DISSERTATION

CHARACTERIZATION OF WALLEYE DERMAL SARCOMA VIRUS ORF B DURING TUMOR DEVELOPMENT

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY CANDELARIA CHRISTINA DANIELS ENTITLED CHARACTERIZATION OF WALLEYE DERMAL SARCOMA VIRUS ORF B DURING TUMOR DEVELOPMENT BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

CHARACTERIZATION OF WALLEYE DERMAL SARCOMA VIRUS ORF B DURING TUMOR DEVELOPMENT

Walleye dermal sarcoma virus is a complex retrovirus associated with walleye dermal sarcomas (WDS). These sarcomas develop and regress on a seasonal basis, providing a unique model to study mechanisms of tumor development and regression in vertebrates. WDS is experimentally transmissible to walleye with cell-free, regressing tumor homogenates. During the fall, low levels of spliced accessory gene transcripts, *A* and *B,* are present in developing tumors suggesting that their encoded proteins, rv-cyclin and Orf B, may play a role in oncogenesis. Infectious virus and high levels of full-length viral RNA and spliced accessory and *env* transcripts are expressed during tumor regression, the following spring. The three accessory proteins Orf A (rv-cyclin), Orf B, and Orf C function in tumor development and regression.

In explanted tumor and mammalian cells stably expressing the 35kDa Orf B protein, Orf B is localized at the cell periphery in structures similar to focal adhesions and along actin stress fibers. Results from these studies demonstrate Orf B interacts directly or in a complex with several cellular proteins important in signal transduction pathways: receptor for activated C kinase (RACK1), protein kinase C alpha (PKCα), Src,

phosphatidylinositol-3-kinase (PI3K), and protein phosphatase 2A (PP2A). The cellular proteins BAD, 90kDa ribosomal S6 kinase (p90RSK), PKCα, and protein kinase B (AKT), which are important in controlling apoptosis and/or proliferation, are activated in Orf B-expressing cells. Orf B protects cells from staurosporine-induced apoptosis and induces cell proliferation of Orf B-expressing cells under serum-deprived conditions suggesting a mechanism of action for tumor development. Expression of Orf B induces transformation of NIH3T3 cells *in vitro* and a PI3K and mTOR inhibitor prevented transformation, providing the first evidence that Orf B induces a transformed phenotype. The regulation of cell signaling pathways is one way in which viruses induce oncogenesis. Orf B ensures the establishment of dermal sarcoma by activating signal transduction pathways that control cell survival and proliferation such as PKC and Akt.

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DEDICATION

I dedicate this dissertation to my family members who have supported me through out my graduate career. My husband and best friend, Tony Daniels, was especially instrumental in keeping our family together and provided unconditional love at each step of this journey. The endless love and encouragement my parents, Leon and Virginia Deolloz, have given me made all my lifetime goals achievable. My precious sons, Antonio and Mario, bring absolute joy every day and reminded me how precious life is. Finally, I dedicate this to my late Tiá, Amanda Ysla. The memory of her reminded me that being able to obtain a higher education is a privilege and should never be taken for granted.

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CHAPTER 1

INTRODUCTION

Retroviruses

Retroviruses were originally known as RNA tumor viruses due to their involvement in cancer and tumor formation and RNA genome. Evidence of retroviruses are documented in the earliest human populations and it is postulated that retroviruses were one of the first viruses to adapt a long-term relationship with their host due to their unique viral replication cycle and integration into the host genome (Flint et al., 2004). Vilhelm Ellerman and Oluf Bang were the first to document transmission of a neoplasm with cell-free filtrates, which provided evidence that a virus was the etiologic agent. The oncogenic agent, avian leukosis virus (ALV), caused leukosis in chickens (Ellerman and Bang, 1908).

The retrovirus virion ranges from 80-100 nm in diameter. A cell-derived lipid bilayer forms the lipid envelope incorporating and displaying viral glycoproteins, which surrounds the capsid (Coffin, Hughes, and Varmus, 1997). Retroviruses have a diploid genome; they are positive-stranded RNA viruses with a double stranded DNA intermediate that integrates into the genome of the host cell. Retroviruses are divided by the organization of their genome, classifying them as either simple or complex. All retroviral genomes contain four genes encoding structural proteins: *gag* directs synthesis

of internal virion proteins, matrix, capsid, and nucleocapsid; *pro* encodes viral protease; *pol* encodes viral enzymes, reverse transcriptase and integrase; and *env* encodes surface and transmembrane proteins (Flint et al., 2004). Complex retroviruses encode additional proteins for other virus-specific functions such as transcriptional regulation (Flint et al., 2004).

The uniqueness of retroviruses is characterized by reverse transcription and integration in the host genome, making the retroviral infection permanent. Reverse transcription occurs in the cytoplasm, where a linear DNA duplex is generated. Reverse transcriptase (RT) has two enzymatic activities: A DNA polymerase that uses DNA or RNA as a template for replication and a nuclease (RNase H) that degrades the RNA portion of the RNA: DNA hybrid, resulting in a single-stranded (ss) DNA product. Two strand-transfer reactions occur during reverse transcription, producing minus-strand strong stop DNA and plus-strand strong stop DNA. The DNA that is copied from RNA is unique in that in contains terminal duplications known as long terminal repeats (LTR) (Coffin, Hughes, and Varmus, 1997).

Diseases that affect walleye

Neoplastic diseases have long been recognized in teleosts and, recently, appear with increasing frequency. A feature of some neoplastic diseases in fish, unlike those of mammals, is the seasonal appearance and regression of lesions (Poulet, Bowser, and Casey, 1994). Two naturally occurring tumors of fish are associated with the presence of retroviruses. (Hart et al., 1996; Holzschu et al., 1995; Martineau et al., 1992; Paul et al., 2006). One of these, walleye dermal sarcoma (WDS), is best characterized and, is

associated with walleye dermal sarcoma virus (WDSV) and provides a unique non-mammalian model with which to study mechanisms of viral-induced tumorigenesis. Two other retroviruses are associated with proliferative lesions in walleye, walleye epidermal hyperplasia viruses type 1 and type 2 (WEHV-1, WEHV-2) (LaPierre, Holzschu, and Casey, 1997; LaPierre et al., 1999; LaPierre et al., 1998).

WDS is a benign cutaneous neoplasm that arises multicentrically from the superficial surface of the scales and can affect up to 27% of adult walleyes in North American lakes (Bowser et al., 1988; Walker, 1969). These neoplasms have been described as highly cellular and non-metastatic (Martineau et al., 1990b; Yamamoto, Kelly, and Nielsen, 1985b). There is a seasonal prevalence of this disease; tumors develop during the fall, regress in the spring, and are rarely seen in the summer (Bowser et al., 1988). A naturally occurring exogenous complex retrovirus, WDSV, is associated with these tumors (Bowser et al., 1988; Martineau et al., 1992; Martineau et al., 1991a; Martineau et al., 1991b). WDSV was molecularly cloned from tumor tissue and found to be 12.7 kb in length (Martineau et al., 1992). WDSV contains three open reading frames (orfs) in addition to *gag*, *pol*, and *env*: *orf a* and *orf b* are located in the 3' proximal region of the genome, and *orf c* lies between the 5' long terminal repeat and *gag* (Holzschu et al., 1995) (Fig. 1.1). It is assumed that these orfs would encode regulatory/accessory proteins similar to those of other complex retroviruses (Cullen, 1992; Cullen, 1991).

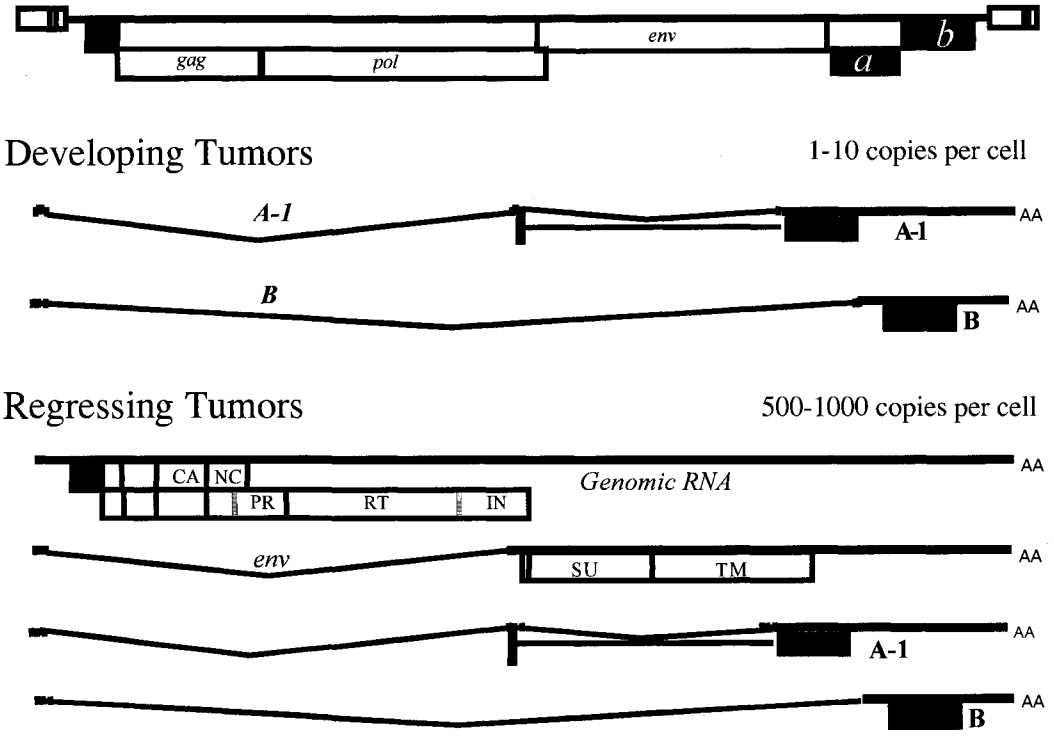

Fig 1.1 Representation of the WDSV genome, open reading frames, genomic RNA, and the products of spliced transcripts. Orfs encoding proteins A, B, and C, are shaded.

There are quantitative and qualitative differences in the amount of viral DNA and the extent of virus expression between developing and regressing tumors. Developing tumors contain ≤1 copy of viral DNA per cell whereas cells of regressing tumors have 10 to 50 copies, most of which are unintegrated (Fig.1.1) (Bowser et al., 1996; Martineau et al., 1992; Martineau et al., 1991b). Northern blot analysis of mRNA isolated from regressing tumors identified full-length viral mRNA and several subgenomic RNAs (12.7, 7.4, 2.8, and 1.8 kb), whereas in developing tumors only subgenomic RNAs of 2.8

and 1.8 kb were detectable (Bowser et al., 1996). Nine different subgenomic mRNAs from developing and regressing tumors have been identified (Quackenbush et al., 1997), indicating that WDSV undergoes an elaborate pattern of mRNA splicing similar to that of other complex retroviruses (Arrigo et al., 1990; Ciminale et al., 1992; Davis, Molineaux, and Clements, 1987; Muesing et al., 1985). In developing tumors two small subgenomic mRNAs are detectable: *orf a and orf b* at low levels (1-10 copies of viral DNA per cell), thus the encoded proteins, Orf A and Orf B, are likely to be involved in tumor development (Fig. 1.1) (Quackenbush et al., 1997). Regressing tumors contain 10-50 copies of viral DNA per cell, most of which is unintegrated viral DNA (UVD). Full-length, genomic RNA encompasses the coding region for *orf c*, spliced accessory transcripts *orf a and orf b*, and infectious virus (Fig. 1.1) (Bowser et al., 1996; Martineau et al., 1991a; Quackenbush et al., 1997).

orf a encodes a retroviral cyclin (rv-cyclin or Orf A) protein, which localizes to the nucleus and interacts with proteins necessary for transcription (LaPierre, Casey, and Holzschu, 1998; Rovnak, Casey, and Quackenbush, 2001; Rovnak et al., 2005; Rovnak and Quackenbush, 2002; Rovnak and Quackenbush, 2006). The homology of rv-cyclin to host cyclins is limited to the cyclin box motif (LaPierre, Casey, and Holzschu, 1998). Rv-cyclin negatively regulates viral gene expression through the direct interaction of its transcription activation domain with TATA binding protein-associated factor 9 (TAF9) (Rovnak et al., 2005; Rovnak and Quackenbush, 2006). WDSV rv-cyclin can induce cell-cycle progression in cyclin deficient yeast and induce hyperplasia in transgenic mice after wound healing (Lairmore et al., 2000; LaPierre, Casey, and Holzschu, 1998). These results support a role in tumor development.

Full-length RNA transcripts encoding *orf c,* which is 5' proximal to *gag*, are only found during tumor regression. The Orf C protein is localized to the mitochondria in naturally infected and cultured cells, and induces apoptosis by disrupting mitochondria membrane potential (Nudson et al., 2003). These results suggest Orf C initiates tumor regression by acting on the mitochondria.

The predicted protein for the *orf b* transcript, Orf B, does not demonstrate homology to known proteins in the GenBank database. Analysis of the Orf B polypeptide identifies a carboxy PEST sequence (a polypeptide enriched in proline (P), glutamic acid (E), serine (S), and threonine (T)). PEST sequences target proteins for degradation by the 26S proteasome or by calpain (Rodgers, Wells, and Rechsteiner, 1986). Orf B also has a consensus nuclear export signal (NES). The Orf B NES is a leucine rich region (a.a. 117 to 126) like those found in other complex retrovirus accessory proteins, such as HIV Rev and HTLV Rex (Cullen, 1998). Unlike Rev and Rex, Orf B does not contain an arginine rich region that targets these proteins to the nucleus and interacts with specific sequences in viral RNAs.

Oncogenesis

Tumor development takes place due to deregulated cell growth and suppressed cell death. Oncogenesis is a multistep process of transforming normal cells to tumor cells. Oncogenesis occurs when there is a disruption between the balance of cell proliferation and death. Genetic mutations in several genes controlling these processes are often disrupted by various means: chemical, environmental, bacterial and viral. Viruses have employed several mechanisms to induce transformation and oncogenesis.

The study of retroviruses has greatly enhanced the knowledge of the mechanisms involved in the development of neoplastic diseases. Oncogenic retroviruses are classified into three groups: (i) Transducing or acutely transforming oncogenic retroviruses cause malignancies in infected animals in a very short period of time (i.e. days) and the efficiency of tumor formation is 100% of infected animals. These retroviruses carry transduced cellular genes that, when expressed, become viral oncogenes (*v-onc*) and are directly capable of transforming cells. Acute transforming retroviruses are replication defective due to the loss of viral genes (*gag*, *pol*, and *env)* during capture of normal cellular genes (proto-oncogenes). Cellular sequences are captured in a provirus during non-homologous recombination between read-through or hybrid transcripts and wild type transcripts that are packaged together in virions (Coffin, Hughes, and Varmus, 1997; Flint et al., 2004). Retroviruses acquire oncogenes by read-through transcription from the provirus to the cellular gene. This generates a large RNA transcript containing viral and cellular sequences. In addition, retroviruses acquire oncogenes through deletions of viral and cellular sequences resulting in a hybrid transcript where provirus and cellular genes fuse. The avian Rous sarcoma virus, which harbors the *v-src* oncogene, is a well characterized oncogenic retrovirus that causes tumors in birds (Rous, 1911). (ii) nontransducing or slow transforming oncogenic retroviruses induce tumors in infected animals within weeks to months at high to moderate efficiency. These retroviruses do not encode oncogenes and instead transformation occurs by the virus's ability to integrate near cellular proto-oncogenes (Bishop and Varmus, 1984). The retrovirus is then able to irregularly activate transcription of cellular proto-oncogenes. The isolation of these cellular genes has provided the basis for studying and understanding normal cellular

growth and differentiation. Avian leukosis virus (ALV) is a well known example of a nontransducing retrovirus. ALV provirus was found integrated into chromosomal DNA adjacent to the *c-myc* proto-oncogene (Colby et al., 1983). (iii) Lastly, nontransducing, long-latency retroviruses are composed of complex retroviruses that encode accessory proteins, which control cellular processes to their advantage. An example is the oncogenic, complex, delta retrovirus HTLV, which causes adult T cell lymphocytic leukemia (ATLL). These retroviruses do not contain oncogenes nor is there a common site of integration, suggesting that these viruses induce tumorigenesis through other mechanisms (Deschamps, Kettman, and Burny, 1981). The deltaretroviruses, as well as the lentiviruses and foamyviruses, encode proteins involved in the regulation of viral gene expression and virus replication (Cullen, 1992; Felber et al., 1989; Sodroski, Rosen, and Haseltine, 1984). This trans-activation may involve DNA or RNA binding by viral proteins or their interaction with host transcription factors. One example is the viral transcriptional activator HTLV-Tax, which has been shown to function via interactions with host transcription factors and to be essential for transformation (Grossman et al., 1995; Matsumoto et al., 1997; Nerenberg et al., 1987; Ross, Pettiford, and Green, 1996; Tanaka et al., 1990). Tax does not bind DNA itself, but it activates transcription by binding to cellular transcription factors such as cyclic-AMP responsive element (CREB), TATA binding protein (TBP), and nuclear factor-κB (NF-κB) (Coffin, Hughes, and Varmus, 1997). Further investigations have demonstrated that HTLV-Tax is responsible for the stabilization and transcriptional inactivation of the tumor suppressor protein, p53 (Pise-Masison et al., 1998). Tax is known to contribute to oncogenesis by altering the expression of cellular genes whose protein function regulates T-cell physiology.

Genomic instability in host cells is suggested as an alternate mechanism for HTLV-1 induced transformation. HTLV-1 genes are not expressed in ATLL cells; therefore the expression of viral regulatory proteins is suggested to initiate but not maintain transformation (Flint et al., 2004).

DNA viruses are also associated with oncogenesis. DNA viral proteins function as oncoproteins capable of promoting cell survival by encoding growth factors, growth factor receptors, and signaling proteins. The human herpesvirus 8 (HHV-8) genome contains several homologues of cellular genes that encode signaling proteins (Flint et al., 2004). For instance, HHV-8 *v-gpcr* encodes a guanine nucleotide-binding protein-coupled receptor, that is constitutively activated in HHV-8 infections resulting in activation of the Map kinase signaling pathway (Vischer, Leurs, and Smit, 2006). In addition, the bovine papillomavirus (BPV), a DNA tumor virus that causes warts, encodes the E5 protein that mimics hormone-mediated receptor dimerization. BPV E5 forms a stable complex with the endogenous platelet derived growth factor receptor (PDGFR), causing receptor aggregation and activation and cell transformation (Borzacchiello et al., 2005; Hunter, 1997).

Viruses affect apoptosis

The initiation of apoptosis is controlled by a family of cysteine proteases known as caspases that are stimulated by external and internal stimuli. The extrinsic pathway initiates apoptosis upon binding of a proapoptotic ligand to a cell surface receptor. The intrinsic pathway responds to intracellular signals of stress and DNA damage. The control of apoptosis is evolutionarily conserved across various species. The first

apoptotic gene to be cloned was *bcl-2* and was later discovered to suppress the apoptotic pathway (Lodish et al., 2000). The Bcl-2 family of proteins consists of anti-apoptotic (Bcl-2, Bcl-XL) and pro-apoptotic (Bax and Bad) proteins. Their activation and deactivation are controlled by a variety of cell signaling pathways that activate enzymes capable of producing post-translational modifications, such as phosphorylation. Inappropriate regulation of phosphorylation and dephosphorylation events can have extreme effects on how the cell responds to stimuli. For instance, the anti-apoptotic function of BAD relies on the phosphorylation at serine residues 112, 136, and 155 (Zha et al., 1996). Phosphorylation of BAD at serine 112 and serine 136 results in BAD binding to the phosphoserine/threonine binding protein, 14-3-3. In contrast, dephosphorylated BAD dissociates from 14-3-3 and promotes cell death by binding and inactivating the survival function of anti-apoptotic proteins, Bcl2 and Bcl-XL (Chiang et al., 2003). BAD is phosphorylated on serine residues 112 and 155 by p90RSK (90kDa ribosomal S6 kinase) (Tan et al., 1999). Protein kinase C (PKC) directly phosphorylates and activates p90RSK. Alternatively, p90RSK is phosphorylated by the MAP kinases, p42 and p44 (Erk1/2, respectively), of the mitogen-activated protein kinases (MAPK) cell signaling pathway (Bonni et al., 1999). Protein kinase B (Akt) phosphorylates BAD on serine 136 (Datta et al., 1997; Zha et al., 1996) (Fig 1.2).

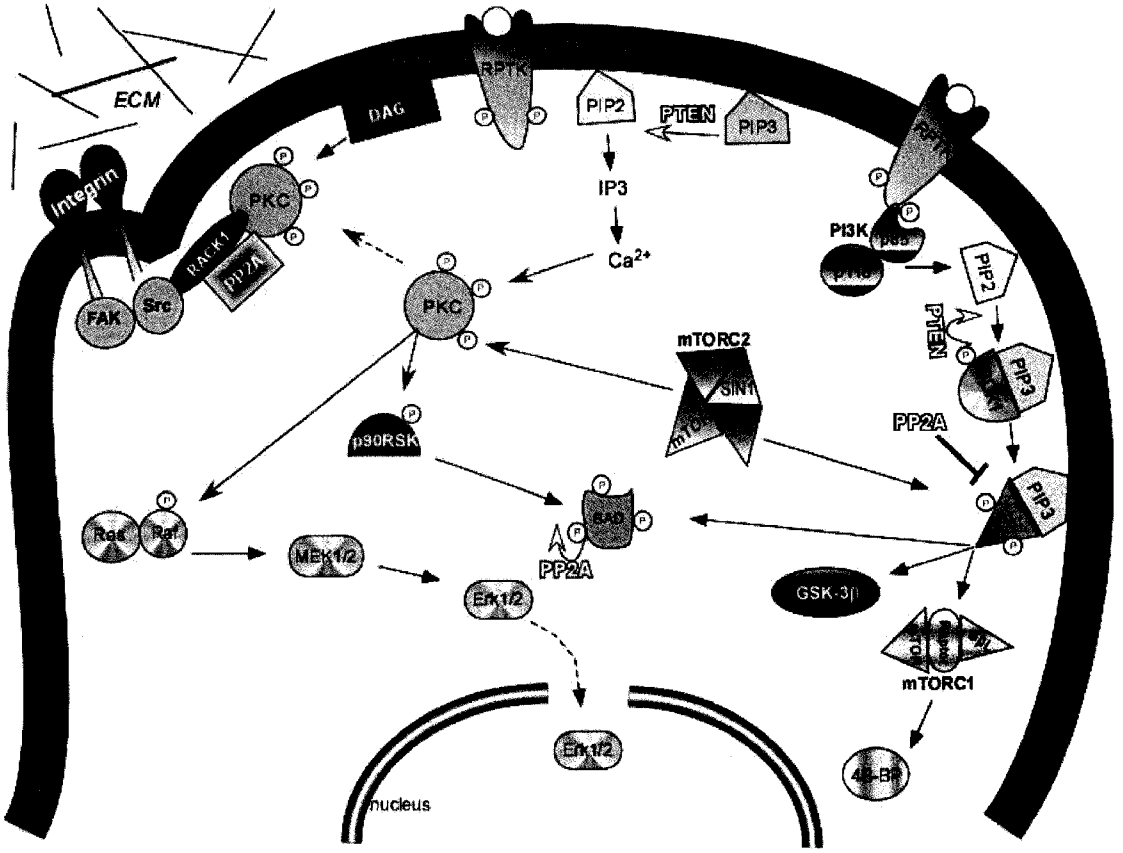

Fig 1.2 Schematic illustration of the signal transduction pathways introduced and which WDSV Orf B influences. A table of acronmyms is listed below.

Abbreviation	*Name*	*Abbreviation*	*Name*
Akt	Protein Kinase B	PI3K	Phospatidylinositol-3-kinase
BAD	Pro-apoptotic Bcl2 family member	PKC	Protein kinase C
Erk1/2	p44/42 MAPK	PTEN	Phosphatase and tensin homologue deleted on chromosome 10
DAG	Diacylglcerol	PP2A	Protein phosphatase type 2A
ECM	Extra-cellular matrix	p85	Regulatory subunit of PI3K
FAK	Focal adhesion kinase	p110	Catalytic subunit of PI3K
GSK-3β	Glycogen synthase kinase 3 beta	p90RSK	90 kDa ribosomal S6 kinase
GβL	Small Gβ-like protein	RACK1	Receptor for activated C kinase 1
IP3	Inositol triphosphate	Raf	Map kinase kinase kinase
MEK1/2	MAP or Erk Kinases	Raptor	Regulatory associated protein of mTOR
mTOR	Mammalian TOR	Ras	Guanine nucleotide binding protein
MTORC1	mTOR complex 1	Rictor	Rapamycin insensitive companion of mTOR
mTORC2	mTOR complex 2	RPTK	Receptor protein tyrosine kinase
PDK1	Phosphoinositide-dependent kinase 1	4B-BP	eIF4E binding protein
PIP3	Phospatidylinositol-3,4,5 triphosphate	SIN1	Stress-activated map kinase interacting protein 1
PIP2	Phospatidylinositol-4,5 bisphosphate		

Table 1.1 Abbreviations used in Figure 1.2.

Viruses employ an arsenal of proteins to interfere with pro-apoptotic signaling pathways, affording a selective advantage for them to maintain a persistent infection or to prolong the survival of lytically infected cells, allowing maximum virus progeny production (O'Brien, 1998). Mechanisms for interfering with apoptosis include expression of viral anti-apoptotic proteins. Examples include inhibitors of apoptosis proteins (IAP), which are encoded by insect baculoviruses and are anti-apoptotic by binding to activated caspases (Clem and Duckett, 1997), and Bcl2 viral homologues, which block the progression of apoptosis and are encoded by lymphotrophic herpesviruses such as EBV and human herpesvirus-8 (Cheng et al., 1997; Henderson et al., 1993; Tschopp et al., 1998; Wang et al., 2002). Alternatively, viral gene products affect apoptosis by interacting with components of conserved cell signaling pathways that regulate cell death (O'Brien, 1998). For instance, the adenoviral E1B 55K protein cooperates with E1A to inhibit p53-mediated apoptosis resulting in cell transformation (O'Brien, 1998). Studies of viral-induced inhibition of apoptosis via alteration of normal cellular pathways will provide a better understanding of the cell signaling processes involved in the execution of cell death.

Oncogenic viruses have capitalized on the strategy of evading cellular defense mechanisms to ensure target cell survival and to promote tumor progression. SV40 large T antigen (LT) and adenovirus E1A and E1B encode proteins that directly interact with regulators of apoptosis (i.e.: p53 and Rb). These viruses are not normally associated with oncogenesis since their replication results in lytic infection and only the integration of the viral genome into cells is responsible for inducing transformation. However, additional studies with SV40 and adenovirus in cultured mammalian cells identified mechanisms for

infected cells surviving infection and becoming transformed. Specifically, E1A promotes cell cycle progression from G1 to S by binding and inactivating Rb important for virus replication while E1B binds and inactivates p53. Similarly, SV40 LT binds to Rb and p53 resulting in loss of their tumor suppressor activity. Proteins that bind to p53 inhibit its normal function of arresting cells with DNA damage at the G1 checkpoint and thereby block the normal initiation of apoptosis (Hartwell, 1992).

Downstream targets of Akt

Akt influences cell survival by phosphorylating and inactivating the pro-apoptotic function of BAD. Akt was originally identified as an oncogene isolated from a transforming murine leukemia retrovirus, AKT8 (Staal, Hartley, and Rowe, 1977b). The viral oncogene, *v-akt*, contained transduced sequences of cellular origin (Staal, 1987). Akt is phosphorylated by two independent pathways. Akt is phosphorylated on threonine 308 (T308) by phosphoinositide-dependent kinase 1 (PDK1), which is activated by the lipid kinase, phosphatidylinositol-3-kinase (PI3K). PI3K, when stimulated by growth factors, phosphorylates and converts phosphatidylinositol-4, 5 bisphosphate (PIP2) to phosphatidylinositol-3, 4, 5-triphosphate (PIP3). PIP3 recruits PDK1 and Akt to the membrane through their pleckstrin homology (PH) domain where PDK1 phosphorylates Akt on T308 (Vara et al., 2004). The phosphatase, PTEN (phosphatase and tensin homologue deleted on chromosome 10, terminates PI3K signaling through dephosphorylation and conversion of PIP3 to PIP2 (Vara et al., 2004). T308 lies within the kinase T loop of Akt and when phosphorylated causes a conformational change, permitting access to substrates (Scheid, Marignani, and Woodgett, 2002). Akt is also

phosphorylated on serine 473 (S473) by the mammalian TOR (target of rapamycin) kinase complex containing rictor, sin, and GβL (mTOR-rictor) (Sarbassov et al., 2005). S473 lies within the carboxy terminus hydrophobic motif (HM). Initially, T308 and S473 phosphorylation were thought necessary for full Akt activation (Testa and Bellacosa, 2001). Tyrosine phosphorylation is also suggested as important for Akt activation and function (Chen et al., 2001). However, recent data indicates phosphorylation of Akt at S473 is important for the recognition and phosphorylation of Akt at T308 and indicates Akt full activation (Buchkovich et al., 2008; Sarbassov, Ali, and Sabatini, 2005; Sarbassov et al., 2005; Scheid, Marignani, and Woodgett, 2002). Activation of Akt results in either activation or inhibition of downstream targets. For instance, Akt inactivates several pro-apoptotic factors such as BAD, procaspase-9, Forkhead transcription factors (FKHR), and p53 (Brunet et al., 1999; Hennessy et al., 2005). Akt also inactivates the catalytic activity of glycogen synthase kinase 3β (GSK-3β) (Diehl et al., 1998). In contrast, Akt activates other substrates such CREB and NF-κB, which are important transcription factors, and the mammalian TOR (mTOR) complex containing regulatory-associated protein of TOR (raptor) (mTOR-raptor) (Wendel et al., 2004).

mTOR complexes

Two mTOR complexes activate cell growth in response to nutrient and growth factors (Polak and Hall, 2006). The more extensively studied complex, mTORC1 consists of mTOR, raptor, and the small Gβ-like protein (GβL). mTORC1, activated by Akt, controls cap-dependent translation by phosphorylating and activating p70S6 kinase (S6K) and the eIF4E binding protein (4E-BP) (Hara et al., 2002; Kim et al., 2002; Kim et

al., 2003). The mTORC2 complex consists of the mTOR kinase, GβL, rictor (rapamycin-insensitive companion of mTOR), and SIN1 (Jacinto et al., 2006; Polak and Hall, 2006; Sarbassov, Ali, and Sabatini, 2005). The function and mechanism of action for mTORC2 are less well understood. The only known substrate of mTORC2 is S473 of Akt (Sarbassov et al., 2005). However recent studies indicate mTORC2 phosphorylates the turn and hydrophobic motifs of PKC and Akt (Facchinetti et al., 2008; Ikenoue et al., 2008). As a result, mTORC2 is suggested to regulate actin cytoskeleton reorganization through activation of protein kinase C (PKC) (Jacinto et al., 2006; Loewith et al., 2002). Raptor, rictor and GβL contain WD40 repeat sequences suggesting involvement in protein-protein interactions (Sarbassov, Ali, and Sabatini, 2005).

PP2A phosphatase influences BAD pro-apoptotic activity

Mitochondrial serine/threonine phosphatase, PP2A reverses the action of kinases in several major signaling cascades (Lodish et al., 2000). The core of PP2A is comprised of a catalytic C subunit, PP2Ac that interacts with a structural A subunit, PP2Aa or PR65 (Westermarck and Hahn, 2008). The third subunit, B, binds the PP2Aa-c heterodimer and regulates substrate specificity and localization of the heterotrimeric PP2A holoenzyme (Eichhorn, Creyghton, and Bernards, 2008). Various PP2A complexes are implicated in controlling cell proliferation and survival. Viral oncoproteins alter the localization and/or function of PP2A by binding to the different PP2A subunits. For instance SV40 small t, polyomavirus middle and small t antigens, and the adenoviral E4orf4 proteins bind the PP2Aa-c heterodimer and displace the B subunit of PP2A (Chen et al., 2007; Cho et al., 2007). Furthermore, interaction of viral proteins with the PP2A

holoenzyme also contributes to transformation. The polyomavirus middle T antigen induces transformation in NIH3T3 cells through activation of the MAPK pathway interaction in a PP2A dependent manner (Eichhorn, Creyghton, and Bernards, 2008).

PP2A regulates the dephosphorylation of BAD and Bcl-2. For instance, PP2A activates the pro-apoptotic function of BAD by dephosphorylating BAD on serine 112 in interleukin 3 (IL-3) dependent lymphoid cells (Chiang et al., 2003; Chiang et al., 2001). In addition, PP2A is implicated in inactivating the anti-apoptotic function of Bcl2 by dephosphorylating Bcl2 on Ser70 (Ruvolo et al., 1999).

Signal transduction through PKC and its role in tumor formation

The family of PKCs is involved in tumor formation and progression by their ability to enhance multiple cellular signaling pathways affecting a wide variety of biological functions such as cell proliferation, differentiation, survival, and apoptosis (Martiny-Baron and Fabbro, 2007; Nakashima, 2002; Nishizuka, 1995). PKCs were identified as the major intracellular receptor for the tumor-promoting phorbol esters (Castagna et al., 1982). PKCs are ubiquitously expressed and comprise a family of 11 serine-threonine isozymes classified into three groups: (i) conventional iosforms α, βI, βII, and γ are calcium-dependent and require binding to diacylglycerol (DAG) for activation; (ii) novel iosforms δ, ε, η, and θ are activated by DAG, but are calcium independent; (iii) atypical iosforms ζ and ι/κ are activated by phosphatidylserine and independent of both calcium and DAG (Newton, 2003; Nishizuka, 1984). PKCs are first synthesized in the cytosol where the immature enzyme undergoes a series of autophosphorylation events to render the enzyme catalytically mature. PKCs are then

recruited to the membrane where they are activated by factors specific to the PKC isoform. The conventional PKC iosforms are shuttled to their appropriate subcellular activation site by a scaffolding protein known as receptor for activated C kinase (RACK), which anchors and stabilizes PKC to the membrane in an active conformation (Ron, Luo, and Mochly-Rosen, 1995).

RACK1 is a ubiquitously expressed protein composed of seven internal Trp-Asp (WD40) repeats, domains first identified in the β subunit of the heterotrimeric G proteins that are important in protein-protein interactions (Fong et al., 1987; Neer et al., 1994). RACK1 interacts with 21 different cellular, bacterial, and viral proteins (McCahill et al., 2002). Therefore, RACK1 is suggested to have a diverse range of functions and is involved in a variety of cell signaling pathways. In addition, RACK1 is well conserved across eukaryotic species. One interacting protein with RACK1, which is important in cell growth, is Src tyrosine kinase (Chang et al., 1998). RACK1 functions as a binding partner, substrate, and inhibitor of Src (Miller et al., 2004) that results in a lower growth rate of NIH3T3 cells caused by prolonged G0/G1 stage (Chang et al., 1998; Mamidipudi et al., 2004b).

Src tyrosine kinases participate in a wide array of cell signaling pathways controlling receptor-induced biological activities such as cell proliferation, differentiation, adhesion and survival (Thomas and Brugge, 1997). For instance, Src interacts with focal adhesion kinase (FAK) and is important in the phosphorylation of additional proteins in focal adhesions (Schaller and Parsons, 1994). Src also binds to receptor protein tyrosine kinase receptors such as platelet derived growth factor receptor

(PDGFR) resulting in phosphorylation and activation of itself (Kypta et al., 1990; Su, Muranjan, and Sap, 1999).

Several viral proteins are known to interact with RACK1 and result in a variety of functional consequences such as enhanced infectivity and disturbance of signal transduction cascades. For instance HIV-1 Nef, a viral protein important in the maintenance of high viral loads and pathogenesis, binds RACK1 resulting in enhanced Nef phosphorylation by PKCs in vitro (Gallina, Rossi, and Milanesi, 2001b). HIV-1 Nef is a substrate for PKCθ (Smith et al., 1996). The matrix M1 protein of avian, swine, and human influenza A interacts with RACK1 and is suggested to be important in M1 phosphorylation by PKC (Reinhardt and Wolff, 2000). The M1 protein of influenza A virus is the major structural virion component and mediates nuclear export of viral ribonucleoproteins (Fujiyoshi et al., 1994). The adenoviral E1A oncoprotein, important in promotion of proliferation and transformation, interacts with RACK1 and contributes to the oncogenic effect of E1A by counteracting RACK1s inhibition of Src activity (Sang et al., 2001; Severino, 2004).

MAPK signaling pathway

The MAPK cell signaling pathway coordinates signaling that regulates several cellular activities such as gene expression, cell cycle machinery, motility, survival, apoptosis, and differentiation (Pearson et al., 2001). MAPK cell signaling pathway is activated by a wide variety of stimuli including hormones, growth factors, cytokines, environmental stresses, and pathogens such as viruses. MAPK is organized into four

cascades: ERK, JNK, p38, and ERK5, which all are activated by phosphorylation mediated by an upstream kinase (Krishna and Narang, 2008).

Both DNA and RNA viruses are known to induce signaling via the MAPK pathway in order to utilize host DNA synthesis machinery for viral replication. For instance, influenza virus infection induces ERK activation that supports viral replication and contributes to airway inflammation and virus-induced cytokine production (Mizumura et al., 2003; Noah, Twu, and Krug, 2003). Lentiviruses, HIV-1 and Visna virus are also known to activate the MAPK pathway that contributes to viral infectivity and viral neurodegenerative pathogenecity, respectively (Barber et al., 2002; Toschi et al., 2006).

Hypothesis

Based on the presence of Orf B transcripts in developing tumors, my hypothesis was that Orf B is involved in tumorigenesis. Thus, the goal of this study was to determine the function of Orf B in order to understand its role in tumor development. The following chapters detail results from experiments studying my hypothesis and provide functional possibilities by which Orf B is involved in tumor development. The results from Chapter 2, published in *Virology*; 375: 550-560 (2008), identifies an interaction with RACK1 and PKC and suggests a functional consequence for this interaction with Orf B. The results from Chapter 3, submitted for publication on September 1, 2008 to *Virology,* demonstrate constitutive activation of Akt and that the expression of Orf B induces transformation of NIH3T3 cells. Results from these studies suggest a mechanism of action for the transformed phenotype observed in Orf B-

expressing cells. Chapter 4 investigates alternative means by which Orf B-expressing cells are protected from staurosporine-induced apoptosis. Specifically, Chapter 4 examines the expression levels of apoptotic genes in Orf B-expressing cells, identifies additional Orf B-interacting cellular proteins, and assesses cell proliferation of silenced PKCα in Orf B-expressing cells. Chapter 5 concludes with final analyses of Orf B's involvement in tumorigenesis, discusses the role of Orf B within our WDSV model, and outlines future experiments.

CHAPTER 2*

WALLEYE DERMAL SARCOMA VIRUS ORF B FUNCTIONS THROUGH RECEPTOR FOR ACTIVATED C KINASE (RACK1) AND PROTEIN KINASE C

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ABSTRACT

Walleye dermal sarcoma virus is a complex retrovirus that is associated with walleye dermal sarcomas that are seasonal in nature. Fall developing tumors contain low levels of spliced accessory gene transcripts *A* and *B*, suggesting a role for the encoded proteins, Orf A and Orf B, in oncogenesis. In explanted tumor cells the 35 kDa Orf B accessory protein is localized to the cell periphery in structures similar to focal adhesions and along actin stress fibers. Similar localization was observed in mammalian cells. The cellular protein, receptor for activated C kinase 1 (RACK1), bound Orf B in yeast two-hybrid assays and in cell culture. Sequence analysis of walleye RACK1 demonstrated high conservation to other known RACK1 sequences. RACK1 binds to activated protein

kinase C (PKC). Orf B associates with PKCα, which is constitutively activated and localized at the membrane. Activated PKC promoted cell survival, proliferation, and increased cell viability in Orf B-expressing cells.

INTRODUCTION

Dermal sarcoma in walleye (*Sander vitreus*) is etiologically associated with the complex retrovirus, walleye dermal sarcoma virus (WDSV) (Martineau et al., 1992; Martineau et al., 1991b; Walker, 1969; Yamamoto, Kelly, and Nielsen, 1985b; Yamamoto et al., 1976). Walleye dermal sarcoma (WDS) is exceptional in its seasonal nature. Tumors first appear on fish in the fall and are present throughout the winter. During the spawning period, the following spring, the tumors naturally regress (Bowser et al., 1988; Bowser and Wooster, 1991). WDS is efficiently transmitted experimentally to walleye fingerlings by topical, oral, or intramuscular administration of filtrates prepared from regressing tumors. In contrast, filtrates from developing tumors are unable to transmit disease (Bowser, Martineau, and Wooster, 1990; Bowser et al., 1996; Martineau et al., 1990a). Associated with the seasonal nature of disease are differences in viral gene expression (Bowser et al., 1996; Quackenbush et al., 1997). The developing fall tumors only express the spliced accessory gene transcripts, *A* and *B*, encoding *orf a* and *orf b,* while regressing spring tumors were found to express spliced transcripts, the full-length genomic RNA, and infectious virus (Bowser et al., 1996; Martineau et al., 1992; Quackenbush et al., 1997). The presence of only the *A* and *B* transcripts during tumor development led to the hypothesis that the Orf A and Orf B proteins mediate oncogenesis.

orf a encodes a retroviral cyclin (rv-cyclin or Orf A) protein, which localizes to the nucleus and interacts with proteins necessary for transcription (LaPierre, Casey, and Holzschu, 1998; Rovnak, Casey, and Quackenbush, 2001; Rovnak et al., 2005; Rovnak and Quackenbush, 2002; Rovnak and Quackenbush, 2006). Rv-cyclin functions to

negatively regulate viral gene expression through the direct interaction of its transcription activation domain with TATA binding protein-associated factor 9 (TAF9) (Rovnak et al., 2005; Rovnak and Quackenbush, 2006). WDSV rv-cyclin can induce cell-cycle progression in cyclin deficient yeast and induce hyperplasia in transgenic mice after wound healing (Lairmore et al., 2000; LaPierre, Casey, and Holzschu, 1998).

orf b encodes a protein of 306 amino acids with a molecular mass of 35 kDa and has limited sequence homology to the rv-cyclin (LaPierre et al., 1999) but no homology with other, known proteins. WDSV Orf B was found to localize in the cytoplasm and at the plasma membrane in explanted tumor cells (Rovnak et al., 2007). Cellular proteins that interact with Orf B were identified in a yeast 2-hybrid assay, one of which is the receptor for activated C kinase (RACK1)(see below).

RACK1 is a 36 kDa protein (Ron et al., 1994) composed of seven WD-repeats, domains first identified in the β subunit of the heterotrimeric G proteins that are important in protein-protein interactions (Fong et al., 1987; Neer et al., 1994). RACK1 binds to activated conventional isoforms of protein kinase C (PKC) and functions as an anchoring protein to stabilize PKC at the membrane in an active conformation (Dorn and Mochly-Rosen, 2002; Ron et al., 1994).

PKC comprises a large family of serine-threonine isoenzymes that contain a regulatory domain and a kinase core. PKCs are classified into three groups based on the domain composition of the regulatory component: conventional (α, βI, βII, and γ), novel (δ, ε, η, and θ), and atypical (ζ and ι/λ) (Newton, 2003). PKCs are involved in a broad array of biological functions such as cell proliferation, differentiation, survival and apoptosis (Nakashima, 2002; Nishizuka, 1988; Nishizuka, 1995). All PKC isoforms

associate with phospholipids in the membrane, and each group has additional cofactor requirements for full activation. The conventional PKCs are calcium-dependent (Ca^{+2}) and require binding to diacylglycerol (DAG) for activation (Newton, 2003; Nishizuka, 1986). Novel PKCs are Ca^{+2}-independent but require DAG for activation, and the atypical isoforms only require phosphatidylserine for activation (Newton, 2003; Nishizuka, 1984).

In this study we demonstrate a direct interaction of Orf B with RACK1 and constitutive activation of PKCα in Orf B-expressing cells. Further, activation of the PKC signaling pathway is responsible for the ability of Orf B-expressing cells to survive and proliferate under serum deprived conditions.

RESULTS

Expression of WDSV Orf B in explanted tumor cells and cell lines.

Cells from a regressing tumor were established in vitro after explanting tumor fragments in culture dishes (Rovnak et al., 2007). The adherent cells, which include cells of mixed lineages, were transferred to glass microscope slides, and expression of WDSV Orf B was evaluated by an immunofluoresence assay using rabbit anti-Orf B serum. Orf B localized to the plasma membrane in structures consistent with focal adhesions and lamellapodia, and along actin stress fibers (Fig. 2.1, upper panels). Stable expression of Orf B was established in NIH3T3 cells, Chinese hamster ovary cells (CHO), and canine fibroblast cells (Cf2Th) by transfection with an Orf B expression construct (pKH3-Orf B) and selection for neomycin resistance. Orf B expression in the cell lines is localized to the cytoplasm and associated with the membrane and stress fibers, similar to that observed in the explanted tumor cells (Fig. 2.1, NIH3T3-Orf B cells-lower panel and data not shown). Orf B is also present in the nucleus of these cells.

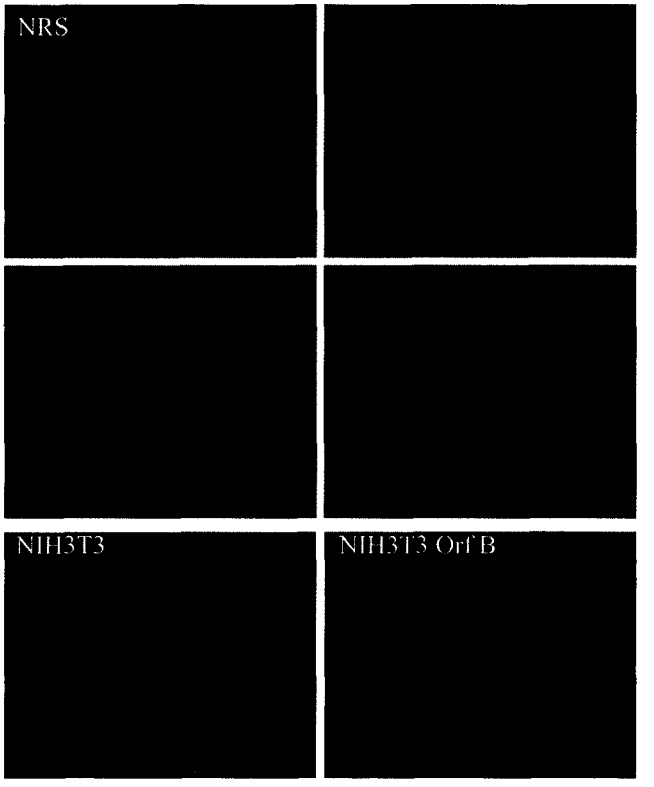

Fig. 2.1. Expression of WDSV Orf B in explanted tumor cells and NIH3T3 cells. (Upper panels) Explanted spring tumor cells were labeled consecutively with rabbit anti-Orf B sera and FITC-conjugated goat anti-rabbit IgG (green). 400 X magnification. (Lower panel) NIH3T3 (left) and NIH3T3-Orf B (right) cells were labeled with anti-HA mAb (HA.11) and FITC-conjugated anti-mouse IgG (green). 400 X magnification. Nuclei were stained with DAPI (blue).

WDSV Orf B interacts with RACK1

A yeast two-hybrid assay was used to identify cellular proteins that interact with WDSV Orf B. WDSV Orf B was fused to the GAL4 DNA binding domain (GAL4 DBD) and used as the bait for screening a human cDNA library. The sequence of the walleye genome is not known, so a human cDNA library was used to facilitate identification of Orf B interacting proteins. *Saccharomyces cerevisiae* strain Y190, harboring the *HIS3* and *lacZ* reporter genes under the control of the GAL1 UAS, were co-transformed with a HeLa cDNA library and the GAL4 DBD-Orf B construct. Positive *HIS3* transformants were screened for β-galactosidase activity by colony-lift filter assay. Sequence analysis of DNA isolated from ten β-galactosidase positive colonies revealed that two clones contained partial cDNAs of the human RACK1 gene encoding amino acids 87-317 or 139-317, limiting potential Orf B interaction to the last 178 amino acids of RACK1. This region of RACK1 encompasses WD repeats 5 through 7.

To verify the interaction of WDSV Orf B with RACK1 in vertebrate cells, immunoprecipitation (IP) assays were used. Cell lysates were subjected to IP with mouse anti-RACK1 antibody or normal mouse serum (NMS) followed by western blot analysis with anti-HA antibody (12CA5) to detect expressed, HA-tagged Orf B. Orf B was co-immunoprecipitated (coIP) with RACK1 from cells that stably express WDSV Orf B (NIH3T3-Orf B) (Fig. 2.2). In addition, Orf B was coIPed with RACK1 from lysates of Cf2Th and CHO cells that stably express Orf B and HeLa cells transiently transfected with an Orf B expression construct, pKH3-Orf B (data not shown).

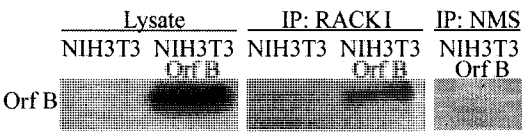

Fig. 2.2. WDSV Orf B interacts with RACK1. Lysates from NIH3T3 cells and 3T3 cells stably expressing Orf B (NIH3T3-Orf B) were immunoprecipitated with mouse anti-RACK1 antibody or normal mouse serum (NMS). HA-tagged Orf B was detected with anti-HA antibody (12CA5). Lysate represents 4% of the total amount used for immunoprecipitation.

The walleye orthologue of human RACK1 was cloned to confirm its interaction with Orf B. RACK1 is highly conserved across species (Chou et al., 1999; Kwak et al., 1997). Therefore, degenerate polymerase chain reaction (PCR) primers, based on highly conserved regions of human, mouse, fugu, and zebrafish RACK1, were used in a reverse transcriptase polymerase chain reaction (RT-PCR) reaction followed by 5' rapid amplification of cDNA ends (5' RACE) to amplify walleye RACK1. Walleye RACK1 was found to be 317 amino acids in length and predicted to encode a 36 kDa protein. The amino acid sequence of walleye RACK1 is 97% identical to zebrafish and 96% identical to mouse and human RACK1 (Fig. 2.3). The seven conserved WD repeats were identified in walleye RACK1 by amino acid alignment with human, mouse, and zebrafish RACK1 (Fig. 2.3). The WD repeats in RACK1 are predicted to form a seven-bladed propeller structure similar to that described for the β subunit of G proteins (Garcia-Higuera et al., 1996). The WD repeats serve as docking sites for binding of multiple proteins (Ron and Mochly-Rosen, 1994; Schechtman and Mochly-Rosen, 2001).

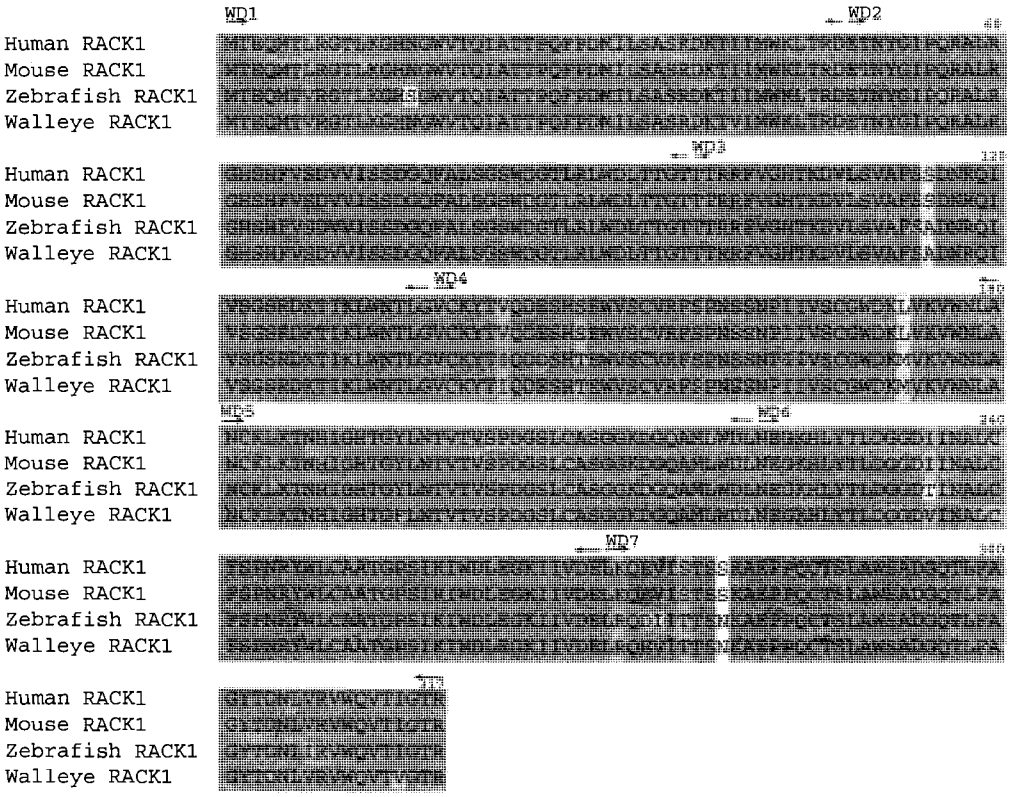

Fig. 2.3. Amino acid sequence alignment of walleye RACK1 with human, mouse, and zebrafish RACK1. Identical residues are darkly shaded, similar residues are lightly shaded, and non-identical residues are unshaded. Positions of the seven WD repeats (WD1-7) are indicated with arrows.

coIP assays were used to test for the interaction of walleye RACK1 with WDSV Orf B. Cell lysates from HeLa cells co-transfected with FLAG-tagged walleye RACK1 (pFLAG-wRACK1) and HA-tagged Orf B (pKH3-Orf B) expression constructs or empty vectors were incubated with anti-HA (12CA5) or anti-FLAG antibodies and protein G sepharose. IP protein complexes were separated by SDS-PAGE and western blots probed with antibodies to HA to detect Orf B or to FLAG to detect walleye RACK1. Results in Fig. 4A demonstrate coIP of Orf B and walleye RACK1 from HeLa cell lysates. Further, an interaction of Orf B with walleye RACK1 was confirmed in walleye fibroblast cells (W12). Cell lysates from W12 cells transiently transfected with pKH3-Orf B or the pKH3 empty vector were subjected to IP with an anti-RACK1 monoclonal antibody. Western blot analysis demonstrates coIP of HA-tagged Orf B with endogenous walleye RACK1 (Fig. 2.4B). Together, these data show that WDSV Orf B interacts with RACK1 in these cells.

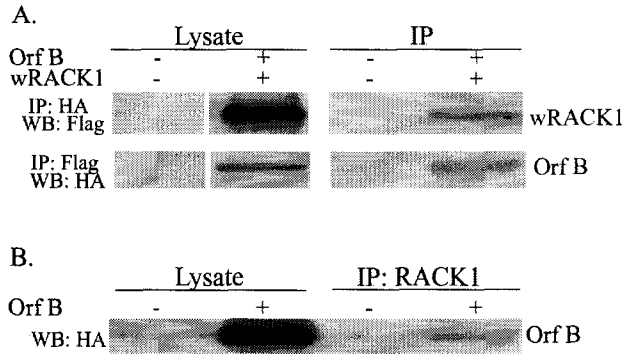

Fig. 2.4. A. Co-immunoprecipitation of Orf B and walleye RACK1 from HeLa cell lysates. Lysates from HeLa cells co-transfected with HA-tagged Orf-B (pKH3-Orf B) and FLAG-tagged walleye RACK1 (pFLAG-wRACK1) or empty vectors pKH3 and pFLAG were immunoprecipitated with anti-HA or anti-FLAG antibodies. HA-tagged Orf B was detected with anti-HA MAb (12CA5) and walleye RACK1 was detected with anti-FLAG MAb (M2). HA-tagged Orf B and FLAG-tagged walleye RACK1 run at approximately 39 kDa and 37 kDa, respectively. Lysates represent 4% of the total amount used for immunoprecipitation. **B**. Immunoprecipitation of Orf B with endogenous walleye RACK1. Lysates from walleye cells (W12) transfected with pKH3-Orf B or pKH3 empty vector were immunoprecipitated with MAb to human RACK1. Orf B was detected with anti-HA (12CA5).

WDSV Orf B associates with PKCα

Based on the known interaction of RACK1 with conventional PKC isoforms and the data above demonstrating binding of RACK1 to WDSV Orf B, we investigated the possibility that Orf B may be in a complex with PKC in the NIH3T3-Orf B cell line. NIH3T3 mouse fibroblast cells only express PKC isozymes α, δ, ε, and ζ (Mischak et al., 1993; Olivier, 1992). Therefore, we tested possible interaction of Orf B with the conventional isoform, PKCα. Lysates from NIH3T3-Orf B and NIH3T3 control cells were subjected to IP with anti-PKCα antibody and Orf B was detected with anti-HA antibody by western blot analysis of the anti-PKCα IP complexes (Fig. 2.5). In contrast, antibody that recognizes one of the novel PKC isoforms, PKCδ, was unable to coIP Orf B from these lysates (Fig. 2.5). CoIP of Orf B with another conventional isoform of PKC, PKCβII, has been demonstrated with lysates harvested from Orf B expressing HeLa and CHO cells (data not shown).

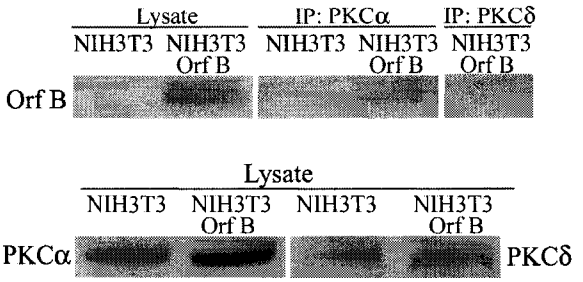

Fig. 2.5. WDSV Orf B interacts with PKCα. Lysates from NIH3T3 cells stably expressing Orf B (NIH3T3-Orf B) and NIH3T3 control cells (NIH3T3) were immunoprecipitated with rabbit anti-PKCα antibody or with rabbit anti-PKCδ antibody. HA-tagged Orf B was detected with anti-HA (12CA5) MAb. Lysate represents 4% of total amount used for immunoprecipitation.

The PKC family of serine/threonine kinases functions to transduce signals that control cell proliferation, differentiation, motility, and apoptosis. Activation of PKCα protects cells from apoptosis (Nakashima, 2002; Ruvolo et al., 1998). The interaction of Orf B with RACK1 and PKCα suggested that PKC function might be altered. To evaluate whether Orf B expression affects PKC function in NIH3T3-Orf B cells, we first tested the ability of cells to survive after exposure to staurosporine, a known PKC inhibitor and inducer of apoptosis. Treatment of cells with concentrations of staurosporine that induce apoptosis significantly reduced the number of viable NIH3T3 cells compared with NIH3T3-Orf B cells ($P< 0.004$). Exposure to 25, 50, or 100 nM staurosporine for 16 hrs reduced NIH3T3 viability to 81% (mean ±8.22), 79% (±5.07), and 71% (±10.84) of untreated cells, respectively (Fig. 2.6). In contrast, the viability of staurosporine-treated NIH3T3-Orf B cells was not reduced relative to untreated cells (mean = 104% at 25 nM (±5.4), 102% at 50 nM (±15.3), and 100% at 100nM (±14.6)). Comparable results were obtained when Orf B expressing Cf2Th cells were treated with staurosporine (data not shown). These results suggest that Orf B associates with conventional PKC isoforms and may affect PKC signaling to promote cell survival.

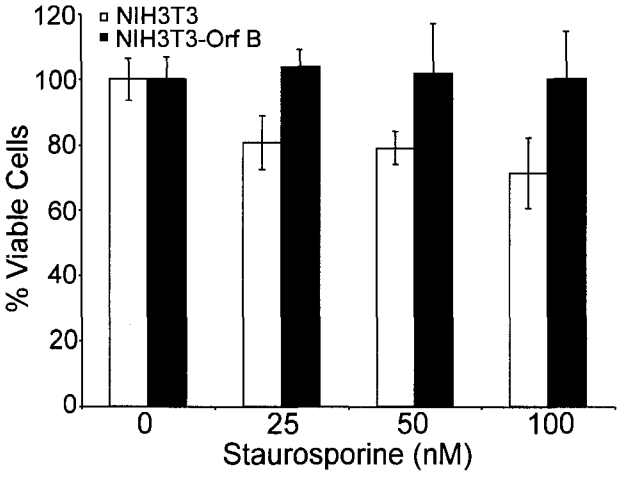

Fig. 2.6. WDSV Orf B expressing cells remain viable after treatment with staurosporine. NIH3T3 and NIH3T3-Orf B cells were treated with 25, 50, or 100 nM staurosporine, and cell viability was measured by MTS assay after 16 hrs of incubation. The mean ± standard deviation of OD_{490} readings from replicates of six wells was determined and normalized to % viability. The data represent one of three independent experiments. Statistically significant differences ($P< 0.004$).

PKC is activated in WDSV Orf B expressing cells

Newly synthesized PKC associates with cellular membranes through weak interactions with phospholipids. PKC then undergoes phosphorylation at three sites rendering it mature and catalytically competent but not yet fully active. Phosphorylated PKC is released into the cytosol where it remains in an inactive conformation until elevated levels of Ca^{+2} and DAG are present, at which time PKC translocates to the membrane. High affinity interactions of PKC with the membrane result in the release of the PKC pseudosubstrate from the substrate binding site and the establishment of an active conformation of PKC (Newton, 2003). Interaction of RACK1 with activated PKC targets PKC to different subcellular sites close to PKC substrates and is thought to maintain PKC in its active conformation.

To determine whether Orf B affects activation of PKCα due to its interaction with RACK1 the activation status and localization of PKCα was investigated. We focused on the NIH3T3 fibroblast cell line for these studies, because, when cultured under serum-deprived conditions, PKC is not activated and the effect of overexpressed RACK1 on cell growth has been extensively investigated (Chang, Chiang, and Cartwright, 2001; Chang et al., 1998; Mamidipudi et al., 2004a). Cell lysates were isolated from NIH3T3 and NIH3T3-Orf B cells that were serum deprived for 24 hrs and either treated with 200 nM phorbol myristic acid (PMA) in dimethyl sulfoxide (DMSO), a phorbol ester known to activate PKC, or with an equal volume of DMSO as control. Cytosolic and membrane fractions were separated by ultracentrifugation and subjected to western blot analysis with anti-phospho-PKC α/βII antibody specific for the autophosphorylated sites of PKCα (Thr 638) and PKCβII (Thr641). As expected, full activation and translocation of PKCα

to the membrane fraction was only detected in serum-deprived NIH3T3 cells after PMA treatment (Fig. 2.7A). In contrast, activated PKCα is present in the membrane fractions from serum-deprived NIH3T3-Orf B cells treated with PMA or with DMSO alone (Fig. 2.7A). The same blot was probed with antibody against the membrane glycoprotein cadherin as a loading control. The blot was then reprobed for total PKCα. The majority of PKCα detected in the membrane fraction from serum-deprived NIH 3T3-Orf B cells appears to be phosphorylated whereas only unphosphorylated PKCα was detected in membranes of NIH3T3 cells. Equivalent levels of phosphorylated PKCα were detected in NIH3T3 and NIH3T3-Orf B cells treated with PMA (Fig. 2.7A), and phosphorylated PKCα was detected in cytosolic fractions isolated from NIH3T3 and NIH3T3-Orf B cells (Fig. 2.7B). Treatment of cells with PMA resulted in reduced levels of cytosolic, phosphorylated PKCα, suggesting that PKCα translocated to the membrane. Expression of Orf B and RACK1 in membrane and cytosolic fractions was confirmed by staining the lower portion of the blots with anti-HA and anti-RACK1 and with anti-β actin as a loading control (Fig. 2.7A and 2.7B).

Next, we assessed the effect of treatment with the PKC inhibitor bisindolymaleimide I hydrochloride (Bim) on PKCα activation in NIH3T3-Orf B cells. Bim binds to the catalytic site of PKC and functions as a competitive inhibitor of ATP (Toullec et al., 1991). Cells were serum deprived for 24 hrs and then treated with Bim for 2 hrs, and lysates collected and processed as above. Treatment with Bim resulted in greatly reduced levels of activated PKCα in membrane preparations from serum-deprived NIH3T3-Orf B cells (Fig. 2.7A). These data indicate that PKCα is constitutively activated in Orf B-expressing cells.

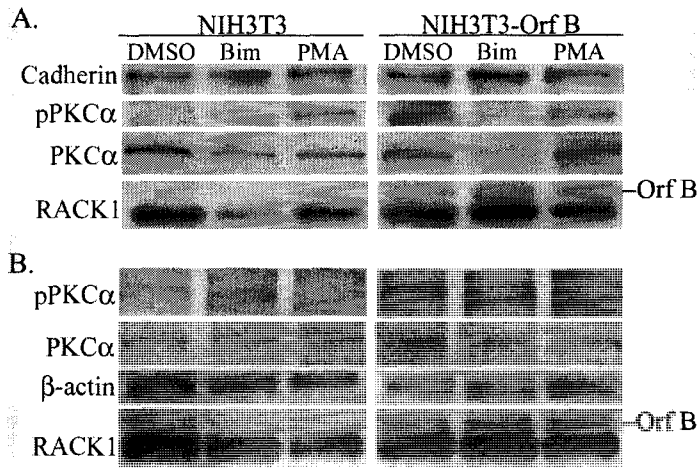

Fig. 2.7. PKCα is constitutively activated in Orf B-expressing cells. NIH3T3 and NIH3T3-Orf B cells were grown without serum for 24 hours and then treated with dimethyl sulfoxide (DMSO), bisindolymaleimide I (Bim), or phorbol ester (PMA). Cell lysates were harvested, separated into membrane **(A)** and cytosolic **(B)** fractions by ultracentrifugation, and analyzed by western blot with the indicated antibodies; anti-pPKCα detects PKCα when phosphorylated at threonine 638, anti-PKCα detects total PKCα, anti-RACK1 detects endogenous RACK1, and anti-HA (12CA5) detects HA-tagged Orf B. β-actin and cadherin serve as loading controls. The data presented in this figure is representative of four independent experiments.

Activation of PKC in NIH3T3-Orf B cells promotes cell survival.

PKC regulates several signaling pathways that mediate cell proliferation and survival. Since PKCα is constitutively activated in Orf B-expressing cells under serum-deprived conditions, we investigated whether activation of PKCα in NIH3T3-Orf B cells influences cell survival. After culture of NIH3T3-Orf B and NIH3T3 control cells for 24 hours in the presence of serum, cells were washed with phosphate buffered saline (PBS) and further cultured in media without serum. An MTS assay was used to measure cell growth. The MTS reagent was added to the culture each day and OD_{490} readings were taken after 3 hrs of further incubation. Total cell input at day 0, when serum was removed, was normalized to a value of 100% viability and adjusted accordingly each day thereafter for 7 days. The growth of NIH3T3 cells declined progressively through day 7 without serum (Fig. 2.8A). The phase contrast image presented in Fig. 2.8B illustrates substantial cell loss by day 3. In contrast, proliferation of NIH3T3-Orf B cells was observed through day 3, during which time 1.7 cell doublings occurred (Fig. 2.8A). A slight decrease in cell viability was observed at days 4 and 5, after which there was increased proliferation of NIH3T3-Orf B cells through day 7 without serum. NIH3T3-Orf B cell proliferation is shown in the phase contrast images at day 0 and 3 days after serum deprivation (Fig. 2.8B).

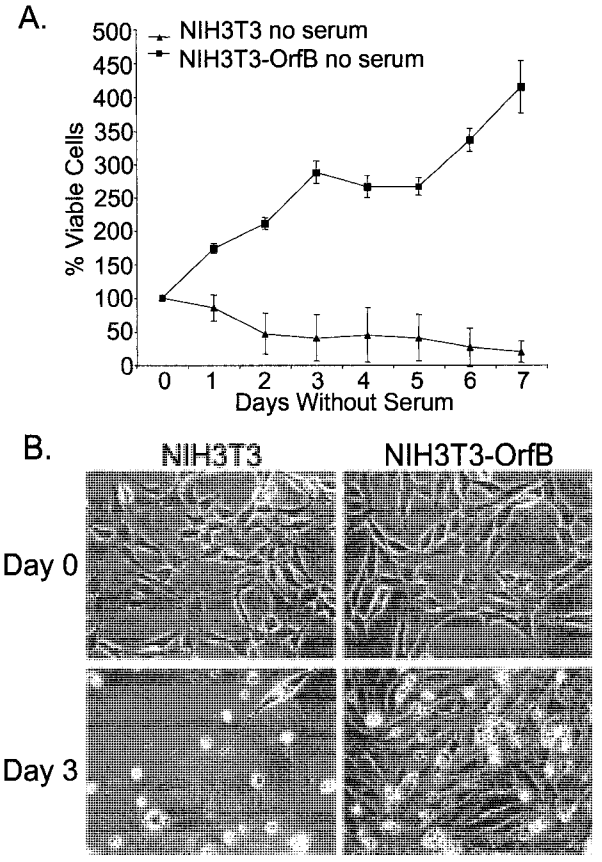

Fig. 2.8. Orf B-expressing cells proliferate and survive in the absence of serum. **A.** NIH3T3 cells and NIH3T3-Orf B cells were grown under serum-free conditions and viable cells measured each day for 7 days by MTS assay. The mean of six replicate wells was determined for each time point. Viable cells at Day 0 were normalized to 100% and ODs from subsequent days were adjusted accordingly. The data presented in this figure is representative of three independent experiments. **B.** Phase contrast images of NIH3T3 and NIH3T3-Orf B cells illustrate cell morphology at day 0 and day 3 of culture without serum. Magnification was 200X

To determine whether activated PKC is responsible for the proliferation and survival of NIH3T3-Orf B cells shown in Figure 2.8, cells were cultured in the presence of the PKC inhibitor, Bim. The growth of serum-deprived NIH3T3-Orf B cells was significantly diminished in the presence of Bim (Fig. 2.9A, open squares, $P< 0.001$). After 3 days of treatment there were 60% fewer NIH3T3-Orf B cells than in cultures without Bim. By day 7 the number of viable cells in the Bim treated cultures was reduced to input levels (mean = 90% ±7.9), whereas the cultures without Bim remained elevated (mean = 316% ±39). The decrease in cell growth is evident in the phase contrast images shown in Fig. 2.9B (compare middle panels vs. right panels). Addition of Bim to NIH3T3-Orf B cells cultured in the presence of serum had no effect on the ability of these cells to proliferate (Figs. 2.9A and B, left panels). Overall, these results support the conclusion that PKCα is activated in NIH3T3-Orf B cells and that this activation contributes to cell proliferation and survival.

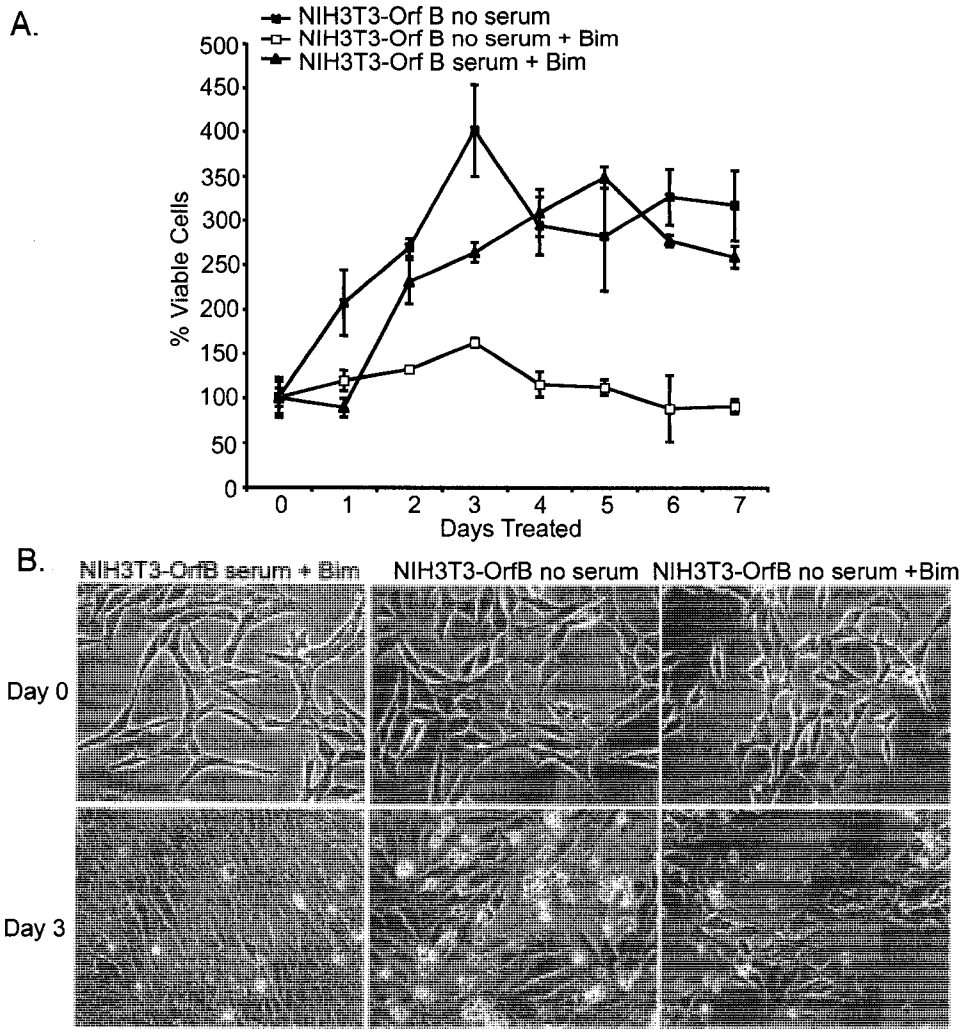

Fig. 2.9. Activated PKC drives proliferation and survival of Orf B-expressing cells. **A.** NIH3T3-Orf B cells were grown without serum in the presence of 5 μM Bim in DMSO (NIH3T3-Orf B no serum+Bim) or DMSO only (NIH3T3-Orf B no serum). NIH3T3-Orf B cells were also cultured with serum and 5μM Bim (NIH3T3-Orf B serum+Bim). Viable cells were measured each day for 7 days by MTS assay. The mean of six replicate wells was determined for each time point. Viable cells at Day 0 were normalized to 100% viability and the ODs from subsequent days were adjusted accordingly. The data presented in this figure is representative of three independent experiments. **B**. Phase

contrast images illustrate the morphology NIH3T3-Orf B cells cultured with serum and treated with 5 μM Bim (left), cells without serum and treated with DMSO (middle), and cells without serum and with 5 μM Bim (right) at day 0 and day 3. Magnification was 200X.

DISCUSSION

The results from these studies demonstrate the interaction of WDSV Orf B with RACK1 and PKCα. RACK1 was identified as a binding partner of WDSV Orf B by a yeast 2 hybrid screen and this interaction was confirmed by coIP assays. RACK1 is known to interact with over 20 cellular and viral proteins with varying functions (McCahill et al., 2002). Cloned walleye RACK1 exhibits 96% amino acid identity to human and mouse RACK1 and 97% identity to zebrafish RACK1, illustrating the highly conserved nature of this protein. The functional importance of RACK1 is exemplified by this conservation across eukaryotic species (McCahill et al., 2002). RACK1 was first identified as an anchoring protein for the conventional PKCs and activated PKC βII was the preferred binding partner of RACK1 (Csukai and Mochly-Rosen, 1999; Mochly-Rosen, Khaner, and Lopez, 1991; Mochly-Rosen et al., 1991; Mochly-Rosen et al., 1995; Ron et al., 1994; Stebbins and Mochly-Rosen, 2001). One of the novel PKC isoforms, PKCε, is known to interact with RACK2, also known as the coatomer protein β'COP (Csukai et al., 1997). The expression pattern for each PKC isoform varies among different cell types (Jaken, Leach, and Klauck, 1989; Mochly-Rosen, 1990). NIH3T3 cells express abundant levels of only one conventional PKC isoform, PKCα, and low levels of the novel forms, PKCδ and PKCε (McCaffrey et al., 1987; Mischak et al., 1993)

The interaction of Orf B with RACK1 suggested that PKC may be present in an Orf B/RACK1 complex, and coIP assays with lysates from NIH3T3-Orf B cells confirmed an interaction of Orf B with PKCα. Orf B was also coIPed with PKCβII from lysates isolated from cells that express this isoform. An association of Orf B and PKC is further suggested by the localization of Orf B in tumor cells at the plasma membrane and

along actin stress fibers. Goodnight et al. (Goodnight et al., 1995) showed that PKCα is diffusely distributed in the cytoplasm in NIH3T3 cells and, upon TPA stimulation, is redistributed to the plasma membrane with marked localization to membrane ruffles and accumulation at focal adhesion contacts. RACK1 is also targeted to focal adhesions where it affects cell motility (Kiely et al., 2006; Vomastek et al., 2007). When over-expressed in NIH3T3 cells, PKCβII associates with actin-rich microfilaments (Goodnight et al., 1995). The localization of Orf B in explanted tumor cells and cultured cell lines exhibited all of these characteristics.

Still unresolved is the question of whether there is direct contact between Orf B and PKCα or whether coIP is dependent on the interaction of Orf B and RACK1. RACK1 holds PKC in an active conformation, and the structural nature of RACK1 indicates that more than one protein may bind to it concomitantly (McCahill et al., 2002). The presence of Orf B in a RACK1/PKC complex may aid in the formation of a more stable, active complex. Another possibility is that Orf B is a substrate for PKC and RACK1 targets PKC to subcellular locations in which Orf B resides. A ProSite Motif analysis (Hulo et al., 2008) identified three predicted PKC phosphorylation sites within Orf B. Binding of viral proteins to RACK1 may be a means to target interaction with PKC. The Epstein-Barr virus protein, ZEBRA, is a transcriptional activator that functions to disrupt latency. ZEBRA binds RACK1 and is phosphorylated by PKC at residue S186. However, this phosphorylation does not appear to be responsible for the disruption of latency (El-Guindy et al., 2002). PKC activity was inhibited in EBV-infected monocytes, likely due to impaired translocation of PKC from the cytosol to the plasma membrane (Tardif et al., 2002). The M1 protein from avian, swine, and human

influenza A viruses interacts with RACK1 and is phosphorylated by PKC (Reinhardt and Wolff, 2000). HIV Nef also binds RACK1 and is phosphorylated by PKC, which results in enhanced viral replication (Gallina, Rossi, and Milanesi, 2001a; Wolf et al., 2008). The phosphorylation status of Orf B will be the subject of future investigations.

After synthesis, PKCs associate with membranes and are targeted for phosphorylation at the activation loop by PDK-1, followed by autophosphorylation at the turn and hydrophobic motifs, and release into the cytosol in an inactive conformation. Extracellular stimuli elevate the levels of intracellular Ca^{+2} and DAG, which facilitates translocation of cytosolic PKC to the membrane and results in high affinity interactions (Newton, 2003). Activated PKC was only detected after stimulation of serum-deprived NIH3T3 cells with PMA. PMA binds PKC and recruits it to the membrane. Detection of phosphorylated PKCα in membranes of serum-deprived NIH3T3-Orf B cells, without PMA treatment, indicates that it is in a constitutively active conformation. Activated PKC functions to phosphorylate and activate substrate proteins that affect cell survival and growth.

Tethering of active PKC to the membrane allows binding to substrates, which affect downstream targets important in cell proliferation such as Raf-1 and the mitogen-activated protein kinase kinase-extracellular signal-regulated kinase (MEK-ERK) (Cai et al., 1997). In HL60 cells PKCα co-localizes with and phosphorylates Bcl-2 in mitochondria, resulting in increased cell survival following chemotherapy (Ruvolo et al., 1999). Constitutive expression of Orf B resulted in cell survival after treatment with staurosporine and proliferation and survival of cells cultured under serum-deprived conditions. The constitutive activation of PKCα in these cells suggests that it is

responsible for Orf B's effects. The reversal of these effects by treatment of serum deprived cells with Bim confirmed the requirement for the PKC signaling pathway in this process.

WDSV transcripts encoding the Orf B protein are present during the period of tumor development, and results from these studies suggest that Orf B is tumorigenic. Constitutive activation of PKCα in WDSV infected cells could lead to deregulated cell signaling pathways critical for both apoptosis and cell proliferation and resulting in tumor formation. The evidence presented here demonstrates alterations of cell proliferation and response to apoptotic stimuli, specifically subject to a PKC inhibitor, in cells expressing Orf B. Identification of the downstream targets activated in Orf B-expressing cells will provide valuable insight into the mechanism(s) of WDSV oncogenesis.

MATERIALS AND METHODS

Cells and transfection.

The WDSV *orf B* coding sequence was cloned into the pKH3 vector (a generous gift from Dr. Jun-Lin Guan, Cornell University) as previously described (Rovnak, Casey, and Quackenbush, 2001). The pKH3 vector contains a CMV promoter and fuses three influenza virus hemagglutinin (HA) tags onto the amimo terminus of the expressed protein (Chen et al., 1996; Mattingly et al., 1994).

NIH3T3 cells (ATCC CRL 1658), Cf2Th cells (ATCC CRL 1430), and HeLa cells (ATCC CCL 2) were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) at 37°C in 5% CO_2. CHO cells (CHO-K1, ATCC CCL 61) were maintained in Ham's F12 medium supplemented with 10% FBS. Cells were transfected using FuGENE6 (Roche) according to the manufacturer's instructions. Stable cell lines were established by cotransfection of pKH3-Orf B and a plasmid encoding neomycin resistance pMC1neo (Stratagene). Cells were selected with G418 and single colonies were expanded and assayed for Orf B expression by western blot analysis.

Fluorescence microscopy

Cells cultured from a regressing dermal sarcoma and NIH3T3 cells were grown on glass slides for immunofluorescence assays (Cel-Line; Erie Scientific, Inc) (Rovnak et al., 2007). Slides were fixed in 2% buffered formalin for 15 min, rinsed in phosphate buffered saline (PBS), and fixed for 5 min in cold acetone:methanol (1:1 v/v). Fixed cells were incubated with a 1:100 dilution of affinity purified rabbit anti-Orf B sera

(kindly provided by Volker Vogt, Department of Molecular Biology and Genetics, Cornell University) or 1:1000 dilution of mouse monoclonal anti-HA antibody (HA.11, Covance) for 1 hr at 37°C in a humidified chamber. Slides were washed in PBS and incubated with a 1:40 dilution of affinity purified goat anti-rabbit IgG or goat anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC). After washing in PBS, slides were stained for 1 min in 300μM 4',6'-diamidino-2-phenylindole (DAPI). Photomicrographs were captured digitally at a magnification of 200X or 400X and images were assembled with Adobe Photoshop 7.0 software.

Yeast 2-hybrid screening

WDSV *orfb* was amplified by PCR from the plasmid clone pKH3-Orf B using 5' and 3' primers that incorporate *Eco*RI (5'GCCGAATTCATGTTTTCAGACTCAGATTCCT-3') and *Bam*HI (5'-GCCGGATCCTTACTCCGTAGGGCTGGGCTCT-3') restriction sites, respectively. The amplified product was digested and ligated into the GAL4 DNA binding domain vector pAS2-1 and used as the bait to screen a pACT2 GAL4 AD fusion HeLa cDNA library (MATCHMAKER GAL4 Two-Hybrid System, Clontech). *Saccharomyces cerevisiae* strain Y190 was cotransformed with the bait plasmid, pAS2-1-Orf B and the pACT2 cDNA library. Positive clones were initially selected based on the ability to grow in the absence of histidine, leucine, and tryptophan in the presence of 25 mM 3-amino 1, 2, 4-triazole (3-AT). Positive colonies were further screened for β-galactosidase activity by a colony lift assay. Plasmid DNA was isolated from clones positive for β-galactosidase activity and sequenced. The specificity of the Orf B and RACK1

interaction was shown by transformation of yeast with (1) pAS2-1-Orf B and pACT2 plasmid, (2) pAS2-1 and pACT2RACK1 plasmid, which were negative for b-galactosidase activity. In addition, screening of the same cDNA library with another viral protein, WDSV rv-cyclin did not result in any interaction with RACK1 (Quackenbush, unpublished).

Cloning of walleye RACK1

RNA was isolated from a walleye fibroblast cell line (W12) with RNAzol (Tel-Test, Inc.) and cDNAs were prepared with oligo dT primer and Superscript II according to instructions of the manufacturer (Life Technologies). An internal walleye RACK1 (wRACK1) sequence was amplified from W12 cDNA by PCR using degenerate primers designed from highly conserved regions of zebrafish, pufferfish, human, and mouse RACK1 sequences (5' primer- ATGACYGAGCARATGACMST; 3' primer-CKNGTDCCRATRGTBCACCTG). Additional walleye RACK1 5' sequences were amplified using a walleye specific RACK1 primer A (5'-AGTTGGCCAGATTCCACACCTTCAC-3') and a primer designed from zebrafish RACK1 (5'-ATGACCGAGCAGATGACAGTAAGGG-3'). The remaining unknown 5' walleye RACK1 sequence was amplified by RNA ligase-mediated and oligonucleotide capping rapid amplification of cDNA ends (RACE) using the 5'RACE primer and the wRACK1 specific primer B (5'-GTCTGGAAACTGCGGGGTCGTGGC-3') according to manufacturers instructions (GeneRacer Kit, Invitrogen). The 3' end of wRACK1 was amplified with an oligo dT primer and wRACK1 specific primer C (5'-GATGGACAGGCCATGCTTTGGGAT-3').

Walleye RACK1 was amplified by PCR from oligo dT primed W12 cDNA using walleye RACK1 specific primer D (5'-GCCGAATTCGATGACCGAGCAGATGACCGTGAGA-3') and primer E (5'-CGGGGATCCTTATCGGGTTCCGACGGTCACCTG-3') that incorporate *Eco*RI and *Bam*HI restriction sites. The amplified product was digested with *Eco*RI and *Bam*HI and ligated into p3XFLAG-CMV™-10 expression vector (Sigma).

Nucleotide sequence accession number

The final cDNA sequence for walleye RACK1 has been assigned GenBank accession number EU290652.

Immunoprecipitation and western blot analysis.

NIH3T3 control cells and NIH3T3 cells stably expressing Orf B (NIH3T3-Orf B) were lysed with immunoprecipitation (IP) buffer (1% Triton X-100, 0.5% NP-40, 150 mM NaCl, 10 mM Tris-HCl [pH 7.5], 1 mM EDTA [pH 8.0], 1 mM EGTA, 0.2 mM sodium orthovanadate, 0.2 mM PMSF, 2 μg/ml of leupeptin and aprotinin, 1 μg/ml pepstatin). Lysates were centrifuged at 21,000 x g for 10 minutes and protein concentration of supernatants was determined with the Micro BCA Kit (Pierce). 500 μg of cell lysates were diluted to a concentration of 1 μg/ml in IP buffer and precleared for 3 hours with 50 μl of a suspension of Protein G-Sepharose (Pharmacia Biotech). One microgram of antibody was added to the precleared lysates and incubated overnight at 4°C with rotation. Fifty microliters of a Protein G suspension was added and incubated for an additional 3 hours at 4°C with rotation. Protein G pellets were washed three times with cold IP buffer, suspended in 20 μl of SDS-PAGE loading buffer, and heated to 70°

for 10 minutes. Samples were separated under denaturing conditions in a 10% polyacrylamide gel. Cell lysates (15 μg of protein) were loaded in control lanes. Denatured proteins were transferred to Immobilon-P-membrane (Millipore) and incubated with primary antibody overnight at 4°C. Blots were washed, incubated with anti-rabbit IgG or anti-mouse IgM antibodies conjugated with horseradish peroxidase, and developed with LumiGLO Chemiluminsescent Substrate Kit (Kirkegaard and Perry Laboratories).

Membrane Protein Fractionation.

NIH3T3 and NIH3T3-Orf B cells were plated at 7.5 x 10^5 cells/ml and grown in DMEM containing 10% FBS supplemented with 4 mM glutamine. Twenty four hours after plating, the growth medium was replaced with DMEM without serum for 24 hours. Cells were then treated with DMSO, for 45 minutes, 200 nM phorbol 12-myristate 13-acetate (PMA) for 45 minutes, or 5 μM Bisindolymaleide I (Bim) for 2 hrs and then cells were immediately placed on ice. Cells were scraped into protein lysis buffer lacking detergent (20 mM Tris [pH7.5], 2 mM EDTA, 2mM EGTA, 1mM PMSF, 2 ug/ml leupeptin, 2 ug/ml aprotinin, 0.1% 2-mercaptoethanol) then sonicated briefly as described in Goodnight et al. (Goodnight et al., 1995). Membrane and cytoplasmic fractions were separated by ultracentrifugation at 100,000 x *g* for 1 hour at 4°C. The supernatant (cytoplasmic fraction) was collected. The pellet (membrane fraction) was resuspended in protein lysis buffer containing 1.2% Triton X-100, sonicated briefly and centrifuged at 20,000 x *g* for 10 minutes at 4°C and the supernatant collected and analyzed as the membrane fraction. Protein concentrations were determined using the Micro BCA Kit

(Pierce). Fifteen micrograms of cytoplasmic proteins were precipitated in 1/10 volume of trichloroacetic acid (Sigma) and pellets were dissolved in 100 mM NaOH.

Cell viability assays

NIH3T3 and NIH3T3-Orf B cells were plated at 5.0 x 10^5 cells/ml in 96 well plates and incubated for 24 hours in DMEM containing 10% FBS. Staurosporine was added to final concentrations of 25, 50, and 100 nM, incubated for 16 hours at 37°C, and cell viability was measured using CellTiter 96 AQueous One Solution (Promega) according to manufacturer's instructions. Briefly, 20 µl of CellTiter 96 solution was added to each well, incubated for 3 hours at 37°C, and then OD readings were taken at 490 nm.

NIH3T3 and NIH3T3-Orf B cells were plated at 5.0 x 10^5 cells/ml in 96 well plates containing DMEM with 10% FBS and incubated at 37°C in 5% CO_2. After 24 hours of culture cells were washed with PBS then further cultured in DMEM without serum. Bim was added daily to maintain a final concentration of 5 µM. Cell viability was measured daily for 7 days using the CellTiter 96 solution (Promega) as described above. Student's *t* test and 95% confidence intervals based on a *t* distribution were used for statistical analyses. A *P* value of less than 0.01 was considered significant.

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CHAPTER 3*

WALLEYE DERMAL SARCOMA VIRUS ORF B ACTIVATES AKT AND INDUCES TRANSFORMATION OF NIH3T3 CELLS

*** Submitted for publication as:**

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ABSTRACT

Walleye dermal sarcoma virus is a complex retrovirus associated with dermal sarcomas in walleye fish. During the fall, spliced accessory gene transcripts, *A* and *B,* are present in developing tumors suggesting that their encoded proteins, rv-cyclin and Orf B, may play a role in oncogenesis. The regulation of cell signaling pathways is one way in which viruses induce oncogenesis. We previously demonstrated constitutively activated PKC in Orf B-expressing cells (Daniels et al., 2008). This study demonstrates that the 90

kDa ribosomal S6 kinase (p90RSK) and protein kinase B (Akt) are also constitutively activated in Orf B-expressing cells.

Akt is phosphorylated at serine 473 and activation of Akt promoted cell survival and proliferation of Orf B-expressing cells. Expression of Orf B induces transformation of NIH3T3 cells *in vitro* and a PI3K and mTOR inhibitor prevented transformation. These results suggest that the rictor-mTOR complex may be a common target for the activation of Akt and PKCα by Orf B.

INTRODUCTION

Walleye dermal sarcoma virus (WDSV) is a complex retrovirus and is etiologically associated with dermal sarcoma in walleye fish (*Sander vitreus*) (Martineau et al., 1992; Martineau et al., 1991b; Walker, 1969; Yamamoto, Kelly, and Nielsen, 1985a; Yamamoto et al., 1976). The disease is characterized by the development of tumors in the fall that grow throughout the winter and regress during the spawning period the following spring. Differences in viral gene expression are associated with tumor development and regression; low levels of spliced accessory gene transcripts, *A* and *B,* are present during tumor development, whereas tumor regression is characterized by high levels of spliced transcripts, full-length genomic RNA, and infectious virus (Bowser, Martineau, and Wooster, 1990; Bowser et al., 1996; Martineau et al., 1992; Martineau et al., 1990a; Martineau et al., 1991a; Quackenbush et al., 1997; Rovnak, Casey, and Quackenbush, 2001). Infectious virus can only be isolated from regressing tumors.

The *orf b* transcript encodes a 35 kDa protein that is localized in the cytoplasm, at the plasma membrane, and along actin stress fibers in naturally infected, explanted tumor cells and when expressed in recombinant form in mammalian and piscine cells (Daniels, Rovnak, and Quackenbush, 2008b; Rovnak et al., 2007). Orf B directly interacts with the cellular receptor for activated C kinase (RACK1) and is associated with protein kinase C alpha (PKCα) (Daniels, Rovnak, and Quackenbush, 2008b). PKCα is constitutively activated in Orf B-expressing cells and promotes cell survival and proliferation (Daniels, Rovnak, and Quackenbush, 2008b). Furthermore, Orf B-expressing cells remain viable after exposure to staurosporine (Daniels, Rovnak, and Quackenbush, 2008b), a known PKC inhibitor and inducer of apoptosis.

A variety of cell signaling pathways control the activation and deactivation of the Bcl-2 family of pro-apoptotic and anti-apoptotic proteins. The pro-apoptotic function of one such protein, BAD, is eliminated upon phosphorylation at serine residues 112, 136, or 155 (Zha et al., 1996). Staurosporine reduces phosphorylation of BAD at serine residues 112 and 136 (Tafani et al., 2001). BAD is phosphorylated on serine residues 112 and 155 by p90RSK (90 kDa ribosomal S6 kinase) (Tan et al., 1999). PKC directly phosphorylates p90RSK at serine 380 to activate it. The MAP kinases, p42 and p44 (Erk1/2, respectively), of the MAPK cell signaling pathway also activate p90RSK (Dalby et al., 1998). The constitutive activation of PKCα in Orf B-expressing cells (Daniels, Rovnak, and Quackenbush, 2008b) implicates it in the activation of p90RSK and the subsequent phosphorylation of BAD in Orf B protection against apoptosis.

Protein kinase B (Akt) phosphorylates BAD at serine 136 (Peso et al., 1997). Phosphatidylinositol-3-kinase (PI3K) is a lipid kinase that, when stimulated by growth factors, phosphorylates and converts phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3), thereby resulting in recruitment of phosphoinositide-dependent kinase 1 (PDK1) and Akt to the membrane, where PDK1 phosphorylates Akt on threonine 308 (T308) (Vara et al., 2004). Akt is also phosphorylated at serine 473 (S473) by the mammalian TOR kinase (mTOR) complex containing rictor and GβL (rictor-mTOR complex, also called mTORC2) (Sarbassov et al., 2005). The phosphatase, PTEN (phosphatase and tensin homologue deleted on chromosome 10) terminates PI3K signaling through dephosphorylation and conversion of PIP3 to PIP2 (Vara et al., 2004), and phosphorylation of PTEN results in inactivation of its phosphatase activity (Wu et al., 1998).

Akt was originally discovered as an oncogene isolated from a transforming murine leukemia retrovirus, AKT8 (Bellacosa et al., 1991; Staal, Hartley, and Rowe, 1977a). Many downstream targets of Akt are inhibited by their phosphorylation. For instance, glycogen synthase kinase 3β (GSK-3β) is constitutively active in unstimulated cells, and phosphorylation by Akt inactivates its catalytic activity upon stimulation of cell proliferation.

In this study we demonstrate the phosphorylation of both Akt and p90RSK in Orf B-expressing cells. We further show that phosphorylation of Akt contributes to the ability of Orf B-expressing cells to survive and proliferate under serum-deprived conditions. Orf B-expressing cells also exhibit properties of transformed cells, and we provide evidence for a significant decrease in the transformed phenotype with the use of specific kinase inhibitors. Finally, we show inactivation of an important Akt regulator in lysates of transformed foci.

RESULTS

BAD is fully phosphorylated in Orf B-expressing cells

PKCα is constitutively active in Orf B-expressing cells, and treatment with staurosporine, at concentrations that induce apoptosis, does not cause death of these cells (Daniels, Rovnak, and Quackenbush, 2008b). Stable expression of Orf B was established in NIH3T3 and Chinese hamster ovary cells (CHO) by transfection with an Orf B expression construct (pKH3-Orf B) (Daniels, Rovnak, and Quackenbush, 2008b). To investigate the phosphorylation status of BAD in control vs. Orf B-expressing cells, NIH3T3, NIH3T3-Orf B, CHO, and CHO-Orf B cells were transfected with the pEBG-mBAD expression construct and cultured without serum. Cell lysates were then examined by western blot with antibodies to detect BAD and specific phospho-epitopes on BAD (Fig. 3.1A) and HA-tagged Orf B (Fig. 3.1B). An increase in the phosphorylation of BAD at serine residues 112, 136, and 155 was detected in Orf B-expressing NIH3T3 and CHO cells cultured under serum-deprived conditions (Fig. 3.1A). Overall, apparent phosphorylation at the targets of p90RSK, serine 112 and 155, was greater than at the Akt target, serine 136, in both control and in Orf B-expressing cells, but both pathways are activated in the presence of Orf B.

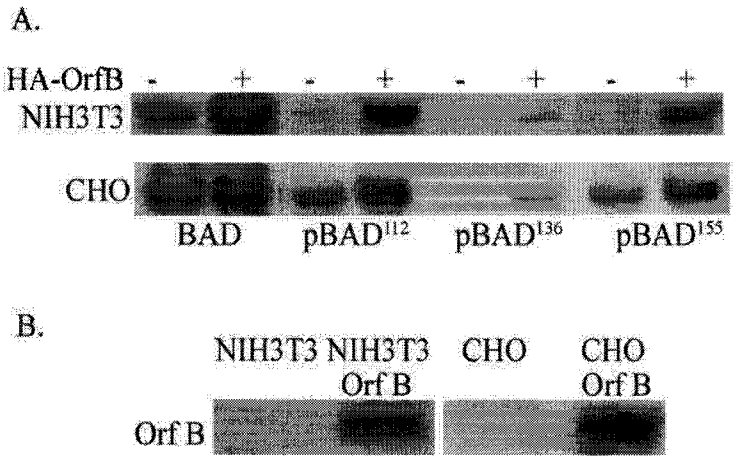

Fig 3.1. BAD is phosphorylated in Orf B-expressing cells. A. NIH3T3, NIH3T3-Orf B, CHO, and CHO-Orf B cells were transfected with a pEBG-BAD construct then serum starved for 48 hrs. Cell lysates were harvested and analyzed by western blot with the indicated antibodies: anti-BAD detects total BAD, anti-pBAD^{112} detects BAD phospho-serine 112, anti-pBAD^{136} detects BAD phospho-serine 136, and anti-pBAD^{155} detects BAD phospho-serine 155. B. HA-tagged Orf B was detected with anti-HA (12CA5) MAb.

p90RSK is activated in Orf B-expressing cells

Phosphorylation of BAD at serine residues 112 and 155 indicate that p90RSK is also activated and contributes to the block of apoptosis by Orf B. To determine if p90RSK is activated, lysates were collected from NIH3T3 and NIH3T3-Orf B cells that were serum-deprived for 24 h and then either left untreated or treated with 200 nM phorbol myristic acid (PMA) or 10% fetal bovine serum (FBS) to stimulate p90RSK activation. Lysates were subjected to western blot analysis with antibodies detecting p90RSK phosphorylation at serine 380 (Fig. 3.2, top). Phosphorylated p90RSK^{380} was detected in NIH3T3 cells only after treatment with PMA or serum. In contrast phosphorylated p90RSK^{380} was detected in lysates from untreated, serum-deprived NIH3T3-Orf B cells (Fig. 3.2, top), confirming its role in the phosphorylation of BAD (Fig. 3.1).

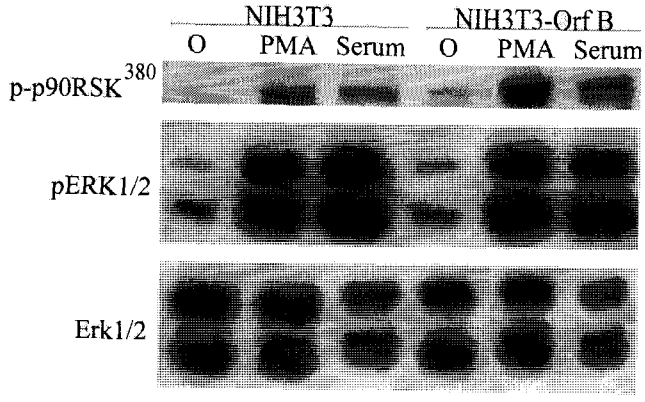

Fig 3.2. p90RSK is activated in Orf B-expressing cells. NIH3T3 and NIH3T3-Orf B cells were serum starved (O) for 24 hrs and then treated with phorbol ester (PMA) or 10% FBS (Serum). Cell lysates were harvested and analyzed by western blot: anti-p-p90RSK detects phospho-serine 380 of p90RSK, anti-pERK1/2 detects phospho-threonine 202 and phospho-tyrosine 204 of ERK1/2, and anti-ERK1/2 detects total ERK1/2.

p90RSK can be activated either by the MAPK cell signaling pathway (Erk1/2) or directly by PKC. PKC was shown previously to be constitutively active in Orf B-expressing cells (Daniels, Rovnak, and Quackenbush, 2008b). To determine if Erk1/2 is also active in p90RSK phosphorylation, the lysates from serum-starved NIH3T3 and NIH3T3-Orf B cells either untreated or stimulated with PMA or FBS were subjected to western blot analysis with antibodies detecting ERK 1/2 phosphorylation at threonine 202 and tyrosine 204, and total ERK1/2. NIH3T3 and NIH3T3-Orf B cells had the same low levels of phosphorylated Erk1 and Erk2 in untreated, serum-deprived cells and the same increase in phosphorylation after PMA or serum stimulation (Fig. 3.2, middle). These data indicate that MAPK pathway activation is no different in serum-deprived NIH3T3 or NIH3T3-Orf B cells. Combined, these data indicate that the apparent p90RSK phosphorylation is a result of PKC activity.

Akt is phosphorylated in Orf B-expressing cells at serine 473.

Akt phosphorylation at serine 473 (S473) is an important indicator of Akt activation (Buchkovich et al., 2008; Sarbassov et al., 2005). Phosphorylation of BAD at serine residue 136 suggests that Akt is activated and may contribute to the anti-apoptotic mechanism of Orf B. To determine if Akt is constitutively activated, CHO and CHO-Orf B cells were serum-deprived for 24 h and then either left untreated or treated with 50 ng/ml platelet derived growth factor (PDGF) to activate Akt. Total lysates were subjected to western blot analysis with antibody specific for phosphorylated Akt at S473. Akt was minimally phosphorylated at S473 under serum-deprived culture conditions in control CHO cells and increased phosphorylation was apparent in CHO-Orf B cells. Akt

phosphorylation was greatly enhanced upon PDGF-stimulation of either cell type, but to a greater degree in CHO-Orf B cells (Fig. 3.3). Akt is also phosphorylated at S473 in 3T3-Orf B cells when cultured under serum-deprived conditions (Fig. 3.7B). These data implicate constitutive activation of Akt as a contributing factor in Orf B protection from apoptosis, a pathway distinct from the previously defined PKCα activation.

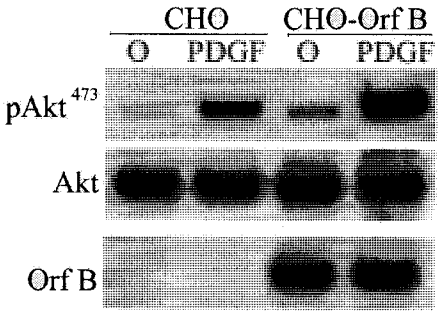

Fig 3.3. Akt is phosphorylated in Orf B-expressing cells. CHO and CHO-Orf B cells were grown without serum (O) for 24 hrs and then treated with 50 ng/ml PDGF. Cell lysates were harvested and analyzed by western blot with the indicated antibodies: anti-pAkt^{473} detects phospho-serine 473 of Akt, anti-Akt detects total Akt, and anti-HA (12CA5) detects HA-tagged Orf B.

Activation of Akt in NIH3T3-Orf B cells promotes cell proliferation

Previously, a specific inhibitor of PKC, bisindolymaleimide I hydrochloride (Bim), was used to demonstrate PKC function in the continued proliferation of NIH3T3-Orf B cells without serum (Daniels, Rovnak, and Quackenbush, 2008b). To determine if the activated Akt in these cells could contribute to this survival and proliferation, cells were cultured without serum and with or without kinase inhibitor, LY294002. LY294002 is a competitive inhibitor of ATP binding to the kinase domains of mTOR and PI3K (Brunn et al., 1996), which phosphorylate Akt either directly (mTOR at S473) or indirectly (PI3K via PDK1 at T308). NIH3T3 and NIH3T3-Orf B cells were washed with phosphate buffered saline (PBS) and cultured in media without serum and an MTS assay was employed daily in order to measure cell growth. The growth of NIH3T3 cells declined progressively through day 7 in the absence of serum (Fig. 3.4, closed squares). In contrast, the numbers of NIH3T3-Orf B cells doubled 3.5 times through day 7 in serum-free media (Fig. 3.4, closed triangles). The proliferation of NIH3T3-Orf B cells was significantly diminished in the presence of LY294002 (Fig. 3.4, open triangles dashed line, $p<0.001$). After 3 days of treatment there were 63% fewer NIH3T3-Orf B cells than in untreated cultures (Fig. 3.4). These data indicate that upstream activators of Akt are also necessary for NIH3T3-Orf B cell proliferation without serum in addition to known PKC dependence.

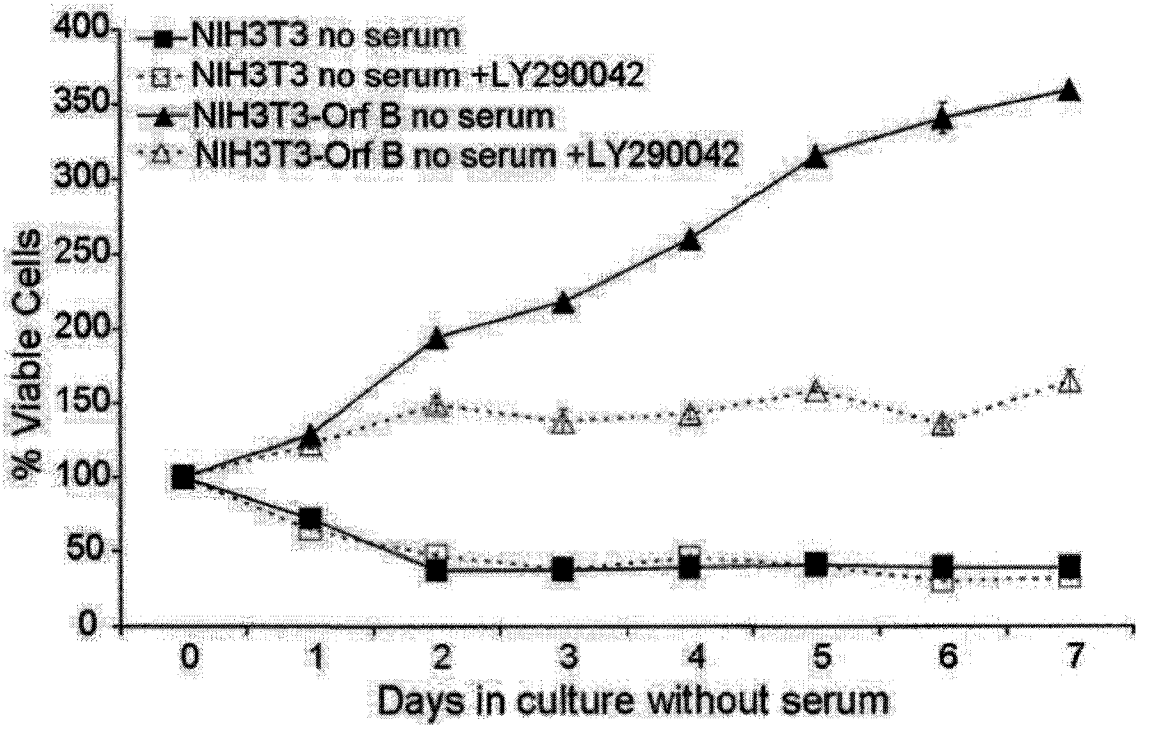

Fig 3.4. Activation of Akt in NIH3T3-Orf B cells promotes cell proliferation. NIH3T3 and NIH3T3-Orf B cells were grown under serum-free conditions in the presence of 5 μM LY294002 or DMSO. Viable cells were measured daily for 7 days by an MTS assay. The mean of six replicate wells was determined for each time point. Viable cells at Day 0 were normalized to 100% viability and the ODs for subsequent days were adjusted accordingly. The data presented in this figure is respresentative of three independent experiments.

Orf B-expressing cells demonstrate properties of transformed cells

Both Akt and PKC are known to be involved in oncogenesis. Activation of Akt has frequently been associated with cell transformation (Hennessy et al., 2005; Testa and Bellacosa, 2001). When allowed to come to confluency and cultured for 25 days, NIH3T3-Orf B cells formed foci characteristic of morphologically transformed cells and were also able to form anchorage-independent, transformed colonies when plated in agarose (Fig. 3.5). To determine if the constitutive activation of PKC or of Akt contributes to this transformed phenotype, we cultured NIH3T3 and NIH3T3-Orf B cells in the presence of the PI3K and mTOR kinase inhibitor, LY294002, or the PKC inhibitor, Bim. In NIH3T3-Orf B cells no significant decrease of the number of transformed foci was observed with Bim treatment (Table 3.1). However, the number of transformed foci in NIH3T3-Orf B cells was significantly decreased in the presence of LY294002 (Table 3.1, $P<0.01$). A limited number of transformed foci developed when the NIH3T3 cells were cultured under the same conditions, however the number of transformed foci in the NIH3T3-Orf B cells was significantly higher (Table 3.1, $P<0.001$). NIH3T3 will undergo spontaneous neoplastic transformation when cultured under contact inhibition conditions (Rubin, 2005). This result implicates the rictor/mTOR kinase or PI3K in the downstream activation of Akt and the maintenance of the Orf B-transformed phenotype.

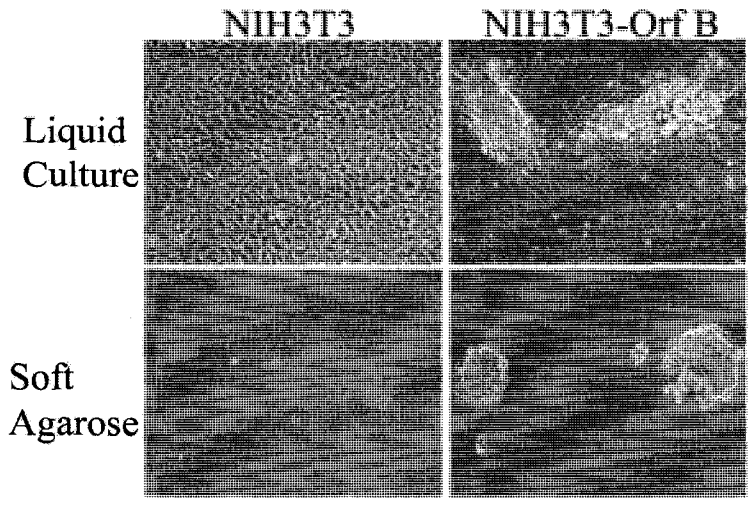

Fig 3.5. Orf B-expressing cells demonstrate properties of transformed cells. Phase contrast images of NIH3T3 control cells and NIH3T3-Orf B cells that were allowed to come to confluency in DMEM with 10% FBS (liquid culture, top panels) or were plated in agarose (bottom panels). Plates were incubated at 37°C for 26 days.

Table 3.1. Effect of PI3K and PKC inhibitors on transformation by NIH3T3-Orf B expressing cells [a]

Cell Type	Number of foci observed		
	DMSO	LY294002	Bim
NIH3T3	11 ± 4	0	9±6
NIH3T3-Orf B	157 ± 17	1 ± 1	141±3

[a]NIH3T3 and NIH3T3-Orf B cells were cultured in 10 cm dishes for 25 days in 10% FBS DMEM treated with vehicle alone (DMSO), 5 μM LY294002, or 20 μM Bim for 25 days. Data represents the average number of foci from duplicate samples ± standard error of the mean for each sample.

Akt is activated in lysates of transformed foci

To confirm the activation of Akt in cells transformed by Orf B expression, the phosphorylation of Akt at S473 and the activation of a down-stream target, GSK-3β, were examined. The activation status of an important negative regulator of Akt, PTEN, was also investigated. Lysates were prepared from isolated foci of transformed NIH3T3-Orf B cells and not from the surrounding monolayer of cells. For comparison, lysates were also prepared from monolayers of NIH3T3 cells and from monolayers of NIH3T3-Orf B cells prior to foci formation and grown in 10% serum. Each preparation was

analyzed by western blot with antibodies that detect PTEN phosphorylation at serine 380, Akt phosphorylation at S473, total Akt, GSK-3β phosphorylation at serine 9, total GSK-3β, and HA-tagged Orf B. The results demonstrate phosphorylation of Akt at S473 in lysates from NIH3T3 and NIH3T3-Orf B cells cultured in the presence of serum as well as in lysates from the transformed foci of NIH3T3-Orf B cells (Fig. 3.6). Lysates from monolayers of NIH3T3 and NIH3T3-Orf B cells demonstrate basal levels of phosphorylated PTEN and GSK-3β. In contrast, phosphorylation of PTEN and GSK-3β were markedly increased in lysates from transformed foci of NIH3T3-Orf B cells (Fig. 3.6). These data indicate that PTEN is inactive, and unable to negatively regulate Akt due to its inhibitory effect on PI3K kinase activity, and that GSK-3β is phosphorylated by active Akt and is itself inactivated and unable to negatively regulate its downstream targets.

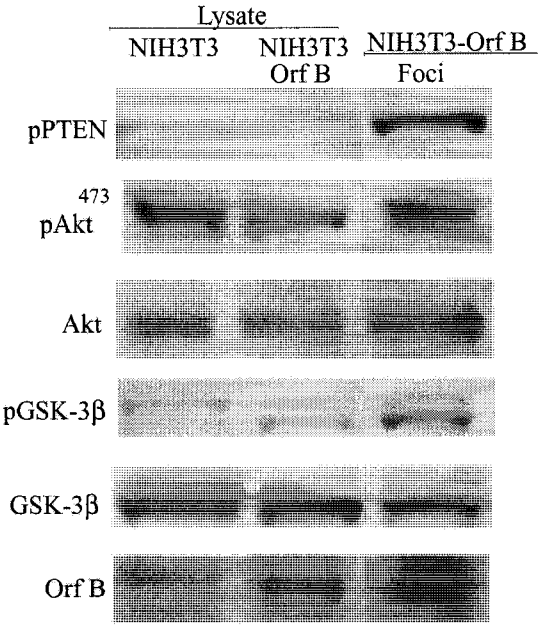

Fig 3.6. Akt is activated in lysates of transformed foci. Transformed foci were collected from NIH3T3-Orf B cells. Lysates from monolayers of NIH3T3 and NIH3T3-Orf B cells and lysates from isolated foci of transformed NIH3T3-Orf B cells were analyzed by western blot with the indicated antibodies: anti-phospho PTEN detects PTEN phospho-serine 380, anti-phospho Akt^{473} detects Akt phospho-serine 473, anti-Akt detects total Akt, anti-phospho GSK-3β detects GSK-3β phospho-serine 9, anti-GSK-3β detects total GSK-3β, and anti-HA detects HA-tagged Orf B.

mTOR is responsible for Akt phosphorylation and activation in Orf B-expressing cells

Akt can be activated by two independent cell signaling pathways, PI3K and rictor/mTOR. Both are inhibited by LY294002. To determine the signaling pathway responsible for the Akt activation associated with Orf B expression, NIH3T3 and NIH3T3-Orf B cells were serum-deprived for 24 h and then either untreated or treated with PDGF to activate Akt via PI3K or with PMA as a negative control. Lysates were subjected to western blot analysis with antibodies detecting Akt phosphorylation at threonine 308, total Akt, and HA-tagged Orf B (Fig. 3.7A). Phosphorylated Akt^{308} was only detected in NIH3T3 and NIH3T3-Orf B cells after treatment with PDGF stimulation of the PI3K cell signaling pathway (Fig. 3.7A). There was no apparent phosphorylation of Akt at T308 in untreated or PMA-treated NIH3T3 and NIH3T3-Orf B cells.

In a similar experiment, NIH3T3 and NIH3T3-Orf B cells were serum-deprived for 24 h, and then treated with DMSO as control, with LY294002 to inhibit mTOR and PI3K, or with PDGF to activate Akt. Cell lysates were subjected to western blot analysis with antibodies specific for phospho Akt^{473}, Akt, and HA-tagged Orf B. Control, DMSO-treated cells were positive for phosphorylation of Akt at S473 only in NIH3T3-Orf B cells and this Akt^{473} phosphorylation was inhibited by treatment with LY294002 (Fig. 3.7B). PDGF treatment stimulated Akt^{473} phosphorylation in NIH3T3 and NIH3T3-Orf B cells. The rictor-mTOR complex directly phosphorylates Akt on S473 and is important in Akt recognition by PDK1, which phosphorylates Akt on T308 (Jacinto et al., 2006; Scheid, Marignani, and Woodgett, 2002). These data indicate that the apparent Akt^{473} phosphorylation is a result of rictor-mTOR activity.

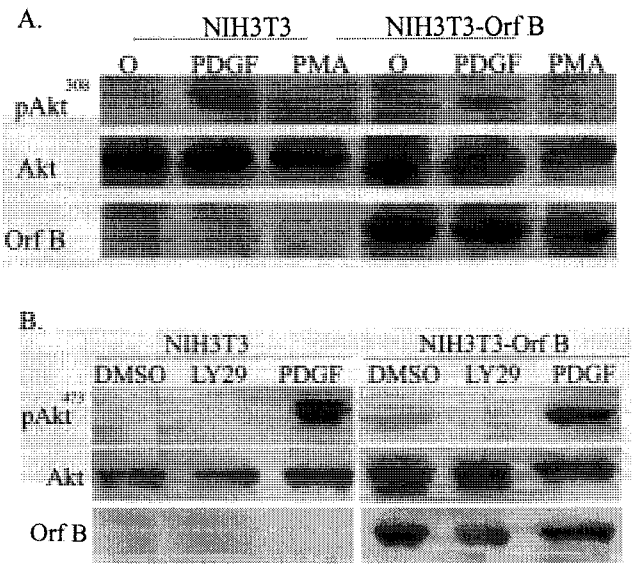

Fig 3.7. LY294002 decreases Akt phosphorylation in Orf B-expressing cells.

A. NIH3T3 and NIH3T3-Orf B cells were serum starved for 24 hrs and then treated with PDGF or PMA. Cell lysates were harvested and analyzed by western blot with the indicated antibodies: anti-pAkt^{308} detects phospho-threonine 308 of Akt, anti-Akt detects total Akt, and anti-HA (12CA5) detects HA-tagged Orf B. B. NIH3T3 and NIH3T3-Orf B cells were serum starved for 24 hrs and then treated with dimethyl sulfoxide (DMSO), LY294002 (LY29), or PDGF. Cell lysates were harvested and analyzed by western blot with the indicated antibodies: anti-pAkt^{473} detects phospho-threonine 473 of Akt, anti-Akt detects total Akt, and anti-HA (12CA5) detects HA-tagged Orf B.

DISCUSSION

The results from these studies identify the capacity of the WDSV Orf B protein to induce a transformed phenotype in murine NIH3T3 cells, and further show that the mechanism of this transformation is the activation of Akt by the rictor-mTOR complex.

NIH3T3-Orf B cells formed foci of morphologically transformed cells as well as anchorage-independent, transformed colonies. We demonstrated that Akt was activated in lysates from transformed foci. Akt is a downstream target of the PI3K cell signaling pathway that is activated by growth factors (Brazil and Hemmings, 2001). Activated PI3K catalyzes the production of PIP3 that triggers translocation of Akt and PDK1 to the plasma membrane. The proximity of Akt to PDK1 at the membrane results in its phosphorylation at T308 by PDK1. The rictor-mTOR (mTORC2) complex is independent of PI3K and phosphorylates Akt at S473, an indicator of activated Akt (Jacinto et al., 2006; Sarbassov et al., 2005; Scheid, Marignani, and Woodgett, 2002). We demonstrated Akt phosphorylation at S473 in serum-deprived and PDGF-stimulated NIH3T3-Orf B cells but not at T308, distinguishing rictor-mTOR not PDK1 in Orf B-mediated activation. Initially T308 phosphorylation was thought necessary for Akt activation and S473 phosphorylation required for full activation (Testa and Bellacosa, 2001). However, recent data indicates phosphorylation of Akt at S473 indicates full activation of Akt (Buchkovich et al., 2008; Sarbassov, Ali, and Sabatini, 2005; Sarbassov et al., 2005). We confirmed Akt activation in lysates of NIH3T3-Orf B transformed foci by demonstrating increased phosphorylation of GSK-3β, a downstream target of Akt. GSK-3β is a serine/threonine kinase that phosphorylates and inactivates important

cellular targets such as glycogen synthase and cyclin D1 (Diehl et al., 1998). Orf B does not have a predicted kinase domain; therefore its role in Akt activation must be due to an association with the rictor-mTOR (mTORC2) complex, a subject for further investigation.

Activation of AKT has been demonstrated in erythroid cells following infection with Friend spleen focus-forming virus, in human T-cell leukemia virus-type I (HTLV-1) transformed cells, and Jaagsietke sheep retrovirus (JSRV) infected cells. (Jeong et al., 2005; Maeda et al., 2005; Maeda et al., 2003; Nishigaki et al., 2000; Palmarini et al., 2001). The JSRV *env* gene was identified as the oncogene capable of inducing transformation in mammalian and avian fibroblast cell lines (Allen et al., 2002; Maeda et al., 2001; Rai et al., 2001). JSRV envelope activates Akt independently of PI3K (Liu, Lerman, and Miller, 2003; Maeda et al., 2003). The Ras-MEK-MAPK and Akt-mTOR signaling pathways were subsequently discovered to be important in JSRV-induced transformation in experiments with inhibitors to MEK, MAPK, PI3K, and mTOR (Maeda et al., 2005). Similarly, the envelope protein of the closely related ovine β-retrovirus enzootic nasal tumor virus (ENTV) functions as an oncogene and the Akt-mTOR signaling pathway has been implicated in its transformation of rodent fibroblast and epithelial cells (Dirks et al., 2002).

Another mechanism that is commonly involved in oncogenesis and inhibited in various tumors/cancers is the PI3K negative regulator, PTEN. This negative regulator of the PI3K/Akt signaling pathway is inactive in its phosphorylated form. PTEN dephosphorylates PIP3, which is important in Akt translocation and activation at the membrane. Loss-of-function mutations in this phosphatase are often associated with

cancer (Li et al., 1997). We demonstrated phosphorylation and loss of PTEN phosphatase activity in transformed foci of NIH3T3 Orf B cells.

These investigations began with the observation of Orf B's ability to protect cells from death. Akt is important in the control of apoptosis. The Bcl-2 family consists of anti-apoptotic and pro-apoptotic proteins that are carefully regulated by cell signaling pathways. One of the pro-apoptotic proteins, BAD, forms a heterodimer with Bcl-2 and Bcl-XL thereby inhibiting their anti-apoptotic function (Datta, Brunet, and Greenberg, 1999; Datta et al., 1997). However, in the presence of survival factors, intracellular signaling pathways are activated resulting in the phosphorylation of BAD and inhibition of its pro-apoptotic activity through interaction with 14-3-3 proteins. Phosphorylated p90RSK was detected in NIH3T3-Orf B cells that were serum-deprived or stimulated with PMA or serum, indicating that p90RSK is activated and capable of BAD phosphorylation at serines 112 and 155, but upstream activators, Erk 1 and 2, were not differentially activated Orf B-expressing cells, suggesting an alternate means of p90RSK phosphorylation and activation. PKC is constitutively activated in Orf B-expressing cells (Daniels, Rovnak, and Quackenbush, 2008b) and is the most likely kinase for p90RSK phosphorylation.

Akt is also important in the control of cell proliferation. The activation of Akt in serum-deprived NIH3T3-Orf B cells suggests it can contribute to Orf B's affects on survival and proliferation. Treatment of serum-deprived cells with LY294002 resulted in decreased proliferation indicating Orf B's effect on proliferation is dependent in part on activated Akt. Similar effects had been observed previously with the PKC-specific inhibitor, Bim, but Bim had no apparent effect on establishment of the transformed

phenotype in Orf B cells, indicating that Akt functions independently of PKC in this process. Recently, the mTORC2 complex was shown to be required for phosphorylation of the turn motif site of PKCα (Facchinetti et al., 2008; Ikenoue et al., 2008). We previously demonstrated that the PKCα turn motif was constitutively phosphorylated in Orf B expressing cells, suggesting that the rictor-mTOR complex may be a common target for the activation of Akt and PKCα. These results support the hypothesis that WDSV Orf B functions as an oncogene in the development of dermal sarcoma, and, its action is through the constitutive activation of cell signaling pathways, PKC and Akt.

MATERIALS AND METHODS

Cells, transfections, and western blot analysis

NIH3T3 cells (ATCC CRL 1658) were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) (Gibco), 4mM glutamine, 100 units penicillin ml^{-1}, and 100μg streptomycin ml^{-1} at 37°C in 5% CO2. CHO cells (CHO-K1, ATCC CCL 61) were maintained in Ham's F12 medium supplemented with 10% FBS. The NIH3T3-Orf B and CHO-B stable cell lines were previously described (Daniels, Rovnak, and Quackenbush, 2008b). Briefly, NIH3T3 cells were transfected with pKH3 or pKH3-Orf B and a neomycin construct (pMC1neo) and selected with G418. WDSV Orf B is expressed as a fusion protein from the pKH3 vector with three influenza virus hemagglutinin (HA) tags onto the amino terminus of the expressed protein (Rovnak, Casey, and Quackenbush, 2001).

NIH3T3 control cells, NIH3T3 cells stably expressing Orf B (NIH3T3-Orf B), CHO control cells, and CHO cells expressing Orf B (CHO-B) were plated in DMEM or Ham's F-12 containing 10% FBS and incubated at 37°C in 5% CO_2. Twenty-fours after plating, cells were transfected with the pEBG-BAD plasmid (Cell Signaling). Twenty-four hours after transfection, the growth medium was replaced with DMEM or Ham's F-12 without serum. Forty-eight hours after culture without serum cells were lysed with immunoprecipitation (IP) buffer (1% Triton X-100, 0.5% NP-40, 150 mM NaCl, 10 mM Tris-HCl [pH 7.5], 1 mM EDTA [pH 8.0], 1 mM EGTA, 0.2 mM sodium orthovanadate, 0.2 mM PMSF, 2 μg/ml of leupeptin and aprotinin, 1 μg/ml pepstatin). Lysates were centrifuged at 21,000 x *g* for 10 minutes at 4°C, and supernatants collected. Protein concentrations were determined with the Micro BCA Kit (Pierce). Fifteen micrograms of

protein were separated under denaturing conditions in a 10% polyacrylamide gel. The proteins were transferred to Immobilon-P-membrane (Millipore) and incubated with primary antibody overnight at 4°C. Blots were washed, incubated with anti-rabbit IgG or anti-mouse IgG antibodies conjugated with horseradish peroxidase, and developed with LumiGLO Chemiluminsescent Substrate Kit (Kirkegaard and Perry Laboratories).

NIH3T3 control cells, NIH3T3 cells stably expressing Orf B (NIH3T3-Orf B), CHO control cells, and CHO cells expressing Orf B (CHO-B) were plated in DMEM or Ham's F-12 containing 10% FBS and incubated at 37°C in 5% CO_2. Twenty-four hours after plating, the growth medium was replaced with DMEM or Ham's F-12 without serum for 24 hours. Cells were then treated with DMSO, 10% FBS, 200 nM phorbol 12-myristate 13-acetate (PMA) or 50ng/ml PDGF. Cells were lysed in IP buffer and processed as above.

The following antibodies were obtained from Cell Signaling, BAD, phospho-pBAD (Ser 112), phospho-pBAD (Ser 136), phospho-pBAD (Ser 155), AKT, phospho-AKT (Ser 473), phospho-AKT (Thr 308), phospho-p90RSK (Ser 380), p44/42 MAP kinase (Erk1/2), phospho-p44/42 Map kinase (Thr 202/Tyr 204) (pERK 1/2), phospho-pTEN (Ser 380), GSK-3β, phospho-GSK-3β (Ser 9). HA-tagged Orf B was detected with mouse monoclonal reactive to HA (12CA5, Roche).

Cell viability assays

NIH3T3 and NIH3T3-Orf B cells were plated at 5.0 x 10^5 cells/ml in 96 well plates containing DMEM with 10% FBS and incubated at 37°C in 5% CO2. After 24 hours of culture, cells were washed with PBS then further cultured in DMEM without

serum. LY294002 was added daily to maintain a final concentration of 5 μM. Cell viability was measured using CellTiter 96 AQueous One Solution (Promega) according to manufacturer's instructions. Briefly, 20 μl of CellTiter 96 solution was added to each well, incubated for 3 hours at 37°C, and then OD readings were taken at 490 nm. Total cell input at day 0 was normalized to a value of 100% viability and adjusted accordingly each day thereafter for 7 days. Student's t test and 95% confidence intervals based on a t distribution were used for statistical analyses. A P value of less than 0.05 was considered significant.

Transformation assays

NIH3T3 control and NIH3T3-Orf B expressing cells were seeded in replicate at 5 x 10^5 cells per 10-cm dishes in DMEM and 10% FBS and grown at 37°C in 5% CO_2. Cells were treated with 5 μM LY294002 (Calbiochem), 20μM Bisindolymaleide I hydrochloride (Alexis Biochemicals) or vehicle alone (DMSO) and cultured with daily media changes for 21 days followed by media changes containing inhibitor every 3 days thereafter. The numbers of transformed foci were counted under phase contrast microscopy.

For anchorage independence assays, 5 x 10^4 NIH3T3 and NIH3T3-Orf B cells were suspended in 5 ml of 0.66% agarose in DMEM with 10% FBS. The cell suspensions were plated on top of a 1.4% agarose layer in DMEM with 10% FBS and grown at 37°C in 5% CO2.

Acknowledgements

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CHAPTER 4

FURTHER CHARACTERIZATION OF ORF B FUNCTION

WDSV *orf b* is one of two transcripts identified in developing tumors and is thought to be important in the development of WDS. Results from my studies demonstrated that Orf B-expressing cells were protected from staurosporine-induced apoptosis (Daniels, Rovnak, and Quackenbush, 2008b). In addition, expression of Orf B induced transformation of NIH3T3 cells. The cellular proteins PKCα and RACK1 interacted in a complex and directly with Orf B, respectively, which resulted in constitutive activation of PKCα in Orf B-expressing cells. In addition, p90RSK and Akt are activated in Orf B-expressing cells, suggesting their activation promotes cell survival and/or transformation. This chapter further examines the direct interaction of Orf B and RACK1 and the mechanism for protection from apoptosis and re-examines the functional consequence of constitutive PKCα activation in Orf B-expressing cells.

Bcl-2 pro- and anti-apoptotic mRNA levels are upregulated in Orf B-expressing cells

Orf B-expressing cells remain viable after treatment with increasing concentrations of staurosporine (Daniels, Rovnak, and Quackenbush, 2008b). I began the investigation into the mechanisms by which Orf B inhibits apoptosis by quantifying mRNA expression levels of Bcl-2 family members. Examples of Bcl-2 family of proteins

include pro-apoptotic proteins Bax and Bad and anti-apoptotic proteins Bcl-2 and Bcl-XL. The quantitative relationship of pro- versus anti-apoptotic Bcl-2 family members in the cell determines cell survival or death (W. Wei-Lynn Wong, 2008); this balance is regulated at the transcriptional and posttranslational level. A multi-probe ribonuclease protection assay (RPA) was used to quantify mRNA levels of apoptosis-related genes in Orf B-expressing cells. Probes were synthesized from a human apoptosis multi-probe template set (Pharmingen) using T7 RNA polymerase and labeled with α ^{32}P dUTP. These radio-labeled probes were then hybridized to mRNA isolated from cells that stably expressed Orf B: canine fibroblast (Cf2Th-Orf B) and mouse fibroblast (NIH3T3-Orf B) or human epithelial cells transiently transfected with an Orf B expression vector (HeLa-Orf B). Control cells for each cell line were transfected with an empty vector. The appropriately sized, protected fragment identified each apoptosis-related gene and densitometry quantified their expression levels. Results demonstrated increased levels of anti-apoptotic genes *mcl-1, bcl2, bcl-XL, bcl-w* and the pro-apoptotic gene *bax* in Orf B-expressing cells (Fig 4.1A and B). The pro-apoptotic gene, *bad,* was not quantified in HeLa and HeLa-Orf B cells; however there appear to be relatively equal levels of the *bad* transcript (Fig. 4.1A).

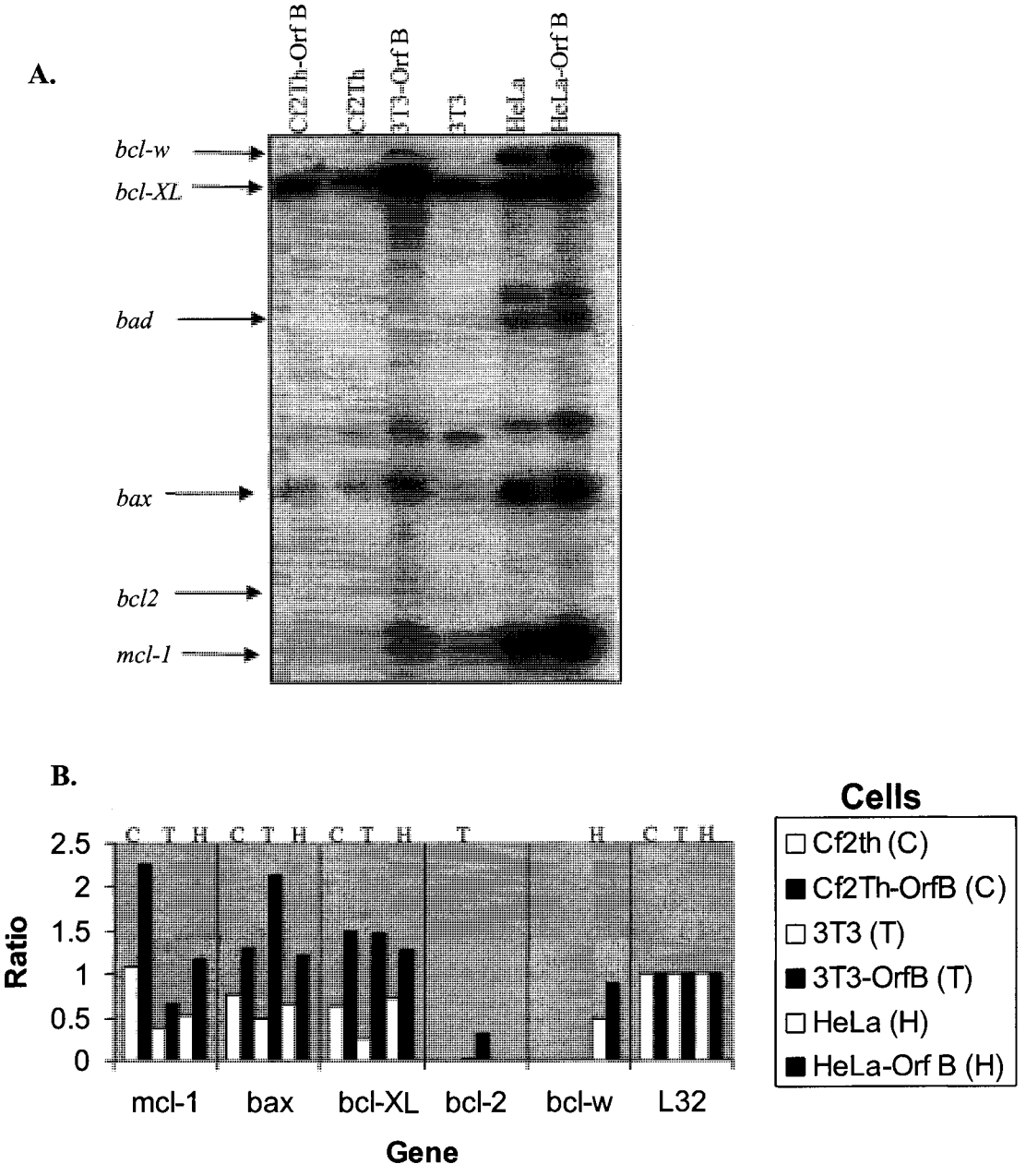

Fig 4.1 Ribonuclease Protection Assay (RPA). A. Autoradiograph of protected gene transcripts separated on a denaturing polyacrylamide gel. B. Densitometer readings normalized against the housekeeping L32 control gene.

Because the probes were synthesized from a human apoptosis multi-probe template set, better cross-reactivity and protection of hybridized genes was observed in HeLa and HeLa-Orf B samples (Fig 4.1A). A mouse apoptosis multi-probe template set also protected transcripts from NIH3T3 and NIH3T3-Orf B samples. However, results were unclear and unable to be quantified (data not shown). A multitude of cellular signals can regulate the quantity of pro- and anti-apoptotic transcripts. These results did not clearly identify increased expression of a specific apoptotic or anti-apoptotic gene or genes that might explain the protection of apoptosis in Orf B-expressing cells.

Staurosporine reduces BAD phosphorylation and promotes its pro-apoptotic activity (Tafani et al., 2001). The results demonstrated relatively equal levels of protected *bad* transcripts in HeLa and HeLa-Orf B samples (Fig 4.1A). No distinguishable bands were visible in each of the other cell types probably due to sequence variation of these transcripts between human, canine and murine species (Fig 4.1A). As an alternative to analyzing Bcl-2 family mRNA levels, we investigated the phosphorylation status of BAD in Orf B-expressing cells and showed that BAD is constitutively phosphorylated (Chapter 3), indicating a mechanism for the protection from staurosporine-induced apoptosis.

Web-based software prediction program, SOSUI, predicts Orf B is a transmembrane protein

Results from immunofluorescence assays demonstrated Orf B localization in the cytoplasm and the plasma membrane in walleye explanted tumor cells and in cells that stably express Orf B (Daniels, Rovnak, and Quackenbush, 2008a; Rovnak et al., 2007).

The localization of Orf B at the membrane and its association with membrane proteins (PKC, Akt, Src, and PI3K) suggests Orf B is a membrane associated protein. Sequence analysis does not indicate that Orf B contains a predicted signal sequence that would target it to the membrane; however, other possibilities exist to explain how Orf B associates with the membrane. Membrane proteins are characterized by the existence of long hydrophobic transmembrane helices. There are two types of membrane proteins: integral and peripheral. Integral membrane proteins are also known as transmembrane proteins because they span the entire lipid bilayer one or several times. For instance, the protein found in photosynthetic bacteria, bacteriorhodopsin contains seven, membrane-spanning alpha helices (Lodish et al., 2000). Orf B was analyzed by various web-based protein structure prediction applications to determine if Orf B contains a potential membrane-spanning region or transmembrane domain. One such prediction application, SOSUI, predicts transmembrane helices for membrane proteins. The accuracy of the SOSUI system for classifying membrane proteins is 99%, and the corresponding value for transmembrane helix prediction is 97% (Hirokawa, Boon-Chieng, and Mitaku, 1998). Results from SOSUI sequence analysis of Orf B predicted a membrane protein with four transmembrane helices (Hirokawa, Boon-Chieng, and Mitaku, 1998) (Fig 4.2). Orf B is predicted to have a primary transmembrane helix (Fig 4.2, amino acids 215-236) that is capable of penetrating into membranes and three secondary transmembrane helices (Fig 4.2, amino acids 98-120, 126-148, and 167-189), which are stabilized by the interaction with other transmembrane segments. These Orf B predicted transmembrane helices remain to be tested.

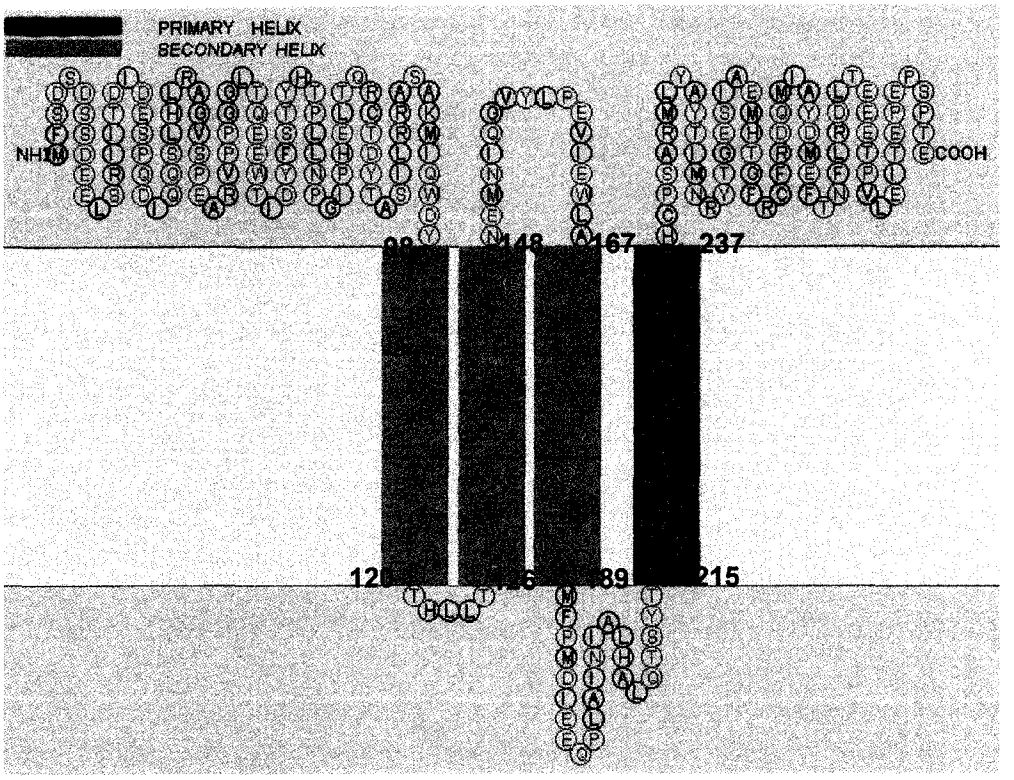

Fig. 4.2 Predicted transmembrane regions within Orf B. SOSUI generated model identified four transmembrane helices: One primary (far right, dark green) and three secondary helices (light green) with the entry and exit amino acids of each helix indicated in black numbers (Hirokawa, Boon-Chieng, and Mitaku, 1998). The WDSV Orf B amino acids 1-306 are circled and indicated as follows: hydrophobic residues are black, polar residues are blue, and negatively charged residues are red.

Amino acids 134-198 of Orf B interact with RACK1

A yeast-two hybrid assay demonstrated a direct interaction of Orf B with RACK1 (Daniels, Rovnak, and Quackenbush, 2008b). Results from an IP experiment with the walleye orthologue of human RACK1 confirmed its interaction with Orf B (Daniels, Rovnak, and Quackenbush, 2008b). To identify the interacting regions of Orf B and walleye RACK1 (wRACK1), additional yeast-two hybrid experiments were performed. WDSV Orf B deletion mutants, generated by PCR, were fused to the GAL4 DNA binding domain vector, pAS2-1, while the GAL4 activation domain vector, pACT2, contained the entire wRACK1 open reading frame. *Saccharomyces cerevisiae* (Y187), harboring the *lacZ* reporter gene, were transformed with individual pAS2-1-Orf B deletion mutants and pACT2-wRACK1 and then tested for protein-protein interactions. Positive yeast transformants were screened for β-galactosidase activity by a colony-lift filter assay. Results identified amino acids 134-198 as the minimal region tested within Orf B to interact with wRACK1 (Fig. 4. 3, Orf BΔ6). In contrast, Orf B deletion mutants 7 and 8 (Orf BΔ7 and 8), the only constructs tested that do not contain amino acids 134-198, did not interact with wRACK1 (Fig. 4.3). Surprisingly, Orf BΔ1 (amino acids 1-256) did not interact with wRACK1 as expected. It is possible a problem existed with Orf B expression in yeast and thus an interaction between Orf B and wRACK1 was not detected; this possibility was not tested.

Posttranslational modifications such as phosphorylation are important in the regulation of protein interactions and biological controls. Orf B has three predicted PKC phosphorylation motifs (P) (Fig. 4.3). These predicted PKC sites on Orf B do not seem to be important in the interaction with wRACK1 since they are not contained within

amino acids 134-198. Orf B directly interacts with RACK1 and I would not expect PKC phosphorylation sites to be important in the interaction of Orf B with RACK1. A direct interaction of PKC with Orf B has not been tested and instead the association of Orf B with PKC is perhaps due to its direct interaction with RACK1, which remains to be determined.

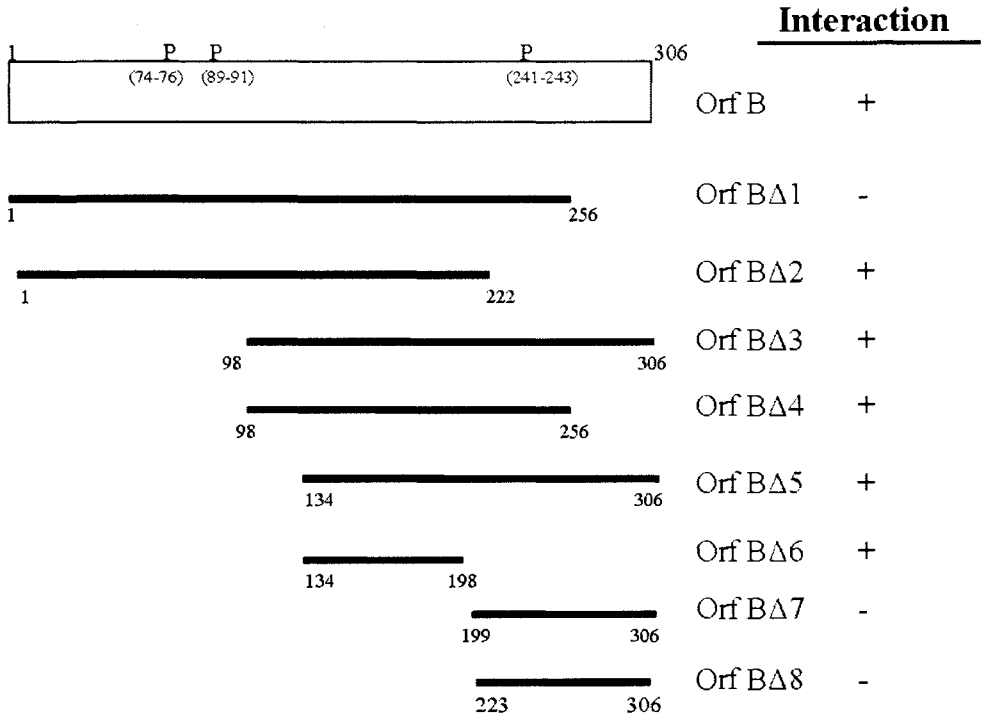

Fig 4.3 The yeast two hybrid system identified the regions of interaction between Orf B and walleye RACK1. The box illustrates WDSV Orf B amino acids 1-306 and predicted PKC motifs (P). Solid black lines illustrate Orf B deletion mutants (Orf B Δ1-8) used to test for an interaction with walleye RACK1. A positive (+) or negative (-) protein-protein interaction, determined by expression of the *lacZ* reporter gene, is indicated on right side of figure.

Orf B associates with PI3K

Orf B is found in membrane fractions with activated PKC and RACK1 (Daniels, Rovnak, and Quackenbush, 2008b). Furthermore, PKC and Akt are constitutively activated in Orf B-expressing cells (Daniels, Rovnak, and Quackenbush, 2008a; Daniels, Rovnak, and Quackenbush, 2008b). PI3K is a known regulator of Akt activation, therefore I investigated the possibility that Orf B associates with PI3K. Cell lysates from NIH3T3 and NIH3T3-Orf B cells were subjected to IP with anti-PI3K p85 antibody or normal rabbit serum followed by western blot analysis with anti-HA antibody. Figure 4.4 demonstrates co-immunoprecipitation of Orf B with PI3K from NIH3T3-Orf B cells. The PI3K-PDK1 pathway results in phosphorylation of Akt at T308. However, we were unable to detect phosphorylation of Akt at T308 in Orf B-expressing cells (Chapter 3). It is possible that the interaction of Orf B with PI3K functions to negatively regulate the PI3K-PDK1 pathway, but this remains to be tested.

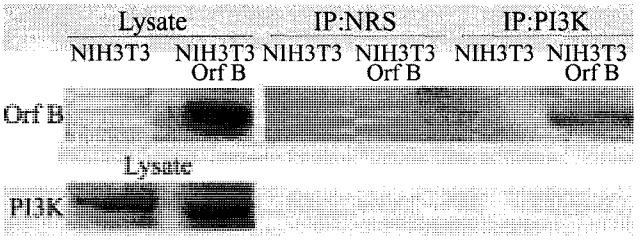

Fig. 4.4 Orf B co-immunoprecipitates with PI3K. Lysates from NIH3T3 and NIH3T3 Orf B cells were immunoprecipitated with rabbit PI3K p85 antibody or normal rabbit serum (NRS). Anti-HA (12CA5) MAb (top, Orf B) detected HA-tagged Orf B and anti-PI3K, detects total PI3K, (bottom, PI3K). Lysate represents 4% of total amount used for immunoprecipitation.

Orf B associates with the serine/threonine protein phosphatase 2A (PP2A)

Co-immunoprecipitation had previously demonstrated an association of Orf B with PP2A. The composition of the Orf B-PP2A complex was determined with antibodies that recognize different PP2A subunit configurations: free A subunit (MAb 6G3), core enzyme (A&C subunits; MAb 5H4), and holoenzyme (A, B, & C subunits; MAb 6F9). Cell lysates from NIH3T3 and NIH3T3-Orf B were subjected to immunoprecipitation with anti-6G3, anti-5H4, and anti-6F9 followed by western blot analysis with anti-HA antibody. Orf B co-immunoprecipitated with the free subunit of PP2A, the core enzyme, and the holoenzyme (Fig. 4.5).

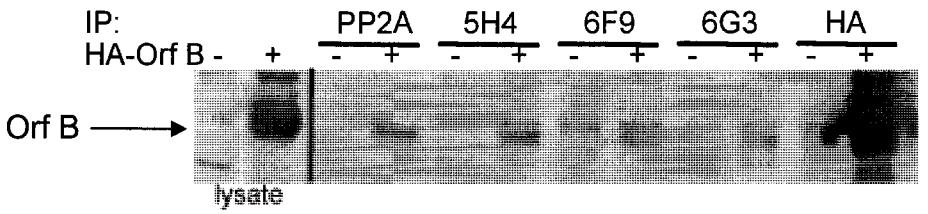

Fig. 4.5 Orf B associates with all three forms of PP2A. Co-immunoprecipitation of HA-tagged Orf B with polyclonal anti-PP2A or with monoclonal antibodies that distinguish PP2A core enzyme (5H4), holoenzyme (6F9), and free A subunit, PR65 (6G3).

The results from this experiment characterize the interaction of OrfB and PP2A. PP2A reverses the action of kinases in several major signaling cascades through its phosphatase activity. Deregulation of the PP2A holoenzyme by Orf B may result in overall increases in the activation of cell signaling components and/or prolonged phosphorylation of substrates, such as BAD. BAD is regulated by reversible phosphorylation (Zha et al., 1996). Perhaps, the functional consequence of an Orf B association with PP2A is the deregulation of BAD pro-apoptotic activity. This remains to be determined. Similarly, Akt is regulated by the phosphatase activity of PP2A. Thus, the constitutive phosphorylation of Akt in Orf B-expressing cells may be due to deregulation by PP2A.

PP2A is also known as a tumor suppressor for its role in regulating the phosphorylation state of specific substrates that suppress cell transformation. Tumor-promoting viral proteins such as polyoma small and middle T antigens (pyST and pyMT, respectively) displace B regulatory subunits from the core enzyme resulting in PP2a

inhibition and activation of cellular pathways (Pallas et al., 1990; Rodriguez-Viciana, Collins, and Fried, 2006). Orf B associates with the PP2A free A subunit, core enzyme, and holoenzyme (Fig 4.5). The possibility of Orf B displacing or altering normal substrate specificity by the B subunit has not been tested.

Orf B associates with c-Src

RACK1 is a substrate and inhibitor of c-Src activity and NIH3T3 cell growth (Chang, Chiang, and Cartwright, 2001; Chang et al., 1998; Chang, Harte, and Cartwright, 2002). In addition, RACK1 partially reverses anchorage and serum-independent growth of v-Src-transformed cells, identifying RACK1 as an endogenous inhibitor of oncogenic Src tyrosine activity (Mamidipudi et al., 2004a). Orf B directly interacts with RACK1 and is localized to structures resembling focal adhesions, actin stress fibers, and membrane ruffling in explanted tumor cells (Daniels, Rovnak, and Quackenbush, 2008b). The Src family of proteins and focal adhesion kinase (FAK) are important components in cell adhesion, shape, motility and division. Therefore, an interaction of Orf B with Src was investigated. Cell lysates from NIH3T3 and NIH3T3-Orf B cells were subjected to immunoprecipitation with anti-Src antibody followed by western blot analysis with anti-HA antibody. Figure 4.6 shows that anti-Src antibody immunoprecipitated Orf B from NIH3T3-Orf B cells. Interaction of Orf B with Src may be important in the regulation of cell adhesion and motility and contribute to the localization of Orf B in focal adhesions as seen in explanted tumor cells. Alternatively, the interaction of Orf B with Src and RACK1 could result in the inhibition of Src activity; these remain to be determined.

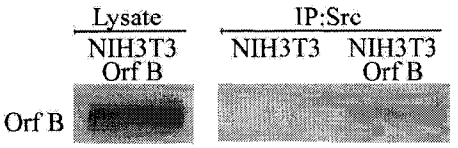

Fig. 4.6 Orf B associates with Src. Rabbit anti-Src antibody immunoprecipitated Orf B from lysates of NIH3T3-Orf B cells. Anti-HA (12CA5) MAb detected HA-tagged Orf B. Lysate represents 4% of total amount used for immunoprecipitation.

Inhibition of PKC in Orf B-expressing cells does not result in cell death

The interaction of Orf B with RACK1 and PKCα and the constitutive activation of PKC promoted cell survival, proliferation, and viability of serum-deprived Orf B-expressing cells (Daniels, Rovnak, and Quackenbush, 2008b). Bim, a PKC inhibitor, reduced the levels of activated PKC at the membrane and significantly diminished the growth of serum-deprived NIH3T3-Orf B cells (Daniels, Rovnak, and Quackenbush, 2008b). To more precisely evaluate the role of PKCα in survival and proliferation, PKCα was silenced in NIH3T3-Orf B cells. Lentiviral particles containing shRNA to PKCα were used to transduce NIH3T3-Orf B cells (Mission shRNA Constructs, Sigma-Aldrich). Cells were selected with 5 μg/ml puromycin for 7 days. Lysates from NIH3T3-Orf B cells that were transduced with individual shRNAs from four different PKCα shRNA constructs were subjected to western blot analysis with antibody to PKCα to detect total PKCα (Fig. 4.7, top 74-76, 78). NIH3T3-Orf B cells transduced with shRNA demonstrated a decrease in expression of PKCα (Fig 4.7). PKCα shRNA-74 was most effective in knocking down PKCα while PKCα shRNA-75 and 76 greatly reduced

PKCα expression (Fig 4.7) PKCα shRNA-78 also reduced levels of PKCα (Fig. 4.7). HA-tagged Orf B was detected with anti-HA (12CA5) antibody (Fig. 4.7, bottom).

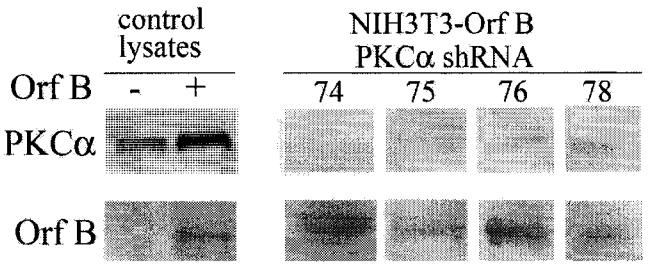

Fig 4.7. PKCα expression was reduced in NIH3T3-Orf B cells using the Mission® shRNA lentiviral packaging system. Lentiviral particles containing four different shRNA PKCα constructs (74-76, and 78) were used to transduce NIH3T3-Orf B cells. Lysates from transduced Orf B cells were harvested and analyzed by western blot with anti-PKCα antibody, which detects total PKCα (74-76 and 78, top). HA-tagged Orf B was detected with anti-HA (12CA5) MAb (bottom).

Since PKCα is constitutively activated in Orf B-expressing cells and contributes to cell proliferation and survival, I investigated more precisely the effect of PKCα in NIH3T3-Orf B cells. Cell growth was measured under serum-deprived conditions for NIH3T3-Orf B cells with reduced expression of PKCα (shRNA constructs 74, 75, 76 and 78), NIH3T3-Orf B cells (positive control), and NIH3T3 cells (negative control). Each cell type was plated at 5.0 x 10^5 cells/ml in DMEM containing 10% FBS for 24 hours, washed with PBS, and then cultured in media without serum. An MTS assay measured cell growth daily for 5 days. Total cell input at day 0, when serum was removed, was normalized to a value of 100% viability and adjusted accordingly each day thereafter. As expected, the growth of NIH3T3 cells declined through day 5 (Fig. 4.8). In contrast, NIH3T3-Orf B cells and all NIH3T3-Orf B cells with reduced expression of PKCα proliferated through five days of culture (Fig. 4.8). NIH3T3-Orf B-74, which had the best reduction of PKCα proliferated through day 3 without serum, during which time 1.7 cell doublings occurred (Fig. 4.8). A slight decrease in viability was observed from days 4 to 5 in NIH3T3-Orf B cells and in all NIH3T3-Orf B cells with reduced expression of PKCα, which was likely due to the confluency of cells after four days of cell growth (Fig 4.8).

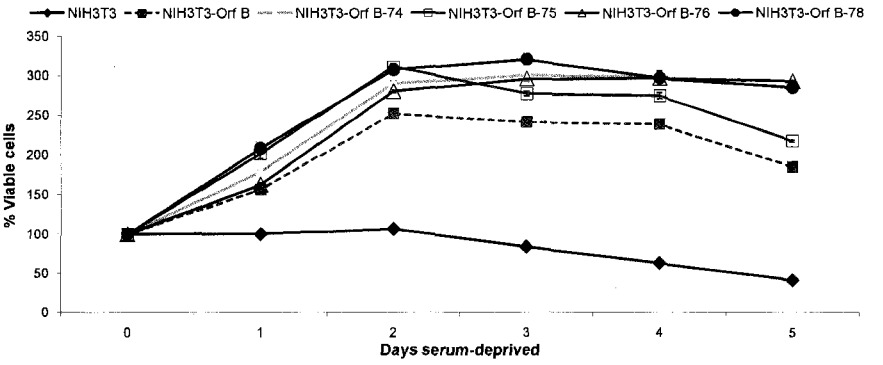

Fig. 4.8 NIH3T3-Orf B cells with reduced expression of PKCα proliferate in the absence of serum. NIH3T3, NIH3T3-Orf B, and NIH3T3-Orf B cells with reduced expression of PKCα (NIH3T3-Orf B 74-76, 78) were grown under serum-deprived conditions and cell viability was measured daily for 5 days with an MTS assay. Viable cells at day 0 were normalized to 100% and ODs from subsequent days were adjusted accordingly. The mean of six replicate wells was determined for each time point.

The results from this experiment differed from our previous experiment, which demonstrated that Bim inhibition of PKC activity significantly reduced cell proliferation of NIH3T3-Orf B cells (Daniels, Rovnak, and Quackenbush, 2008b). A possible explanation for this observed difference could be due to non-specific activity of Bim. Bim inhibits PKC activity exclusively via the ATP binding site; therefore it is possible that Bim inhibits other enzymes that use ATP as a substrate, such as cAMP-dependent protein kinase (PKA) (Toullec et al., 1991).

By Day 2, all the NIH3T3-Orf B cells with reduced expression of PKCα proliferated significantly better than NIH3T3-Orf B control cells ($p<0.001$, Fig 4.8), suggesting the proliferation of Orf B-expressing cells under serum-starved conditions is not dependent on PKC activity. In addition, these results even suggest that functional PKCα inhibits the proliferation of Orf B-expressing cells. Perhaps the lack of PKCα kinase activity is compensated by an increase in the activity of other important enzymes such as Src, PI3K, or Akt kinases and/or phosphatases such as PP2A. This experiment was performed only once, therefore additional experiments are needed to confirm these results.

The four individual shRNA PKCα constructs used to silence PKCα in NIH3T3-Orf B cells did not silence PKCα expression (Figure 4.7). My conclusions assessing the effect of reduced PKCα in Orf B-expressing cells assume the remaining PKCα that is still expressed is not sufficient for normal PKC function.

NIH3T3 cells are unable to survive in serum-deprived conditions; therefore the assessment of silenced PKCα could not be tested under these conditions. Instead, cell growth of silenced PKCα in NIH3T3 cells would need to tested under cell culture conditions with 10% FBS. I would predict PKCα knockdown in NIH3T3 cells would not cause proliferation. Only NIH3T3-Orf B cells contain constitutive activation of other important kinases such as Akt that could potentially compensate for PKCα knockdown; NIH3T3 cells do not have this functional alternative, therefore would not be able to proliferate.

CHAPTER 5

CONCLUSIONS

The results from these studies identify several proteins that are associated with WDSV Orf B: a direct interaction with RACK1 and the presence in a complex with PKC, PI3K, Src, and PP2A. In addition, results from these studies demonstrate that BAD is phosphorylated suggesting a mechanism of action for the protection from apoptosis in Orf B-expressing cells. PKCα and Akt are constitutively activated in Orf B-expressing cells resulting in increased cell proliferation when cultured under serum deprived conditions. Initial experiments with PKC inhibitors indicated activated PKCα is important for cell growth of NIH3T3-Orf B cells (Daniels, Rovnak, and Quackenbush, 2008b). However, a more precise experiment with diminished levels of PKCα demonstrated PKCα is not necessary for cell growth in NIH3T3-Orf B cells (Chapter 4). Results from Chapter 3 demonstrated that expression of Orf B induced transformation of NIH3T3 cells in vitro. Experiments using a PI3K and mTOR inhibitor prevented the transformed phenotype in NIH3T3-Orf B cells suggesting PI3K or mTOR as mechanisms of action for transformation. Results demonstrated Akt was not phosporylated at T308 indicating the PI3K-Akt cell signaling pathway is not activated and instead suggests the mTOR signaling pathway as the mechanism of action for the transformed phenotype in NIH3T3-Orf B cells.

The Orf B and wRACK1 interaction

Results from a yeast two-hybrid experiment identified Orf B amino acids 134-198 as the smallest region tested that directly interacts with walleye RACK1 (Chapter 4). A portion of these amino acids are predicted to lie within the membrane by a web-based prediction program, SOSUI (Chapter 4, Fig 4.8, amino acids 134-148 and 167-189). However, amino acids 149-166 and 190-198 are located outside of the predicted transmembrane helices, suggesting these regions are critical for a direct interaction with wRACK1 (Fig 4.8).

Additional experiments are needed to establish Orf B as a transmembrane protein

The SOSUI system only predicts transmembrane regions and does not make any predictions on topology or subcellular localization. Therefore, it is not known if Orf B associates with the plasma membrane or a subcellular membrane. Results from immunofluorescence assays demonstrated Orf B localization in the cytoplasm and the plasma membrane in walleye explanted tumor cells and in cells that stably express Orf B (Daniels, Rovnak, and Quackenbush, 2008a; Rovnak et al., 2007). In addition, Orf B associates with cellular proteins associated with the plasma membrane such as Src. I predict Orf B directly associates with the plasma membrane via its predicted primary transmembrane helix. Furthermore, alternate mechanisms exist for direct insertion in to the plasma membrane. Integral proteins are also associated with the membrane by lipid-anchors. These proteins do not interact directly with the hyrdrophobic core of the lipid bilayer, instead they anchor to the cytosolic face of membranes by a hydrocarbon moiety (prenyl and farnesyl groups) covalently attached to a cysteine near the C-terminus

(Lodish et al., 2000). Fatty acyl palmitate groups provide additional anchors to the membrane by forming thioester bonds to nearby cysteine residues or by forming an amide bond to the N-terminal glycine residue (Lodish et al., 2000). For instance, v-Src interacts with the membrane by a myristate fatty acyl group linked to the N-terminal glycine residue. Palmitolyation enhances surface hydrophobicity and membrane affinity of proteins and is important in protein trafficking, stability, and sorting (Draper, Xia, and Smith, 2007; Linder and Deschenes, 2007). Ras is linked to the membrane by a palmitate group attached to a C-terminal cysteine residue (Lodish et al., 2000). Web-based prediction programs for post-translational modifications did not identify N-terminal myristate residues or other anchors such as prenyl, farnesyl, and geranylgeranyl groups. However, one program that predicts palmitolyation sites using a clustering and scoring strategy, CSS-Palm, identified two predicted sites within Orf B for palmitolylation (CXXC): amino acids 236 and 239 (Ren et al., 2008) In order to determine if the predicted palmitolyation sites are important for membrane attachment, I would change one of the cytsteines in the predicted palmitolyation motif a serine (i.e. CXXC→SXXC) by point mutation, then analyze Orf B expression by IFA to determine if the mutations affected Orf B localization.

Peripheral proteins indirectly bind the membrane by interacting with integral membrane proteins or by directly binding to the polar head group of membrane phospholipids (Lodish et al., 2000). For example, PKC is a peripheral protein and localizes to the cytosolic face of the plasma membrane by binding to PIP3. The association Orf B with membrane proteins such as PKC and PI3K may provide a mechanism for Orf B's attachment to the membrane.

Functional consequence of Orf B interacting with proteins from different cell signaling pathways

Viral transforming proteins that activate cell signaling pathways result in their constitutive activation, which promotes cell survival and proliferation. Transduced cellular genes of transforming retroviruses are classical examples of viral proteins capable of altering cell signaling pathways. For instance, Rous sarcoma virus protein product, v-Src mimics cell signaling molecules by possessing protein tyrosine kinase activity. Src harbors two domains that mediate protein-protein interactions important in signaling from receptors such as receptor tyrosine kinases and integrins: SH2 binds to phosphotyrosine-containing residues and SH3 binds to proline-rich sequences (Abram and Courtneidge, 2000; Giancotti and Ruoshlahti, 1999; van der Geer, Hunter, and Lindberg, 1994).

Several other viruses encode proteins that function to activate signal transduction pathways. For instance, expression of Epstein-Barr virus (EBV) latent proteins contributes to EBV properties of transformation (Bornkamm and Hammerschmidt, 2001). LMP1, the major transforming protein of EBV, promotes cell proliferation by activating the cellular transcriptional regulator NF-κB and the PI3K-Akt pathway (39, 54). Jaagsiekte sheep retrovirus (JSRV) envelope (env) protein is a viral oncogene that induces transformation of rodent and chicken fibroblasts and rodent epithelial cells in culture (Allen et al., 2002; Maeda et al., 2001; Rai et al., 2001). Initial studies demonstrated the mechanism of action for JSRV-induced transformation is through the activation of PI3K (Ikenoue et al., 2008; Palmarini et al., 2001). Additional studies demonstrated Akt is phosphorylated in JSRV transformed cells and the PI3K inhibitor,

LY294002 reduced phosphorylation of Akt at serine 473, concluding that Akt activation was PI3K-dependent (Alberti et al., 2002). Subsequent studies utilizing JSRV-transformed PI3K-negative NIH3T3 cells determined Akt was activated, indicating transformation by JSRV envelope induces PI3K-independent activation of Akt (Maeda et al., 2003). LY294002 also inhibits the mTOR kinase (Brunn et al., 1996) and likely inhibited the mTOR kinase resulting in PI3K-independent Akt activation. mTOR is a serine/threonine kinase that forms two different protein complexes: mTORC1 consists of mTOR, GβL, and raptor; mTORC2 consists of mTOR, GβL, SIN1, and rictor. mTORC1 and 2 function differently; mTORC1 is activated by Akt and controls cap-dependent translation; mTORC2 phosphorylates Akt at serine 473 and its activation is not completely understood.

Similarly, the envelope protein of the closely related ovine β-retrovirus enzootic nasal tumor virus (ENTV) is 88.8% identical at the nucleotide level to JSRV envelope, functions as an oncogene, and transforms cells in vivo and in vitro (Dirks et al., 2002). The PI3K-Akt pathway is also implicated in ENTV-1 transformation (Alberti et al., 2002; Liu, Lerman, and Miller, 2003). Additional studies extend the involvement of PI3K-Akt to PI3K-Akt-mTOR pathway as rapamcyin, an mTOR-raptor complex inhibitor, inhibited ENTV transformation in rat kidney epithelial cells (Maeda and Fan, 2008).

Overall, the mechanism of transformation for Orf B in NIH3T3 cells is not completely understood. The complexity of signal transduction pathways and their crosstalk adds to the difficulty of determining the mechanism of action for Orf B-induced oncogenesis. It is likely different pools of Orf B interact with discrete sets of cellular proteins and thus function at multiple locations within the cell. Figure 5.1 illustrates the

known interactions of Orf B with other signaling proteins. Orf B directly interacts with RACK1 and associates with PKCα. The interaction with the scaffolding protein RACK1 is of particular interest since most protein-protein interactions with RACK1 are directed towards one or more WD repeats. The seven WD repeats in RACK1 form a propellar-like structure that binds activated PKC and proteins with PH domains concurrently (Rodriguez et al., 1999). Experiments by yeast-two hybrid identified the region through which Orf B interacts with wRACK1 (Chapter 4). Further experiments are necessary, utilizing a different approach such as pull-down assays with purified proteins, to identify the WD repeat motif in walleye RACK1 that binds directly to Orf B. Purified proteins, would allow testing of walleye RACK1 binding to Orf B and additional proteins simultaneously. The potential of Orf B associating with PKC and RACK1 in the same complex may alter the localization and/or function of PKC.

Orf B associates with PP2A, but it remains unknown if this association is independent of RACK1. PP2A regulates the activity of a vast array of proteins. Two proteins that are regulated by PP2A dephosphorylation are BAD and Akt. PP2A dephosphorylates BAD at serine 112 and Akt at threonine 308, resulting in the activation of BADs pro-apoptotic activity and inhibition of Akt, respectively(Chiang et al., 2001; Kuo et al., 2008). Orf B could promote cell survival and proliferation by inhibiting the phosphatase activity of PP2A; this would result in constitutive phosphorylation of BAD and Akt.

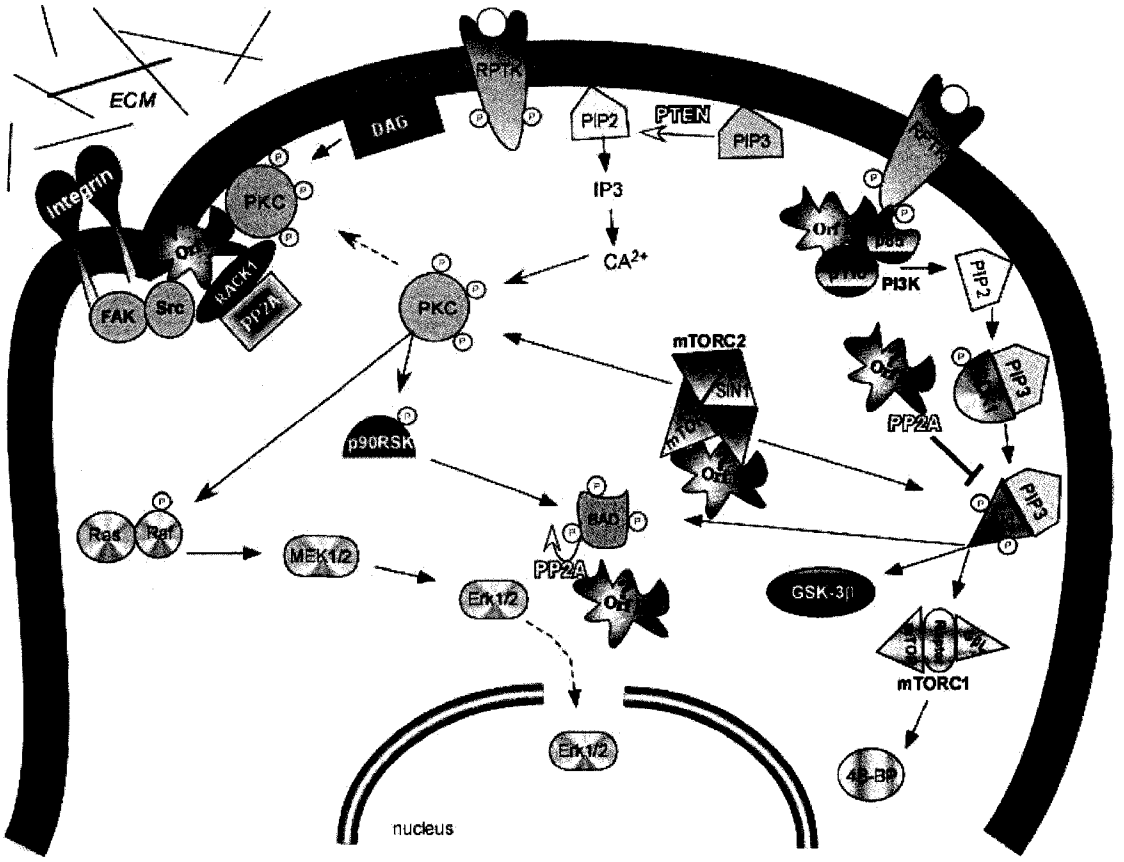

Fig 5.1. Signaling pathways involved in proliferation and/or transformation of Orf B-expressing cells. The known involvements of Orf B with different signaling molecules and pathways in proliferation and/or transformation of Orf B are shown. Question marks indicate where an association of Orf B and the indicated protein is unknown.

Orf B associates with Src and it is unknown whether this association is direct or perhaps in a complex with other proteins. The complexity of known Src interactions with proteins from various signaling pathways makes it difficult to predict the functional consequence of Orf B's interaction with Src. One known function of Src is to interact with and phosphorylate FAK. Focal adhesions and actin stress fibers observed by IFA in explanted tumor cells and Orf B-expressing cells suggest Orf B influences Src activity resulting in cytoskeletal rearrangement. Src also interacts with RACK1, which results in inhibition of its tyrosine kinase activity (Miller et al., 2004). The inclusion of Orf B with RACK1 and Src may result in the recovery of Src kinase activity or possibly altered Src localization.

Future experiments for Orf B

Observably, the mechanisms for transformation of immortalized cells lines do not necessarily reflect the mechanisms of cell transformation in vivo. Additional mechanisms might be necessary for WDSV Orf B-induced oncogenesis to occur. Further experiments are needed to determine the mechanism of action for cell transformation via signal transduction. This information would provide the basis for understanding Orf B-induced transformation. The interaction of Orf B with PI3K does not result in activation of Akt by the PI3K-PDK1 pathway, since phosphorylation of Akt at T308 was not observed, and instead suggests an alternate mechanism of action for Akt activation. The mTOR-rictor complex (mTORC2) is a viable candidate for Akt activation and cell transformation in Orf B-expressing cells. Recent studies identified mTORC2 in phosphorylation of Akt at serine 473 (Sarbassov et al., 2005) and phosphorylation of the

turn motif of PKCα, which is important for PKCα maturation, stability, and signaling (Facchinetti et al., 2008; Ikenoue et al., 2008). The factors controlling activation of mTORC2 are unknown and additional biological targets of mTORC2 remain to be identified. I propose an interaction or association of Orf B with mTORC2 as the source for PKCα and Akt activation resulting in increased proliferation and transformation in Orf B-expressing cells. Experiments to test this hypothesis include: IPs with specific components of mTORC2 (i.e. rictor and SIN) and silencing of mTORC2 proteins in Orf B-expressing cells for transformation assays.

I also realize the mechanism of action for transformation could potentially be different in walleye. Therefore, all of the experiments reported here would need to be repeated in walleye cells. In addition, I have not analyzed the protein expression in fall tumors for the same proteins that are associated with Orf B in mammalian cells. The cross reactivity of antibodies to fish cells is a challenge, with the exception of proteins that demonstrate high conservation such as walleye RACK1. We have screened several antibodies for cross reactivity to fish cells and determined Erk1/2 and PKCα/β cross react. Many cellular proteins are well conserved across eukaryotic species. Therefore, I might expect walleye cell signaling pathways to be somewhat conserved. Recent studies done by others in our lab demonstrated rv-cyclin inhibits NF-κB-dependent transcription, suggesting conservation of the NF-κB pathway (unpublished data).

WDSV is a unique model to study the mechanisms of tumor development and regression in vertebrates. The differential expression of *orf a, b, and c* correlates with the stage of disease and although the protein products for these viral transcripts each have individual functions, their cumulative activity are critical for the WDSV disease cycle.

Both *rv-cyclin* and *orf b* are expressed during tumor formation. Rv-cyclin was able to rescue G1/S cyclin-deficient yeast from growth arrest (LaPierre, Casey, and Holzschu, 1998) and depending on the cell type, can inhibit or enhance promoter activity in reporter gene assays (Rovnak et al., 2005; Rovnak and Quackenbush, 2002).

I believe Rv-cyclin and Orf B function together to provide an optimal environment or condition for tumor formation. Rv-cyclin, through down regulation of the WDSV promoter, inhibits the production of other viral proteins that might trigger a host immune response causing destruction of infected cells. Rv-cyclin also has the capacity to enhance gene transcription, which would disrupt the delicate balance of cellular transcription factors and promote cell proliferation. Orf B ensures the establishment of dermal sarcoma by activating signal transduction pathways that control cell survival and proliferation such as PKC and Akt.

The Orf C protein completes the infection cycle by inducing apoptosis, resulting in the release of virus through loss of tumors and/or during viral budding. This would ensure infection of naïve fish by direct physical contact with fish containing tumors during spring spawning or ingestion of tumors floating in the water. The WDSV infection cycle would then be restarted in newly infected fish that will develop tumors the following fall.

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