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Molecular characterization of the gene products encoded by open reading frames 34 and 59 of equine herpesvirus type 1 (EHV-1)

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Abdelrahman Fekry Abdelrahman Said
Tierarzt
aus Ägypten/Kafer Elsheikh

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Dekan:	Univ.-Prof. Dr. Jürgen Zentek
Erster Gutachter:	Univ.-Prof. Dr. Klaus Osterrieder
Zweiter Gutachter:	Prof. Dr. Benedikt Kaufer
Dritter Gutachter:	Univ.-Prof. Dr. Robert Klopfleisch

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Dedication

Dedicated to my Father´s soul, to my Mother, to my brothers, to my dearest wife, to my lovely daughters and son.

Abdelrahman

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List of abbreviations

Act-D	Actinomycin D
BAC	Bacterial artificial chromosome
BHV-1	Bovine herpesvirus type 1
β2M	β2-microglobulin
CX	Cycloheximide
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
E	Early
E1	Ubiquitin-activating enzyme
ECL	Enhanced chemoluminscence
E.coli	Escherichia coli
EGFP	Enhanced green fluorescence protein
EHV-1	Equine herpsvirus type 1
EHV-2	Asinine herpesvirus 2
EHV-3	Equine coital exanthema virus
EHV-4	Equine rhinopneumonitis virus
EHV-5	Asinine herpesvirus 2
EHV-6	Asinine herpesvirus 1
EHV-7	Asinine herpesvirus 2
EHV-8	Asinine herpesvirus 3
EHV-9	Gazelle herpesvirus 1
EMEM	Earle’s minimum essential medium
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FW	Forward
gM	Glycoprotein M
gp	Glycoprotein P
hpi	Hour post infection
HSV	Herpes simplex virus

IE	Immediate early
IF	Indirect immunofluorescence
IgG	Isotype immune globulin G
IMDM	Iscove′s modified Dulbecco′s medium
IR_S	Internal repeat short
IR_L	Internal repeat long
Kan^R	kanamycin resistance gene
KDa	Kilodalton
L	Late
LB	Luria-Bertani
MAb	Monoclonal antibody
MEM	Modified eagle's medium
MHCI	Major histocompatibility class I
m.o.i	Multiplicity of infection
mRNA	Messenger Ribonucleic acid
NBL6	Equine dermal cells
ORFs	Open reading frames
PAA	Phosphonoacetic acid
pAb	Polyclonal antibody
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
pCDNA_34-HA	pCDNA3 plasmid with HA tag at C-terminus of ORF34
PCR	Polymerase chain reaction
PEI	Polyethylenimine
PFU	Plaque forming units
pORF34	ORF34 protein
pORF59	ORF59 protein
PRV	Pseudorabies virus
PVDF	Polyvinylidene fluoride
qPCR	quantitative polymerase chain reaction

R	Revertant
r	recombinant
rAb4Δ34	recombinant Ab4 strain of EHV-1 lacking ORF34
rAb4Δ59	recombinant Ab4 strain of EHV-1 lacking ORF59
rAb4_34-HA	recombinant Ab4 with an HA-tagged ORF34 protein
rAb4_59-HA	recombinant Ab4 with an HA-tagged ORF59 protein
RFLP	Restriction fragment length polymorphism
RIPA	Radioimmunoprecipitation assay
RK13	Rabbit kidney cells
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
RT	Room temperature
RV	Reverse
SDS	Sodium dodecyl sulfate
λ-PPase	λ-protein phosphatase
TR_L	Terminal repeat long
TR_s	Terminal repeat short
UL	Unique long
US	Unique short
VN	Virus neutralizing
VZV	Varicella-zoster virus

Zusammenfassung

Titel: Molekulare Charakterisierung der Genprodukte des ORF34 sowie ORF59 des Equinen Herpesvirus Typ 1 (EHV-1)

Das Equine Herpesvirus Typ 1 (EHV-1) ist ein wichtiger Erreger, der weltweit Populationen von Pferden bedroht und weitreichende, wirtschaftliche Verluste durch häufige Ausbrüche von respiratorischen und neurologischen Erkrankungen sowie Aborten verursacht.
Die Primärinfektion mit EHV-1 führt zur Etablierung einer latenen Infektion während der ersten Wochen nach der Geburt. Strikte Hygienemaßnahmen und Impfungen sind daher die beiden Hauptstrategien zur Bekämpfung von EHV-1 Infektionen. Obwohl einige Impfstoffe gegen EHV-1 die schwersten Folgen der Infektion verhindern können, besteht jedoch kein ausreichender Langzeitschutz. Daher sind bisher viele Anstrengungen unternommen worden, um die Replikation und die Wachstumseigenschaften des EHV-1 sowie die Funktion einzelner Genprodukte zu verstehen und diese Informationen zur Entwicklung effektiver Impfstoffe zu nutzen. Die Funktionen der EHV-1 Genprodukte ORF34 und ORF59 (pORF34 und pORF59) sind bisher unbekannt. Die Hauptziele dieser Arbeit waren daher (i) die Identifizierung und Charakterisierung der EHV-1 pORF34 und pORF59 Genprodukte und (ii) die Untersuchung ihrer Rolle bei der Pathogenese des EHV-1.

Im Kapitel 2 dieser Arbeit wird die Identifizerung des ORF34 Genprodukts, welches früh während des Replikationszykluses exprimiert wird, beschrieben. Das Genprodukt lokalisiert im Zytosol der EHV-1 infizierten Zelle. Analysen mit Hilfe von Immunoblots erlaubten die Detektion eines Proteins mit einer molekularen Masse von 28 KDa, die maßgeblich von der auf der Sequenz beruhenden Vorhersage der Größe abwich (18 kDa). Interessanterweise zeigte sich in Expressionsstudien des pOF34, dass das Protein bis 10 Stunden nach der Infektion (h p.i.) stabil war. Während späterer Zeitpunkte der Infektion zeigten sich jedoch zusätzliche Banden, deren molekulare Massen zwischen 17- bis 28 kDa rangierten. Diese Ergebnisse implizieren einerseits eine zeitabhängige Stabilität des pORF34 während der Infektion und andererseits, dass pORF34 möglicherweise nur während frühen Zeitpunkten nach Infektion benötigt wird oder seine Funktion zu späteren Zeitpunkten durch die Modifizierung des Proteins im Ubiquitin/Proteasome -Weg beinflußt wird.
Des Weiteren ergab die Untersuchung der Wachstumseigenschaften einer ORF34 deletierten

EHV-1-Mutante (rAb4Δ34), dass das pORF34 für das effiziente Wachstum während der frühen Stadien der Infektion *in vitro* notwendig ist.

In Kapitel 3 dieser Arbeit werden Details über das EHV-1 Genprodukt pORF59 und seiner Rolle in der Pathogenese des Viruses beschrieben. pORF59 wird in den frühen Phasen der Infektion gebildet und lokalisiert im Zytosol der infizierten Zelle. Analysen mit Hilfe von Immunoblots ermöglichten die Detektion des pORF59 (mit inseriertem Epitope) als Bande mit einer molekularen Masse von ca. 21 kDa, in Übereinstimmung mit der theoretisch vorhergesagten Sequenz.

Des Weiteren zeigte eine ORF59 deletierte EHV-1 Mutante (rAb4Δ59) eine verringerte Größe der viralen Plaques und konnte nicht propagiert werden. Diese Ergebnisse implizieren, dass das ORF59 Protein eine entscheidene Rolle beim Wachstum des EHV-1 *in vitro* spielt.

Die entscheidenen Ergebnisse dieser Arbeit können wie folgt zusammengefasst werden:

► Das EHV-1 ORF34 Protein wird während der frühen Phase der Infektion gebildet und lokalisiert im Zytosol der infizierten Zelle.

► Das ORF34 Protein wird durch Ubiquitinierung modifiziert und während später Phasen der Infektion durch das Proteasom abgebaut.

► Das ORF34 Protein wird für die optimale Replikation des EHV-1 *in vitro* und in *ex vivo* infizierten Zielzellen in frühen Phasen der Infektion benötigt.

► Das EHV-1 ORF59 Protein ist ein 21-kDa Protein, welches früh während der Infektion gebildet wird.

► Das Produkt des EHV-1 ORF59 lokalisiert im Zytosol von EHV-1-infizierten Zellen.

► Das EHV-1 ORF59 Protein ist essentiell für das EHV-1 Wachstum *in vitro*.

Schlüsselwörter

EHV-1, ORF34, ORF59, Rekombination, Proteinlokalisation, Ubiquitination, Proteindegradation

Summary

Title: Molecular characterization of the gene products encoded by open reading frames 34 and 59 of equine herpesvirus type 1 (EHV-1)

Equine herpesvirus type 1 (EHV-1) is an important pathogen that threatens horse populations worldwide and causes spacious economic losses through recurrent outbreaks of respiratory, abortion and neurological disease. Primary infections with EHV-1 lead to the establishment of latent infection within the first weeks of life. The two main strategies for controlling EHV-1 infection and disease are strict hygiene and management practices as well as vaccination. Several vaccines exist to prevent the most severe outcomes of EHV-1 infection, but they are insufficient for long-term protection. Therefore, considerable efforts have been developed to understand the replication and growth properties of EHV-1 and the function of its gene products in order to generate information that may be useful in the design of an effective vaccine. Since the functions of the EHV-1 ORF34 and ORF59 protein (pORF34 and pORF59) products are unknown, the overall targets of this thesis are to (i) identify and characterize the EHV-1 pORF34 and pORF59 products and (ii) examine whether they have a role in pathogenesis of EHV-1.

In Chapter 2 of this thesis, we focused on the identification of ORF34 product that is expressed with early kinetics and its product is localized within the cytosol of EHV-1- infected cells. Immunoblot analysis of EHV-1 pORF34 detected a protein with the apparent molecular mass of 28-kDa, which is larger than that predicted based on the sequence of the protein (18-kDa). Interestingly, the kinetics of pORF34 expression had shown protein stability until 10 h p.i., however, additional bands with apparent molecular masses ranging between 17- and 28-kDa appeared at later times after infection. Our findings suggested that stability of pORF34 is dependent on the time after infection and that pORF34 may only be required at early times after infection or that its function at later times after infection requires the protein be modified by ubiquitin/proteasome pathway. Furthermore, examination of the replication properties of an EHV-1 lacking ORF34 (rAb4Δ34) revealed that pORF34 is required for efficient virus growth *in vitro* at early stages of infection.

In Chapter 3 of this thesis, we addressed more details about the identification of EHV-1 pORF59 and its role in the EHV-1 pathogenesis. We found that pORF59 is expressed at the early stage of infection and its product is localized in the cytosol of the infected cells. Immunoblot analysis of

EHV-1 pORF59, including the inserted epitope, detected a protein with a molecular mass of approximately 21-kDa, which is similar to the predicted sequence of the protein. Moreover, EHV-1 lacking ORF59 (rAb4Δ59) exhibited a small-plaque phenotype and could not be propagated even after serial passages in cell culture. Our findings suggest that the ORF59 protein plays a major role in EHV-1 growth in cultured cells.

Generally, a concise list below can show the highlights points of this thesis:

► The EHV-1 ORF34 protein is expressed at early stage of infection and its product is localized in cytosol of EHV-1 infected cells.

► The ORF34 protein is modified by ubiquitination and degraded in the proteasome at late times of infection

► The ORF34 protein is required for optimal replication of EHV-1 in cultured cells and target cells taken directly *ex vivo* at early times of infection.

► The EHV-1 ORF59 protein is a 21-kDa protein expressed with early kinetics.

► The product of the EHV-1 ORF59 protein localizes to cytosol of EHV-1-infected cells.

► The EHV-1 ORF59 protein is essential for EHV-1 growth in cultured cells.

Key word

EHV-1, ORF34, ORF59, recombination, protein localization, ubiquitination, protein degradation

CHAPTER 1

General Introduction

Chapter 1: General Introduction

1.1. Taxonomy and genome characters

The Herpesviridae comprises a large family of enveloped double-stranded DNA viruses, with a broad host spectrum ranging from mammals to birds and reptiles. Herpesviruses represent one of the largest known virus groups. In equid populations, nine herpesviruses have been isolated so far and are divided into two subfamilies: (i) the alphaherpesvirinae, which is included the following viruses, equine abortion virus (EHV-1), equine coital exanthema virus (EHV-3), equine rhinopneumonitis virus (EHV-4), asinine herpesvirus 1 (EHV-6), asinine herpesvirus 3 (EHV-8) and gazelle herpesvirus 1 (EHV-9). (ii) The gammaherpesvirinae that is included equine cytomegalovirus (EHV-2), equine gamma herpesvirus 5 (EHV-5) and asinine herpesvirus 2 (EHV-7). Based on clinical and economical effects, Equine herpesvirus type 1 and 4 (EHV-1 and 4) are the most pathogens of equine herpesviruses (Patel and Heldens, 2005). Although both viruses show a high degree of antigenic and genetic similarity, but they have different clinical symptoms. EHV-1 has a systematic distribution causing different diseases that range from mild rhinopneumonitis to abortion and lethal myeloencephalopathy. While the EHV-4 infection is limited mainly to the upper respiratory tract in the most of the cases. EHV-1 is the most important to all horse populations worldwide and causes wide economic losses through frequent outbreaks of respiratory, neurological and abortogenic disease (Crabb and Studdert, 1995; Reed and Toribio, 2004; Slater et al., 2006). Equine herpesvirus type 1 (EHV-1) is a member of the genus Varicellovirus in the subfamily Alphaherpesvirinae. (Allen and Bryans, 1986; Davison et al., 2009). The complete genome sequences of EHV-1 was reported (Telford et al., 1992). It is a double-stranded DNA genome of 150-Kbp in length (Davison et al., 2009; Telford et al., 1992). It is divided into a unique long (UL) and a unique short (US) region, both flanked by an inverted internal and terminal repeat sequence (IRS and TRS or IRL and TRL; respectively). The genome contains 80 open reading frames (ORFs), which encode 76 unique genes; four ORFs are duplicated in the TRS (Telford et al., 1992) (Fig. 1.1).

Viral replication requires a coordinated gene expression program in which immediate-early (IE) gene expression progresses to early (E) and finally late (L) gene expression (Caughman et al., 1985; Gray et al., 1987).

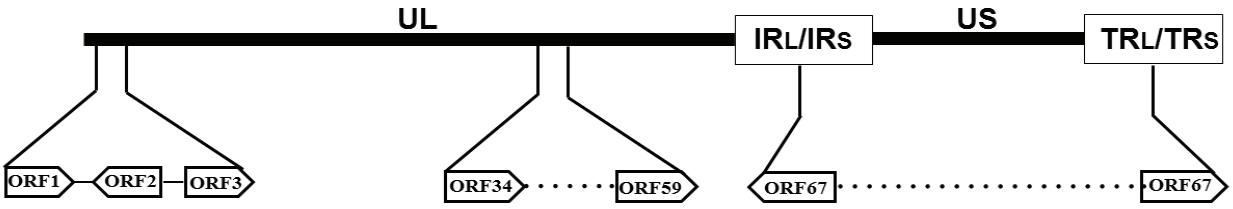

Fig. 1.1. Schematic diagram of sequence arrangement of the EHVs genome. The genome of EHVs divided into unique-long (UL) and unique-short (US) sequences, the latter being flanked by inverted repeat sequences termed internal and terminal repeats (IR and TR, respectively). Moreover, schematic map illustrating the position of the genes (ORF 1, 2, 3, 34, 59 and 67) that do not have a homolog in HSV-1.

1.2. Conserved and non-conserved genes of EHV-1

The genomes of *Alphaherpesvirus* show a generally collinear gene arrangement and most of the genes are conserved between different subfamily members. However, comparison of EHV-1and herpes simplex virus type 1 (HSV-1) sequences revealed exceptions to this rule, with six of the 76 unique genes in EHV-1, namely ***ORFs 1, 2, 3, 34, 59 and 67***, lacking positional and/or sequence homologues in HSV-1 (Telford et al., 1992). Although those six genes do not have a homolog in HSV-1, they share positional and limited sequence homology with proteins encoded by members of the Varicellovirus genus.

1.2.1. Open reading frame 1 (ORF 1)

The EHV-1 ORF1 (UL56) gene is predicted to encode a protein containing 180 amino acids localizes to Golgi membranes and that is dispensable for virus growth *in vitro*. ORF1 protein (pORF1) is an early, phosphorylated type II transmembrane protein expressed with early kinetics (Ma et al., 2012). The transmembrane domain of pORF1 is indispensable for proper subcellular localization of pORF1 and function. Moreover, pORF1 plays an important role in the downregulation of cell surface MHC-I in the early stages of infection. In addition, it was shown by (Said et al., 2012) that EHV-4 leads to a dramatic downregulation of MHC-I expression on cell surface at early stage of infection through at least two viral immune evasion proteins: pORF1 (pUL56) and pORF10 (pUL49.5).

1.2.2. Open reading frame 2 (ORF 2)

The EHV-1 ORF2 gene is another gene lacking an HSV-1 counterpart. While, complete sequence analysis of the VZV revealed that VZV ORF1 product has an overall amino acid identity with EHV-1pORF2 (Cohen and Seidel, 1995). VZV ORF1 protein (VZV pORF1) is early expressed during virus infection *in vitro* and is not essential for replication of VZV in cell culture. VZV pORF1 is located in the membrane of infected cells. However, EHV-1 ORF2 gene is predicted to encode a protein containing 206 amino acids (Telford et al., 1992). In addition, (Hussey et al., 2011) evaluated immune responses of an EHV-1 ORF1/2 deletion mutant in ponies and their findings demonstrated that the ORF1/2 genes of EHV-1 are important for disease outcome and modulation of cytokine responses. In general, little is known about EHV-1 ORF2 gene, therefore, several groups have started to study the role of this gene in virus infection and immunity.

1.2.3. Open reading frame 3 (ORF 3)

EHV-1 ORF3 share a limited sequence homology with proteins encoded by VZV ORF2 (Davison and Scott, 1986; Sato et al., 2002) and bovine herpesvirus type 1 (BHV-1) circ gene (Fraefel et al., 1994; Schwyzer et al., 2002), but EHV-1 ORF3 homolog is not present in the genome of HSV-1. EHV-1 ORF3 encodes a 33-kDa protein that is localized in the perinuclear region of the cytoplasm of infected cells (Harty et al., 1993a). EHV-1 deleted ORF3 gene is termed with EHV-1ΔUL3, exhibited properties of host cell tropism, plaque size, and growth kinetics similar to those of the parental virus. Intranasal infection of the mice with EHV-1ΔUL3 and parental virus showed that no significant difference in mortality or virus lung titers among mutant and parental viruses. Taken together, ORF3 (UL3) protein does not play an essential role in the EHV-1 biology in cell culture or virulence in a murine infection model (Ahn et al., 2011).

1.2.4. Open reading frame 34 (ORF 34)

The gene is located in the unique-long region of the genome and predicted to encode a polypeptide of 161 amino acids (Telford et al., 1992). Alignment of the amino acid sequence of EHV-1 pORF34 to its EHV-4 pORF34 and varicella zoster virus (VZV) pORF32 homologues revealed that EHV-1pORF34 has an overall amino acid identity of 72% with its EHV-4 counterpart but only 24% with VZV pORF32. VZV pORF32 is predicted to encode a protein of 16 kDa, phosphorylated and located in the cytosol of virus-infected cells (Reddy et al., 1998). Moreover,

the same authors demonstrated that VZV pORF32 is dispensable for virus replication in tissue cultures. In chapter 2 of this thesis, we showed that pORF34 is an early viral protein with an apparent molecular mass of 28-kDa and located in the cytoplasm of infected cells. Moreover, we showed that an ORF34 gene deletion mutant exhibited a significant growth defect in equine dermal cells (NBL-6) and equine peripheral blood mononuclear cells taken directly *ex vivo* during early but not late times of infection. Our findings in chapter 2 suggested that the pORF34 is required for optimal replication of EHV-1 *in vitro* at early times of infection.

1.2.5. Open reading frame 59 (ORF 59)

EHV-1 ORF59 is predicted to encode a protein containing 180 amino acids (Telford et al., 1998). Although EHV-1 ORF59 does not have a homolog in HSV-1, it does share positional and limited sequence homology with proteins encoded by members of the Varicellovirus genus. These include VZV ORF57 (Cox et al., 1998), EHV-4 ORF59 (Telford et al., 1998), pseudorabies virus (PRV) UL3.5 (Dean et al., 1993), BHV-1 UL3.5 (Khattar et al., 1995), and infectious laryngotracheitis virus UL3.5 proteins (Fuchs et al., 1996). The VZV ORF57 gene is predicted to encode a protein of 71 amino acids, localized in the cytosol of VZV-infected cells and shown to not play a major role in replication of the VZV *in vitro* (Cox et al., 1998). The PRV pUL3.5 is predicted to encode a 224 amino acid protein that is critical for virus growth in cell culture (Fuchs and Mettenleiter, 1996). Schikora and co-workers (Schikora et al., 1998) who reported that the BHV-1 pUL3.5 is late viral protein with apparent molecular mass of 13.2 -kDa and localizes to the cytoplasm but not the nucleus of BHV-1-infected cells. In chapter 3 of this thesis, we reported that ORF59 protein (pORF59) is an early protein that localizes to the cytosol of virus-infected cells and indispensable for growth of EHV-1 in cultured cells.

1.2.6. Open reading frame 67 (ORF 67)

The both copies of ORF67 gene (IR6) of EHV-1 is absent in the modified live EHV-1 vaccine strain RacH during its attenuation (Meyer et al., 1992; Hübert et al., 1996; Osterrieder et al., 1994, 1996a) and is still present in the virulent EHV-1 strains; e.g. Ab4 strain. EHV-1 ORF67 protein (pORF67) was identified and its product characterized by O'Callaghan et al. (1994) who reported that the ORF67 gene is encoded an early phosphorylated protein with a molecular mass of a 31 to

33 KDa and associated with purified EHV-1 virions and nucleocapsids. Moreover, it was demonstrated that EHV-1 pORF67 is a major determinant of EHV-1 virulence and that the IR6 protein may play a role in virus maturation and/or egress (Osterrieder et al., 1996).

1.3. Epidemiology and pathogenesis

Equine herpesvirus type 1 (EHV-1) is the one of the most important causes of infectious abortion and nervous system disorders with frequent fatal outcome in horses worldwide with symptoms that vary in severity from sub-clinical to severe and include fever, lethargy, anorexia, nasal discharge, and cough (Crabb and Studdert, 1995; Reed and Toribio, 2004; Slater et al., 2006). In horses, EHV-1 and EHV-4 viruses spread by the respiratory route, with primary replication occurring in respiratory epithelia. The EHV-1 is distributed in the lymphatic tissues associated with upper respiratory tract, and infected peripheral blood mononuclear cells (PBMC) that ultimately reach the bloodstream and lead to cell-associated viremia (Kydd et al., 1994; Wilson et al., 1997). EHV-1 can be carried by the infected PBMC, reached the vasculature of other tissues such as the pregnant uterus or the central nervous system, where the virus can replicate in endothelial cells and result in abortion or nervous system disorders (Tearle et al., 1996). While EHV-4 is an upper respiratory disease restricted to the infection of the respiratory tract epithelium and its associated lymph nodes. EHV-4 rarely causes abortion in infected pregnant mares unlike its EHV-1 counterpart (Matsumura et al., 1992; Reed and Toribio, 2004). Although both viruses cause respiratory disease, only EHV-1 has been established as a major cause of epidemic and sporadic abortion, prenatal mortality and neurological disorders that can range in severity but often including complete paralysis (Allen and Bryans, 1986; Crabb and Studdert, 1995; Edington et al., 1991). EHV-1 and its close relative EHV-4 are clinically and pathologically indistinguishable and the extensive antigen cross-reactivity between two viruses does not allow differentiation between them. The development of type-specific serologic tests has in recent years allowed investigation of the seroprevalence of EHV-1 and EHV-4 infections in different populations of horses and in different age categories (Crabb and Studdert, 1995).

1.4. Establishment of latent infection

EHV-1 life cycle involves two infection states: active (lytic) and quiescent (latent) infection.

During the lytic, productive phase, viral replication requires a coordinated gene expression program in which immediate early (IE) progresses to early (E) and finally late (L) gene expression (Caughman et al., 1985; Gray et al., 1987). Upon entry of the viral nucleocapsid into the infected cell, the gene expression cascade is initiated with the synthesis of the sole IE gene, which is activated by viral protein (VP) 16, also referred to as alpha-transinducing factor (alpha-TIF) contained in the virion and IE mRNA is transcribed by the cellular RNA polymerase II. E genes require IE protein activation to be transcribed and translated E proteins. IE and E proteins

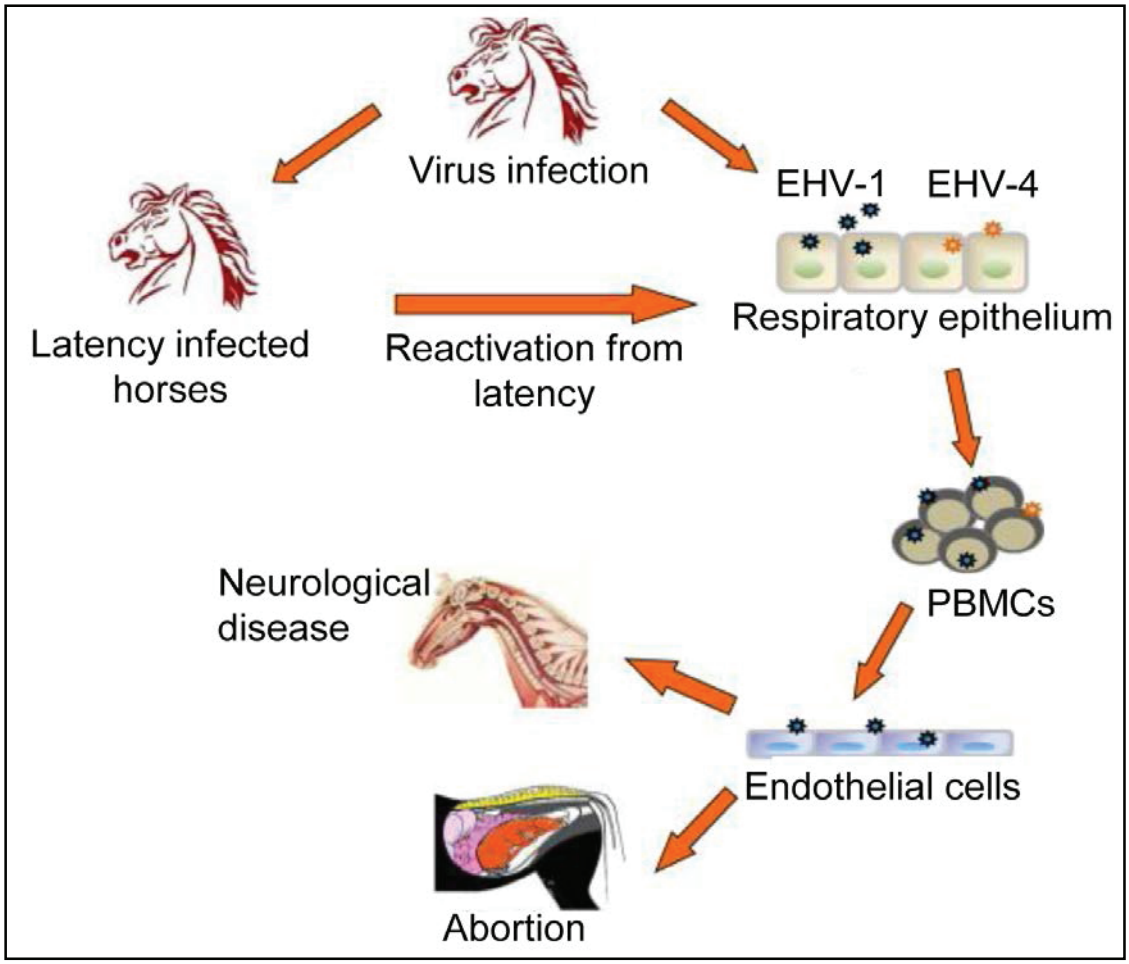

Fig. 1.2. The EHV-1 life cycle. After primary infection or reactivation from latency, EHV-1 replicates in the nasal epithelium, then infects PBMC and establishes viremia. Finally, vascular endothelia are infected in the CNS and pregnant uterus, which leads to neurological disease and abortion. Figure adopted from Ma et al., 2013 with a permission (Ma et al., 2013).

negatively regulate and suppress translation of IE mRNA, and induce replication of viral DNA,

transcription and translation of L genes, which also downregulate expression of IE and E proteins (Gray, 1987; O'Callaghan and Osterrieder, 2008). After the establishment of the latent state in as yet unknown cell populations in the host, EHV-1 can periodically reactivate and shed large amounts of virus. Herpesviruses have evolved very specific mechanism not only to evade host immune defences, but also to shut down lytic replication cycle during latency. This is achieved by preventing the synthesis of viral IE proteins, which appears to be the critical step for maintaining the latent state and direct repression of viral lytic gene expression and by inhibition of apoptosis. We know that, like other herpes viruses, EHV-1 causes a lifelong latent infection in affected animals and infected horses are usually the source for new infection for young horses from weanling to 2 years of age. Reactivation and shedding of the virus through nasal secretions after stressful events is believed to maintain the virus in populations (Fig. 1.2) (Edington et al., 1985; Gibson et al., 1992; Ma et al., 2013).

1.5. Immune response against EHVs infection

Several host immune mechanisms are activated during the course of infection with both EHV-1 and EHV-4 and recovery from infection is accompanied by the appearance of virus-specific antibody, cell-mediated immune responses and interferon (Bridges et al., 1988; Edington et al., 1989; Fitzpatrick and Studdert, 1984). Following natural or experimental infection, the immune response induces a solid but transient protective immune response to EHV that protects against reinfection for 3 to 6 months. But with repeated infection, the height and duration of the antibody response will increase (Bryans et al., 1969). The reasons for the apparently poor protection following infection considering the absence of marked antigenic and genetic variation in EHV-1 and EHV-4 isolates is poorly understood (Allen and Bryans, 1986). The development of type-specific serologic tests has in recent years allowed investigation of the seroprevalence of EHV-1 and EHV-4 infections in different populations of horses and in different age categories (Crabb and Studdert, 1995). Humoral immune responses alone do not provide protective immunity against EHVs infection. An effective immune response to the EHVs infection requires a combination of humoral and cellular immune responses. The humoral immune response is mediated by complement-fixing (CF) antibodies that appear at the fourth day of infection and virus neutralizing (VN) antibodies, which tends to be slower to form but persists for longer periods than (FC) antibodies (Doll and Bryans, 1962). The envelope glycoproteins of EHV-1 and EHV-4 play

important roles in viral replication by mediating virus attachment to and entry into cells and are critical determinants of tropism, cell-to-cell spread, pathogenesis and the induction of host humoral immune responses. They have been shown to play a major role in the immunogenicity of EHV-1 and EHV-4, serving as major targets for neutralization of virus infectivity by antibodies (Allen and Bryans, 1986; Bridges and Edington, 1987; Crabb et al., 1991; Crabb and Studdert, 1990). The glycoproteins B, C, and D have been identified as immunodominant antigens for generating antiviral serological responses to EHV-1 and EHV-4 in infected horses (Crabb et al., 1991). Cellular immunity mediated by CD8+ cytotoxic T lymphocytes (CTLs) is an essential defense mechanism against many virus infections. EHVs infection induces both tissues (mucosal and lymphoid) and circulating cellular immune response mediated by CD8+ CTLs (Kydd et al., 2003).

1.6. Diagnosis of equine herpesvirus infection

There are many types of equine herpesviruses, but the most one that affect the domestic horse are EHV-1 and -4. The EHV-1 and EHV-4 can cause more serious problems in equine. For diagnosis of EHV-1 or EHV-4 infection, virus isolation from nasopharyngeal swabs, blood sample, or tissue from the aborted fetus is attempted. A four-fold rise in antibody titre between acute and convalescent samples indicates infection by EHV, although it cannot discriminate between EHV-1 and EHV-4 infections due to the extensive antigenic cross-reactivity (Crabb et al., 1995; Patel and Heldens, 2005, Azab and Osterrieder, 2012). Moreover, EHV-1 and -4 can be differentiated by their DNA restriction enzyme patterns, which requires DNA isolation and is not feasible for routine diagnostics. In recent years, a number of PCR-based assays have been developed for diagnosis of EHV-1 and EHV-4 infection from different clinical samples. A PCR assay is the one of the sensitive and fast method to detect of EHV-1 infection (OIE 2015). Most of published PCR methods target different conserved genes of EHV-1 and -4 In order to differentiate between lytic and latent phase of EHVs infection, Chesters and co-workers (Chesters et al., 1997) investigated the presence of EHV-1 LATs in total RNA derived from equine peripheral blood mononuclear cells (PBMC) by constructing a CDN library by a random PCR technique. LAT transcripts were found and antisense to and partially overlapping with EHV-1 IE gene 64, the homologue of ICP4 of HSV.

1.7. Vaccination against equine herpesvirus infection

The currently available vaccines for protection against EHV-1 and EHV-4-induced diseases are modified live virus (MLV) and inactivated vaccines. The modified live vaccine containing exclusively the attenuated EHV-1 RacH strain are commercially available as Rhinomune in the United States and as Prevaccinol in Europe. The MLV vaccines have an excellent safety record and can reduce the clinical symptoms after infection. However, their efficacy in the preventing of clinical symptoms of the disease is still unclear (Kydd et al., 2006b). In general, inactivated vaccines are able to induce high levels of virus neutralizing antibodies, which play an important role in neutralizing of equine herpesvirus. But it still has some disadvantage in compared to MLV vaccines including (i) humoral immunity is not considered to be the most effective mechanism in protection against abortion or neurological symptoms. It has been shown that abortions occur in the presence of high levels of VN antibodies in pregnant mares (Mumford et al., 1994), (ii) vaccinated animals exposed to infection can become asymptomatic carriers, (iii) lack of long-term of protection, (v) and finally, vaccines can contain traces of non-structural proteins making it difficult to distinguish between vaccinated and infected animals. To date, there is no vaccine licensed for protection against the EHV-1-induced neurological form of infection. Current efforts focus on available infectious bacterial artificial chromosome clones of various EHVs strains to develop a new generation of modified live virus vaccines against EHVs infection (Azab et al., 2009; Rudolph et al., 2002; Trapp et al., 2003).

1.8. Outline of the thesis

Equine herpesvirus type 1 (EHV-1) is one of the common viral agents causing diseases in horses worldwide. It causes high economic and animal welfare losses through frequent outbreaks of respiratory disease, abortion, and neonatal foal death as well as neurological disease (Crabb and Studdert, 1995; Reed and Toribio, 2004; Slater et al., 2006). Infections with EHV-1 have been recognized as a significant impediment to breeding, competition, and recreational horse operations. Therefore, the overall objectives of this thesis are to address more details about characterization and identification of EHV-1 genes that will help us to understand EHVs pathogenesis. As there is no information on the function of the protein encoded by EHV-1 ORF34

and ORF59, we aimed in this thesis to characterize and identify EHV-34 and-59 genes that do not have a homolog in herpes simplex virus type 1.

The specific aims of this study are:

1- To generate recombinant EHV-1 Ab4 strain mutant viruses (rAb4ΔORF34 and ORF59).
2- To study *in vitro* characterization of the ORF34 and ORF59 proteins
3- To study growth properties of ORF34- or ORF59-negative virus in culture cells.
4- To identify subcellular localization of ORF34 or ORF59 protein in infected cell culture.

1.9. References

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CHAPTER 2

Ubiquitination and degradation of the ORF34 gene product of equine herpesvirus type 1 (EHV-1) at late times of infection.

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Said A[1], Damiani A[2], Osterrieder N[3].

[1]Institut für Virologie, Zentrum für Infektionsmedizin-Robert von Ostertag-Haus, Freie Universität Berlin, Robert-von-Ostertag-Str. 7-13, 14163 Berlin, Germany; Parasitology and Animal Diseases Department, National Research Center, Dokki, Giza, Egypt.

[2]Institut für Virologie, Zentrum für Infektionsmedizin-Robert von Ostertag-Haus, Freie Universität Berlin, Robert-von-Ostertag-Str. 7-13, 14163 Berlin, Germany.

[3]Institut für Virologie, Zentrum für Infektionsmedizin-Robert von Ostertag-Haus, Freie Universität Berlin, Robert-von-Ostertag-Str. 7-13, 14163 Berlin, Germany. Electronic address: no34@cornell.edu.

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CHAPTER 3

Equine herpesvirus type 1 (EHV-1) open reading frame 59 encodes an early protein that is localized to the cytosol and required for efficient virus growth.

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Said A[1], Osterrieder N[2].

[1]Institut für Virologie, Zentrum für Infektionsmedizin - Robert von Ostertag-Haus, Freie Universität Berlin, Robert-von-Ostertag-Str. 7-13, 14163 Berlin, Germany; Parasitology and Animal Diseases Department, National Research Center, Dokki, Giza, Egypt.

[2]Institut für Virologie, Zentrum für Infektionsmedizin - Robert von Ostertag-Haus, Freie Universität Berlin, Robert-von-Ostertag-Str. 7-13, 14163 Berlin, Germany. Electronic address: no.34@fu-berlin.de.

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CHAPTER 4

General Discussion

Chapter 4: General Discussion

4. 1. General features of equine herpesvirus

Equine herpesvirus type 1 (EHV-1) is an *Alphaherpesvirus* of the genus *Varicellovirus* (Davison et al., 2009) and is the one of the most important respiratory pathogen in the horse population, causing respiratory, and can also cause abortion and nervous manifestations with frequently fatal outcome (Brosnahan et al., 2010; Ma et al., 2013; Rosas et al., 2006).The EHV-1 genome is approximately 150-kbp in size and contains at least 76 distinct genes, four genes of which (64, 65, 66 and 67 gene) are duplicated resulting in a total of 80 ORFs. EHV-4, with a slightly smaller genome size of 146-kbp, also contains 76 genes, but only three genes (64, 65 and 66 gene) are duplicated. Sequence analysis showed that there is from 55-84 % DNA homology between EHV-1 and -4 depending on the gene and that the homology at the amino acid level ranged from 55-96 % (Telford et al., 1992; Telford et al., 1998). Infections of horses with EHV-1 are clinically difficult to be distinguished from those caused EHV-4, the close relative of EHV-1. Although both viruses cause respiratory disease, only EHV-1 has been established as a major cause of abortion, prenatal mortality and neurological disorders that can range in severity.

Herpesviruses are important pathogens for animals and humans, and have the largest DNA and complicated genomes among mammalian viruses (Roizmann et al., 1996). Generation and genetic manipulation of recombinant viruses have been hardly difficult. But functional characterization of these viral genes by generating virus mutants is important for understanding the biological properties and pathogenesis of herpesvirus in compare to parental virus, and to provide basis for the rationale development of new vaccines and gene therapy. Although there are available conventional methods for generation of a variety of herpesviruses mutants with (i) chemical mutagenesis, (ii) site-directed mutagenesis by homologous recombination in eukaryotic cells (iii) or cosmid technology, but their construction is often inefficient, laborious and time consuming (Brune et al., 1999; McGregor and Schleiss, 2001; Wagner et al., 2002). The limitations of previous methodologies for generation of herpesvirus mutants have stimulated the development of new methodology, bacterial artificial chromosomes (BACs) that have been represented one of the useful and powerful genetic tools for generating herpesvirus mutants to study molecular aspects

of herpesvirus replication and pathogenesis.

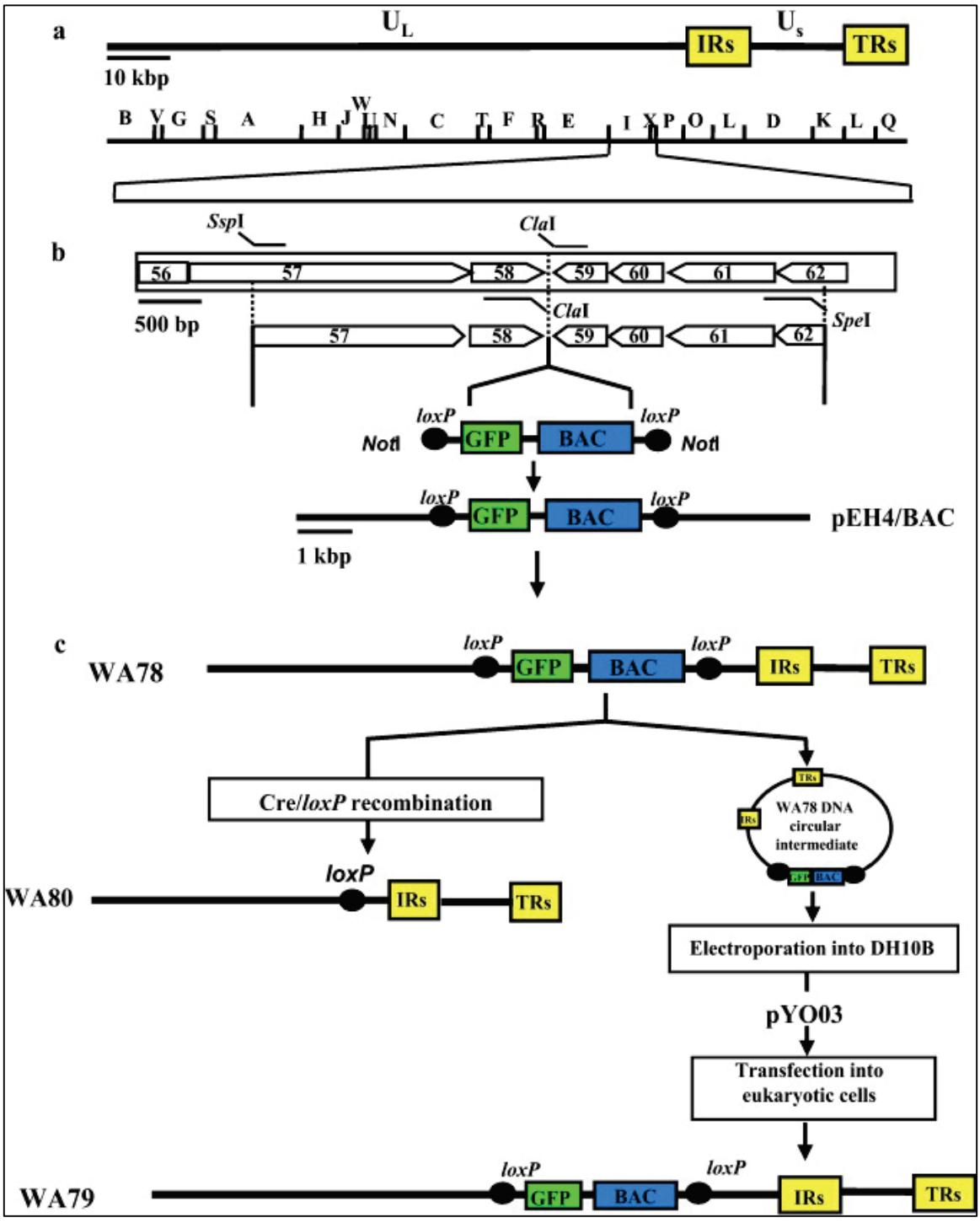

Fig. 4.1. Schematic diagram of the strategy used to construct infectious herpesvirus BAC (e.g. EHV-4). Adapted with permission from (Azab et al., 2009).

Recently, many laboratories have been succeed to clone the large herpesvirus genomes as BACs (Adler et al., 2000; Azab et al., 2009; Borst et al., 1999; Chang and Barry, 2003; Domi and Moss, 2002; Horsburgh et al., 1999; Mahony et al., 2002; Messerle et al., 1997; Rudolph et al., 2002; Saeki et al., 1998; Tanaka et al., 2003; Trapp et al., 2003; Yu et al., 2002; Zhou et al., 2002).

In the Fig. 4.1, Azab et al (Azab et al., 2009) have been illustrated the strategy that they used it to construct infectious EHV-4 BAC plasmid.

The EHV-1 vaccine strain RacH has been cloned as an infectious BAC (Rudolph et al., 2002; Rudolph and Osterrieder, 2002) and developed as a modified live virus vector against various pathogens. The EHV-1 genome is the one of the largest DNA and that allows with the insertion of foreign DNA and in this case the EHV-1 can easily be manipulated using infectious genomes cloned in E.coli (Trapp et al., 2005). The MLV vaccine of EHV-1 RacH strain is commonly used to vaccinate horse against EHV-1 disease in Europe. The EHV-1 RacH strain is capable of entering a wide variety of cell types of different origins and induction of protective immune responses in various species and laboratory animals. Attenuation of EHV-1 RacH strain has been happened during its passages on primary kidney, attributed to deletion of both copies of gene 67 (IR6). In addition, other genomic modification such as truncation of the glycoprotein B also contributes to its attenuation in a variety of species (Hubert et al., 1996; Neubauer et al., 1999; Osterrieder et al., 1996). To date, there is no vaccine licensed for protection against the neurological form of Ab4 strain of EHV-1 infection. The sequences of the genome and infectious bacterial artificial chromosome clones of EHV-1 Ab4 strain is now available and this information should aid in the understanding of the biology of virus and in the control of infections through immunoprophylaxis. Therefore, the main objective of this thesis is to gain more insight into the functions of an uncharacterized EHV-1 protein that has been shown to play an important role in the pathogenesis of related herpesviruses. The generated information may contribute to a better understanding of EHV-1 pathogenesis and the design of a better EHV-1 vaccine.

To date, the function of the EHV-1 ORF34 and ORF59 protein products are unknown. Therefore, the overall goals of this thesis are to

(i) identify and characterize the EHV-1 ORF34 and ORF59 gene product

(ii) examine whether they have a role in EHV-1 pathogenesis.

4. 2. Identification and characterization of the EHV-1 ORF34 gene product

EHV-1 ORF34 gene is the one of six genes that does not have homolog with HSV but it has positional and sequence homology with two herpesvirus proteins, EHV-4 gene 34 and VZV gene 32. The CLUSTAL alignment of the predicted amino acid sequence of the EHV-1 pORF34 show a 72% and 24% overall amino acid identity with its orthologues of EHV-4 and VZV, respectively. As there is no information on the function of the protein encoded by EHV-1 ORF34, in the chapter 2 of this thesis, we aimed to identify the ORF34 gene product of EHV-1. To achieve the goals of this study, the authors generated an EHV-1 recombinant virus lacking ORF34 (Ab4Δ34) based on wild-type EHV-1 Ab4 strain, which had previously been cloned as an infectious BAC clone, in which mini-F sequences along with an eGFP marker was inserted into the viral genome instead of gp2-encoding gene 71 sequences (Goodman et al., 2007). The correct mutant clones were confirmed with PCR, RFLP, and nucleotide sequencing. Reconstitution of parental rAb4 and mutant viruses were achieved by transfection of BAC DNA into RK13 cells (Rudolph et al., 2002). To characterize the EHV-1 pORF34 product, western blot analysis was used to detect expression of pORF34 as described before (Osterrieder et al., 1996; Said et al., 2012). The anti-HA antibody and the pORF34-specific peptide antibody were used to detect pORF34 expression in infected and transfected cells and our results revealed that both antibodies able to detect a 28-kDa pORF34-specific band and did not exhibit any cross-reactivity with other viral or cellular proteins. Furthermore, our data show that pORF34 is expressed at early stage of infection (1 h p.i.) Our findings are in agreement with previous results showing that the VZV pORF32 is also expressed at early times after infection (Reddy et al., 1998). On the other hand, additional bands of 17- to 28-kDa were observed when we used the anti-HA MAb and the polyclonal antibody targeting the N-terminus of pORF34 at late stage of infection. We concluded from these observations that the ORF34 protein undergoes degradation that is initiated at its N-terminus but not at its C-terminus. It was previously reported that the VZV orthologue of pORF34 (VZV pORF32) is a phosphoprotein (Reddy et al., 1998), but our data revealed that phosphorylation was not responsible for the observed effect on EHV-1 pORF34. On the other hand, glycosylation is also one of the possible reasons for posttranslational modification, we used enzymatic digestion with PNGaseF and EndoH to exclude that glycosylation is responsible for the posttranslational

modification of the EHV-1 pORF34. To test whether the proteasome has a role in the posttranslational modification of EHV-1 pORF34, cell lysates that treated and non-treated with proteasome inhibitor (lactacystin) were examined with western blot analysis revealed only a 28-kDa band in treated cells with lactacystin, while additional bands of 17- to 28-kDa were observed in non-treated cells. We concluded that pORF34 is degraded through the proteasomal pathway. In order to address whether pORF34 degradation through the proteasome was dependent on ubiquitination, our results showed that pORF34 is ubiquitinated at late stage of infection and targeted for degradation by the proteasome.

The immunofluorescence analysis and fractionation studies of infected cells indicated that the pORF34 is located in the cytosol of infected cells. Our findings are in agreement with previous results for the VZV orthologue, which was reported to also be present in the cytosol of infected cells (Reddy et al., 1998). Furthermore, examination of the growth and replication properties of an EHV-1 lacking ORF34 (rAb4Δ34) revealed a significant reduction of one-step growth kinetics and virus genome copies in cell culture when compared to those of parental (rAb4) and revertant (rAb4Δ34R) virus at early but not late stages of infection.

Taking all data presented in the chapter 2 of this thesis together, we conclude that EHV-1 pORF34 is:

(i) expressed with early kinetics.

(ii) localized in the cytoplasm of infected cells.

(iii) degraded in the proteasome following ubiquitination, a process that requires a late viral gene product(s).

(iv) replicated with reduced efficiency *in vitro* at early but not late stages of infection.

4.3. Identification and characterization of the EHV-1 ORF59 gene product

Equine herpesvirus type 1 (EHV-1) encodes six genes that do not have homologs in herpes simplex virus. One of these genes, EHV-1 ORF59, is predicted to encode a protein containing 180 amino acids in length (Telford et al., 1992). EHV-1 ORF59 share other herpesviruses in a positional and limited sequence homology; e.g. VZV gene 57 (Cox et al., 1998) and equine herpesvirus type 4 (EHV-4) gene 59 (Telford et al., 1998) as well as the UL3.5 genes of pseudorabies virus (PRV) (Dean and Cheung, 1993), bovine herpesvirus type 1 (BHV-1) (Khattar et al., 1995), and infectious

laryngotracheitis virus (Fuchs and Mettenleiter, 1996). The CLUSTAL alignment of the predicted amino acid sequence of the EHV-1 pORF59 show overall amino acid identities between EHV-1 pORF59 and the related proteins were 48.4 (EHV-4), 30.3 (BHV-1), 26 (PRV) and 12.2% (VZV), respectively.

As there is no available information on the role of the EHV-1 pORF59 in EHV-1 pathogenesis, in the chapter 3 of this thesis, we aimed to characterize and identify the ORF59 gene product of EHV-1. To fulfill the aim of this study, the authors generated an EHV-1 recombinant virus lacking ORF59 (Ab4Δ59) and a recombinant with an HA-tagged ORF59 protein (rAb4_59-HA) based on wild-type EHV-1 Ab4 strain. The PCR, RFLP, and nucleotide sequencing were used to confirm the correct of all mutant clones construction. The parental rAb4 and mutant viruses were reconstituted by transfection of BAC DNA into RK13 cells (Rudolph et al., 2002; Said et al., 2012). Western blot analysis was used to detect expression EHV-1 pORF59 as described before (Osterrieder et al., 1996; Said et al., 2012). The monoclonal anti-HA tag antibody detected a 21-kDa pORF59-specific band in rAb4_ORF59-HA-infected cells and in pcDNA_59-HA-transfected cells. Our findings demonstrated that the anti-HA antibody was able to detect a pORF59-specific band and did not exhibit any cross-reactivity with other viral or cellular proteins.

Kinetics expression of pORF59 was determined by western blot analysis using lysates of cells infected with the rAb4_59-HA virus harvested at different time points after infection in the presence or absence of PAA to differentiate weather pORF59 is early or late protein. For more differentiation whether pORF59 is an immediate early (IE) or early (E) protein, NBL6 cells were either mock-infected or infected with parental or rAb4_59-HA virus in the presence of the CX for 5 h followed by Act-D. Our findings suggested that the EHV-1 pORF59 is expressed with early kinetics. Moreover, our data strongly suggested a cytoplasmic localization of EHV-1 pORF59, which supported with immunofluorescence analysis and fractionation studies.

Another interested finding was that the EHV-1 lacking ORF59 gene was able to grow on equine dermal monolayer cells with morphology and average plaques sizes that were significantly smaller when compared to parental and revertant viruses and only consisted of only very few cells. This finding strongly suggested that EHV-1 pORF59 is the one of the EHV-1 genes that required for efficient growth of EHV-1cell culture.

The data presented in the chapter 3 of this thesis concluded that pORF59 is:

(i) an early viral protein.

(ii) localized in cytosol of infected cells.

(iii) efficiently required in the growth of the EHV-1 in cell culture.

4.4. References

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List of puplications

Said A, Damiani A, Osterrieder N. Ubiquitination and degradation of the ORF34 gene product of equine herpesvirus type 1 (EHV-1) at late times of infection. **Virology 460-461: 11-22.** http://dx.doi:10.1016/j.virol.2014.05.009

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Selbeständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen Anspruch genommen habe.

Berlin, den 10.06.2016 **Abdelrahman Said**