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CHARACTERIZING CHICKEN STEM CELL ANTIGEN 2, A PUTATIVE MAREK'S DISEASE RESISTANT GENE

By

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ABSTRACT

CHARACTERIZING CHICKEN STEM CELL ANTIGEN 2, A PUTATIVE MAREK'S DISEASE RESISTANT GENE

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Marek's disease virus (MDV) is an alpha-herpesvirus that is the causative agent of Marek's disease (MD), a lymphomatous disease of chicken. MD is a serious chronic disease of concern to the poultry industry worldwide, although it has been controlled through the use of vaccination since the 1970's. MD vaccines prevent the formation of lymphomas but are not sterilizing and do not prevent MDV infection or replication in the bird. Consequently, the observed increase in MDV virulence over the years may be an undesirable response to the widespread use of MD vaccines. With increases in MDV virulence expected in the future and the most effective MD vaccine (Rispens) already in use, additional approaches such as genetic resistance to control MD are needed to augment vaccinal control of MD. The overall purpose of this project is to characterize stem cell antigen 2 (SCA2), the product of the putative MD resistant gene, and its role in MDV biology

To analyze the biological properties of chicken SCA2, SCA2 protein was expressed and purified in *E. coli*, and a polyclonal antibody was developed. Utilizing this anti-SCA2 antibody, SCA2 was identified to be a 13 kDa cell surface protein anchored by a GPI moiety as is the case for most other Ly6 family members. *In vivo* studies showed unique SCA2 expression pattern in bursal cortical medullary epithelial cells (CMEC), which are surrounded by MHC class II presenting cells. Expression profiles of

bursal cells induced by contact with SCA2-expressing cells *in vitro* demonstrated down-regulation of numerous genes that are involved in the B cell receptor and immune response signaling pathways. These data suggest that SCA2 plays a role in regulating chicken B lymphocytes.

The viral US10 protein was previously demonstrated to interact with SCA2 in an *E. coli* two hybrid screen followed by confirmation using an *in vitro* binding assay. To analyze the functional interaction of SCA2 and US10 for MDV growth properties, two recombinant MDVs were developed in which viral *US10* gene was fused to or replaced with an enhanced green fluorescent protein (EGFP) coding region. Over-expressing SCA2 impairs both MDV plaque size and the percent of fibroblasts infected *in vitro* but this effect is dependent on the presence of US10. We conclude that MDV US10 is both sufficient and required for this growth impairment via association with SCA2.

A missense point mutation in MDV *UL41* gene was found out when cloning the viral genome into bacterial artificial chromosome (BAC). Monitoring the frequency of each SNP by pyrosequencing during virus passages determined the ratio of each viral genome in a single monolayer. This point mutation in *UL41* gene enhanced the viral fitness in the competitive growth assay *in vitro*, but abolished the virion host shutoff (vhs) activity of UL41. This result suggests that the enhanced fitness *in vitro* for MDV with inactive vhs was due to reduced degradation of viral transcripts.

Dedicated to:

Yunli Liu
Eric Mao

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ADOL	Avian Disease and Oncology Laboratory
BAC	Bacterial artificial chromosome
BCR	B cell receptor
BLNK	B cell linker
CEF	Chicken embryo fibroblasts
CMEC	Cortico-medullary epithelial cells
CTL	Cytotoxic T lymphocyte
EGFP	Enhanced green fluorescent protein
EID	Embryonic incubation day
ERC	Epithelial reticular cells
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPI	Glycosylphosphatidyl-inositol
HC	Hematopoietic cells
HSC	Hematopoeitic stem cells
HSV-1	Herpes simplex virus 1
HSV-2	Herpes simplex virus 2
IE	Immediate-early genes
IFN	Interferon
IL-2	Interleukin 2
LATs	Latency-associated transcripts

LM	Liebovitz's L-15 and McCoy 5A
Ly-6	Lymphocyte complex antigen 6
mAb	Monoclonal antibody
MD	Marek's disease
MDV	Marek's disease virus
MHC	Major histocompatibility complex
ORFs	Open reading frames
pAb	Polyclonal antibody
PCR	Polymerase chain reaction
PFU	Plaque-forming units
PI-PLC	Phosphatidylinositol-specific phospholipase C
PLCG2	Gamma 2-phospholipase C
qRT-PCR	Quantitative reverse transcription PCR
QTL	Quantitative trait loci
RMA	Robust multichip average
SAM	Significance analysis of microarrays
SCA2	Stem cell antigen 2
SNP	Single nucleotide polymorphism
SYK	Spleen tyrosine kinase
T2ECs	Transforming growth factor-induced erythrocytic cells
TCR	T cell receptor
UL	Unique long

US10EGFP	rMd5-B40-US10EGFP
ΔUS10EGFP	rMd5-B40-ΔUS10EGFP
US	Unique short
vhs	Virion host shutoff
vv+MDV	Very virulent plus MDV
vvMDV	Very virulent MDV
VZV	Varicella-zoster virus

Chapter 1

Introduction and literature review

Introduction

Marek's disease virus (MDV) is a highly oncogenic alpha-herpesvirus that induces Marek's disease (MD), a lymphomatous disease of chicken. MD is considered the most serious persistent infectious disease of concern to the poultry industry worldwide, although it has been primarily controlled through vaccination and changes in animal husbandry since the 1970s. Even with vaccines, MD still has a significant economic impact to the poultry industry as there are sporadic outbreaks, which require the continuous vaccination and monitoring of birds. Consequently, there is a need for alternative methods, such as enhanced genetic resistance to MD, to augment vaccinal control of MD.

This chapter reviews the pathogenesis of MDV infection, the genomic structure of MDV, and genetic resistance to MD, while also discussing the prevention and control of the disease. MDV *UL41, ICP4, US10* genes, as well as chicken *SCA2*, a putative MD resistant gene, are discussed. The possibility that SCA2 plays role in MDV spread through regulating MDV maturation and cell-cell adhesion is hypothesized.

The overall purpose of this thesis was to determine and characterize the biological function of chicken SCA2, and through its interaction with MDV US10, investigate the role of SCA2 in MDV infection. By identifying the properties of SCA2 and its putative functions, we hoped to obtain a better understanding of the host protein and its involvement in the MDV life cycle. The impact of single nucleotide polymorphisms (SNPs) in MDV *UL41* and *ICP4* genes were also elucidated in this thesis.

1. History of Marek's disease (MD)

The first report of what is later called Marek's disease (MD) occurred in 1907 by Dr. Joseph Marek, an eminent veterinary clinician and pathologist at the Royal Hungarian Veterinary School in Budapest (Marek, 1907). In the report, Dr. Marek described a disease in four adult cockerels that were affected by paralysis of the legs and wings, and the pathological changes that occurred in the central and peripheral nervous system. The disease is characterized by a mononuclear infiltration of the peripheral nerves, gonads, various viscera, muscles, skin, and partial or complete paralysis as a result of the accumulation of tumor cells in peripheral nerves. In the 1940s and 1950s, diagnosis relied on pathological examination and MD was confused with other leukotic diseases under the term "avian leukosis complex" because of the difficulty of distinguishing between the visceral lymphoma of MD and lymphoid leukosis. Subsequently, on the basis of the studies in the field and transmission experiments, it was concluded that this avian lymphomatous disease would more appropriate to MD than leukosis. Biggs and Payne (1963, 1967) described that the transmission of this disease with blood or lymphoma cells from diseased birds and the transmission was dependent on whole cells as the destruction of cells resulted in loss of infectivity. The behavior of MD in chickens suggests that it was an infectious disease, and the causative agent was likely to be a cell-associated virus. In 1967, a cell-associated herpesvirus was isolated from blood and kidney tumor cells of infected chickens (Churchill and Biggs, 1967), and then in infected duck embryo fibroblasts (Nazerian et al. 1968; Solomon et al. 1968). Because the herpesvirus was associated with cases of MD, the ability of the herpesvirus-infected cells to produce cytopathic effects in cell culture was quantitatively associated with lesions of MD, and the virus was highly cell-associated similar to the infectious agent of MD, this cell-associated herpesvirus was concluded to be the aetiological agent of MD and came to be referred to as the Marek's disease virus (MDV).

2. Marek's disease vaccines

MDV and its close relatives are grouped by serotype. Serotype 1 viruses include MDV (gallid herpesvirus type 2, GaHV-2) and are the only ones that are oncogenic. Serotype 2 MDVs (GaHV-3, MDV-2) are naturally occurring strains that are non-oncogenic. Serotype 3 is turkey herpes virus 1 (HVT; MDV-3), a close relative (Osterrieder et al., 2006). Serotype 1 is further categorized into very virulent plus (vv+MDV), very virulent (vvMDV), virulent (vMDV), mild (mMDV) and attenuated strains based on their ability to induce disease in unvaccinated and vaccinated flocks (Witter, 1997).

MDV can lead to tremendous economic losses to the poultry industry. After the introduction and widespread use of vaccines in the early 1970s, the disease was first controlled. The first widely used MDV vaccine was the antigenically similar HVT. HVT has been widely used in the United States, and appears to offer good protection (Witter et al., 2000). The current and most effect MD vaccine is CV1988 (also referred to as Rispens), a cell culture-attenuated serotype 1 MDV strain that offers effective protection against challenge with the very virulent pathotypes of MDV. The principle underlying successful immunization remains unknown. One speculation is "substrate deprivation" where the targeted B and T cells infected by the live attenuated vaccine are no longer susceptible to wild type virus infection or transformation. The major concern of MD vaccines is that they are not sterilizing, i.e., the vaccines block tumor formation but do not prevent viral replication or spread in birds. MDV continues to replicate and potentially evolve in MD vaccinated birds, consequently, the co-existence of the vaccines and wild type viruses in vaccinated flocks may speed up the virus recombination and viral evolution. The evolution of MDV to greater virulence (Witter, 1997; Nair, 2005) in the near future is one of the major driving forces to develop new alternative approaches to augment vaccinal control of MD. For example, recombinant MDV vaccines such as a *Meq*-deleted MDV serotype 1 strain, has shown improved vaccine protection (Lee et al., 2008).

Apart from its economic impact, MDV is of interest to biomedical researchers in that it is the only naturally-occurring alpha-herpesvirus that induces tumors in its host and is controlled by vaccination. These characteristics of MDV make it a good model for the study of the mechanisms of viral induced T cell lymphoma.

3. Marek's disease virus

3.1. Pathogenesis of MDV infection

MDV is a highly cell-associated herpesvirus, and horizontal transmission occurs through enveloped virion particles produced at the superficial layers of the feather follicle epithelium. Poultry dusts containing these epithelial cell-enveloped MDV are infectious to both chickens and cultured cells (Biggs, 2000). These results provide a reasonable explanation for why the cell-associated virus can be highly contagious. Lymphomagenesis results from MDV infection in susceptible birds. Lymphomas may develop in most of the visceral organs: spleen, liver, kidney, heart, pancreas, gonads, and also in other organs including muscle, peripheral nerves, eye, and skin. Lymphomas in visceral organs may be the most proliferative but the lesions in other tissues may also be obvious. The involvement of lymphoma in various organs is influenced to some extent by both the virus strain and the genotype of the host.

The pathogenesis of MDV in susceptible chickens has been divided into four phases: (1) early cytolytic, (2) latent, (3) late cytolytic and (4) transforming. The initial infection of chicken occurs via the respiratory tract following the inhalation of the cell-associated MDV from the contaminated environment. In the respiratory tract, macrophages or dendritic cells are infected and presumed to carry the virus to lymphoid organs such as the bursa of Fabricius (bursa), thymus, and spleen (Calnek et al., 2000). A cytolytic infection in these lymphoid organs occurs between 3 and 6 days postinfection. During the lytic phase, the virus is able to infect B cells and activated CD4+ T cells, and rarely CD4-CD8- T cells or CD8+ T cells (Calnek et al., 2000). After 7 to 8 days, the infection in lymphoid organs switches from lytic to latent infection mostly in CD4+ T cells. No extracellular virus can be detected in the blood in any infected bird, so it is concluded that virus spread is cell-to-cell. Interestingly, despite significant efforts, the putative receptor for MDV attachment or entry has not been resolved. The latent phase is defined as the presence of the viral genome without production of infectious virions. In contrast to other members of the alpha-herpesvirus subfamily, MDV goes latent in activated T cells but not in sensory nerve ganglia (Calnek et al., 2000). During latency, latency-associated transcripts (LATs) are among the few active viral transcripts. Latently infected cells can become transformed cells leading to lymphoma formation; lymphomatous tumors caused by MDV can appear as early as 12-14 days postinfection.

Various potentially important genes and latency-related transcripts that could contribute to oncogenicity of MDV have been reported. The most studied one to date is *Meq*. *Meq* is a gene that encodes a protein with 339 amino acids and is completely contained in the MDV *EcoR*I Q fragment, hence the name *Meq*. Overexpressed Meq is associated with lymphocytic tumors and confer these cells with anti-apoptotic activities. Anti-sense transcripts of *Meq* inhibit the growth of MDV-transformed T cells (Xie et al., 1996). Thus, the evidence strongly supports *Meq* as a significant viral oncogene for transformation. Further studies have shown Meq has transactivating activity, and binds to Jun/Fos cellular oncogenes to activate oncogenic pathways (Jones et al., 1992; Liu et al., 1999; Shamblin 2004; Levy et al., 2005; Brown et al., 2009). In addition to the viral oncogene, certain host cell genes, such as the tumor suppressor gene, *p53*, and proto-oncogene, *Bcl-2* could also play roles in the pathogenesis of MD (Venugopal and Payne, 1995).

3.2. The genomic structure of MDV

Like other herpesviruses, MDV contains a linear, double-stranded DNA genome surrounded by an icosahedral protein capsid core, which in turn is surrounded by tegument proteins and a lipid-containing envelop to enclose the mature virion (Silva et al., 2000). The 180 kb MDV genome consists of unique long (UL) and unique short (US) regions, each flanked by inverted repeats: terminal repeat long (TRL), internal repeat long (IRL), internal repeat short (IRS), and terminal repeat short (TRS). The genomic structure resulted in MDV being classified as an alpha-herpesvirus (Roizman et al., 1992) although it was originally thought to have been a gamma-herpesvirus due to its biological characteristics, especially the ability to produce lymphoid tumors. MDV genomic structure is very similar that of herpes simplex virus 1 (HSV-1; the prototype alpha-herpesvirus) and human herpesvirus 3 (varicella-zoster virus, VZV) (Osterrieder et al., 2006). As a herpesvirus, MDV genes are classified as immediate-early genes (IE), early genes and late genes based on the timing of expression of their homologs during the lytic cycle. The IE proteins migrate to the nucleus to transactivate other MDV IE genes. The IE genes in turn regulate the expression of early and late genes. Some of the early genes are enzymes required for viral DNA replication, while the late genes include most of the structural genes such as capsid, envelope, and tegument proteins (Lupiani et al., 2000).

3.2.1. MDV virion host shutoff gene (vhs; *UL41*)

One viral defense mechanism common to alpha-herpesviruses is to degrade the host mRNA and inhibit processing of new synthesized host mRNA. This degradation is an important feature of the early stage of infection, and this activity is contained in the virion host shutoff (vhs or *UL41*) gene of HSV-1 (Read and Frenkel, 1983; Schek and Bachenheimer, 1985; Kwong et al., 1988). It was later demonstrated that UL41 is an endoribonuclease that degrades mRNA and does not discriminate between cellular or viral origin (Jones et al., 1995; Pak et al., 1995; Taddeo and Roizman, 2006). However, because the viral mRNA is much more abundant, the effect is to downregulate host gene expression. HSV *UL41* is a late gene and the gene product is packaged into the virion as a part of the tegument. Immediately after ingress of mature virions into cells, UL41 is released from the virion and degrades host mRNA in the cytoplasm. As a result, there is down regulation of MHC class I and class II proteins, and the degradation of Jak1 and Stat2 to block IFN signaling, suggesting that UL41 modulates the host's immune defense to viral infection. UL41 also contributes to the ability of the virus to evade the host adaptive immune response due to reduced cytotoxic T-lymphocyte recognition (Suzutani et al., 2000; Kopper-Lalic et al., 2001; Murphy et al., 2003). In addition to evasion of the host immune response, HSV UL41 also facilitates the transition from IE to E (Kwong and Frenkel, 1987; Oroska and Read, 1987). While UL41 represents a significant virulence factor in virus latency and pathogenesis, deletion of UL41 in HSV-1 did not alter one-step growth curves *in vitro* but impaired growth in corneas, trigeminal ganglia, and brains of mice. Viral DNA within trigeminal ganglia infected with the UL41-deleted virus was reduced by 30-fold compared to wild type HSV-1 (Strelow and Leib, 1995, Strelow et al., 1997). In herpes simplex virus 2 (HSV-2), UL41 also plays an important role *in vivo* by interfering with the IFN-alpha/beta-mediated antiviral response and is a determinant in HSV-2 pathogenesis (Smith et al., 2002, Murphy et al., 2003).

The central region of MDV UL41 is highly conserved among all MDV serotypes as well as exhibiting high protein sequence identity with herpes simplex (29%) and varcella-zoster (33%) virus homologs (Gimeno and Silva, 2008). A recombinant MDV lacking *UL41* replicated in cell culture as well as the parental MDV, but resulted in a longer early cytolytic infection in the lymphoid organs. The deletion did not affect either levels of latency or the ability to reactivate from latency and did not decrease the replication of the virus in lymphoid organs or feather follicle epithelium. Thus, MDV UL41 may facilitate the normal cascade of immediate early, early, and late gene expression but the role of MDV UL41 in MDV pathogenesis is not nearly as great compared to HSV vhs, although the MDV UL41 is functionally equivalent to the HSV-1 vhs.

3.2.2. MDV *ICP4* gene

MDV *ICP4* is a homologue of HSV *ICP4* and lies in the inverted repeat that flanks the unique short region of the genome (Anderson et al., 1992). HSV ICP4 protein is a transcriptional regulator required for the induced transcription of most of the remaining HSV genes and is essential for viral replication (Compel and DeLuca, 2003; Su et al., 2006). The exact role of ICP4 in MDV replication has not been completely determined although it is speculated to play a role similar to HSV ICP4, is associated with maintenance of transformation, and is essential for MDV growth in chicken embryo fibroblasts (CEF) (Endoh, 1996; Chattoo et al., 2006). A number of studies demonstrated that LATs of MDV include a 10 kb RNA that is antisense to *ICP4*, and the suppression of *ICP4* by LATs plays an important role in MDV latency as well as maintenance of transformation (Cantello et al., 1994; Xie et al., 1996; Cantello et al., 1997; Li et al., 1998).

3.2.3. MDV *US10* gene

HSV-1 US10 is a phosphorylated tegument protein that copurifies with the nuclear matrix (Yamada et al., 1997). Homologues of US10 protein are also encoded by other alpha-herpesviruses including HSV-2, VZV, and MDV (Brown and Harland, 1987; Davison and Scott, 1986; Sakaguchi et al., 1992). MDV US10 is not essential for virus replication in CEF but a ~4.8 kb deletion in the MDV US region that includes *US10* and adjacent MDV reading open frames (*US1, US2, SORF1, SORF2, SORF3*) decreased early cytolytic infection, mortality, and tumor incidence *in vivo* (Parcell et al., 1994; Parcell et al., 1995). The role of US10 in the MDV life cycle remains uncertain. Liu et al. (2003) demonstrated that MDV US10 interacts with chicken SCA2 protein through a *E. coli* two-hybrid screen followed by an *in vitro* binding assay, suggesting that US10 may be involved in MD genetic resistance via SCA2 (Liu et al., 2003; Niikura et al., 2004).

3.2.4. Bacterial artificial chromosome-cloned MDV genomes

The development of recombinant MDV DNA using infectious bacterial artificial chromosome (BAC)-cloned MDV genomes has contributed greatly to the ability of researchers to study viral genes and gene function including viral morphogenesis. Through recombinant MDVs, viral *gB, gE, gI,* and *UL49-VP22* genes have been demonstrated to be indispensible for MDV growth *in vitro* (Schumacher et al., 2000; Schumacher et al., 2001; Dorange et al., 2002). The viral genes *Meq, vLIP, gC, pp38, US3, vIL-8, vTRm* are involved in pathogenesis and immune evasion *in vivo* (reviewed in Osterrieder et al., 2006).

3.2.5. The morphogenesis of MDV

MDV morphogenesis is proposed to be like a typical alpha-herpesvirus. The assembly process begins in the nucleus, where the viral genome is packaged into capsids. Then, nucleocapsids which follow with the envelope at the nuclear membrane exit from the nucleus to the cytoplasm, bind to tegument proteins, and are enveloped a second time at the trans-Golgi network or endosomal membranes. The final egress step is assumed through exocytosis of vesicles (reviewed in Johnson and Huber, 2002; Mettenleiter 2002; Campadelli-Fiume and Roizman, 2006; Sugimoto et al., 2008). Using a recombinant MDV that expresses a structural protein (VP22, which is encoded by *UL49*)-EGFP fusion protein, an ultrastructural study of MDV morphogenesis showed that, compared to other alpha-herpesviruses, MDV seems deficient in a few crucial steps of viral morphogenesis, i.e., release from the nucleus, secondary envelopment and the final egress (Denesvre et al., 2007). The number of enveloped MDV virions in the cytoplasm is very low, which may partially explain why infectious MDV cannot be recovered from cell lysates by freeze-thawing or sonication. However, due to its strict cell association, MDV morphogenesis is still not well understood.

3.3. Immune responses to MDV infection

The innate and acquired immune responses of the chicken play important roles in different phases of MDV infection. After the virions are engulfed by macrophages, they are delivered to replicate in bursal follicles, in which MDV mostly replicates in B lymphocytes. Later, activated T lymphocytes are infected through intimate contact between B and T cells (Schat and Marlowski-Grimsrud, 2000). A complex set of innate and acquired immune responses are consequences of the maintenance of viral latent infection and tumor formation.

3.3.1. Innate immune responses

Innate immune responses of MDV-infected chickens include activation of macrophages, natural killer (NK) cells, cytokines, and other soluble mediators. The role of macrophages in MDV pathogenesis is significant. Phagocytosis of MDV by macrophages is important to the transportation of MDV from lung, the initial entry point, to other lympoid tissues. However, MDV does not replicate in these macrophages and, in fact, activation of macrophage inhibits viral DNA replication in chicken spleen cells (Lee et al., 1979). *In vivo* studies also have shown an increasing number of activated macrophages reduces virus replication and delays the onset of MD tumors (Gupta et al., 1989). These results demonstrate that macrophages play an important role in inhibiting MDV replication.

MDV lytic infection, as well as the establishment of latency in lymphocytes, causes the differential expression of cytokines and other soluble mediators. The up-regulation of IFN-gamma is one of the first immune responses after MDV infection (Schat and Xing, 2000). Some expression of IE, early, and late MDV genes is down-regulated by IFN-alpha (Volpini et al., 1996). Buscaglia and Calnek demonstrated that two soluble factors maintained MDV latency in spleen cell culture, but these factors have not been characterized (Buscaglia and Calnek, 1988). The increased NK cells activities induced by MDV suggests its role in protection against MDV infection (Lessard et al., 1996).

3.3.2. Adaptive immune responses

Adaptive immunity to MDV consists of the development of viral antigen-specific antibodies and cytotoxic T lymphocyte (CTL) responses. During infection, virusneutralizing antibodies are generated to protect against infection. Antibodies to several MDV glycoproteins have been demonstrated but only antibodies to MDV gB are believed to play an important role in MDV protection based on its ability to neutralize MDV (Ikuta et a., 1984; Nazerian et al., 1992). The non-neutralizing antibodies also play a role in adaptive immunity. Maternal antibodies passed from vaccinated hens or transferred by injection can reduce the severity of morbidity, mortality, and tumor formation of MD (Schat and Markowski-Grimsrud, 2000).

The CTL response is of particular importance in immunity to MDV due to its highly cell-associated nature. Effector cells were characterized as $CD4^-CD8^+TCR\alpha\beta1^+$ (Omar and Schat, 1997). MDV glycoproteins have been implicated as determinants of CTL responses. MDV gB was shown to induce a significant CTL response (Omar and Schat, 1996). The down-regulation of MHC class I on the surface of infected cells by MDV is involved in the immune evasion of MDV in lytic infection (Hunt et al., 2001). This escape from MHC class I may restrict cytotoxicity and facilitate lytic MDV spread and the maintenance of latency. The hypothesis of MHC class I down-regulation and antigen processing blocked by MDV proteins has been proposed, but the mechanism of CTL evasion through MDV proteins is still inclusive.

4. Genetic resistance to MD

Chickens resistant to MD are those that fail to develop characteristic symptoms upon exposure to MDV. Genetics resistance to MD is controlled by multiple genes or QTL (Schat and Xing, 2000; Vallejo et al., 1998; Yonash et al., 1999). The United States Department of Agriculture (USDA), Avian Disease and Oncology Laboratory (ADOL), has generated inbred chicken lines selected for resistance and susceptibility to MD since the 1940s. Three of the original lines are presently maintained. These include line 6 which is MD resistant, and lines 7 and 15 which are MD susceptible. Less than 1% of line 6 chickens compared to 76-80% of line 7 chickens were shown to develop MD after infection with GA or JM strains of MDV (Bacon et al., 2000).

The best understood mechanism for the involvement of genetic resistance to MD involves the MHC or, as it is known in the chicken, the *B* complex. The MHC contains three tightly linked regions known as *B-F* (class I), *B-G* (class II), and *B-L* (class IV), which control cell surface antigens. The *B-G* locus is expressed in erythrocytes, which enables convenient typing of blood groups. By measuring the allelic frequency of specific blood groups, it has been observed that certain *B* alleles are associated with resistance or susceptibility. Chickens with the B*21 allele have been found to be more resistant than those with other *B* haplotypes (Bacon and Witter, 1994). Other studies have allowed for the relative ranking of the other *B* alleles: moderate resistance, B*2, B*6, B*14; susceptibility, B*1, B*3, B*5, B*13, B*15, B*19, B*27 (Bacon, 1987; Bacon and Witter, 1992) The MHC also influences vaccinal immunity as some haplotypes develop better protection with vaccines of one serotype than of a different serotype (Bacon and Witter, 1992; 1994).

ADOL lines 6 and 7 chickens share the same MHC, B haplotype, yet differ with respect to resistance to MD (Lee et al., 1981; Hunt and Fulton, 1998). Thus, non-MHC factors also play a major role in genetic resistance to MD. Using genetic markers spaced throughout the chicken genome, 14 QTL (7 significant and 7 suggestive) were discovered that explain one or more MD-associated traits (Vallejo et al., 1998; Yonash et al., 1999).

Also, a few non-MHC genes have been correlated with resistance to MD. The BU1 allele (antigens on bursal lymphocytes) of line 6 was associated with resistance to MD in progeny of a (6×7)×7 backcross population (Tregaskes et al., 1996; Bacon et al., 2000). Chicken SCA2 was significantly associated with length of survival and the indicence of proventricular tumors following MDV challenge in a commercial layer resource population (Liu et al., 2003). The chicken growth hormone allele is linked with MD QTL and polymorphism in the growth hormone gene is associated with the number of tissues with tumors in commercial White Leghorn chickens with the MHC B*2/B*15 genotype (Liu et al., 2001).

DNA microarrays have been employed to identify candidate genes for QTL and pathways involved in MD resistance. Host genes that were reproducibly induced by infection of CEF with MDV included macrophage inflammatory protein (MIP), interferon response factor 1 (IRF1), interferon-inducible protein, SCA2, MHC class I, and MHC class II (Morgan et al., 2001). To sufficiently resolve the QTL containing the genes conferring genetic resistance to MD, the expression of genes in peripheral blood lymphocytes from uninfected and MDV-infected line 6 and line 7 chickens were analyzed through microarray. The microarray results were refined by genetic mapping of the differentially-expressed genes. Microarray results showed that up to 25% of the genes detectable show differential expression between the resistant and susceptible lines. Integration of microarrays with genetic mapping data was achieved with a sample of 15 genes in which the SCA2, growth hormone, and MHC class I were involved (Liu et al., 2001). DNA microarrays provided a complementary approach for identifying positional candidate MD resistant genes. Aside from the the DNA and RNA variation associated with MD resistance, host and MDV protein interactions associated with MD resistance were examined through a bacterial two-hybrid system (Niikura et al., 2004). One example is chicken growth hormone which interacted with MDV SORF2 and combined with linkage and differential expression, was defined as a MD resistant gene (Liu et al., 2000). Another putative MD resistant gene is SCA2 which is associated with the genetic resistance QTL mapping, differentially transcribed between Line 6 and Line 7 following MDV infection, and interacts with MDV US10 (Liu et al., 2003).

In addition to defining host genes associated with MD resistance, other alternatives to enhancing MD resistance include the transgenics approach such as RNA interference to MDV. A successful report of RNA interference that inhibits MDV *in vitro* and *in vivo* has been described (Mo et al., 2008).

5. Stem cell antigen 2 (SCA2; Ly-6E/TSA1)

Chicken SCA2 has been proposed as a MD resistance gene due to the following criteria: (1) association with MD resistance in a commercial resource population, (2) transcriptional differences between MD resistant and susceptible experimental chicken lines following challenge with MDV, and (3) a direct protein interaction with MDV US10 as determined by a two-hybrid screen followed by an *in vitro* binding assay. These potential effects in MD encouraged us to investigate the properties of chicken SCA2 and its role in MDV infection.

5.1. Lymphocyte complex antigen 6 (Ly-6) family

The majority of research on Ly-6 family members has been conducted using mice. The mouse Ly-6 locus encodes a family of 10-12 kDa proteins are characterized by their remarkable conservation of cysteine residues and, with few exceptions, are linked to the cell surface via a glycosylphosphatidyl-inositol (GPI) anchor. Ly-6 proteins have a wide range of functions but, in general, are thought to be involved in cell signaling and cell adhesion (reviewed in Bamezai, 2004). Ly-6 proteins are broadly expressed in tissues of hematopoietic stem cells (HSC) origin and, consequently, are often used as differential markers for developmental stages in these cells (reviewed in Rock et al., 1986; Shevach et al., 1989; Gumley et al., 1995). Ly-6A.2 (also known as stem cell antigen 1 and T cell-activating protein) is expressed on the murine stem cells of bone marrow and T cell lineages, and contributes to regulating HSC renewal and development of progenitor cells (Uchida et al., 1994; Ito et al., 2003). Ly-6C is expressed on a majority of bone marrow cells, some subsets of immature T cells and B cells, and was shown to promote differentiation of B cells into antibody-generating plasma cells (reviewed in Gumley et al., 1995; Schlueter et al., 1997; McHeyzer-Williams and McHeyzer-Williams, 2004). Ly-6E (also known as stem cell antigen 2, SCA2; thymic shared antigen 1, TSA1) is expressed on the majority of immature double negative thymocytes, B cells, and also expressed in lymphoid epithelial cells (Godfrey et al., 1992; MacNei et al., 1993; Classon and Coverdale, 1996; Antica et al., 1997; Zammit et al., 2002). Ly-6G is a granulocyte-differentiation antigen and serves as a marker for identifying mature granulocytes (Fleming et al., 1993). Ly-6M is expressed on hematopoietic cells (HC), especially monocytes and myeloid precursors (Patterson et al., 2000).

In the chicken genome, there are 3 annotated genes showing similar molecular size and conserved cysteine residues with mice Ly-6 genes, but it is not certain whether these chicken genes belong to the same Ly-6 family.

5.2. Chicken stem cell antigen 2

Chicken SCA2 transcript was first identified through a subtraction cDNA library of v-Rel (a NF-kappaB family member) – transformed avian bone marrow cells regulated by a conditional v-Rel estrogen receptor (Petrenko et al., 1997). Bresson-Mazet et al. reported that SCA2 was involved in the self-renewal of chicken erythroid progenitor (Bresson-Mazet et al., 2008). But the protein properties of chicken SCA2 were not defined due to the lack of antibodies to SCA2.

Chicken SCA2 has the 10 cysteine residues conserved among Ly-6 members (Fig. 1-1), however, it is not known if this chicken homolog has the same biological characteristics of mice SCA2 (Ly-6E) or other Ly-6 members. While the biological role of chicken SCA2 protein is not defined, its homologues in the mouse Ly-6 family provide clues for investigation. Mature mice SCA2 is a cell surface protein of 82 amino acids anchored on the cell membrane by a C-terminal GPI moiety; highly expressed in immature T cells, bone marrow cells, and peripheral B cells, with low levels in peripheral mature T cells. SCA2 is implicated in regulating intracellular signaling events via TCR and cell-cell adhesion as a receptor on the lymphocyte membrane. The ability of anti-SCA2 mAb to block thymopoiesis in fetal thymic organ culture indicates that SCA2 functions in T cell development (Randle et al., 1993). Fleming and Malek (1994) demonstrated that anti-SCA2 mAb inhibited anti-CD3-induced interleukin 2 (IL-2) production and this inhibition is independent on the GPI anchor (Fleming and Malek, 1994. This finding was further supported by Kosugi et al. (1994). Saitoh et al. (1995) demonstrated that SCA2 plays an important role in the TCR signaling pathway in that the tyrosine phosphrylation of CD3ξ-chains following TCR stimulation was reduced by anti-SCA2 mAb. Anti-TCR/CD3-induced T cell apoptosis was blocked by anti-SCA2 mAb, which confirmed the function of SCA2 through the TCR signaling pathway (Noda et al., 1996), and the physical interaction between SCA2 and CD3 may clarify their functional interaction (Kosugi et al., 1998). Aside from lymphocytes, SCA2 is also expressed in other tissues, including the embryonic adrenal gland and liver and adult kidney and liver and in various epithelial cells (MacNeil et al., 1993; Classon and Coverdale, 1996). SCA2 deficient (SCA2-/-) mice exhibited abnormal development, impaired function of the embryonic adrenal gland and midgestational lethality at embryonic day 15 due to the cardiac abnormalities, but the lymphoid development is not impaired, suggesting SCA2 is not an obligate requirement for normal differentiation of lymphocytes (Zammit et al., 2002).

One common expression pattern of SCA2 shared within different species is that SCA2 transcripts are overexpressed in malignant cells which suggest it may play role in tumorigenesis (Eshel et al., 1995; Petrenko et al., 1997; He and Chang, 2004; Damiola et al., 2004).

```
Mice Ly-6E    LECYQCYGVPFETSCP----SITCPYPDGVCVTQEAAVIVGSQT-RKVKNNLCLPI--CPPNIESMEILGTKVNVKTSCCQEDLCN
Mice Ly-6C    LQCYECYGVPIETSCP----AVTCRASDGFCIAQNIELIEDSQR-RKLKTRQCLSF--CPA---GVPIKDPNIRERTSCCSEDLCN
Mice CD59     LQCYNCPNPTA--DCKT---AVNCSSDFDACLITKA---------GLQVYNKCWKFEHCNFNDVTTRLREN--ELTYYCCKKDLCN
Chicken SCA2 LICFSCSDASSNWACLTPV---KCAENEEHCVTTYYGYGIG-GKSGQSISKGCSPV--CPS----AGINLGIAAASVYCCDSFLCN
```

Figure 1-1. Protein sequences of chicken SCA2 with homologous mice Ly-6 family members.
The conserved cystein residues are marked by red.

6. Avian lymphoid tissues

Avian lymphoid tissues include the bursa of Fabricius, thymus, spleen, and other tissues including bone marrow and blood system. The avian bursa of Fabricius is the site for hematopoiesis, B lymphocyte differentiation and proliferation, and lies between the cloaca and the sacrum. The avian thymus is where T lymphocytes differentiate and proliferate, and lies parallel to the vagus nerve and internal jugular veins on each side of the neck where there are 7-8 separate lobes. The spleen is not a primary site for lymphocyte antigen-independent differentiation and proliferation but has an important role in embryonic lymphopoiesis (Olah et al., 2008). Here we only discuss the structure of bursa and thymus as these are the organs where SCA2 may potentially function.

6.1. Avian bursa of Fabricius

The bursa is unique to birds and has a critical role in development of antibody responses. The bursa is an epithelial and lymphoid organ, and contains 15-20 longitudinal folds in the lumen. The bursal folds are filled with hundreds of bursal follicles. Each bursal follicle consists of a medulla and cortex with the supporting epithelium cells between the medulla and the cortex. The cellular composition of the medulla consists of epithelial cells and blood-borne HC including dendritic cells, lymphoid cells, and macrophages. The cortex is separated from the medulla by the cortico-medullary epithelial cells (CMEC). Once the cortical and medullary regions have been separated, bursal B cell proliferation continues in the cortex but the rate of cell division in the medulla is reduced more than 10 fold (Reynolds, 1987). This result suggests that medullary and cortical regions have functional as well as structural differences (Ratcliffe, 2008).

The function of the CMEC in bursa follicles is not understood but it is suggested to have a kind of "nursing "or regulatory function for B cell development. The murine Notch 1 plays important roles in T and B lymphocyte development (reviewed in Schmitt and Zuniga-Pflucker, 2006). The avian Notch ligand, Serrate2, is expressed exclusively in the CMEC and is suggested to regulate B cell differentiation by association with Notch displayed on the B cells (Morimura, et al., 2001). Ramm et al., (1991) suggested that the CMEC represent a macrophage subpopulation. The CMEC express neither B cell antigen nor dendritic cell antigen and are resistance to infectious bursal disease virus (Ramm et al., 1991).

6.2. Avian thymus

The avian thymus lies parallel to the vagus nerve and internal jugular veins. The avian thymus begins to develop as an epithelial outgrowth of the pharyngeal pouches at the age of 5 days of embryonic development, and the thymic epithelium begins to attract blood-borne HC at embryonic incubation days (EID) 6, 12 and 18 (Coltey et al., 1989). At these stages, some molecules expressed on the surface of the bone marrow T cell progenitor have been identified, including MHC class II, c-kit, and αIIβ3integrin (Ody et al., 2000). During embroyonic development, the thymic mass gradually increases with colonization of HSC and results in the appearance of the medulla. Blood-borne HC invade the epithelial analage of the thymus, and immature and proliferating T lymphocytes are present in the subcapsular zone from where they migrate to the medulla during T cell maturation. The cortex and medulla are established by EID 12 and stromal epithelial cells form a network containing lymphoid cells (Coltey et al., 1987). In the thymic cortex, the epithelial reticular cells (ERC) are densely packed with thymocytes and may have a nursing function (Rieker et al., 1995). The development of the T cell lineage was traced using mAb recognizing different chains of the T cell receptor (TCR). At EID 12, a subpopulation of thymocytes begins to express the γδTCR-CD3 complex (TCR^{+}1) on the thymoctye surface (Sowder et al., 1988). The expression patterns of TCR^{+}2 and TCR^{+}3 are similar to mammalian αβTCR cells where the dual expression of the CD4 and CD8 molecules are on the surface of both of TCR^{+}2 and TCR^{+}3 cortical thymocytes (Coltey et al., 1989). These CD4^{+}CD8^{+} double-positive cells mature to become either single positive CD4^{+} or CD8^{+} T cells (Davidson and Boyd, 1992). The histological structure in the thymic medulla is an epithelial cell aggregation but it is not certain if they are actual epithelial cells or stages of differentiation of lympoid cells, and their functional significance is not known, but an endocrine function has been suggested (Isler, 1976; Audhya et al., 1986; Watanabe et al., 2005). The cortico-medullary border contains a dendritic cell barrier expressing the MHC class II and a large number of peroxidase-positive cells, which may play a role in the negative selection of T cells (Guillemot et al., 1984).

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Chapter 2

Cloning of and functional characterization of chicken stem cell antigen 2

Abstract: Stem cell antigen 2 (SCA2) is a Ly-6 family member whose function is largely unknown. To characterize biological properties and tissue distribution of chicken SCA2, SCA2 was expressed in *E. coli,* purified, and a polyclonal antibody developed. Utilizing the polyclonal antibody, SCA2 was detected to be a 13 kDa cell surface protein anchored by a glycosyl-phosphatidylinositol (GPI) moiety. SCA2 is expressed in connective tissues of thymus and bursa based on immunohistochemistry, immunoprecipitation, and western blots. In bursal follicles, SCA2 is specifically expressed on the cortical-medullary epithelial cells (CMEC) surrounded by MHC class II presenting cells. Expression profiles of bursal cells induced by contact with SCA2-expressing cells show down-regulation of numerous genes including *CD79B*, B cell linker (*BLNK*), spleen tyrosine kinase (*SYK*), and gamma 2-phospholipase C (*PLCG2*) that are involved in the BCR and immune response signaling pathways. These results suggest chicken SCA2 plays a role in regulating B lymphocytes.

Introduction

Stem cell antigen 2 (SCA2), also known as lymphocyte antigen 6 complex, locus E (Ly-6E) and thymic shared antigen-1 (TSA-1), is a member of the lymphostromal cell membrane Ly-6 superfamily. Ly-6 family members are low molecular weight (10-12 kDa) proteins with 8-10 conserved cysteine residues, anchored to the cell surface via a glycosyl-phosphatidylinositol (GPI) moiety. In mouse, SCA2 is expressed on thymic medullary epithelial cells and immature T lymphocytes, B lymphocytes, and activated T cells (Godfrey et al., 1992; Classon et al., 1994; Antica and Scollay, 1997). Aside from lymphoid organs, SCA2 is also expressed in other mouse tissues including embryonic adrenal gland and liver, and adult kidney and liver (MacNeil et al., 1993; Classon and Coverdale, 1996; Zammit et al., 2002). SCA2 has been suggested to function in cell-cell adhesion (Classon and Boyd, 1998), signaling transduction via T cell receptor, and T cell development (Randle et al., 1993; Fleming and Malek, 1994; Saitoh et al., 1995; Noda et al., 1996). Some studies showed mouse SCA2 expression was highly associated with tumorigenicity (Eshel et al., 1995; He and Chang, 2004) although the role of SCA2 in tumor progression is not well defined.

The *SCA2* homologue in chicken was initially identified as a transcript highly expressed in v-Rel-transformed avian bone marrow cells (Petrenko et al., 1997). In self-renewing avian erythrocytic progenitor cells called T2ECs (transforming growth factor (TGF)-α, TGF-β induced erythrocytic cells), *SCA2* mRNA is up-regulated T2ECs in comparison to differentiated T2ECs, and inhibition of SCA2 expression through RNA interference substantially reduced cell proliferation and increased differentiation of T2ECs (Gandrillon et al., 1999; Damiola et al., 2004; Bresson-Mazet et al., 2008). These results imply that SCA2 supports the self-renewal of chicken lymphoid cells, and the up-regulation of SCA2 may promote the transformation of lymphoid cells. We previously reported that the allele of the chicken SCA2 can influence resistance to Marek's disease (MD), a viral-induced T cell lymphoma, based on linkage to MD resistance and related traits, induced transcription in Marek's disease virus (MDV)-infected chicken embryo fibroblasts (CEF), transcriptional differences between MD resistant and susceptible experimental chicken lines following challenge with MDV, and direct protein interaction with MDV US10 (Liu et al., 2003). Although studies (Gandrillon et al., 1999; Damiola et al., 2004; Bresson-Mazet et al., 2008) have shown that chicken SCA2 may participate in cell proliferation, differentiation, or tumorigenesis, the biochemical and biological properties of SCA2 are not understood. These studies are further hindered by the lack of a specific antibody to SCA2.

In this report, we describe the purification of chicken SCA2 protein in *E. coli* and development of a polyclonal antibody to SCA2. Utilizing this antibody, the biological properties of chicken SCA2 were investigated. Chicken SCA2 is a 13 kDa protein that is anchored on the cell surface by a GPI moiety. *In vivo* studies show chicken SCA2 is expressed in liver cells, and cells in connective tissues of thymus and bursa. Within bursa follicles, the MHC class II presenting cells are adjacent to the cortical-medullary epithelial cells (CMEC) expressing SCA2. Bursal cells incubated with a SCA2-expressing cell line down-regulate many genes involved in BCR and immune response signaling pathways. These results suggest a role of SCA2 as a microenvironment factor regulating B cell development, and it may act as a functional regulator in lymphomatous disease.

Materials and methods

Cells and animals

DF1, an immortalized cell line from chicken embryonic fibroblasts (Himly et al., 1998), was used for transfection of the SCA2Flag-pSELECT-zeo-mcs vector (Invivogen, San Diego, CA, USA). Chicken lines 0 (lacks endogenous avian leucosis viruses), 6_3 (inbred and MD resistant), and 7_2 (inbred and MD susceptible) were maintained at the Avian Disease and Oncology Laboratory (ADOL), USDA. White rabbits were purchased from Harlan Laboratories, Inc. (Indianapolis, IN, USA).

Development of anti-SCA2 rabbit antisera

Primers (SCA2F: 5' GC GAA TCC CAC TCA TCT GCT TTT CGT GCT CG 3'; SCA2R: 5' CG CTC GAG TGG GCC GCC TAA TAG CTG GCT TTA ACG C 3'; restriction sites are underlined) based on GenBank accession no. NM_204775 were designed to amplify chicken SCA2 without the signal peptide and GPI anchor (bases 115 to 375). Using a chicken spleen cDNA library as template, the amplicon was cloned into the pET-28c prokaryotic expression vector between the *EcoR*I and *Xho*I sites (Novagen, Madison, WI, USA), forwarded by DNA sequencing to verify the construct. The recombinant vector containing both N- and C-terminal His tags was transformed into *E. coli* strain BL21 (DE3) (Novagen). The expression and purification of the SCA2-His recombinant protein followed the pET system protocol (Novagen). The expressed SCA2 was examined through western blotting using anti-His tag monoclonal antibody (mAb; Invitrogen, Carlsbad, CA, USA) and purified through His. Bind kits (Novagen). Purified chicken SCA2 was electrophoresed through a 10% SDS-PAGE gel, and the 13 kDa band removed and mixed with Imject Alum (Pierce, Rockford, IL, USA). The antigen-alum mixture was administered into multiple intramuscular, intradermal, or subcutaneous sites of two rabbits. Four weeks after the primary immunization, booster immunizations were given and the animals were bled 7 days later. The anti-SCA2 sera were obtained after the fifth boost.

Generation of chicken SCA2 eukaryotic expression vector and stable transfection cell line

Primers (SCA2SalIF: 5' ATCG <u>GTC GAC</u> ATG AAG GCG TTT CTG TTC GC 3'; SCA2NCOIR: 5' ATCG <u>CCA TGG</u> TCA CTC ACG AGC CCT GAG G 3'; restriction sites are underlined) were designed to amplify the entire coding portion of chicken SCA2 (bases 55 to 435). The amplicon was inserted between the SalI and NcoI sites of the pSELECT-zeo-mcs vector (Invivogen) to generate SCA2-pSELECT-zeo-mcs. A Flag tag was inserted immediately downstream of the signal peptide of SCA2 by PCR using SCA2-pSELECT-zeo-mcs as the template with the primers (SCA2SalIF; Sca2FlagNsiIR: 5' GTT GGA GG <u>ATG CAT</u> CCG AGC ACG AAA AGC AGA TGA *GCT TAT CGT CGT CAT CCT TGT AAT C*CG TGT GGG CTC TCT CCA CG 3'; restriction sites are underlined, Flag tag sequences are in italic). The amplicon was inserted between the *Sal*I and *Nsi*I sites of SCA2-pSELECT-zeo-mcs to construct SCA2Flag-pSELECT, which was confirmed by DNA sequencing. The SCA2Flag-pSELECT-zeo-mcs and empty pSELECT-zeo-mcs vector were independently transfected into the DF1 cell line using the Basic Nucleofector Kit (Amaxa Inc., Gaithersburg, MD, USA). The transfected cells were incubated in medium containing Liebovitz's L-15 and

McCoy 5A media (1:1) supplemented with 5% fetal bovine serum (FBS), penicillin, streptomycin, and zeocin (300 ng/ml) until single colonies appeared. The expression of SCA2 was tested by western blots using anti-Flag mAb and anti-SCA2 antisera. The single colony with the best SCA2 expression was maintained and called the SCA2Flag-DF1 cell line. The single colony with the empty vector was maintained and named the Vector-DF1 cell line.

Flow cytometry analysis

Cell surface expression of SCA2 was measured as previously reported (Hunt et al., 2001). SCA2Flag-DF1 cells and DF1 cells were trypsinized and harvested (1000xg, 5 min.). Thymocytes were collected from 2 to 4-week old ADOL Line 7_2 and Line 6_3 chicken thymus, followed by centrifugation (1000xg, 5 min.). 10^5 cells were washed with cold flow cytometry buffer (phosphate buffered saline containing 1% FBS and 0.1% sodium azide). Anti-Flag M2 mAb (1:100; Sigma-Aldrich Inc, Milwaukee, WI, USA) and anti-SCA2 antisera (1:100) were diluted in flow cytometry buffer and reacted with cells for 20 min. at 4 °C, followed by washing and incubation with goat anti-mouse and goat anti-rabbit IgG conjugated with FITC (1:100; Zymed, South San Francisco, CA, USA). The cells were analyzed using a Becton-Dickinson FACScaliber flow cytometer and CellQuest Pro software (BD Bioscience, San Jose, CA, USA).

Cleavage of GPI-linked cell surface SCA2 molecules

10^5 SCA2Flag-DF1 and DF1 cells were incubated with 0.2-0.5 unit of phosphatidylinositol-specific phospholipase C (PI-PLC; Sigma-Aldrich Inc.) in 200 µl flow cytometry buffer at 37 °C for 30 min. Cells were analyzed by flow cytometry analysis as described in section 2.4.

Western blots and immunoprecipitation

Cells and tissues were lysed in Cellytic M Mammalian Cell lysis reagent (Sigma-Aldrich Inc., Milwaukee, WI, USA), supplemented with 1% P8340 protease inhibitor cocktails (Sigma-Aldrich Inc.), followed by sonication and centrifugation. Cell lysates were mixed with loading and reducing buffer, heated to 70°C for 10 min. and resolved using 10% SDS-PAGE gels (Invitrogen). The samples were blotted on Immobilon-P^{SQ} PVDF transfer membranes (Millipore, Billerica, MA, USA). After blocking in PBS containing 5% non-fat milk for 1 hour at room temperature, membranes were probed with anti-Flag M2 mAb (1:100) or anti-SCA2 antisera (1:100) at 4 °C overnight. Membranes were washed three times in PBS before incubation with goat anti-mouse or goat anti-rabbit IgG conjugated with horseradish peroxidase (Zymed) at room temperature for 1 hour. Proteins were detected using ECL Western blotting substrate (Pierce) after washing three times. Immunoprecipitation using anti-SCA2 antisera followed the instructions of the Immunoprecipitation Kit (Roche, Indianapolis, IN, USA).

Immunohistochemistry

Multiple tissues were collected from 4-week old ADOL Line 7_2 birds. The tissues were soaked into compound optimum cutting temperature embedding medium (Tissue-Tek, Torrance, CA, USA) and stored at -80 °C. Samples of flash frozen chicken bursa were sectioned at 5 μm on a Cryotome FSE (Thermo Fisher, Pittsburgh, PA, USA) at -

20 °C. Slides were placed in -70 °C for storage until staining. Reagent incubation was as follows: anti-SCA2 antisera (1:1000) in normal antibody diluent (NAD) (Scytek Laboratories, Logan, UT, USA) for 60 min. at room temperature; biotinylated goat anti-rabbit IgG (h+l) (Vector Laboratories, Burlingame, CA, USA) in NAD for 30 min.; Alkaline Phosphatase Complex Reagent (Kirkeguard & Perry Laboratories, Gaithersburg, MD, USA) for 60 min.; and developed using Vector Red substrate I (Vector Laboratories, Burlingame, CA, USA) for 8 min.

Double staining was performed on the frozen bursa follicles. Tissue sections were incubated with anti-SCA2 antisera (1:1000) in NAD for 60 min. at room temperature; biotinylated goat anti-rabbit IgG(h+l) in NAD for 30 min.; Vectastain Elite ABC Reagent (Vector Laboratories) for 30 min.; and developed using DAB (Vector Laboratories) for 15 min.; followed with the second antibody anti-MCH II mAb TAP1 (1:200; Developmental Studies Hybridoma Bank, Iowa City, IA, USA) in NAD for 60 min. at room temperature; biotinylated horse anti-mouse IgG(h+l) (Vector Laboratories) in NAD for 40 min.; Alkaline Phosphatase Complex Reagent for 60 min.; and developed using Vector Red substrate I for 8 min.

Affymetrix array processing and data analysis

Bursa of 3-week old line 0 chickens were collected and manually teased to harvest the bursal cells. 2×10^6 bursal cells were spread on the monolayers of SCA2Flag-DF1 cells or control Vector-DF1 cells in triplicate. After growth in the mixture of Liebovitz's L-15 and McCoy 5A media (1:1) supplemented with 4% FBS, penicillin, and streptomycin for overnight at 37 °C in 5% CO_2, the bursal cells were recovered by washes from the adhesive DF1 cells followed by gentle centrifugation at 1000×g for 10 min. Total RNA was isolated using the absolutely RNA microprep kit (Stratagene, La Jolla, CA, USA). The quantity and quality of total RNA were analyzed on an Agilent 2100 Bioanalyzer using the RNA 6000 Nano Chip Kit according to the manufacturer's instructions (Agilent Technologies, Santa Clara, CA, USA). The cDNA synthesis, labeling, hybridization and scanning of Affymetrix GeneChip Chicken Array (Affymetrix, Inc., Santa Clara, CA, USA) were carried out at the Michigan State University Research Support Technology Facility (RSTF). The .CEL files (GEO accession no.GSE18506) were provided and normalized at the probe level using the robust multichip average (RMA) method (Irizarry et al., 2003) implemented into the Affymetrix Expression Console software; RMA is a robust linear model analyzing data for background adjustment, quantile normalization, and log2-transformation of the perfect match probe intensities for each probe set. Statistical comparison of differences between the normalized mRNA expression in SCA2-induced and control bursal cells was performed through significance analysis of microarrays (SAM) software (Tusher, et al., 2001). The significant differential expressions between SCA2-induced and control samples were filtered based on the following criteria: 1) false discovery rate<5% and 2) fold change ≥2. Gene ontology annotations associated to Affymetrix DNA probe sets were analyzed through Ingenuity Pathways Analysis software (Ingenuity Systems Inc, Redwood, CA, USA). Biological processes were identified by inputting differentially expressed gene sets into the Metacore database (GeneGo Inc., St. Joseph, MI, USA) and the biological processes were ranked according to their P value.

Real-Time quantitative reverse transcription PCR

Selected chicken transcripts were measured using real time quantitative reverse transcription PCR (qRT-PCR). Briefly, 1 µg of total RNA was used for cDNA synthesis with the Improm-II Reverse Transcription System according to the manufacturer's protocol (Promega, Madison, WI, USA). The primers were designed using Primer Express Software v3.0 (Applied Biosystems, Foster City, CA, USA). Primers listed in Table 2-1 were synthesized by Operon Biotechnologies, Inc (Huntsville, Al, USA). 2.5 µM of each forward and reverse primer were used in the presence of 25 µl SYBR GREEN PCR master mix (Applied Biosystems, Foster City, CA, USA) in a final volume of 50 µl. The real-time PCR program was as follows: 95 °C for 10 min., 40 cycles at 95 °C for 15 sec followed by 60 °C for 1 min., in an ABI 7500 Sequence Detection System (Applied Biosystems). The expression of each gene was normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) through relative quantification using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Table 2-1. Primers used for quantitative real-time PCR validation of microarray data

Gene	Primer	Sequence (5' to 3')
GAPDH	Forward	TGCCATCACAGCCACACAGAAG
	Reverse	ACTTTCCCCACAGCCTTAGCAG
CD79B	Forward	AGGAGTTCCACGTGCTGGAT
	Reverse	GAAGCTGATGCGGTCATTTGT
SYK	Forward	CCAAAAACCTCAGCTGGAGAA
	Reverse	TCTGACTCTTCCCGAGAGATCCT
BTK	Forward	CCCAGAGCTCATCACGTACCA
	Reverse	GACACGGGATACTTCAGTCTGGAT
BLNK	Forward	GCCTTGCCCAGACCAAAGA
	Reverse	CCCTCGGCTTCAGTGGTAATT
PLCG2	Forward	GAATTTGATCTGCGTCTCACTGA
	Reverse	CCTCGACTCAAGTTGCTATAGTACCA
THY1	Forward	CAGCGTCTCCGAGAACGTCTA
	Reverse	GAGGCACACCAGGTTCTTGTG

Results

Chicken SCA2 is a 13 kDa cell surface protein anchored by a GPI moiety

The eukaryotic expression vector SCA2Flag-pSELECT-zeo-mcs includes the entire SCA2 cDNA sequence and a Flag tag immediately downstream of the SCA2 signal peptide. In SCA2Flag-DF1 stably transfected cells, SCA2Flag ran with a mobility of about 13 kDa on denatured SDS-PAGE gels as measured by western blot analysis utilizing both anti-Flag mAb and anti-SCA2 polyclonal antisera (Fig. 2-1). *SCA2* encodes 126 amino acids and the mature SCA2 protein is predicted to possess 85 amino acids. The 13 kDa molecular size suggests potential posttranslational modifications of SCA2 in DF1 cells. In SCA2Flag-DF1 cells, SCA2 is displayed on the cell surface as determined by flow cytometry using both anti-Flag mAb and anti-SCA2 antisera (Fig. 2-2). When the viable SCA2Flag-DF1 cells were incubated with PI-PLC, a phospholipase that specifically cleaves the GPI anchor, the SCA2 molecule is cleaved from the surface of SCA2Flag-DF1 cells in a dose dependent manner. The surface expression of control DF1 cells were not affected by the enzyme. These results confirm the surface expression of SCA2 with a direct attachment of the GPI anchor to the C terminus of SCA2.

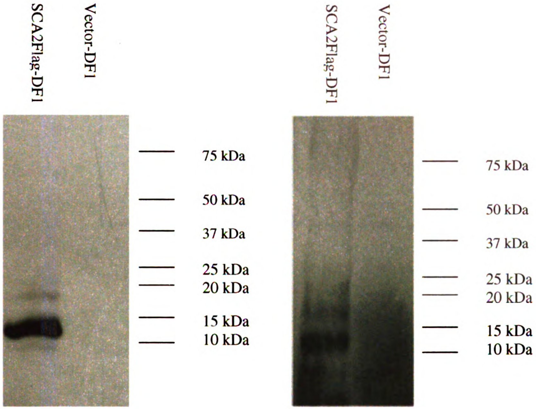
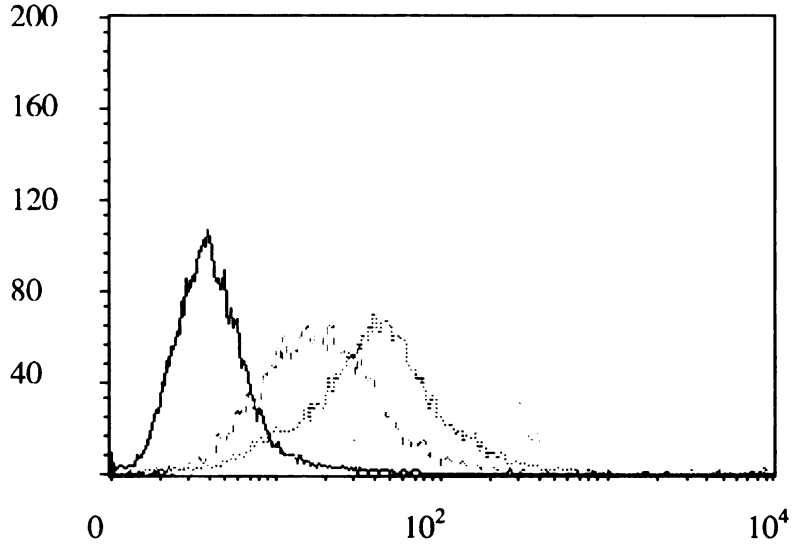


Figure 2-1. Western blots using anti-Flag mAb or anti-SCA2 antisera. DF1 cells transfected with SCA2Flag or empty vector probed with a) anti-Flag mAb or b) anti-SCA2 antisera. The size of the protein standards are indicated. The molecular size of SCA2 is about 13 kDa.

a)



b)

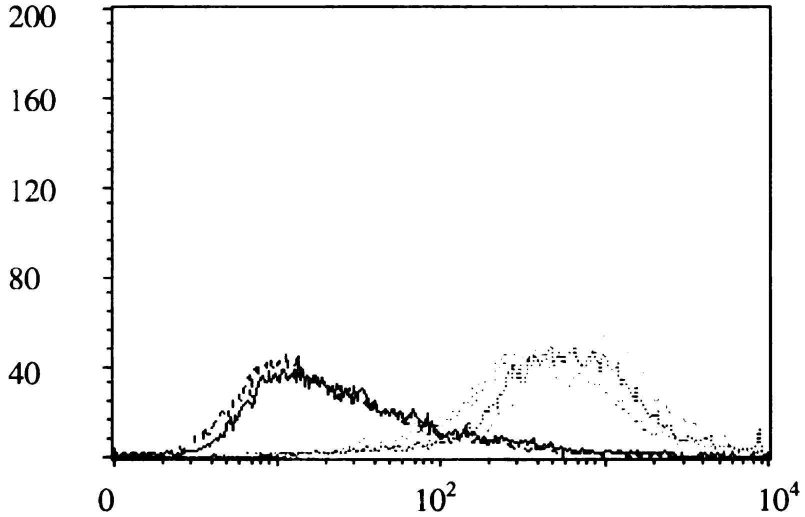


Figure 2-2. Flow cytometry analysis of SCA2Flag-DF1 and vector-DF1 with anti-Flag mAb and anti-SCA2 antisera. a) SCA2Flag-DF1 (green line) and Vector-DF1 (black line) stained with anti-Flag mAb; SCA2Flag-DF1 treated with 0.2 units and 0.5 units of PI-PLC stained with anti-Flag mAb (pink and blue dotted lines, respectively). b) SCA2Flag-DF1 stained with anti-SCA2 antisera (green line) and prebleed antisera (black line); and SCA2Flag-DF1 treated with 0.2 units and 0.5 units of PI-PLC and stained with anti-SCA2 antisera (pink and blue dotted lines, respectively), SCA2Flag-DF1 treated with 0.5 units of PI-PLC and stained with prebleed antisera (purple dotted line).

Tissue distribution of chicken SCA2

Frozen sections of selected organs were stained with the anti-SCA2 polyclonal antisera or prebleed antisera as a negative control. As shown (Fig. 2-3), SCA2 is strongly expressed in liver cells and connective cells in thymus and bursa. In the liver, hepatocytes were stained uniformly for SCA2 and the endothelial cells also stained strongly. In the thymus, SCA2 is expressed on connective epithelial cells. In bursa, SCA2 is specifically expressed on CMEC and medullary reticular epithelial cells. In the spleen, weak staining was observed in red pulp areas. Weak staining was also observed in gonad, heart and lung tissues. The molecular weight of SCA2 expression in these organs was determined by western blot. SCA2 ran with a mobility of 13 kDa, consistent with SCA2Flag expressed in SCA2Flag-DF1 cells (Fig. 2-4). The results show abundant SCA2 expression in thymus, bursa, liver and spleen and relatively sparse expression in gonads, heart and lung.

In bursal follicles, SCA2 is specifically expressed in CMEC and reticular epithelial cells in the medulla (Fig. 2-5a). In the immunohistochemistry image of bursa follicles stained with both anti-SCA2 antisera and anti-MHC class II mAb, the majority of MHC class II presenting cells are located adjacent to the CMEC on which SCA2 is strongly and specifically stained (Fig. 2-5b, c). Under the fluorescent microscope, the areas positive for MHC class II and the areas positive for SCA2 do not overlap. The immunohistochemistry results show CMEC and MHC class II presenting cells are two distinct types of cells, and the MHC class II presenting cells are probably attracted to the CMEC on both sides of the medullar cortex.

Figure 2-3. Immunohistochemical analysis of chicken SCA2 expression in chicken tissues. Tissues from 4-week old line 7_2 birds were stained with anti-SCA2 antisera. Magnification of the bursal follicle is 400x. All the other magnifications are 100x.

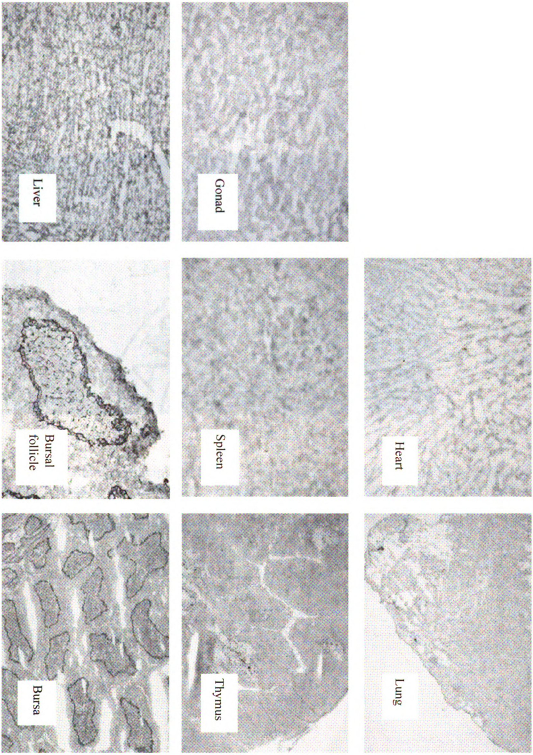

Liver
Gonad
Bursal follicle
Spleen
Heart
Bursa
Thymus
Lung

Figure 2-3

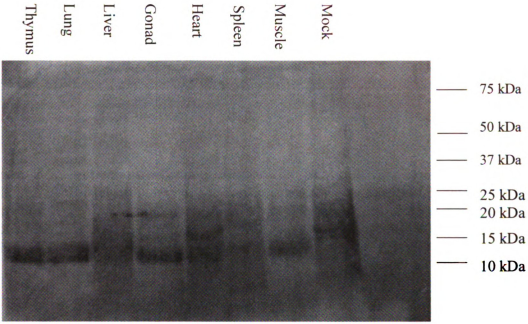


Figure 2-4. Immunoprecipitation and western blot analysis of SCA2 expression in chicken tissues. Cell lysates from chicken tissues were immunoprecipitated with anti-SCA2 antisera and detected with anti-SCA2 antisera through western blots. The size of the protein standards are indicated.

Figure 2-5. Immunohistochemical analysis of SCA2 expression in bursal follicles. a) Bursal follicles were doubly stained with anti-SCA2 antisera (gray/black) and anti-MHC class II mAb (red). An identical section viewed under b) bright and c) fluorescent lighting; with the fluorescent microscope, only MHC class II is illuminated in red.

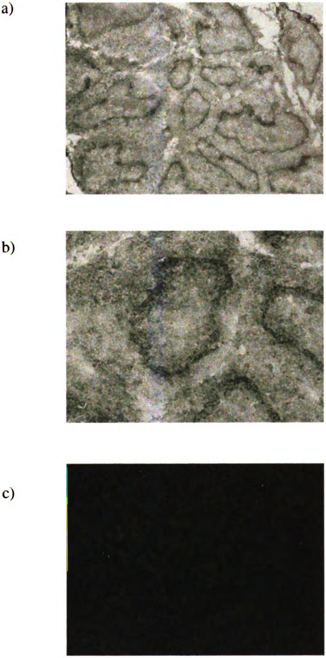


Figure 2-5

Gene expression profiling of bursal cells in contact with SCA2-expressing cells

Bursal cells incubated with SCA2-expressing (SCA2Flag-DF1) or negative control (Vector-DF1) cells were profiled using Affymetrix chicken genome arrays and analyzed. 95 genes are significantly under-expressed (Table 2-2) and 68 genes are over-expressed (Table 2-3) in the SCA2-induced bursal cells in comparison to the control. In contrast to the over-expressed genes, many of the genes with decreased expression were related to B cell proliferation, development, and immune response. The results of biological process analysis through the MetaCore database showed the set of down-regulated genes is induced in a statistically significant manner in comforment of several pathways including the BCR signaling pathway ($P \leq 1.6 \times 10^{-7}$) and immune response signaling in B lymphocytes ($P \leq 3.2 \times 10^{-11}$). To validate the microarray results, selected under-expressed (*CD79B*, *SYK*, *BLNK BTK*, *PLCG2*) and over-expressed (*THY-1*) genes were analyzed by qRT-PCR. Using the chicken GAPDH gene for normalization, all the queried genes showed an expression pattern similar to the results from the microarray (Table 2-4).

Table 2-2. Genes under-express in SCA2 induced bursal cells

Probe ID	Gene Symbol	Gene Name
Gga.5273.1.S1_at	ADRBK2	adrenergic, beta, receptor kinase 2
GgaAffx.9065.1.S1_at	AICDA	activation-induced cytidine deaminase
GgaAffx.21699.1.S1_at	ANKRD44	ankyrin repeat domain 44
Gga.7334.1.S1_at	APBB1IP	amyloid beta (A4) precursor protein-binding, family B,
member 1	2.86	
GgaAffx.7881.1.S1_s_at	ARHGAP15	Rho GTPase activating protein 15
Gga.19387.1.S1_at	ATXN7	ataxin 7
GgaAffx.20426.1.S1_at	BCL11A	B-cell CLL/lymphoma 11A (zinc finger protein)
Gga.3738.1.S1_at	BLNK	B-cell linker
GgaAffx.11885.1.S1_s_at	BTK	Bruton agammaglobulinemia tyrosine kinase
Gga.10069.1.S1_at	CBFA2T3	core-binding factor, runt domain, alpha subunit 2;
translocated to, 3	2.25	
Gga.5128.1.S1_at	CCL20	chemokine (C-C motif) ligand 20
GgaAffx.11849.1.S1_s_at	CD36	CD36 molecule (thrombospondin receptor)
Gga.998.2.S1_a_at	CD3D	CD3d molecule, delta (CD3-TCR complex)
Gga.14078.1.S1_s_at	CD72	CD72 molecule
GgaAffx.151.1.S1_s_at	CD79B	CD79b molecule, immunoglobulin-associated beta
Gga.17250.1.S1_at	CDKN1B	cyclin-dependent kinase inhibitor 1B (p27, Kip1)
Gga.9816.1.S1_s_at	CLIC2	chloride intracellular channel 2
GgaAffx.12918.1.S1_s_at	CYTH4	cytohesin 4
GgaAffx.21308.1.S1_s_at	DCBLD1	discoidin, CUB and LCCL domain containing 1
Gga.19843.1.S1_s_at	DENND5B	DENN/MADD domain containing 5B
Gga.3573.2.S1_a_at	DKK3	dickkopf homolog 3 (Xenopus laevis)
Gga.6002.1.S1_a_at	DNMT3A	DNA (cytosine-5-)-methyltransferase 3 alpha
Gga.6032.1.S1_at	DOCK8	dedicator of cytokinesis 8
Gga.445.1.S1_at	EG667604	predicted gene, EG667604
Gga.8031.2.S1_a_at	FAM18A	family with sequence similarity 18, member A
Gga.13533.1.S1_at	FAM65B	family with sequence similarity 65, member B
Gga.4564.2.S1_a_at	FMO6	flavin containing monooxygenase 6 pseudogene
Gga.588.1.S1_at	GFRA1	GDNF family receptor alpha 1
GgaAffx.13238.1.S1_s_at	GLCCI1	glucocorticoid induced transcript 1
Gga.9138.1.S1_at	GMIP	GEM interacting protein
Gga.5289.1.S1_at	GPD1	glycerol-3-phosphate dehydrogenase 1 (soluble)
Gga.1092.1.S1_s_at	GPR174	G protein-coupled receptor 174
Gga.10880.1.S1_at	GRAP	GRB2-related adaptor protein
GgaAffx.11705.1.S1_at	HAGHL	hydroxyacylglutathione hydrolase-like
GgaAffx.11387.1.S1_s_at	HCLS1	hematopoietic cell-specific Lyn substrate 1
Gga.11016.1.S1_at	HLA-DMA	major histocompatibility complex, class II, DM alpha
Gga.13336.2.S1_x_at	HLA-DRB1	major histocompatibility complex, class II, DR beta 1
Gga.16655.1.S1_at	HMHA1	histocompatibility (minor) HA-1
Gga.14571.1.S1_s_at	HPS5	Hermansky-Pudlak syndrome 5
Gga.11539.1.S1_at	IFI30	interferon, gamma-inducible protein 30
Gga.3366.1.S1_at	IL20RA	interleukin 20 receptor, alpha
Gga.4326.1.S1_at	JAK3	Janus kinase 3 (a protein tyrosine kinase, leukocyte)
GgaAffx.26566.1.S1_at	IP6K2	inositol hexakisphosphate kinase 2
Gga.1235.2.S1_at	KCNAB2	potassium voltage-gated channel, shaker-related
subfamily, beta member 2	2.70	
Gga.9912.1.S1_at	KLHL6	kelch-like 6 (Drosophila)
GgaAffx.11727.1.S1_at	LCP1	lymphocyte cytosolic protein 1 (L-plastin)
GgaAffx.13207.1.S1_s_at	LIMD2	LIM domain containing 2

Continued Table 2-2. Genes under-express in SCA2 induced bursal cells

GgaAffx.5229.1.S1_at	LRRN1	leucine rich repeat neuronal 1	
Gga.4491.1.S1_s_at	LSP1	lymphocyte-specific protein 1	
Gga.4354.1.S1_at	M6PR	mannose-6-phosphate receptor (cation dependent)	
GgaAffx.6232.1.S1_s_at	MDM1	Mdm1 nuclear protein homolog (mouse)	
Gga.5323.1.S1_at	MEF2C	myocyte enhancer factor 2C	
GgaAffx.12551.1.S1_at	MGC33894	transcript expressed during hematopoiesis 2	
Gga.8736.1.S1_at	MICAL1	microtubule associated monoxygenase, calponin and LIM domain containing 1	2.28
Gga.8880.1.S1_at	MME	membrane metallo-endopeptidase	
Gga.16267.1.S1_at	MR1	major histocompatibility complex, class I-related	
GgaAffx.11413.1.S1_s_at	NCOA7	nuclear receptor coactivator 7	
GgaAffx.11420.1.S1_s_at	NECAP2	NECAP endocytosis associated 2	
Gga.17430.1.S1_at	NUP210	nucleoporin 210kDa	
Gga.13291.1.S1_at	OIT3	oncoprotein induced transcript 3	
Gga.12076.1.S1_at	P2RX5	purinergic receptor P2X, ligand-gated ion channel, 5	
Gga.11516.1.S1_at	P2RY6	pyrimidinergic receptor P2Y, G-protein coupled, 6	
GgaAffx.9707.1.S1_s_at	PCMTD1	protein-L-isoaspartate (D-aspartate) O-methyltransferase domain containing 1	2.10
Gga.4916.1.S2_s_at	PDCD4	programmed cell death 4 (neoplastic transformation inhibitor)	2.40
Gga.71.1.S1_at	PDGFB	platelet-derived growth factor beta polypeptide (simian sarcoma viral oncogene homolog)	2.27
Gga.10030.1.S1_s_at	PIK3R5	phosphoinositide-3-kinase, regulatory subunit 5	
Gga.7148.2.A1_at	PLCG2	phospholipase C, gamma 2 (phosphatidylinositol-specific)	
Gga.19667.1.S1_at	PREX1	phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor 1	3.33
Gga.8329.1.S1_at	PRKCH	protein kinase C, eta	
Gga.9379.1.S1_s_at	PTPN6	protein tyrosine phosphatase, non-receptor type 6	
GgaAffx.12720.1.S1_at	RABGAP1L	RAB GTPase activating protein 1-like	
GgaAffx.11475.1.S1_s_at	RASSF2	Ras association (RalGDS/AF-6) domain family member 2	
Gga.9386.1.S1_at	RBP7	retinol binding protein 7, cellular	
Gga.15728.1.S1_at	RCSD1	RCSD domain containing 1	
Gga.352.1.S1_at	RGS20	regulator of G-protein signaling 20	
Gga.4113.1.S1_at	RHOF	ras homolog gene family, member F (in filopodia)	
GgaAffx.2351.2.S1_s_at	RORA	RAR-related orphan receptor A	
Gga.408.1.S1_at	RPE65	retinal pigment epithelium-specific protein 65kDa	
GgaAffx.12264.1.S1_s_at	SASH3	SAM and SH3 domain containing 3	
Gga.7956.1.S1_at	SH3BGRL	SH3 domain binding glutamic acid-rich protein like	
Gga.6286.1.S1_at	SIAH2	seven in absentia homolog 2 (Drosophila)	
Gga.8508.1.S2_s_at	SLA	Src-like-adaptor	
GgaAffx.11711.1.S1_s_at	SNAP91	synaptosomal-associated protein, 91kDa homolog (mouse)	3.29
Gga.6976.1.S1_at	SPINK5	serine peptidase inhibitor, Kazal type 5	
GgaAffx.4780.1.S1_at	STAT4	signal transducer and activator of transcription 4	
GgaAffx.943.1.S1_s_at	SPNS3	spinster homolog 3 (Drosophila)	
Gga.1148.1.S2_at	ST6GAL1	ST6 beta-galactosamide alpha-2,6-sialyltranferase 1	
GgaAffx.12873.1.S1_at	STRBP	spermatid perinuclear RNA binding protein	
GgaAffx.12646.1.S1_at	SYK	spleen tyrosine kinase	
Gga.11781.1.S1_at	TM6SF1	transmembrane 6 superfamily member 1	
Gga.16833.1.S1_at	TP53INP1	tumor protein p53 inducible nuclear protein 1	
GgaAffx.12557.1.S1_s_at	TPST2	tyrosylprotein sulfotransferase 2	
GgaAffx.12475.1.S1_at	TRAF5	TNF receptor-associated factor 5	
Gga.15780.1.S1_at	WDR66	WD repeat domain 66	
Gga.17034.1.S1_s_at	YPEL2	yippee-like 2 (Drosophila)	

Table 2-3. Genes over-express in SCA2 induced bursal cells

Probe ID	Gene Symbol	Gene Name
GgaAffx.6058.1.S1_at	AADAT	aminoadipate aminotransferase
Gga.10984.1.S1_at	AIDA	axin interactor, dorsalization associated
Gga.12321.1.S1_at	ALG11	asparagine-linked glycosylation 11, alpha-1,2-
mannosyltransferase homolog (yeast)		2.02
Gga.2301.1.S1_at	APCDD1	adenomatosis polyposis coli down-regulated 1
Gga.11793.1.S1_at	ATG10	ATG10 autophagy related 10 homolog (S. cerevisiae)
Gga.8363.1.S1_at	AXIN2	axin 2
Gga.2840.1.S1_at	B4GALT1	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase,
polypeptide 1	2.59	
Gga.12194.1.S1_at	CBY1	chibby homolog 1 (Drosophila)
GgaAffx.21817.1.S1_s_at	CDH13	cadherin 13, H-cadherin (heart)
Gga.2592.1.S1_at	COL5A1	collagen, type V, alpha 1
Gga.4257.1.S1_at	COL6A2	collagen, type VI, alpha 2
Gga.2584.1.S1_s_at	CPNE2	copine II
Gga.4211.1.S1_at	CRTAP	cartilage associated protein
Gga.184.1.S1_a_at	CSPG5	chondroitin sulfate proteoglycan 5 (neuroglycan C)
Gga.16050.1.S1_s_at	DNAJC3	DnaJ (Hsp40) homolog, subfamily C, member 3
Gga.9493.1.S1_at	DPYSL3	dihydropyrimidinase-like 3
GgaAffx.11017.5.S1_s_at	DYNC2H1	dynein, cytoplasmic 2, heavy chain 1
Gga.17040.2.S1_a_at	FGF2	fibroblast growth factor 2 (basic)
Gga.8858.1.S1_s_at	FKBP7	FK506 binding protein 7
Gga.2663.1.S1_at	FKBP9	FK506 binding protein 9, 63 kDa
GgaAffx.1557.1.S1_at	GPC1	glypican 1
GgaAffx.4054.1.S1_at	HECTD2	HECT domain containing 2
Gga.13785.1.S1_at	HNRPLL	heterogeneous nuclear ribonucleoprotein L-like
Gga.3066.1.S1_at	HS1BP3	HCLS1 binding protein 3
Gga.4724.1.S2_at	HSP90B1	heat shock protein 90kDa beta (Grp94), member 1
Gga.892.1.S1_at	ID1	inhibitor of DNA binding 1, dominant negative helix-
loop-helix protein	5.11	
GgaAffx.20617.1.S1_at	IFT81	intraflagellar transport 81 homolog (Chlamydomonas)
Gga.14123.1.S1_at	ISM1	isthmin 1 homolog (zebrafish)
Gga.16230.2.S1_a_at	KCTD3	potassium channel tetramerisation domain containing 3
Gga.7898.1.S1_at	LCLAT1	lysocardiolipin acyltransferase 1
Gga.15876.1.S1_at	LRIG3	leucine-rich repeats and immunoglobulin-like domains 3
Gga.13162.1.S1_at	MANEA	mannosidase, endo-alpha
Gga.2550.1.S1_at	MAT1A	methionine adenosyltransferase I, alpha
Gga.6218.1.S1_at	MFAP5	microfibrillar associated protein 5
Gga.10617.1.S1_a_at	MSRB3	methionine sulfoxide reductase B3
Gga.3332.1.S1_at	MXRA8	matrix-remodelling associated 8
Gga.18504.1.S1_at	PARVA	parvin, alpha
Gga.5300.1.S1_at	PDE6D	phosphodiesterase 6D, cGMP-specific, rod, delta
Gga.3398.2.S1_a_at	PITX2	paired-like homeodomain 2
Gga.4726.1.S1_at	PLOD1	procollagen-lysine 1, 2-oxoglutarate 5-dioxygenase 1
Gga.9989.1.S1_at	PLXDC2	plexin domain containing 2
Gga.4856.1.S1_s_at	PMP22	peripheral myelin protein 22
Gga.11468.1.S1_a_at	PPIC	peptidylprolyl isomerase C (cyclophilin C)
Gga.1546.1.S2_a_at	PRRX1	paired related homeobox 1
Gga.5640.1.S1_at	PRSS23	protease, serine, 23
Gga.9612.1.S1_at	RDH14	retinol dehydrogenase 14 (all-trans/9-cis/11-cis)
Gga.5921.1.S1_at	REEP5	receptor accessory protein 5
Gga.8500.1.S1_at	RGS2	regulator of G-protein signaling 2, 24kDa
Gga.10018.1.S1_at	RIN3	Ras and Rab interactor 3

Continued Table 2-3. Genes over-express in SCA2 induced bursal cells

Gga.4516.2.S1_at	SEPT11	septin 11
Gga.7890.1.S1_at	SFRP4	secreted frizzled-related protein 4
Gga.13449.1.S1_s_at	SGCE	sarcoglycan, epsilon
GgaAffx.8046.1.S1_s_at	SLC41A2	solute carrier family 41, member 2
Gga.19099.2.S1_a_at	SPARC	secreted protein, acidic, cysteine-rich (osteonectin)
Gga.17868.1.S1_s_at	SPATA6	spermatogenesis associated 6
Gga.16722.1.S1_at	SRGAP1	SLIT-ROBO Rho GTPase activating protein 1
Gga.485.1.S1_a_at	TEAD4	TEA domain family member 4
Gga.9244.2.S1_a_at	TEKT4	tektin 4
GgaAffx.4539.1.S1_at	TEKT5	tektin 5
Gga.4153.1.S2_a_at	THY1	Thy-1 cell surface antigen
GgaAffx.13062.1.S1_at	TMEM129	transmembrane protein 129
Gga.19252.1.S1_s_at	TMEM184C	transmembrane protein 184C
Gga.10985.1.S1_at	TMEM45A	transmembrane protein 45A
GgaAffx.6948.1.S1_at	TMTC2	transmembrane and tetratricopeptide repeat containing 2
Gga.4975.1.S1_a_at	TPM3	tropomyosin 3
GgaAffx.12952.1.S1_at	USP28	ubiquitin specific peptidase 28
Gga.3250.1.S1_at	VSNL1	visinin-like 1
Gga.11611.1.S1_at	WIF1	WNT inhibitory factor 1

Table 2-4. Real-time quantitative reverse transcription PCR analysis of differential expression for bursal cells incubated with SCA2-expressing or control DF1 cells

Gene	SCA2[1]	Control[1]	Fold Change
CD79B	6.56±0.82	3.21 ±0.39	-10.2
BLNK	6.38 ±0.63	4.82 ±0.36	-2.9
BTK	11.94 ±0.89	7.86 ±0.36	-16.9
PLCG2	10.67 ±0.74	7.62 ±0.41	-8.2
SYK	10.79 ±0.76	7.48 ±0.39	-9.9
THY-1	4.95 ±0.80	8.33 ±0.53	10.4

[1] Ct mean and standard deviation for three replicates.

Discussion

Although chicken *SCA2* gene expression was associated with tumorigenicity and self-renewal of avian erythroid progenitors, the natural biochemical and biological properties of SCA2 protein are unknown. We here describe the development of a polyclonal antibody to chicken SCA2 and its application to the analysis of the biological properties and tissue distribution of SCA2. SCA2 is a 13 kDa small molecular weight, cell surface protein anchored by a GPI moiety, which is similar to homologs in other species. The expression pattern of SCA2 in chicken is relatively wide, and it is abundantly expressed in some tissues. In thymus, strong staining using anti-SCA2 antisera was on the connective cells. In bursa, SCA2 was specifically expressed on the CMEC. SCA2 also shows strongly expression in hepatocytes and endothelial cells in liver. Anti-SCA2 antisera stained weakly in the spleen, lung, heart and gonad. The expression pattern of SCA2 is similar in chicken and mouse in which SCA2 was abundantly expressed in thymic epithelial cells in thymus and in other tissues including the embryonic adrenal gland and liver and adult kidney and liver.

Although the function of mouse SCA2 is not well defined, the literature suggests its participation in cell-cell adhesion, signal transduction, and T cell differentiation. The deletion of SCA2 in mouse resulted in abnormal adrenal gland and midgestational lethality due to cardiac abnormalities (Zammit et al., 2002). Chicken SCA2 has been suggested to have an association with tumorigenesis based on the up-regulation of the SCA2 transcript in transformed cell lines, and the role of SCA2 in self-renewal and proliferation of avian erythroid progenitors (Petrenko et al., 1997; Gandrillon et al., 1999; Damiola et al., 2004; Bresson-Mazet et al., 2008). Based on our anti-SCA2 antisera, chicken SCA2 is specifically expressed on medullary reticular epithelial cells and CMEC that are surrounded by MHC class II presenting cells in bursal follicles. Therefore, SCA2 may function in maintaining or mediating the proliferation or differentiation of B lymphocytes located at the cortex-medullar border.

To provide insights into the biological function induced by SCA2, we performed a microarray experiment on bursal cells in contact with SCA2. After sorting for differential expression at ≥2-fold changes defined by SAM, the expression of 95 genes were reduced in SCA2-induced bursal cells in comparison to control bursal cells, and many of them are related to signaling, proliferation, immunity response and control of transcription of B cells. Although 68 genes were up-regulated in SCA2-induced bursal cells, the majority of these genes are not specific to B cells. Real time PCR assays with a limited number of genes indicate the array results are valid.

The development of B lymphocytes from hematopoietic stem cells is regulated by specific signaling mechanisms and the BCR pathway serves a central role during this progression (Reviewed in Kurosaki, 1998; Kurosaki 1999; Gauld et al., 2002; Jumaa et al., 2005). Of the down-regulated genes profiled and validated in the SCA2-induced bursal cells, *CD79B*, *SYK*, *BLNK BTK*, and *PLCG2* are components of the BCR signaling pathway and play critical roles in B cell development. CD79B is a subunit of the BCR complex, and phosphorylation of CD79B by protein tyrosine kinase results in the recruitment of SYK, another tyrosine kinase, that facilitates down-stream signaling cascades (Sanchez et al., 1993; Pao et al., 1998; Kurosaki et al., 1995). BTK is a nonreceptor tyrosine kinase, and BTK-deficient mice show an X-linked immunodeficiency phenotype with immature peripheral B cells (Kerner et al., 1995;

Khan et al., 1995; Hendriks et al., 1996). Phosphorylated BLNK provides docking sites for various signaling proteins including BTK and PLCG2, and pre-B cells deficient in BLNK show an increased proliferation rate resulting in a pre-B cell leukemia, suggesting that BLNK is a tumor suppressor for limiting the proliferation of pre-B cells (Pappu et al., 1999; Minegishi et al., 1999; Jumaa et al., 2003). Therefore, the ability of chicken SCA2 in down-regulating critical components for normal B cell development and proliferation, such as the putative tumor suppressor BLNK, indicate SCA2 may be functionally associated with transformation by delivering proliferation signals.

The role of CMEC, where SCA2 is specifically localized, is still not clear although these cells are suggested to provide a “nursing” function for regulating the development of bursa cells. Notch family transmembrane receptor proteins function in mediating cell development and differentiation (reviewed by Robey, 1999), and have been known to be expressed in medullary B cells located close to CMEC, and the Notch ligand Serrate2 is expressed exclusively in the CMEC (Morimura et al., 2001). Although the ligands for SCA2 are not known, the tissue localization of SCA2 is closely related to Serrate2 and may also function similarly as a B cell developmental signal. K-1 antigen has also been identified to be expressed in CMEC and reticular epithelial cells in bursa (Kaspers et al., 1993; Olah et al., 2002). K-1 antigen is a cell surface antigen consisting of a heterodimer with polypeptide chains of molecular weights of 135 kDa and 61-68 kDa, and it is expressed on chicken thrombocytes, macrophages, and a virus transformed phagocytic cell line, but not on T and B cells. Although both K-1 and SCA2 are located on CMEC, no apparent homology is observed between SCA2 and K-1 antigen.

Mice Ly-6 family members are broadly used as a differential marker for hemotopoeitic stem cells. In this report, we did not thoroughly investigate SCA2 as a differential marker of chicken hemotopoeitic stem cells. We did not obtain the detectable level of cell surface expression of SCA2 on thymocyte cells from 1-4 week old birds. Therefore, either the expression of SCA2 is restricted to certain stages of lymphoid cells, or it is mainly expressed in epithelial cells.

In conclusion, chicken SCA2 has been identified as a cell surface antigen anchored by GPI moiety, and the tissue distribution of SCA2 was determined. SCA2 is specifically expressed on CMEC in bursal follicles, which are surrounded by MHC class II expressing cells. The down-regulation of genes in the BCR signaling pathway induced by contact with SCA2-expressing cells suggests SCA2 may function in mediating B cell proliferation and differentiation via interaction with receptors on the B cell surface. Further analysis of the role of SCA2 in B cell proliferation is warranted and may elucidate its function in B cell development.

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Chapter 3

Chicken stem cell antigen 2 modulates Marek's disease virus growth properties *in vitro* via US10

Abstract: Marek's disease virus (MDV) is a highly cell-associated oncogenic alphaherpesvirus and the causative agent for Marek's disease (MD), a lymphoproliferative disease in chickens. Understanding the molecular mechanisms for pathogenesis is of both academic and commercial interest. Chicken stem cell antigen 2 (SCA2) is a putative MD resistance gene based on (1) genetic linkage of *SCA2* to MD resistance and related traits, (2) differential transcription of *SCA2* between MD resistant and susceptible chickens following MDV challenge, and (3) a confirmed and direct protein interaction of SCA2 with MDV US10 *in vitro*. The functional relevance of the interaction between SCA2 and US10 proteins has not been elucidated. Two recombinant MDVs were developed: rMd5-B40-US10EGFP (referred to as US10EGFP) expresses a US10-enhanced green fluorescent protein (EGFP) fusion protein, and rMd5-B40-ΔUS10EGFP (referred to as ΔUS10EGFP) replaces *US10* with *EGFP*. Neither modification impacted viral replication rate *in vitro*. These two mutant viruses were employed to examine the sub-cellular localization of US10 and SCA2, and the potential influence of their interaction on viral growth properties. As judged by EGFP expression, US10 is found exclusively in the cytoplasm of DF1 cells, an immortalized chicken embryo fibroblast cell line. SCA2, which is normally found on the cell surface, co-localizes with US10 in DF1 cells stably expressing a SCA2-Flag fusion protein. The expression of SCA2 in DF1 cells results in MDV plaques that are significantly smaller at 6 days post-infection but only when US10 is present. The ability of SCA2 to impact MDV growth properties is in agreement with the percentage of US10EGFP or ΔUS10EGFP-infected DF1 cells as measured using flow cytometry. Over-expression of SCA2 impairs MDV spread *in vitro* but this influence is dependent on the presence of

US10. This is the first report showing a direct influence of SCA2 on MDV, and it provides insights on how this chicken protein may impact MDV replication and MD pathogenesis, and helps to confirm its role as an MD resistance gene.

Introduction

Marek's disease (MD) is a lymphoproliferative disease of chickens characterized by T cell lymphomas and nerve enlargements. The causative agent is the highly oncogenic alphaherpesvirus Marek's disease virus (MDV). The 180 Kb MDV genome has a typical alphaherpesvirus structure with a linear double-stranded DNA genome consisting of unique long (UL) and unique short (US) regions, each flanked by inverted repeats (Tulman et al., 2000; Silva et al., 2000). The viral genome is enclosed by viral nucleocapsids surrounded by a tegument protein layer and an outer viral glycoprotein envelope (Kato and Hirai, 1985). Viral products and host elements participate within different structures of the cell for virion packaging and egress. We have reported nine specific chicken-MDV protein-protein interactions via a bacterial two-hybrid screen following by *in vitro* binding assays for confirmation (Niikura et al., 2004).

MD is considered the most serious persistent infectious disease of concern to the poultry industry worldwide, although it has been controlled through vaccination since 1970s. Even with vaccines, MD still has a significant economic impact to the poultry industry as there are sporadic outbreaks, which require the continuous vaccination and monitoring of birds. Consequently, there is a need for alternative methods, such as enhanced resistance to MD, to augment vaccinal control of MD. Chicken stem cell antigen 2 [SCA2, also known as lymphocyte antigen 6 complex, locus E (LY6E) and thymic shared antigen 1 (TSA1)], which directly interacts with MDV US10, was further investigated and shown to be a putative MD resistance gene based on its genetic association with MD incidence and related traits, and differential transcription following MDV challenge between MD resistant and susceptible chicken lines (Liu et al., 2006). To address the putative function(s) of SCA2 in MDV biology through association with viral US10 product, validating their co-localization in chicken cells and determining the relevance of this interaction is important.

MDV US10 is located in the US region of the viral genome and encodes a 213 amino acid protein. US10 homologues are encoded by other herpesviruses including herpes simplex virus type 1 (HSV-1), varicella-zoster virus (VZV), and equine herpesvirus type 1 (EHV-1) (McGeoch et al., 1985; Brown and Harland, 1987; Davison and Scott, 1986; Telford et al., 1992; Sakaguchi et al., 1992). Based on properties of HSV-1, MDV US10 in virions is predicted to be a capsid/tegument phosphoprotein (Yamada et al., 1997). MDV US10 in lymphoblastoid cell lines derived from MD tumors show US10 is localized in the cytoplasm near patches of glycoprotein B (gB) on the cell surface, suggesting US10 is associated with virion assembly (Parcells et al., 1999). The deletion of the MDV US region containing US10 and adjacent MDV open reading frames (ORFs) indicates that US10 is not essential for MDV growth *in vitro*, however, this deletion decreases early cytolytic infection, mortality, and tumor incidence *in vivo* (Parcells et al, 1994; Parcells et al, 1995).

Chicken SCA2 transcription is induced by MDV infection in cultured chicken embryo fibroblast (CEF) cells (Morgan et al., 2001). However, the function of chicken SCA2, as in other species, is not well defined. Chicken SCA2 is associated with v-Rel-transformed avian bone marrow cells and plays a role in self-renewal of avian erythroid progenitors (Petrenko et al., 1997; Bresson-Mazet et al., 2008). The majority of work characterizing SCA2 has been done in mice. Mouse SCA2 is a member of the lymphostromal cell membrane Ly6 superfamily, and it localizes on the cell surface via a glycosyl-phosphatidylinositol (GPI)-anchor (Classon et al., 1994). Mouse SCA2 functions in regulating intracellular signaling events via T cell receptor and cell-cell adhesion as a receptor on the lymphocyte membrane (Randle et al., 1993; Fleming and Malek, 1994; Saitoh et al., 1995; Noda et al., 1996).

Here we report the development of recombinant MDVs that either lack or have US10 fused with EGFP. Utilizing chicken cell lines stably expressing SCA2, we find that SCA can co-localize with US10 in the cytoplasm. Furthermore, SCA2 alters the size and percentage of MDV infected cells but this effect is dependent on the MDV genome containing *US10.*

Materials and methods

Cells

MDV was propagated on secondary CEF from 10-day old line 0 chicken embryos and cultivated on a mixture of Liebovitz's L-15 and McCoy 5A (LM) media (1:1) supplemented with 4% fetal bovine serum (FBS), penicillin, and streptomycin. DF-1 cells were cultured in LM media with 4% FBS, penicillin and streptomycin. The Vector-DF1 cell line is the DF-1 stably transfected with the pSELECT-zeo-mcs vector (Invivogen, CA, USA). The SCA2Flag-DF1 cell line is identical to Vector-DF1 except the vector was engineered to constitutively express a SCA2Flag recombinant protein with the Flag tag inserted immediately downstream of the signal peptide of SCA2. In brief, the entire coding portion of chicken SCA2 (bases 55 to 435 based on GenBank accession no. NM_204775) was amplified from a chicken spleen cDNA library by PCR using primers (SCA2SalIF: 5' atcg gtc gac atg aag gcg ttt ctg ttc gc 3'; SCA2NcoIR: 5' atcg cca tgg tca ctc acg agc cct gag g 3'; restriction sites are underlined). The amplicon was inserted between the *Sal*I and *Nco*I sites of the pSELECT-zeo-mcs vector to generate SCA2-pSELECT-zeo-mcs, and DNA sequenced to confirm that the construct was correct. A Flag tag was inserted immediately downstream of the signal peptide of SCA2 by PCR using SCA2-pSELECT-zeo-mcs as the template with the primers (SCA2SalIF; Sca2FlagNsiIR: 5' gtt gga gg atg cat ccg agc acg aaa agc aga tgag *ctt atc gtc gtc atc ctt gta atc* cgt gtg ggc tct ctc cac g 3'; restriction sites are underlined, Flag tag sequences are in italic). The amplicon was inserted between the *Sal*I and *Nsi*I sites of SCA2-pSELECT-zeo-mcs to produce SCA2Flag-pSELECT-zeo-mcs. The SCA2Flag-pSELECT-zeo-mcs and pSELECT-zeo-mcs vectors were each transfected into the DF1 cell line using the

Basic Nucleofector Kit (Amaxa Inc., Gaithersburg, MD, USA). The transfected cells were incubated in medium containing LM media supplemented with 5% FBS, penicillin, streptomycin, and zeocin (300 ng/ml) until single colonies appeared. The expression of SCA2 was tested by western blots using anti-Flag mAb and anti-SCA2 antisera. The single colony with the best SCA2 expression was maintained as the SCA2Flag-DF1 cell line.

Recombinant BAC clones

To insert EGFP into MDV US10 gene, two step Red-mediated recombination techniques were applied (Tischer et al., 2006). All PCR amplifications used in BAC recombineering were conducted with Expand High Fidelity PCR system (Roche, Indianapolis, IN, USA). Briefly, the kanamycin gene (*aphA1*) and *I-SceI* cassette from pEP-kanS (kindly provide from Niklaus Osterrieder, Cornell University) were amplified with pEPEGFP.F (5'-TCC *TGT ACA* <u>AGT AAA GCG GCC GCG ACT CTA GAT CAT AAT CAG CCA TAC CAC ATT</u> **TAG GGA TAA CAG GGT AAT CGA TT**-3'; italicized sequence for *BsrG1* restriction sites, underlined sequence for Red recombination, bold sequence to amplify *I-SceI* cassette from pEP-kanS) and pEPEGFP.R (5'-TCC *TGT AC*A CTA **GCC AGT GTT ACA ACC AAT TAA CC**-3'; italicized sequence for *BsrG1* restriction sites, bold sequence to amplify *I-SceI* cassette from pEP-kanS). The primer sets were synthesized by Integrated DNA Technologies (IDT, Coralville, IA, USA). The PCR amplicon and pEGFP-N1 (Clontech, E Palo Alto, CA, USA) were digested with *BsrG1* and the resulting fragment (~1053 bp) cloned into pEGFP-N1 to create the pEP-EGFP-N1 construct. The EGFP, *aphA1* and *I-SceI* cassette was amplified from the pEP-EGFP-N1 construct with the primers, US10-EGFP-fus.F (5'-TAT CTG ACA AAT CTT CGG GAA TCG CCA ACA GGA GAC GGG GAA TCC TAC TTA **ATG GTG AGC AAG GGC GAG GAG CTG T**-3'; underlined sequences for homologous recombination, bold sequences to amplify EGFP, *aphA1* and *I-SceI* cassette) and US10-EGFP.R (5'-CGT TAG TAG CAG TTT TTC CTA AAA TCC TAT TAA TAA TTG TGC GAT TAG TTA **AAA GCA AGT AAA ACC TCT ACA AAT**-3'; underlined sequences for homologous recombination, bold sequences to amplify EGFP, *aphA1* and *I-SceI* cassette). The PCR amplicon with US10-EGFP-fus.F and US10-EGFP.R was used for Red recombination in SW105 *E. coli* as earlier described (Warming et al 2005, Tischer et al, 2006) to insert between the 212th amino acid sequence and the downstream stop codon of US10 (155616-155617 nucleotide in *Gallid herpes virus 2* genome, GenBank accession number: NC_002229) of rMd5-B40 BAC (Niikura et al, 2006).

To replace the MDV US10 gene with EGFP, the EGFP, *aphA1* and *I-SceI* cassette was amplified from pEP-EGFP-N1 construct with US10-EGFP-rep.F (5' TTT GAA TAC TGG AGA CGA GCG CCG TGT AAG ATT AAA ACA TAT TGG AGA GGT **ATG GTG AGC AAG GGC GAG GAG CTG T**-3'; underlined sequences for homologous recombination, bold sequences to amplify EGFP, *aphA1* and *I-SceI* cassette) and US10-EGFP.R. The PCR amplicon with US1-EGFP-rep.F and US10-EGFP.R was used for Red recombination to replace the US10 from the start to stop codons (154978-155617 nucleotide in *Gallid herpes virus 2* genome, GenBank accession number: NC_002229) of rMd5-B40 BAC (Tulman et al., 2000; Niikura et al, 2006). After Red recombination using the above PCR fragment, kanamycin-resistant BAC clones were selected, then *I-*

SceI induced from pBAD-I-SceI plasmid by treatment with arabinose-containing media. The digestion of *I-SceI* recognition site in MDV-BAC and subsequent second Red recombination at duplicated sequences were conducted in SW105 cells. After the second Red recombination, the positive MDV-BAC clone was kanamycin sensitive, thus, screened via PCR amplification using US10fl.F (5'-GAGAGGAGA ACGTGTTTGAATAC-3') and EGFP.R (5'-CTGCTTCATGTGGTCG GGGTAG-3') primers.

The resulting MDV-BAC clone containing the US10-EGFP fusion protein was named US10EGFP and the MDV-BAC clone with the replacement of *US10* by *EGFP* was named ΔUS10EGFP. Both BAC clones were purified, digested with *Bam*HI, and their digestion patterns analyzed by gel electrophoresis.

Generation of viruses

To generate MDVs from all three BAC clones, purified plasmids (rMd5-B40 BAC, US10EGFP and ΔUS10EGFP) were individually transfected into CEF. The nucleofector II (Amaxa Biosystems, Gaithersburg, MD, USA) was used to transfect MDV-BAC into CEF. All CEF cultures were incubated at 37 C°, 5% CO_2 incubator in LM media supplemented with 4% FBS, penicillin, and streptomycin. The FBS was reduced from 4 % to 1 % after virus inoculation until MDV plaques were observed.

One step growth curve assay and plaque area determination

Plaque growth assays were performed as previously reported (Mao et al., 2008). Briefly, 100 PFU of various viruses were inoculated onto CEF cells seeded on 35-mm dishes. On 1, 2, 3, 4, and 5 days after inoculation, the infected cells were trypsinized, serial dilutions inoculated onto fresh cells, and plaques of the different dilutions counted 7 days later in triplicate to determine growth characteristics of rMd5-B40-derived, US10EGFP and ΔUS10EGFP viruses.

500 PFU US10EGFP or ΔUS10EGFP were inoculated onto 5×10^5 Vector-DF1 and SCA2Flag-DF1 cells seeded on 35-mm dishes, and the cell cultures incubated with LM supplemented with 1% FBS, penicillin, and streptomycin. Virus plaques on Vector-DF1 and SCA2Flag-DF1 cells 7 days post-infection in three replicates were examined under a LEICA DM IRB/E inverted fluorescence microscope (Leica Wetzlar, Germany). 7-10 plaques were counted on each dish and 21-30 plaques for each virus were counted in total. Images were captured by a DEL-750CE digital system (Optronics, Goleta, CA, USA), and plaque sizes determined using the ImageJ software (rsb.info.nih.gov/ij/index.html). Statistical significance of plaque areas were performed using the Student's T-test.

Immunofluorescence and confocal image analysis

SCA2Flag-DF1 and fresh CEF cells grown on glasscovers were inoculated with 1000 PFU of recombinant viruses, and incubated in LM medium with 4% FBS, penicillin, and streptomycin at 37°C. The CEF with recombinant viruses were fixed with 100% acetone, washed 3 times with PBS , and stained with Hoechest 33342 (Invitrogen, Carlsbad, CA, USA). SCA2Flag-DF1 cells with recombinant virus were incubated with anti-Flag M2 mAb (1:100) for 20 min at 4°C, then Rhodamine-conjugated anti-mouse goat IgG (1:400; Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) as second Abs for 20 min at 4°C, and stained with Hoechest 33342 after 3 washes with PBS. The stained cells were analyzed with Olympus fluoview 1000 confocal microscope (Olympus, Tokyo, Japan) and the images analyzed using FV10-ASW software (Olympus, Tokyo, Japan).

Western blot analysis

CEF with recombinant viruses were lysed in Cellytic M Mammalian Cell lysis reagent (Sigma-Aldrich Inc., Milwaukee, WI, USA), supplemented with 1% P8340 protease inhibitor cocktails (Sigma-Aldrich Inc., Milwaukee, WI, USA) and followed with sonication and centrifugation. Cell lysates were mixed with loading and reducing buffer, heated at 70°C for 10 min and loaded on 10% SDS-PAGE gel (Invitrogen, Carlsbad, CA, USA). The samples were blotted on Immobilon-P^{SQ} PVDF transfer membrane (Millipore, Billerica, MA, USA). After blocking in PBS containing 5% non-fat milk for 1 hour at room temperature, membranes were probed with anti-GFP mAb (Sigma-Aldrich Inc., Milwaukee, WI, USA) at 4°C overnight. Membranes were washed three times in PBS before incubation with a goat anti-mouse IgG conjugated with horseradish peroxidase (Zymed, South San Francisco, CA, USA) at room temperature for 1 hour. Proteins were detected using ECL Western blotting substrate (Pierce, Rockford, IL, USA) after washing for three times.

Flow cytometry analysis

Three replicates of SCA2Flag-DF1 and Vector-DF1 cells infected with US10EGFP or ΔUS10EGFP viruses were trypsinized and harvested (1000×g, 5 min). After 7 days post-infection, 10^6 cells were washed with cold flow cytometry buffer (phosphate buffered saline containing 1% FBS and 0.1% sodium azide). The cells were analyzed using a Becton-Dickinson FACScaliber flow cytometer and CellQuest Pro software (BD Bioscience, San Jose, CA, USA).

Results

Construction of US10EGFP and ΔUS10EGFP MDV-BAC clones

Insertion of *EGFP* to generate either a fusion protein with US10 on the C-terminal end or to delete *US10* were performed using the rMd5-B40-BAC clone, which generates virulent MDV, and BAC modification techniques (Fig. 3-1). To produce a recombinant MDV-BAC that expresses a US10-EGFP fusion protein, the EGFP-containing amplicon was inserted between the 212th amino acid and the stop codon of *US10* resulting in rMd5-B40-US10EGFP (referred to as US10EGFP). Likewise, the MDV-BAC clone lacking *US10* was produced by replacing *US10* with *EGFP* from the start to stop codons to generate rMd5-B40-ΔUS10EGFP (referred to as ΔUS10EGFP). Both US10EGFP and ΔUS10EGFP BAC clones retain the natural *US10* promoters for downstream expression of US10-EGFP and ΔUS10-EGFP recombinant genes. Nucleotide analysis of the two BAC clones confirmed the genetic modifications. Analysis of the BAC clones compared to the parental rMd5-B40 also indicates that no unexpected modifications occurred in the generation of the recombinant MDV-BAC clones (Fig. 3-2); unfortunately, the US10-containing *Bam*HI fragment is too large to detect a change in size after the *EGFP* insertion into rMd5-B40 (21.6, 21.5, and 20.9 kb for rMd5-B40, US10EGFP, and ΔUS10EGFP, respectively).

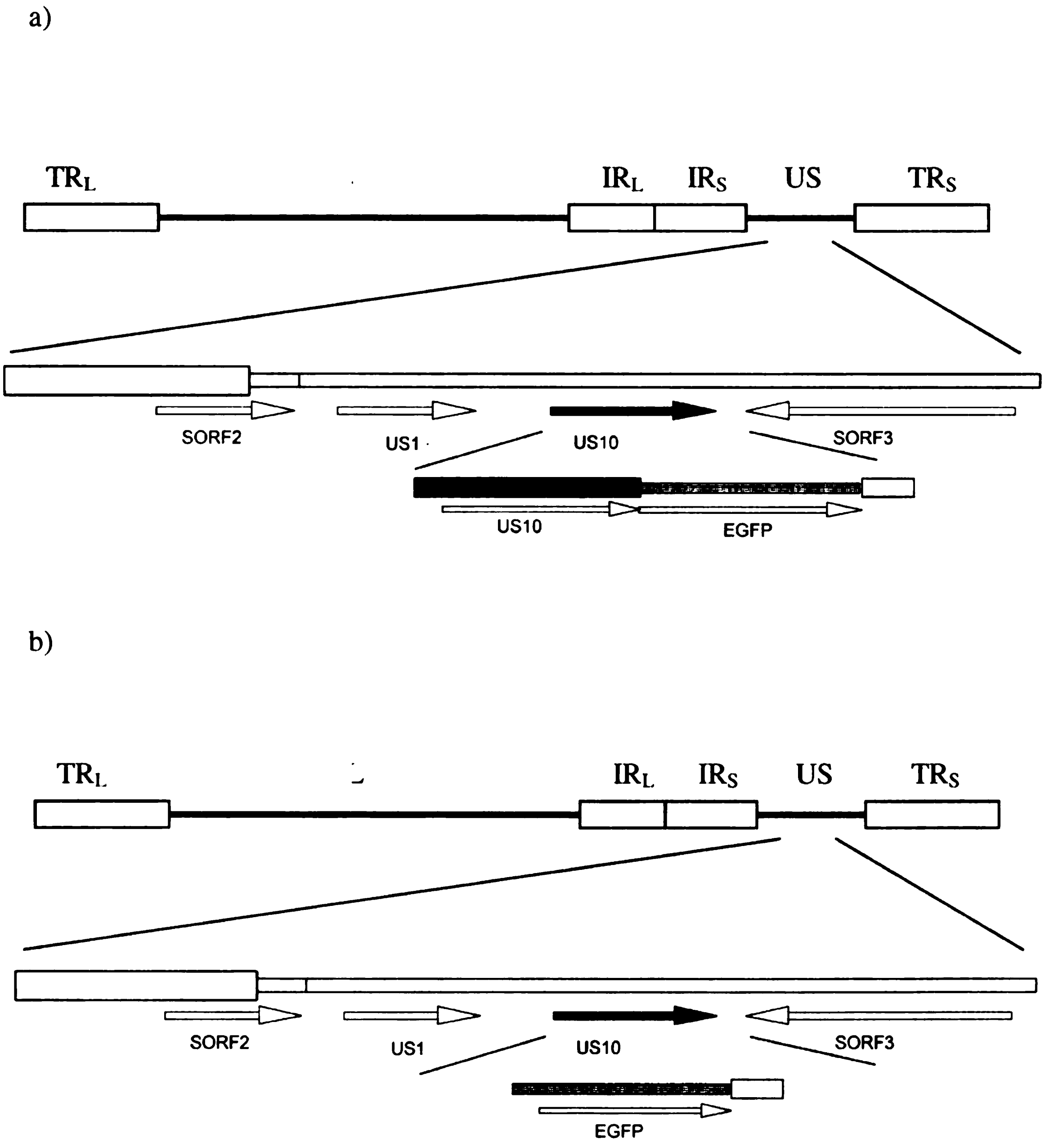


Figure 3-1. Schematic diagram of recombinant MDV-BAC clones. a) Schematic diagram of MDV US10 and neighboring MDV ORFs in US10EGFP-BAC. The insertion of *EGFP* to generate a fusion protein at the C-terminus of MDV US10. b). Schematic diagram of MDV US10 and neighboring MDV ORFs in ΔUS10GFP-BAC where *US10* is replaced with *EGFP*.

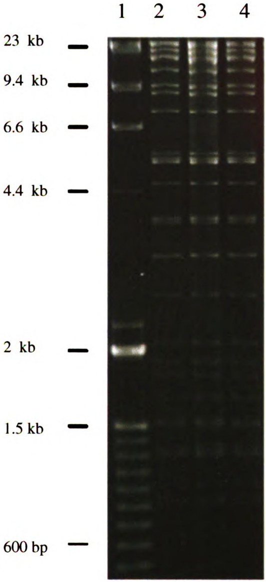


Figure 3-2. *Bam*HI digestion pattern of purified US10EGFP-BAC and ΔUS10EGFP-BAC clones. MDV-BAC clones are purified and digested with *Bam*HI. Digestion patterns are compared with rMd5-B40 BAC clone. Lane 1, DNA size marker; Lane 2, rMd5-B40; Lane 3, US10EGFP; and Lane 4, ΔUS10EGFP.

Generation and growth properties of the mutant viruses in CEF

Recombinant MDVs were generated by transfection of the US10EGFP and ΔUS10EGFP-BAC clones into CEF, and grown until virus plaques were visualized. The expression of the US10EGFP fusion protein or EGFP protein in CEF from the US10EGFP and ΔUS10EGFP viruses, respectively, were detected by western blots using anti-GFP mAb. As predicted, the US10EGFP and EGFP proteins migrated to 52 and 28 kDa, respectively (Fig. 3-3).

After determination of virus titers, 100 PFU of each virus was inoculated onto CEF cells to compare growth properties of the parental and recombinant viruses. The plaque assay showed that neither US10EGFP or ΔUS10EGFP were impaired for virus growth properties *in vitro* (Fig. 3-4), which confirmed that US10 is dispensable for virus growth *in vitro* (Parcell, et al., 1994).

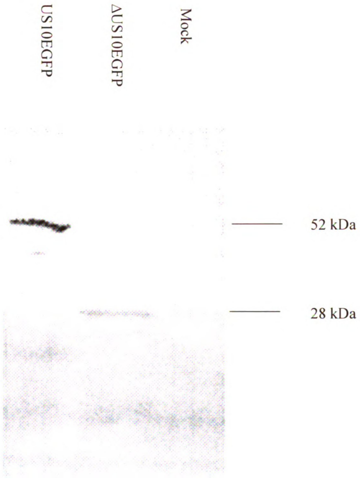


Figure 3-3. Western blot analysis of US10EGFP and ΔUS10EGFP viruses. CEF infected with US10EGFP and ΔUS10EGFP viruses, as well as an uninfected control CEF (mock), were detected using anti-GFP mAb. The molecular size of US10EGFP and ΔUS10EGFP are 52 kDa and 28 kDa respectively.

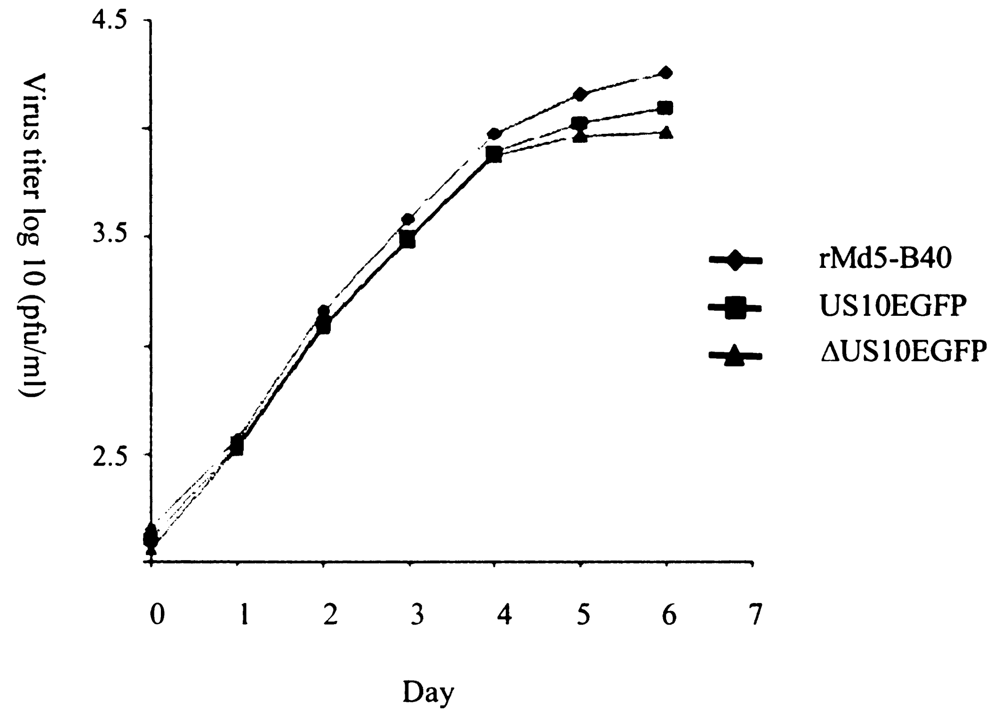


Figure 3-4. One step growth curve assay of rMd5-B40, US10EGFP, ΔUS10EGFP viruses in CEF cells. 100 PFU of various viruses were inoculated onto CEF cells seeded onto 35-mm dishes. On 1, 2, 3, 4, and 5 days after inoculation, the infected cells were trypsinized, serial dilutions inoculated onto fresh cells, and plaques of the different dilutions were counted 7 days later in triplicate to determine growth characteristics of rMD5-B40, US10EGFP and ΔUS10EGFP viruses.

Cellular localization of US10-EGFP and SCA2 proteins

CEF infected with US10EGFP virus were grown on cover glass slides for 3 days, and the sub-cellular localization of US10 were analyzed by visualization of EGFP under the fluorescent microscope. US10 is exclusively localized in the cytoplasm in infected cells (Fig. 3-5). Some co-localization of US10 and the cytoplasmic DNA fragment (stained by Hoechest 33342, and marked with an * in Fig. 3-5a) suggests US10 is associated with viral DNA and virion assembly.

SCA2Flag is expressed on the cell surface of SCA2Flag-DF1 cell lines through immunofluorescence using anti-Flag M2 mAb (Fig. 3-6). After inoculation of US10EGFP virus on the SCA2Flag-DF1 cells, the sub-cellular localization of US10 and SCA2 was observed through EGFP and immunofluorescence using anti-Flag M2 mAb. In the SCA2Flag-DF1 cells infected with US10EGFP, SCA2 can co-localized with US10 in the cytoplasm (marked by an * in Fig. 3-7c). In the uninfected SCA2Flag-DF1 cells, the majority of SCA2 is on the cell surface.

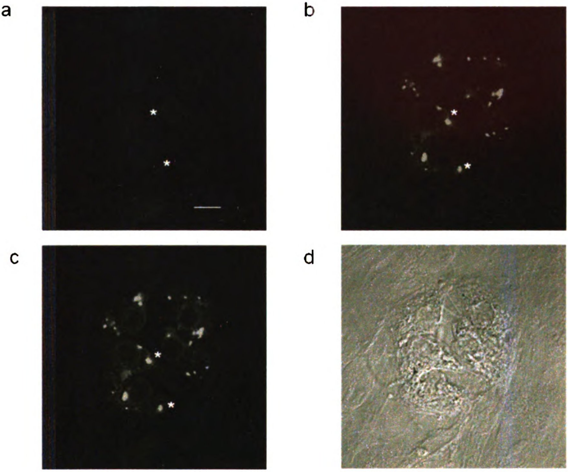


Figure 3-5. Confocal imaging to determine sub-cellular localization of US10 in CEF infected with US10EGFP virus. a) US10EGFP-infected CEF stained with Hoechst 33342 (blue). Bar, 40 μm. b) US10EGFP visualized as green fluorescence. c) Merged image of a and b. Co-localization of US10-EGFP and cytoplasmic viral DNA are marked by asterisks. d) Infected CEF visualized through differential interference contrast (DIC) imaging.

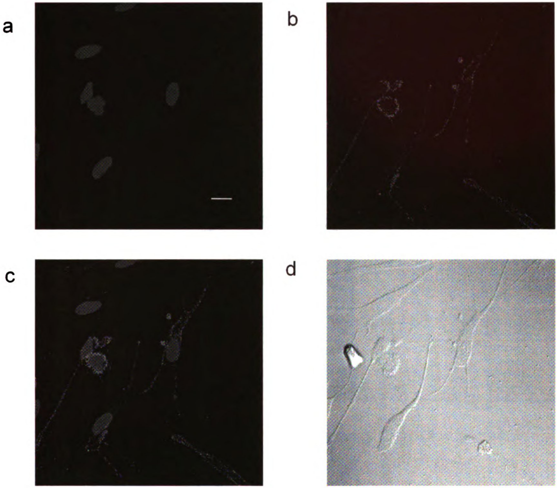


Figure 3-6. Confocal images of SCA2 in SCA2Flag-DF1 cells. a) SCA2Flag-DF1 strained with Hoechst 33342 (blue). Bar, 20 μm. b) SCA2Flag-DF1 stained with anti-Flag M2 mAb and goat anti-mouse IgG conjugated with Texas Red (red). c) Merged image of a and b. d) SCA2Flag-DF1 cells visualized through DIC imaging.

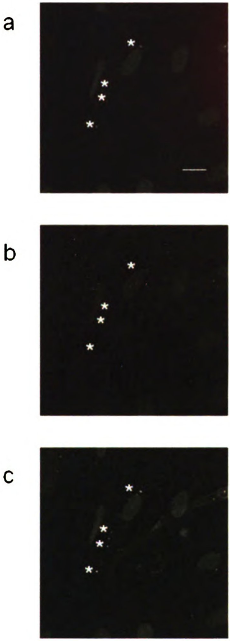


Figure 3-7. Confocal immunofluoresence images of US10-EGFP and SCA2 in SCA2Flag-DF1 cells infected with US10EGFP virus. a) SCA2Flag-DF1 culture image to show US10-EGFP (green) and Hoechst 33342 (blue). Bar, 20 μm. b) SCA2Flag-DF1 culture stained with Hoechst 33342 (blue), and anti-Flag M2 Ab and goat anti-mouse IgG conjugated with Texas Red (red). c) Merged image of a and b. Co-localization of SCA2 and US10-EGFP in the cytoplasm are marked by asterisks.

Growth properties of US10EGFP and ΔUS10EGFP mutant viruses in Vector-DF1 and SCA2Flag-DF1 cells

Two cells lines were produced. Vector-DF1 are DF1 cells stably transfected with the pSELECT-zero-mcs vector only. SCA2Flag-DF1 are identical except the vector was engineered to constitutively express a recombinant SCA2 protein where a Flag tag was inserted immediately downstream of the SCA2 signal peptide. These cell lines allowed us to examine the growth properties of US10EGFP and ΔUS10EGFP recombinant viruses in the presence or absence of SCA2. 500 PFU US10EGFP and ΔUS10EGFP were seeded on both Vector-DF1 and SCA2Flag-DF1 cells. At 6 days post-infection, the plaque areas on Vector-DF1 and SCA2Flag-DF1 cells were determined and analyzed by monitoring fluorescence expressed from the viruses. Plaque sizes of US10EGFP on SCA2Flag-DF1 cells were significantly reduced ($P<0.000001$) to 56% in comparison to the plaque areas of the same virus grown on Vector-DF1 cells (Fig. 3-8, 3-9a). The reduction of plaque area was not observed when the recombinant virus lacked *US10* (Fig 3-8, 3-9b). These results demonstrate that an effect of SCA2 on MDV plaque size is dependent on the presence of *US10*.

To help confirm and extend the results of plaque size determinations, the percent of US10EGFP and ΔUS10EGF-infected cells were analyzed via flow sorting (Fig. 3-10). At 6 days post-infection, the percent of ΔUS10EGFP-infected cells was not significantly different between those grown on SCA2Flag-DF1 or Vector-DF1 cells (1.3% vs. 1.38%). In contrast and consistent with the plaque assays, expression of SCA2 in SCA2Flag-DF1 cells reduced the percent of US10EGFP-infected cells compared to the Vector-DF1 cells.

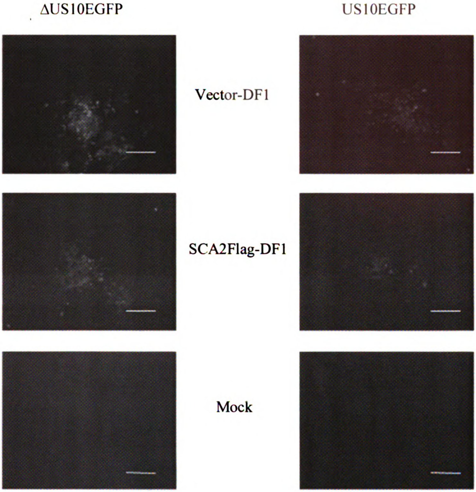
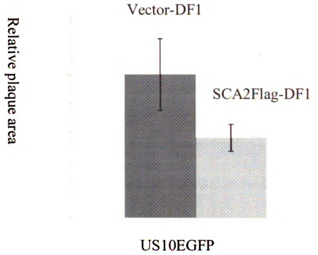


Figure 3-8. Plaques of US10EGFP and ΔUS10EGFP viruses on Vector-DF1 and SCA2Flag-DF1 cells. 500 PFU of US10EGFP and ΔUS10EGFP viruses were inoculated on Vector-DF1 and SCA2Flag-DF1 cells in three replicates. Infected cells with US10EGFP and ΔUS10EGFP viruses were detected with green fluorescence. The plaques after 6 days post-infection were photographed with a fluorescence microscope and captured with a DEL-750CE digital system. Bars, 200 μm.

a)



b)

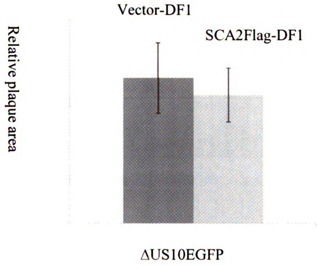
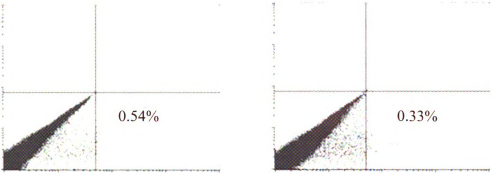
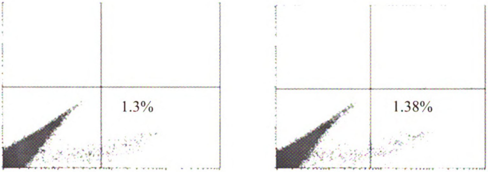


Figure 3-9. Relative plaque sizes of recombinant US10EGFP and ΔUS10EGFP viruses on Vector-DF1 and SCA2Flag-DF1 cells. Vector-DF1 and SCA2Flag-DF1 cells were infected with US10EGFP (a) and ΔUS10EGFP (b) in three replicates, and used for determination of plaque sizes. Plaque areas of US10EGFP and ΔUS10EGFP viruses were detected through the green fluorescence of EGFP. The plaques after 6 days post-infection were photographed with a fluorescence microscope, captured with a DEL-750CE digital system, and measured with the Image J software. Seven to ten plaques were counted in each 35 mm dish, and 21-30 plaques in total for each virus were counted. Means and standard deviations (error bars) of relative plaque area are given.



Vector-DF1 with US10EGFP

SCA2Flag-DF1 with US10EGFP



Vector-DF1 with ΔUS10EGFP

SCA2Flag-DF1 with ΔUS10EGFP

Figure 3-10. The percentage of infected cells within Vector-DF1 and SCA2Flag-DF1 cells infected with US10EGFP and ΔUS10EGFP viruses. The average percentage of three replicates of infected cells within Vector-DF1 and SCA2Flag-DF1 cells with US10EGFP (a) and ΔUS10EGFP (b) were determined by flow cytometry.

Discussion

MDV is a highly cell-associated virus and the aetiological agent of MD. Although MD has been controlled through vaccination since the 1970's, MD is still a significant threat to the poultry industry as there are sporadic outbreaks. Consequently, alternative methods, such as genetic resistance, are needed to augment vaccinal control of MD. We previously reported that chicken *SCA2* is a MD resistance gene due to the following criteria: (1) association to MD incidence and related traits in a commercial resource population, (2) transcriptional differences between MD resistant and susceptible experimental chicken lines following challenge with MDV, and (3) a direct protein interaction with MDV US10 as determined by a two-hybrid screen followed by an *in vitro* binding assay (Liu et al., 2003). The potential role of SCA2 in MD biology encouraged us to investigate its properties and impact on MDV infection.

The recombinant US10EGFP and ΔUS10EGFP viruses were develop to help confirm the interaction of SCA2 and US10 proteins *in vitro*. During the course of these studies, it was noticed that expression of SCA2 altered the size of virus plaques but only for US10EGFP. Specifically, plaque sizes of US10EGFP on SCA2Flag-DF1 cells were significantly reduced in the SCA2 over-expressing cells (Fig. 3-8, 3-9a), however, this effect on plaque sizes was not observed for ΔUS10EGFP between the two cell lines (Fig. 3-8, 3-9b). This suggests that SCA2 can influence MDV replication or spread *in vitro* but this effect is dependent on the presence of *US10* in the viral genome. To further analyse and confirm this result, the percent of infected SCA2Flag-DF1 and Vector-DF1 cells with US10EGFP and ΔUS10EGFP viruses were analyzed via flow cytometry. The results were consistent with those results from the plaque size assays as the percent of infected

SCA2Flag-DF1 and Vector-DF1 cells for US10EGFP virus reflected their plaque size changes (44% reduced plaque areas vs. 39% reduced infected cells), and the ΔUS10EGFP virus did not either form statistically significantly different plaque areas nor different percentages of infected cells (Fig 3-9, 3-10). Based on the results, we concluded that the over-expression of SCA2 impairs MDV growth *in vitro*, and this impairment is dependent on the expression of MDV US10.

Based on the putative function of mouse SCA2, we hypothesize three putative roles of SCA2 in MD resistance. These putative roles are summarized as: (1) SCA2 promotes cell-cell adhesion for MDV spread, (2) SCA2 transduces signals for the transformation of lymphocytes with latent MDV, and (3) SCA2 interferes in virion assembly and egress via association with MDV US10. While our results do not rule out the first two possible roles, the impact of SCA2 on MDV plaque size and number of infected cells supports a role for SCA2 in influencing MDV assembly.

Although the expression of SCA2 in our results is associated with inhibition of viral replication, this is likely an artifact of unregulated and over-expression of *SCA2*. All other lines of evidence suggest that SCA2 expression promotes viral replication. Specifically, in naturally-occurring lymphocytes of MD susceptible chicken lines where there are high levels of MDV replication and in cultured CEF infected with MDV, SCA2 transcription is induced (Liu et al., 2003, Morgan et al., 2001). The confirmed protein-protein interaction between SCA2 and US10 would also support a favorable action of SCA2 on MDV as it is highly unlikely that an unfavourable interaction would be evolutionarily conserved. It is interesting to note that CD59, the closest Ly6 superfamily member to SCA2, was found packaged in HSV-1 virions (Loret et al., 2008), which indicates other Ly-6 family members may play roles in replication of other alpha-herpesviruses.

The requirement of US10 for impaired MDV growth due to the over-expressed SCA2 strongly supports the physical interaction of SCA2 and viral US10 proteins (Liu et al., 2003). The localization of US10 and SCA2 was examined to provide additional support for this protein interaction. US10 is only localized to the cytoplasm (Fig. 3-5). In the presence of US10, SCA2 can be found in the cytoplasm and co-localized with US10 (marked by * in Fig. 3-7c), but the majority of SCA2 remains on the cell surface (Fig. 3-6). We did not observe cell surface expression of US10 protein. These observations, though not conclusive, support the protein interaction and our result that over-expression of SCA2 protein interferes in virus morphogenesis via its direct association with MDV US10 in the cytoplasm.

HSV US10 is a capsid/tegument phosphoprotein and it co-purifies with the nuclear matrix (Yamada et al., 1997). We did not find US10 protein in the nucleus. The accumulation of US10 in the cytoplasm sometimes co-localized with DNA fragments (marked by * in Fig. 3-5c), which could be the viral DNA genome as no cytoplasmic DNA staining was observed in adjacent uninfected cells. As an alpha-herpesvirus, MDV capsid particles containing viral DNA are transported from the nucleus to cytoplasmic organelles for envelopment with the various tegument proteins. Consequently, these observations suggest MDV US10 is a viral tegument protein and plays a role in virion assembly in the cytoplasm.

In examining the expression patterns of SCA2 in chicken tissues, we found a unique pattern in the bursa. SCA2 is specifically expressed on the cortical-medullary epithelial cells (CMEC) surrounded by MHC class II presenting cells. Furthermore, expression profiling of bursal cells induced by contact with SCA2-expressing cells shows down-regulation of numerous genes including *CD79B*, B cell linker (*BLNK*), spleen tyrosine kinase (*SYK*), and gamma 2-phospholipase C (*PLCG2*) that are involved in the B cell receptor (BCR) and immune response signaling pathways. Combined, these results support the impact of SCA2 in MDV-induced transformation of lymphocytes though the mechanism(s) is unknown. However, while *SCA2* mRNA is induced by MDV infection, SCA2 protein has not been detected in MDV-infected CEF using polyclonal antibodies against SCA2. These somewhat contradictory results indicate that further studies are needed to fully elucidate the role of SCA2 in MDV replication and pathogenesis.

In summary, we developed two recombinant viruses to investigate the sub-cellular localization of MDV US10 and its potential influence on SCA2. MDV US10 was localized in the cytoplasm and could not be seen in the nucleus. In the cytoplasm, MDV US10 could co-localize with SCA2, which is normally found on the cell surface. US10EGFP virus formed significantly smaller plaques on cells in which SCA2 was over-expressed. The plaque areas of ΔUS10EGFP were not significantly different between Vector-DF1 and SCA2Flag-DF1 cells. Therefore, we conclude over-expression of SCA2 impairs MDV spread via association with MDV US10 protein in a cultured cell line but it is likely that under natural conditions, expression of SCA2 favors viral morphogenesis.

Acknowledgements

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Chapter 4

Mao, W., M. Niikura, R. F. Silva, and H. H. Cheng. (2008) Quantitative evaluation of viral fitness due to a single nucleotide polymorphism in the Marek's disease virus *UL41* gene via an in vitro competition assay. J Virol Methods 148:125-31.

Abstract: Marek's disease, a T cell lymphoma, is an economically important disease of poultry caused by the Marek's disease virus (MDV), a highly cell-associated alpha-herpesvirus. A greater understanding of viral gene function and the contribution of sequence variation to virulence should facilitate efforts to control Marek's disease in chickens. To characterize a naturally-occurring single nucleotide polymorphism (SNP; AY510475:g.108,206C>T) in the MDV *UL41* gene that resulted in a missense mutation (AAS01683:p.Arg377Cys), bacterial artificial chromosome (BAC)-derived MDVs that differed only in the *UL41* SNP were evaluated using a head-to-head competition assay *in vitro*. Monitoring the frequency of each SNP by pyrosequencing during virus passage determined the ratio of each viral genome in a single monolayer, which is a very sensitive method to monitor viral fitness. MDV with the *UL41**Cys allele showed enhanced fitness in vitro. To evaluate the mechanism of altered viral fitness caused by this SNP, the virion-associated host shutoff (vhs) activity of both *UL41* alleles was determined. The *UL41**Cys allele had no vhs activity, which suggests that enhanced fitness in vitro for MDV with inactive vhs was due to reduced degradation of viral transcripts. The in vitro competition assay should be applicable to other MDV genes and mutations.

Introduction

Marek's disease virus (MDV; family *Herpesviridae*, genus *Mardivirus*, species *Gallid herpesvirus 2*) is an infectious pathogen and the cause of Marek's disease, which is characterized by T cell lymphomas, neurological disorders, and death in susceptible chickens (Osterrieder et al. 2006). Periodic yet unpredictable disease outbreaks that vaccines cannot adequately protect make Marek's disease a serious problem for the poultry industry worldwide. As the molecular basis of the increasing virulence is unknown, there is a need to understand the role of naturally-occurring mutations in MDV genes on virulence (Witter, 1996). In this report, a head-to-head competition assay is described that contributes to the understanding of the molecular basis for viral fitness variance, and should enable more efficacious vaccines and other methods that provide improved disease control.

MDV has an alpha-herpesvirus structure that is similar to herpes simplex virus type-1 (HSV-1) with ~100 genes contained within its 175 kb DNA genome (Silva et al., 2001). Recently, MDV genomes have been cloned into bacterial artificial chromosome (BAC) vectors (Schumacher et al., 2000; Petherbridge et al., 2004; Niikura et al., 2006). Besides providing a platform for infectious molecular clones, MDV-BACs can be readily manipulated even at the nucleotide level, which enables the direct association of specific sequences to phenotypes. Ideally, recombinant MDVs would be engineered in sites where mutations may account for the observed variation in virulence (e.g., Spatz and Silva, 2007; Spatz et al., 2007).

During the cloning and full genome sequencing of virulent MDV Md11 strain (Niikura et al., 2006), a nonsynonymous single nucleotide polymorphism (SNP;

AY510475:g.108,206C>T) was found in the *UL41* gene that resulted in Arg to Cys substitution in residue 377 (AAS01683:p.Arg377Cys). *UL41* encodes the virion-associated host shutoff (vhs) protein, which degrades both host and viral mRNA during the viral cytolytic infection and assists the virus in evading the host immune response (Read and Frenkel, 1983; Kwong and Frenkel, 1988; Read et al., 1993; Suzutani et al., 2000; Murphy et al., 2003; Barzilai et al., 2006). The *UL41* point mutation identified in the MDV clone is interesting because it is located in a critical amino acid that is conserved among alpha-herpesvirus genomes (Everly and Read, 1999). Thus, questions arise as to whether this point mutation would affect viral fitness or activity of the MDV *UL41* gene product, and is there any relationship between the MDV *UL41* gene and virulence?

The straightforward and preferred way of quantifying viral fitness is by a head-to-head competition experiment using stable genetic markers. This is because the two different viruses must compete in the same environment, and assuming the viruses do not recombine, the genetic markers provide proxies for each genome. Competition experiments have applied in other viruses including vesicular stomatitis virus (Novella, 2003), human immunodeficiency virus type-1 (Holland et al., 1991; Yuste et al., 2005), and tobacco etch virus (Carrasco et al., 2007). This method might be particularly useful for MDV as the traditional plaque assay counts infectious foci generated from a single infected cell rather than a single virion due to the strict cell-associated nature of this virus. Furthermore, many studies examining defined recombinant MDVs have shown that growth rate measurements are not very sensitive and, more importantly, are not good predictors of *in vivo* effects (Reddy et al., 2002; Silva et al., 2004; Prigge et al., 2004;

Kamil et al. 2005; Schumacher et al., 2005), thus, a more sensitive *in vitro* method is desired.

For MDV, pyrosequencing was used to monitor the *UL41* SNP. Pyrosequencing is a DNA sequencing technology based on a 4-enzyme real-time monitoring of DNA synthesis, and accurately measures the relative abundance of each SNP nucleotide (Ronaghi et al., 1998; Ronaghi, 2001). This technology has been applied to determine the influence of specific HIV SNPs on resistance to protease inhibitors among others (O'Meara et al., 2001; Lahser et al., 2003). In this study, viral fitness was quantified by evaluating the relative SNP frequency for MDV populations that differed only in *UL41* alleles following repeated passages in cell culture. Also, the *UL41* vhs activity for each allele was measured in an attempt to help explain the affect on viral fitness.

Materials and methods

Culture method for MDV and DNA purification

MDV was propagated on secondary CEF from line 0 chicken embryos cultivated on a mixture of Liebovitz's L-15 and McCoy 5A media (1:1) supplemented with 4% FBS, penicillin, and streptomycin. DNA of MDV-infected cells was extracted in Tris buffer (0.1 M, pH 8.0) containing 1% SDS, 1 mM EDTA, 0.1 M NaCl and 100 μg/ml proteinase K, followed by phenol extraction and ethanol precipitation.

Mini-λ mutagenesis to generate pMDV-UL41*Arg and pMDV-UL41*Cys-rev

As shown in Fig. 4-1, recombinant BAC clones containing the entire MDV strain Md11 genome were generated that differ only in the genomic sequence that encodes UL41 amino acid residue 377 (GenBank no. AAS01683) using the mini-λ mutagenesis as described by Court et al. (2003) and Costantino and Court (2003). Briefly, the oligonucleotides UL41mut (5' GCTGTCTCATCACGTCCCTCTTGCATTATAGGGAACCGTTTCAAAATACTAAC CCTGGAAGTTCTGCGCT 3') and UL41mutRev (5' GCTGTCTCATCACGTCCCTCTTGCATTATAGGGAAGCACTTCAAAATACTAAC CCTGGAAGTTCTGCGCTT 3') were designed to introduce specific point mutations and synthesized (Operon, Huntsville, AL, USA). UL41mut was mixed with competent cells containing the Md11gDloxP5-12 MDV-BAC clone (Niikura et al., 2006), which has the *UL41**Cys allele (Cys at amino acid residue 377) and referred to as pMDV-UL41*Cys, and screened by PCR using primers 97556F (5' CGATAGTGCTAGCTTCATCTAC 3') and 97628AtGR (5' GAACTTCCAGGGTTAGTATTTTGAAACGG 3') to generate pMDV-UL41*Arg. Similarly, UL41mutRev was mixed with the competent cells containing pMDV-UL41*Arg, subjected to electroporation, and screened by PCR using primers 97556F and 97628UL41rev (5' GAACTTCCAGGGTTAGTATTTTGAAGTGC 3)' to generate pMDV-UL41*Cys-rev. pMDV-UL41*Cys and pMDV-UL41*Cys-rev encode the same UL41 amino acid sequence but differ for a silent SNP (AY510475:g.108,207A>G), which allows molecular tracking of both viral genomes in mixed populations. The new BAC clones were purified using a Large-Construct Kit (Qiagen, Valencia, CA, USA) and a ~500 bp region that encompasses the UL41 SNP was confirmed by DNA sequencing. To generate MDV, BAC DNA (50-100 ng) was added to a CEF cell suspension and electroporated using a cell electroporation system at a setting of 330 μF and 400 V (Life Technologies, Rockville, MD, USA).

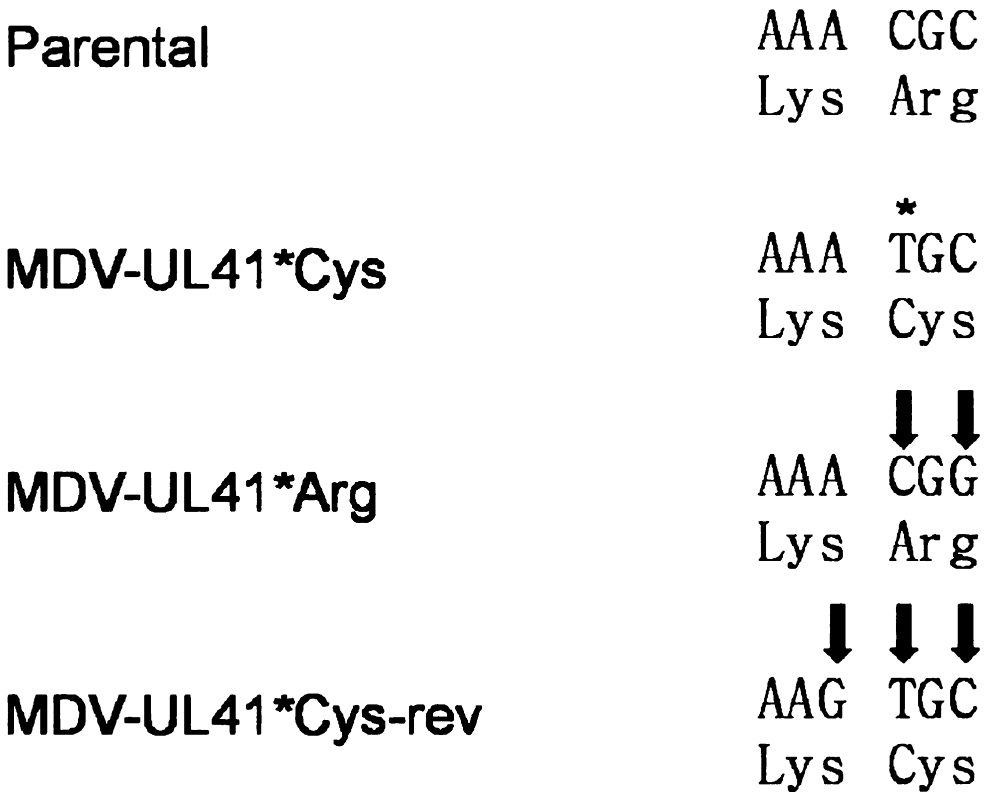

Figure 4-1. Partial *UL41* DNA and amino acid sequences for the parental Md11 strain, the original MDV-BAC clone (MDV-UL41*Cys), and the two recombinant viruses (MDV-UL41*Arg and MDV-UL41*Cys-rev). The position of the given sequences for the first and last bases are 108,209 and 108,204 based on the Md11 strain sequence (GenBank no. AY510475). The asterisk (*) indicates the SNP between the parental Md11 and MDV-UL41*Cys that resulted in an amino acid difference at amino acid residue 377 (GenBank no. AAS01683). In addition to altering this position by the mini-λ mutagenesis procedure, the third base of the same codon (position 108,204) was mutated to increase the efficiency of recovering the correct recombinants. Plus, the third base of the Lys codon was changed (AY510475:g.108207A>G) in MDV-UL41*Cys-rev to allow tracking and allele quantification by pyrosequencing between MDV-UL41*Cys and MDV-UL41*Cys-rev.

Growth rates of individual viruses

Growth characteristics of each individual cloned virus was determined. Briefly, 100 PFU were inoculated onto CEF cells seeded on 35-mm dishes. On 1, 2, 3, 4, and 5 days after inoculation, the infected cells were trypsinized, serial dilutions inoculated onto fresh cells, and plaques of the different dilutions were counted 7 days later in triplicate.

Standard curve

UL41-containing fragments were cloned from MDV-BAC clones containing the UL41*Arg and UL41*Cys alleles by PCR using primers 97628F (5' TTCAACTGCTGTCTCATCAC 3') and 97628R (5' TCCCAGAGCGCAGAACTTCC 3'). Amplicons containing the *UL41**Arg and *UL41**Cys alleles were cloned individually into the TOPO TA Vector (Invitrogen, Carlsbad, CA, USA) and the entire segment verified by DNA sequencing. DNA pools containing the two vectors in 10% increments were generated. The artificial DNA pools were analyzed by pyrosequencing using primers 97628fBiotin (5' [BioTEG] TTCAACTGCTGTCTCATCAC 3') and 97628R for PCR amplification, primer 97628Rseq (5' CCAGGGTTAGTATTTTGAA 3') for sequencing, and the PSQ 96M software (Biotage, Uppsala, Sweden).

Competitive assay

Three experimental groups were established. Viruses with the *UL41**Arg, *UL41**Cys, *UL41**Cys-rev alleles at amino acid residue 377 (see Fig. 4-1), referred to as MDV-UL41*Arg, MDV-UL41*Cys, and MDV-UL41*Cys-rev, respectively, were generated by transfections into CEF individually from pMDV-UL41*Arg, pMDV-

UL41*Cys, and pMDV-UL41*Cys-rev, respectively. Viral stocks of all three MDV types were made and the titers determined. CEF containing 1,000 unit PFU of MDV-UL41*Arg and MDV-UL41*Cys each, MDV-UL41*Arg and MDV-UL41*Cys-rev each, or MDV-UL41*Cys and MDV-UL41*Cys-rev each were mixed in triplicate. MDV-UL41*Arg, MDV-UL41*Cys, or MDV-UL41*Cys-rev were passed individually as controls. Virus DNA was extracted on every passage for SNP frequency determination and each sample was measured once by pyrosequencing.

UL41 activity assay in vitro

Chicken DF1 cell line (Himly et al., 1998) was used for UL41 activity assays, and were cultured using the same conditions described for CEF. *UL41* alleles from pMDV-UL41*Arg and pMDV-UL41*Cys were amplified using the primers UL41HindIIIF (5'ATCGAAGCTTCGCCACCATGGGAGTATATGGATGTATG 3') and UL41EcoRIR (5' ATCGGAATTCTCAGTCATTAGATCGTGTTG 3'). The PCR products were digested with *Hin*dIII and *Eco*RI, cloned into the *Hin*dIII and *Eco*RI sites of the pcDNA3.1/Zeo vector (Invitrogen, Carlsbad, CA, USA), and the entire region verified by DNA sequencing. DF1 cells were co-transfected with 0.5 pmoles of the β-galactosidase expression plasmid pON249 (Geballe et al., 1986) along with 0.01 to 0.5 pmoles of the UL41*Arg or UL41*Cys expressing plasmid. Two HSV-1 UL41-containing constructs, pCMVvhs and pCMVvhs-1, which express functional and inactive forms of the UL41 protein, respectively, were used as positive and negative controls (Jones et al., 1995); kindly provided by Jeffery Cohen (NIAID, NIH). The pcDNA3.1/Zeo vector was added to each co-transfection reaction to maintain a constant amount of the CMV promoter and total DNA. All transfections were performed in duplicate. Two days after co-transfection, the DF1 cells were lysed by alternate cycles of freeze-thaw. β-galactosidase activity was measured at an OD of 420 nm in conjunction with the β-Gal Assay Kit (Invitrogen, Carlsbad, CA, USA).

Results

Generation of pMDV-UL41*Arg and pMDV-UL41*Cys-rev by mini-λ mutagenesis

The mini-λ phage recombinant mutagenesis system was used to introduce a point mutation into the original MDV-BAC clone to alter a specific UL41 amino acid (residue 377). Furthermore, additional point mutations were utilized to increase the efficiency of recovering the correct recombinant (AY510475:g.108,204C>G), or for differentiating between two viruses with the same amino acid sequence (AY510475:g.108,207A>G). A recombinant BAC clone that generates MDV with a UL41*Arg allele was generated from pMDV-UL41*Cys, the original MDV-BAC clone. pMDV-UL41*Cys-rev was generated from pMDV-UL41*Arg, which contains the same amino acid but a different nucleotide sequence compared to pMDV-UL41*Cys (Fig. 4-1). This reverse mutant was generated as a negative control for the head-to-head competitive assay. All of the MDV-BAC clones were confirmed by restriction enzyme digestions using *Bam*HI or *Hin*dIII for the intact viral genome, and sequencing of the UL41 SNP and flanking regions.

Growth rates of individual viruses in vitro

MDV-UL41*Cys, MDV-UL41*Arg, and MDV-UL41*Cys-rev were generated from the appropriate MDV-BAC clones. To determine potential effects of the UL41 polymorphisms, each virus was grown individual in vitro. As shown in Fig. 4-2, there was no significant difference between any of the three MDVs.

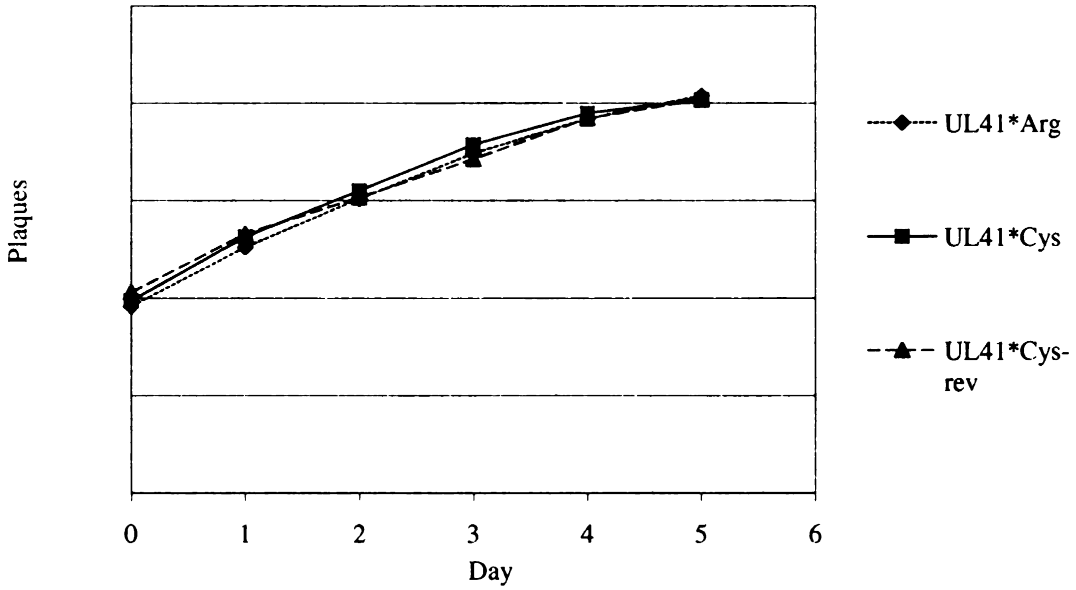

Figure 4-2. Growth rates of individual viruses *in vitro*. MDV-UL41*Cys, MDV-UL41*Arg, and MDV-UL41*Cys-rev were grown on CEF, harvested and titered on the days indicated. The experiment was performed in triplicate and the average is given.

Standard curve for pyrosequencing

A standard curve was generated to calibrate the pyrosequencing technology using DNA pools containing a known mixture of cloned *UL41**Arg and *UL41**Cys alleles in 10% increments. The standard curve showed that the pyrosequencing signals for the

SNPs accurately reflected the original proportion of the two recombinant vectors without a need for extrapolation (Fig. 4-3).

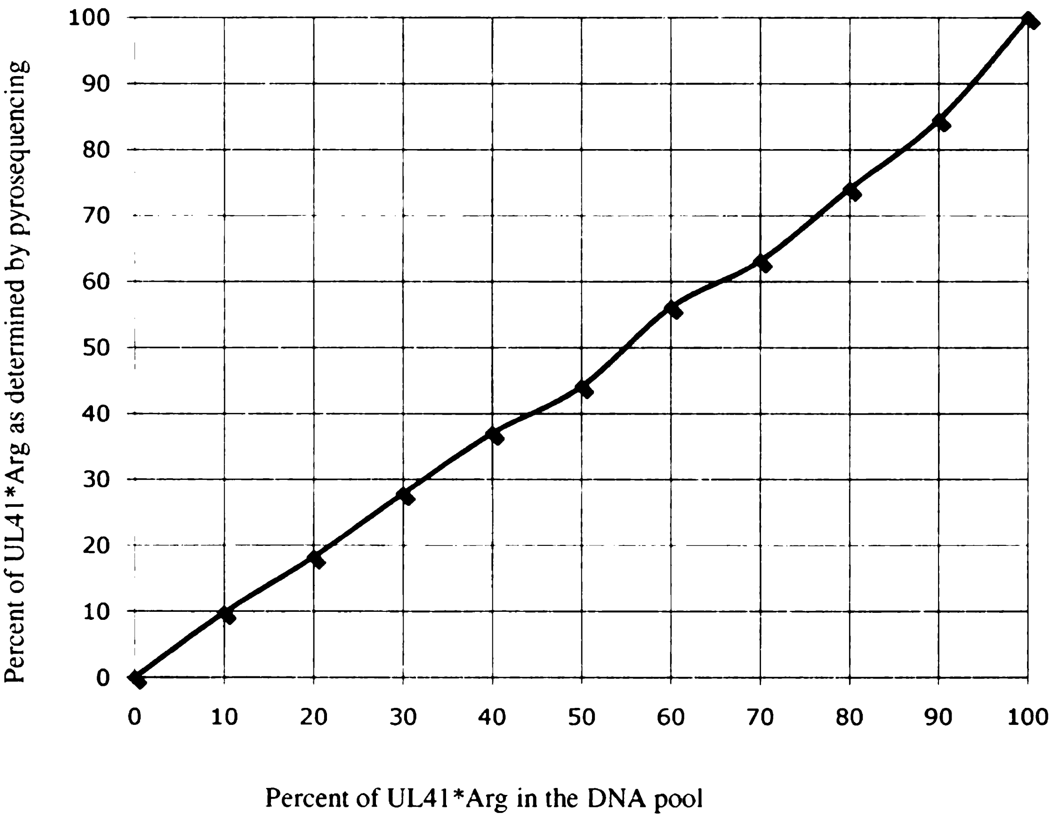

Figure 4-3. Standard curve of pyrosequencing for *UL41* alleles. DNA fragments containing the *UL41**Arg and *UL41**Cys alleles were mixed at known amounts to generate DNA pools that differed in 10% increments (X axis). The percent of *UL41**Arg was determined by pyrosequencing (Y axis).

Increasing virus fitness of MDV-UL41*Cys in virus populations *in vitro*

In the group containing MDV-UL41*Arg and MDV-UL41*Cys, the average percentage of MDV-UL41*Arg steadily decreased from 52% to 5% after 20 passages, while the average percentage of MDV-UL41*Cys increased from 48% to 95% (Fig. 4-4a).

In the MDV-UL41*Arg and MDV-UL41*Cys-rev mixed group, the average percentage of MDV-UL41*Arg decreased from 50% to 5%, and the percentage of MDV-UL41*Cys-rev increased from 50% to 95% relatively over 20 passages (Fig. 4-4b). Note that control groups that contained MDV-UL41*Cys and MDV-UL41*Arg separately, the SNP frequency was constant at 100%. To exclude the possibility that these results were attributed to unexpected mutations during the mutagenesis approach itself, the virus growth in a mixed group containing the same amino acid (Cys) but different SNPs were evaluated. In contrast to other mixtures, following 20 passages, the proportion of MDV-UL41*Cys and MDV-UL41*Cys-rev genomes remained at a similar proportion (Fig. 4-4c).

Figure 4-4. The percentage of MDVs genomes in the competition assay. A. The percent of MDV genomes with the *UL41**Arg alleles among a mixture of CEF infected with viruses containing either *UL41**Arg or *UL41**Cys alleles as determined by pyrosequencing. EXP 1, EXP2 and EXP3 represent three independent replicates. MDV-UL41*Cys Control is the result where CEF were infected with MDV containing the *UL41**Cys allele only. B. Results from the same experimental design as A except that MDVs differed in having either *UL41**Arg or *UL41**Cys-rev alleles. MDV-UL41*Arg Control results are from infected CEF where MDV containing the *UL41**Arg allele only were passaged. C. Results from the same experimental design as A and B except that MDVs differed in having either *UL41**Cys or *UL41**Cys-rev alleles. MDV-UL41*Cys-rev Control results are from infected CEF where MDV containing the *UL41**Cys-rev allele only were passaged.

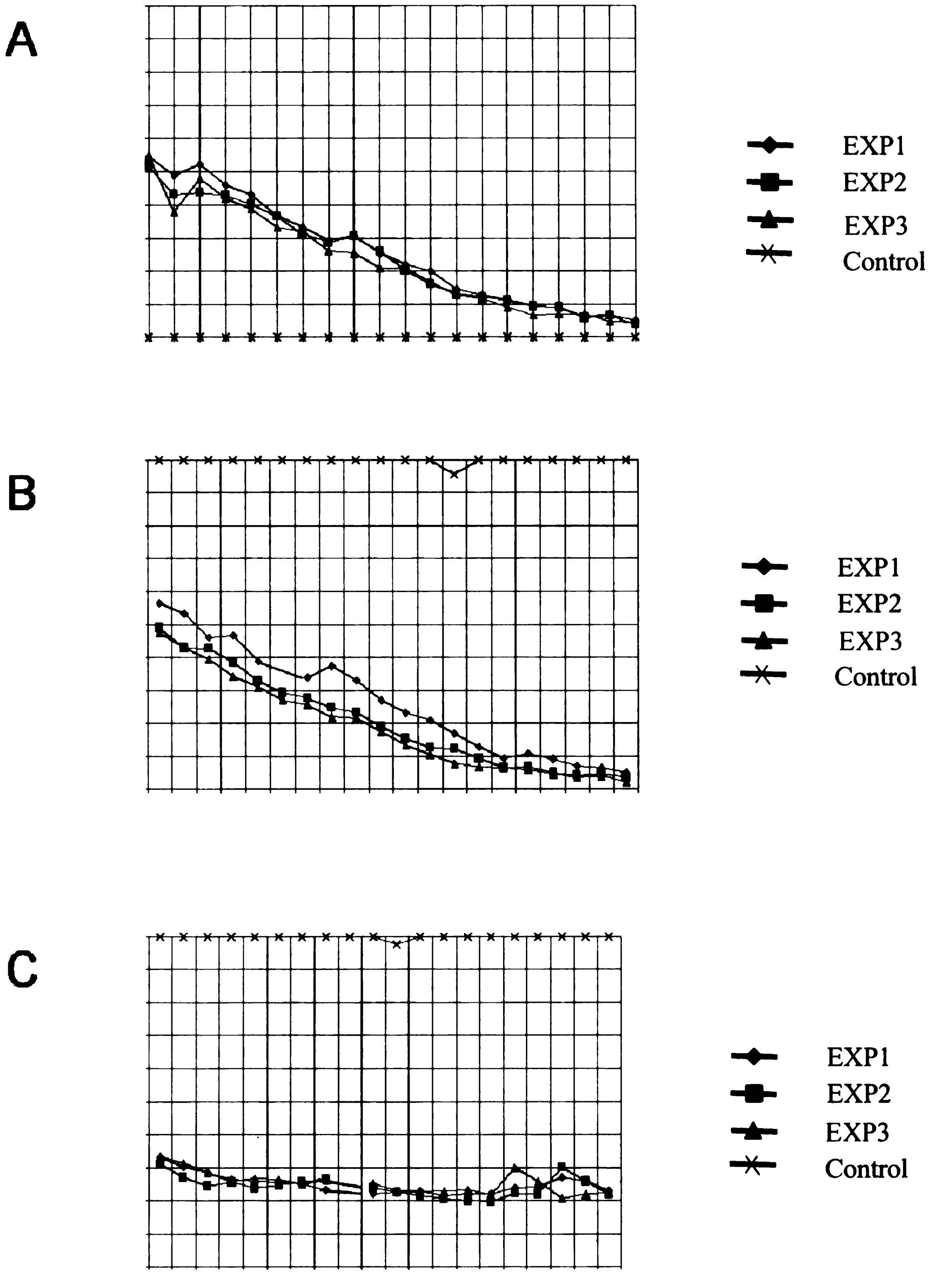
A
EXP1
EXP2
EXP3
Control
B
EXP1
EXP2
EXP3
Control
C
EXP1
EXP2
EXP3
Control

Figure 4-4

Wild type MDV UL41 (MDV-UL41*Arg) has vhs activity and this enzyme activity is abolished in MDV-UL41*Cys

When DF1 cells were co-transfected with plasmids expressing MDV UL41*Arg and β-galactosidase, β-galactosidase activity decreased as the concentration of the UL41-expressing plasmid increased (Fig. 4-5). On the other hand, the β-galactosidase activity did not change with varying MDV UL41*Cys expressing plasmid amounts when co-transfected with a β-galactosidase expression plasmid into the DF1 cells (Fig. 4-5). The HSV-1 vhs plasmid, the positive control, showed vhs activity that was dependent on the amount of plasmid DNA while the HSV-1 vhs-1 plasmid, the negative control, did not show any detectable vhs activity (Fig. 4-5). The background β-galactosidase activity of DF1 cells only was less than 0.15 when measured at an OD of 420 nm.

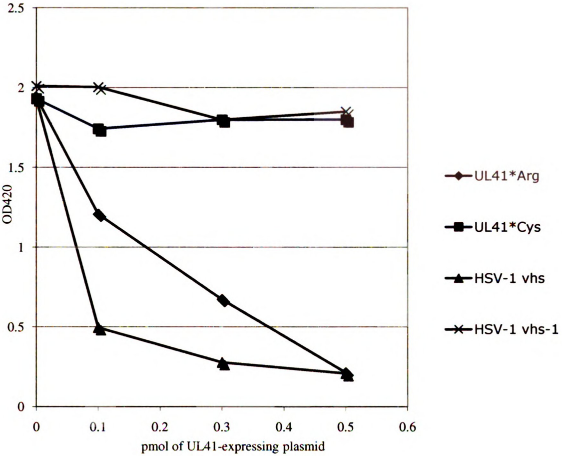

Figure 4-5. The β- galactosidase assay for MDV UL41*Arg, UL41*Cys activity and the controls. Plasmids expressing MDV UL41*Cys, MDV UL41*Arg, HSV-1 UL41 (vhs, positive control), or a mutant form of HSV-1 UL41 (vhs-1, negative control) were co-transfected into chicken DF1 cells along with the β-galactosidase expression plasmid pON249, and after two days, the cell lysates were measured for β-galactosidase activity. The values on the X axis indicate the amount of the UL41 expressing plasmid that was transfected; in addition, 0.5 pmole of pON249 was added along with varying amounts of pcDNA3.1/Zeo vector to keep CMV promoter constant. This figure shows the result from one of two replicates, which were consistent.

Discussion

Understanding gene function in MDV has been greatly facilitated by the ability to make defined recombinant viruses through MDV-BAC clones. However, it is often the case that recombinant MDVs lacking a gene of interest show little to no altered phenotype in vitro but significant changes *in vivo* (e.g., Reddy et al., 2002; Silva et al., 2004; Prigge et al., 2004; Kamil et al. 2005; Schumacher et al., 2005). This suggests that the *in vitro* assays are not very sensitive in detecting relevant mutations, which may be compounded by the fact that there are different environmental conditions and selective pressures *in vitro* compared to those *in vivo*. To provide an alternative and more efficient screening method for evaluating recombinant MDVs *in vitro*, a head-to-head competition assay was employed, which is very sensitive to detect differences in viral fitness, combined with pyrosequencing to quantify each viral genome.

The missense mutation in MDV-UL41 that results in Cys at amino acid 377 was found to enhance the viral fitness in cell culture by the head-to-head competition assay but not with the traditional *in vitro* growth curves of individual viruses. In addition, the growth advantage was not shown in the mixed viral population with MDV-UL41*Cys and MDV-UL41*Cys-rev, viruses that have identical UL41 amino acids. Thus, mixed MDV populations favor the growth of viruses with inactive forms of vhs in cell culture, and the pyrosequencing approach could quantify this difference in viral fitness.

During 20 passages, the MDV-UL41*Arg was almost replaced by MDV-UL41*Cys in the viral population when the initial ratio of the two viruses was 1:1. This adaptability of MDV-UL41*Cys is gradual, consistent between replicates, and constant, which suggests the *UL41**Cys allele provides a moderate relative fitness advantage at each passage. Also, a similar fitness advantage was observed in viral populations where the ratio of MDV-UL41*Arg to MDV-UL41*Cys was 4:1 suggesting that viral fitness depends on the number of passages rather than the initial ratio. The separate MDV-UL41*Arg and MDV-UL41*Cys viruses did not show any SNP frequency change after 20 passages. These controls provide evidence that MDV with different UL41 alleles are capable of growing in cell culture, there is no contamination among plates, and the SNPs are stable.

As the fitness advantage was dependent on the UL41 amino acid composition, UL41 activity was evaluated. The Arg in the variant amino acid site was reported to be conserved in all alpha-herpesviruses, and the replacement of Arg to His in HSV-1 abolishes endoribonuclease activity of UL41 (Everly and Read, 1999). Using the same β-galactosidase functional assay, the effect of the Arg to Cys substitution in MDV UL41 was determined. *UL41**Cys lacks any detectable endoribonuclease activity. However, the wild type MDV UL41 containing Arg has endoribonuclease activity at levels similar to HSV-1 UL41. Thus, in the competitive assay, the null MDV *UL41* gene is beneficial to MDV replication when competing with MDV with the *UL41* wild type allele. It has been reported that HSV with a non-functional UL41 showed impaired growth comparing with wild type HSV in mice though there is no obvious growth difference in cell culture (Smith et al., 2002). The HSV-1 *UL41* gene had been demonstrated to play an important role in the virus life cycle as it is capable of degrading the host mRNA although this degradation, for the most part, does not discriminate among host and viral mRNAs (Kwong and Frenkel, 1987; Esclatine et al., 2004). One possible hypothesis for this contradictory result is that a functional MDV *UL41* gene is advantageous for virus growth and spread *in vivo* where its vhs may interfere with the host immune response, however in vitro, a null *UL41* allele confers a slight viral replication advantage as the viral transcripts are not degraded. This slight difference might not be detected by classical plaque assay as a previous report demonstrated that the effect of vhs deletion on viral replication in tissue is minimal (Smith et al., 2002). If true, then these results support a role for MDV UL41 in interfering with the host immune system although further studies are required to determine the detail mechanism of UL41 gene in MDV biology.

In summary, this study presents a quantitative approach for head-to-head viral competition assays using SNPs as genetic markers for MDV. This approach is capable of directly detecting the ratio of virus genomes represented by the SNP on genomes and providing very sensitive monitoring at every passage. The use of this approach should help evaluate whether other MDV genes and genetic variation have functional roles in vitro and potentially in vivo.

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Chapter 5

Future work

This thesis described the cloning and functional characterization of chicken SCA2, and its influence on MDV growth properties *in vitro* via association with viral US10 protein. To investigate the biological properties of chicken SCA2, chicken SCA2 was expressed and purified in *E. coli*, and a polyclonal antibody was developed. Utilizing this anti-SCA2 antibody, SCA2 was identified to be a 13 kDa cell surface protein anchored by a GPI moiety as is the case for most other Ly-6 members (Classon and Coverdale, 1994). *In vivo* studies showed that SCA2 is expressed in a number of chicken tissues. Of particular interest was the unique SCA2 expression pattern in bursal CMEC that were often surrounded by MHC class II presenting cells. Expression profiles of bursal cells induced by contact with SCA2-expressing cells shows down-regulation of numerous genes including *CD79B*, B cell linker (*BLNK*), spleen tyrosine kinase (*SYK*), and gamma 2-phospholipase C (*PLCG2*) that are involved in the BCR and immune response signaling pathways. These results suggest chicken SCA2 plays a role in regulating B lymphocytes.

Based on the identified functions and expression patterns of SCA2, we hypothesize three putative roles for SCA2 in MDV biology. First, SCA2 promotes MDV spread as SCA2 may function in cell-cell adhesion with direct cell-cell contact required for MDV spread. Second, SCA2 transduces signals for cell proliferation that could affect transformation of cells harboring latent MDV. Third, SCA2 promotes virion maturation or egress via interacting with viral proteins. Some reports have demonstrated that chicken SCA2 is associated with v-Rel-transformed avian bone marrow cells and plays a role in self-renewal of avian erythroid progenitors (Petrenko et al., 1997; Bresson-Mazet et al., 2008). We also found the cell surface SCA2 was up-regulated in a MDV transformed T cell line (RP1) when induced for MDV replication (Fig. 5-1). An important question that one could address is whether up-regulation of SCA2 is specific for MDV or it is a response from host immune system. The preliminary results suggest that cell surface SCA2 was not induced by a reticuloendotheliosis virus in RP1-transformed T cell line. Consequently, the up-regulation of SCA2 could be induced by MDV gene products. SCA2 transcription was induced by MDV infection in CEF, a fibroblast cell line. Therefore, these results support the conclusion that MDV gene products, probably US10, induce the SCA2 transcription and further SCA2 cell surface expression. Interestingly, SCA2 transcription was up-regulated in many malignant tumor cell lines (Bresson-Mazet, 2008). Consequently, the question arises as to whether virus-induced SCA2 promotes virus-induced lymphomogenesis. The expression profiling results from B lymphocytes upon contact with SCA2-expressing cells indicated that SCA2 is involved in BCR and immune response signal pathways. Therefore, future efforts should be directed to investigate the role of SCA2 in signal transduction in chicken lymphocytes.

While we are the first to show a biological role for MDV US10, the role of MDV US10 is stil not well defined. The interaction of US10 and SCA2, and the putative function of SCA2 in lymphocytes proliferation suggest that US10 may regulate chicken lymphocyte via interacting with SCA2. Therefore, to elucidate the role of MDV US10 protein and the functional interaction of SCA2 and US10 for transformation or immune responses of lymphocytes, we could profile the expression of host genes of lymphocytes infected with MDV and ΔUS10-MDV to find out the immune response pathways in which US10 is involved. Parcell et al. (1994) reported that MDV lacking *US1, US2 SORF1, SORF2, SORF3* and *US10* showed decreased early cytolytic infection, mortality, tumor incidence, and horizontal transmission, but the role of US10 alone in MDV pathogenicity was not identified. A ΔUS10-MDV recombinant BAC has been generated, which we could use to characterize US10 with respect to MDV pathogenicity.

The US10EGFP-MDV we developed could also be employed for studies on MDV morphogenesis. An ideal assay for MDV morphogenesis analysis would be to label MDV membrane, tegument, and capsid proteins each with unique fluorescent tags. By utilizing this recombinant MDV, one could track the MDV dynamics of viral replication in living fibroblast cells. This would allow one to address areas such as: 1) The timing of new virion maturation and egress to the neighboring cells. 2) Is an intact virion required for MDV spread between cell-cell? 3) Does the virion transmit between cells mainly through cell-cell fusion? 4) The efficiency of super-infection. Due to the highly cell-associated characters, we would use MDVs labeled with fluorescent proteins to synchronize MDV infection. Furthermore, MDVs with fluorescent proteins might also be employed for tracking and collecting infected-lymphocytes *in vivo.*

Finally, on an unrelated subject, during the cloning and full genome sequencing of virulent MDV Md11 strain (Niikura et al., 2006), another nonsynonymous single nucleotide polymorphism (SNP; AY510475:g.160,319 A>G) was found in the *ICP4* gene that resulted in a Leu to Ser substitution in residue 256 (AAS01711.1:p.Leu256Ser; Fig. 5-2). Monitoring the frequency of each SNP by pyrosequencing during virus passages determined the ratio of each viral genome in a single monolayer, which is a very sensitive method to monitor viral fitness. MDV with the *ICP4**Ser allele showed enhanced fitness in vitro (Fig. 5-3). HSV ICP4 protein is a transcriptional regulator required for the induced transcription of most of the remaining HSV genes and essential for viral replication (Compel and DeLuca, 2003; Su et al., 2006). The exact role of ICP4 in MDV replication has not been well determined although it is speculated to play a role similar to the ICP4 of other alpha-herpesviruses as a transcription regulator, and it is associated with maintenance of transformation and is essential for MDV growth in CEF (Endoh, 1996; Chattoo et al., 2006). Therefore, we would identify the effect of the SNP on the role of ICP4 as a transcriptional activator, and further on the virus evolution.

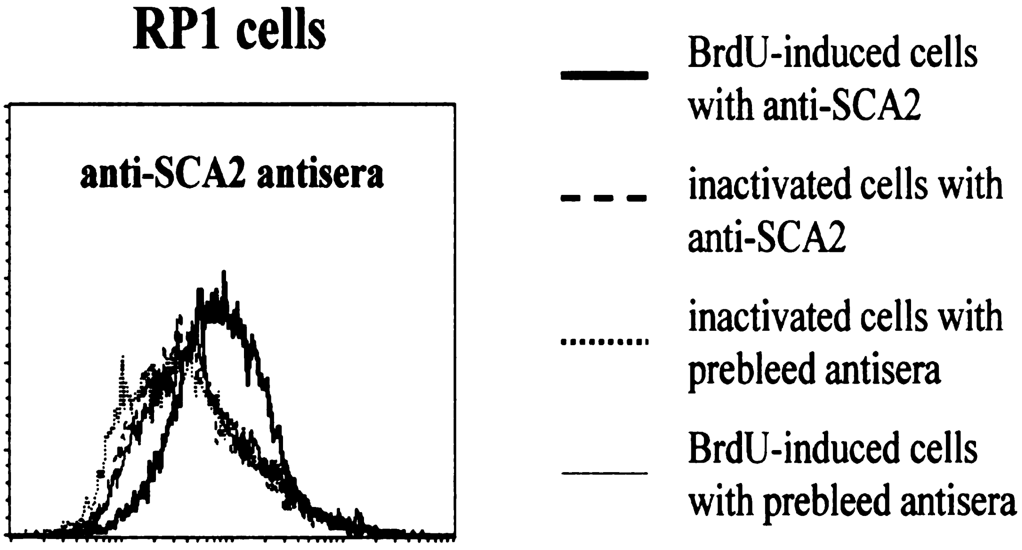

Figure 5-1. SCA2 is up-regulated on the surface of BrdU-induced RP1 (MDV transformed) T cell line.

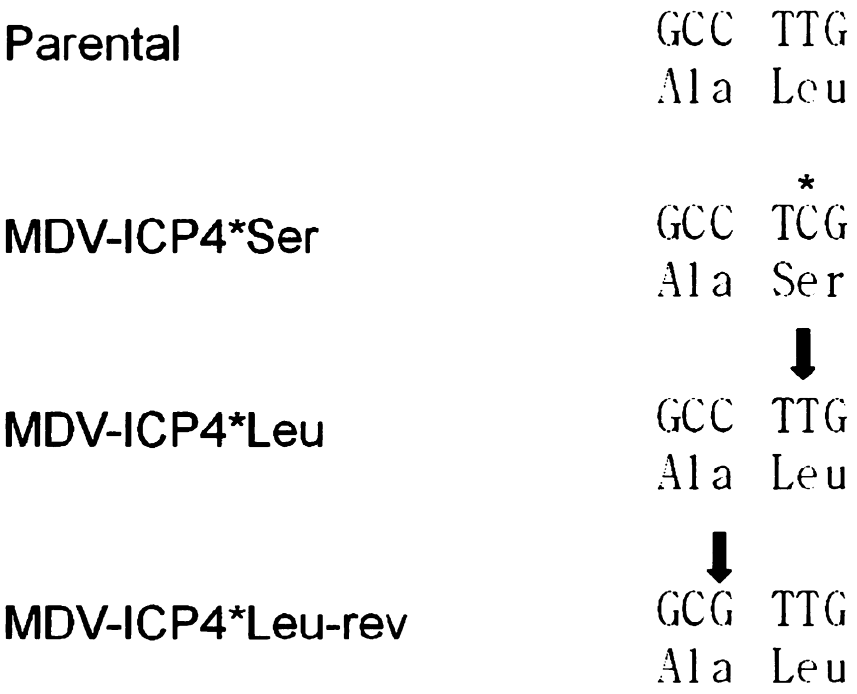

Figure 5-2. Partial *ICP4* DNA and amino acid sequences for the parental Md11 strain, the recombinant virus (MDV-ICP4*Ser), the original MDV-BAC clone (MDV-ICP4*Leu), and the recombinant viruse (MDV-ICP4*Leu-rev).

Figure 5-3. The percetange of MDVs genomes containing SNPs in *ICP4* alleles in a competition assay. A. The percent of MDV genomes with the *ICP4**Leu alleles among a mixture of CEF infected with viruses containing either *ICP4**Leu or *ICP4**Ser alleles as determined by pyrosequencing during 8 passages. Exp 1, Exp 2 and Exp 3 represent three independent replicates. B. Results from the same experimental design as A except that MDVs differed in having either *ICP4**Ser or *ICP4**Leu-rev alleles. C. Results from the same experimental design as A and B except that MDVs differed in having either *ICP4**Leu or *ICP4**Leu-rev alleles.

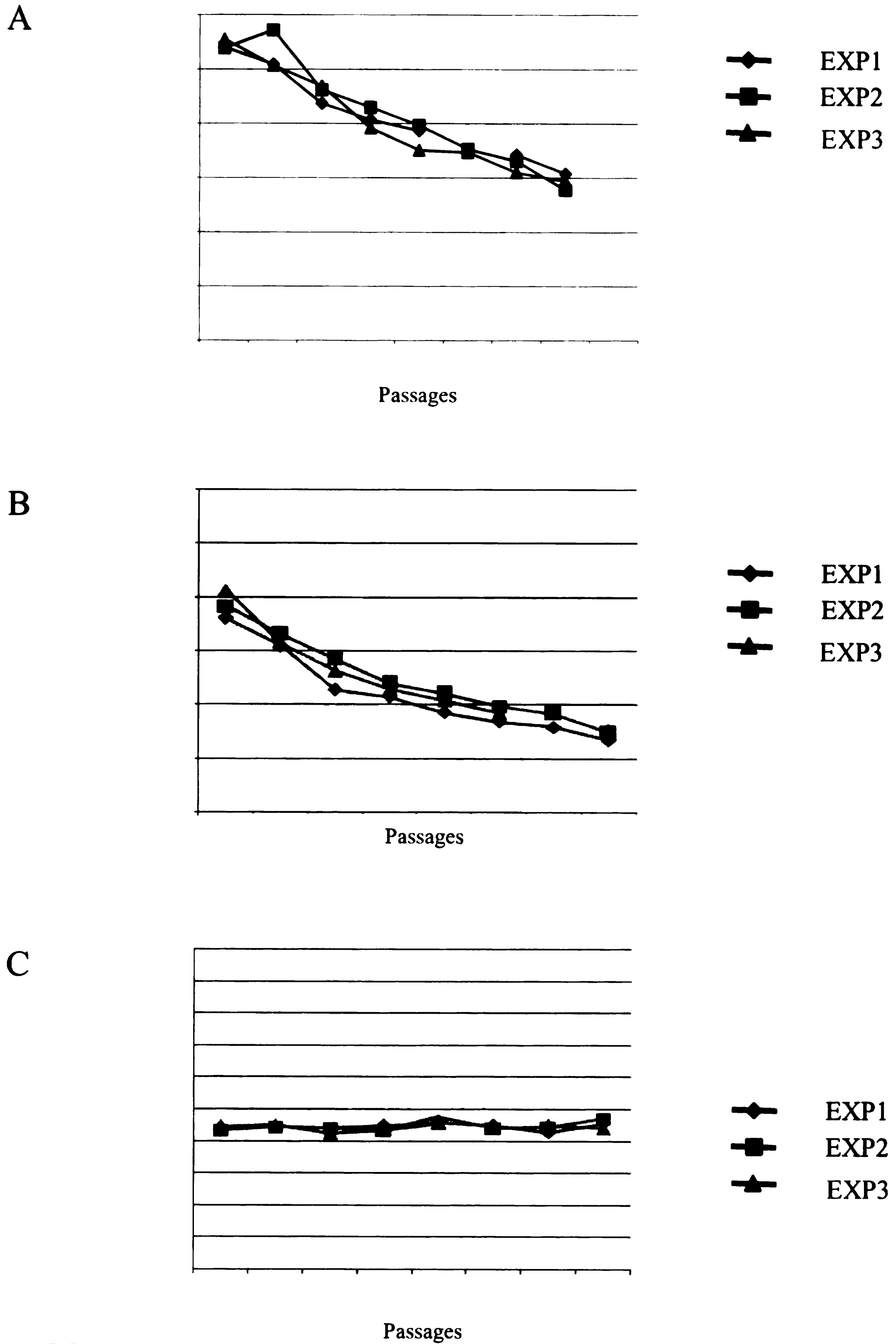
A
EXP1
EXP2
EXP3
Passages
B
EXP1
EXP2
EXP3
Passages
C
EXP1
EXP2
EXP3
Passages

Figure 5-3

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