a 12% SDS-polyacrylamide gel stained with Coomassie blue (see Figure 7). Protein from the pGEX-B1R plasmid was never successfully isolated.

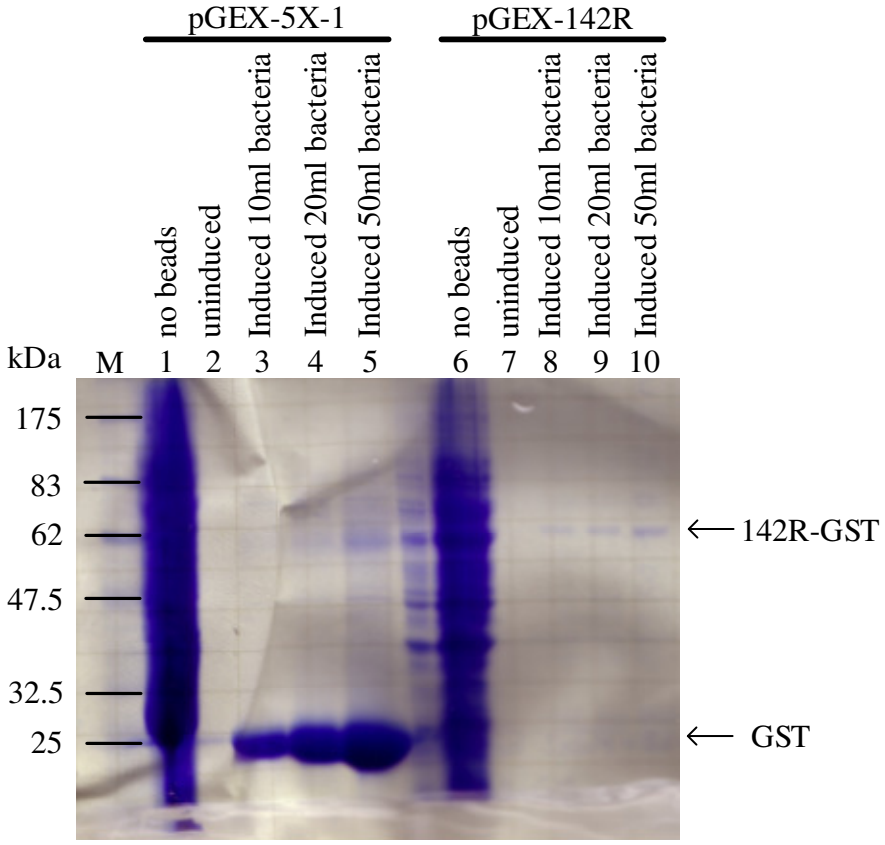

Figure 7. Isolation of Protein Expressed From the pGEX-5X-1 and pGEX-142R Plasmids. Lane M is the marker lane containing protein standards. Lanes 1-5 show the protein induction of pGEX-5X-1 plasmid at varying culture volumes isolated with a glutathione bead slurry. Lanes 7-11 show the protein induction of pGEX-142R plasmid at varying bacterial volumes isolated with a GST bead slurry. While the control plasmid yields more protein, both plasmids show a correlation of protein levels with the culture volume.

pET Plasmids

With the potential problems that a large GST tag may have on protein activity, we attempted to express 142R as a fusion protein with the smaller 6x-His tag using the pET series of vectors. Both 142R and B1R coding regions were cloned into pET-45b(+) vector. Using BL21-A1 cells protein expression was attempted at 21ºC, 30ºC and 37ºC (see Figure 8). In addition protein from pGEX-142R plasmid was also expressed in BL21-A1 cells at the same three temperatures. Protein expressed from the pET-142R plasmid was purified by column chromatography using either nickel or cobalt columns. pGEX-142R expressed protein was isolated using glutathione columns. Although a band that was of the predicted molecular weight for the fusion protein produced from the pET-142R plasmid could be visualized in whole cell lysates, it failed to adhere to either column. For these samples whole cell lysate was used in addition to the column isolates in case the protein was not abundant enough to be seen with Coomassie staining.

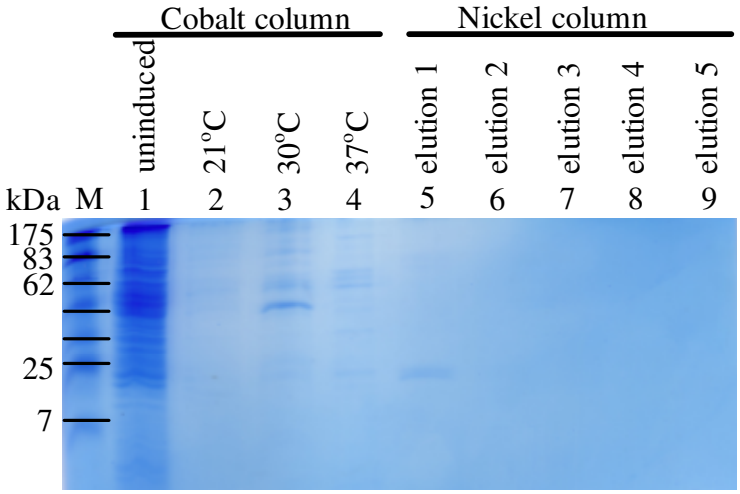

Figure 8. Protein Isolation Comparing Nickel and Cobalt Columns. Lane M is the marker lane containing protein standards. Lanes 2-4 are elutions from a cobalt column of protein expressed from the pET45-142R plasmid at a variety of temperatures. Lanes 5-9 are five different fractions of protein expressed from the pET45-142R plasmid from the elution off of a nickel column.

In order to produce a protein that can interact with cobalt columns, pET21-142R plasmid was created. In this construct the His tag is on the C-terminus, whereas the pET45-142R plasmid the His tag is on the N-terminus which can often inhibit the ability of chimeras to interact with the column. pET21-142R and pET21-B1R plasmids were both induced for protein expression in BL21-A1, BL21 Star, pLysS and Rosetta2 cells. Lysates were subjected to cobalt column chromatography, the eluted protein separated on a 12% SDS-PAGE and the gel stained with Coomassie blue (see Figure 9). The lane that was loaded with the elution 2 fraction showed a band of the expected molecular weight.

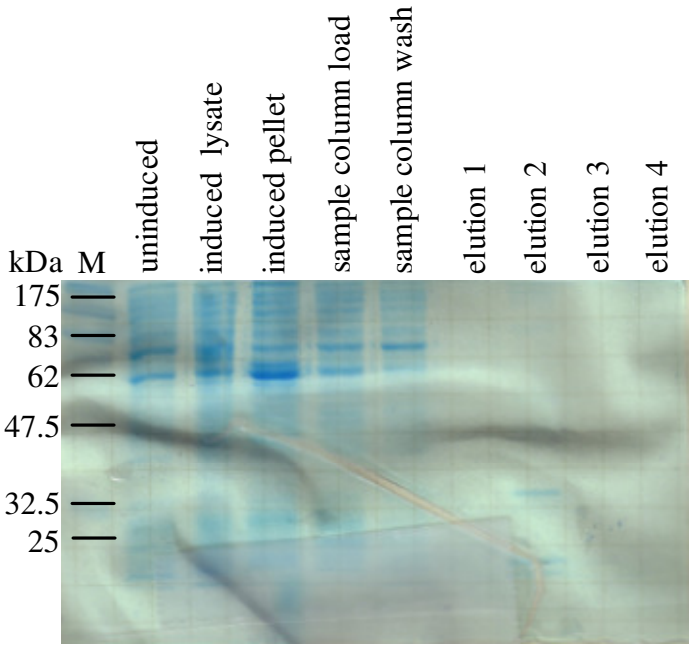

Figure 9. Protein Expressed From the pET21-142R Plasmid Isolated Using Cobalt Column Affinity Chromatography. Lane M is the marker lane containing protein standards. Elution 2 fraction does show some protein at the correct molecular size.

To determine if protein levels could be increased by codon optimization, the GeneArt program from Invitrogen was used to design an optimized version of 142R. This DNA with appropriate restriction sites was purchased from Invitrogen and cloned into the pET-21a(+) vector to create pET21-GA which was confirmed by sequencing. Protein was expressed at 21°C, 30°C and 37°C, and along with protein expressed from the pET-142R plasmid was isolated as previously described (see Figure 10).

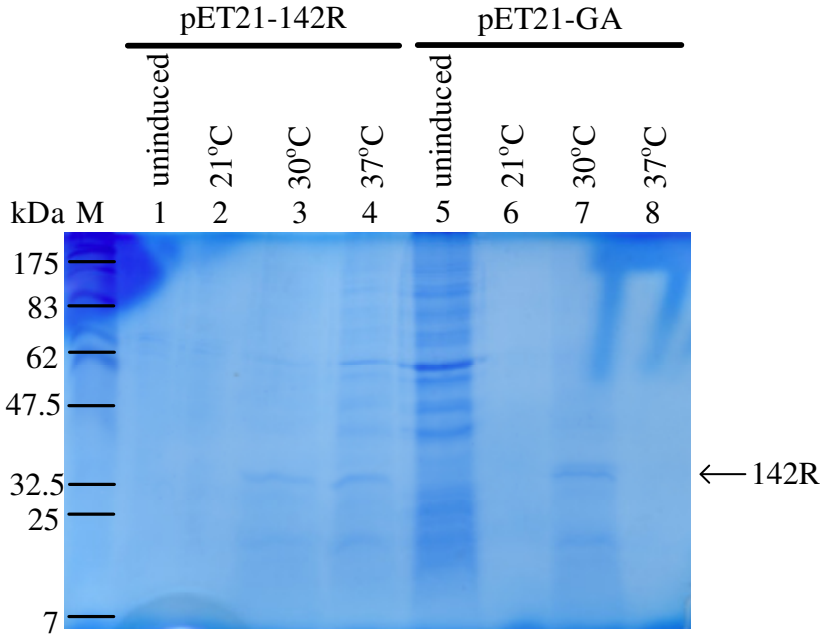

Figure 10. Protein Expression From the pET21-142R and pET21-GA Plasmids. Lane M is the marker lane containing protein standards. Lane 1 has protein isolated from uninduced bacteria containing the pET21-142R plasmid. Lanes 2-4 has protein expressed from the pET21-142R plasmid at 21°C, 30°C and 37°C. Lane 5 has protein isolated from uninduced bacteria containing the pET21-GA plasmid. Lanes 6-8 has protein expressed from the pET21-GA plasmid at 21°C, 30°C and 37°C. Lanes 3, 4 and 7 all show protein at the predicted molecular weight of 142R protein.

pPICZA Plasmids

To circumvent any potential problems such as misfolding in expressing protein in a prokaryotic system, 142R was cloned into the yeast expression vector pPICZ A (Invitrogen) to create pPICZA-142R. However, upon attempting to express the protein using the Easy Select Pichia Expression Kit (Invitrogen), the yeast failed to grow in repeated attempts. The few times yeast did grow, no 142R protein could be detected from cell lysates (data not shown).

pcDNA3.1myc/his Plasmids

Protein expression was also attempted in a mammalian system. Cos-7 cells are derived from an African green monkey kidney cell line, CV-1, that have been transformed with the SV40 virus. They express nuclear large T antigen as well as all proteins necessary for replication of DNA that has an SV40 origin of replication (Hancock, 1991). Any plasmid with the SV40 origin of replication in Cos-7 cells will result in rapid plasmid replication to high copy number along with expression of genes driven by an appropriate promoter. In this manner Cos-7 cells can quickly express protein that undergoes normal posttranslational processing making them ideal for transient expression of mammalian protein. The 142R ORF was cloned into the pcDNA3.1myc/his vector for expression in Cos-7 cells creating pcDNA-142R. This plasmid then had the GFP region of the pTracer-CMV plasmid cloned within the 142R region creating pcDNA-142R-GFP. Cos-7 cells were plated in 60mm dishes and transfected with pcDNA-142R, pcDNA-142R-GFP or pcDNA3.1myc/his plasmids. Dishes were scraped, centrifuged, sonicated and subjected to cobalt column chromatography. Protein expression was visualized by SDS-PAGE and Coomassie blue staining (see below).

Kinase Assays

Bacterial Expressed Protein

Kinase assays yielded no kinase activity using protein derived from pGEX plasmids (see Figure 11). Attempts were made at altering conditions, which included

fixing and drying the gel for a range of time, varying the pH of the reaction, digestion of GST away from 142R (see Figure 12) and exposing film for up to a week. There was no activity that could be detected under any of these conditions.

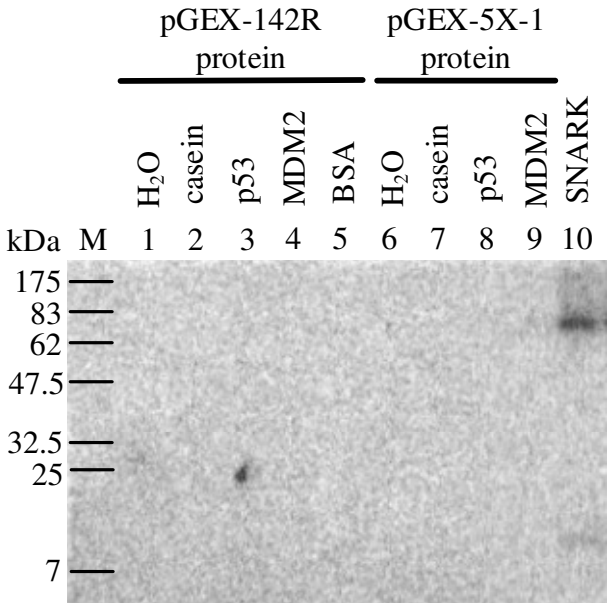

Figure 11. pGEX Plasmid Derived Protein Kinase Assay. Representative kinase assay obtained using the GST fusion 142R protein. Lane M is the marker lane containing protein standards. The first five lanes are proteins expressed from the pGEX-142R plasmid with a variety of substrates. Lanes 6-9 are the proteins expressed from pGEX-5X-1 plasmid with the same variety of substrates. The far right lane is the positive control, which is a SNARK protein (kind gift of Dr. P. Hoppe).

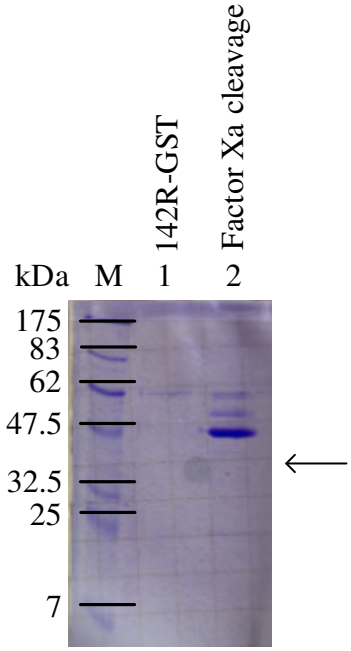

Figure 12. Protein Produced with the pGEX Vector is Refractile to Digestion with Factor Xa. Lane M is the marker lane containing protein standards. Lane 1 shows protein expressed from the pGEX-142R plasmid. Lane 2 shows protein from lane 1 cleaved with Factor Xa. The arrow represents the predicted size of 142R without a GST tag.

Since expression of 142R using the pGEX vector was not successful in providing an active protein we next tried expressing 142R using the pET series of vectors. We also tried expressing the pGEX plasmid at different temperatures. Kinase assays were performed with proteins expressed with the pET45-142R (with a His tag on the N-terminus) and pGEX-142R plasmids, but again no activity was detected (data not shown). The type of casein (casein from bovine milk and α-casein), concentration of casein, pH, amount of 142R derived protein (1-10 µg), concentration of ATP, temperature and time of incubation were all varied, but no assays demonstrated activity.

The next set of kinase assays used the protein fused to the His tag on the C-terminus of the pET-21 vector. A positive control casein kinase I was used to ensure the assay was performing correctly, but no samples showed kinase activity (see Figure 13).

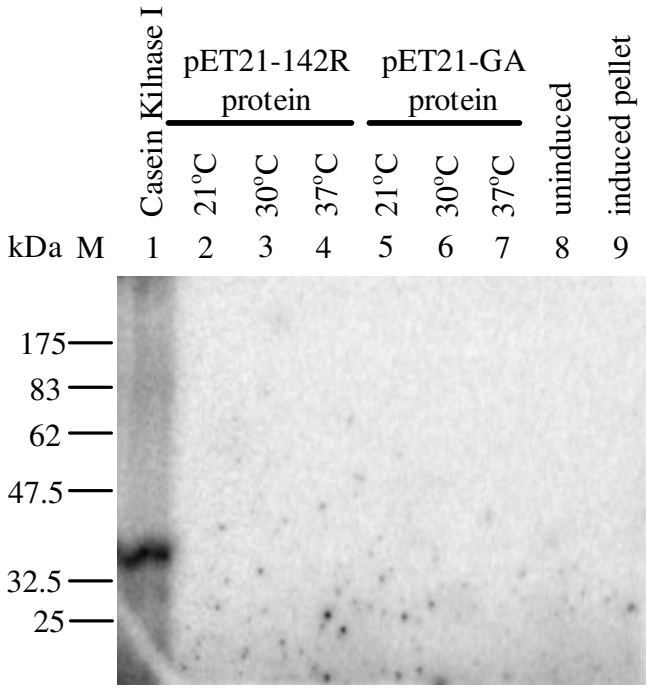

Figure 13. Protein Kinase Assay Using Protein Expressed From the pET-21 Plasmid. This assay is a representation of results obtained with protein produced using the pET-21 expression system. Lane M is the marker lane containing protein standards. Lane 1 is the positive control, which is casein kinase I. Lanes 2-4 has protein expressed from the pET21-142R plasmid from 21ºC, 30ºC and 37ºC with substrate p53. Lanes 5-7 has protein expressed from the pET21-GA plasmid from the same three temperatures with substrate p53.

Cos-7 Cell Expressed Protein

Protein expressed in transfected Cos-7 cells from the pcDNA-142R, pcDNA-142R-GFP or pcDNA3.1myc/his empty vector plasmids were visualized on

Coomassie blue stained gels. These proteins were also used in protein kinase assays. Proteins expressed from pcDNA-142R-GFP and pcDNA3.1myc/his are not easily visualized on this gel due to a very low concentration of protein, as determined by the Nanodrop. Whole cell lysates of any of these samples demonstrated phosphorylation of p53 and was therefore used as a positive control. The 142R protein was able to phosphorylate p53, but did not phosphorylate casein, BSA or MDM2. This indicated that whatever role 142R plays in regulating p53 it is due to direct interaction with p53 and not due to effects on other p53 pathway components. The multiple bands visualized in the lanes containing p53 correlate with the multiple bands that were in the p53 preparation. Neither protein from the vector alone nor 142R protein disrupted by GFP are able to phosphorylate p53.

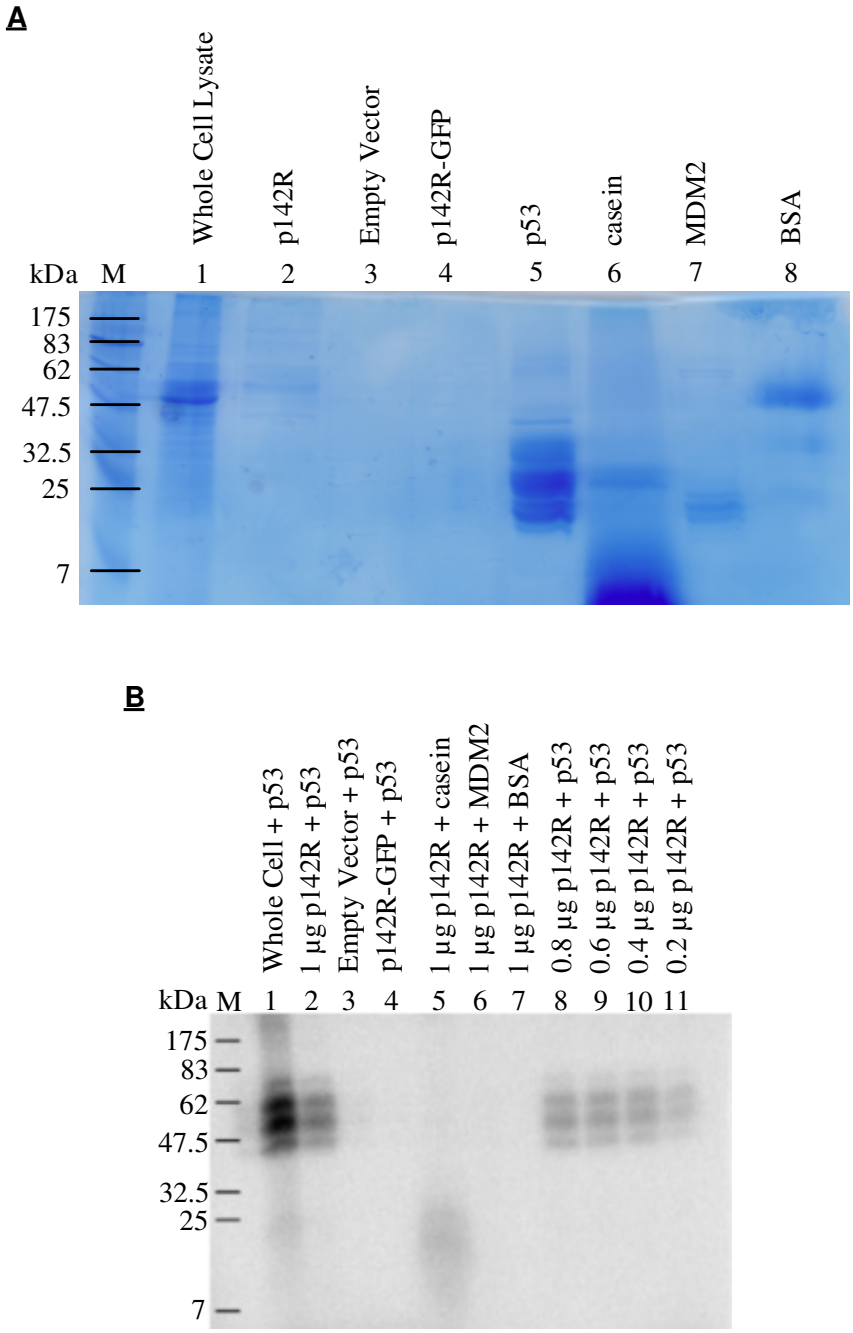

Figure 14. Protein Kinase Assay Using Protein Derived From the pcDNA Plasmids. Panel A – Coomassie blue stained gel showing all protein samples used in the kinase assay. Panel B – Protein kinase assay. Lane M is the marker lane containing protein standards. Lanes 1-4 are the expressed proteins with p53. Lanes 5-7 are the pcDNA-142R plasmid expressed protein with a variety of substrates. Lanes 8-11 are the pcDNA-142R plasmid expressed protein in decreasing concentrations with p53.

Creation of a Knockout Virus

pcDNA-142R-GFP Plasmid

The first plasmid that was used to try to create a 142R knockout virus was the pcDNA-142R-GFP plasmid. The cloning of a GFP region within 142R allowed enough TPV-142R sequence on either side of the GFP to allow for recombination with the wild-type virus at the 142R locus and detection of recombinant plaques exhibiting green fluorescence. However, after multiple transfection/infections no fluorescent plaques were observed. Upon further investigation it was determined that the CMV promoter would not be transcriptionally active and a different approach was needed.

p2KOΔ142R Plasmid

To change the promoter that was used to express the fluorescent protein the p2KOΔ142R plasmid was created. This plasmid has pox virus promoters driving expression of mCherry. Around four days post transfection/infection red plaques began to appear with many red plaques seen at seven days post infection (see Figure 15).

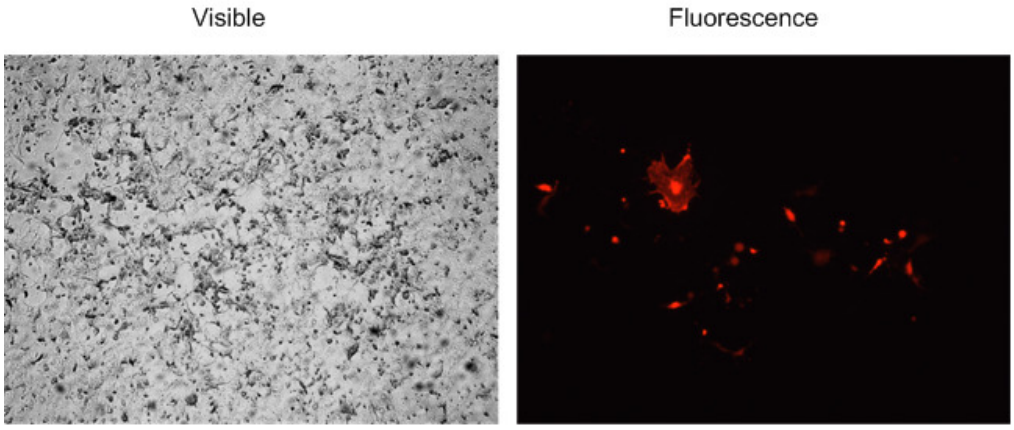

Figure 15. Transfection/Infection of TPV-Kenya and p2KOΔ142R. The panel on the left shows infected OMK cells 4 days postinfection under visible light. The panel on the right shows the same area under illumination at 580nm.

Viral plaques were isolated, diluted and reinoculated into new plates until individual plaques could be isolated based on the expression of the mCherry proteins red (see Figure 16).

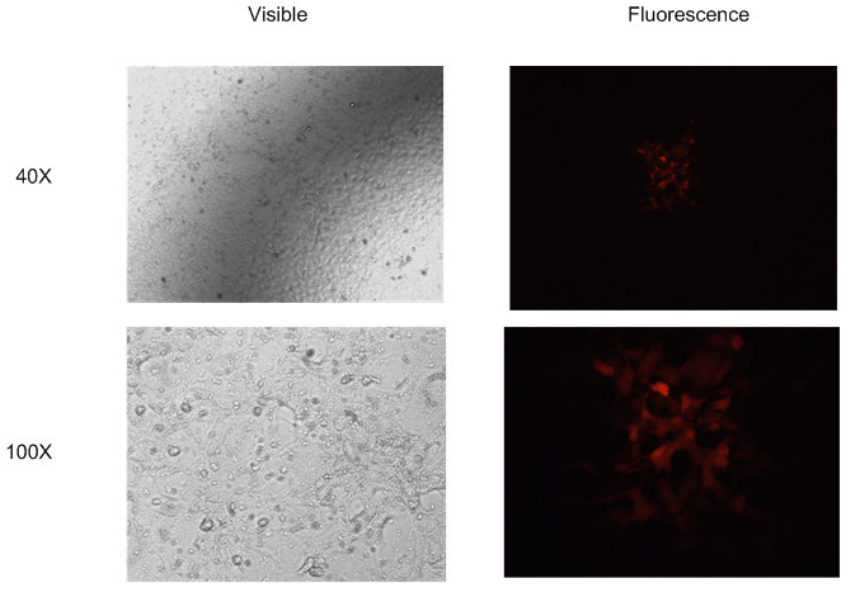

Figure 16. Plaque Purification of TPVΔ142R. The top pictures are at 40X and the bottom pictures are at 100X magnification of the same plaque. The panels on the left shows infected OMK cells 7 days postinfection under visible light. The panels on the right shows the same area under illumination at 580 nm.

Once recombinant virus appeared visually pure, based on the absence of non-fluorescent plaques, PCR was used to verify that the recombination took place at the 142R locus. Although plaques appeared pure, PCR demonstrated that isolates contained detectable wild-type virus. This indicated that the mutant virus had not been fully isolated from the wild-type virus which had been done in another knockout virus lacking the 66R gene (Conrad and Essani, unpublished).

In a further attempt at purifying the mutant virus, three mutants were chosen (all from separate transfection/infections), reinoculated at dilutions of 0.5 – 5 µl in 60 mm plates and new plaques picked at 3 days postinfection. This was done to try to isolate plaques that were small to get good separation and no contamination from wild-type plaques. These plaques were reinoculated at 5 µl in 60 mm plates for about 17 days. No wild-type TPV-Kenya plaques were visualized. Out of the 10 separate viruses obtained only 4 replicated sufficiently for DNA to be obtained. This DNA was used for PCR using 4 different primer sets (see Figure 17). The two wild-type primer sets only show bands if wild-type TPV-Kenya is present as the forward primer is located in the region of 142R that is removed in the knockout virus. The two mutant primer sets only show a band if TPVΔ142R is present as the forward primer is located in the mCherry region. All four of these viruses contained both wild-type DNA and mutant DNA (see Figure 17). This indicates that while the mutant virus is present, it has not been successfully separated from the wild-type virus.

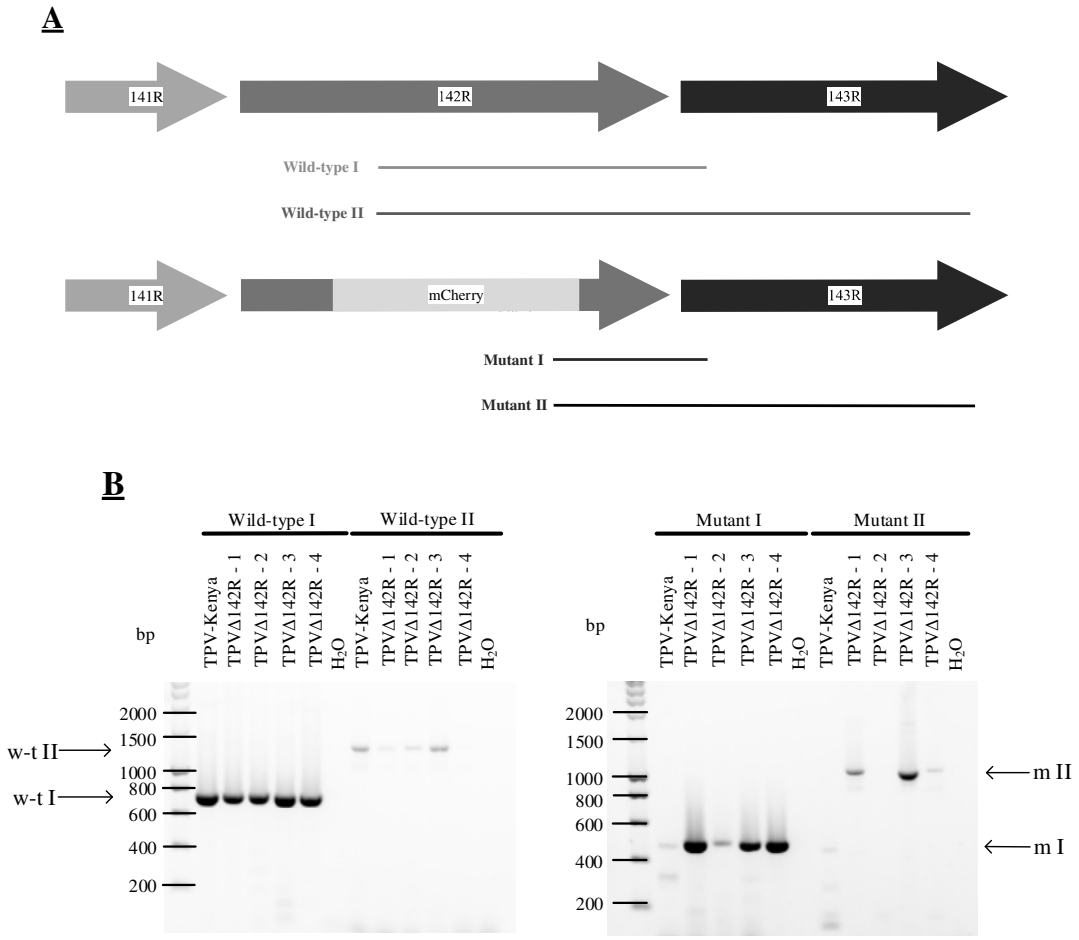

Figure 17. PCR of Potential TPVΔ142R. Panel A – Size and Location of PCR Products. Panel B - The gel on the left are the results using the 2 wild-type primer sets. The gel on the right are the results using the 2 mutant primer sets. A forward primer is shared by wild-type I and II PCR reactions. Another forward primer is shared by mutant I and II PCR reactions. A reverse primer is shared by wild-type I and mutant I PCR reactions. Another reverse primer is shared by wild-type II and mutant II PCR reactions.

One mutant virus was selected and rediluted 1:1000 in order to obtain well isolated plaques. Individual plaques were picked and used for PCR (see Fig. 18). The wild-type virus primers should only amplify a product if wild-type TPV-Kenya is present as the forward primer is located in the region of 142R that is removed in the knockout virus. The mutant virus primers should only amplify a product if TPVΔ142R is

present as the forward primer is located in the mCherry region. The ampicillin resistance primers are for the ampicillin resistance gene which could be present in a single cross-over event. All the plaques appear to be coinfected with TPV-Kenya and TPVΔ142R with no single cross-over event. This indicates that the presence of the 142R protein is required for viral replication.

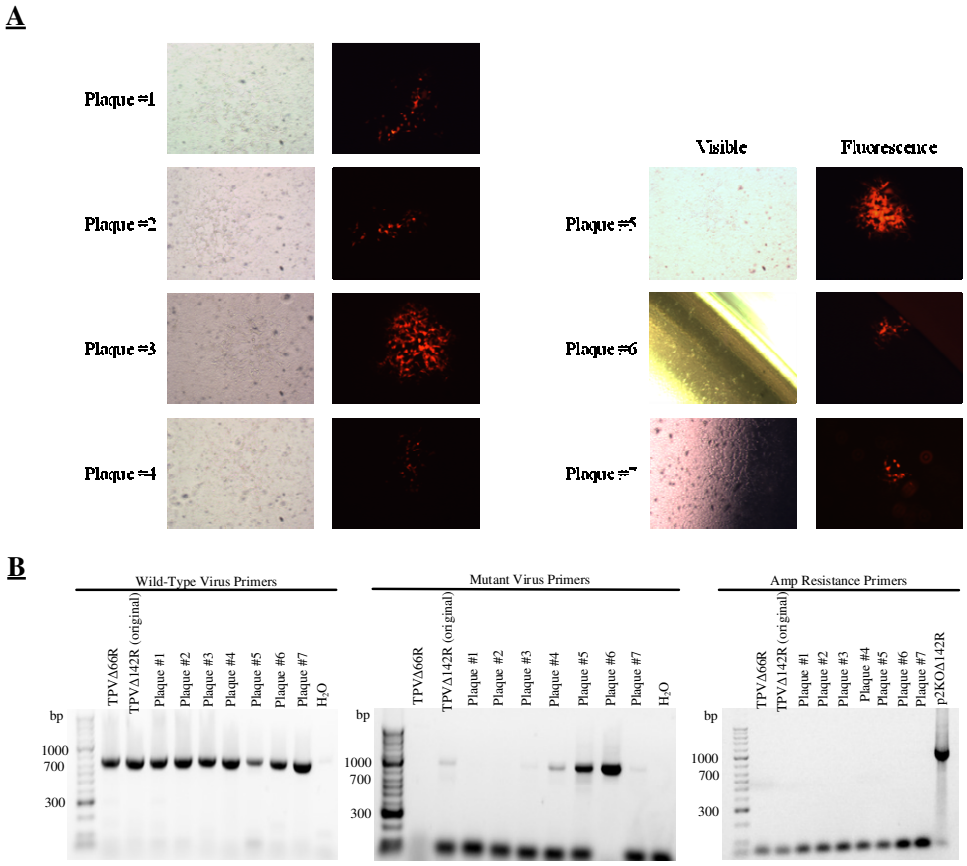

Figure 18. Individual Plaque PCR. Panel A - The pictures are at 40X magnification with the panels on the left showing infected OMK cells 7 days postinfection under visible light. The panels on the right shows the same area illuminated at 580 nm. Panel B - The gel on the left uses the wild-type virus primers. The gel in the middle uses mutant virus primers. The gel on the right uses primers that are for the ampicillin resistance gene.

142R is Necessary for Viral Replication

To determine if 142R was necessary for viral replication shRNAs that were designed to target 142R mRNA starting at 464 bp or 652 bp of the 142R gene were cloned into the pSuperior.neo vector. The empty vector was used as a control, along with two plasmids pSUP-c-Jun and pSUP-Fra that have FRA and cJUN scrambled sequence (Smith and Bejcek, 2011). Cells were photographed 4 days post-inoculation (see figure 19). The virus appeared evenly distributed throughout all of the dishes containing control plasmids, but the samples transfected with shRNA 464 and 652 plasmids contained large areas without viral plaques. Virus was harvested and quantitated (see figure 20). After performing Student's t-Test on the viral titer CPE, both shRNA 464 and 652 plasmids showed significant ($P < 0.05$) reduction with P values of 0.0351 and 0.0231 respectively. The three control plasmids had varying levels of decreased CPE, but it was not significant with P values of 0.0537 for pSuperior.neo, 0.3475 for pSUP-c-Jun and 0.8793 for pSUP-Fra plasmids.

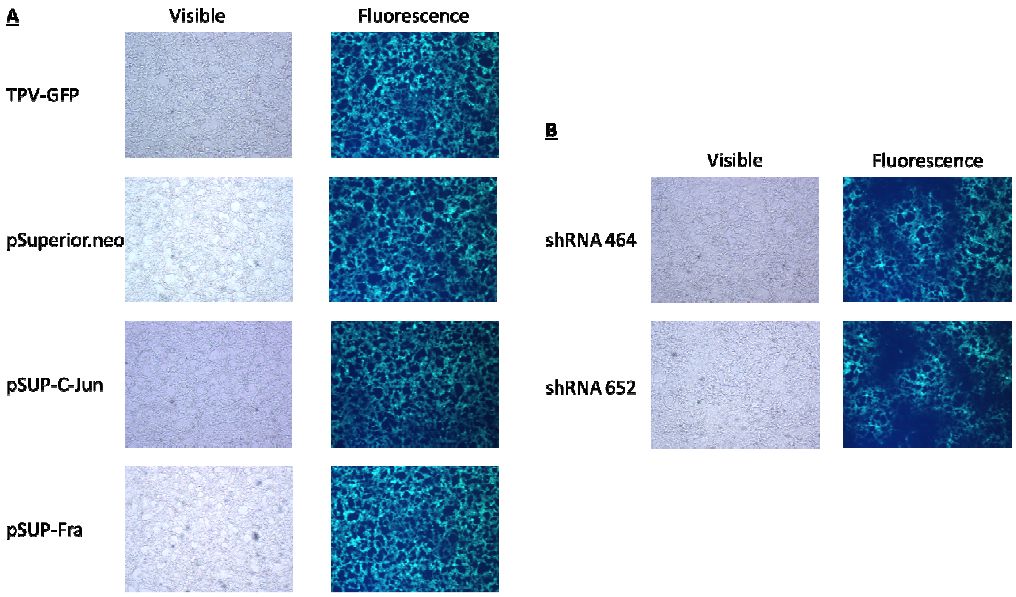

Figure 19. TPV-GFP in the Presence of shRNA Targeting 142R. The pictures are at 40X magnification with the panels in the visible columns showing infected OMK cells 4 days postinfection under visible light. The panels in the fluorescence columns shows the same area under UV fluorescence illuminated at 510 nm. Panel A - Pictures of the four controls that did not target 142R. Panel B - Pictures of the two shRNA samples targeting 142R that have marked decrease in fluroescence with 1 µg transfections.

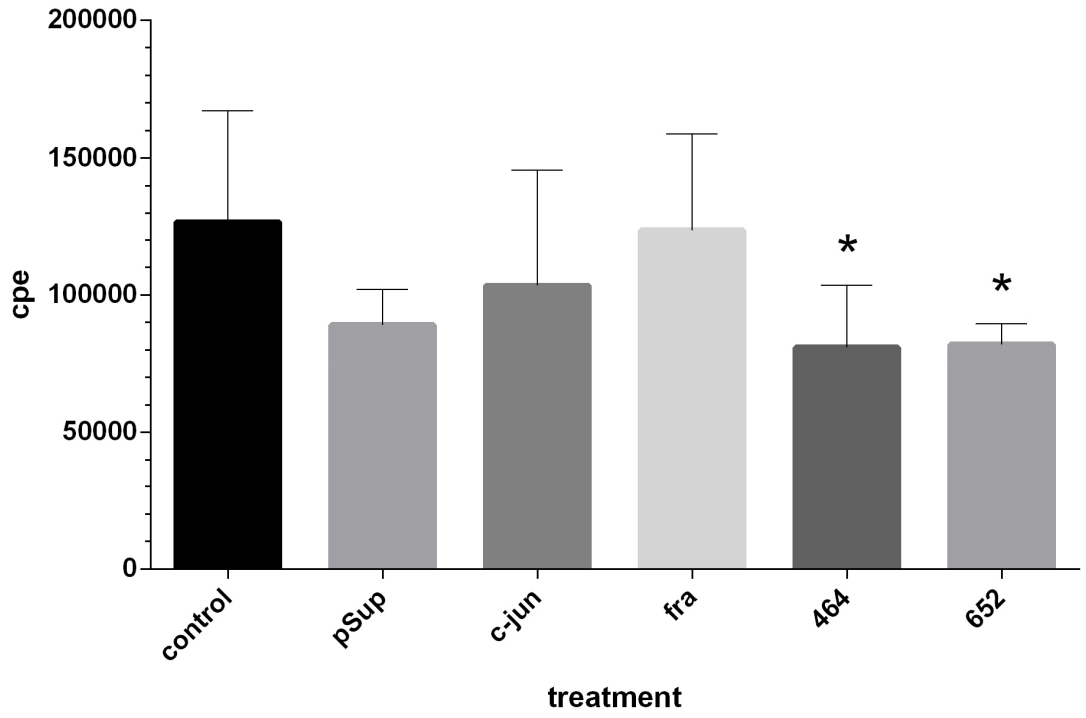

Figure 20. TPV-142R shRNA Treatment Reduces Virion Production in OMK Cells. Unpaired Student's t-Test was performed comparing populations to a control sample that received no plasmid transfection. Both shRNA 464 and 652 were significantly reduced (indicated by *), while empty vector and nonsense controls were not. The standard deviation is indicated by the error bars.

DISCUSSION

Protein Expression and Kinase Activity

Production of active kinase in bacteria failed even though multiple tags at both the N and C termini were tried. It is not known why no active protein could be produced but it was most likely due to misfolding. Although it is the method other laboratories have used to produce the related protein, B1R, for kinase studies (Rempel & Traktman, 1992b). To increase the chances of getting protein into the correct conformation, three temperatures were used during the induction of protein expression. Often proteins require a specific temperature in which they will fold correctly so by producing protein under different conditions a direct comparison of protein could be performed in the expression of protein. However, none of these samples exhibited kinase activity and it was concluded that none of these alterations to protein expression corrected the folding problem. Further evidence that there was misfolding occurring is the inability to cleave the GST tag with Factor Xa.

Other *E. coli* strains were used in an attempt to perhaps overcome a codon bias (Gustafsson et al., 2004, Hilterbrand et al., 2012), and an optimized version of 142R for expression in BL21-Star was created as well. It has been shown that by optimizing codon usage in poxvirus genes it can improve the transient expression in mammalian cells (Barrett et al., 2006). However, this did not produce kinase function either.

Changes post protein expression were also done to determine if the kinase assay was the problem including altering pH, incubation time, cation, concentration of all components with no change in results. A buffer exchange was undertaken to remove the imidazole after elution from the cobalt column. Since the imidazole can cause interference with the His tag, so it seemed possible that an effect could also be seen in the kinase assays. When introducing imidazole into the previously functioning positive control, kinase activity diminished (data not shown). Despite the fact that imidazole interfered with kinase activity, removing it still did not repair the kinase function with the 142R protein. At this point further exploration into prokaryotic expression of the 142R protein seemed ineffectual, so eukaryotic expression was examined in mammalian cell culture.

Cos-7 cells are an ideal way to express a large amount of protein in a short amount of time in an eukaryotic system (Hancock, 1991). By utilizing the large T antigen, protein is highly expressed from plasmids containing a SV40 promoter to the extent that the protein will destroy the cells with the overexpression. The kinase assay showed phosphorylation of p53 by both whole cell lysate and column purified 142R protein. The fact that there are several bands around the 53 kDa area is most likely due to either protein degradation, the p53 sample was not completely pure, and/or multiple phosphorylation states. The reaction with p53 is specific, as MDM2 does not show phosphorylation. If 142R phosphorylates p53 on multiple residues, then this could account for several sizes of p53. This circumstance seems quite likely

in light of fact that B1R is known to hyperphosphorylate p53 on several residues (Santos et al., 2004).

The expression of the 142R protein from this system may have shown activity while the other systems did not for a variety of reasons. One reason is that the protein is normally expressed in a eukaryotic system so it is possible it could not fold correctly in a prokaryotic system. Although this would be a departure from B1R that was produced in bacteria and had activity (Rempel & Traktman, 1992b). Another reason is that after each step a mixture of protease inhibitors was added to the protein. This was done when isolating B1R protein from vaccinia virus due to its apparent instability to enable further experimentation with the protein (Traktman, personal communication). Added precautions were taken by performing the kinase assays immediately after isolation of the protein to ensure freezing the sample would not affect kinase activity.

Knockout Virus Creation

Prior to this study, it was unknown whether 142R was necessary for replication of tanapox virus as B1R is for vaccinia virus (Traktman et al., 1989). If this was indeed the case, then a knockout would not be able to replicate in the absence of wild-type virus. The original experimental design was to create a cell line that constitutively expressed 142R protein so that the lack of a virally encoded 142R could be overcome by the presence of the protein in the cell. However, repeated attempts at creating a cell line that expressed 142R in OMK, U373 MG and U87 MG proved

unsuccessful. OMK cells would appear to simply stop growing. U373 MG and U87 MG cells would become G418 resistant, but 142R could not be visualized by western blot.

Other strategies included utilizing the presence of human homologue VRK to overcome a 142R deficiency. VRK is known to be upregulated in certain cell types, including kidney cells (Vega et al., 2003). By using OMK cells it was possible that the presence of VRK may overcome the lack of 142R, in a similar fashion to the rescue effect it has on B1R (Boyle & Traktman, 2004). Several transfection/infections were performed, all resulting in red colonies; however, after several rounds of plaque purifications the mutant virus was still not pure as determined by PCR. Despite the absence of wild-type plaques by microscopy, PCR repeatedly showed that both wild-type and mutant viruses were both present. This implies that no pure mutant virus could be isolated or produced. The fact that a virus lacking 142R has not been able to be isolated without the presence of the wild-type virus indicates that 142R is a necessary gene that can be rescued with the presence of the protein. Throughout the purification steps viral replication appeared to decrease which suggests that as the amount of wild-type decreases so does the ability of the mutant to replicate.

To further support this assertion, shRNA were designed to target 142R to determine if knocking down the protein expression would also decrease viral production. Both shRNA plasmid transfections resulted in a significant decrease in virus production. Despite having a P value above the significant threshold, the empty

vector control does appear to also have a decrease in virion production. While it is unclear why this is occurring, the FRA and c-JUN plasmids do not show this effect. These plasmids are arguably better controls in that they are actually expressing a shRNA unlike the empty vector, thus being more indicative of how the shRNA is acting upon the system.

Due to both shRNAs showing a decrease in virion production, while targeting separate regions of the gene, it strongly suggests that by disrupting the 142R protein expression the virus production decreases as well. Together with the data from the attempted knockout virus creation, it is highly indicative of 142R being an essential gene for tanapox viral replication.

Future Directions

With this knowledge, it is unlikely that a pure virus lacking 142R can be obtained without a plasmid that can constitutively express 142R protein and possibly using different cell line that is easily transfected but does not have issues growing when sparsely seeded. Even if this virus was obtained the ability to use it as an oncolytic virus to target p53- cells may have other constraints to overcome. While the protein does show phosphorylation of p53, there are other functions of 142R that are essential for virus replication and therefore use as an oncolytic therapy will be problematic. B1R also has several other viral and cellular interactions (Banham et al., 1993, Beaud et al., 1995, Santos et al., 2006a, Webb et al., 2006) and 142R should be further characterized as to its role in the replication cycle of tanapox virus.

To create a 142R knockout virus a plasmid that expresses the protein in a stable cell line will need to be created and used to establish a 142R expressing cell line that is capable of viral production.

If a cell line that constitutively expresses 142R protein cannot be created it may be necessary to try to create a temperature sensitive mutant as was done for studying B1R (Rempel & Traktman, 1992a). In regards to vaccinia virus, this was the only way to study the gene because making a knockout was impossible due to it being necessary for replication (Traktman, personal communication). This would allow exploration of the interaction of 142R protein with proteins and genes to determine if they are the same as B1R. To determine if VRK1 would restore replication of tanapox virus a cell line with an increased concentration of VRK1 would need to be created as well. If VRK1 cannot rescue the replication deficiency, then a knockout or temperature sensitive mutant may be a possible oncolytic virus to target tumors that have an upregulation of VRK1 and with a mutation in p53. Due to the fact that VRK1 and p53 are in an autoregulatory loop, this happens regularly. In human lung carcinomas, when p53 is mutated, it is unable to downregulate VRK1 (Valbuena et al., 2007). In this situation a knockout of 142R that was rescued by VRK1 would only be able to replicate in the tumor.

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