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Activation of the ERK1/2 signalling molecule by the gE glycoprotein of pseudorabies virus

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Dissertation submitted in fulfillment of the requirements for the degree of Doctor of Philosophy (PhD) in Veterinary Sciences, 2017

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LIST OF ABBREVIATIONS

Ab	Antibody
AP-1	Activator Protein-1
BCA	bicinchoninic acid
Bcl-2 protein	B-cell lymphoma 2 protein
BH-3 domain	Bcl-2 homology 3 domain
BMK	big MAPK
BoHV-1, BoHV-5	Bovine Herpesvirus 1, 5
CB	citrate buffer
CBP	CREB-binding protein
cd	cytoplasmic domain
CHV-1	Canine Herpesvirus 1
CREB	cAMP response element-binding
CXCR4	C-X-C Chemokine receptor 4
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
ECL	Enhanced Chemiluminescence
EGF	Epidermal Growth Factor
EHV1, EHV-4	Equine Herpesvirus 1, 4
ER	Endoplasmic Reticulum
ERK1/2-5	Extracellular signal-regulated kinase 1 and 2-5
ET/P	Early Transcription/Protein
FACS	Fluorescence-activated cell sorting
FAK	focal adhesion kinase
FBS	Fetal Bovine Serum
FHV-1	Feline Herpesvirus 1
FITC	Fluorescein isothiocyanate
FRET	Förster resonance energy transfer
gE	glycoprotein E
GFP	Green Fluorescent Protein
gp120	glycoprotein 120
HCMV	Human cytomegalovirus
HIV-1	Human Immunodeficiency virus 1
hpi	hours post-infection
HRP	Horseradish peroxidase
HSV-1, HSV-2	Herpes Simplex Virus 1, 2
HTLV1	Human T-cell leukaemia type 1 virus
HVEM	herpesvirus entry mediator
IE180	immediate early 180
IET/P	immediate Early Transcription/Protein
IEX-1	immediate early response gene X-1
IgG	immunoglobulin G
IL	interleukin
INM	inner nuclear membrane
IP	Immunoprecipitation
ITAM	immunoreceptor tyrosine-based motifs
JNK	c-Jun N-terminal kinase
kDa	kiloDalton

KIF1A	Kinesin Family Member 1A
KSHV	Kaposi's sarcoma associated-herpesvirus
KSR	kinase suppressor of Ras
Lck	lymphocyte-specific protein tyrosine kinase
LL	dileucine motifs
LMP1/2A	latent membrane protein 1/2A
LT/P	Late transcription/protein
mAb	monoclonal Antibody
MAP3K	mitogen-activated protein kinase kinase kinase
MAPK	mitogen-activated protein kinase
MAPKAPK	MAP Kinase Activated Protein Kinases
Mcl-1	myeloid cell leukaemia 1
MDV	Marek's Disease virus
MEK1/2	MAPK/ERK kinases
MEKK1	mitogen-activated protein kinase kinase 1
MLC	myosin light chain
MLCK	myosin light chain kinase
MNK	MAPK-interacting kinase
MOI	Multiplicity of infection
MP1	MEK partner 1
MSK	mitogen- and stress-activated kinases
NF-kB	nuclear factor kappa B
NGF	Nerve Growth Factor
NK cells	Natural Killer cells
NMHC-IIA/B	non-muscle myosin heavy chain IIA/B
ONM	outer nuclear membrane
ORF12	open-reading frame 12
PBMC	Peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PC12 cells	pheochromocytoma cells
pDC	plasmacytoid dendritic cells
PDL-1	programmed death-ligand 1
PEA-15	phosphoprotein enriched in astrocytes
PILRα	paired immunoglobulin-like type 2 receptor alpha
PK15 cells	Pig Kidney cells
PKC, PKCθ	protein kinase C
PRV	pseudorabies virus
PVR	Poliovirus Receptor
RSK	ribosomal S6 kinase
SAPK1-4	Stress-activated protein kinase 1-4
SDF-1	stromal cell-derived factor 1
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SHV-1	Suid Herpesvirus 1
ST cells	Swine Testicle Cells
STAT3/5	Signal transducer and activator of transcription 3/5
Tat	Trans-Activator
TBS	Tris-buffered saline
TCR	T cell receptor
TGN	*trans*-Golgi network
TIF-IA	transcription initiation factor
TNF	tumor necrosis factor
UL	Unique Long segment

US	Unique Short segment
VP1/2	Viral Protein 1/2
Vpr	Viral protein R
VZV	Varicella zoster virus
WT	Wild type

Chapter 1

INTRODUCTION

1.1 PSEUDORABIES VIRUS

1.1.1 Introduction

Pseudorabies virus (PRV), a swine pathogen also known as Suid Herpesvirus 1 (SHV-1) or Aujeszky's disease virus, is a member of the *Herpesviridae* family. All herpesviruses share common biological characteristics such as a double-stranded DNA genome, comparable size (200 to 250 nm) and structure (envelope, tegument and capsid), and undergo a latent stage at some point during their life cycle, ensuring long-term survival of the virus in the host population.

The *Herpesviridae* family (including viruses of mammal, bird and reptile), along with two other families - *Alloherpesviridae* (containing fish and frog viruses) and *Malacoherpesviridae* (containing a bivalve virus) - belongs to the *Herpesvirales* order. The *Herpesviridae* family can be further divided in three subfamilies: *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae* (Davison et al., 2009). Subdivision of the family is based on biological criteria, genome characteristics and virus structure. For example, the three subfamilies differ with regard to the length of their productive replication cycle and typically establish latency in different cell types: neurons for most of the *Alphaherpesvirinae*, the monocyte lineage for the *Betaherpesvirinae* and lymphocytes for the *Gammaherpesvirinae* (Arvin et al., 2007).

PRV is classified as an alphaherpesvirus, and like several other members of this subfamily, PRV is able to infect different species and replicate rapidly, generating new viral particles in a matter of hours. Human alphaherpesviruses include the human herpes simplex virus 1 and 2 (HSV-1, HSV-2) and varicella zoster virus (VZV). Besides PRV, there are other widely studied animal alphaherpesviruses, like bovine herpesvirus 1 and 5 (BoHV-1, BoHV-5), equine herpesvirus 1 and 4 (EHV1, EHV-4), canine herpesvirus 1 (CHV-1) and feline herpesvirus (FHV-1). The chicken herpesvirus Marek's Disease virus (MDV) is also classified as an alphaherpesvirus, although it establishes latency in T lymphocytes (Calnek et al., 1984a; Calnek et al., 1984b; Shek et al., 1983; Spencer and Calnek, 1970).

To some, the focus in virology entails the search for solutions of practical problems. (…) To others, virology offers the opportunity to study fundamental problems in biology (Lynn Enquist, in *Life beyond eradication: veterinary viruses in basic science*). In addition to being instrumental in the eradication of the pathogen in several industrialized

countries, PRV research has provided valuable and general insights in the alphaherpesvirus life cycle and the complex virus-host interactions. Therefore, PRV remains of major interest to virologists for several reasons: PRV has a broad host range, being able to cause (lethal) infection in different animals, but it poses no threat to humans; this virus is easy to manipulate and grows well in culture; purified DNA is infectious, and the techniques to replace and manipulate its genes are thoroughly described; bacterial artificial chromosomes encoding the entire viral genome have been constructed (Kopp et al., 2003; Smith and Enquist, 1999; Smith and Enquist, 2000); and finally, animal experiments can be carried out in the natural host of PRV, the pig, as well as in other animal models, such as mice, rats and rabbits.

1.1.2 Structure

The complete genome of PRV (± 144 kbp) has been entirely sequenced (Klupp et al., 2004; Szpara et al., 2011) and is similar in arrangement to other alphaherpesviruses, containing a unique long segment (UL) and a unique short region (US). The US region is flanked by inverted repeat sequences, resulting in the formation of two possible PRV genome isomers with oppositely oriented US regions. Designation of the different genes depends on their position on the different regions, UL or US, and on their place within the specific region, represented by a number. The PRV genome contains 72 open reading frames (ORF) and encodes 70 different proteins, and all of these proteins have orthologs in other alphaherpesviruses (Klupp et al., 2004; Pomeranz et al., 2005).

The virion architecture of PRV resembles others in the *Herpesviridae* family. The mature virion consists of four distinct structural components: a DNA genome in its central core, a protective capsid enclosing the genome, the tegument and a lipid envelope (Figure 1). Together, the linear double-stranded DNA genome and the icosahedral capsid (formed by 162 capsomers) compose the nucleocapsid (Mettenleiter, 2000; Newcomb et al., 1999). The nucleocapsid is surrounded by a protein matrix known as the tegument, which contains proteins that are important for viral entry and morphogenesis (Mettenleiter, 2002).

A **B**

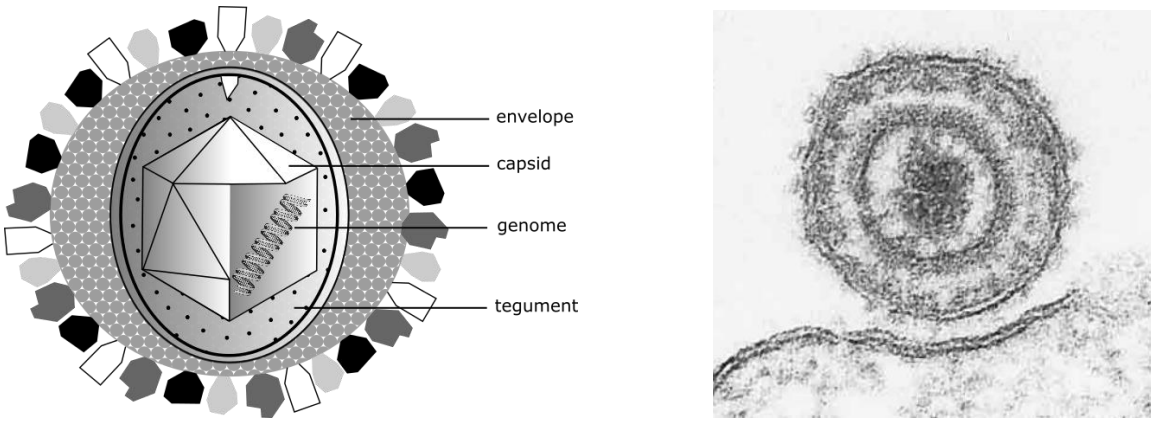

Figure 1. A Schematic representation of the PRV virion. **B** Transmission electron microscopic picture of the PRV virion (Granzow et al., 1997).

Finally, the tegument is surrounded by the envelope, a double phospholipid membrane embedded with several viral (glyco)proteins (gB, gC, gD, gE, gH, gI, gK, gL, gM, gN, UL20, UL43, US9). The envelope is acquired by budding of the capsid in vesicles derived from the *trans*-Golgi network (Mettenleiter and Minson, 2006; Mettenleiter et al., 2006). The envelope proteins play essential or important roles in viral entry, egress, cell-to-cell spread, induction of protective immunity and immune evasion (reviewed in (Nauwynck et al., 2007; Pomeranz et al., 2005)).

1.1.3 Infection cycle

Figure 2 offers an overview of the virus productive replication cycle, which can be roughly subdivided into 5 stages: (1) virus attachment and entry, (2) transport of the nucleocapsid to the nucleus, (3) gene expression, (4) replication and virus assembly and (5) egress. The following information is mostly based on the replication cycle of HSV, the prototype of the *Alphaherpesvirinae* subfamily. Information that is specific for the PRV replication cycle will be indicated as such in the text.

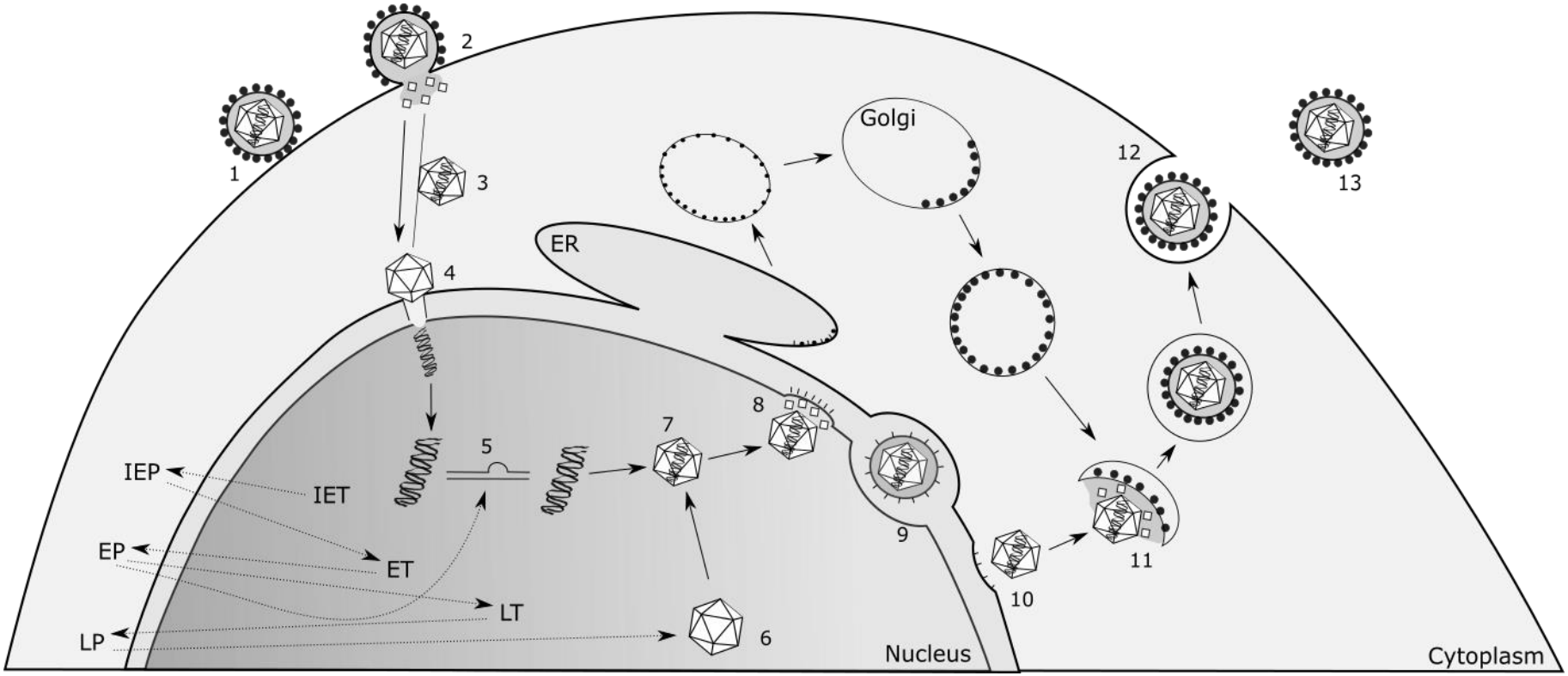

Figure 2. Replication cycle of PRV. Virions attach to **(1)** and subsequently fuse **(2)** with the plasma membrane. Capsids are released in the cytoplasm and transported along microtubules to the nucleus **(3)**, where viral DNA is released into the nucleus **(4)**. After DNA replication **(5)** and capsid assembly **(6)**, DNA is packaged into the preformed capsids **(7)**. Capsids bud into the inner nuclear membrane **(8)** and primary enveloped virus particles are found in the perinuclear space **(9)**. The primary envelope is lost during fusion with the outer nuclear membrane **(10)**. Subsequently, a secondary envelope is acquired by budding into the *trans*-Golgi network **(11)**. Virus particles are released **(13)** by fusion with the plasma membrane (**12)** (adapted from (Mettenleiter, 2004). IET/P: immediate-early transcription/proteins; ET/P: early transcription/proteins; LT/P: late transcription/proteins.

Entry

Attachment of the virion to the plasma membrane is divided into two steps: a first labile interaction of gC (and, to a lesser extent, gB) with heparan sulfate proteoglycans in the cell membrane, followed by a more stable interaction between gD and specific cellular receptors, stabilizing the virion-cell interaction. The first binding, although non-essential, greatly enhances the efficiency of infection (Immergluck et al., 1998; Karger et al., 1995; Mettenleiter et al., 1990; Shukla and Spear, 2001; Spear and Longnecker, 2003). With regard to the second and more stable binding, three classes of gD-associated receptors have been identified (Spear et al., 2000): (i) HVEM (herpesvirus entry mediator), a member of the TNF receptor family; (ii) nectins and nectin-like molecules (nectin 1, nectin 2 and poliovirus receptor (PVR, CD155)), members of the immunoglobulin super family; and (iii) specific heparan sulfate moieties generated by certain 3-O-sulfotransferases (Geraghty et al., 1998; Heldwein and Krummenacher, 2008; Montgomery et al., 1996; Shukla et al., 1999; Warner et al., 1998). Alphaherpesviruses differ in their affinity for the different described receptors. HSV-1 and HSV-2 share their preference for HVEM and nectin-1; concerning the other two receptors, only HSV-2 makes use of nectin-2 for entry (although it has a weak activity), while HSV-1 favours 3-O-sulphate-modified heparan sulfate. In the case of PRV, three entry receptors have been described so far: nectin-1, nectin-2 and PVR (Campadelli-Fiume et al., 2000; Spear and Longnecker, 2003; Spear et al., 2000).

However, it has been reported that nectin-1, despite binding to gD, is not necessary for PRV entry in fibroblasts (Ch'ng et al., 2007). For HSV-1 and PRV, gB was also shown to interact with receptors like paired immunoglobulin-like type 2 receptor alpha (PILRα) (Arii et al., 2009; Satoh et al., 2008) and non-muscle myosin heavy chain IIA (NMHC-IIA) (Arii et al., 2010; Arii et al., 2015). Interaction between gB and NMHC-IIB has been described for HSV-1 (Arii et al., 2010).

Following virus binding to host cells, the viral envelope fuses with the plasma membrane, a process that requires four viral proteins: gB, gD, gH and gL (Atanasiu et al., 2010; Fan et al., 2015; Klupp et al., 1997; Rauh and Mettenleiter, 1991). These glycoproteins are absolutely essential for viral entry, as depletion of either one of them abrogates viral entry (Klupp et al., 1997; Mata et al., 2001; Peeters et al., 1992; Rauh and Mettenleiter, 1991; Reske et al., 2007; Subramanian et al., 2007). Depending on the cell

type, the virus may also enter the cell via endocytosis, although it remains unclear how the virus selects its entry pathway (Clement et al., 2006; Frampton et al., 2005; Gianni et al., 2004; Hambleton et al., 2007; Komala Sari et al., 2013; Nicola and Straus, 2004; Nicola et al., 2003; Nicola et al., 2005) .

Transport to the nucleus

Upon arrival in the cytoplasm, incoming nucleocapsids are rapidly transported through the cytosol towards the nuclear pore complexes, where the viral genomes are injected into the nucleoplasm. Evidence indicates that the tegument proteins UL36 (also known as VP1/2) and UL37 are essential for transport of the capsid to the nucleus. To propel the capsids from the periphery to the nucleus, the virus travels along microtubules via an interaction with dynein (Antinone and Smith, 2010; Dodding and Way, 2011; Granzow et al., 2005; Luxton et al., 2005; Luxton et al., 2006; Sodeik et al., 1997), a cellular microtubule-associated motor protein. UL36 has been found to associate with dynein, thereby acting as a potent effector of microtubule-dependent transport (Zaichick et al., 2013). UL36 was also reported to play a role in docking of the nucleocapsid at the nuclear pores and release of the viral genome in the nucleus (Copeland et al., 2009; Jovasevic et al., 2008). UL37 has been shown to be required for rapid nuclear translocation (Krautwald et al., 2009). Also, a more recent study reported dystonin/BPAG1, an important cytoskeleton cross-linker involved in microtubule-based transport, as a binding partner of the HSV-1 UL37 protein (Pasdeloup et al., 2013).

Gene expression

After injection into the nucleus, the genome circularizes and the transcription cascade starts, a process that is tightly regulated (Ben-Porat and Kaplan, 1985). At a first stage, the virus uses the host cell machinery to transcribe the immediate-early (IE) genes. For PRV, only one IE gene has been reported so far, IE180, which is homologous to HSV-1 ICP4 (Cheung, 1989; Vlček et al., 1989).The IE180 protein is a transactivator and drives the expression of several viral genes, including US4 (gG), UL12 (alkaline nuclease), UL22 (gH), UL23 (thymidine kinase) and UL41 (viral host shutoff). The early genes are subsequently transcribed and translated, and their protein products often regulate

expression of cellular and viral promotors (EP0, UL48, UL54) and produce protein determinants for nucleotide synthesis (UL23, UL39/UL40, UL50) and DNA replication (UL5, UL9, UL30, UL42, UL52) (Berthomme et al., 1995; Fuchs et al., 2002; Jöns et al., 1997; Kaliman et al., 1994; Kit et al., 1987; Lehman and Boehmer, 1999; Ono et al., 1998; Schwartz et al., 2006). Finally, transcription of late genes, which for the most part encode structural proteins, is prompted by DNA replication (Ben-Porat and Kaplan, 1985).

Replication and assembly

DNA replication is initiated by circularization of the linear viral genome, proceeding towards a rolling-circle mechanism of replication. This process produces replicated DNA in the form of long linear concatemeric genomes that serve as the substrate for genome encapsidation. The newly formed capsid proteins enter the nucleus for assembly of the progeny capsids (Copeland et al., 2009). Encapsidation of PRV DNA requires two linked events: cleavage of the replicated concatemeric DNA into monomeric units and packaging of the linear monomeric genomes into capsids, by pulling the genome through a cylindrical entry pore encoded by the protein UL6 (Ben-Porat and Kaplan, 1985; Cardone et al., 2007; Kwong and Frenkel, 1989; Ladin et al., 1982; White et al., 2003).

Egress

Most enveloped viruses assemble their capsids in the cytoplasm, acquiring their envelope later by budding at the plasma membrane or at cytoplasmic membranes. Since nucleocapsids are assembled in the nucleus, herpesviruses first have to face the challenge of transporting their capsids across the nuclear envelope. They achieve this via a peculiar two-step process, consisting of envelopment at the inner nuclear membrane (INM) (Hofemeister and O'Hare, 2008), leaving a virion with a primary envelope in the perinuclear space between both nuclear membranes. This is followed by fusion of the primary envelope with the outer nuclear membrane (ONM), releasing the naked capsid in the cytoplasm. Very few enveloped particles are observed in the perinuclear space, suggesting that this process occurs quickly and efficiently

(Mettenleiter, 2002; Skepper et al., 2001). Three proteins (US3, UL31, and UL34) have been reported to contribute to this 'nuclear escape process' (Funk et al., 2015; Lorenz et al., 2015; Paßvogel et al., 2014, 31; Reynolds et al., 2002; Reynolds et al., 2004; Schulz et al., 2014; Schumacher et al., 2005; Sherry et al., 2017; Simpson-Holley et al., 2004).

Once in the cytoplasm, tegument proteins start to interact with the capsid, which is then directed to the site of secondary envelopment, (vesicles derived from) the *trans*-Golgi network (TGN). At this stage, the different envelope proteins are already expressed in the membrane of the TGN. It is likely that an intricate interaction between tegument proteins, capsid and envelope proteins aid the tegumented capsid to bud in the TGN (Granzow et al., 1997; Mettenleiter et al., 2006). This process confers the virion its envelope, although this secondary envelopment is still poorly understood. Several viral envelope and tegument proteins have been linked to this process, including UL11, gE/gI, gD, gM, and more recently UL36 and UL37 (Baines and Roizman, 1992; Brack et al., 2000; Fulmer et al., 2007; Kelly et al., 2014; Kopp et al., 2003; Kopp et al., 2004; Leege et al., 2009). For PRV, gE/gI and gM were proven to be particularly important, since double-deletion of gE (or its cytoplasmic region alone) and gM largely impaired secondary envelopment (Brack et al., 2000).

In a final step, infectious particles are then released via exocytosis (Granzow et al., 1997; Granzow et al., 2001). Hogue and co-workers have visualized the final transport and exocytosis of PRV particles using a live-cell microscopy-based method. In short, virus particles travel to the plasma membrane inside small, acidified secretory vesicles which undergo fast, directional transport directly to the site of exocytosis. Vesicles are tightly docked at the site of exocytosis for several seconds, and membrane fusion occurs, displacing the virion a small distance across the plasma membrane. After exocytosis, particles remain tightly confined on the outer cell surface (Hogue et al., 2014).

1.1.4 Intercellular spread

Spread of herpesviruses may occur via two distinct routes: cell-free dissemination and cell-to-cell or cell-associated spread. Cell-free dissemination enables the virus to transit rapidly through the blood, lymph and cerebrospinal fluid to infect distant tissues. However, by exiting the cell to infect another cell or host, viral particles are exposed to

various biophysical, kinetic and immunological barriers. Typically, cell-to-cell spread is rapid and efficient, partly due to viruses and their cell surface receptors being in close proximity. Also, when moving across the narrow spaces between cells, the viral particles are protected from the effects of neutralizing antibodies and other immune system components by cell junctions. Still, whether the virion is released to diffuse and infect new host cells, or spread directly between adjacent cells, depends on the target cell or tissue (Mothes et al., 2010; Sattentau, 2008; Zhong et al., 2013).

The simplest mechanism of cell-associated spread consists of the fusion of infected and uninfected cells, resulting in giant multinuclear cells (syncytium). Alphaherpesviruses induce syncytium formation *in vitro* and *in vivo* (Cole and Grose, 2003; Spear, 1993), and express a range of glycoproteins that have a role in this process. Cell–cell fusion by HSV-1 requires the coordinated action of glycoproteins gB, gD and gH–gL on the virus-infected cell, with a further role for the gD receptor on the target cell (Campadelli-Fiume et al., 2007; Reske et al., 2007).

There are other, more sophisticated, mechanisms of cell-to-cell virus spread used by alphaherpesviruses, like transfer of virions across neuronal synapses and direct viral spread across cell junctions. Viral spread between unconnected cells through formation of viral-induced projections which connect distant cells has also been described. These mechanisms are explained more in detail below.

PRV and HSV-1 infection lead to formation of varicosities (synaptic boutons) in sensory neurons of the trigeminal ganglion and rat hippocampal neurons, respectively. These varicosities form synaptic contacts which appear to be sites for cell-to-cell transmission to surrounding neuronal and non-neuronal cells. In both viruses, the formation of varicosities is driven by gD and its receptor nectin-1 (De Regge et al., 2006; Mizoguchi et al., 2002), suggesting a possible role for gD in promoting cell-associated spread from neurons to neighbouring cells. Nevertheless, for PRV (but not HSV), this glycoprotein is not absolutely essential for neuronal cell-to-cell spread (Ch'ng et al., 2007; Peeters et al., 1992; Rauh and Mettenleiter, 1991).

Alphaherpesviruses are also able to spread across cell junctions. The glycoprotein E plays an important part in this process (Johnson et al., 2001a; Zsak et al., 1992), and as this glycoprotein is one of the key subjects of this thesis, this topic will be discussed in detail later,

The highly conserved viral US3 protein kinase also plays a role in efficient viral spread. This protein is responsible for the formation of long actin- and microtubule-containing cellular projections, which can connect otherwise unconnected nearby cells. The virus was found to move across these filaments, being able to successfully infect neighbouring uninfected cells, even in the presence of virus-neutralizing antibodies (Favoreel et al., 2005).

1.1.5 Pathogenesis

Pigs are the natural host of PRV. These animals are able to survive PRV infection whilst most mammals (with the exception of higher primates and humans that are non-susceptible) succumb to the virus. Severity of infection can vary, depending on virus strain-specific virulence as well as on the age and immunological status of the animal. In neonatal piglets, PRV causes severe to fatal affliction of the nervous system, whereas older pigs typically suffer from relatively mild respiratory complications, like coughing, sneezing and pneumonia. Pregnant sows may suffer from reproductive failure upon infection, including abortion, stillbirths and mummified foetuses.

PRV reaches sensory neurons via the respiratory tract, which is the primary site of infection upon oronasal infection (Pomeranz et al., 2005). Initial replication in respiratory epithelia is followed by infection of neurons and depends on the essential entry glycoproteins gB, gD and gH–gL (Mettenleiter, 2003). Upon entry in nerve cells, the virus is transported by retrograde traffic towards the cell body of the sensory neurons, where it replicates in the nucleus and/or becomes latent. Specific stimuli, which are often stress-related, may trigger reactivation of the virus and virus dissemination through anterograde axonal transport (Kramer and Enquist, 2013; Tomishima et al., 2001). The virus may also spread via the blood and lymph, allowing it to reach and replicate in other internal organs, like reproductive organs (Pomeranz et al., 2005).

1.2. THE GLYCOPROTEIN E OF PSEUDORABIES VIRUS

1.2.1 Introduction

The glycoprotein E of pseudorabies virus (gE, initially called gI) was first characterized in 1984 by Hampl and coworkers. This glycoprotein is an envelope protein and can be divided in three distinct domains: a 428-amino-acid N terminal (extracellular domain), a 26-amino-acid hydrophobic transmembrane domain, and a 123-amino-acid, highly charged C terminal (cytoplasmic domain) (Figure 3) (Tirabassi et al., 1997).

Homologs of PRV gE can be found in all alphaherpesviruses. Although there is little amino acid identity between the homologous proteins, two clusters (one at the N-terminal and one at the C-terminal end) of closely spaced cysteine residues can be found in PRV, HSV-1, bovine herpesvirus type 1 (BHV-1), EHV-1, VZV, and other alphaherpesviruses. Whilst the cluster at the N-terminal region is only partly conserved in some alphaherpesviruses, the cysteine cluster at the C-terminal region, consisting of five cysteine residues, is extremely well conserved in all members of this subfamily (reviewed in Jacobs, 1994).

Shortly after protein translation, gE binds to another glycoprotein, the glycoprotein I (gI, initially called gp63). Both gE and gI are transmembrane proteins that form a heterodimer in the endoplasmic reticulum (ER) via noncovalent interactions between their ectodomains and the complex is often referred to as one functional entity (Tirabassi et al., 1997; Whealy et al., 1993; Zuckermann et al., 1988). The formation of this heterodimer facilitates the maturation and intracellular transport of both proteins to the plasma membrane of cells (Whealy et al., 1993; Zuckermann et al., 1988). Nevertheless, PRV gE and gI can reach the cell surface independently of each other, albeit with lower efficiency (Tirabassi and Enquist, 1998; Whealy et al., 1993). Some studies have also shown that PRV gE appear to have a bigger impact in the function of the heterodimer: deletion of the gene encoding PRV gE caused the virus to be less virulent and spread with lower efficiency than gI-negative virus (Jacobs, 1994; Kimman et al., 1992; Kritas et al., 1994; Kritas et al., 1995). The absence of gE also caused a more severe decrease in antibody-induced capping observed in infected cells (Favoreel et al., 1997). Therefore, without disregarding the contribution /importance of gI, this thesis

will focus mainly on gE, although it is implied that some of the functions discussed further are common to (or depend on) both gE and gI.

After leaving the ER, PRV gE localizes primarily to the Golgi apparatus, cytoplasmic vesicles and the plasma membrane (Tirabassi and Enquist, 1998; Tirabassi et al., 1997). Labeling of cell surface proteins either with biotin or using specific antibodies demonstrated that gE undergoes constitutive endocytosis up until 6 hours post infection (Tirabassi and Enquist, 1998). Endocytosis of gE depends on its cytoplasmic domain, which contains several potential endocytosis/trafficking motifs: two tyrosine-based YXXΦ motifs (a membrane proximal at amino acid positions 478–481 and another at amino acid positions 517-510), clusters of acidic residues, and dileucine motifs (LL) (Alconada et al., 1996; Alconada et al., 1999; Fölsch et al., 1999; Tirabassi and Enquist, 1999; Zhu et al., 1996). The YXXΦ and LL motifs may interact with μ and β subunits of clathrin adapters (Le Borgne and Hoflack, 1998; Molloy et al., 1999) and these interactions might affect intracellular trafficking in endosomes and the *trans*-Golgi Network (TGN), as well as endocytosis. In addition, such motifs also determine basolateral versus apical sorting in polarized cells (Bonifacino and Dell'Angelica, 1999; Brideau et al., 2000; Fölsch et al., 1999; Tirabassi and Enquist, 1999). Both HSV-1 and VZV gE contain similar tyrosine-based motifs within their cytoplasmic domains that are important for endocytosis and sorting (Brideau et al., 2000). The endocytosis motifs in PRV gE do not appear to contribute to the efficiency of gE incorporation in the envelope of progeny virions (Tirabassi and Enquist, 1999). Despite several studies on the topic, the exact role of endocytosis of gE during infection is yet to be fully understood.

1.2.2 Functions of PRV gE

Glycoprotein E is not essential for replication (*in vitro* or *in vivo)* of either PRV or other alphaherpesviruses, like HSV-1 (Longnecker and Roizman, 1986; Neidhardt et al., 1987), EHV-1 (Flowers and O'Callaghan, 1992) and BHV-1 (Engelenburg et al., 1995; Jacobs, 1994). The only exception is VZV. This virus does not express gD, which is essential in most other alphaherpesviruses, and VZV gE appears to take over the function of gD, being vital for viral entry (Cohen and Nguyen, 1997; Moffat et al., 2002). On the other hand, conservation of gE among the different *Alphaherpesvirinae* subfamily members

strongly indicates that this protein may be essential for virus survival in the host population. The functions of PRV gE have been studied for many years and several roles have been described for this glycoprotein. Most notably, gE is involved in mediating full virulence in animal infections and cell-to-cell spread. Other functions like virion assembly (mentioned above) and species-specific binding of immunoglobulin G (IgG) through its Fcγ receptor-like activity have also been reported. Later in this chapter we describe these functions with greater detail (Figure 3).

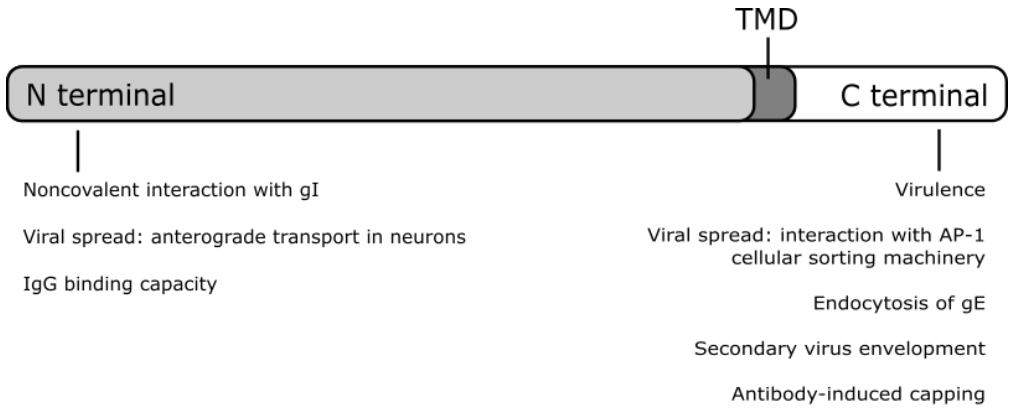

Figure 3. Representation of the different domains of the glycoprotein gE: N terminal (extracellular domain) = 428 aa; transmembrane domain (TMD) = 26 aa; C terminal (cytoplasmic domain) = 123 aa. The two major regions of gE appear to have different contributions in the different functions reported for the glycopretin.

Virulence

Early reports on the characterization of PRV gE showed that this viral protein is a determinant factor for PRV's virulence (Card and Enquist, 1995; Card et al., 1992; Jacobs et al., 1993b; Jacobs et al., 1993b; Kimman et al., 1992; Lomniczi et al., 1984; Mettenleiter et al., 1987). Virulence is usually quantified by the time after infection when symptoms begin to appear or by the mean time to the animal's death. Animals, both pigs and mice, infected with PRV gEnull mutants live longer, develop symptoms later, and are symptomatic for a significantly shorter time than are animals infected with wild-type virus (Card and Enquist, 1995; Jacobs, 1994; Jacobs et al., 1993b; Kritas et al., 1994; Kritas et al., 1995; Tirabassi et al., 1997). Different studies suggested that, in the absence of gE, the transport of the virus to certain parts of the nervous system is impaired to some extent (Jacobs, 1994; Jacobs et al., 1993b; Kimman et al., 1992; Kritas et al., 1994; Kritas et al., 1995; Tirabassi and Enquist, 1999; Tirabassi et al., 1997).

Deletion of the gene encoding HSV-1 gE drastically decreased neurovirulence in mice (Meignier et al., 1988); also, gE-negative BHV-1 mutants were avirulent in calves (Kaashoek et al., 1994).

Impaired virulence of PRV in the absence of gE has been attributed, at least partially, to the reduced ability of these viruses to spread from cell to cell and from neuron to neuron (discussed later in this chapter) (Card et al., 1992; Kimman et al., 1992; Mettenleiter et al., 1987; Whealy et al., 1993). However, several data have shown that gE-mediated virulence and spread appear to reflect two possibly independent functions. For example, the attenuated PRV vaccine strain, Bartha, which lacks gE amongst other proteins, spreads remarkably well in the central nervous system (CNS) (Card and Enquist, 1995; Card et al., 1990; Rinaman et al., 1993). Despite this, when infected with the Bartha strain, pigs remain symptom free, and mice live several days longer than wild-type virus-infected animals (Rinaman et al., 1993). Similarly, in the rodent eye infection model, gEnull viruses are not completely defective in neuronal spread, even though these mutants appear to be less virulent (Card et al., 1992; Whealy et al., 1993). Tirabassi *et al.* have shown that the cytoplasmic domain of PRV gE is necessary for full virulence, but not for neurotropism in the rat eye model, suggesting that virulence and neurotropism defects seen in infection with gE mutants may indeed reflect separate functions (Tirabassi and Enquist, 1998; Tirabassi and Enquist, 1999; Tirabassi et al., 1997).

Considering all the reports mentioned above, it is evident that PRV gE greatly influences virulence, although the underlying mechanisms are not entirely clear.

Viral spread

HSV and PRV cell-to-cell spread mediated by gE has been observed primarily or exclusively in polarized cells or in cells that form extensive junctions with one another (i.e., keratinocytes, epithelial cells, and neurons) (Dingwell and Johnson, 1998; Dingwell et al., 1995; Enquist et al., 1998; Mettenleiter et al., 1987; Tirabassi and Enquist, 1998; Wisner et al., 2000; Zsak et al., 1992). In experimental animal models, both HSV and PRV mutants lacking gE are restricted for spread in epithelial tissues and in the nervous system (Card et al., 1992; Dingwell et al., 1994; Dingwell et al., 1995; Tirabassi and Enquist, 1998; Tirabassi and Enquist, 1999). In cell culture, infection with PRV gE mutant viruses results in smaller plaques, emphasizing the role of this protein during cell-to-cell

spread. Other studies have confirmed these findings for HSV and VZV (Cohen and Nguyen, 1997; Cole and Grose, 2003; Dingwell et al., 1994; Jacobs et al., 1993a; Jacobs et al., 1993b; Johnson and Huber, 2002; Johnson et al., 2001a; Tirabassi and Enquist, 1998). In addition, deletion of the cytoplasmic domain of gE resulted in a similar small plaque phenotype, suggesting that this domain may be involved in gE-mediated viral spread (Tirabassi et al., 1997; Tirabassi and Enquist, 1999). However, as mentioned above, PRV mutants lacking the cytoplasmic region of gE were shown to be able to spread in the rat CNS (Tirabassi et al., 1997; Tirabassi and Enquist, 1999).

PRV mutants lacking gE also showed reduced anterograde neuronal spread *in vitro* (Ch'ng and Enquist, 2005a; Ch'ng and Enquist, 2005b). The importance of gE in cell-to-cell spread in non-neuronal cells and anterograde spread in neurons has been thoroughly studied, and it is not clear if these two phenotypes reflect common or distinct mechanisms.

Regarding gE-mediated cell-to-cell spread in polarized epithelial cells, Johnson and Huber proposed a model based on various observations (Figure 4). Briefly, gE accumulates in the TGN/endosomes, which promotes accumulation of other viral components (glycoproteins and tegument) in the same compartments. This accumulation and ultimate acquisition of the viral envelope occurs in specific TGN/endosome membranes that will then be sorted specifically to the basolateral surfaces. These processes depend on the interaction between the cytoplasmic domain of gE and the AP-1 cellular sorting machinery (specifically µ1B, a component of the AP-1 clathrin adapter complexes). As virus replication continues, there is directed delivery of nascent particles to cell junctions; virus particles are delivered into the space between adjacent cells and can initiate infection of a neighbouring cell (Brack et al., 2000; Farnsworth and Johnson, 2006; Granzow et al., 2001; Harley et al., 2001; Johnson and Huber, 2002; Johnson et al., 2001a; McMillan and Johnson, 2001).

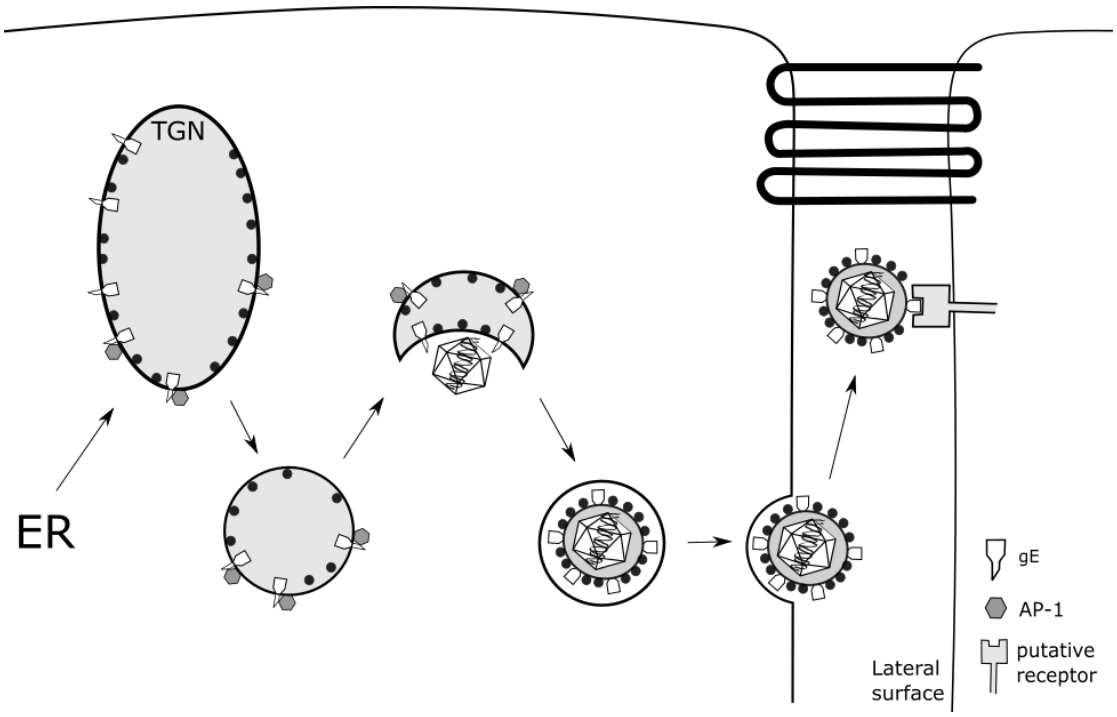

Figure 4. gE mediates directed egress in polarized epithelial cells, facilitating cell-to-cell virus spread (adapted from Johnson and Huber, 2002). gE accumulates in the TGN/endosomes, promoting aggregation of other viral components. The interaction between gE and the AP-1 cellular sorting machinery is important for directing nascent virions to lateral cell surfaces

Furthermore, the authors speculated that not only the cytoplasmic domain, but also the extracellular domain of gE might play a role in cell-to-cell spread, perhaps by binding to proteins that are components of cell junctions, e.g., cell adhesion molecules (Dingwell and Johnson, 1998; Johnson and Huber, 2002; McMillan and Johnson, 2001). This may explain the preferential localization of gE to cell junctions (McMillan and Johnson, 2001; Wisner et al., 2000).

The role of gE in anterograde spread in neurons has been further elucidated more recently, although the mechanism is still far from being fully comprehended. Kratchmarov et al. suggested that gE is required for efficient anterograde transport of viral particles by indirectly facilitating or stabilizing the interaction between US9 and the kinesin-3 motor KIF1A, a cellular motor protein that mediates axonal movement of viral particles (Kratchmarov et al., 2013). Another viral envelope protein, US9 has been shown to be essential for initial sorting of virions to axons, although the subsequent step

of fast axonal transport is carried out independently from this viral protein (Daniel et al., 2015).

Virion assembly

As mentioned above, gE accumulates in the TGN and endosomes, which may contribute to virion assembly in these organelles. PRV mutants lacking both gE, or its cytoplasmic domain, and gM caused the accumulation of large numbers of cytosolic nucleocapsids (Brack et al., 2000). This is consistent with a role for gE and gM in secondary envelopment of virus particles. These and other data have led to the hypothesis that gE promotes the initial accumulation of virus structural components in the TGN or endosomes, likely in tandem with other virion components, such as gM, which contributes to secondary envelopment of progeny virus particles (Brack et al., 2000; Granzow et al., 2001; McIntosh and Smith, 1996).

Fcγ receptor-like activity and cell signalling

Similar as described for HSV and VZV, PRV gE has been shown to exhibit IgG binding (Favoreel et al., 1997; Johnson et al., 1988; Litwin et al., 1992). This Fcγ receptor-like activity of gE was shown to be involved in viral immune evasion, both in HSV and PRV (Nagashunmugam et al., 1998; Van de Walle et al., 2003a), probably by shielding the Fc domain of virus-specific antibodies from immune effectors like complement, NK cells and phagocytes.

Adding PRV-specific antibodies to PRV-infected epithelial cells resulted in antibody-antigen capping characterized by the polarization of viral glycoproteins and associated antibodies on the surface of an infected cell. This capping process was mediated by gE (Favoreel et al., 1999). Similar antibody-induced and gE-mediated capping of viral glycoproteins was also reported for HSV (Rizvi and Raghavan, 2003) . Although the exact function of gE-mediated antibody-antigen capping is not entirely clear, it resembles capping of immunoreceptors on leukocytes. The latter process is observed upon crosslinking of immunoreceptors with antibodies or antigens, and typically leads to leukocyte activation (D'Ambrosio et al., 1996). The cytoplasmic domain of gE was reported to take part in this capping, since deleting the cytoplasmic domain of gE

strongly reduced the phenomenon, and since capping was associated with Src tyrosine kinase-mediated tyrosine phosphorylation of the cytoplasmic domain of gE (Desplanques et al., 2007; Favoreel et al., 1999). Mutation of the YXXΦ motifs in the cytoplasmic domain of gE reduced tyrosine phosphorylation of gE and suppressed antibody-induced capping (Desplanques et al., 2007; Favoreel et al., 1999). These YXXΦ motifs resemble immunoreceptor tyrosine-based motifs (ITAM) found in the cytoplasmic region of immunoreceptors. ITAM domains are crucial for antigen-induced signalling and activation of immune cells. Furthermore, it was reported that gE associates with lipid rafts, that this lipid raft association increases during capping, and that gE-mediated capping may depend on lipid raft-associated signalling machinery, as disruption of these structures inhibits capping, which again shows strong similarity to the mechanism of immune receptor signalling and capping during leukocyte activation (Desplanques et al., 2007; Favoreel et al., 2004). Taken together, these studies suggest that gE, in addition to its Fcγ receptor-like activity, may also trigger cell signalling in the infected cell. However, gE-mediated effects on particular cell signalling pathways have not been directly demonstrated thus far.

1.3. MITOGEN-ACTIVATED PROTEIN KINASES (MAPK)

1.3.1 Introduction

Cells respond to their surroundings in a highly efficient manner, translating extracellular messages into an appropriate cellular response. Such outside-in communication usually begins with the interaction between extracellular stimuli (like mitogens or hormones) and specific molecules present on the cell membrane, which subsequently transmit the different signals through the signalling network of the cell. These signalling processes, or signalling pathways, may also be modulated by pathogens.

Over the years, numerous intracellular signalling pathways have been identified, including the mitogen-activated protein kinase (MAPK) signalling pathways that are at the heart of many signalling networks (Ahn, 1993; Bogoyevitch and Court, 2004; Chang and Karin, 2001; Johnson and Lapadat, 2002; Pearson et al., 2001; Seger and Krebs, 1995). The MAPK signalling pathways consist of several tiers of protein kinases that sequentially activate each other by phosphorylation and each tier is composed of several isoforms. This increases the complexity of the signalling pathways, conferring a broader range of activity and specificity to the MAPK system. The MAPK signalling cascades are often referred to as the central building blocks of the intracellular signalling network, as they regulate a diversity of cellular processes like proliferation, differentiation, migration, development, stress response, and apoptosis (Bogoyevitch and Court, 2004; Kuida and Boucher, 2004; Raman and Cobb, 2003; Rubinfeld and Seger, 2005).

The four MAPK cascades currently known are named according to the subgroup of their MAPK components: (1) extracellular signal-regulated kinase 1 and 2 (ERK1/2) (Boulton et al., 1991; Sturgill and Ray, 1986); (2) c-Jun N-terminal kinase (JNK), also known as stress-activated protein kinase 1 (SAPK1)(Dérijard et al., 1994; Kyriakis et al., 1994); (3) p38 MAPK, also known as SAPK2-4 or p38 α−δ (Freshney et al., 1994; Han et al., 1994; Rouse et al., 1994); and (4) ERK5, also known as Big MAPK (BMK) (Lee et al., 1995; Zhou et al., 1995).

The distinct MAPK pathways differ in their physiological activities: ERK1/2 in general plays a role in cell survival, proliferation and differentiation, whilst JNK and p38 are often involved in stress responses and apoptosis. The ERK5 cascade seems to transmit both mitogenic and stress signals. However, there are many exceptions to these broad

function subdivisions and specific MAPK-dependent functions may be cell type- and cell condition-dependent. For example, ERK1/2 signalling has also been reported to participate in apoptotic responses under certain conditions (as described later in this chapter), whereas JNK signalling may take a part in cell survival and proliferation (Hess et al., 2002). Despite their distinct physiological activity, cross-talk between the MAPK different signalling pathways often occurs and contributes to the ultimate fate of the cell in response to different stimuli (Junttila et al., 2008).

This thesis will focus on one particular branch of the MAPK signalling cascade, the ERK1/2 pathway, which controls a myriad of cellular events, making it a very attractive target for various pathogens, including viruses.

1.3.2 The ERK1/2 signalling pathway

The ERK1/2 signalling pathway was the first of the MAPK system to be elucidated (Boulton et al., 1991; Seger and Krebs, 1995; Sturgill and Ray, 1986). This signalling pathway is evolutionary conserved and is involved in the control of many fundamental cellular processes that include cell proliferation, survival, differentiation, apoptosis, motility, metabolism, morphology determination and oncogenic transformation (Chong et al., 2003; O'Neill and Kolch, 2004; Seger and Krebs, 1995; Torii et al., 2004; Viala and Pouysségur, 2004; Wellbrock et al., 2004; Yoon and Seger, 2006).

Two ERK genes have been described for mammals and these genes encode a 44 kDa protein (ERK1) and a 42 kDa protein (ERK2) (Boulton et al., 1991; Seger and Krebs, 1995; Sturgill and Ray, 1986). Because of the high degree of similarity between ERK1 and ERK2, these proteins are considered to be functionally redundant and are mostly being referred to as ERK1/2, although some differences in their substrate specificity have been reported (Seger and Krebs, 1995).

Figure 5 depicts a general overview of the ERK1/2 signalling pathway.

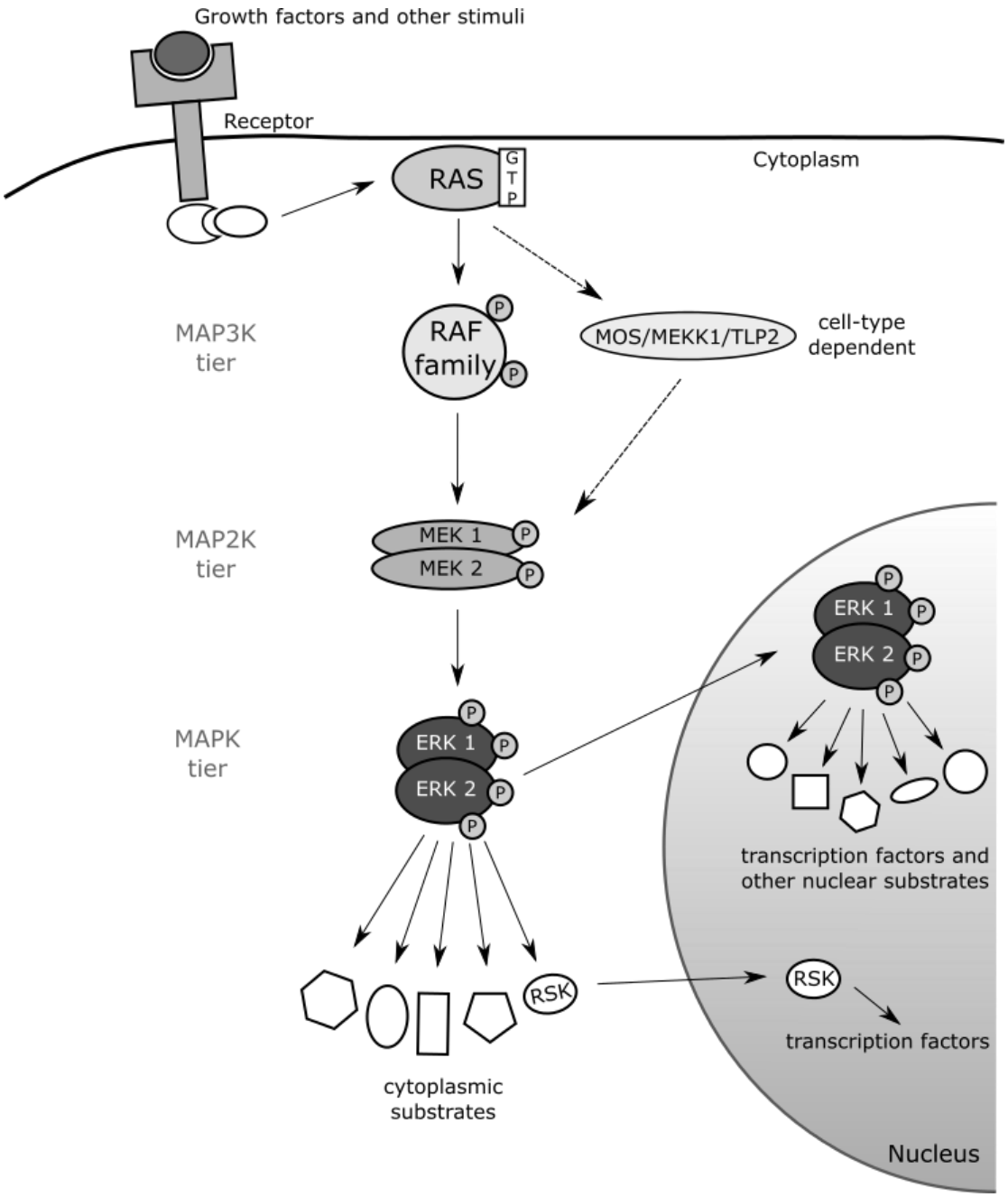

Figure 5. The MAPK/ERK1/2 signalling pathway. The first pathway of the MAPK system to be elucidated, this signalling cascade is composed by several tiers of protein kinases that sequentially activate each other by phosphorylation, controlling many fundamental cellular processes. P – Phosphoryl group.

ERK1/2 can be activated by a wide variety of extracellular agents, which include growth factors, hormones, and neurotransmitters (Chang et al., 2003; Chuderland and Seger, 2005; Yao and Seger, 2004; Zhang and Liu, 2002). These extracellular factors, acting through several receptors like G protein-coupled receptors (Naor et al., 2000), tyrosine kinase receptors (Marmor et al., 2004) and ion channels (Rane, 1999), initiate a variety of intracellular downstream events. The ERK1/2 signalling cascade is usually triggered

by the activation of small G proteins (e.g. Ras), which transmit the signal further to the next tier of the cascade, composed of MAPK kinase kinases (MAP3K) (Ahn, 1993). The three isoforms of the Raf kinase family (A-Raf, B-Raf and Raf-1) are considered to be the primary MAP3K for ERK1/2 activation, although additional MAP3K (MOS, MEKK1 and TLP2) may also lead to ERK1/2 phosphorylation under more restricted cell type- and stimulation-specific situations (Gotoh and Nishida, 1995; Lange-Carter et al., 1993; Raman et al., 2007; Salmeron et al., 1996). All these MAP3K transmit the signal further by phosphorylating and activating the MAPK kinase tier composed of MAPK/ERK kinases (MEK1/2; (Ahn et al., 1991; Gómez and Cohen, 1991). MEK1/2 is a dual-specificity protein kinase that mediates the phosphorylation of tyrosine as well as threonine residues in ERK1/2, its only known physiological substrate (Payne et al., 1991; Ray and Sturgill, 1988; Roskoski Jr., 2012; Seger et al., 1992).

ERK1/2 is a ubiquitous serine/threonine kinase and due to the broad nature of its substrate recognition, ERK1/2 can phosphorylate numerous proteins (Yoon and Seger, 2006). Some of these proteins are localized in the cytoplasm, whilst others are phosphorylated in the nucleus after nuclear translocation of activated ERK1/2 (Yoon and Seger, 2006). Notably, ERK1/2 activates a series of transcription factors such as Elk1 (Gille et al., 1992), c-Fos (Murphy et al., 2002), p53 (Milne et al., 1994), Ets1/2 (Yang et al., 1996), and c-Jun (Morton et al., 2003), which are important for the initiation and regulation of proliferation and oncogenic transformation. Alternatively, ERK1/2 may activate the family of MAP Kinase Activated Protein Kinases (MAPKAPKs). The main MAPKAPK activated by ERK1/2 is the 90 kDa ribosomal S6 kinase, RSK (Sturgill et al., 1988), which can translocate independently into the nucleus where it phosphorylates a set of transcription factors, playing its own role in transcriptional regulation. Additional MAPKAPKs are the mitogen- and stress-activated kinases (MSK; (Deak et al., 1998)) and the MAPK-interacting kinases (MNKs; (Fukunaga and Hunter, 1997; Waskiewicz et al., 1997), which are equally activated by the related p38 MAPK cascade (Kant et al., 2003). All the above substrates change their activity upon phosphorylation by ERK1/2, and may contribute to ERK1/2-dependent processes.

1.3.3 Regulation of the ERK1/2 signalling pathway

The ERK1/2 signalling cascade is able to activate different, sometimes opposing, processes within the same cell. This raises the question how the cell regulates this signalling pathway in order to guarantee a specific effect. Several parameters that determine the signalling outcome have been proposed in the past years. Duration and strength of ERK1/2 phosphorylation is one of these factors (Ebisuya et al., 2005; Marshall, 1995; Murphy and Blenis, 2006). Several studies investigating ERK1/2 activation have shown that transient ERK1/2 activation leads to different effects than sustained activation (Ebisuya et al., 2005; Kolch, 2005; Marshall, 1995). As a classical example, in rat PC12 cells, transient Ras-dependent activation of the ERK1/2 signalling pathway by Epidermal Growth Factor (EGF) induces proliferation, whilst sustained ERK1/2 activation by Nerve Growth Factor (NGF) induces differentiation (Dumaz and Marais, 2005; Gotoh and Nishida, 1995; Marshall, 1995; Murphy et al., 2002; Nguyen et al., 1993; York et al., 1998). York et al. have reported that transient and sustained ERK activation in PC12 cells are regulated by the small GTPases Ras and Rap1, respectively (Sasagawa et al., 2005; York et al., 1998). Duration of ERK1/2 activation can also be influenced by protein kinase C (PKC)/Raf signalling (Bhalla and Iyengar, 1999; Bhalla et al., 2002). Another temporal regulator of ERK activity is Sprouty – a negative feedback inhibitor of the ERK pathway (Hanafusa et al., 2002). Overexpression of Sprouty led to a transient ERK1/2 activation, suppressing the differentiation of PC12 cells. In contrast, overexpression of a dominant-negative form of Sprouty results in sustained ERK1/2 activation and promotes PC12 cells differentiation.

The strength of ERK1/2 activity is also a determinant of cell fate. The regulation of the magnitude of ERK1/2 signalling can be carried out by scaffold proteins, such as KSR (kinase suppressor of Ras) and MP1 (MEK partner 1). KSR interacts with Raf, MEK and ERK, thereby potentiating ERK activation (McKay et al., 2009; Morrison, 2001; Therrien et al., 1996). KSR forms a ternary complex with MEK and ERK and the absence of KSR significantly diminishes MAPK signalling (Nguyen et al., 2002). MP1 facilitates coupling of ERK to MEK (Schaeffer et al., 1998), and reduction of MP1 expression results in decreased ERK activation in response to growth factor stimulation (Teis et al., 2002).

Subcellular localization of ERK1/2 also plays a role in its regulation (Ebisuya et al., 2005; Kolch, 2005; Pouysségur et al., 2002), leading to different protein-protein interactions

and therefore defining which substrates will be activated. Translocation of ERK1/2 to the nucleus allows its access to several transcription factors, like Elk-1, Sap-1a, and TIF-IA (Chen et al., 1992; Gille et al., 1992; Lenormand et al., 1993; Zhao et al., 2003). On the other hand, cytosolic ERK1/2 may phosphorylate different substrates such as pro-apoptotic Bim, and MLCK and m-calpain, two pro-migration proteins. Interaction with various scaffold proteins appears to regulate spatial distribution of ERK1/2 (Chuderland and Seger, 2005; Ebisuya et al., 2005; Kolch, 2005; Morrison and Davis, 2003). Proteins like β-arrestin-2, PEA-15 (phosphoprotein enriched in astrocytes) and Sef bind to ERK1/2, which blocks its translocation to the nucleus (Formstecher et al., 2001; Torii et al., 2004; Whitehurst et al., 2004). Sef resides on the Golgi apparatus and binds active MEK/ERK complexes, permitting signalling to cytosolic substrates but not nuclear targets (Torii et al., 2004). PEA-15 binds ERK1/2, abolishes its nuclear translocation, and thereby inhibits the phosphorylation of Elk-1. Also, PEA-15 contains a nuclear export sequence that mediates the relocation of ERK1/2 from the nucleus to the cytoplasm (Formstecher et al., 2001). A similar regulatory mechanism is mediated by β-arrestin-2, as this adaptor protein also contains a nuclear export sequence. Thus, ERK1/2 activation caused by β-arrestin-2-mediated assembly of complexes comprising Raf-1, MEK and ERK/MAPK is majorly restricted to the cytoplasm (DeFea et al., 2000; Luttrell et al., 2001; Tohgo et al., 2003; Wei et al., 2003).

Other elements contribute to the regulation of ERK1/2 signalling, including extensive cross-talk and interplay with other intracellular signalling pathways (Dumaz and Marais, 2005; Raman and Cobb, 2003), and the presence of several similar but functionally different isoforms in each tier of the cascade (Lloyd, 2006), including alternatively spliced forms (Aebersold et al., 2004). In addition, the particular ERK1/2-dependent effect of a given ligand on different cells may also be influenced by other mechanisms, such as the cell type-specific expression of ERK1/2 substrates (Yoon and Seger, 2006). Another layer of ERK1/2 regulation complexity is generated by the many phosphatases that can downregulate ERK1/2 activity (Krishna and Narang, 2008; Muthumani et al., 2005; Wang et al., 2006). Once dephosphorylated in the nucleus, ERK1/2 is rapidly exported to the cytosol via an active mechanism that is mediated, at least in part, by MEK which enters the nucleus independently from ERK1/2 (Adachi et al., 2000).

1.3.4 The role of ERK1/2 activation in different cellular processes

As mentioned above, the ERK1/2 signalling pathway is involved in a broad range of different cellular processes. To keep the extensive information within the scope of this thesis, only the cellular processes that are of particular relevance for the current work were selected for a more detailed description in the present chapter, namely cell motility and cell survival.

ERK1/2 in cell motility

The process of cell migration involves a continuous cycle of adhesion and detachment (adhesion turnover) both at the leading edge and the rear end of cells (Ridley et al., 2003; Webb et al., 2002). The ERK1/2 signalling pathway has been reported to regulate different stages in the process of cell motility. As consequence, inhibition of the ERK1/2 signalling pathway prevents the migration of diverse cell types in response to various stimuli (Anand-Apte et al., 1997; Cheresh et al., 1999; Cuevas et al., 2003; Degryse et al., 2001; Eliceiri et al., 1998; Jo et al., 2002; Klemke et al., 1997; Krueger et al., 2001; Lai et al., 2001; Nguyen et al., 1999; Shono et al., 2001; Webb et al., 2000; Xie et al., 1998). Several ERK1/2 substrates, including various protein kinases, transcription factors and nuclear proteins have been linked to cell migration (Deak et al., 1998; Fukunaga and Hunter, 1997; Glading et al., 2004; Hunger-Glaser et al., 2003; Klemke et al., 1997; Waskiewicz et al., 1997). The focal adhesion kinase (FAK)/paxillin complex, the myosin light chain kinase (MLCK) and calpains are the most likely candidates to be involved in ERK1/2-mediated cell migration (Figure 6).

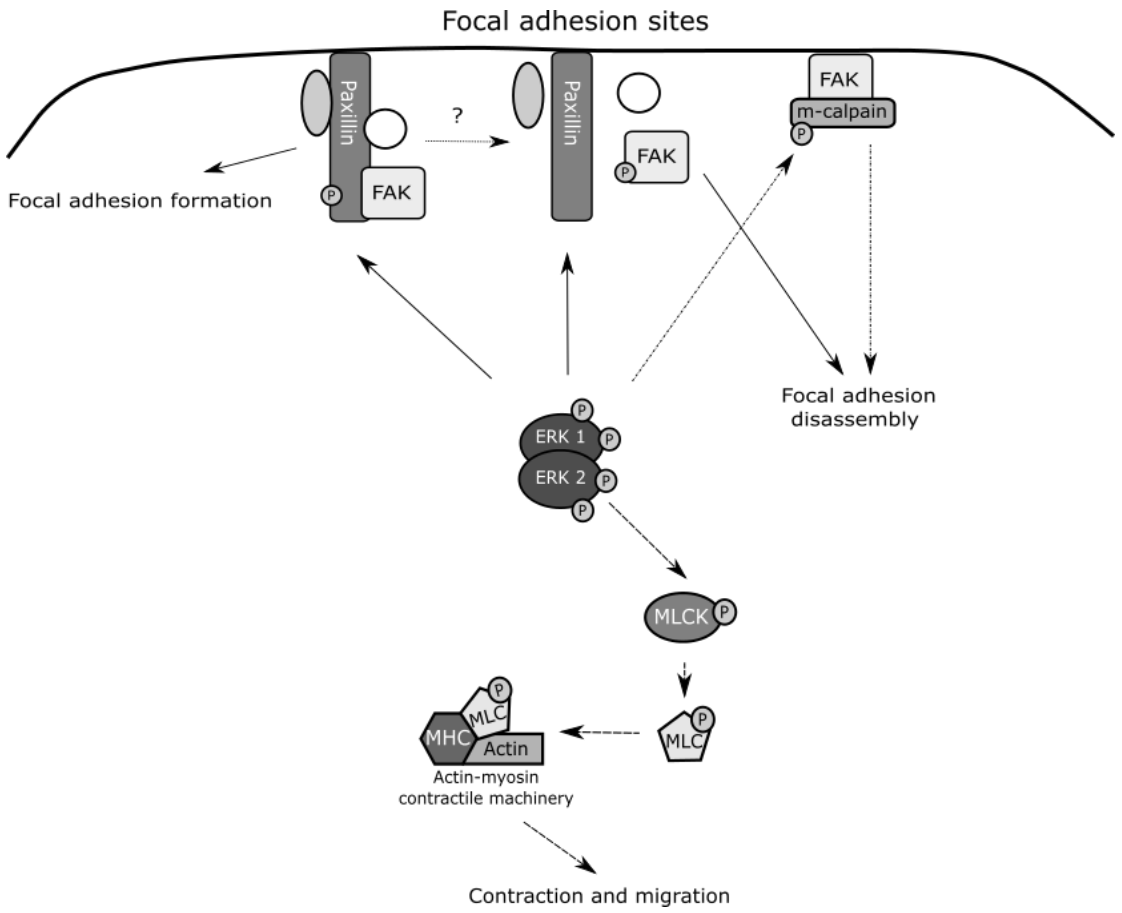

Figure 6. The role of the ERK1/2 signalling pathway in cell motility. The ERK1/2 signalling pathway can regulate different stages in the process of cell motility by phosphorylating different substrates that have been shown to be important for cell motility. ERK1/2 substrates, focal adhesion kinase (FAK)/paxillin complex, the myosin light chain kinase (MLCK) and calpains, were reported to mediate key events in cellular motility such as focal adhesion formation, adhesion turn-over and lamellipodium extension. P – Phosphoryl group.

FAK is a non-receptor protein tyrosine kinase that localizes at focal adhesions or focal contacts (Schaller, 2001a). FAK mediates the phosphorylation of paxillin, a well-known scaffolding molecule (Bellis et al., 1995; Schaller, 2001b), that can recruit other molecules to focal adhesions and regulate the organization of the actin cytoskeleton. FAK and paxillin have been found to be critical for cell spreading and migration (Deramaudt et al., 2014; Lai et al., 2000; Liu et al., 2002) and the interaction between these two proteins is involved in focal adhesion turnover (Webb et al., 2004), although the precise role and mechanism remain to be fully clarified. It has been shown that ERK1/2 activity mediates phosphorylation of both FAK and paxillin, as well as regulating the association of FAK with paxillin (Hunger-Glaser et al., 2003; Ishibe et al., 2004; Jiang et al., 2007). Phosphorylation of paxillin by ERK1/2 has been shown to enhance the FAK-

paxilin association (Liu et al., 2002), whereas ERK1/2-mediated phosphorylation of FAK at Ser910 was shown to block the interaction of FAK with paxillin (Hunger-Glaser et al., 2003). These intriguing observations may suggest a sophisticated regulation of the FAK-paxillin complex, in which ERK1/2 might initially promote complex-assembly by phosphorylation of paxillin and then promote disassembly by subsequent phosphorylation of FAK, leading to inactivation of the latter, promoting adhesion turnover and cellular migration (Zheng et al., 2009).

MLCK controls actin-myosin contractility through the phosphorylation of myosin light chain (MLC). Phosphorylation of MLC allows the formation of a functional actin-myosin motor. Interestingly, MLC phosphorylation by MLCK occurs at the leading edge of migrating cells, suggesting an important role for MLCK in the control of lamellipodium extension (Mitchison and Cramer, 1996; Totsukawa et al., 2004). In this context, it was shown that ERK1/2 directly phosphorylates and activates MLCK, leading to MLC phosphorylation and cell migration (Klemke et al., 1997; Mansfield et al., 2000; Nguyen et al., 1999; Webb et al., 2004).

Calpains are a family of Ca^{2+}-activated proteolytic enzymes that are involved in cell migration (Dourdin et al., 2001; Huttenlocher et al., 1997). ERK1/2 phosphorylates m-calpain both *in vitro* and *in vivo*, and phosphorylation of m-calpain is required for adhesion turnover and cell migration (Glading et al., 2004). However, the exact molecular mechanism of regulation of motility by ERK1/2 and calpain remains to be determined.

It is also well known that the Ras-Raf-MEK-ERK pathway regulates the affinity of integrins for their substrate (Chou et al., 2003; Hughes et al., 1997), although the molecular mechanism remains to be elucidated. Because dynamic integrin activation is required for cell migration (Chou et al., 2003; Huttenlocher et al., 1996; Palecek et al., 1997), ERK1/2 might also play an important role in the regulation of cell migration by modulating integrin activity (Ishibe et al., 2003).

ERK1/2 and cell survival

ERK1/2 activation has generally been associated with cell survival and proliferation. Nonetheless, a number of studies have shown that depending on the stimuli and cell types involved, activation of ERK1/2 can also mediate cell death. Some studies suggest that the balance between the intensity and duration of pro- versus anti-apoptotic signals mediated by the ERK1/2 cascade determines whether a cell survives and proliferates or undergoes apoptosis (Ebisuya et al., 2005; Lu and Xu, 2006; Pearson et al., 2001; Shaul and Seger, 2007). However, the molecular mechanisms that define the conditions for ERK1/2-mediated cell death remain poorly understood.

The ERK1/2 signalling pathway modulates cell survival by different mechanisms like post-translational modification and regulation of components of the cell death machinery, and controlled transcription of anti- and pro-survival genes (Figure 7) (Kolch, 2005).

As mentioned earlier in this chapter, ERK1/2 activates the RSK (ribosomal S6 family kinases) kinase family. This kinase family is usually present in the cytoplasm in quiescent cells (Anjum and Blenis, 2008; Zhao et al., 1996) and catalyses the phosphorylation and subsequent degradation of the pro-apoptotic Bcl-2 family member protein Bad. Furthermore, the pro-apoptotic BH-3 only protein Bim, another member of the Bcl-2 family, is phosphorylated on multiple sites by ERK1/2 and targeted for poly-ubiquitination, followed by degradation via the proteasome pathway (Ewings et al., 2007; Hübner et al., 2008; Ley et al., 2003). These and other studies have shown that phosphorylation and degradation of Bim and/or Bad by ERK1/2 through multiple mechanisms can contribute to reduced sensitivity of cells to apoptosis (Bonni et al., 1999; Ley et al., 2003; Sheridan et al., 2008; Zha et al., 1996a). Evidence of repressed activation of more downstream events, such as inhibition of caspase activity, has also been reported. ERK1/2 has been shown to phosphorylate, and consequently inhibit, caspase 9, blocking subsequent downstream events, such as caspase 3 activation, culminating in inhibition of cell death (Allan et al., 2003).

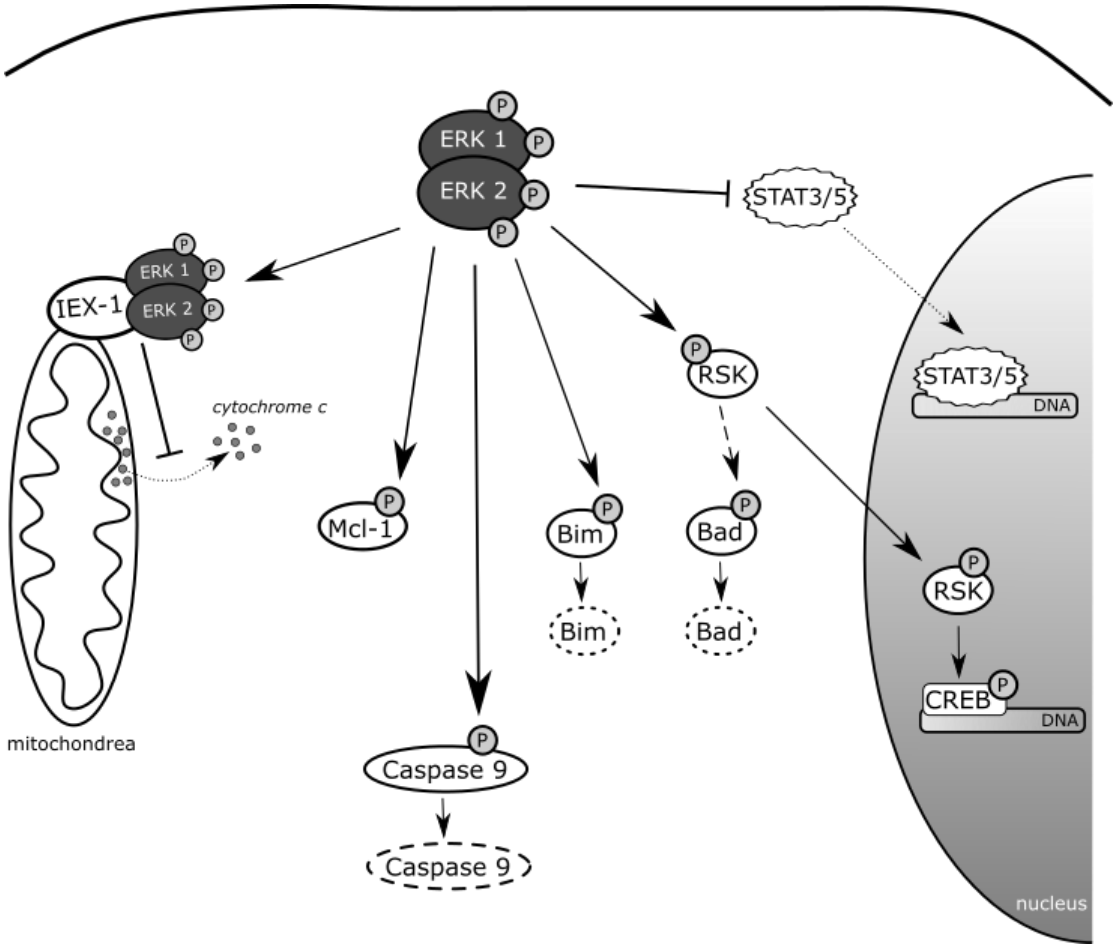

Figure 7. The role of the ERK1/2 signalling pathway in promoting cell survival. The ERK1/2 signalling pathway modulates cell survival by different mechanisms like post-translational modification and regulation of components of the cell death machinery, and controlled transcription of anti- and pro-survival genes. ERK1/2 triggers phosphorylation of apoptosis mediators, like Bcl-2 family members Bad and Bim, as well as Caspase 9, leading to the degradation of these proteins and protection against apoptosis. Additionally, ERK1/2 potentiates the activity of pro-survival proteins Mcl-1 and IEX-1. ERK1/2 also interferes with transcription factors like STAT3/5 and CREB to promote cell survival. P – Phosphoryl group.

In addition to suppressing the functions of pro-apoptotic proteins, ERK1/2 can promote cell survival by enhancing the activity of anti-apoptotic molecules. Mcl-1, an anti-apoptotic member of the Bcl-2 family, is phosphorylated by ERK1/2, thus increasing its stability and enhancing its anti-apoptotic activity (Domina et al., 2004). ERK1/2-mediated activation of IEX-1, an early response and nuclear factor kappa B (NF-kB) target gene, leads to prevention of cytochrome c release from the mitochondria and to inhibition of cell death induced by various stimuli (Garcia et al., 2002).

ERK1/2 may also control cell survival by regulating the activity of anti- and pro-apoptotic transcription factors. ERK1/2-activated RSK phosphorylates the transcription factor

cAMP response element-binding protein (CREB) (Bonni et al., 1999), which allows recruitment of the co-activators CREB-binding protein (CBP) and p300, strongly enhancing CREB-dependent transcription (Janknecht and Nordheim, 1996; Johannessen et al., 2004). Activated CREB promotes cell survival, and inhibition of CREB phosphorylation triggers neuronal apoptosis (Bonni et al., 1999). In addition, it has been also shown that inhibition of ERK1/2 upregulates STAT3/5 phosphorylation and promotes cell apoptosis (Krasilnikov et al., 2003).

Despite the progress in identifying the mechanisms that control ERK1/2-mediated cell survival and proliferation, as indicated, there is also convincing but poorly understood evidence supporting a role for the MEK-ERK signalling cassette in cell death (Mebratu and Tesfaigzi, 2009). Additional studies are required to define which conditions allow ERK1/2 activation to be responsible for both cell survival/proliferation and apoptosis. The complexity of the ERK1/2 signalling network undoubtedly contributes to this proliferation/apoptosis conundrum, and likely depends on particular yet to be identified cell type- and stimulus-dependent ERK1/2 substrates and regulators.

1.3.5 Herpesviruses interaction with the ERK1/2 signalling cascade

As mentioned above, the ERK1/2 signalling pathway is involved in an impressive array of different cellular processes, making it highly attractive for viruses to manipulate this signalling cascade for their own benefit. Many different viruses have been reported to modulate (and often activate) the ERK1/2 signalling cascade. In addition, for some viruses, multiple viral proteins belonging to the same virus trigger this signalling pathway. One good example is the human immunodeficiency virus 1 (HIV-1). Several groups have shown that ERK1/2 activation is required for HIV replication (Hemonnot et al., 2004; Mettling et al., 2008; Yang and Gabuzda, 1999) and at least three HIV-1 viral proteins have been shown to trigger ERK1/2 signalling, Nef, gp120 and Tat. HIV Nef increases ERK1/2 activity in primary CD4 T lymphocytes through the recruitment of Lck and PKCθ to lipid rafts, potentially enhancing HIV transcription (Schrager et al., 2002; Witte et al., 2008). Popik et al. have shown that activation of Raf-1 and the MAP kinase pathway by binding of HIV-1 envelope glycoproteins to CD4 receptors leads to

the transcriptional activation of expression of the early inflammatory genes (Popik et al., 1998). Lastly, Tat, a viral immunosuppression factor, upregulates programmed death-ligand 1 (PDL-1, also known as B7-H1) in an ERK1/2-dependent manner, leading to expression of the anti-inflammatory cytokine IL10, which may contribute to disease progression (Planès et al., 2014; Shi et al., 2011; Trabattoni et al., 2003).

Several members across the large family of herpesviruses have also been described to modulate the ERK1/2 signalling cascade (Table 1). ERK1/2 modulation in host cells has been described for alpha-, beta- and gamma-herpesviruses, although the responsible viral factors, the underlying molecular mechanism and/or biological consequences are often poorly understood. Moreover, how the virus manipulates the ERK1/2 signalling pathway and the way how subsequent effects manifest often seems to depend on the cell type.

Within the *Alphaherpesvirinae* subfamily, HSV-1 has been reported to either induce or inhibit ERK1/2 activity, once again apparently dependent on the cell type. In epithelial cells, HSV-1 leads to ERK1/2 activation in the early stages of infection, which seems to contribute to viral replication (Torres et al., 2012). HSV-1 also induced ERK1/2 in B cells (lymphoma cell line), and this activation is partially responsible for HSV-1-induced Kaposi-Sarcoma herpesvirus (KSHV) replication (Qin et al., 2013). More recently it has also been shown that HSV-1-mediated ERK1/2 activation in neuronal cells (neuroblastoma cell line) controls virus-induced filopodia formation and possibly contributes to viral entry (Zheng et al., 2014). Whilst these studies demonstrated ERK1/2 activation caused by the virus, others reported that HSV-1 suppresses ERK1/2 activity in fibroblasts, an effect mediated by the viral US3 protein kinase (Chuluunbaatar et al., 2012). Although the precise consequences of this ERK1/2 suppression were not investigated, the authors hypothesized that it may take part in US3-associated functions during HSV-1 infection, like its anti-apoptotic effect and/or effect on gene expression (Chuluunbaatar et al., 2012).

Table 1 - Herpesviruses interaction with the ERK1/2 signalling cascade

Virus	Cell type	Viral factors/ mechanism of action	Consequences of ERK1/2 viral manipulation	References
HSV-1	Epithelial cells	NS	Effective viral replication	Torres et al., 2012
	B cells	NS	HSV-1-induced KSHV replication	Qin et al., 2013
	Neuronal cells	NS	Virus-induced filopodia formation and contribution to viral entry	Zheng et al., 2014
	Fibroblasts	US3 inhibits ERK1/2 activation	NS	Chuluunbaatar et al., 2012
HSV-2	Vero cells	ICP10 activates ERK1/2	Timely onset of virus growth expression of viral immediate-early proteins	Smith et al., 2000
	Neuronal cells	ICP10 activates ERK1/2	Inhibition of caspase 3-mediated apoptosis	Perkins et al., 2002; Perkins et al., 2003
	HEK 293 cells	Virus activates ERK1/2 in a sustained manner	Effective viral replication	Zhang et al., 2010
VZV	Epithelial cells	ORF12 activates ERK1/2	Effective viral replication Degradation of pro-apoptotic proteins Bim and Bad	Liu and Cohen, 2014; Liu et al., 2012
PRV	Epithelial cells	UL46 (VP11/12) activates ERK1/2	NS	Schulz et al., 2014
	Fibroblasts	US2 binds and sequesters ERK1/2	Prevention of ERK1/2 nuclear translocation	Kang and Banfield, 2010; Lyman et al., 2006
	Plasmacytoid DCs	gE supresses ERK1/2 phosphorylation	Suppression of TI-IFN production	Lamote et al., 2017
BHV-1	MDBK cells	Bi-phasic ERK1/2 activation	NS	Zhu et al., 2011
MDV	Fibroblasts	Meq activates ERK1/2	Cell proliferation	Subramaniam et al., 2013
HCMV	Fibroblasts	Early and sustained ERK1/2 activation	Activation of transcription factors that control early viral gene expression and DNA replication	Chen and Stinski, 2002; Rodems and Spector, 1998
	Endothelial cells	NS	Pro-survival signalling and protection of the cell against free radical-induced apoptosis	Wang et al., 2006
	Myeloid cells	gB activates ERK1/2 upon virus binding and entry	Activation of ERK1/2 triggers the upregulation of key cell survival protein Mcl-1	Reeves et al., 2012
	Dendritic cells	Interleukin 6 signals through the ERK1/2	Reactivation of IE gene expression	Reeves and Compton, 2011

Virus	Cell type	Viral factors/ mechanism of action	Consequences of ERK1/2 viral manipulation	References
EBV	Epithelial cells	Rta activates ERK1/2	Maintenance of the lytic state and induction of autophagy	Hung et al., 2014; Lee et al., 2008
	Epithelial cells B lymphocytes	LMP2A activates ERK1/2	Modulation of Bim and protection against cell death	Clybouw et al., 2005; Iwakiri et al., 2013
	Epithelial cells	LMP1 activates ERK1/2	Promotion of malignant transformation	Meckes et al., 2013; Roberts and Cooper, 1998
	Burkitt's lymphoma cell line and epithelial cells	Virus activates ERK1/2 in a transient manner	Contribution to cell-cell transmission from infected lymphoma cells to EBV-negative epithelial cells	Nanbo et al., 2012
KSHV	B cell lymphoma	Bi-phasic ERK1/2 activation, first upon virus binding entry and later during gene expression	Essential for establishment of lytic infection	Kuang et al., 2008; Naranatt et al., 2003; Ford et al., 2006
	Fibroblasts Endothelial cells	gpK8.1A and gB activate ERK1/2 upon virus binding to the cell	Essential for to viral gene expression and establishment of infection	Sharma-Walia et al., 2005
	Endothelial cells	Virus inhibits cellular ERK1/2 phosphatases	Latent viral gene expression, induction of promigratory factors, and cell invasiveness	Qin et al., 2013
	B lymphocytes	ORF45 mediates activation of RSK by ERK1/2	Accumulation of transcription factors that control the virus lytic life cycle	Fu et al., 2015; Kuang et al., 2008; Kuang et al., 2009; Li et al., 2015

NS= Not specified

Studies on HSV-2 have shown that the viral ICP10 protein kinase (ICP10 PK), which is unique for HSV-2, activates ERK1/2 in non-neuronal (Vero cells) and neuronal (hippocampal primary cultures) cells (Perkins et al., 2002; Perkins et al., 2003; Smith et al., 2000). In Vero cells, activation of the ERK1/2 signalling pathway by ICP10 PK is essential for a timely onset of HSV-2 growth. This activation may also play an important role in the expression of viral IE proteins (Smith et al., 2000). In neuronal cells, ICP10 PK activates ERK1/2 leading to the inhibition of caspase 3-mediated apoptotic cell death (Perkins et al., 2002; Perkins et al., 2003). In human embryonic kidney cells (HEK 293), HSV-2 infection was shown to activate ERK1/2 in a sustained manner and inhibition of ERK1/2 signalling severely impaired viral growth, a process that is mainly dependent on MEK1 rather than MEK2 (Zhang et al., 2010). Similarly, ERK1/2 signalling induced by VZV ORF12 was required for effective virus replication during infection of epithelial cells (Liu et al., 2012). In addition, ERK1/2 activation in VZV-infected epithelial cells is associated with degradation of the pro-apoptotic proteins Bim and Bad, therefore contributing to cell survival during infection (Liu and Cohen, 2014; Liu et al., 2012).

For PRV, viral infection has been reported to activate ERK1/2 and three viral proteins have been described to tamper with the ERK1/2 signalling pathway. In PRV-infected fibroblasts, the US2 protein, although not affecting the activation of ERK1/2 *per se*, has been shown to bind and sequester ERK1/2, targeting it to the plasma membrane. Furthermore, binding of US2 to ERK1/2 did not prevent activation of the latter, which suggests that US2 may serve as a spatial regulator of ERK1/2. The authors argued that the interaction of US2 with ERK1/2 results in preventing this kinase from being translocated to the nucleus, where it may activate undesirable transcription factors, and also that the virus may use this interaction to recruit ERK1/2 where it is needed during virus replication (Kang and Banfield, 2010; Lyman et al., 2006). The interaction between US2 and ERK1/2 was not observed during HSV-2 infection, suggesting that the two homologues (PRV US2 and HSV-2 US2) may interact with a different subset of proteins (Kang et al., 2013). Another PRV protein, UL46 (or VP11/12), was reported to activate ERK1/2 in epithelial cells (Schulz et al., 2014). Contrary to what was observed in infected fibroblasts, active ERK1/2 was able to reach the nucleus, where it activated Elk-1, a well-known ERK1/2 substrate. The function of UL46-mediated ERK1/2 activation remains elusive (Schulz et al., 2014). Additionally, and very recently, it has been demonstrated

that the absence of the glycoprotein gE leads to ERK1/2 phosphorylation in plasmacytoid dendritic cells (pDC), which correlates with an increase in TI-IFN production (Lamote et al., 2017).

Other alphaherpesviruses were also found to modulate the ERK1/2 signalling pathway, like BHV-1 and Marek's disease virus. BHV-1 induces ERK1/2 activation in a bi-phasic manner, although the biological relevance for viral replication was not clear (Zhu et al., 2011). In Marek's disease, Meq, a viral protein critically involved in viral-induced tumorigenesis, activates ERK1/2 signalling pathway, consequently inducing cell proliferation. Meq is also responsible for the downregulation of certain phosphatases, which are known to deactivate MAPK (Subramaniam et al., 2013).

Betaherpesvirinae appear to equally depend on and/or modulate the ERK1/2 signalling cascade. Human cytomegalovirus (HCMV) triggers an early and sustained ERK1/2 activation in infected human fibroblasts (up to 8 hours post inoculation), and this activation contributes to the activation of transcription factors that control early viral gene expression and DNA replication (Chen and Stinski, 2002; Rodems and Spector, 1998). HCMV infection also activates ERK1/2 in endothelial cells, contributing to pro-survival signalling and protecting the cell against free radical-induced apoptosis (Wang et al., 2006). Moreover, HCMV activates ERK1/2 in myeloid cells upon virus binding and entry, upregulating the key cell survival protein myeloid cell leukaemia 1 (Mcl-1). This activation of the ERK1/2 signalling pathway is mediated by the viral envelope protein gB (Reeves et al., 2012). ERK1/2 has also been shown to be one of the key players in reactivation of HCMV after dendritic cell differentiation (Reeves and Compton, 2011).

For the *Gammaherpesvirinae*, Epstein-Barr virus (EBV) and the Kaposi's sarcoma associated-herpesvirus (KSHV) have been shown to activate ERK1/2. Three viral proteins belonging to EBV have been reported to be involved in activation of ERK1/2 and each one is linked to different cellular/viral events. ERK1/2 activation by the IE protein Rta was shown to be essential for maintenance of the lytic state and induction of autophagy in EBV-infected epithelial cells (Hung et al., 2014; Lee et al., 2008). Phosphorylation of ERK1/2 in EBV-infected cells mediated by another viral protein, the latent membrane protein 2A (LMP2A), has been shown to correlate with modulation of Bim and protection against cell death in both epithelial cells and B lymphocytes (Clybouw et al., 2005; Iwakiri et al., 2013). Finally, the latent membrane protein 1 (LMP1) activates ERK1/2 signalling

to promote malignant transformation of epithelial cells (Meckes et al., 2013; Roberts and Cooper, 1998). The ERK1/2 pathway also plays a role in the trafficking of virions to the intercellular space, contributing to cell-cell transmission from infected lymphoma cells to EBV-negative epithelial cells (Nanbo et al., 2012).

In KSHV-infected fibroblasts, the ERK1/2 signalling pathway was found to be essential during primary infection. In these cells, ERK1/2 is activated in a biphasic manner, firstly after virus binding and entry and later during gene expression (Kuang et al., 2008; Naranatt et al., 2003). ERK1/2 activation has also been described as a trigger for reactivation of the virus in B lymphocytes (Ford et al., 2006). Also, *de novo* infection of KHSV suppresses ERK1/2 phosphatases in endothelial cells, leading to an effective ERK-dependent latent viral gene expression, induction of promigratory factors, and cell invasiveness (Qin et al., 2013). Two proteins have been implied in the KSHV-mediated ERK1/2 activation that contributes to viral gene expression: gpK8.1A and gB (Sharma-Walia et al., 2005). Furthermore, a third KSHV protein, ORF45, mediates activation of RSK by ERK1/2, leading to accumulation of transcription factors that will take part in the control of the KHSV lytic life cycle in B lymphocytes (Fu et al., 2015; Kuang et al., 2008; Kuang et al., 2009; Li et al., 2015).

The overview above describes that herpesviruses interfere with the ERK1/2 signalling pathway throughout different stages of the virus replication cycle and the consequences of this manipulation are diverse. Herpesviruses seem to have evolved to take full advantage of this pleitropic signalling pathway, re-directing ERK1/2 function to contribute to the regulation of gene expression, effective viral replication, protection against apoptosis, cell proliferation, virus reactivation from latency and cell-cell spread. For several viruses, viral proteins have been described to mediate virus-induced ERK1/2 signalling (including glycoproteins, like gB), however information regarding the molecular details how these viruses trigger ERK1/2 signalling (e.g corresponding cellular ligands) is often lacking. To this date, investigation of PRV interference with the ERK1/2 signalling in different cellular contexts and potential biological outcomes have been fairly limited. As presented earlier in this chapter, the glycoprotein E (gE) has been suggested to be involved in cellular signalling events, however no concrete link to a specific signalling pathway has been demonstrated so far. Considering this, we

questioned whether PRV effect on ERK1/2 signalling pathway could be further elucidated and whether gE would play a role in this interference.

Chapter 2

AIMS

Herpesviruses are outstanding manipulators of the host cell machinery, ensuring successful viral replication and spread, as well as achieving life-long persistence in their hosts. Over the years, our group has been studying the role of different pseudorabies virus (PRV) viral proteins in different aspects of virus-host interaction, including virus interference, either direct or indirect, with different cellular signalling pathways. One of these proteins, the glycoprotein E (gE), was reported to display potential signalling capabilities, such as species-specific Fcγ receptor-like activity (Favoreel et al., 1997; Johnson et al., 1988; Litwin et al., 1992) as well as driving signalling-dependent processes like cell surface antigen capping (Desplanques et al., 2007; Favoreel et al., 1997; Rizvi and Raghavan, 2003). Despite these earlier studies, there are no reports connecting gE with a particular signalling pathway.

The central ERK1/2 signalling pathway has been widely studied in the context of viral manipulation of the host, as it controls a large variety of cellular events (Chong et al., 2003; O'Neill and Kolch, 2004; Seger and Krebs, 1995; Torii et al., 2004; Viala and Pouysségur, 2004; Wellbrock et al., 2004; Yoon and Seger, 2006). Many herpesviruses have been reported to interfere with the ERK1/2 signalling pathway, including PRV, which has been shown to activate ERK1/2 in infected epithelial cells and fibroblasts (Lyman et al., 2006; Kang and Banfield, 2010; Schulz et al., 2014).

In the present thesis, the main aim was to investigate the potential ability of gE to interfere with the host signalling machinery and consequent biological effects that may contribute to viral replication and spread. Specifically, we focused on exploring a potential connection between gE and the modulation of the ERK1/2 signalling pathway during PRV infection.

Immune evasion is one of the key elements to the success of herpesvirus infections. The ERK1/2 signalling cascade can control several aspects of T cell activity. Since little has been reported about the interaction between PRV and T lymphocytes, the first objective of this thesis was therefore to explore the effect of PRV infection on ERK1/2 activation in the Jurkat T cell line, as well as the role of gE in this process (**Chapter 3a**). Going forward, we investigated whether PRV gE modulates ERK1/2 activation in primary porcine T lymphocytes, and potential changes in cellular behaviour due to the activation of this signalling pathway mediated by gE (**Chapter 3b**). Together with the objectives

mentioned above, we also aimed at unravelling part of the mechanism used by gE to modulate this central signalling pathway (**Chapters 3.1 and 3.2**).

ERK1/2 is not only involved in T cell activity, but also in promoting cell survival. PRV has been described to suppress apoptosis of infected cell, although thus far, only one anti-apoptotic PRV protein has been described (US3). Therefore, in the last experimental chapter of this thesis (**Chapter 4**), we aimed at understanding whether PRV- and/or gE-mediated ERK1/2 signalling modulation can also be observed in epithelial cells and whether this may interfere with the apoptotic signalling network.

Chapter 3

PSEUDORABIES VIRUS TRIGGERS GLYCOPROTEIN gE-MEDIATED ERK1/2 ACTIVATION AND ERK1/2-DEPENDENT MIGRATORY BEHAVIOR IN T CELLS

Adapted from:

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Journal of Virology (2015); 89(4): 2149-56

Chapter 3a

PSEUDORABIES VIRUS gE TRIGGERS THE ERK1/2 SIGNALLING PATHWAY IN JURKAT T CELLS

ABSTRACT

The interaction between viruses and immune cells of the host may lead to modulation of intracellular signalling pathways, and subsequent changes in cellular behavior that may have important consequences for both virus and host. The ERK1/2 (Extracellular signal Regulated-Kinase 1/2) signalling pathway is a key cellular signalling axis that controls many fundamental cellular processes. In this chapter we show that the porcine alphaherpesvirus pseudorabies virus (PRV) activates the ERK1/2 signalling pathway in infected Jurkat T cells. Furthermore, experiments using virus mutants and recombinant proteins show that the glycoprotein E (gE) of PRV plays a key role in PRV-triggered ERK1/2 activation. In summary, we show that PRV induces ERK1/2 activation in Jurkat T-cells, which is mediated by gE, thereby revealing a new function for this viral protein.

INTRODUCTION

Alphaherpesviruses are the largest subfamily of the Herpesviruses. This subfamily contains closely related pathogens, including herpes simplex virus 1 and 2 (HSV-1 and HSV-2) and varicella zoster virus (VZV) in man. Another member of the alphaherpesvirus subfamily is the porcine pseudorabies virus (PRV) that is often used as a model to study general features of alphaherpesvirus biology (Pomeranz et al., 2005).

PRV encodes 11 envelope glycoproteins (Mettenleiter, 2000) that are incorporated in different host membranes of the infected cell, including the plasma membrane. One of these glycoproteins is the glycoprotein E (gE), which is important for virulence and viral (neuronal) spread (Babic et al., 1996; Banfield et al., 1998; Enquist et al., 1994; Kratchmarov et al., 2013; Kritas et al., 1994; Lomniczi et al., 1984; Mettenleiter et al., 1985; Mettenleiter et al., 1987). For both PRV and HSV-1 there are indications that gE may have a signalling function in immune cells, as it drives signalling-dependent processes like cell surface antigen capping (Desplanques et al., 2007; Favoreel et al., 1997; Rizvi and Raghavan, 2003). However, to date, there are no reports that gE indeed triggers any particular signalling pathway.

The ERK1/2 MAPK signalling pathway (ERK for extracellular signal-regulated kinase and MAPK for mitogen-activated protein kinase) is an evolutionary conserved pathway, controlling many fundamental cellular events (Chong et al., 2003; O'Neill and Kolch, 2004; Wellbrock et al., 2004). It may come as no surprise that many viruses, including alphaherpesviruses, modulate the ERK1/2 signalling pathway (Chuluunbaatar et al., 2012; Kang and Banfield, 2010; Liu et al., 2012; Lyman et al., 2006; Schulz et al., 2014).

Several studies have described alphaherpesvirus modulation of ERK1/2 signalling in fibroblasts and/or epithelial cells, but relatively little is known about such modulation in immune cells. PRV has been reported to interact with T lymphocytes (Page et al., 1992; Wang et al., 1988a). ERK1/2 is involved in key aspects of T cell activation, therefore investigating the modulation of this signalling pathway may provide valuable insights to understand certain aspects of immune evasion and the pathogenesis of the virus (Aubert et al., 2009; Naci and Aoudjit, 2014; Sen et al., 2014; Sloan and Jerome, 2007; Sloan et al., 2006; Zerboni et al., 2014a). In this chapter we describe that PRV activates ERK1/2 signalling in the Jurkat T cell line and that PRV gE plays an important role in this process.

These data provide additional insights in the interaction of PRV with T lymphocytes and reveal a new role for gE during PRV infection, which may contribute to our understanding of the complex interaction that alphaherpesviruses like PRV have developed with the host immune system.

MATERIALS AND METHODS

Cells and viruses

The Jurkat T cell line was derived from a patient with acute T-cell leukemia (Gillis and Watson, 1980) and analysis of cell surface markers revealed that these cells express CD7 (pan-T-cell-specific marker, widely expressed from pro-T-cell to mature T-cell), CD2 and CD5 (expressed from early thymocyte to mature thymocyte), and CD3 and CD4 (mature T-cell marker) (Inoue et al., 2014). The cells, clone E6-1, were purchased from the American Type Culture Collection (www.atcc.com) and grown in RPMI 1640 (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Life Technologies), 100 U/mL penicillin and 0.1 mg/mL streptomycin (Life Technologies). PRV wild type (WT, Becker strain) and isogenic mutants, gEnull PRV 91 (PRV ΔgE, (Tirabassi et al., 1997)) and PRV107, lacking the cytoplasmic domain of gE (gEΔcd, (Tirabassi and Enquist, 1999)), were grown and titrated on ST cells, and stored at −80°C. PRV Becker and its isogenic mutant viruses were a kind gift from L. Enquist, Princeton University.

Infection and T cell stimulation

Jurkat T cell infection was performed in 24-well plates. Cells ($1.5x10^6$) were inoculated with either PRV WT or the isogenic mutants PRVΔgE and PRV gEΔcd at a multiplicity of infection (MOI) of 10 for 24 hours (except when mentioned otherwise). For TCR stimulation, mock- or PRV-infected cells were incubated with anti-CD3 antibody (OKT3, 10 µg/ml) for 30 min on ice and subsequently incubated with a secondary antibody (goat anti-mouse IgG) again on ice for 30 min. The cells were then placed at 37 °C for 5 min, and lysed immediately. For T cell stimulation assays using gE recombinant protein, Jurkat T cells ($1x10^6$/ml) were resuspended in medium containing 10 µg/ml of gE recombinant protein (Gut et al., 1999) and incubated for different time points at 37°C. To block gE-triggered stimulation, gE recombinant protein was incubated prior to stimulation with two different antibodies raised against gE, a rabbit polyclonal (1/100) and a mouse monoclonal (1/100) antibody, for 30 min at 37 °C. Both gE recombinant protein and polyclonal antibody against gE were a kind gift of K. Bienkowska-Szewczyk, University of

Gdansk. Monoclonal antibody 5F8 against gE was a kind gift of H. Nauwynck, Ghent University.

Western Blotting

After infection or gE stimulation, cells were washed with PBS, resuspended in ice-cold lysis buffer (TNE buffer, 10% NP40, 1 mM Na_3VO_4, 10 mM NaF, protease inhibitor cocktail) and incubated for 20 min at 4 °C. SDS-PAGE and Western blotting were performed as described previously (Deruelle et al., 2007). Protein concentrations of 20 to 30 µg were used for all experiments. Protein concentration was determined using the bicinchoninic acid (BCA) protein assay reagent (Thermo Scientific) according to the manufacturer's instructions. Blots were blocked in 5% nonfat dry milk in phosphate-buffered saline (PBS)–Tween 20 for 1 h at room temperature and incubated with primary antibodies for 1 h or overnight (according to the manufacturer's instructions). After several washes in 0.1% Tris-buffered saline (TBS)–Tween 20 (TBS-T), blots were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature and developed using enhanced chemiluminescence. Phospho-specific ERK1/2 antibody (1/1000; Cell Signalling) signal was detected with ECL Plus (GE Healthcare). Total ERK1/2 antibody (1/1000; Cell Signalling) signal and viral protein antibody – gE (1/500), US3 (1/500) and gB (1/200) – signals used as loading and infection controls, respectively, were detected with Pierce ECL (Thermo Scientific).

RESULTS

PRV modulates ERK1/2 activation in Jurkat T cells

We first analysed whether PRV affects ERK1/2 signalling in T cells. To this end, Jurkat T cells were used, a cell line widely utilized for signalling and functional studies in T cells (Abraham and Weiss, 2004). Cells were either mock-inoculated or inoculated with PRV wild type (WT) and ERK1/2 phosphorylation was assessed by Western Blot. Figure 1A indicates that at 24 hours post-inoculation (hpi), levels of ERK1/2 phosphorylation were substantially increased in infected Jurkat T cells when compared to mock-infected cells. A time course assay showed that PRV induces ERK1/2 phosphorylation at a relatively late stage of infection, from 12 hpi onwards (Figure 1B), suggesting the potential involvement of late/structural viral proteins. Onset of ERK1/2 phosphorylation coincided with expression of the viral gE protein (Figure 1B). A common method of triggering ERK1/2 signalling in T lymphocytes occurs via the T cell receptor (TCR) (Sloan and Jerome, 2007; Sloan et al., 2006; Walker et al., 1987) and antibody-induced crosslinking of the CD3 T cell co-receptor complex typically results in robust ERK1/2 signalling (Koike et al., 2003; Poltorak et al., 2013). To investigate whether CD3-mediated ERK1/2 activation is affected in PRV-infected cells, CD3 was crosslinked on mock- or PRV-infected Jurkat T cells (at 24 hpi) and ERK1/2 activation was assessed. Interestingly, in contrast to mock-infected cells, stimulating PRV-inoculated Jurkat T cells with an anti-CD3 antibody did not trigger ERK1/2 activation (Figure 1C). These data indicate that PRV triggers ERK1/2 activation during infection of Jurkat T cells and at the same time interferes with CD3-mediated activation of ERK1/2.

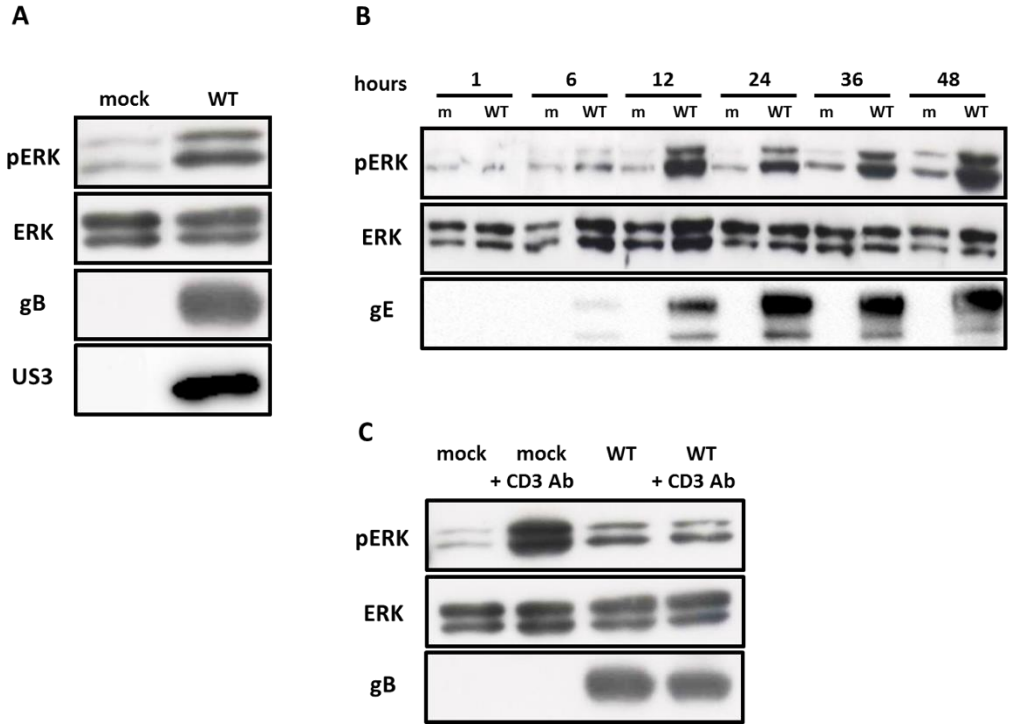

Figure 1. PRV infection induces ERK1/2 phosphorylation in Jurkat T cells. **(A)** Cells were either mock-inoculated or inoculated with PRV WT (MOI 10) and lysed at 24 hpi. ERK1/2 activation was detected via Western Blot using a phospho ERK1/2-specific antibody. Loading and infection controls were performed by detecting for total ERK1/2 and viral proteins gB and US3, respectively. **(B)** A time course of infection was performed, where cells were either mock-inoculated or inoculated with PRV WT (MOI 10) and lysed at different time points post inoculation. Phospho and total ERK1/2 and viral protein gE were detected. **(C)** At 24 hpi, mock- or PRV-infected cells were left untreated or stimulated with an anti-CD3 antibody for 5 min to induce TCR-dependent ERK1/2 activation. m: mock.

Glycoprotein E is required for PRV-induced ERK1/2 activation in Jurkat T cells

Alphaherpesvirus gE is involved in viral cell-cell spread (Babic et al., 1996; Banfield et al., 1998; Johnson et al., 2001b) as well as other functions. It has been suggested that gE of different alphaherpesviruses may affect cell signalling, although gE-mediated modulation of a particular signalling axis has not been demonstrated before (Desplanques et al., 2007; Favoreel et al., 1997; Favoreel et al., 1999; Favoreel et al., 2004; Olson et al., 1997; Riteau et al., 2006; Rizvi and Raghavan, 2003). To investigate if gE is involved in PRV-induced ERK1/2 activation, Jurkat T cells were inoculated with either PRV WT or an isogenic PRV mutant that lacks gE expression (PRVΔgE). ERK1/2 phosphorylation was assessed at 24 hpi (Figure 2A). Contrary to PRV WT, infection with PRVΔgE did not cause ERK1/2 phosphorylation, indicating that gE is required for PRV-mediated ERK1/2 activation. The cytoplasmic domain of PRV gE contains potential

signalling domains, i.e. tyrosine-based YXXΦ motifs (Desplanques et al., 2007). We used another isogenic PRV mutant, lacking the cytoplasmic domain of gE (PRV gEΔcd), to study the involvement of putative cytoplasmic signalling domains in ERK1/2 activation (Figure 2B). Jurkat T cells were inoculated with PRV WT, PRVΔgE or PRV gEΔcd, and ERK1/2 phosphorylation was detected at 24 hpi. The absence of the cytoplasmic domain of gE did not affect PRV-mediated ERK1/2 activation, as ERK1/2 phosphorylation levels were similar when cells were infected with either PRV gEΔcd or PRV WT. These results show that ERK1/2 activation upon PRV infection depends on gE, but does not require the cytoplasmic domain of gE.

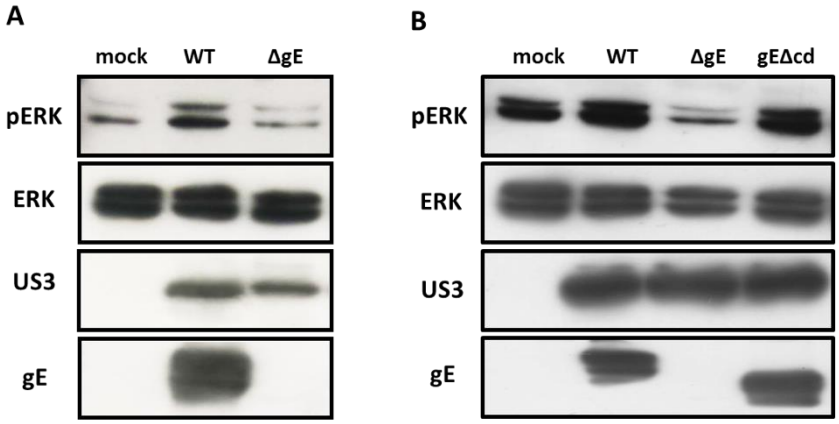

Figure 2. The gE glycoprotein is required for PRV-induced ERK1/2 phosphorylation in Jurkat T cells. **(A-B)** Cells were mock-inoculated or inoculated with PRV WT **(A, B)**, PRV ΔgE **(A)** or PRVgEΔcd **(B)** (MOI 10) and lysed at 24 hpi. ERK1/2 activation was detected via Western Blot using a phospho ERK1/2-specific Ab. Loading and infection controls were performed by detecting for total ERK1/2 and US3, respectively. Glycoprotein gE was also detected to confirm absence of the entire protein or the cytoplasmic domain.

Addition of recombinant gE to Jurkat T cells leads to rapid and transient ERK1/2 activation

To further confirm that gE affects ERK1/2 phosphorylation through its extracellular domain, Jurkat T cells were incubated with recombinant gE protein for several time points. Already at 2 minutes post incubation there was a clear increase in ERK1/2 phosphorylation (Figure 3A) that quickly decreased, reaching background levels at 5 minutes post incubation. Pre-incubation of recombinant gE with gE-specific antibodies prior to Jurkat T cells stimulation abrogated its ability to induce ERK1/2 phosphorylation, confirming that the effect is gE-dependent (Figure 3B). Thus, binding of PRV gE to Jurkat T cells causes a rapid and transient activation of ERK1/2.

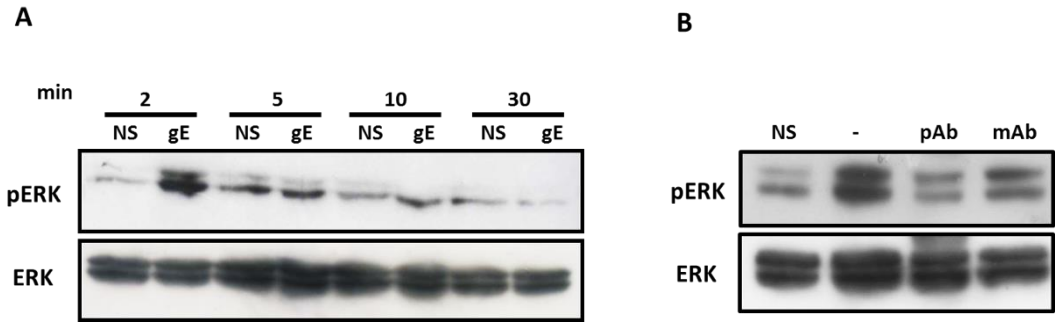

Figure 3. Addition of gE recombinant to Jurkat T cells results in rapid and transient ERK1/2 phosphorylation. **(A)** Cells were stimulated for different time points with recombinant gE (10 µg/ml), lysed and ERK1/2 phosphorylation was detected. **(B)** Recombinant gE was pre-incubated for 30 min with two blocking antibodies (mAb and pAb, 1/100) raised against gE, prior to stimulation of Jurkat T cells for 2 min at 37 °C. NS, non-stimulated Jurkat T cells.

DISCUSSION

Interaction between alphaherpesviruses and ERK1/2 signalling has been studied extensively over the past years (Chuluunbaatar et al., 2012; Kang and Banfield, 2010; Liu et al., 2012; Lyman et al., 2006; Schulz et al., 2014; Sloan and Jerome, 2007). This signalling axis controls various fundamental cellular events, making it an attractive target for the virus to subvert the host, as it has the potential to promote viral replication and survival of infected cells.

The current data show that PRV activates the ERK1/2 signalling pathway in infected Jurkat T cells. In addition, the virus appears to actively regulate this signalling cascade, since at the same time PRV interfered with ERK1/2 activation via classical T cell stimulation through CD3. Regulation or modulation of the ERK1/2 signalling pathway in T cells has been reported before for alphaherpesviruses. Similarly to what we observed for PRV, HSV-1 seems to "hijack" the signalling cascade, inhibiting T cell receptor (TCR)-induced ERK1/2 activation (Sloan and Jerome, 2007; Sloan et al., 2006). The T cell-tropic VZV modulates TCR-dependent signalling by triggering the depletion of CD3 (Sen et al., 2014).

PRV-induced ERK1/2 activation occurred during later stages of virus infection, suggesting the involvement of late/structural viral proteins. This was confirmed by our finding that ERK1/2 phosphorylation is triggered by the glycoprotein E (gE). Moreover, infection with the virus mutant PRV gEΔcd and incubation with a gE recombinant protein provided evidence that ERK1/2 activation is mediated by the extracellular domain of gE. One possible interpretation of these results is that gE may bind to an unidentified cellular receptor on the surface of T lymphocytes, thereby triggering ERK1/2 activation. This way of ERK1/2 activation has been reported before for HIV-1, where extracellular binding of gp120 recombinant protein to its receptor CXCR4 transiently activates ERK1/2 in T cells (Popik et al., 1998). A cellular interaction partner for the extracellular domain of gE has only been reported for VZV (insulin-degrading enzyme), but not for any of the other alphaherpesviruses, including PRV (Li et al., 2006).

We noticed that addition of recombinant gE triggered transient ERK1/2 activation, whereas gE-dependent ERK1/2 signalling during productive infection of Jurkat T cells appeared to be continuous for several hours. We propose that differences in transient

versus continuous ERK1/2 activation can possibly be explained by dynamic cell-cell interactions and gE availability. T cell interactions, either homotypic or with antigen presenting cells, can be transient, allowing the T cells to establish contact with different cells during a given time period (Hugues et al., 2004; Sabatos et al., 2008). In infected cells, there is an ongoing expression of gE on the plasma membrane, and dynamic interactions between cells may cause gE to bind (and detach) repeatedly to its possible receptor, resulting in almost continuous repetitive, rather than truly sustained, ERK1/2 activation. On the other hand, binding of recombinant gE led to a rapid but transient activation, possibly due to the non-dynamic nature of the interaction. Although speculative, upon binding, gE recombinant may remain attached, preventing binding of soluble gE still present in the medium.

It would be interesting to study if the novel function of gE reported here for PRV is conserved in other alphaherpesviruses. In HSV-infected Jurkat T cells, there are indications that gE may take part in the inhibition of TCR-dependent cell activation (measured by calcium flux). However, a direct correlation between gE and ERK1/2 activity was not assessed (Aubert et al., 2009; Sloan and Jerome, 2007; Sloan et al., 2006). During PRV infection, we could observe virus-induced modulation of TCR-mediated ERK1/2 activation, although gE appeared not to be involved in this event (data not shown). Future research may clarify whether the observed differences are virus-dependent or reflect differences in experimental setup. In any case, previous data on PRV and HSV together with our current data provide strong evidence for an involvement of alphaherpesvirus gE with the cell signalling machinery. Further studies would help to elucidate if these signalling features of gE can be observed in primary T lymphocytes or other cell types. Together, our results shed light on a potential new function for gE during PRV infection, possibly taking part in the virus-immune system crosstalk.

Chapter 3b

PRV gE TRIGGERS ERK1/2-DEPENDENT CELL AGGREGATION AND MIGRATION OF PORCINE PRIMARY T LYMPHOCYTES

ABSTRACT

Herpesviruses are known to be highly successful in evading the immune system of their hosts, subverting cellular signalling cascades to their own advantage. The ERK1/2 signalling pathway, being involved in many cellular processes, represents a particularly attractive target for viral manipulation. Pseudorabies virus, a member of the *Alphaherpesvirinae* subfamily, has been reported previously to interfere with this signalling pathway in different cell types. We have shown in the previous chapter that PRV activates ERK1/2 in infected Jurkat T cells and that this is mediated by the extracellular domain of the glycoprotein E (gE). Here, we report that gE triggers ERK1/2 phosphorylation in porcine primary T lymphocytes, leading to cellular aggregation and increased migration of these cells, two hallmarks of T cell activation. This study further unravels the contribution of gE in the crosstalk between PRV and T lymphocytes, and points to a novel mechanism how gE can contribute to virus spread.

INTRODUCTION

Members of the large *Herpesviridae* family have been highly successful in manipulating the immune system, i.e. by perturbing recognition of virus infected cells by virus-specific T cells and consequent T cell activation (Adhikary et al., 2006; Wiertz et al., 2007; Zerboni et al., 2014b). In addition, some of these viruses have acquired the ability to use immune cells as a vehicle for viral dissemination, which may contribute importantly to viral pathogenesis (Arvin et al., 2010; Gryspeerdt et al., 2010; Laval et al., 2015; Nauwynck and Pensaert, 1992; Nauwynck and Pensaert, 1995b; Smith et al., 2004). The interaction between herpesviruses and immune cells of the host may lead to modulation of specific intracellular signalling pathways, and subsequent changes in cellular behavior that may be of benefit for the virus.

The ERK1/2 signalling cascade controls many fundamental cellular events, representing a very attractive target for viruses (Chong et al., 2003; Hindley and Kolch, 2002; Naci and Aoudjit, 2014; O'Neill and Kolch, 2004; Wellbrock et al., 2004). Investigating ERK1/2 modulation in T lymphocytes may be of particular interest as this signalling pathway is involved in T cell activation, aggregation and motility (Khunkaewla et al., 2008; Layseca-Espinosa et al., 2003; Naci and Aoudjit, 2014; Sloan and Jerome, 2007) and since T lymphocytes may be involved in virus spread and transmission of some alphaherpesviruses. The latter is particularly evident for the *Varicellovirus* VZV, whose tropism for T cells contributes to several central aspects of its pathogenesis, including viral dissemination in the body, transmission to skin cells and spread to new hosts (Arvin et al., 2010; Sen et al., 2014; Zerboni et al., 2014b). Other members of the *Varicellovirus* genus like PRV have also been reported to interact with T lymphocytes (Page et al., 1992; Wang et al., 1988a).

In the previous chapter, we have demonstrated that PRV activates the ERK1/2 signalling pathway in the Jurkat T cell line, a process mediated by the viral virulence factor glycoprotein E (gE). In the present chapter, we report that PRV gE-induced ERK1/2 activation also occurs in primary porcine T lymphocytes and that, *in vitro*, this signalling leads to T lymphocyte aggregation and migration, and that migratory T lymphocytes may transfer the virus to susceptible host cells. These data provide additional insights in the interaction of PRV with T lymphocytes and reveal a new role for gE during PRV infection,

suggesting that PRV/gE-induced ERK1/2 activation may trigger PRV-carrying T lymphocytes to migrate and infect other cells that are susceptible to PRV replication.

MATERIALS AND METHODS

Cells and viruses

Porcine peripheral blood mononuclear cells (PBMC) were isolated from whole blood obtained from healthy pigs from 6 to 15 weeks using Lymphoprep™ density centrifugation. Porcine primary CD6+ T lymphocytes were isolated as described before (Devriendt et al., 2013). Cells were enriched from PBMC to a purity of >95% by positive immunomagnetic selection with an α-CD6 mAb (IgG_1, clone a38b2 (Saalmüller et al., 1994)). A percentage of 39–76% of peripheral blood T cells express CD6. From this population the following subsets have been characterized: $CD4^+CD8^+$ double positive T lymphocytes, $CD4^+CD8^-$ T helper cells and $CD4^-CD8^{high}$ T cytolytic cells. $CD4^-CD8^{low}$ and $CD4^-CD8^-$ are majorly devoid of CD6, and a small percentage shows very low CD6 density. CD6 is not expressed by B cells or by cells of the myeloid lineage (Pauly et al., 1996; Piriou-Guzylack and Salmon, 2008). CD6+ T lymphocytes were cultured overnight prior to stimulation in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 0.1 mg/mL streptomycin and 0.2 mM HEPES (Life Technologies). Swine Testicle (ST) cells were grown in Eagle's minimal essential medium (MEM, Life Technologies) supplemented with 10% FBS, glutamine (0.3 mg/ml), and antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin). PRV wild type (WT, Becker strain) and isogenic mutant gEnull PRV 91 (PRV ΔgE, (Tirabassi et al., 1997)) were grown and titrated on ST cells, and stored at −80°C. PRV Becker and PRV91 (PRV ΔgE) were a kind gift of L. Enquist, Princeton University.

Transfection and co-cultures

ST cells were transfected according to the manufacturer's guidelines (Jet Prime®, Polyplus Transfection™) with a plasmid encoding wild type gE (pIB2, kind gift of Lynn Enquist, (Tirabassi and Enquist, 1998)) for 48 hours. As a control, cells were transfected with either empty vector or a GFP-expressing plasmid (pcDNA-GFP, made in house). Rested T lymphocytes (2.5 $x10^6$ cells per well) were added to transfected ST cells and co-cultured for different time periods. After co-culture, T lymphocytes were gently collected for analysis via SDS-PAGE and Western blot. To guarantee that the majority of

T lymphocytes would be collected, the wells were washed carefully with PBS, assuring that ST cells remained adherent.

To determine transmission of infection from T cells to ST cells, primary porcine T lymphocytes were inoculated with PRV WT for 24 hours, treated with citrate buffer for 1 min (40 mM Na citrate, 10 mM KCl, 135 mM NaCl, pH 3.0), washed 3 times with PBS-1%FBS, and co-cultured with ST cells for another 24 hours. In some experiments, virus-neutralizing anti-PRV polyclonal antibodies were added prior and during co-culture, neutralizing possibly remaining virus attached to the T lymphocyte cell surface. ST cells were then fixed with 3% paraformaldehyde and stained with an anti-PRV FITC conjugated antibody.

Western Blotting

Cells were washed with PBS, resuspended in ice-cold lysis buffer (TNE buffer, 10% NP40, 1mM Na_3VO_4, 10 mM NaF, protease inhibitor cocktail) and incubated for 20 min at 4 °C. SDS-PAGE and Western blotting were performed as described previously (Deruelle et al., 2007). Protein concentrations of 20 to 30 µg were used for all experiments. Protein concentration was determined using the bicinchoninic acid (BCA) protein assay reagent (Thermo Scientific) according to the manufacturer's instructions. Blots were blocked in 5% nonfat dry milk in phosphate-buffered saline (PBS)–Tween 20 for 1 h at room temperature and incubated with primary antibodies for 1 h or overnight (according to the manufacturer's instructions). After several washes in 0.1% Tris-buffered saline (TBS)–Tween 20 (TBS-T), blots were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature and developed using enhanced chemiluminescence. Phospho-specific ERK1/2 antibody (1/1000; Cell Signalling) signal was detected with ECL Plus (GE Healthcare). Total ERK1/2 antibody (1/1000; Cell Signalling) signal was detected with Pierce ECL (Thermo Scientific).

Homotypic T cell aggregation assay

Primary T lymphocytes ($0.5x10^6$) were placed in a 96-well plate and inoculated with either PRV WT or PRV ΔgE (MOI 100). When assessing the involvement of ERK1/2 signalling, the ERK1/2 phosphorylation inhibitor U0126 (10 µM, Cell Signalling) was

added to the T lymphocytes 30 minutes prior to inoculation with the virus and at 2 hours post inoculation (hpi). U0126 was dissolved in DMSO at 10 mM and diluted 1,000 x in medium to reach the working concentration. As a control, the same experiments were performed in the presence of the corresponding dilution of DMSO, and cell viability was tested for both the inhibitor and the solvent. At 24 hpi, 20 randomly selected fields per condition were observed under the microscope and pictures were taken. Cell aggregates were counted, and their surface area was measured using ImageJ 1.48. Background aggregation levels observed in mock-infected cells were used as threshold to distinguish small from large aggregates. This threshold was determined as the surface area at which ≤2% of the identified aggregates in mock-infected samples had a larger surface area. For the other conditions, aggregates with a surface area above this threshold value were considered as large aggregates.

Transwell® migration assay

Primary T lymphocytes were inoculated with either PRV WT or PRV ΔgE (MOI 100). After 24 hours of virus inoculation, primary T lymphocytes ($0.5x10^6$) were placed onto a 5 µm Corning® Transwell® (24-well plate, Sigma-Aldrich) and incubated further to allow migration. To determine levels of migration, a collagen-coated coverslip was placed at the bottom of each well, so that migrating cells would be able to attach to the coverslip. At 24 hours post incubation, Transwells were removed and the adherent cells were counted. When assessing the involvement of ERK1/2 signalling, U0126 was added to the T lymphocytes 30 minutes prior to virus inoculation and at 2 hpi. As a control, the same experiments were performed in the presence of DMSO-based U0126 diluent.

Statistics

Statistical analysis was performed on GraphPad Prism 5 (GraphPad Software, Inc). Data sets (n=3) were analysed using One-way ANOVA ($p<0.05$) combined with Tukey's Multiple Comparison Test (95% confidence interval).

RESULTS

Providing gE in trans *leads to ERK1/2 activation in primary porcine T lymphocytes*

In the previous chapter we found that PRV gE activates ERK1/2 in the Jurkat T cell line, a widely used cell line for signalling and functional studies in T cells. To investigate whether PRV gE also leads to ERK1/2 activation in primary T cells of its natural host, in a first experimental assay, primary porcine T lymphocytes were incubated with recombinant gE for different time points (Figure 1A). Similar to our observations in Jurkat T cells, recombinant gE led to a fast increase in ERK1/2 phosphorylation, decreasing back to basal levels at 30 minutes. Since ERK1/2 activation appeared slightly less pronounced in primary porcine T lymphocytes compared to Jurkat T cells, a second experimental assay was used to confirm these findings. ST cells were transfected with a gE-expressing plasmid or, as control, empty vector or a GFP-expressing plasmid. At 48h post transfection, T lymphocytes were co-cultured with transfected ST cells for different time points. T lymphocytes incubated with gE-expressing ST cells showed an increased ERK1/2 activation already at 5 minutes post co-cultivation, followed by a slow decrease to almost basal levels at 30 minutes post co-incubation, which was not observed in T lymphocytes incubated with control plasmid-transfected ST cells (Figure 1B) or empty vector-transfected ST cells (data not shown). To ensure that the observed ERK1/2 phosphorylation was not derived from co-cultured ST cells that might have detached from the bottom of the well during T lymphocyte collection, the same assay was performed without addition of T lymphocytes. Neither total ERK1/2 nor phosphorylated ERK1/2 could be detected (data not shown). Finally, in a third experimental assay, primary porcine T lymphocytes were brought into contact with PRV WT or PRV ΔgE (Figure 1C). PRV WT induced rapid and substantial ERK1/2 phosphorylation, whereas PRV ΔgE led to a less prominent ERK1/2 phosphorylation compared to WT. Hence, also in primary porcine T lymphocytes, PRV gE triggers ERK1/2 phosphorylation.

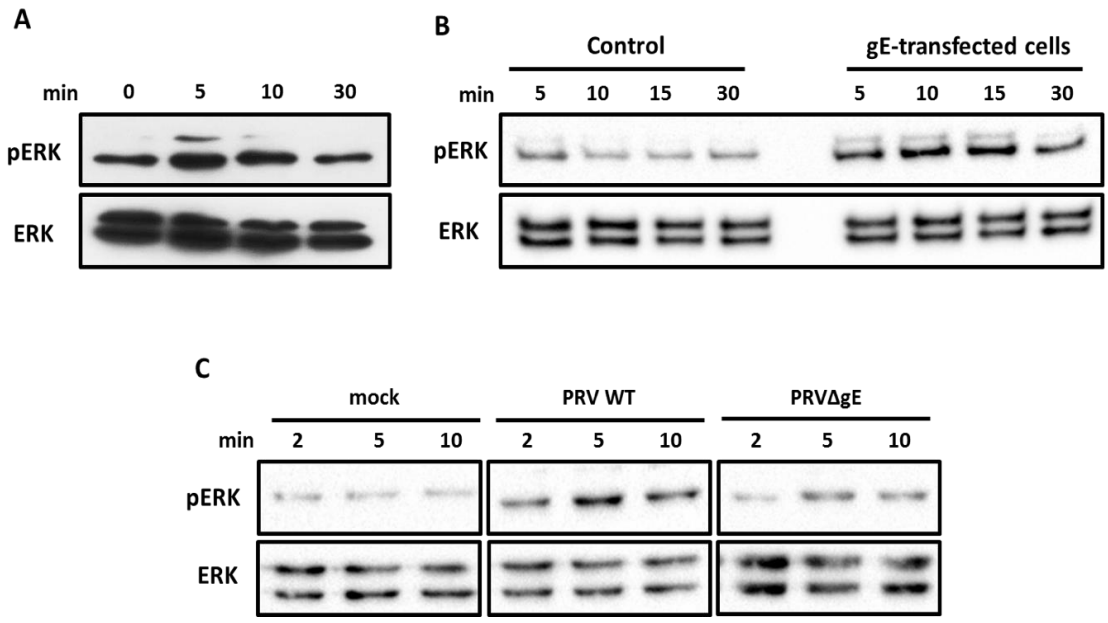

Figure 1. Providing gE *in trans* triggers ERK1/2 phosphorylation in primary porcine T lymphocytes. **(A)** Primary porcine T lymphocytes were stimulated with gE recombinant (100 µg/ml) for different time points, lysed and ERK1/2 phosphorylation was detected via Western Blot. **(B)** ST cells were transfected with either a control or a gE-encoding plasmid and incubated for 48 hours. The first four lanes (left) correspond to primary T lymphocytes co-cultured with control-transfected ST cells; the last four lanes (right) correspond to T lymphocytes co-cultured with gE-transfected ST cells. After co-incubation with transfected ST cells for different time points, primary T lymphocytes were collected and lysed. ERK1/2 phosphorylation was assessed via Western Blot. **(C)** Primary T lymphocytes were inoculated with either PRV WT or PRV ΔgE for different time points, collected and lysed. Figures are representative of three independent biological replicates (primary T lymphocytes were isolated from 3 different porcine blood donors).

Primary T lymphocytes can transmit PRV to susceptible cells

To be able to determine possible biological consequences of gE-mediated ERK1/2 phosphorylation in T lymphocytes, we first investigated whether PRV WT and PRVΔgE showed obvious differences in their ability to productively infect primary T lymphocytes. It has been described previously that the percentage of T lymphocytes productively infected by PRV is very low, both *in vivo* and *in vitro* (Nauwynck and Pensaert, 1994; Wang et al., 1988b). In line with this observation, using immunofluorescence to detect different viral antigens, we did not observe obvious productive infection in T lymphocytes upon PRV inoculation, either with PRV WT or PRVΔgE (data not shown). Nonetheless, despite the absence of obvious productive infection, it has been demonstrated that T lymphocytes isolated from PRV-inoculated pigs carry infectious virus and can transmit infection to other cells (Page et al., 1992). To corroborate this, primary T lymphocytes were inoculated with PRV WT or PRVΔgE for 24 hours. After citrate buffer (CB) treatment to remove extracellular infectious virus, T lymphocytes were co-cultivated with ST cells. At 24 hours post co-culture, the formation of plaques was evident, indicative for direct spread of virus from T lymphocytes to ST cells (Figure 2A). In support of direct intercellular spread of the virus, addition of PRV-neutralizing antibodies to CB-treated T lymphocytes did not prevent plaque formation in the co-cultures (Figure 2B). In agreement with the established role of gE in cell-associated spread in cell cultures, plaque sizes in ST cells were smaller using PRVΔgE compared to PRV WT, but the number of plaques was similar for both viruses (Figure 2C). Hence, confirming earlier reports, although PRV does not lead to obvious productive infection in primary porcine T lymphocytes, PRV-inoculated T cells can transmit infection to susceptible cells. Despite the well-established role of gE in cell-to-cell spread, this glycoprotein appears not to play a substantial role in transmission of infection from T lymphocytes to susceptible cells.

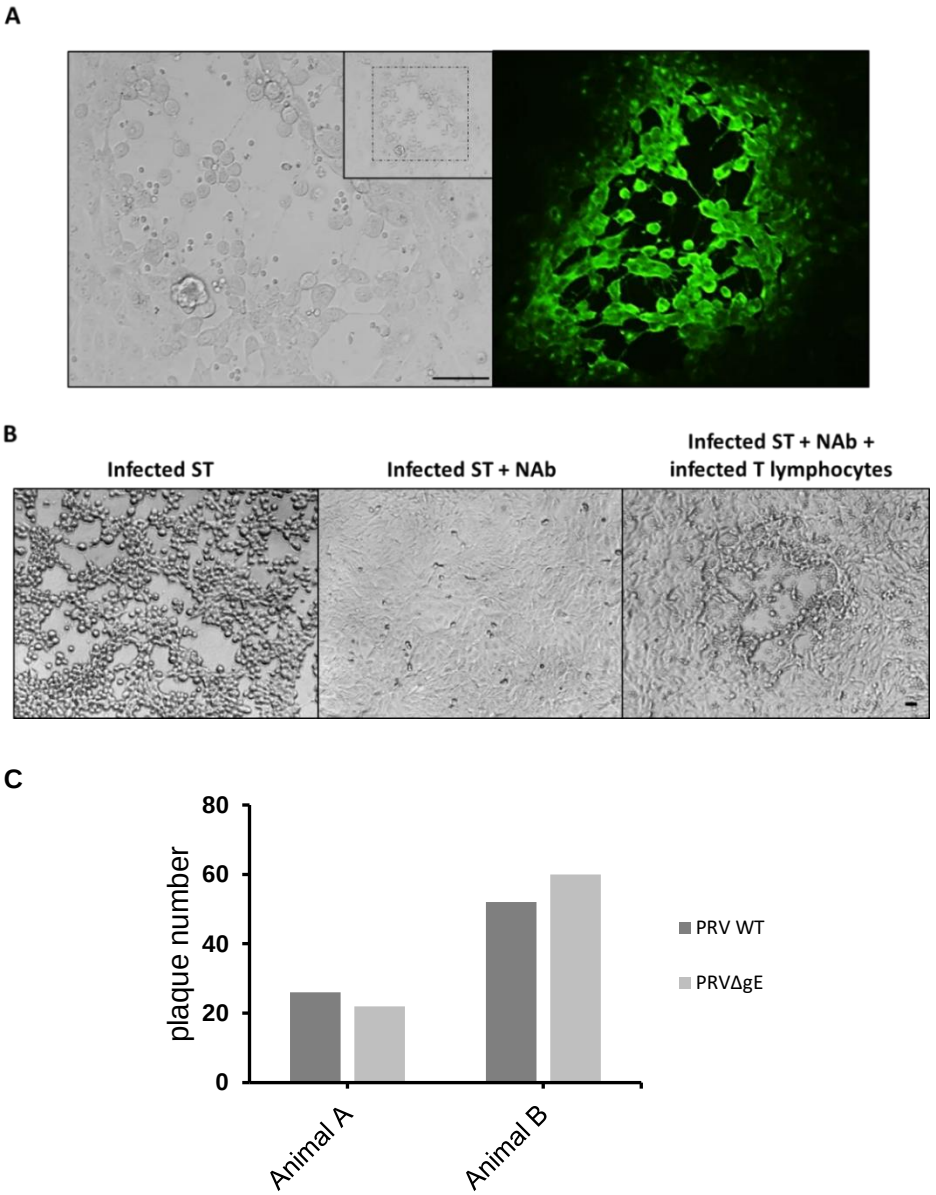

Figure 2. Primary T lymphocytes transmit PRV to susceptible cells. **(A)** Primary T lymphocytes were inoculated with PRV WT for 24 hours, treated with citrate buffer and co-cultured with ST cells for another 24 hours. After co-culture, ST cells were either observed directly by light microscopy (left) or were fixed, permeabilized, stained for PRV antigens and observed by fluorescence microscopy (right). Typical PRV plaques in ST cells can be observed. **(B)** Primary T lymphocytes transmit PRV to susceptible cells in the presence of PRV-neutralizing antibodies. ST cells were inoculated directly with PRV in the absence (left) or presence (center) of neutralizing polyclonal anti-PRV antibodies for 24 hours, confirming the neutralizing capacity of the polyclonal antibodies. Primary T lymphocytes were inoculated with PRV WT for 24 hours, treated with citrate buffer and co-cultured with ST cells (0.5 lymphocyte/ST cell ratio) for another 24 hours in the presence of neutralizing antibodies (right). Size bar = 100 µm. **(C)** Although gE is necessary for cell-cell virus spread, our experiments showed no significant differences between PRV WT and PRVΔgE infection when inoculated T-lymphocytes were co-cultivated with ST cells, with both viruses causing a similar amount of plaques. However, in agreement to previous observations, infection with PRVΔgE yielded smaller plaques in comparison to PRV WT infection.

PRV-mediated ERK1/2 signalling in primary T cells leads to cell aggregation and migration

Since gE appears not to affect productive infection in T cells or virus transmission from T cells to susceptible cells, we investigated whether the ability of PRV gE to trigger ERK1/2 activation would have any biological consequences on T lymphocyte behavior. One of the biological consequences of ERK1/2 signalling in T cells is homotypic cell aggregation, which is used as readout for T cell activation, adhesion and migration (Layseca-Espinosa et al., 2003). T cell aggregation was therefore assessed in primary porcine T lymphocytes that were exposed to either PRV WT or PRVΔgE. Aggregation was analysed at 24 hpi, and identified aggregates were categorized into small or large aggregates. When compared to mock-infected cells, PRV WT caused a significant increase in the formation of large aggregates (from 2% to 23%, respectively) (Figure 3), whereas PRVΔgE caused significantly less cell aggregates (11%). The ERK1/2 phosphorylation inhibitor U0126 strongly reduced PRV-induced aggregation close to background levels (3% of large aggregates). Addition of the U0126 diluent did not visibly affect cell aggregate formation (data not shown). These data confirm that T cell aggregation triggered by PRV depends on ERK1/2 signalling and to a large extent on gE.

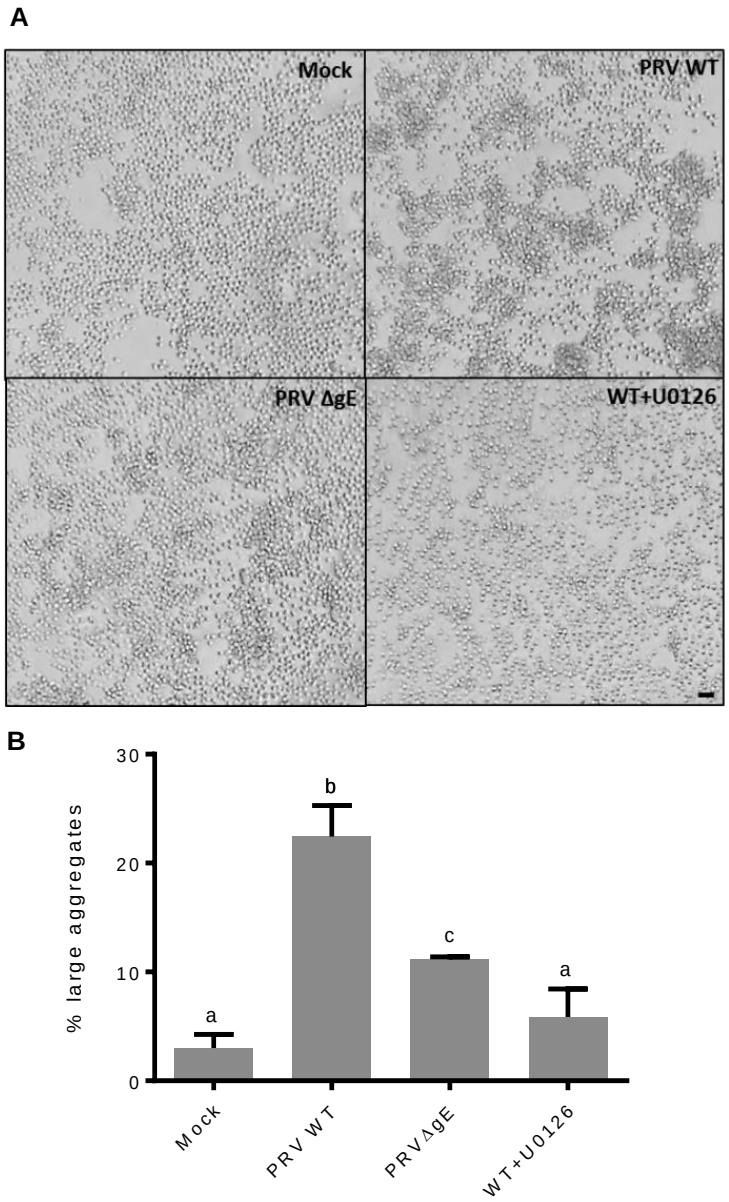

Figure 3. PRV-induced ERK1/2 activation leads to T cell aggregation. Primary T lymphocytes were inoculated with either PRV WT (in the absence or presence of 10 µM ERK1/2 inhibitor U0126) or PRV ΔgE for 24 hours. At 24 hpi, cells were analysed by microscopy and pictures were taken. Representative images are shown in **(A)**. **(B)** Twenty random fields per condition were analysed, aggregates were measured and classified as large based on their surface area (see Materials and Methods). Plot depicts the average percentage (± SD, three independent biological replicates) of large aggregates in each condition. Different letters classify datasets to compare statistically significant differences ($p<0.05$). Significance levels: a-b: ****, b-c: ***; a-c: **. Size bar = 100 µm.

T cell aggregation is often associated with increased cellular motility (Jevnikar et al., 2008; Pike et al., 2011). We therefore assessed whether PRV gE triggers T cell migration. To this end, T lymphocytes were inoculated with either PRV WT or PRV ΔgE and subsequently placed in a Transwell® system with a collagen-coated coverslip in the lower chamber, and incubated further for 24 hours (Figure 4A). Afterwards, cells that

migrated through the Transwell® system onto the collagen-coated coverslip were counted. In line with what we observed for T cell aggregation, cell migration was significantly increased in PRV-inoculated T lymphocytes compared to mock-inoculated cells (Figure 4B). PRVΔgE led to a consistent trend of decreased T cell migration compared to PRV WT, though not statistically significant (p value = 0.077). In line with what we observed for cell aggregation, addition of U0126 reduced migration of PRV-inoculated T lymphocytes to the level seen in mock-inoculated cells. As a control, addition of the DMSO-based U0126 diluent did not affect cell migration (Figure 4C). To determine whether migrating PRV-inoculated T lymphocytes may transmit the virus to other cells, PRV-inoculated T lymphocytes that migrated to the bottom chamber of the Transwell® system were treated with citrate buffer to remove extracellular virus. Subsequent co-cultivation with ST cells resulted in obvious infection of the ST cells, indicating that migrating PRV-inoculated T lymphocytes can transmit infection to susceptible cells (Figure 4D). Altogether, these results show that PRV triggers aggregation and migration of primary porcine T lymphocytes *in vitro*, and that these effects are ERK1/2-signalling dependent and partially mediated by gE.

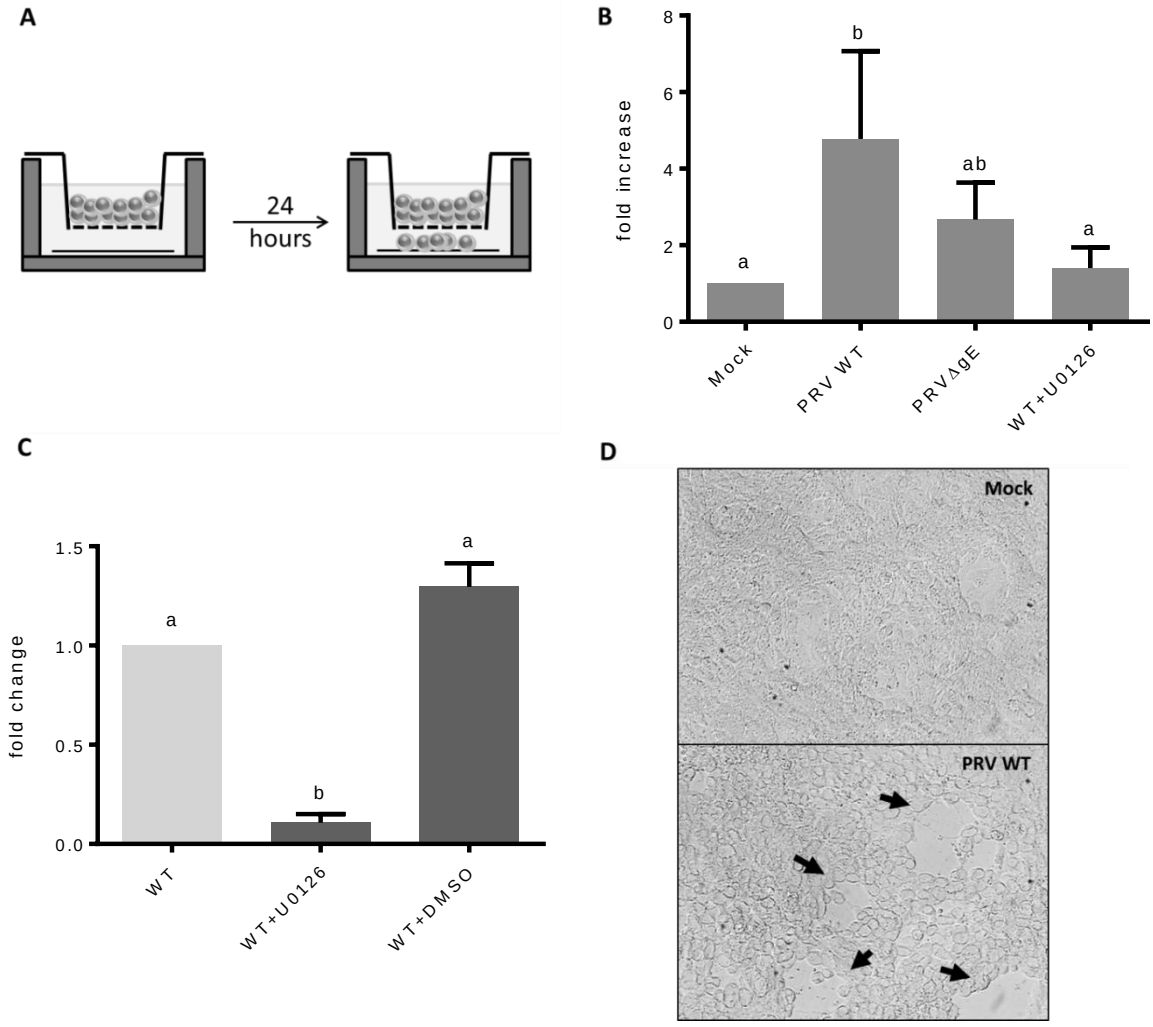

Figure 4. PRV-induced ERK1/2 activation leads to increased motility of T lymphocytes. Primary T lymphocytes were inoculated with either PRV WT (in the absence or presence of 10 µM U0126) or PRV ΔgE for 24 hours. **(A)** Afterwards, cells were placed onto a Transwell® and incubated 24 hours further to allow migration of the cells to the lower chamber, followed by microscopic quantification of migrated cells. **(B)** Plot shows the fold increase (mean ± SD, three independent biological replicates) in the number of migrated cells for the different conditions compared to mock-inoculated cells. Different letters classify datasets to compare statistically significant differences ($p<0.05$). Significance levels: a-b: *. 'ab' represents a set of data, of which, when compared to either data set 'a' or data set 'b', the statistical p-value is greater than 0.05. **(C)** Migration of PRV-inoculated primary T lymphocytes in the presence of 10 µM U0126 or DMSO-based diluent. Primary porcine T lymphocytes, at 24 hpi with PRV WT, were placed on a Transwell® and cultivated further for 24 hours to allow cell migration. Migratory cells were collected on a collagen-coated glass insert placed on the bottom of the well (see Materials and Methods and Figure 7A). Plot represents mean + SD of three independent biological replicates. Different letters classify datasets to compare statistically significant differences ($p<0.05$). Significance levels: a-b: *** **(D)** T lymphocytes that had migrated to the lower chamber were treated with citrate buffer and incubated for 24 hours with ST cells. Cells were analysed by light microscopy and showed numerous PRV plaques (arrowheads). Size bar= 100 µm.

DISCUSSION

In the previous chapter, we demonstrated that PRV gE has the previously uncharacterized ability to trigger ERK1/2 phosphorylation in the Jurkat T cell line. The present study shows that PRV activates the ERK1/2 signalling pathway in porcine T lymphocytes and gE again contributes to this ERK1/2 activation. Moreover, PRV-mediated ERK1/2 activation in primary porcine T lymphocytes results in T cell aggregation and migration, which is in part mediated by gE.

Primary porcine T lymphocytes have been reported before to display limited susceptibility to productive PRV infection *in vitro* and *in vivo* (Nauwynck and Pensaert, 1994; Wang et al., 1988b). Still, co-cultivation of T lymphocytes isolated from PRV infected pigs with highly susceptible cells resulted in the formation of plaques, indicating that T lymphocytes may function as carrier cells to transmit virus to susceptible cells (Page et al., 1992). We confirmed these earlier findings *in vitro* in primary T lymphocytes. In our assays, no differences in transmission efficiency of virus from T lymphocytes to ST cells were observed between PRV WT and ΔgE inoculations, suggesting that gE (and gE-mediated ERK1/2 signalling) does not substantially contribute to this process. Despite this, gE and ERK1/2 signalling were found to significantly affect T lymphocyte behavior.

Homotypic T cell aggregation correlates with T cell activation. Formation of T cell aggregates upon contact with either infected cells or virus has been described before (Takahashi et al., 2002; van Velzen et al., 2013). PRV WT caused a strong increase in T lymphocyte aggregation, whereas PRV ΔgE was significantly impaired in triggering cell aggregation. As others before, we observed that T cell aggregation depends on ERK1/2 signalling (Guo et al., 2014; Layseca-Espinosa et al., 2003), as the addition of an inhibitor of ERK1/2 signalling abrogated the formation of large aggregates. T cell aggregation has been described to correlate with T cell migration (Jevnikar et al., 2008; Pike et al., 2011). Other viruses, like Human T-lymphotropic Virus 1 (HTLV-1) and the betaherpesvirus Human Cytomegalovirus (HCMV), trigger migration of virus-inoculated leukocytes (Chevalier et al., 2014; Smith et al., 2004). In the current report, PRV inoculation also resulted in an increased migration of T lymphocytes. In line with our results on T cell aggregation, gE contributed to some extent to PRV-induced migration of T lymphocytes and inhibition of ERK1/2 signalling abrogated migration. Nevertheless, residual ERK1/2

signalling, T cell aggregation and T cell migration could be observed with PRVΔgE. This may indicate that additional viral proteins may be involved in these processes. Interestingly, other viral proteins of PRV, like US2 and UL46, have been reported to modulate ERK1/2 signalling in other cell types (Lyman et al., 2006; Kang and Banfield, 2010; Schulz et al., 2014; Nogalski et al., 2013). Future research will show whether these or other viral proteins may also be involved in the ERK1/2-dependent processes that we describe here. It will also be important to consider the involvement of gI, which forms a hetero-dimer with gE (Whealy et al., 1993; Zuckermann et al., 1988). The gE-gI complex mainly acts as one functional entity, although gI is able to reach the cell surface in the absence of gE and vice versa (Whealy et al., 1993). Although our data using recombinant gE and gE-transfected cells indicate that gE, in the absence of gI, can trigger ERK1/2 phosphorylation, it will be interesting to determine whether complex formation with gI affects the efficiency of ERK1/2 phosphorylation and whether gI on itself may possibly affect this signalling pathway.

In our assays, migrating T lymphocytes were able to transmit PRV to ST cells. Some viruses utilize cells that are less permissive to viral replication as carrier cells, subverting antiviral signalling pathways of the host to facilitate viral spread. For instance, HIV-1 uses dendritic cells as transporters to reach one of its major target cell populations, CD4+ T cells (Cavrois et al., 2008). HCMV infects non-permissive monocytes, prompting these cells to migrate to target tissues, where the virus then initiates replication upon differentiation of the monocytes to macrophages (Smith et al., 2004). Our study shows that PRV, and in particular gE, activates ERK1/2 in non-permissive T lymphocytes, leading to an increase in homotypic T cell aggregation and migration, and that migrating cells are able to transmit the virus to highly susceptible cells. Hence, our results suggest a hypothetical novel role for ERK1/2 signalling and gE in viral spread, where (gE-mediated) ERK1/2 activation triggers PRV-carrying T lymphocytes to migrate and infect other cells susceptible to PRV replication.

Chapter 4

PRV gE TRIGGERS ERK1/2 PHOSPHORYLATION AND DEGRADATION OF THE PRO-APOPTOTIC PROTEIN BIM IN EPITHELIAL CELLS

Adapted from:

Maria S. Pontes, Cliff Van Waesberghe, Hans Nauwynck, Bruno Verhasselt & Herman W. Favoreel

Virus Research 213 (2016) 214–218

ABSTRACT

ERK1/2 activation is generally associated with cell survival and proliferation. Activation of ERK1/2 may trigger anti-apoptotic responses such as the phosphorylation and consequent degradation of Bim, a pro-apoptotic member of the Bcl-2 protein family (Ewings et al., 2007; Ley et al., 2003). ERK1/2-dependent host cell survival may be beneficial for intracellular pathogens and different viruses have been shown to benefit from ERK1/2-dependent Bim degradation. In the previous chapters, we have described that the viral glycoprotein gE mediates PRV-induced activation of ERK1/2 in T lymphocytes. In the present chapter, we show that PRV gE-mediated ERK1/2 phosphorylation also occurs in epithelial cells and that in these cells, gE-mediated ERK1/2 signalling is associated with the degradation of Bim. For the first time, our results link the viral glycoprotein gE, an important virulence factor, with the apoptotic signalling network.

INTRODUCTION

Apoptosis of virus-infected cells is an intrinsic anti-viral host response and consequently many viruses have acquired various strategies to evade this process. Large DNA viruses like alphaherpesviruses typically encode several proteins that interfere with apoptotic signalling. The alphaherpesvirus subfamily contains closely related pathogens of man and animal, including herpes simplex virus (HSV) and varicella-zoster virus (VZV) in man and pseudorabies virus (PRV) in pig. For PRV, thus far, only the viral protein US3 has been described to interfere with apoptotic signalling (Chang et al., 2013; Geenen et al., 2005; Munger and Roizman, 2001; Ogg et al., 2004).

Many viruses interact with the ERK1/2 signalling cascade as a method to control cell survival. The ERK1/2 signalling pathway promotes cell survival by different mechanisms like post-translational modification and regulation of components of the cell death machinery, and controlled transcription of anti- and pro-survival genes (Kolch, 2005). The ERK1/2 signalling pathway is the only signalling pathway so far implicated in the degradation of Bim, a pro-apoptotic member of the Bcl-2 protein family (Ewings et al., 2007; Hübner et al., 2008; Ley et al., 2003; Luciano et al., 2003). Phosphorylation and degradation of Bim by ERK1/2 contributes to reduced sensitivity of cells to apoptosis (Bonni et al., 1999; Ley et al., 2004; Puthalakath and Strasser, 2002; Sheridan et al., 2008; Zha et al., 1996b). Bim has been shown to have a central role in the regulation of immune responses, particularly of cytotoxic T lymphocytes, upon chronic viral infection (Grayson et al., 2006; Pellegrini et al., 2003).

We have recently described that the viral glycoprotein E (gE) of PRV, an important virulence factor, triggers activation of ERK1/2 in T lymphocytes (Chapters 3a and 3b). In the current study, we wanted to investigate whether PRV gE-mediated ERK1/2 signalling also occurs in epithelial cells and whether this correlates with degradation of the ERK1/2-regulated pro-apoptotic Bim protein.

MATERIALS AND METHODS

Cells, viruses and infections

PK15 (pig kidney) cells were grown in Eagle's minimal essential medium (MEM, Life Technologies) supplemented with 10% FBS, glutamine (0.3 mg/ml), and antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin). PRV wild type (WT, Becker strain), gEnull PRV 91 (PRV ΔgE, (Tirabassi et al., 1997)) and PRV107, lacking the cytoplasmic domain of gE (gEΔcd, (Tirabassi and Enquist, 1999), were grown and titrated on PK15 cells, and stored at −80°C. PRV Becker and PRV 91 (PRVΔgE) were a kind gift of L. Enquist, Princeton University. Prior to inoculation, cells were seeded at 400,000 cells/ml and were inoculated with either PRV WT or the isogenic mutants PRVΔgE and PRV gEΔcd at a multiplicity of infection (MOI) of 2 for different time points, as indicated in the Results section below. To assess the involvement of ERK1/2 signalling, the ERK1/2 phosphorylation inhibitor U0126 (100 µM, Cell Signalling) was added to the medium 30 minutes prior to inoculation of the cells, as well as at 2 hours post inoculation (hpi) with PRV WT at an MOI of 2. Cells were collected 1, 4 and 6 hpi for Western Blot analysis.

Western Blotting

Cells were washed with PBS, resuspended ice-cold lysis buffer (TNE buffer, 10% NP40, 1mM Na_3VO_4, 10 mM NaF, protease inhibitor cocktail) and incubated for 20 min at 4°C. SDS-PAGE and Western blotting were performed as described previously (Deruelle et al., 2007). Protein amounts of 20 µg were used for all experiments. Protein concentration was determined using the bicinchoninic acid (BCA) protein assay reagent (Thermo Scientific) according to the manufacturer's instructions. Blots were blocked in 5% nonfat dry milk in phosphate-buffered saline (PBS)–Tween 20 for 1 h at room temperature and incubated with primary antibodies for 1 h or overnight (according to the manufacturer's instructions). After several washes in 0.1% Tris-buffered saline (TBS)–Tween 20 (TBS-T), blots were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature and developed using enhanced chemiluminescence. Phospho-specific ERK1/2 antibody (1/1000; Cell Signalling) signal and Bim-specific antibody (1/1000; ab32158, Abcam), were detected with ECL Plus (GE

Healthcare). Total ERK1/2 antibody (1/1000; Cell Signalling) signal and viral protein antibody – rabbit polyclonal gE antibody (1/500), and mouse monoclonal US3 (1/500) and gB (1/200) antibodies – signals used as loading and infection controls, respectively, were detected with Pierce ECL (Thermo Scientific).

Cell surface expression of gE and gB

After inoculation with the different viruses, PK15 cells were detached from the wells using trypsin at different time points indicated in the Results section below. The cells were washed in ice cold PBS, fixed in 3% paraformaldehyde for 10 minutes at room temperature and finally washed twice with PBS. The cells were then resuspended in PBS containing primary antibody, gE (1/100; mAb 5F8, Nauwynck & Pensaert, 1995) or gB (1/100; mAb 1C11, Nauwynck & Pensaert, 1995), and incubated for 30 minutes at room temperature. After washing twice with PBS, the cells were incubated with secondary antibody, FITC-conjugated goat anti-mouse, for 30 minutes. Expression of gE and gB on the cell surface was measured by flow cytometry (FACS Canto, Beckton-Dickison Biosciences). As a first step, viable cells were selected using Forward Scatter (FSC)/ Side scatter (SSC), excluding debris and dead cells. From the population of viable cells, doublet discrimination was performed using FSC Height versus FSC Area and SSC Height versus SSC Area, ensuring only single cells are considered. Finally, mean fluorescence was measured (minimal event count=10,000) using the FL1 channel.

RESULTS

PRV gE leads to ERK1/2-dependent Bim degradation in epithelial cells

To analyze whether gE PRV mediates ERK1/2 activation on epithelial cells, PK15 cells were inoculated either with PRV WT or PRVΔgE, for 1, 4 and 6 hours. At 6 hours post inoculation (hpi), PRV WT caused a pronounced increase in ERK1/2 activation, whereas PRVΔgE infection led to similar ERK1/2 phosphorylation levels as seen in mock-infected cells (Figure 1A&B). As indicated earlier, it has been reported before that ERK1/2 signalling triggers the phosphorylation of the pro-apoptotic protein Bim, leading to its degradation via the proteasome (Ewings et al., 2007; Ley et al., 2003). ERK1/2-induced Bim modulation during alphaherpesvirus infection has only been shown for VZV (Liu and Cohen, 2014). Phosphorylation and consequent degradation of Bim can be identified by Western blot analysis via a slight increase in apparent molecular weight, followed by a loss of Bim detection (Ley et al., 2003). We observed that PRV WT infection triggers Bim degradation and that this process is suppressed in PRVΔgE-infected cells (Figure 1A&B). Addition of an ERK1/2 phosphorylation inhibitor (100 µM U0126) during PRV WT infection fully prevented Bim degradation (Figure 1C), confirming that PRV-induced ERK1/2 activation results in Bim degradation. Western blot analysis of viral proteins showed that differences in ERK1/2 phosphorylation or Bim degradation could not be attributed to differences in replication efficiency of PRV WT versus PRVΔgE, neither due to a suppressive effect of the inhibitor on virus replication (Figures 1D and 1E, respectively). In summary, our results show that PRV triggers gE-mediated ERK1/2 activation in epithelial PK15 cells and that phosphorylation of ERK1/2 in these cells is associated with Bim degradation.

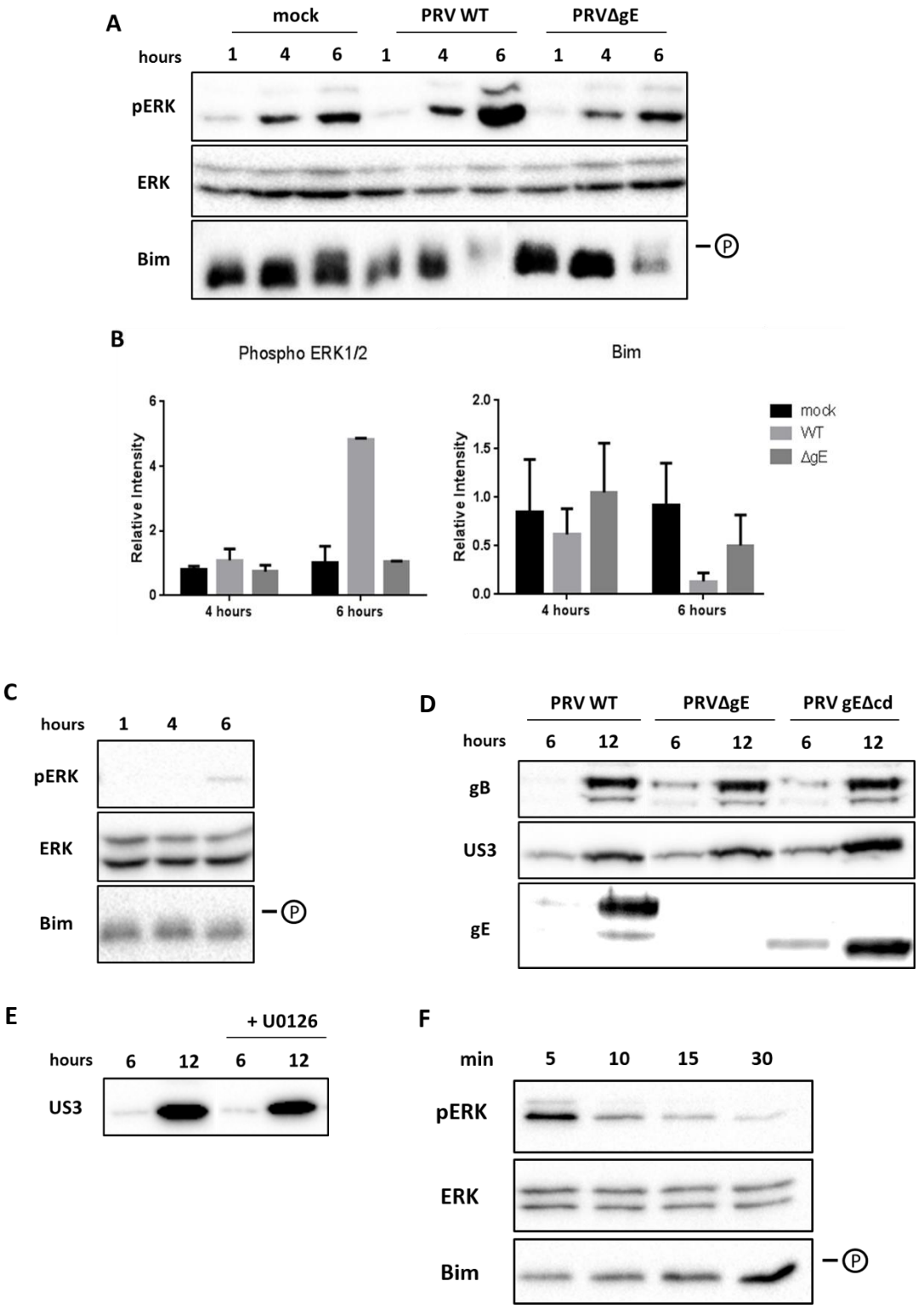

Figure 1. PRV gE triggers ERK1/2 phosphorylation in PK15 cells, causing degradation of Bim. (**A)** PK15 cells were either mock-inoculated or inoculated with PRV WT or PRVΔgE at an MOI of 2. At different time points post inoculation, cells were collected, lysed and analysed via Western blot, detecting phospho-ERK, total ERK and Bim. (**B)** ERK1/2 activation and Bim degradation detected by Western Blot were quantified by measuring the relative intensity of the bands (Bio-Rad Image Lab™ system). **(C)** PK15 cells were inoculated with PRV WT in the presence of the ERK1/2 phosphorylation inhibitor U0126, collected and analyzed at different time points as indicated above. **(D)** Protein expression of PRV gB, US3 and gE. PK15 cells were

inoculated with PRV WT, PRVΔgE or PRV gEΔcd (MOI 2) and expression of the different viral proteins was detected by Western Blot. Cells infected with PRV gEΔcd express gE without cytoplasmic domain and therefore show a reduced gE band size. **(E)** Inhibition of ERK1/2 phosphorylation does not affect expression of PRV US3. PK15 cells were inoculated with PRV WT (MOI 2), and incubated in the presence or absence of the ERK1/2 phosphorylation inhibitor U0126. Expression of US3 was detected by Western Blot. **(F)** Primary porcine T lymphocytes were co-cultivated with gE-transfected ST cells as described in Chapter 3b. After stimulation, the T lymphocytes were carefully collected and pERK, ERK and Bim were detected by Western Blot.

Infection with gEΔcd results in earlier ERK1/2-activation and Bim degradation

In infected cells, gE, like other viral glycoproteins, is expressed in different cell membranes, including the plasma membrane (Favoreel et al., 2004). The cytoplasmic domain of PRV gE contains endocytosis motifs that retrieve the protein from the plasma membrane during early stages of infection (up to 6 hpi), a process with a relatively unclear function (Tirabassi and Enquist, 1998; Tirabassi and Enquist, 1999). Genetic variants of PRV that lack the cytoplasmic domain of gE (PRV gEΔcd), and therefore the intracytoplasmic endocytosis motifs, lose the capacity to internalize from the plasma membrane (Tirabassi and Enquist, 1999). Interestingly, when PK15 cells were inoculated with PRV gEΔcd, we could observe a more rapid ERK1/2 phosphorylation (at 4hpi) and a more dramatic Bim degradation at 6hpi when compared to PRV WT (Figure 2). Western blot analysis of viral proteins showed that differences in ERK1/2 phosphorylation or Bim degradation could not be attributed to differences in virus replication efficiency (Figure 1D). Hence, whereas PRVΔgE showed an impaired ability to trigger ERK1/2 activation and Bim degradation, PRV gEΔcd showed an increased ability to trigger these processes compared to PRV WT.

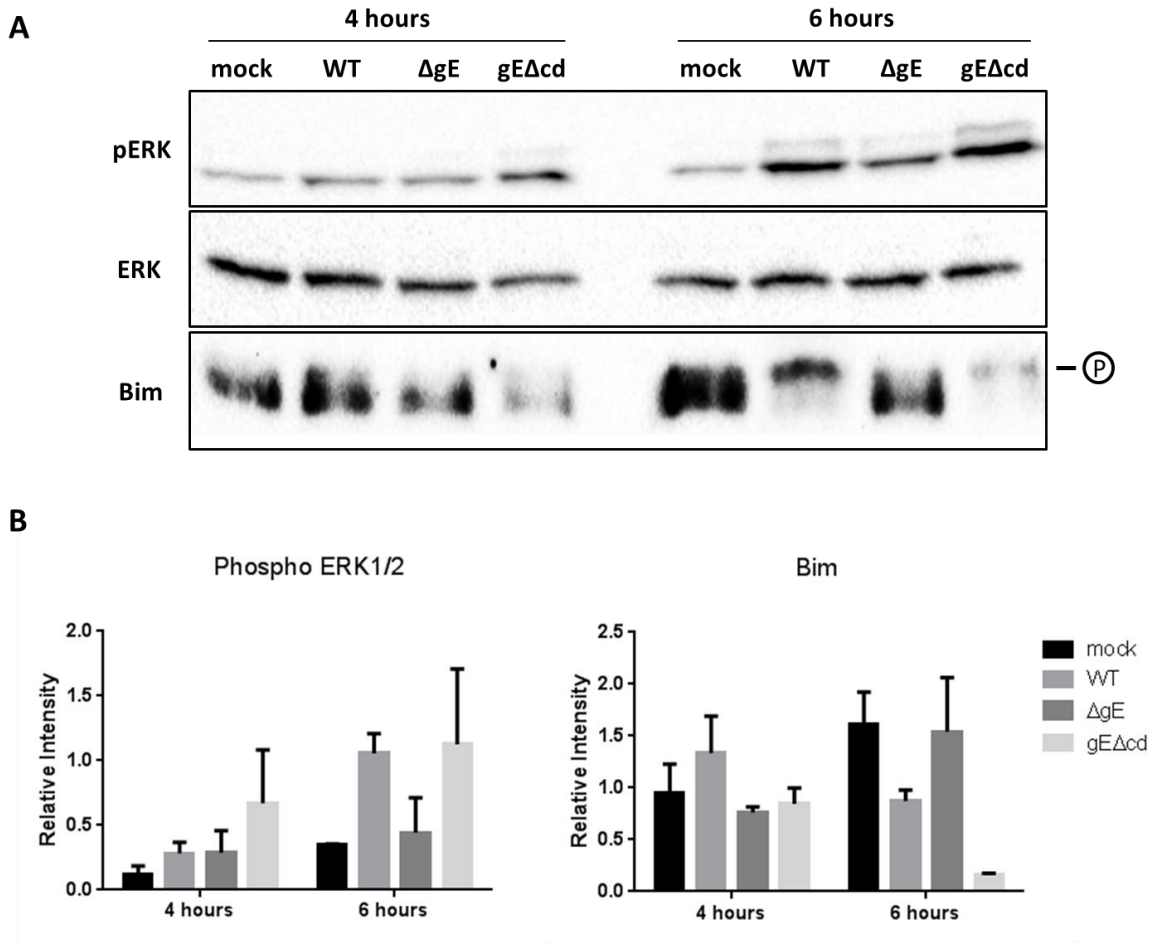

Figure 2. (A) A PRV mutant that lacks the cytoplasmic domain of gE triggers enhanced ERK1/2 phosphoryrlation and Bim degradation. PK15 cells were inoculated with PRV WT, PRVΔgE or PRV gEΔcd at an MOI of 2. Cells were collected and lysed at different time points. Phospho-ERK, total ERK and Bim were detected by Western Blot. **(B)** ERK1/2 activation and Bim degradation detected by Western Blot were quantified by measuring the relative intensity of the bands (Bio-Rad Image Lab™ system).

We wanted to confirm whether there were any obvious variances in gE cell surface expression levels between cells inoculated with PRV WT and PRV gEΔcd that could contribute to understand the results described above. Infected PK15 cells were collected at 4 and 6 hpi, and gE expression levels at the plasma membrane were analysed using flow cytometry. Cell surface levels of glycoprotein B at 6 hpi were determined as a control. Differences in gE cell surface expression were noticeable by 4 hpi, and even more obvious at 6 hpi, clearly showing greater levels of gE in the plasma membrane of PRV gEΔcd-infected cells (Figure 3). Although further research will be required to investigate this mechanistically, these results appear to be in line with our findings in T lymphocytes (Chapter 3b), which suggested that gE-dependent ERK1/2 activation may

occur via interaction of the extracellular domain of cell surface-expressed gE with a putative receptor(s) on the plasma membrane.

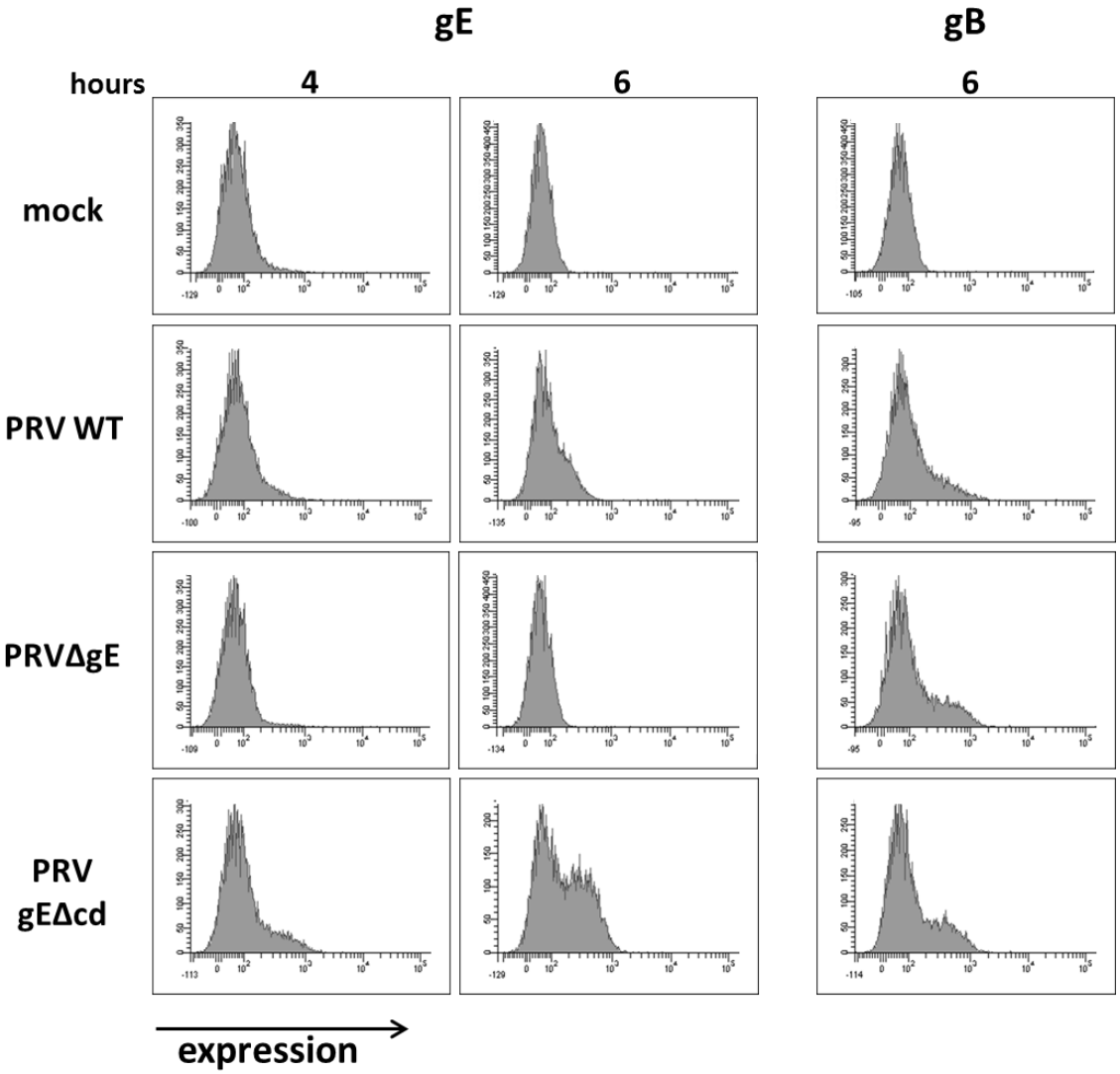

Figure 3. Cells infected with a PRV mutant that lacks the cytoplasmic domain of gE show increased cell surface expression of gE. Cells were inoculated for 4 or 6 hours with PRV WT, PRVΔgE or PRV gEΔcd at an MOI of 2 and fixed with paraformaldehyde. Expression of cell surface gE was assessed by flow cytometry (minimal event count=10000). Surface levels of gB were detected as a control.

DISCUSSION

Apoptosis of virus-infected cells is an intrinsic anti-viral host response and many viruses have acquired the ability to evade this process. The ERK1/2 signalling cascade promotes cell survival by different mechanisms (Lu and Xu, 2006), making this signalling pathway an ideal target for viruses to modulate host-driven apoptosis. One of the anti-apoptotic mechanisms that ERK1/2 has been associated with is the phosphorylation and consequent degradation of Bim, a pro-apoptotic member of the Bcl-2 protein family (Ewings et al., 2007; Ley et al., 2003).

In this chapter, we reveal that PRV infection leads to the sustained activation of ERK1/2 in epithelial cells, which coincides with the phosphorylation and consequent degradation of Bim. VZV (Liu and Cohen, 2014) also interferes with ERK1/2-dependent Bim degradation, highlighting the potential importance of this signalling axis for the biology of the *Alphaherpesvirinae* subfamily. Several other viruses have also been described to trigger Bim degradation in an ERK1/2-dependent manner, like the gammaherpesvirus Epstein-Barr virus (EBV) and the Human T-cell leukaemia type 1 virus (HTLV1) (Clybouw et al., 2005; Higuchi et al., 2014; Trushin et al., 2012). Aside viral factors that trigger ERK1/2-mediated Bim degradation, some viruses (including EBV, Kaposi's Sarcoma-associated herpesvirus (KSHV) and vaccinia virus) also encode proteins that directly bind and sequester Bim, thereby interfering with its activity (Campbell et al., 2014; Choi et al., 2012; Desbien et al., 2009). On the contrary, adenovirus infection has been associated with Bim upregulation and consequent virus-mediated cell death (Subramanian et al., 2007). Moreover, infection with HIV 1 triggers Bim-mediated apoptosis by interference of the Tat protein with the cell microtubule dynamics (Chen et al., 2002); additionally, the binding of gp120 protein with its partner, CXCR4, leads to the upregulation of Bim (Trushin et al., 2012). This multitude of strategies to interfere with Bim activity indicates that this pro-apoptotic protein possibly represents a pivotal player in the biology and pathogenesis of many viruses.

As demonstrated in the previous chapters for T lymphocytes, we show that also in epithelial cells, PRV gE is associated with PRV-triggered ERK1/2 activation. Furthermore, our data indicate that in this cell type, gE-triggered ERK1/2 phosphorylation appears to be associated with the degradation of Bim, linking gE with the apoptosis signalling

network for the first time. In support of the hypothesis that gE may trigger these signalling events, infection with a PRV mutant encoding an endocytosis-defective gE variant (gEΔcd PRV) showed higher plasma membrane levels of gE, earlier ERK1/2 phosphorylation and Bim degradation, when compared to infection with PRV WT. Noticeably, gE-mediated ERK1/2 phosphorylation did not cause Bim degradation in primary porcine T lymphocytes (Figure 1F), pointing out that PRV-induced ERK1/2 activation may trigger different down-stream events depending on the cell type.

In conclusion, our study illustrates a potential novel link between gE and the anti-apoptotic properties of PRV. To this date only the US3 viral kinase had been reported to inhibit programmed cell death during PRV infection (Deruelle et al., 2007; Geenen et al., 2005; Ogg et al., 2004) Additionally, our results are in line with the hypothesis that gE may trigger ERK1/2 activation via interaction with a yet unidentified receptor(s).

Chapter 5

General Discussion

Viruses are highly competent in hijacking and redirecting various signalling pathways of the host cell machinery, ensuring successful virus replication and propagation. One signalling pathway that has been widely studied in the context of viral manipulation of the host is the ERK1/2 signalling cascade. This central signalling pathway coordinates a myriad of cellular events, making it an attractive target for viruses to manipulate for their own benefit.

Being able to establish life-long infections, herpesviruses excel at effective host manipulation. Many herpesviruses have been reported to interfere with the ERK1/2 signalling pathway, and this signalling cascade seems to play a role at different stages of the life cycle of these viruses. Some herpesviruses activate ERK1/2 at a very early stage during infection, which seems to contribute to viral entry (HSV-1, KSHV, HCMV) (Naranatt et al., 2003; Sharma-Walia et al., 2005; Reeves et al., 2012; Zheng et al., 2014) and replication (HSV-1, HSV-2, VZV, HCMV, KSHV) (Rodems and Spector, 1998; Smith et al., 2000; Chen and Stinski, 2002; Kuang et al., 2009; Liu et al., 2012; Torres et al., 2012; Fu et al., 2015). ERK1/2 activity also seems to take part in viral-induced protection against apoptosis (HSV-2, VZV, HCMV, EBV) (Perkins et al., 2003; Clybouw et al., 2005; Wang et al., 2006; Oussaief et al., 2009; Liu et al., 2012; Reeves et al., 2012; Iwakiri et al., 2013; Liu and Cohen, 2014; Li et al., 2015), either by suppressing pro-apoptotic molecules or activating anti-apoptotic agents. The establishment of latency is a major characteristic of herpesviruses, which makes these viruses highly successful pathogens. ERK1/2 has been shown to be involved in both establishing of and reactivation from latency for some herpesviruses (HCMV, KSHV) (Ford et al., 2006; Reeves and Compton, 2011). Being a key signalling pathway in tumour growth, some tumour-inducing herpesviruses activate ERK1/2 to induce cell proliferation, malignant transformation, migration and cell invasiveness (MDV, EBV, KSHV) (Roberts and Cooper, 1998; Dawson et al., 2008; Meckes et al., 2010; Qin et al., 2013; Subramaniam et al., 2013).

Pseudorabies virus (PRV) has been shown to activate ERK1/2 in infected epithelial cells (Lyman et al., 2006; Kang and Banfield, 2010; Schulz et al., 2014), and the viral UL46 protein has been shown to play a role in this process (Schulz et al., 2014). Also in fibroblasts PRV was shown to activate ERK1/2; additionally, the PRV protein US2 was described to bind and sequester activated ERK1/2, targeting the kinase to the plasma membrane and therefore inhibiting its nuclear translocation (Kang and Banfield, 2010;

Lyman et al., 2006). The present thesis reports new insights on the modulation of the ERK1/2 signalling pathway by PRV. Here we reveal the contribution of the viral glycoprotein E (gE) in PRV-induced ERK1/2 activation and provide hints on the mechanism used by gE to modulate this central signalling pathway. Additionally, we describe physiological consequences linked to PRV gE-mediated ERK1/2 activation in two different cell types, T lymphocytes and epithelial cells.

The role of gE in PRV-induced ERK1/2 activation

One of the first PRV envelope proteins to be described (Hampl et al., 1984), gE is not essential for the replication (*in vitro or in vivo)* of PRV or other alphaherpesviruses (HSV-1, EHV-1, BHV-1), except for VZV (Engelenburg et al., 1995; Flowers and O'Callaghan, 1992; Jacobs, 1994; Longnecker and Roizman, 1986; Neidhardt et al., 1987). Albeit not essential in the replication of most alphaherpesviruses, gE is conserved among the different subfamily members, controlling important aspects of these viruses' pathogenesis. Most noticeably, gE is a determining factor in mediating full virulence during animal infections (Card and Enquist, 1995; Card et al., 1992; Jacobs et al., 1993b; Kimman et al., 1992; Lomniczi et al., 1984; Mettenleiter et al., 1987) and cell-to-cell spread in polarized cells or in cells that form extensive junctions with one another (i.e., keratinocytes, epithelial cells, and neurons) (Dingwell and Johnson, 1998; Dingwell et al., 1995; Enquist et al., 1998; Mettenleiter et al., 1987; Tirabassi and Enquist, 1998; Wisner et al., 2000; Zsak et al., 1992). Other functions that have been reported are involvement in virion assembly (Brack et al., 2000) and species-specific Fcγ receptor-like activity, with gE binding immunoglobulin G (IgG) at their Fc domain (Favoreel et al., 1997; Johnson et al., 1988; Litwin et al., 1992).

The present thesis demonstrates that gE is a key viral factor in PRV-induced activation of the ERK1/2 signalling cascade and that gE-mediated ERK1/2 activation does not depend on the glycoprotein´s C-terminal (cytoplasmic) domain. This observation was surprising, as up until recently, cellular signalling mediated by gE was invariably associated with its cytoplasmic region. In fact, the cytoplasmic region of gE contains tyrosine motifs that once phosphorylated upon antibody-mediated crosslinking may trigger capping of viral glycoproteins (Desplanques et al., 2007; Favoreel et al., 1997).

Capping can be observed in leukocytes upon crosslinking of immunoreceptors, and typically leads to leukocyte activation (D'Ambrosio et al., 1996). Additional experiments described in Chapters 3a and 3b, where we provided gE *in trans*, confirmed that the N-terminal (extracellular) domain is required and sufficient for ERK1/2 phosphorylation.

How is gE able to activate ERK1/2 via its extracellular region? This question prompted us to speculate that this glycoprotein may bind to one (or more) cellular receptor(s), likely present on the cell membrane. A cellular interaction partner for the extracellular domain of gE has only been reported for VZV, namely the insulin-degrading enzyme (Li et al., 2006), but not for any of the other alphaherpesviruses, including PRV.

The lentivirus HIV-1 is frequently associated with MAPK signalling modulation (Furler and Uittenbogaart, 2010), particularly through its gp120 envelope protein, and previous reports have linked HIV-1/gp120-triggered ERK1/2 activation to the chemokine receptor CXCR4 (Kinet et al., 2002; Popik and Pitha, 1996; Popik et al., 1998; Trushin et al., 2012). Gp120-CXCR4 signalling events affects entry and post-entry stages of infection and may modulate cellular functions even in the absence of infection (Borghi et al., 1995; Merrill et al., 1992; Shiratsuchi et al., 1994). CXCR4 is a 7-transmembrane G-protein coupled receptor originally identified in peripheral blood leukocytes (Federsppiel et al., 1993; Nomura et al., 1993; Loetscher et al., 1994) and later described as essential for T-cell tropic HIV-1 entry and for HIV-induced signal transduction (Deng et al., 1996; Feng et al., 1996; Popik and Pitha, 1996). Presently, CXCR4 is reported to be expressed on the surface of a variety of cell-types like T cells (Bleul et al., 1996; Bleul et al., 1997), B cells (Honczarenko et al., 1998), macrophages (Simmons et al., 1998; Verani et al., 1998; Yi et al., 1998) epithelial and endothelial cells (Gupta et al., 1998; Murdoch et al., 1999), and neurons from both the central and peripheral nervous systems (Dwinell et al., 1999; Murdoch, 2000; Ghosh et al., 2012; Hesselgesser et al., 1997). This chemokine receptor is also described to play a pivotal role in several physiological processes such as hematopoiesis, neurogenesis, germ cell development, cardiogenesis, vascular formation and cell migration (Agarwal et al., 2010; Cui et al., 2013; Ghosh et al., 2012; Moser and Loetscher, 2001; Murphy, 1994; Richardson and Lehmann, 2010; Sainz and Sata, 2007; Zhou et al., 1995).

The ability of CXCR4 to modulate the ERK1/2 signalling pathway has not only been described in the context of HIV/gp120, but also of its interaction with its most known

physiological ligand, the stromal cell-derived factor 1 (SDF-1 or CXCL12), where CXCR4-triggered ERK1/2 activation has been shown to lead to cell migration in T and B lymphocytes and myeloid cells (Alsayed et al., 2007; Gorter et al., 2008; Kremer et al., 2013). Considering this, we have begun to explore whether CXCR4 may be involved in gE-mediated ERK1/2 phosphorylation and preliminary results are encouraging (Figure 1).

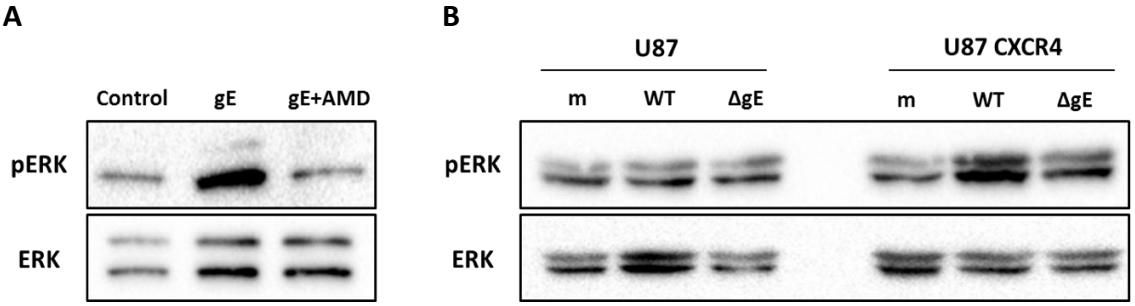

Figure 1 – CXCR4 appears to be involved in PRV gE-mediated ERK1/2 activation **(A)** Using similar methodology as in Chapter 3b, primary porcine T lymphocytes were incubated with epithelial cells transiently transfected with gE, in the presence or absence of the CXCR4 antagonist AMD3100. As a control we used a GFP-expressing plasmid (pcDNA-GFP, made in house) **(B)** Parental and CXCR4-expressing U87 cells were incubated with PRV or PRVΔgE, or mock-inoculated. In both experiments, cell lysates were prepared, and ERK1/2 activation was detected using Western Blot.

Firstly, by pre-incubating primary T lymphocytes with the CXCR4 antagonist AMD3100 (Schols et al., 1997; Donzella et al., 1998), we observed that gE-mediated ERK1/2 activation was impaired (Figure 1.A). In a second set of experiments, we used two cell lines, U87 cells (parental astrocyte cell line, which does not express CXCR4) and U87 cells stably transfected with CXCR4 (U87.CXCR4) (kindly provided by Dr. Dominique Schols, Rega Institute, Leuven). In U87.CXCR4, inoculation with PRV WT led to an increase in ERK1/2 phosphorylation, whereas inoculation with PRVΔgE caused a less pronounced effect on ERK1/2 phosphorylation. As a control, we observed no differences in ERK1/2 activation levels in mock- or virus-infected parental U87 cells (Figure 1.B).

The potential involvement of CXCR4 in gE-mediated ERK1/2 signaling would be in line with the notion that CXCR4 is a particularly attractive target for viruses. Indeed, in a noteworthy review (Arnolds and Spencer, 2014), the authors point out that amongst the well-documented exploitation by pathogens of host chemokines and chemokine receptor functions (Holst and Rosenkilde, 2003; Lalani and McFadden, 1999; Liston and McColl, 2003; Murphy, 2001; Sodhi et al., 2004), CXCR4 in particular appears to be a

prime target for manipulation by a number of different viruses. Besides HIV/gp120 as explained higher, several other viruses like herpesviruses, poxviruses, and papillomaviruses, have been found to interact with CXCR4, altering the receptor´s expression or functional activity. This has direct effects on cell trafficking, immune responses, cell proliferation, and cell survival (Arnolds and Spencer, 2014). For herpesviruses, HSV-1 infection of mature dendritic cells caused a decrease of protein levels of CXCR4 on the cell surface (Prechtel et al., 2005); although the mechanism for the down-regulation of CXCR4 during HSV-1 infection remains unknown. Also for EBV, studies have indicated viral manipulation of CXCR4. EBV-infected primary B lymphocytes displayed a decrease in CXCR4 expression, and this correlated with decreased migration toward SDF-1 (Ehlin-Henriksson et al., 2006). Additionally, transformation of a B-cell line with either LMP1 or EBNA2 also resulted in down-regulation of CXCR4 (Nakayama et al., 2002). For Human Cytomegalovirus (HCMV), two viral proteins (UL33 and UL78) were found to interact directly with CXCR4, interfering with the function of the receptor (Tadagaki et al., 2012). In that report, the authors also show that UL33 and UL78 modulate CXCR4 surface expression in the THP-1 monocytic cell line, as well as modulation of cellular migration.

However promising our initial results on the involvement of CXCR4 in gE-mediated ERK1/2 activation may appear, further research is necessary to assess whether gE (in)directly binds to CXCR4, thereby resulting in ERK1/2 activation. Later in this chapter we elaborate further on future prospects.

PRV-mediated ERK1/2 activation in T lymphocytes

Members of the *Herpesviridae* family are highly successful in manipulating the immune system. (Adhikary et al., 2006; Arvin et al., 2010; Smith et al., 2004; Wiertz et al., 2007; Zerboni et al., 2014b). The interaction between herpesviruses and immune cells of the host can lead to modulation of specific intracellular signalling pathways and subsequent changes in cellular behaviour which benefit viral infection.

In Chapters 3a and 3b, we describe that PRV gE triggers ERK1/2 activation in T lymphocytes, leading to homotypic aggregation, a sign of T cell activation (Guo et al., 2014; Layseca-Espinosa et al., 2003). Homotypic T cell aggregation is often associated

with increased cell motility (Jevnikar et al., 2008; Layseca-Espinosa et al., 2003; Pike et al., 2011). In line with this, our data showed that PRV gE-triggered ERK1/2 activation resulted in an increased T cell migration. It has also been shown for other viruses, like Human T-lymphotropic Virus 1 (HTLV-1) and the betaherpesvirus Human cytomegalovirus (HCMV), that virus-inoculated leukocytes show increased motility (Chevalier et al., 2014; Smith et al., 2004).

Previous reports described that PRV is not able to replicate efficiently in primary porcine T lymphocytes (Nauwynck and Pensaert, 1995b; Wang et al., 1988b). However, some viruses can manipulate and utilize less permissive cells as carriers to facilitate viral spread across the host. Viruses like HIV-1 and HCMV use dendritic cells and monocytes, respectively, as transporters in order to reach and carry out a successful replication in their primary target or more permissive environments (Cavrois et al., 2008; Smith et al., 2004). Consistent with these observations, we describe in the present thesis that apparently uninfected migratory T lymphocytes can transmit the virus to epithelial cells, where the virus replicates with higher efficiency. It is not the first time that cell-associated spread of infection is described for PRV, as the virus may use other blood cells, like monocytes, as carriers to disseminate infection across the host (Nauwynck and Pensaert, 1995b; Page et al., 1992; Van de Walle et al., 2003a; Van de Walle et al., 2003b). During PRV infection, monocytes support a full cycle of replication, and are able to survive for some time in the bloodstream of the infected animal, even in vaccination-immune animals (Nauwynck and Pensaert, 1992). The latter may be due to virus-induced capability of internalizing PRV-specific antibodies bound to viral proteins expressed in the cell membrane of monocytes, thereby avoiding antibody-mediated cell lysis (Van de Walle et al., 2003a). Additionally, these immune-masked PRV-infected monocytes are able to transmit the virus to endothelial cells (Van de Walle et al., 2003b). In the present thesis, we suggest that PRV gE-induced ERK1/2 activation and consequent migration of T lymphocytes could be important for PRV pathogenesis; the virus may be using these cells as carriers alongside infected monocytes to spread throughout the host. Cell-mediated spread of PRV in the blood of infected animals may allow the virus to reach other replication sites more efficiently, such as the reproductive system. It has been observed in pregnant sows that PRV infection may cause reproductive failure, including abortion, stillbirths and mummified foetuses (Akkermans, 1976; Baskerville, 1973), and

that this can be facilitated by cell-mediated viremia of the virus (Nauwynck and Pensaert, 1992).

Viral interaction with T lymphocytes during alphaherpesvirus infection has also been reported for VZV and HSV, human alphaherpesviruses closely related to PRV. VZV, initially classified as a neurotropic herpesvirus, has been described to mainly show T cell tropism. Unlike PRV, VZV efficiently infects T cells, particularly tonsil T cells that express skin homing markers, triggering T cell-mediated viral transport from the initial site of inoculation to the skin (Arvin et al., 2010; Zerboni et al., 2014b). For HSV, virus-positive T lymphocytes have been detected in skin grafts from lesions of patients (Aubert et al., 2009). HSV has evolved the ability to enter and infect T lymphocytes, although these cells may not support efficient viral replication (Westmoreland, 1978; Sloan and Jerome, 2007; Aubert et al., 2009). Nevertheless, infection by HSV profoundly modulates T-cell receptor (TCR) signalling which hinders T-cell cytotoxic function (Sloan and Jerome, 2007; Sloan et al., 2003; Sloan et al., 2006) and alters cytokine production profiles toward an IL10-dominated immunosuppressive phenotype (Sloan and Jerome, 2007). Regarding PRV, we have observed that, in infected Jurkat T cells, the virus modulates CD3/TCR-induced ERK1/2 activation (Chapter 3a) but preliminary observations indicated no significant variations in IL10 secretion between mock and PRV WT-infected cells (Figure 2). However, when using a gE-deleted PRV, there was a significant decrease in secreted levels of this anti-inflammatory cytokine. Although speculative at this stage, these data possibly indicate that, via gE and possibly its effect on ERK1/2 phosphorylation, PRV actively maintains a steady-state level of IL10 secretion, perhaps aiding in virus evasion from a pro-inflammatory antiviral response of the host. Future experiments are necessary to understand whether the observed effect is indeed driven by gE-mediated modulation of ERK1/2 activation, for example by using an inhibitor of ERK1/1 phosphorylation, like U0126.

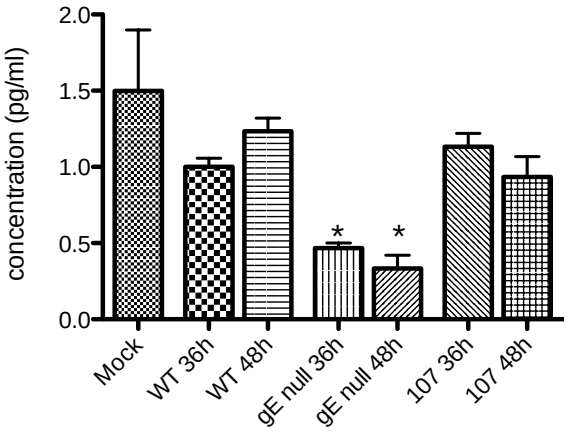

Figure2 - Jurkat T-cells were inoculated with PRV WT, PRVΔgE or PRV gEΔcytoplasmic domain (PRV 107) for 36h or 48h. Medium was analysed using ELISA to detect the levels of the anti-inflammatory cytokine IL10 secreted by the cells. Plot depicts mean concentration (± SD, three independent replicates); * represents statistically significant differences ($p<0.05$). Statistical analysis was performed on GraphPad Prism 5 (GraphPad Software, Inc). Data sets (n=3) were analysed using One-way ANOVA ($p<0.05$) combined with Tukey's Multiple Comparison Test (95% confidence interval).

PRV-mediated ERK1/2 activation in epithelial cells

When infected by a virus, host cells often undergo programmed cell death in an attempt to abort viral production and spread. It is therefore not surprising that many viruses have evolved mechanisms to modulate host cell apoptosis. In chapter 4 we demonstrate that PRV infection leads to the sustained activation of ERK1/2 in epithelial cells, which coincides with the phosphorylation and consequent degradation of pro-apoptotic BH-3 only protein Bim. We also show that, similarly to ERK1/2 activation in T lymphocytes, the process of ERK1/2-dependent Bim degradation is associated with PRV gE. The effect of ERK1/2 signalling on Bim degradation is in line with other reports, showing that Bim is phosphorylated by activated ERK1/2 and targeted for degradation, which contributes to reduced sensitivity of cells to apoptosis (Bonni et al., 1999; Ewings et al., 2007; Hübner et al., 2008; Ley et al., 2004; Marani et al., 2004; Sheridan et al., 2008; Zha et al., 1996b). Bim is an important initiator and regulator of the intrinsic pathway of the apoptotic signalling network, interacting with the anti-apoptotic Bcl-2 proteins as well as the multidomain pro-apoptotic effectors. It has been demonstrated that ERK1/2 is an important regulatory factor of Bim, as ERK1/2-dependent phosphorylation of Bim promotes Bim dissociation from anti-apoptotic Bcl‑2 proteins (Ewings et al., 2007; Harada et al., 2004; Lei and Davis, 2003) as well as its targeting for ubiquitination and

degradation by the proteasome (Ley et al., 2003; Luciano et al., 2003). To date, the only signalling pathway implicated in targeting Bim for ubiquitination and degradation is the ERK1/2 pathway; however, the specific details of how this is achieved are not known (Ewings et al., 2007). Given the specificity of the regulation of Bim, together with its important role within the apoptotic pathway, it may come as no surprise that some viruses target this particular signalling route. Viruses like VZV, EBV and HTLV-1 virus have been shown to manipulate ERK1/2 for Bim regulation (Clybouw et al., 2005; Higuchi et al., 2014; Liu and Cohen, 2014).

Our study demonstrates therefore a possible novel link between gE and the anti-apoptotic properties of PRV. To this date only the US3 viral kinase had been reported to inhibit programmed cell death during PRV infection (Geenen et al., 2005; Ogg et al., 2004). Studies, including from our group, have demonstrated that US3 mediates the phosphorylation and subsequent degradation of another Bcl-2 family member, the pro-apoptotic protein Bad, in PRV-infected epithelial cells (Ogg et al., 2004; Geenen et al., 2005; Deruelle et al., 2007; Deruelle et al., 2010), which likely contributes to the anti-apoptotic role of US3 during PRV infection. Additionally, in a more recent study, US3 has been shown to activate Akt and NF-ĸB anti-apoptotic signalling pathways (Chang et al., 2013).

Together with previous research, our data indicate that PRV encodes at least two proteins that interfere with the apoptotic signalling network. This is not a surprise, as several viruses encode multiple viral proteins that interfere with programmed cell death. As an example, HSV-1 encodes several proteins associated with preventing premature cell death. The viral proteins/transcripts US3, LAT, gJ, gD, ICP4, ICP22 and ICP27 were demonstrated to have anti-apoptotic activity during viral infection (Nguyen and Blaho, 2007). Similarly, the HIV-1 proteins Nef, Tat and Vpr have been shown to use different strategies to interfere with the highly complex apoptotic network (Cummins and Badley, 2010). The diversity of anti-apoptotic mechanisms developed by (persistent) viruses underscores that it is likely of paramount importance for these viruses to target several and/or different checkpoints of the apoptotic network.

Additional research is necessary to understand the implications of gE-associated Bim degradation during PRV infection, particularly in protecting the infected cell from apoptosis. Unfortunately, the redundancy in viral anti-apoptotic proteins complicates

studies to address the importance of preventing premature host cell death in virus infection and pathogenesis. For example, studies using a US3-deleted PRV did not demonstrate conclusive data regarding the anti-apoptotic activity of US3 on virus titers in cell culture (Deruelle et al., 2010). One of the potential explanations for this observation was that, while US3 at the time was the only identified anti-apoptotic PRV protein, it was hypothesized that PRV may encode additional anti-apoptotic proteins with (partially) redundant or overlapping activity (Deruelle et al., 2010). Our current data, which indicate that PRV gE also modulates apoptotic cell signalling, are in support of this hypothesis.

ERK1/2 signalling: the complexity of a tightly regulated cascade

An increasing amount of evidence has shown that cellular signalling pathways are not simply 'on-off' switches, and that spatiotemporal properties are important in determining the downstream effects of these pathways. The ERK1/2 signalling cascade represents remarkably well this concept. As previously mentioned, this signalling pathway controls an array of cellular events and physiological responses to ERK1/2 activation vary greatly depending on a range of factors.

Cellular response to ERK1/2 signalling has been shown to depend on the **cell type** as well as on the **strength** and **duration** of ERK1/2 activation. Differences in strength and duration of ERK1/2 signalling are determined by factors such as cell-surface receptor density, expression of scaffolding proteins, the surrounding extracellular matrix, and the interplay between kinases and phosphatases (Murphy and Blenis, 2006; Murphy et al., 2002). Throughout our research, we could observe differences in duration of gE-mediated ERK1/2 activation. In Jurkat T cells, gE-dependent ERK1/2 activation during productive infection appeared to be continuous for several hours, whereas adding recombinant gE triggered transient ERK1/2 activation. We speculate here that apparently sustained activation of ERK1/2 in Jurkat T cells could in fact be the result of dynamic cell-cell interactions; infected gE-expressing Jurkat T cells may establish several transient homotypic contact with different cells during a given time period (Hugues et al., 2004; Sabatos et al., 2008), and these dynamic interactions between cells may cause gE to bind (and detach) repeatedly to its possible receptor. As a result, it may be possible

to observe an almost continuous repetitive, rather than truly sustained, ERK1/2 activation. On the other hand, binding of recombinant gE led to a rapid but transient activation, possibly due to the non-dynamic nature of the interaction. We speculate that once bound, gE recombinant may remain attached, preventing binding of soluble gE still present in the medium.

Differences in gE-mediated ERK1/2 activation in infection-permissive cells (e.g. porcine epithelial cells and Jurkat T cells) versus cells that show weak susceptibility to infection (e.g. primary porcine T lymphocytes) may also be influenced by the possible effect of other viral factors that are expressed during infection of permissive cells. Successful expression of other viral proteins during PRV infection may further influence downstream ERK1/2 regulation and consequent cellular responses. In particular, PRV US2 was described to bind and sequester ERK1/2, inhibiting its nuclear translocation (Kang and Banfield, 2010; Lyman et al., 2006). **Subcellular location** of activated ERK1/2 has been described as an important regulating factor contributing to the cellular physiological response to ERK1/2 signalling. Spatial distribution of ERK1/2 can be regulated by scaffold proteins, which can impede ERK1/2 nuclear translocation and consequent phosphorylation of its nuclear substrates (Ebisuya et al., 2005; Kolch, 2005; Sharrocks, 2006; Shaul and Seger, 2007; Stork, 2002).

It would be interesting to conduct future experiments in order to understand how ERK1/2 is activated and regulated during PRV infection, including its subcellular localization, using our and other cellular models. The central role of the ERK1/2 signalling pathway in general cell biology aligned with the extensive research focused on this pathway in the various fields could provide valuable insights for future cell-focused antiviral treatments.

GENERAL REMARKS, WEAKNESSES AND FUTURE PROSPECTS

A novel function for Glycoprotein E

The data presented in this thesis show that PRV activation of the ERK1/2 signalling cascade is mediated by gE in at least two cell types, revealing a new aspect of the role of this glycoprotein in the biology of PRV.

For the past years, the role of gE in (neuronal) spread has been studied and characterized (Enquist et al., 1998; Kramer and Enquist, 2013); how gE drives PRV virulence remains, however, largely unknown. Neuronal spread and virulence were thought to be correlated at first, however studies came to reveal that these gE-associated events are, at least partially, regulated independently (Card et al., 1992; Rinaman et al., 1993; Tirabassi et al., 1997; Whealy et al., 1993). Given the central role of the ERK1/2 signalling pathway in many key cellular events, it is tempting to assume that this signalling pathway may play a part in driving gE-associated virulence. Additional research is required to consolidate the contribution of gE-mediated ERK1/2 in the context of PRV infection; however, novel aspects revealed by our work may contribute to fully understand the involvement of gE in disease progression.

We describe here that gE induces ERK1/2-mediated migratory behaviour in virus-carrying T lymphocytes, which are then capable of infecting cells more susceptible to virus replication. Due to the continuous trafficking of lymphocytes through blood, lymphoid organs and non-lymphoid tissue (Jalkanen et al., 1986; Mackay, 1993; Wiedle et al., 2001), the virus may have found in T lymphocytes an effective vehicle to disseminate through the host. This hypothesis may be further elucidated by understanding the migratory pattern of PRV-infected T lymphocytes. Future experiments may include measuring the expression of specific membrane markers on these cells. Upon activation, T lymphocytes may be induced to express a unique set of adhesion molecules and chemokine homing receptors, so that primed T cells acquire the ability to interact with organ-specific endothelial cells and migrate to distinct target tissues (Fu et al., 2016; Jalkanen et al., 1986). The migratory behaviour of T lymphocytes is greatly influenced by their surrounding microenvironment (Butcher and Picker, 1996; Wiedle et al., 2001), in combination with cell activation. As in many *in vitro* systems, our experiments excluded the presence of a surrounding microenvironment containing soluble factors (like chemokines and other cytokines), as well as possible interactions with other cells (such as antigen-presenting cells). Performing similar marker analysis in T lymphocytes isolated from infected animals could potentially provide valuable information to bridge the gap between an *in vitro* and an *in vivo* set up regarding the behaviour of T lymphocytes.

We also describe that gE induces ERK1/2-mediated degradation of pro-apoptotic Bim, hence potentially being involved in cell survival. The next step in this research is to test for protection of infected cells from stimulus-induced apoptosis, possibly by measuring specific apoptosis markers, such as cleaved caspase-3. However, such experiments should consider the demonstrated effect of US3 in protecting PRV-infected cells against apoptosis, which may complicate determining the true effect of gE. A PRV mutant including a double deletion of gE and US3 could be used as a tool for testing the role of gE in preventing premature host cell death. While transfecting epithelial cells with gE could also be regarded as a promising path forward, this method would bring additional challenges. Widely used liposome-based DNA carriers may induce a cellular stress response, including expression of genes engaged in vital cellular functions, mostly maintaining cellular metabolism, cell cycle control and progression, and apoptosis (Fiszer-Kierzkowska et al., 2011; Kim and Eberwine, 2010). In consequence, the effect of gE in ERK1/2 signalling may be masked, leading to a false result. A preliminary evaluation on different transfection methods would be useful to define future research. On a molecular scale, further investigating Bim interactions with its partners could also provide a good indication of the true effect of gE-mediated ERK1/2 signalling.

Interestingly, earlier onset of ERK1/2 activation in infected epithelial cells appeared to correlate with higher gE cell surface expression levels. If higher levels of gE surface expression lead to a putative anti-apoptotic phenotype, we questioned why would this glycoprotein be retrieved from the cell surface at early stages of infection. The role for endocytosis of gE remains unclear to this point. Tirabassi and Enquist have demonstrated that, in similarity to other glycoproteins, gE is internalized from the plasma membrane of infected cells early in infection (up to 6 hours post inoculation). The cytoplasmic domain of PRV gE contains endocytosis motifs which are responsible for the endocytosis of gE. Earlier reports had speculated that glycoproteins expressed in the cell surface would be preferentially incorporated in the virion envelope, but the aforementioned authors did not find any evidence that would support this line of thought. By engineering the PRV mutant that lacks the cytoplasmic domain of gE (gEΔcd, Tirabassi and Enquist, 1999), and studying the impact of this mutation on the virus life cycle, the authors could not find any obvious role for the endocytosis of gE. Considering these earlier studies and our observations, we suggest that PRV may use endocytosis to

regulate the signalling properties of gE. Signalling of plasma membrane receptors can be regulated by endocytosis at different levels, including receptor internalization (Barbieri et al., 2016; Cendrowski et al., 2016). Even if highly speculative, the binding of gE to its putative receptor may trigger the phosphorylation of the endocytosis motifs present in the cytoplasmic region, initiating the endocytic pathway.

As the interference of gE with the ERK1/2 signalling pathway has been observed in at least two different cell types, it would be interesting to understand whether this effect is seen in other relevant cell types. Very recently, our lab has described that gE inhibits the activation of plasmacytoid dendritic cells (pDC) and that this phenomenon is related to gE's ability to modulate ERK1/2 signalling (Lamote et al., 2017). Moreover, given their central role in PRV host infection and pathogenesis, it would be pertinent to investigate a potential interference of gE with ERK1/2 signalling in neuronal cells, the further consequences on neuronal behaviour as well as the overall impact on PRV infection.

gE (potential) receptor

Another future step in our research would be to determine how gE is able to activate ERK1/2 via its extracellular domain. Based on literature and our preliminary data, there is a strong indication that the chemokine receptor CXCR4 is potentially involved in gE-mediated ERK1/2 activation.

To consolidate these findings, future experiments applying proteomic methods would be considered as a logical initial step. The most conventional method is protein isolation via Immunoprecipitation (IP). These isolations can be performed by either using antibodies against gE or CXCR4, or by tagging the interacting proteins of interest and using antibodies against the tag. While straightforward in concept, IPs can be challenging, relying on the strength of protein-protein interactions as well as on the affinity of the antibodies used for the isolations. An alternative approach for validating the gE-CXCR4 interaction is the assessment of potential co-localization of the proteins by immunofluorescence microscopy. Several optical techniques may serve as complementary or alternative approaches (Dunham et al., 2004). For example, Förster resonance energy transfer (FRET) and proximity ligation assay (PLA) can provide insight into direct interactions *in situ*.

CONCLUDING NOTES

Larger viruses often rely on multiple proteins that contribute to several aspects of survival of the virus within the host and/or population, such as host invasion and spread, immune evasion, host gene expression and virus transmission to other hosts.

The main aim of this thesis was to further understand the biological functions of the alphaherpesvirus virulence factor gE and investigate its potential ability to interfere with host cell signalling. With our research, we discovered that gE manipulates a central cellular signalling network, the ERK1/2 signalling pathway, leading to a clear impact on downstream events, including changes in cellular behaviour. We are hopeful that our research could be the starting point for future investigations that may further unravel the importance of these findings and answer some of the questions that are still open, like confirming the role of CXCR4 and/or other putative cellular binding partners of gE in ERK1/2 signalling, exploring PRV/gE-mediated ERK1/2 manipulation in other relevant cellular models including neuronal cells, as well as during *in vivo* PRV pathogenesis.

It remains extraordinary that viruses, via a limited set of viral proteins, can manipulate intricate and highly complex signalling networks to their advantage in multiple stages of the viral replication cycle, which likely is a result of strong evolutionary pressure in order to survive and replicate. One can conclude, albeit arguably, that less (complexity) is more (advantage).

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Summary

The glycoprotein E (gE), an envelope protein conserved amongst the *Alphaherpesvirinae* subfamily members, has been credited with multiple functions throughout the years. The role of gE in virulence, viral intercellular and neuronal spread, virion assembly, species-specific Fcγ receptor-like activity and antibody-induced capping has been reported by several authors, including our group.

Herpesviruses are highly competent in manipulating cellular signalling cascades to their advantage. The ERK1/2 MAPK signalling pathway, a central signalling cascade, represents a particularly attractive target for viruses since this signalling axis controls various fundamental cellular events. Many herpesviruses have been reported to interfere with the ERK1/2 signalling pathway during different stages of the virus life cycle.

In **Chapter 1** we introduce pseudorabies virus (PRV) life cycle and pathology, and a description of the characteristics of gE and its role during PRV infection. We also describe the ERK1/2 signalling pathway and the involvement of this cascade in the cellular events relevant for this thesis, cell motility and cell survival. Finally, we provide an overview of studies that have shown the interaction/effect of different herpesviruses with the ERK1/2 signalling pathway.

The main objective of the present thesis was to investigate whether PRV and particularly gE interfere with the ERK1/2 signalling machinery. Furthermore, we aimed at studying the biological effects resulting from PRV and gE's interference with the ERK1/2 signalling pathway, which may contribute to viral replication and spread (**Chapter 2**).

Immune evasion is one of the key elements to the success of herpesviruses propagation and survival in their hosts. We started by studying the effect of gE PRV on the ERK1/2 signalling pathway in T lymphocytes (**Chapter 3**). Since this signalling cascade is involved in key aspects of T cell activation, PRV may benefit from the modulation of this pathway as part of its virus immune evasion strategies. In **Chapter 3a**, we describe that PRV WT infection leads to the phosphorylation, and therefore activation of ERK1/2 in the Jurkat T cell line. Additionally, we observed that PRV interfered with ERK1/2 activation mediated by CD3/TCR signalling, the classical route used for T cell activation. These results suggest that, besides triggering ERK1/2 activation, PRV actively regulates the ERK1/2 cascade by blocking other external stimuli from activating ERK1/2. Going forward, infecting Jurkat T cells with an isogenic PRV mutant that lacks gE expression

(PRVΔgE) did not cause ERK1/2 phosphorylation. Infection with a second isogenic PRV mutant lacking the cytoplasmic domain of gE (PRV gEΔcd), however, did not affect PRV-mediated ERK1/2 activation. Taken together, these results show that PRV-induced ERK1/2 activation depends on gE, but does not require the cytoplasmic signalling domain of gE. To confirm that gE affects ERK1/2 phosphorylation through its extracellular domain, Jurkat T cells were incubated with a recombinant version of the extracellular domain of gE protein, which caused a rapid and transient activation of ERK1/2.

To validate that PRV gE also leads to ERK1/2 activation in primary porcine T lymphocytes, we describe in **Chapter 3b** that, similarly to what was observed in Jurkat T cells, incubating primary T lymphocytes with recombinant gE led to a fast increase in ERK1/2 phosphorylation. Additionally, two different experimental set-ups were used to corroborate these findings. In one set of experiments, swine testicle (ST) cells were transiently transfected with gE and co-cultured with primary T lymphocytes. In a second set of experiments, primary T lymphocytes were brought into contact with PRV WT or PRV ΔgE. These two experimental set-ups showed similar results: providing gE *in trans* to primary porcine T lymphocytes triggers a rapid and transient ERK1/2 activation. Primary porcine T lymphocytes have been shown, by us and other labs, to display limited susceptibility to productive PRV infection *in vitro* and *in vivo*. We co-cultured inoculated primary T lymphocytes with ST cells, which are highly susceptible to PRV infection. With this experiment, we confirmed earlier findings which have shown that apparently noninfected lymphocytes are able to transmit the virus to more virus-susceptible cells. However, gE (and therefore gE-induced ERK1/2 signalling) appears not to affect virus transmission from T cells to susceptible cells. Thus, we investigated whether the ability of PRV gE to trigger ERK1/2 activation would have any biological consequences on T lymphocyte behavior. Homotypic T cell aggregation correlates with T cell activation and formation of T cell aggregates upon contact with either infected cells or virus has been described before, albeit not in the context of herpesvirus infection. PRV WT caused an increase in T lymphocyte aggregation, whereas PRVΔgE was significantly impaired in triggering cell aggregation. As others before, we observed that T cell aggregation depends on ERK1/2 signalling, as the addition of an inhibitor of ERK1/2 signalling abrogated the formation of large cell aggregates. T cell aggregation has been described

to correlate with T cell migration. Inoculation of primary T lymphocytes with PRV resulted in an increased cell migration and, in line with our results on T cell aggregation, gE contributed to some extent to PRV-induced migration of T lymphocytes and inhibition of ERK1/2 signalling abrogated cell migration.

ERK1/2 is not only involved in T cell activation, aggregation and motility, but also in promoting cell survival. **Chapter 4** describes that PRV also causes ERK1/2 activation in epithelial cells. Infection of Porcine Kidney (PK15) cells with PRVΔgE further showed that gE is also involved in ERK1/2 phosphorylation in this cell type. We observed that PRV WT infection triggered degradation of the pro-apoptotic protein Bim and that this process was suppressed in PRVΔgE-infected cells. Addition of an ERK1/2 phosphorylation inhibitor during PRV WT infection fully prevented Bim degradation, confirming that PRV-induced ERK1/2 activation results in Bim degradation. Interestingly, when PK15 cells were inoculated with PRV gEΔcd, we could observe a more rapid ERK1/2 phosphorylation and a more dramatic Bim degradation that in PRV WT. This result may be explained by differences in gE cell surface expression, which clearly showed greater levels of gE in the plasma membrane of PRV gEΔcd-infected cells compared to cells infected with PRV WT cells.

In **Chapter 5** we discuss further our findings, highlighting hypothetical possibilities how this novel role of gE may contribute to survival and propagation of PRV within the host population, as well as its possible contribution to functions described in previous reports. Moreover, we discuss the mechanism via which gE may activate ERK1/2, as well as suggest CXCR4 as a putative binding partner for this glycoprotein.

In conclusion, we describe for the first time that gE manipulates the cellular machinery of host cells by modulating the ERK1/2 signalling pathway in both T lymphocytes and epithelial cells. This leads to a clear impact on downstream events, such as changes in cellular behaviour like T cell activation and increased motility, or the interference with the apoptotic signalling network. We look forward for future research to further unravel the importance of these findings and answer pending questions including a further assessment of the role of CXCR4 and/or other putative cellular binding partners of gE in ERK1/2 signalling, exploring PRV/gE-mediated ERK1/2 manipulation in other relevant cellular models including neuronal cells, as well as during *in vivo* PRV pathogenesis.

Samenvatting

Aan het glycoproteïne E (gE), een envelop eiwit dat geconserveerd is binnen de leden van de alfaherpesvirus subfamilie, werden over de jaren diverse functies toegewezen. Een rol van gE in virulentie, viraal intercellulair en neuronaal spreiden, virion vorming, species-specifieke Fcγ receptor activiteit en antistof-gemedieerde 'capping' werd gerapporteerd door diversie auteurs, inclusief door onze onderzoeksgroep.

Herpesvirussen zijn zeer gedreven in het manipuleren van cellulaire signalisatiewegen voor hun eigen voordeel. De ERK1/2 MAPK signalisatieweg, een centrale signalisatiecascade, vormt een bijzonder aantrekkelijk doelwit voor virussen aangezien deze signalisatie-as diverse fundamentele cellulaire functies controleert. Voor veel herpesvirussen werd beschreven dat ze interfereren met de ERK1/2 signalisatieweg tijdens verschillende stadia van hun virale vermeerderingscyclus.

In **Hoofdstuk 1** worden de vermeerderingscyclus en de pathologie van het pseudorabies virus (PRV) virus geïntroduceerd, en wordt een overzicht gegeven van de karakteristieken van het gE eiwit en zijn rol tijdens PRV infectie. Daarnaast wordt ook de ERK1/2 signalisatieweg beschreven en de betrokkenheid van deze cascade in cellulaire functies die relevant zijn voor deze thesis, namelijk celmotiliteit en het overleven van de cel. Finaal wordt een overzicht gegeven van de studies die een interactie/effect aangetoond hebben van verschillende herpesvirussen met/op de ERK1/2 signalisatieweg.

De belangrijkste doelstelling van deze thesis was om te onderzoeken of PRV, en in het bijzonder het gE eiwit, de ERK1/2 signalisatie moduleren. Daarnaast was een bijkomende doelstelling om enkele biologische effecten te onderzoeken die voortvloeien uit dergelijke modulatie van ERK1/2 signalisatie door PRV/gE en die een impact kunnen hebben op de vermeerdering en het spreiden van het virus (**Hoofdstuk 2**).

Immuno evasie is een sleutelelement in het succes van herpesvirus vermeerdering en persistentie in de gastheer. Daarom werd in eerste instantie het effect van PRV en gE op de ERK1/2 signalisatie onderzocht in een celpopulatie die van bijzonder belang is in de immuunrespons, de T lymfocyten (**Hoofdstuk 3**). Aangezien deze signalisatieweg betrokken is in diverse aspecten van T cel activatie, is het mogelijk dat PRV deze signalisatie beïnvloedt als één van zijn immuno evasie mechanismen. In **Hoofdstuk 3a** wordt beschreven dat PRV infectie leidt tot fosforylatie, en daardoor activatie, van

ERK1/2 in de Jurkat T cellijn. Daarenboven werd aangetoond dat PRV de ERK1/2 activatie gemedieerd door CD3/TCR signalisatie, de klassieke weg voor T cel activatie, onderdrukt. Deze resultaten suggereren dat, naast het induceren van ERK1/2 activatie, PRV actief de klassieke ERK1/2 activatie via externe stimuli onderdrukt. In een volgende stap werd aangetoond dat een PRV virusmutant die geen gE tot expressie brengt (PRVΔgE) tijdens infectie van Jurkat T cellen geen aanleiding geeft tot ERK1/2 fosforylatie. Infectie met een andere isogene PRV mutant die enkel het cytoplasmatisch domein van gE mist (PRV gEΔcd) vertoonde daarentegen wel ERK1/2 fosforylatie. Deze resultaten tonen aan dat de PRV-geïnduceerde ERK1/2 activatie in Jurkat T cellen afhankelijk is van het gE eiwit, maar dat het cytoplasmatisch domein van dit eiwit hier niet bij betrokken is. Om te bevestigen dat het extracellulair gedeelte van het gE eiwit verantwoordelijk is voor de ERK1/2 fosforylatie werden Jurkat T cellen geïncubeerd met een recombinante versie van het extracellulair domein van gE, wat aanleiding gaf tot een snelle en tijdelijke activatie van ERK1/2.

In **Hoofdstuk 3b** werd nagegaan of PRV gE ook aanleiding geeft tot ERK1/2 activatie in primaire porciene T lymfocyten. Hierbij werd aangetoond dat, net als bij Jurkat T cellen, incubatie van primaire porciene T lymfocyten met recombinant gE aanleiding gaf tot een snelle en tijdelijke ERK1/2 activatie. Om deze bevindingen in primaire cellen te bevestigen werden twee bijkomende experimentele proefopzetten gebruikt. In één set van experimenten werden een ST ('swine testicle') epitheliale cellijn transiënt getransfecteerd met gE en in co-cultuur gebracht met primaire T lymfocyten. In een tweede reeks van experimenten werden primaire T lymfocyten in contact gebracht met PRV WT (wild type) of PRV ΔgE. Beide experimentele proefopzetten gaven een gelijkaardig resultaat: toedienen van gE *in trans* aan primaire porciene T lymfocyten gaf aanleiding tot een snelle en transiënte ERK1/2 activatie. Door onze en andere onderzoeksgroepen werd aangetoond dat primaire porciene T lymfocyten een zeer beperkte gevoeligheid hebben voor PRV infectie, zowel *in vitro* als *in vivo*. Wanneer we echter PRV-geïnoculeerde primaire T lymfocyten in co-cultivatie brachten met ST cellen, die zeer gevoelig zijn voor PRV infectie, konden we echter eerdere resultaten bevestigen dat schijnbaar niet-geïnfecteerde lymfocyten in staat zijn om het virus over te dragen naar meer virus-gevoelige cellen. Hierbij bleek dat gE (en dus gE-geïnduceerde ERK1/2 activatie) niet rechtstreeks betrokken was bij de overdracht van het virus van de T cellen

naar de gevoelige cellen. Daarom werd onderzocht of het vermogen van PRV gE om ERK1/2 activatie te induceren mogelijks andere biologische gevolgen kon hebben op het gedrag van de T cellen, die mogelijks een impact kunnen hebben op het spreiden van het virus. In eerdere studies werd reeds aangetoond dat T cel aggregatie correleert met de activatie en migratie van T cellen. Voor andere virussen werd reeds aangetoond dat ze T cel aggregatie kunnen induceren, maar nog niet voor herpesvirussen. Wij konden aantonen dat PRV WT T cel aggregatie kan veroorzaken, terwijl PRVΔgE significant minder in staat was om dit proces te induceren. Zoals eerder aangetoond in andere experimentele proefopzetten, konden we aantonen dat de T cel aggregatie afhankelijk is van ERK1/2 signalisatie, aangezien de toediening van een inhibitor van ERK1/2 signalisatie het proces sterk onderdrukte. Eerder werd reeds aangetoond dat T cel aggregatie correleert met T cel migratie. In overeenstemming met de resultaten voor T cel aggregatie, konden we aantonen dat PRV WT de T cel migratie verhoogt, dat gE hiertoe tot op bepaalde hoogte bijdraagt en dat inhibitie van ERK1/2 signalisatie deze migratie verhindert. Daarenboven konden we aantonen dat de PRV-geïnoculeerde T cellen die migratie vertonen in staat zijn om het virus over te dragen naar gevoelige cellen. Alhoewel deze resultaten *in vitro* werden bekomen, suggereert dit dat PRV en gE-geïnduceerde, ERK1/2-afhankelijke T cel migratie kan bijdragen tot het efficiënt spreiden van het virus.

ERK1/2 signalisatie is in veel meer processen betrokken dan enkel T cel activatie, aggregatie en motiliteit, waaronder in het verhinderen van apoptotische celdood. In **Hoofdstuk 4** werd aangetoond dat PRV ook ERK1/2 activatie veroorzaakt in epitheelcellen. Infectie van de epitheliale PK15 (‘porcine kidney’) cellijn met PRV WT of PRVΔgE toonde aan dat PRV ook in dit celtype ERK1/2 fosforylatie induceert, en dat ook hier het gE eiwit hierbij belangrijk is. Daarenboven leidde PRV WT infectie in deze cellen tot afbraak van het pro-apoptotische eiwit Bim en deze degradatie was onderdrukt in cellen geïnfecteerd met PRVΔgE. Toediening van een ERK1/2 fosforylatie inhibitor verhinderde Bim degradatie tijdens PRV WT infectie volledig, wat dus aanduidt dat PRV-geïnduceerde ERK1/2 activatie aanleiding geeft tot Bim degradatie in PK15 cellen. Interessant hierbij was dat infectie van PK15 cellen met de PRV gEΔcd virusmutant, die dus het cytoplasmatisch domein van gE mist, aanleiding gaf tot een snellere ERK1/2 fosforylatie en een meer uitgesproken Bim degradatie in vergelijking met PRV WT. Dit

resultaat kan verklaard worden door het feit dat deze virusmutant sneller grotere hoeveelheden van het gE eiwit tot expressie brengt in de plasmamembraan van de geïnfecteerde cel, in vergelijking met PRV WT.

In **Hoofdstuk 5** worden de bevindingen van deze thesis bediscussieerd, waarbij nadruk gelegd wordt op hypothetische mogelijkheden hoe deze nieuwe rol van het virale gE eiwit mogelijks kan bijdragen tot het persisteren en vermeerderen van PRV in de gastheer populatie, en hoe deze nieuwe rol kan bijdragen tot functies die eerder werden beschreven voor gE. Daarenboven worden de mechanismen bediscussieerd hoe gE aanleiding kan geven tot ERK1/2 activatie, waarbij de mogelijke rol van de cellulaire chemokine receptor CXCR4, als mogelijke receptor voor gE, besproken wordt.

Samengevat kan gesteld worden dat in deze thesis voor de eerste maal werd aangetoond dat gE de cellulaire machinerie van gastheer cellen beïnvloedt door modulatie van de ERK1/2 signalisatieweg, en dit zowel in T lymfocyten als epitheelcellen. Het activeren van deze signalisatieweg heeft duidelijke biologische consequenties, waaronder veranderingen in cellulair gedrag zoals T cel aggregatie en motiliteit en interferentie met de signalisatie die aanleiding geeft tot apoptotische celdood. We kijken uit naar toekomstige experimenten die het belang van deze bevindingen verder zullen onderzoeken en antwoord zullen proberen te bieden op diverse openstaande vragen zoals de rol van CXCR4 en eventueel andere potentiële bindingspartners van gE in ERK1/2 signalisatie, het voorkomen en effect van PRV/gE-gemedieerde ERK1/2 manipulatie in andere relevante cellulaire modellen waaronder neuronale cellen, en het belang van deze bevindingen tijdens *in vivo* pathogenese van PRV.

CURRICULUM VITAE

Personalia

Maria José Setas Lopes Pontes was born in Mirandela, Portugal, on 17th of October 1986. In 2004, Maria entered University of Minho, Braga, Portugal. As part of Maria's study program and under the Erasmus program, she attended Wageningen University & Research (WUR), Wageningen, The Netherlands, to develop her master thesis research at the Cell Biology and Immunology Lab. The work developed during this time culminated in the draft of her thesis entitled *Electromagnetic fields effect on health: evaluation using carp macrophages*. After successfully defending her master thesis, Maria graduated in 2008 with a Master degree in Applied Biology. From November 2008 to July 2009, Maria worked as a research assistant at the Cell Biology and Immunology Lab, WUR, co-authoring two peer-reviewed papers published in international journals. In September 2009, Maria enrolled in the Doctoral Program of Ghent University, Ghent, Belgium. After developing her research around the effects of pseudorabies glycoprotein E on the ERK1/2 signalling pathway, Maria published, as first author, two peer-reviewed papers published in international journals. During her research time, Maria presented her work at the renowned International Herpesvirus Workshop twice, both via poster- and oral- presentation. Maria was also selected for oral and poster presentations at the Annual Symposium of the Belgian Society for Microbiology and the Belgian Society for Virology Annual meeting.

Relevant Publications

Desplanques, A. S.**, Pontes, M.,** De Corte, N., Verheyen, N., Nauwynck, H. J., Vercauteren, D. and Favoreel, H. W. (2010). Cholesterol depletion affects infectivity and stability of pseudorabies virus. *Virus Res.* 152, 180–183.

Pontes, M. S., Devriendt, B. and Favoreel, H. W. (2015). Pseudorabies Virus Triggers Glycoprotein gE-Mediated ERK1/2 Activation and ERK1/2-Dependent Migratory Behavior in T Cells. *J. Virol.* 89, 2149–2156.

Pontes, M. S., Van Waesberghe, C., Nauwynck, H., Verhasselt, B. and Favoreel, H. W. (2016). Pseudorabies virus glycoprotein gE triggers ERK1/2 phosphorylation and degradation of the pro-apoptotic protein Bim in epithelial cells. *Virus Res.* 213, 214–218.

Presentations

M. J. S. L. Pontes, K. Bienkowska-Szewczyk, S. Glorieux, and H. W. Favoreel (2011) Pseudorabies virus glycoprotein E induces ERK1/2 activation in T lymphocytes. 36th International Herpesvirus Workshop, Gdansk, Poland. *(oral and poster presentation)*

M. J. S. L. Pontes, K. Bienkowska-Szewczyk, S. Glorieux, and H. W. Favoreel (2011) Pseudorabies virus glycoprotein E induces ERK1/2 activation in T lymphocytes. Annual Symposium of the Belgian Society "Life, Death and Survival of Microorganisms", Brussels, Belgium. *(oral and poster presentation)*

Pontes, M. S., Devriendt, B. and Favoreel, H. W. (2014) Pseudorabies virus gE causes ERK1/2 activation in primary porcine T lymphocytes and subsequent cell aggregation. 39th International Herpesvirus Workshop, Kobe, Japan. *(oral and poster presentation)*

Pontes, M. S., Devriendt, B. and Favoreel, H. W. (2014) Pseudorabies virus gE causes ERK1/2 activation in primary porcine T lymphocytes and subsequent cell aggregation. Belgian Society for Virology Annual meeting Brussels, Belgium. *(oral presentation)*

Acknowledgements

Thank you!

Many people have helped making this journey a happy and successful one, and there are a few who I would like to mention.

First and foremost, I would like to thank my promotor. Herman, there are not many supervisors who support and teach their students with your dedication and interest. Your door was always open to all of us. Thank you for giving me the opportunity to join your group. Also, thanks for all your words: of support and encouragement, of kindness, of reality (which we're not often prepared to hear…). More practically, thanks for making these difficult last steps as swift and easy as you possibly could ☺.

To the members of the exam committee, for reading my thesis, providing valuable comments for its improvement, as well as for the interesting discussion during my internal defence. There are two members I would like to mention in particular. Bert, there are parts of this thesis work that would have not been possible without you (the word 'aggregates' should have been forbidden ☺). Thank you for all your help along the way, for the insightful discussions and valuable reviews. Hans, thanks for your support by gifting us with important resources, as well as the always interesting discussions during our Friday seminars.

I would like to thank to all my colleagues from the Immunology and Virology groups, in particular:

- To Nina, who from day one made me feel at home in the lab. Thank you for all the lab lessons, patience, lengthy science talks, companionship, friendship, numerous quiches and much needed chit-chat (to everyone's annoyance☺). I will never forget all your help in and out of the lab, thank you!
- To Céline, who always had a word of support and encouragement, especially when I doubted myself. Thank you ☺ And I really look forward to continuing being your colleague!
- To Cliff and Jochen, who've made working in the lab infinite times more fun ☺ Jochen, friend of early mornings in the stables and late nights and weekends in the lab. Thanks for your never-ending support, selfless help and friendship. Cliffy, your support in the lab has been extremely important, so thank you very much!
- To Lennert and Marc, colleagues, friends and companions ☺ You have been there for me in so many moments, ready to pick me up whenever things were down! Thanks for your friendship, always positive attitude and support!

To my 5 Madams Ana, Carina, Dina, Gina and Sofia. You have remained by my side through thick and thin, offered kind words of support, said all the right things when necessary and brought me to tears of so much laughter with our late-night Skype calls ☺. No matter how far apart we may be (for now!), our distance made my heart grow fonder of all of you! Gosto muito de vocês e muito muito muito obrigada minhas madams.

To Carla and Wouter, the wonder couple. The love for science (Carla), crazy talk (Wouter) and food (both) brought us together, and I´ve been lucky to call you my friends ever since. Carlinha, obrigada por tudo o que me ensinaste e pelo exemplo de preserverança e força. Wouter, obrigado pela companhia e paciência durante as nossas "lab talks".

To Galbha, Sharat and Minerva, who adopted me as one of their kind ☺ Thank you for all our fun times which kept me sane, for all your (endless) support sessions and for all the insightful science talks. I´m very fortunate to have found you during this journey and I hope many more adventures will come our way!

To Filipa, my favorite flat mate. We found each other by chance and the chemistry was immediate 😊 thank you for all the amazing times we shared in our awesome apartment, our shared (guilty) love for cheesy music, the deep and enlightening talks… Fomos a família uma da outra em alturas boas e más, e nunca mais esquecerei todos os momentos que passámos juntas. Muito obrigada!

To my most recent colleagues turned friends, Jutta and Cristina, who were such a positive influence of work and perseverance in these past months, celebrating each step as a small victory ☺ Thank you!

To the Drost family, thank you for welcoming me as a family member. Your kindness, understanding and words of support have been extremely important for these past, looooong, months!

To my uncle António and aunt Patrícia, who made me feel capable and confident in my own abilities. Depois dos meus pais, nunca senti tanto orgulho vindo de outro lado como sinto com voces. Obrigada pelo vosso apoio, por verem em mim força que eu muitas vezes duvido ter e pelo vosso exemplo!

To my brother, who very kindly designed the cover for this thesis and supported me during this lengthy isolation period.

To my parents, for their humongous support and pride on their daughter. Mamã e papá, obrigada por tudo (tudo mesmo)! Por muito que seja difícil estar longe, senti sempre o vosso apoio incondicional e encorajamento em seguir em frente, sem porquês nem receios. Nunca conseguirei dizer suficientes vezes obrigada e o quão sortuda sou por me terem calhado na rifa ☺

To Arthur… you've arrived later to the craziness and you were not a day too soon. The process of finishing this journey has been challenging to say the least… And you've been there for me for the good, the bad and the ugly (cry ☺), with endless support and love, hugs and laughter, food and water, patience and understanding, cheering me all the way through… and I couldn't be luckier than having you in my life. Dankjewel xxx.

Maria
15th February 2018