Max-von Pettenkofer-Institute and Gene Center,
Ludwig Maximilians University, Munich
Prof. Dr. Karl-Klaus Conzelmann

Institute of Molecular Animal Breeding and Biotechnology, Gene Center
Faculty of Veterinary Medicine of the Ludwig Maximilian University, Munich
Prof. Dr. Eckhard Wolf

Interferon Escape of Respiratory Syncytial Virus: Functional Analysis of the Nonstructural Proteins NS1 and NS2

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by
Sabrina Marozin
from Milan, Italy

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Aus der
Abteilung für Virologie des Max-von Pettenkofer-Institutes,
Genzentrum, München
Arbeitsgruppe Prof. Dr. Karl-Klaus Conzelmann
Leiter Prof. Dr. Ulrich Koszinowski

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Univ.-Prof. Dr. Eckhard Wolf

Unterdrückung der Interferon-vermittelten Immunantwort durch das Respiratorische Synzitial Virus: Funktionelle Analyse der Nicht-Strukturproteine NS1 und NS2

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Sabrina Marozin
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If a man will begin with certainties, he shall end in doubts,
but if he will content to begin with doubts, he shall end in certainties.

Francis Bacon

CONTENTS

Abbreviations

A	Ampere
APS	ammonium persulfate
ATP	adenosintriphosphate
b	bovine
BRSV	Bovine respiratory syncytial virus
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
cm	centimeters
CMV	cytomegalovirus
CoA	coenzyme A
CPE	cytopathic effect
CS	newborn calf serum
C-terminus	carboxy terminus
DEPC	diethylpyrocarbotate
DL	Dual luciferase assay
DMEM	Dulbecco's Modified Eagle Medium
DNA	desoxyribonucleic acid
dNTP	desoxyribonucleoside triphosphates
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
e.g	example given
Et al.	Et alii
FCS	fetal calf serum
FITC	fluoresceinisothiocyanate
FFU	focus forming units
g	gram
GFP	green fluorescent protein
h	human
h, hrs	hour(s)
HRSV	Human respiratory syncytial virus
IFN	interferon
Ig	immunoglobulin
IRF	Interferon regulatory factor
IU	international units
kb	kilobase
kDa	kilodalton
l	liter
LB	Luria Broth
LPS	lipopolysaccharides
Luc	luciferase
M	molar, marker

m	milli
mA	milliampere
mg	milligram
min	minute(s)
ml	milliliter
mM	millimolar
mm	millimeter
MOI	multiplicity of infection
mRNA	messenger RNA
MvP	Max von Pettenkofer
ng	nanogram
nm	nanometers
N	nucleoprotein
N-terminus	amino terminus
NS	nonstructural protein
nt	nucleotide
P	phosphoprotein
p	plasmid
PAGE	polyacrylamide gel elctrophoresis
PBS	phosphate-buffered saline
PBS-T	phosphate-buffered saline/0.05% Tween
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
p.i.	post infection
PKR	RNA-dependent protein kinase
Poly I/C	polyriboinosinic acid/polyribocytidilic acid
PRD	positive regulatory domain
p.t.	post transfection
OD	optical density
ORF	open reading frame
RLU	relative light unit
RNA	ribonucleic acid
RNase	ribonuclease
Rnasin	Rnase inhibitor
RNP	ribonucleoprotein
rpm	revolution per minute
RT-PCR	reverse transcription PCR
RSV	respiratory syncytial virus
RT	reverse transcriptase
RV	Rabies virus
SDS	sodium dodecyl sulfate
STAT	signal transducer and activator of transcription
TAE	Tris-acetate-EDTA
TBS	Tris-buffered saline

TEMED	N,N,N’,N’-tetramethylethylendiamine
U	Unit
UV	ultraviolet
V	volt
wt	wild-type
µg	micrograms
µl	microliters

1 INTRODUCTION AND OBJECTIVES

Respiratory Syncytial Virus (RSV) is recognised as the most frequent cause of severe lower respiratory tract infections in infants and cattle worldwide. Human Respiratory Syncytial Virus (HRSV) infects around 50% of infants during the first year and almost all the children by age of two, resulting in the most common factor of paediatric hospitalizations in particular of subjects between 2 and 6 months of age (Collins P.L. et al., 2001; Hoffman S.J. et al., 2004). Bovine Respiratory Syncytial Virus (BRSV)-associated disease has been observed in young calves at less than 6 months of age and also associated with outbreaks in dairy cows (Elvander M., 1996). Seroprevalence among the adult human population and cattle is around 70% (Van der Poel W.H. et al., 1994). BRSV and HRSV are closely antigenically related and in both cases serological subgroups have been identified (Mallipeddi S.K. et al., 1993; Furze J.M. et al., 1994; Mufson M.A. et al., 1985). Human and bovine respiratory syncytial viruses share common epidemiological, clinical and pathological characteristics. RSV is spread from respiratory secretion via close contact with infected persons or contaminated materials. Infections follow a seasonal periodicity and typical pathological manifestations of RSV-related illness are tracheobronchitis, peri-bronchiolitis, bronchiolitis and pneumonia. Bronchiolitis is associated with long term impairment of pulmonary functions and histamine hyper responsiveness can last for many years after RSV-infection in infancy probably leading to the development of asthma and general allergic sensitisation in children (Sigurs N. et al., 2000).

The presence of maternal antibodies gives neither efficacious protection nor reduces viral-shedding after infection. RSV infection does not lead to a complete and durable immunity and human and cattle of all ages can be re-infected throughout life. Current treatments of RSV are based on supportive care and antiviral therapy. An antiviral compound, which is approved for RSV treatment in humans, is Ribavirin, a nucleoside analog. Ribavirin aereosol can be used in the treatment of some patients with severe disease. However, limited clinical benefits have been observed. Controversy on the significance of passive RSV prophylaxis, using RSV Immunoglobulin (RespiGam) or genetically engineered humanized monoclonal antibodies against F glycoprotein (Palivizumab), is also stated. However, combination of immunoglobulin intravenously (IGIV) with neutralizing RSV antibody (RSV IGIV) and Ribavirin has been used to treat patients with compromised immune system. Development of

an RSV vaccine is a high research priority, but none is yet available. In conclusion, no effective treatments for RSV infection are available at the moment. Moreover the development of a successful vaccine has been hampered by the fact that natural infection does not provide complete protection against re-infections. Besides, previous immunization can enhance the severity of the disease in naturally infected individuals. A safe effective RSV vaccine and/or the development of efficient therapeutic interventions is crucial and relies on a proper understanding of RSV disease pathogenesis and virus host-cell interactions. In this respect, studies of the innate immunity, which plays a critical role at the time of maximum severity of illness and influences the subsequent adaptive response, as well as viral counter measurements must be elucidated.

RSV, as well as many negative strand RNA viruses (e.g. Measles, Mumps, Parainfluenza and Influenza virus) has evolved several strategies to hinder IFN response.
IFN treatment of RSV infected cells does not inhibit viral replication, indicating RSV ability to circumvent the action of IFN. RSV NS1 and NS2 proteins are responsible for the pronounced resistance to exogenous interferon and their ability to inhibit activity of cellular IFN-induced antiviral proteins is exerted in a host-adapted manner (Schlender J. et al., 2000; Bossert B. et al., 2002). RSV nonstructural proteins (NS) as other viral accessory proteins in Influenza or Bunyamwera viruses, are also strong inhibitors of IFN α/β production.
Interferon type I synthesis is regulated at a transcriptional level. In response to viral infection, the transcription factors AP-1, NF-kB and Interferon Regulatory Factor 3 (IRF3) become activated following protein phosphorylation, bind to designated positive regulatory domains (PRD) present in the IFN-β promoter and form a transcriptional enhancer complex which stimulates transient activation of IFN-β transcription. Nonstructural proteins of RSV specifically impair IRF3 activation by preventing its phosphorylation (Bossert B. et al., 2003) but how this occurs is still unclear.

My attempt is to gain more insight into the mechanisms leading to NS protein-mediated inhibition of IRF3 factor. My approach includes analysis of both cellular and viral elements that play an active role in this complex mechanism. The aim of my work is to identify possible cellular targets within the signalling pathway activating IFN production and to determine specific amino acid motifs in the HRSV NS proteins involved in their interferon-inhibitory activity.

2 REVIEW OF THE LITERATURE

2.1 Structure and genome

2.1.1 Virion structure

Bovine (BRSV) and human (HRSV) respiratory syncytial viruses are members of the genus *Pneumovirus,* subfamily *Pneumovirinae*, family *Paramyxoviridae*.

RSV virions have an irregular spherical shape of about 150-300 nm in diameter and consist in a nucleocapsid embedded within a lipid envelope.

The nucleocapsid (RNP complex) is composed of the nucleocapsid protein N, the phosphoprotein P, the large polymerase subunit L and the M2-1 protein and it has a symmetrical helical structure of 12-15 nm.

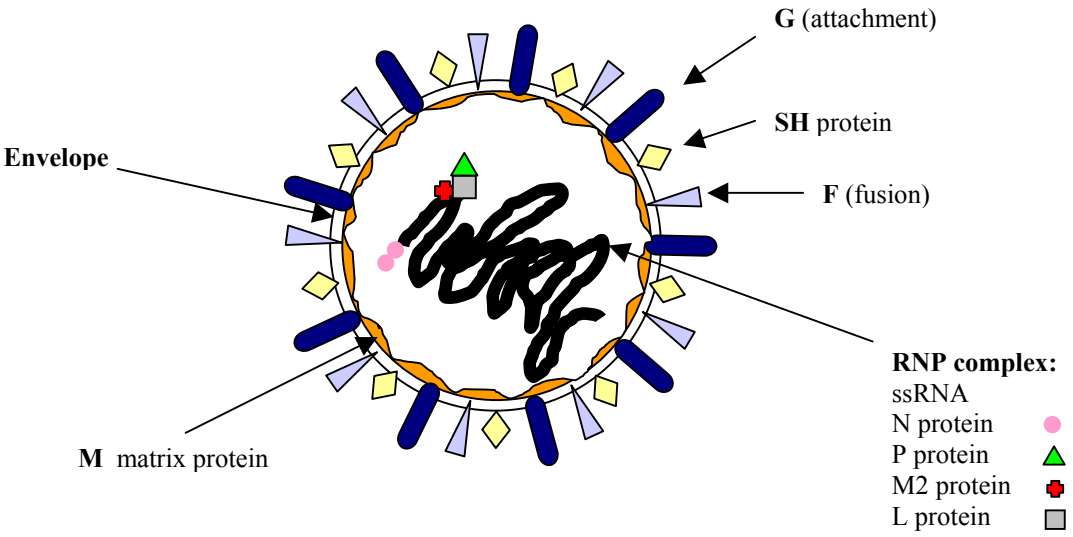

Fig. 1: Respiratory syncytial virus structure. The major nucleocapsid protein N, the phosphoprotein P, the anti-termination factor M2, the large polymerase subunit L and a single-stranded RNA genome of negative polarity compose RNP complex. Three transmembrane surface glycoproteins are present on the surface: the attachment protein G, the fusion protein F and the small hydrophobic SH protein. The matrix protein M forms a scaffold on the inner layer of the envelope connecting the viral membrane with the RNP complex.

The envelope is a lipid bilayer derived from the host plasma membrane and containing on the surface virally encoded transmembrane glycoproteins (G), the fusion protein (F) and the small hydrophobic (SH) protein. The inner layer is scaffold by the matrix protein M.

These viral glycoproteins are assembled in “spikes” closely spaced on the surface and exert attachment functions.

2.1.2 Genome organization

The RSV genome is a non-segmented single-stranded negative RNA of about 15.000 nucleotides. RSV genome contains 10 genes, transcribed in 11 major subgenomic mRNAs. All mRNAs are capped at the 5’-end and polyadenylated at the 3’-end presumably by the viral polymerase. Instead, genomic and anti-genomic RNA, the latter represents the intermediate in RNA replication, are neither capped nor polyadenylated and are component of the nucleocapsids. Transcription is initiated from the 3’-end in an obligatory sequential manner with only a fraction of the polymerase moving to the next gene. This mechanism creates a gradient of transcriptional attenuation with distance from the transcriptional start-site, indicating the required relative abundance of the encoded proteins (fig.2).

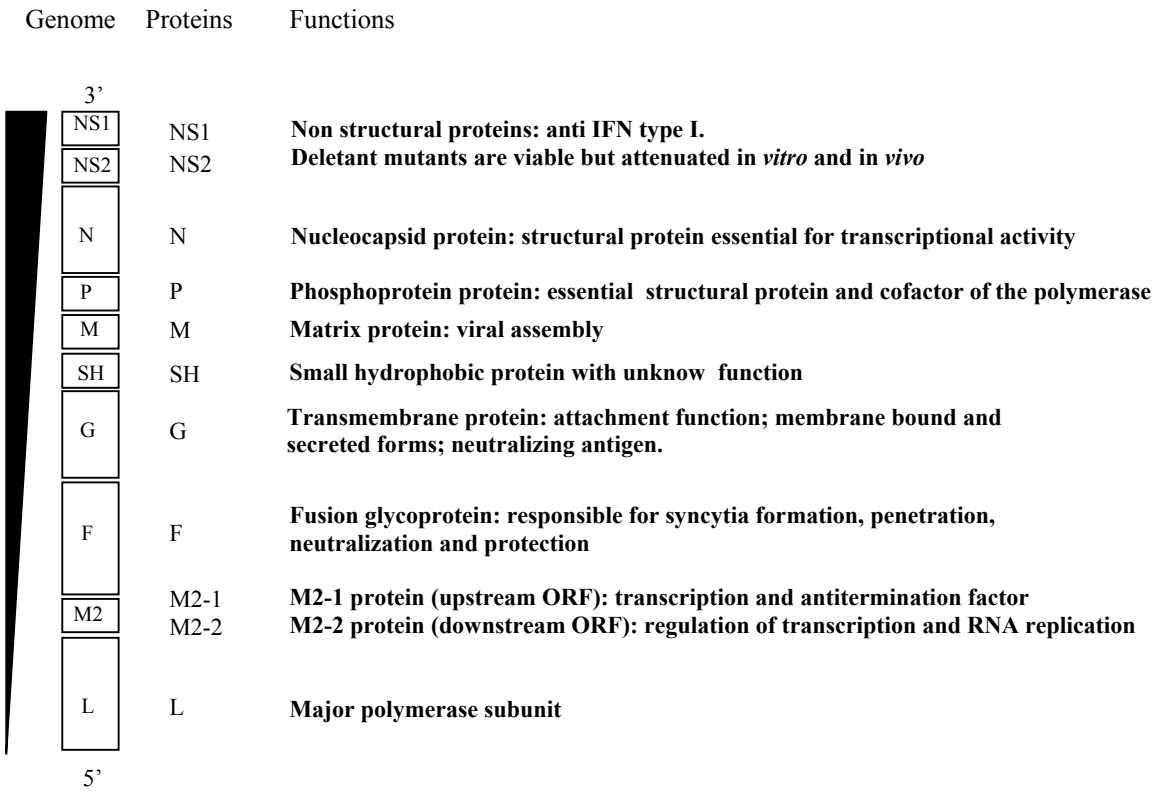

Fig. 2: RSV genes and encoded viral proteins.

At the genes junctions, semi-conserved gene-end (GE signal: 12-13 nucleotides) and conserved gene-start (GS signal: 10 nucleotides) sequences are separated by non-conserved intergenic sequences of different length lacking consensus sequence motifs.
The first 54 nucleotides at the 3' -end of the genome represent a conserved promoter sequence containing the essential signals for transcription and replication. The leader sequence (Le) within this region is necessary, together with the GS signal, for synthesis of the antigenome during RNA replication (McGivern D.R. et al., 2005).

2.1.3 Viral proteins

The RSV F protein is a transmembrane glycoprotein and it is thought to form trimers. It plays a role in viral entry, mediating surface fusion of the virion envelope with the host cellular plasma membrane.

Late in infection, it is also involved in viral spread by syncytia formation. Like in other Paramyxoviruses, the RSV fusion protein is synthesized as a precursor F_0 that is activated by cellular protease, such as furin. The cleavage results in the generation of disulfide-linked F_2 and F_1 subunits. This process leads to the exposure of the hydrophobic "fusion peptide" at the N-terminus of the F_1 subunit which inserts in the cell membrane. Moreover, as a consequence of the furin-cleavage, an intervening peptide of 27 amino acids (pep27) is released. Recent data indicate pep27 importance in intracellular transport, maturation and biological activity of the fusion protein F (Konig P. et al., 2004). Syncytia formation seems to be also coadjuvated by G and SH proteins. Together with the glycoprotein G, it is the major protective and neutralizing agent. In fact, antibodies raised against F or G protein are able to neutralize infectivity in *vitro* and to confer resistance to RSV infection in *vivo* (Taylor G. et al., 1984; Walsh E.E. et al., 1984). Hints of an immunosuppressive activity of the F protein derive from its ability to arrest T cell cycle by contact in a species-specific manner (Schlender J. et al., 2002).

The RSV G protein is a type II transmembrane protein extensively N- and O-glycosylated and seems to form trimers or tetramers. It mediates viral attachment but it is not essential for propagation in *vitro* and in *vivo*. Antibodies raised specifically against the G protein can prevent virus binding to HeLa cells. In addition to a membrane-bound form, a truncated soluble G protein arises from translational initiation at the second AUG in the ORF. This secreted form is involved in counteraction of the immune response. RSV viruses deficient in soluble G protein (ΔsG) induce an increase of certain chemokines, such as IL-8

and Rantes in comparison to wild-type strains (Arnold et al., 2004). Moreover, an activity in trapping RSV-neutralizing antibodies cannot be excluded (Collins P.L. et al., 2001). Deletion mutant RSV viruses for the G protein (ΔG) are viable but display a host range restriction in growth.

The small hydrophobic SH protein is a short integral transmembrane protein and it is present in glycosylated and non-glycosylated species in form of oligomers. Its function is so far unknown. Recombinant RSV viruses lacking SH protein fully replicate in *vitro* but attenuation in the lower respiratory tract in *vivo* has been reported (Jin H. et al., 2000). Since expression of SH protein in bacteria increased permeability of small molecular-weight compounds, it has been speculated its involvement in membrane channels formations (Perez M. et al., 1997).

The matrix protein M is a non-glycosylated protein forming a sheet on the inner side of the viral envelope. It plays an important role in virus assembly and budding by mediating the association between viral nucleoprotein (vRNP) and cell plasma membrane (Peeples M.E., 1991; Ghildyal R. et al., 2002). Apart from inactivating transcription activity of the nucleoprotein before packaging, early in infection M protein it localises into the nucleus where it possibly inhibits host-cell transcription (Ghildyal R. et al., 2003). RSV M protein has also RNA-binding capacity as recently described (Rodriguez L. et al., 2004) but the real function of this interaction is still unclear.

RSV N, P and L proteins co-purify with nucleocapsid. They are necessary and sufficient for RNA replication. The major nucleocapsid protein is the nucleoprotein N. It binds to genomic and antigenomic RNA conferring RNase resistance to the nucleocapsids. The phosphoprotein P is the major phosphorylated species. It functions as a chaperonin for soluble N and it is essential together with N protein for encapsidation activity. P protein is also a polymerase cofactor. It seems to convert initiated polymerase into a stable complex and its phosphorylation is mandatory for this function (Dupuy L.C. et al., 1999). The L protein is the major RNA-dependent RNA polymerase subunit and it is bound to its cofactors, the phosphoprotein P and M2-1 by the N protein.

The M2-1 protein is a transcription processivity factor and it is essential for viral replication. It prevents premature termination during transcription (Fearns R. and Collins P.L., 1999; Zhou et al., 2003) and enhances read-through at the gene junctions (Hardy R.W. and Wertz G.W., 1998; Hardy R.W. et al., 1999). The M2-1 protein interacts with the nucleocapsid N protein through RNA mediation (Cuesta I. et al., 2000; Cartee T.L. and Wertz

G.W, 2001) and with the P protein (Mason S.W. et al., 2003). Phosphorylation of M2-1 appears to be indispensable for the interaction with the P protein. RSV infection is characterised by persistent NF-kB activation and recently M2-1 protein has been identified as inducer of Rel A (p65), a protein of the mammalian NF-kB complex (Reimers K. et al., 2005). The M2 mRNA encodes for the M2-1 protein by the 5'-proximal ORF, while the M2-2 protein originates by the 3'-proximal open reading frame. M2-2 protein has a possible role in RNA synthesis regulation mediating the switch from transcription to genome replication (Collins P.L. et al., 1996). Recombinant viruses lacking M2-2 protein expression (ΔM2-2) are attenuated in *vitro* and in *vivo* compared to wild type (Jin H. et al., 2000 b).

NS1 and NS2 are proteins with an estimated mass of about 14-15 kDa. They are found only in pneumoviruses and they have been classified as nonstructural proteins since they are found only in traces in purified virions (Evans J.E. et al., 1996). Their subgenomic mRNAs are the most abundant among the transcripts due to the typical gradient of transcription being their promoter the most proximal in the RSV genome. From the functional point of view, NS1 and NS2 proteins, despite enhancing growth, are not essential. Single and double deletion mutants are viable although displaying an attenuated phenotype both in *vitro* and in *vivo* (Buchholz U.J. et al., 1999; Jin H. et al., 2000; Teng M.N. and Collins P.L., 1999; Teng M.N. et al., 2000). NS1 protein has been reported to be a potent negative regulatory factor of transcription and synthesis of genome and antigenome RNA, most likely acting at early stages of promoter initiation by the viral polymerase (Atreya P.L. et al, 1998).

2.2 Replicative cycle

Binding and entry of RSV into targeted cells are mediated by the interaction between the G and the F proteins with host cell molecules. The specific RSV cellular-receptor has not been identified so far but there are data indicating an interaction of the RSV G protein with cell surface glycosaminoglycans (GAGs), such as heparan sulphate and chondroitin sulphate B (Feldman S.A. et al., 1999). Cell surface GAGs are essential for RSV binding in *vitro* and are therefore important for infectivity (Martinez I. and Melero J.A., 2000). Interestingly, recombinant RSV viruses lacking the G protein are infectious in cell cultures, however they show attenuation in *vivo*, both in human and mouse airway cells (Karron R.A. et al., 1997; Teng M.N. et al., 2001). These results imply that G protein is dispensable for cell attachment but it has other functions that might influence the efficiency of the process. For example, a

putative binding domain to CX3C receptor (CX3CR1), which could favour infection, has been shown (Tripp R.A et al., 2000 and 2001).

Protein F alone can mediate attachment. It is responsible for fusion of the virus envelope with the host plasmame mbrane and for syncytium formation, therefore it is absolutely required during the virus life cycle.

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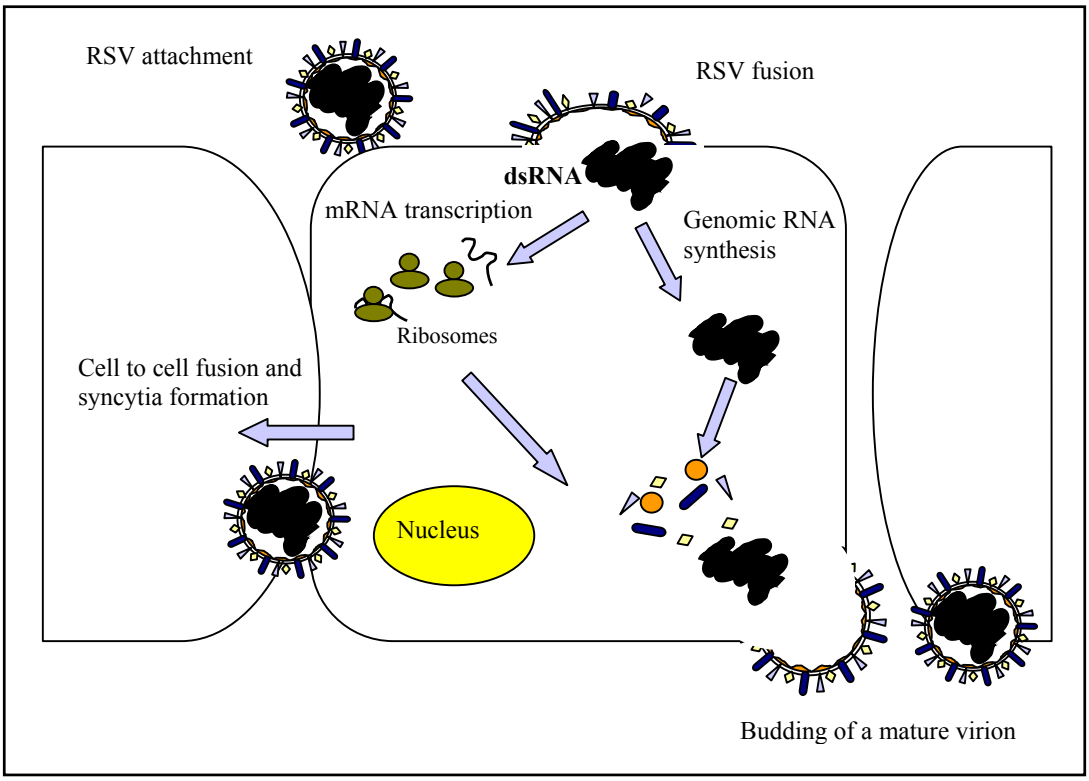

Fig. 3: RSV replicative cycle. The attachment protein G and the fusion protein F mediate RSV entry/uptake. After fusion of the viral envelope with the host plasmamembrane, nucleocapsids are released into the cytoplasm, where the entire replication takes place. RNA transcription and replication is followed by encapsidation and budding on the cell surface of the newly formed virions.

RSV uptake seems to occur by fusion rather than endocytosis although several studies indicate an interaction between RSV and caveolae-enriched membranes (Gingras D. et al., 1998; McCurdy L.H. and Graham B.S., 2003). For example, entry of RSV in bovine dendritic cells (DC) and subsequent antigen presentation to T lymphocytes appears occurs via a caveolae-dependent mechanism (Werling D., 2002a/b).

RSV requires cytoskeletal elements. The microtubule and actin networks play a role in several aspects of the viral life cycle. Perturbation of cytoskeletal functions is negatively reflected on RSV entry, release, cell-cell spreading and syncytia formation (Kallewaard N.L. et al., 2005).
The entire RSV replicative cycle takes place in the cytoplasm and begins with transcription of the genome into 5'-capped and 3'-polyadenylated mRNAs by the viral RNA-dependent polymerase complex. RNA synthesis occurs in a sequential manner from the 3'-end of the genome with the polymerase complex terminating and reinitiating mRNA transcription at each junction. Reinitiating can be occasionally inefficient and this results in a gradient of mRNA decreasing proportionally to the distance of the gene from the 3'-end of the genome.
The polymerase complex is also responsible for antigenomic RNA synthesis. In this case, all the junction signals are completely ignored and the result is a complementary positive-sense copy of the viral genome. The antigenomic RNA represents a replicative intermediate and it is less abundant in comparison to the genomic RNA of about 10-20 fold. Both RNAs are packed into virions in an equal proportion. Antigenomic and genomic RNA synthesis correlates with protein translation indicating a need for cosynthetic encapsidation. In contrast to other Paramyxoviruses, the switch between RSV transcription and RNA replication seems to implicate M2-2 protein and not to depend on the intracellular levels of N and P proteins (Fearns R. et al., 1997). At the early stages of infection, low levels of M2-2 correlates to a high transcriptional rate. Afterwards, when the intracellular levels of M2-2 protein increased, transcription is inhibited in favour of replication (Bermingham A. and Colllins P.L., 1999).
Assembly of the nucleocapsids is entirely cytoplasmatic. N protein associates first with genomic and antigenomic RNA followed by P and L. Once assembled, nucleocapsids are transported close to the cell surface and bud at specific plasma membrane patches where glycoprotein G clusters. Association of the nucleocapsids with the nascent envelope is mediated by M protein, which makes contacts with the cytoplasmatic tails of the viral glycoproteins. However, efficient viral formation in *vitro* is not affected by deletion of G and SH proteins. On the contrary F protein seems to be essential in this context. Assembly is also related to an intact cellular cytoskeleton. (Ulloa L. et al., 1998; Burke E. et al., 2000; Gower T.L. et al., 2001).

2.3 Epidemiology and Pathogenesis

Respiratory syncytial virus (RSV) infection is one of the most important health problems in infancy accounting for about 85% of cases of bronchiolitis and approximately 20% of cases of childhood pneumonia (Wright P.F. and Cutts F.T., 2000). It significantly contributes to hospitalisation of infants in developed countries. Only in the United States, it has been estimated that more than 120.000 children younger than 1 year are hospitalised annually, with about 200 deaths as a results of this illness (Shay D.K. et al., 2001). Of course the scenario in countries with less well-developed medical care programs is even more serious. Bronchiolitis and pneumonia occur most frequently between the 6 weeks and 9 months of age, showing a peak in coincidence of the dropping of maternal antibody titers around the second-seventh month. Fortunately, the risk of severe illness related to RSV is quite low in developed countries. However, several groups of infants might be more predisposed to a severe outcome, like in the case of infants with chronic lung disease of prematurity, congenital heart disease or compromised immunity. In addition RSV is an important pathogen in the elderly.
RSV has a worldwide distribution and it is a seasonal infection, with peaks around winter and/or spring (Stensballe L.G. et al., 2003). Persistence, in *vivo*, has been postulated to explain the apparent absence of the virus between epidemics. There are experimental indications demonstrating that, for example BRSV-infected B lymphocyte can be isolated in calves 10 weeks after infection and that B-lymphocytes cell-lines show persistent infection in *vitro* for 6 months (Streckert H.J. et al., 1996; Valarcher J.F. et al., 2001).
Transmission occurs via contact with respiratory secretions. The incubation period can vary between 2-8 days and it is followed by symptoms related to upper and lower respiratory tract infection.

Veterinary pathogens, belonging to the same subfamily of the human respiratory syncytial virus (HRSV), have been identified. The avian metapneumovirus (APV) is the causative agent of the turkey rhinotracheitis (Njenga M.K. et al., 2003) and probably of the swollen head syndrome in chickens (Cook J.K., 2000). Outbreaks follow a seasonal pattern and wild migratory birds might be involved in virus spreading. APV causes severe upper respiratory infections with high mortality and big economic loss for the industry as seen in the late nineties in USA (Panigrahy B. et al., 2000; Jirjis F.F. et al., 2002). The pneumonia virus of mice (PMV) was isolated for the first time in 1938 and it seems not to be an important disease

in wild mice, with the exception of immunocompromised subjects. PMV represents an alternative model to study pneumovirus-related disease in rodents (Cook P.M. et al., 1998) and recently its full genome has been published (Krempl C.D. et al., 2005). APV is closely related to a newly described human metapneumovirus (HMPV), which is responsible for a clinical syndrome practically indistinguishable from the one related to HRSV (van den Hoogen B.G. et al., 2001; van den Hoogen B.G. et al., 2002; Kahn J.S., 2003).

Sheep and goats have also distinct RSV pathogens (Trudel M. et al., 1989; Brogden K.A. et al., 1998). Bovine pneumovirus (BRSV) has been isolated in the 70s and research on this virus has been encouraged by the fact that BRSV represents the most close phylogenetic-relative of HRSV. BRSV shares with the human counterpart the same way of transmission and the seasonality of the outbreaks, but, unlike HRSV, bovine pneumovirus infections are often complicated by concomitant bacterial infection, e.g. Pasteurella multocida, Haemophilus somnus (Woolums A.R. et al., 2004; Gershwin L.R. et al., 2005).

RSV-illness usually begins with infection of the upper respiratory tract and it is characterised by non-specific symptoms: fever and rhinorrhea that can last several days. When the lower respiratory tract gets involved, the outcome can be pneumonia with tachypnea, difficulties in breathing, wheezing upon auscultation or bronchiolitis that might impair pulmonary functions for long time and/or predispose to asthma (Psarras S. et al., 2004). Morbidity can be increased by many predisposing conditions like premature birth, heart diseases, immunodeficiency (Welliwer R.C., 2002). Host factors, especially related to aberrant inflammatory response, have been associated to severe RSV disease. Particular emphasis has been paid to the potential role of proinflammatory chemokines and cytokines, whose expression is up-regulated in RSV-related disease and, in some cases, contributes to exacerbate the detrimental effects of the primary infection. High levels of IL-1β, IL-6, IL-8, TNF-α, MIP1α (macrophage inflammatory protein), RANTES and adhesion molecule ICAM-1 have been detected in respiratory secretions. The productions of these factors follow a biphasic pattern with an early peak during RSV active infection and a later peak not related to viral replication (Miller A.L. et al., 2004; Kong X. et al., 2005).

Prominent pathological features of severe RSV infections are necrosis of the airway epithelium, interstitial inflammation with lymphocytes, plasma cell and macrophages infiltrations, mucus secretion leading to obstruction of the airways and a general respiratory compromise.

Similarly, BRSV induces mild to severe respiratory signs especially among calves and it has shown to be involved in the paroxystic respiratory distress syndrome (PRDS) (Jolly S. et al., 2004).

2.4 Immunity

Natural infection with RSV does not provide efficient protection against reinfections, indicating that the acquired immunity in this case is neither complete nor durable. Ineffectiveness of the immune response against RSV infection has hampered the development of effective vaccines against BRSV and HRSV. The other major problem is that prior vaccination can enhance the severity of the disease during subsequent natural infection. This has been already observed in children in the 60s during trials with formalin-inactivated (FI) or alum-adjuvant vaccines (Kim H.W. et al., 1969). Similarly, in animals inactivated vaccines were responsible for immunopathological states in calves infected with BRSV (Schreiber P. et al., 2000; Antonis A.F. et al., 2003; Kalina W.V. et al., 2005).

Severe RSV-disease following natural infection or immunization can be attribute to impairment of type 1/2 phenotype balance with a dominance of Th2 lymphocyte response. Infants and mice with severe bronchiolitis show an augmented eosinophilia and an increase in type 2 cytokine levels, e.g. IL-4, IL-5, and IL-13 (Roman M. et al., 1997; Boelen A. et al., 2002; Johnson T.R.et al., 2005).

Other studies have emphasised more the potential role of chemokine production than the Th2 cytokines levels as a key factor of RSV immunopathogenesis. In particular the beta chemokine macrophages inflammatory protein-1alpha (MIP1α), the monocyte chemotactic protein 1 (MCP-1) and the regulated on activation normal T lymphocyte expressed and secreted (RANTES) are known to attract lymphocytes, basophils and eosinophils and they are associated with greater inflammatory response in severe RSV-illness (Hornsleth A. et al., 2001; Garofalo R.P. et al., 2001 and 2005).

2.4.1 Humoral immunity

Partially protective antibodies against F and G proteins are produced during natural infection. Passive immunization with immunoglobulin preparations containing human RSV-specific Ig

or monoclonal anti-F antibodies have shown to be able to attenuate the severity of the disease (Prince G.A. et al., 2000; Simoes E.A. and Groothuis J.R., 2002; Sastre P. et al., 2004).
Unfortunately not all humoral responses are favourable. RSV-specific IgE for example may also contribute to increase the severity of the disease (Welliver R.C. et al., 1981; Tumas D.B. et al., 2001; Dakhama A. et al., 2004). RSV is able to infect infants in the presence of moderate titers of maternal antibodies. Secretory antibodies (IgA) are defective in neutralizing the virus in *vitro* (McIntosh K. et al., 1978), which could explain the failure of natural immunity during early ages. In adults, higher levels of IgA are produced as a result of reinfections and in experimental trials immunity showed to better correlation with RSV-neutralizing secretory antibodies than with serum neutralizing immunoglobulin. Nevertheless secretory IgA gives only partial protection and do not confer resistance to reinfections (Gleeson M. et al., 2004; Walsh E.E. and Falsey A.R., 2004). Humoral immunity seems not to provide complete protection against RSV infection and current hypotheses point the importance of the cell-mediated immune response in viral clearance.

2.4.2 Cell-mediated immunity

Infants with a primary RSV infection develop a cellular immune response within 10 days. In the BALB/c mouse model the first to appear are natural killer (NK) cells followed by $CD8^+$ cytotoxic T cells (CTL), which can further modulate the immunity by secretion of lymphokines, especially IFN γ (Chiba Y. et al., 1989; Graham B.S. et al., 1991; Johnson T.R. et al., 2002). Human cytotoxic T lymphocytes recognize mainly HRSV N protein and also SH, F, M, NS1, M2, and NS2 but not G protein (Cherrie A.H. et al., 1992; Heidema J. et al., 2004). In a mouse model, where BALB/c mice were infected with HRSV, CTLs major target was M2 followed by F and N proteins (Openshaw P.J. et al., 1990; Jiang S. et al., 2002). Similar studies have been carried out also in cattle. The recognition pattern of bovine $CD8^+$ T lymphocytes includes F, N, M2 proteins and, differently from human and mice, the G protein may also elicit CTL activation (Gaddum R.M. et al. 1996 and 2003).
Vaccination with recombinant vaccinia virus (rVV) expressing RSV proteins or with recombinant viral proteins prime $CD8^+$ T cell response and can mediate protection (Connors M. et al, 1992; Taylor G. et al., 1997; Zeng R.H. et al., 2005). However, the derived immunity has a short duration and this is due to the capacity of RSV to interfere with T-cell receptor (TCR)-mediated signalling (Connors M. et al, 1991; Chang J. and Braciale T.J., 2001). In

conclusion $CD8^+$ and also $CD4^+$ T-lymphocytes appear to play important roles in virus clearance but, also may also contribute to lung pathology (Alwan W.H. et al., 1991; Taylor G. et al., 1995; Rutigliano J.A. and Graham B.S., 2004).

2.4.3 Innate immunity

Due to the difficulties to induce an efficient and safe protection towards RSV-infection via induction of the adaptive immune response, many recent studies have been focusing on the innate antiviral host defences. Respiratory epithelial cells, as well as being the principal target of RSV-infection, represent also the first line of defence of the innate immune response before the involvement of professional antigen presenting cells (APC), namely macrophages (MΦ) and dendritic cells (DC).

Respiratory epithelial cells release nitric oxide (NO) upon infection, produce opsonins and collectins, which are important in virus clearance, and secrete inflammatory mediators, such as chemokines, leucotrienes and cytokines (Olszewska Pazdrak B. et al., 1998; Barr F.E. et al., 2000; Hacking D. et al., 2002; LeVine A. et al., 2004). Release of such inflammatory factors initiates maturations of neutrophils, eosinophils, macrophages and $CD4^+$ T helper chemiotaxis. Alveolar macrophages are very important in the innate immune defence against RSV. They regulate the ensuing immune response by releasing proinflammatory cytokines: tumor necrosis factor (TNF) and interleukin 10 (IL-10) which synergistically enhance opsonization; IL-6 and IL-8. Alteration of macrophages (MΦ) and dendritic cells (DC) upon RSV infection has been observed. In particular, RSV-induced release of IL-10 is responsible for a local immunosuppressive activity and a Th2 bias shift. Thus, increased production of IL-10 concomitant with a reduction in IL-12 levels, which instead supports Th1-type immune response, leads to a reduced production of interferon gamma by T cells (Bartz H. et al., 2003; Schauer U. et al., 2004).

In macrophages and epithelial cells, RSV induces activation of NF-kB, which in turn stimulates transcription of genes linked to antiviral response (Bitko V. et al., 1997; Tian B. et al., 2004). NF-kB is an ubiquitously expressed transcription factor that is present in the cell cytoplasm as a complex of homo- and heterodimers of Rel family members (p65/RelA; RelB; c-Rel; p100/p52 and p105/p50). In unstimulated cells, NF-kB is held into an inactive state by the inhibitory IkBα proteins. Phosphorylation and proteasomic degradation of IkBα mediates

NF-kB activation. Activation consists in NF-kB translocation to the nucleus and binding to the promoter/enhancer of targeted genes (Baldwin A.S. et al., 1996).
NF-kB regulates expression of cytokines in response to ligation of many receptors involved in immunity. Numerous pathways lead to NF-kB activation. The so-called "classical" pathway has inputs from tumor necrosis factor receptors (TNFR1/2), T and B cell receptors, Toll-like and IL-1 receptors (TRL/IL-1R). The "alternative or noncanonical" pathway, which goes through NIK (NF-kB inducing kinase) activation, responds to ligands to lymphotoxin-β receptor and CD40 (Bonizzi G. and Karin M., 2004). Interestingly, early in infection RSV induces NIK activity and consequent activation of the "noncanonical" NF-kB activation pathway. This pathway is independent of activation of IKK-β, which occurres only later in the course of the infection with involvement of the "classical" pathway (Choudhary S. et al., 2005). NF-kB plays an essential role in early stages of innate immune response especially via the Toll-like receptor (TLR) signalling pathway (Haeberle H. et al., 2002; Cusson-Hermance N. et al., 2005). Toll-like receptors are evolutionary conserved pattern recognition receptors, which are responding to pathogen-associated molecular patterns (PAMPs). PAMPs include lipopolysaccharids (LPS), nonmethylated CpG DNA and dsRNA (Medzhitov R., 2001; Barton G.M. and Medzhitov R., 2003; Gelman A.E. et al., 2004).
Cytokine and chemokine production in RSV-infected cells involves the Toll-like receptor signalling pathway. RSV is known to express potent activators of Toll-like receptors and the role of several TLRs is currently under extensive examination. Up-regulation of TLR3 in human lung fibroblasts and epithelial cells has been shown, while involvement of TLR4 is still under controversy (Haynes L.M. et al., 2001; Ehl S. et al., 2004 and Rudd B.D et al., 2005). Recently, RSV has been reported to switch off the activation of TLR-7 and -9 in PDCs. (Schlender J. et al., 2005).

2.4.4 The interferon α/β system

The interferons (IFN) are heterogeneous family of inducible cytokines, originally identified on the basis of their biological activity in determining antiviral resistance in cell culture. Interferons are commonly classified into two types, which are functionally not redundant in host antiviral defence. The type II interferon is known as IFN-γ and it is considered to be a regulator of the adaptive immune response. IFNγ is induced upon mitogenic or antigenic stimuli mainly by haematopoietic-derived stemm cells, like T cells ($CD4^+$ Th1 and $CD8^+$) or

natural killer cells (NK).Type I interferons are the main cytokines for innate immune responses against viral infection. They are produced by many types of cells, from leucocytes to fibroblasts, in response to different viruses and their induction is primarily controlled at the transcriptional level. The type I interferons include IFN-α_{I-II}, IFN-ϖ, IFN-τ and IFN-β and their genes cluster on chromosome 9 in humans and chromosome 4 in the mouse. Most of the type I interferons are posttranslationally glycosilated, except human IFN-α, and they can function as mono- or homodimers (reviewed in Samuel C.E., 2001). Spontaneous production of IFN-α/β in absence of viral infection has been reported. There are indications that interferonmight be involved in antitumoral activities and cell-growth regulation (Gresser I., 1990).

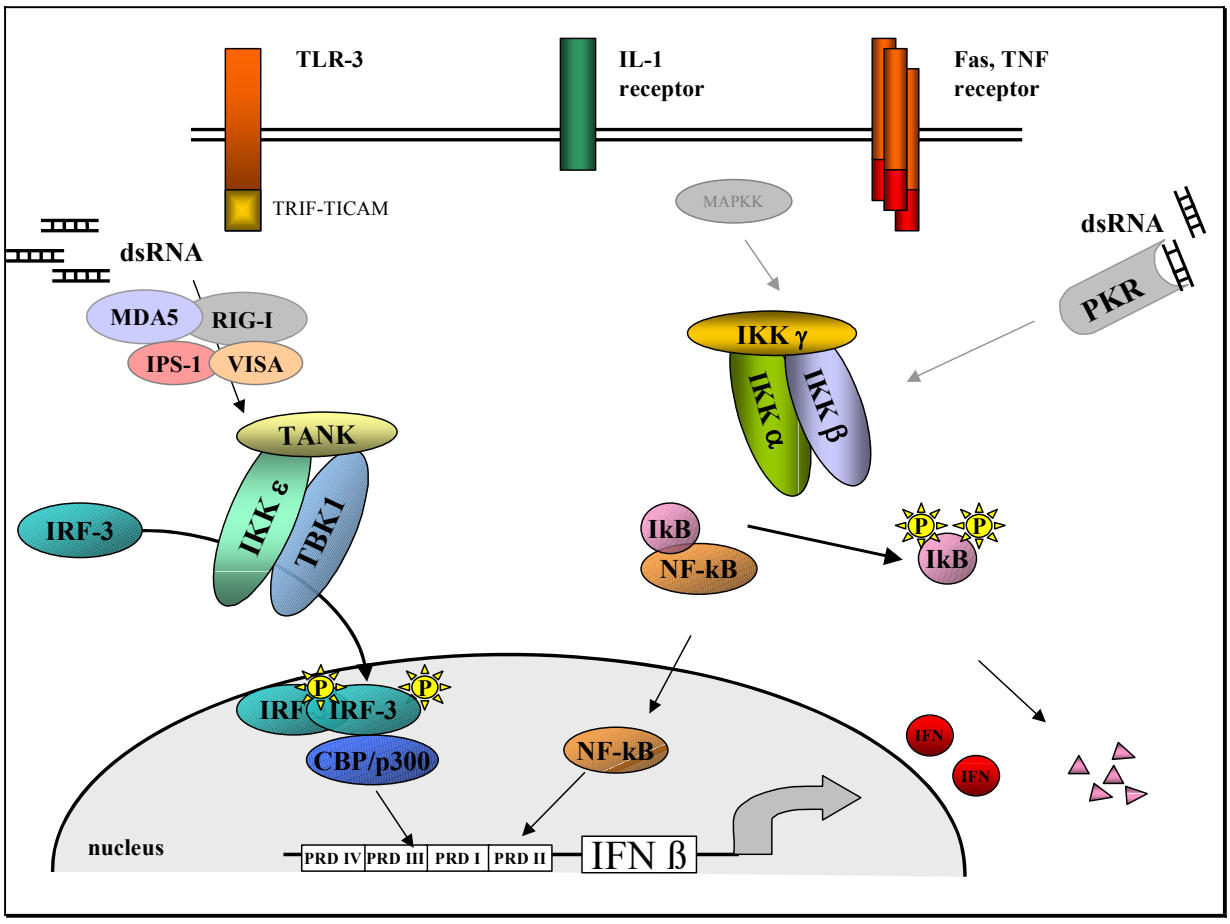

Fig. 4 Transcriptional induction IFNB gene through a Toll-like-receptor-independent signalling pathway. Viral dsRNA is sensed at the intracellular level by the helicases RIG-I and MDA5, which initiate a "downstream" signalling. IPS-1 mediates activation of the IFN-β promoter by TBK1/IKK-ε-dependent IRF3 phosphorylation.

Many viruses can induce activation of IFNA/B genes transcription. Activation occurs when members of the IRF (Interferon Regulatory Factors) family bind to regulatory sequences in the IFN-α/β gene promoters. The IRF family consists in 9 transcriptional activators of which IRF3 and IRF7 are essential for IFN-α/β expression. IRF3 is constitutively expressed in all cells and resides into the cytoplasm of unstimulated cells in a latent form. IRF3 activation is mediated by dsRNA and virus infection. Upon activation, IRF3 undergoes serine/threonine phosphorylation, dimerization, nuclear translocation, and after association with p300/CBP coactivator, DNA-binding at consensus sites (Lin R. et al., 1998). Transcriptional activity of IRF3 is controlled by C-terminal phosphorylation, which is carried out by a newly identified virus-activated kinase complex, whose components are the IKK-related kinases TBK1 and IKK-i/ε (Fitzgerald K.A. et al., 2003; Sharma S.et al., 2003; McWhirter S.M. et al., 2004). Activation of the TBK1/IKK-ε-mediated IFN-β signalling pathway is mediated by the retinoic-acid inducible gene I (RIG-I) and the melanoma differentiation associated gene 5 (MDA5). RIG-I and MDA5 are cytoplasmatic RNA elicases responsible for dsRNA recognition. By gene targeting, it has been shown that RIG-I is essential for induction of type I interferons after infection of fibroblasts and conventional dendritic cells (DCs). RIG-I activates IRF3 via TBK1 and IKK-ε (Yoneyama M et al., 2004; Hiroki K. et al., 2005). Moreover, RIG-I and MDA5 interact with the newly identified interferon-beta promoter stimulator 1 (IPS-1) and the virus-induced signalling adaptor VISA in sensing viral infection and in the activation of IFN-α/β induction signalling pathway (Kawai T. et al., 2005; Xu L.G. et al., 2005).

IFN-α/β signalling pathway is mediated by a common receptor complex IFNAR, which consists in two subunits, IFNR1 and IFNR2. Upon ligand-induced stimulation of IFNAR, two receptor-associated Janus protein tyrosine kinases, Jak1 and Tyk2, become cross-activated. This is followed by tyrosine phosphorylation of two members of the family of signal transducers and activators of transcription (STATs), namely STAT1 and STAT2. Activation of STATs leads to the formation of two transcriptional-activator complexes, IFN-α-activated factor (AAF) and IFN-stimulated gene factor 3 (ISGF3). ISGF3 consists of activated STAT1, STAT2 and the interferon regulatory factor (IRF) 9. This trimeric complex locates into the nucleus and binds to a cis-acting DNA element, designated ISRE, which is present in IFN-α/β inducible genes. Among these IFN-inducible genes, some encode for proteins implicated in

antiviral activities: the RNA-dependent protein kinase (PKR); the 2', 5'-oligoadenylate synthetase (OAS); RNase L and the Mx protein GTPases (Lau J.F. and Horvarth C.M., 2002).

2.5 Viral antagonists of IFN type I response

Both DNA and RNA viruses encode for proteins and have developed strategies to impair IFN response. Circumventing or blocking IFN activity delays the generation of an acquired immunity and allows viruses to successfully establish infection. Viral countermeasures can affect IFN production or target IFN signalling. Several viruses have been reported to prevent IFN induction by sequestration of dsRNA activators of PKR or 2',5'oligoadenylate synthetase/Rnase L system. dsRNA-binding proteins are encoded by vaccinia virus (E3L), reovirus (capsid protein σ3), rotavirus (NSP3) and influenzavirus (NS1) (Chang H.W. et al., 1992; Lu Y. et al. 1995; Bergeron J. et al., 1998). A direct antagonism of PKR has been also observed in poliovirus, adenovirus, SV40 and hepatitis C virus (HCV) (Black T.L. et al.1993; Gale M. and Katze M.G., 1998). Encephalomyocarditis virus (EMCV) and HIV instead downregulate RNase L (Martinand C.et al., 1998 and 1999). Another common strategy to block IFN production consists in repressing transcriptional activation of IFN-α/β promoter. For example, human herpesvirus 8 (HHV8) synthesizes an IRF homologue that is able to block the transcriptional complex CBP/p300-IRF3 (Gao S.J. et al., 1997). The E6 protein of human papillomavirus type 16 (HPV-16) binds IRF3 and therefore inhibits its activity (Ronco L.V. et al., 1998). The nonstructural proteins (NS1 and NS2) of BRSV antagonize IRF3 phosphorylation (Bossert B. et al., 2003).

The IFN signalling pathway can be targeted as well by several viruses and its block can be achieved by multiple mechanisms. Poxviruses, for example, encode soluble IFN receptor homologues (vIFN-Rc). These secreted viral proteins sequester cellular IFNs, antagonizing their binding to natural receptors (Smith G.L. et al., 1998). Simian 5 virus (SV5) or mumps virus (MV) induce degradation of STAT1, while parainfluenza virus type 2 elicits degradation of STAT2 , thereby preventing the formation of ISGF3 complexes (Dideock L. et al., 1999; Young D.F. et al., 2000). Adenovirus affects DNA-binding of ISGF3 via the E1A protein (Leonard G.T. and Sen G.C., 1997). Among Herpesviruses, varicella-zoster virus (VZV) inhibits expression of STAT1 and JAK2, whereas human cytomegalovirus (HCMV) blocks STATs phosphorylation by inducing degradation of JAK1 and IRF9 (Miller D.M.et al., 1998; Abendroth A. et al., 2000).

Respiratory syncytial virus is resistant to type I IFN but conflicting results about its ability to affect type I IFN-signalling have been reported. Most of the data indicate that inhibition of the IFN-signalling pathway is not involved. IFN-mediated MxA expression is maintained in epithelial cells infected with HRSV or BRSV. Besides no decrease of STAT1 and STAT2 levels has been observed (Atreya P.L. and Kulkarni S.,1999; Young D.F. et al., 2000; Bossert B. et al., 2003). Other groups have been arguing that RSV does block JAK/STAT pathway by decreasing STAT2 expression (Ramaswamy M. et al., 2004; Lo M.S. et al., 2005).

2.6 The RSV nonstructural proteins NS1 and NS2

Pneumovirus is the only genus in the Paramyxoviridae family whose members encode the NS1 and NS2 proteins. The two NS genes are located at the 3' end of the negative-strand RNA genome and, due to a characteristic transcriptional gradient, the transcripts of these genes are abundantly expressed in infected cells. NS proteins are not essential for RNA replication, however recombinant RSV lacking NS genes are severely attenuated in *vitro* and in *vivo* (Teng M.N. and Collins P.L., 1999; Schlender J. et al., 2000; Valarcher J.F. et al., 2004). The NS1 and NS2 genes of HRSV subgroup A are 528 and 499 nucleotides long with single open reading frames encoding polypeptides of 139 and 124 amino acids, respectively. Similarly, NS1 and NS2 genes of BRSV strain A51908 have 524 and 489 nucleotides which encode for polypeptides of 136 and 124 amino acids, respectively. Comparison of the sequences of HRSV NS proteins with the corresponding BRSV revealed amino acid identity of 69% for NS1 and 84% for NS2 protein (Collins P.L. and Wertz G.W., 1985; Pastey M.K. and Samal S.K., 1995). HRSV NS1 protein coprecipitates with M protein and interacts with the C-terminal region of phosphoprotein P. The NS2 protein, despite colocalizing with N and P proteins in cytosolic "inclusion bodies", does not coprecipitate with any viral protein (Evans J.E. et al., 1996; Hengst U. and Kiefer P., 2000; Bossert B. et al., personal observation). Nonstructural proteins of pneumoviruses do not show common features of other known proteins or functional domains that would suggest their function. NS proteins are abundantly transcribed in RSV-infected cells and can block induction of IFN-α/β. Conzelmann and colleagues generated BRSV mutants in which NS1 and NS2 genes have been deleted singly (ΔNS1; ΔNS2) or in combination (ΔNS1/NS2). All three mutant viruses display a slight attenuation in their growth in BSR T7/5 and Vero cells. Similar results have been also obtained with the human counterpart HRSV. These results suggest that NS proteins are

dispensable for viral growth but they also indicate their involvement in viral replication (Buchholz U. et al., 1999; Teng M. and Collins P.L., 1999). In cultured cell lines that are competent for type I IFN production, as in *vivo*, deletion HRSV and BRSV viruses are severely impeded in their replication providing evidence that NS1 and NS2 proteins independently or cooperatively subvert IFN-α/β-mediated antiviral state (Jin H. et al., 2000 and 2003; Valarcher J.F. et al., 2003). NS proteins can therefore be considered as potent antagonists of IFN induction.

Our studies demonstrate that BRSV impairs type I IFN production by preventing the activation of IRF3. In wild-type BRSV-infected cells, but not in cells infected with the double deletion mutant BRSV ΔNS1/NS2, phosphorylation of IRF3 is selectively blocked. This leads to the suppression of IRF3 transcriptional activity in infected cells and compromises the subsequent establishment of an IFN-mediated immune response (Bossert B. et al., 2003).

Similarly to BRSV, wild-type HRSV poorly induces type I interferons, in contrast recombinant viruses lacking NS1 and NS2 genes do increase dramatically expression levels of IFN-β (Spann K.M. et al., 2004).

3 MATERIALS AND METHODS

3.1 Cells culture and viruses

3.1.1 Cells

Vero and HEp_2 cells were obtained by the American Type Culture Collection (ATCC; Rockville, MD) and maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) supplemented with 5% fetal calf serum (FCS) and 1% Penicillin/Streptomycin 100x (Sigma cell culture). For Vero-p125Luc cells, stably transfected with the IFN-β promoter/enhancer, 1 mg/ml of G418 were added to the medium. 293 and A549 cells were cultured in DMEM containing 10% FCS and antibiotics. For 293 cells the medium was additionally supplemented with 1% L-glutamine (Gibco BRL) . Baby hamster kidney cells stably expressing T7 RNA polymerase (BSR T7/5) were propagated in BHK-21 medium (Glasgow MEM, Gibco) containing 10% newborn calf serum (CS), 2% MEM amino acids (Gibco BRL), 2% Tryptose phosphate broth 50x (Gibco BRL), 1% Penicillin/Streptomycin and 1 mg/ml of G418 .

3.1.2 RSV propagation and titer determination

Human respiratory syncytial virus (HRSV) subgroup A strains A2 and Long, were obtained by the American Type Culture Collection. Recombinant bovine respiratory syncytial virus (rBRSV) was derived from BRSV strain A51908 (American Type Culture Collection) variant Atue51908 (GeneBank accession no AF092942). For production of RSV stocks, 80% confluent Vero cells grown in a 75 cm^2 tissue culture flask were infected at a multiplicity of infection (MOI) of 0.1 in serum-free DMEM. After absorption for 1-1.5 hours, the inoculum was removed and cells were incubated in DMEM supplemented with 2.5% FCS. When an extensive cytopathic effect (syncytia formation) was observed, virus was released by freezing and thawing. After centrifugation at 3,500 rpm (Heraeus Varifuge 3R) for 5 min to remove cellular debris, the supernatant was aliquoted and stored at −70°C. Virus titers were determined by limiting dilution in microwell plates. A confluent 75 cm^2 flask of Vero cells was trypsinized and resuspended in 20-30 ml of DMEM containing 5% FCS, 100 µl were distributed in each well of a 96-well microtiter plate. Virus stocks were stepwise 10-fold

diluted in serum-free DMEM and 100 µl of each dilution were pipetted into the wells. After 4 days, cells were fixed with 80% acetone for 20 min at 4°C and air-dried. Infected cell foci were stained with a monoclonal antibody recognizing the RSV nucleoprotein N (Serotec, diluted 1:75 in PBS) for 60 min at room temperature. After washing with PBS for three times, a FITC-conjugated anti-mouse antibody (dilution 1:100 in PBS) was applied for 60 min at RT. Wells were washed three times with PBS and once with dH_2O; foci were counted using a fluorescent microscope (Olympus, IX71).

3.1.3 Rabies virus (RV) stocks and titer determination

A recombinant rabies virus carrying nucleotide sequence of Street Alabama Dufferin B19 (rRV SAD L16), a recombinant RV where the P coding sequence was replaced with eGFP-P fusion protein (SAD eGFP-P) and mutant SAD eGFP-Ps harbouring the HRSV NS1 and NS2 genes (SAD eGFP-Ph2/1 and SAD eGFP-Ph2*^{T26I}/1) were propagated in BSR T7/5 cells.
A 25 cm^2 tissue culture flasks 80% confluent were infected at an MOI of 0.1 for 1 hour. Cells were then incubated in Glasgow MEM supplemented with 10% CS and supernatants were harvested after 3 and, when possible, 6 days post infection. Supernatants were centrifuged 5 min at 3.500 rpm, aliquoted and freezed at −70°C. Determination of virus titers was carried out on BSR T7/5 cells by limiting dilutions as described for RSV. Virus foci were visualised by immunostaining with a fluorescein isothiocyanate conjugate (Centocor®) recognizing RV N protein. For recombinant SAD eGFP-P viruses, fluorescence of infected cells was detected directly by fluorescence microscopy.

3.2 General cloning procedures

3.2.1 Restriction enzyme digest

Restriction endonuclease digests were performed according to the supplier's manual using the recommended buffer and 10 units (U) of the chosen enzyme for each microgram of DNA.

3.2.2 Extraction of DNA fragments from agarose gel

Restriction fragments were separated by electrophoresis on 1%-1.5% agarose gel using 1 x TAE buffer containing 0.1 μg/ml of ethidium bromide. Addition of ethidium bromide solution permitted visualization of nucleic acids under UV light.

x TAE buffer:	*Tris*	*40 mM*
	CH_3COONa x 3 H_2O	*5 mM*
	EDTA	*1 mM*

Samples were diluted in 1x DNA loading buffer, loaded into the slots and electrophoresis was performed for 45-60 min at 120 V.

DNA loading buffer:	*Ficoll 400*	*15%*
	TAE	*5%*
	Orange G	

Fragments were visualised under UV light at 366 nm (BIO-RAD, Universal Hood II) and DNA fragments were recovered by gel excision. DNA was purified using QIAquick gel extraction kit (Qiagen) following the supplier's manual. Concentration of purified DNA (μg/μl) was estimated by measuring the OD of 100 μl of a 1:50 dilution of the sample at 260 and 280 nm in a spectrophotometer (BioPhotometer, Eppendorf). Only samples with a ratio between 1.7 and 2.0 were considered as appropriately pure.

3.2.3 DNA ligation

A ratio of vector/insert DNA of 1:5 or 1:10 was used in any ligation. 100 ng of vector were mixed, in a sterile 1.5 ml centrifuge tube, with the appropriate amount of insert DNA, 2 μl of 10x ligation buffer, 2 U/μl of T4 DNA ligase (MBI) and bidistilled water up to 20 μl. The ligation mix was incubated at room temperature for 3-4 hours or alternatively over night at 16°C.

3.2.4 Transformation into competent bacteria

Competent XL-1 Escherichia coli were prepared by calcium chloride method. Bacteria were thawed on ice and 50 µl were transferred to a semisterile centrifuge tube. About 10 ng of plasmid or up to 20 µl of a ligation mix were added. After mixing by pipetting, bacteria were incubated on ice for 20 min. The tube was than transferred for 2 min to a heating block, preheated at 42°C and then rapidly returned to ice for 1-2 min. 250 µl of LB^{++} medium were added to each tube and cultures were incubated at 37°C for 1 hour on a shaker.

LB^{++} Medium:	*LB medium*	*1 l*
	$MgSO_4$	*19 ml of 1 M solution*
	KCl	*3,2 ml of 3 M solution*

An appropriate volume of culture (10-50 µl for plasmids and 250 µl for ligation) was distributed onto agarose-LB plates containing 100 µg/ml of ampicillin. Transformed bacteria were spread over the agarose plate by using a sterile metal rod and incubated over night at 37°C.

Agarose-LB:	*LB medium*	*1 l*
	Agar, in granules	*15 g*

After autoclaving the solution, swirl carefully to dissolved the agarose. When the solution cools down to 50°C, add the antibiotic and pour directly in 90 mm Petri dishes. Wait until the medium has solidified completely, turn the plates up side down and store at 4°C.

3.2.5 Preparation of minipreps and midipreps

To screen for positive clones, colonies were picked with pipette tips from the agarose-LB plate and cultured in 1 ml of LB media supplemented with 100 µg/ml of ampicillin overnight under constant agitation.

LB^{++} Medium:	*NaCl*	*5 g*
	Yeast extract	*5 g*
	Bactotrypton	*10 g*
	$MgSO_4$	*1 ml of 1 M solution*
	Bidistilled water	*up to 1 l*

Dissolved the solute, adjust the pH at 7.5 and autoclave

Cultures were centrifuged for 5 min at 4000 x g (Ependorf table centrifuge). The supernatant was discharged and the pellet resuspended in 0.2 ml of Flexi I buffer. Flexi II buffer (0.2 ml) was added to lyse the cells for 5 min at room temperature, followed by the addition of 0.2 ml of Flexi III buffer to precipitate chromosomal DNA and cellular debris. After an incubation on ice for 5 min, tubes were centrifuged for 10 min at 8000 x g and the supernatant was transferred to a fresh 1.5 ml. A volume of 0.42 ml of isopropanol was added to precipitate plasmid DNA and the solution was mixed by pipetting before centrifugation at 8000 x g for 10 min at room temperature. The supernatant was removed and the pellet was washed with 1 ml of 70% ethanol, centrifuged as before and, after being air-dried, dissolved in 50 µl of dH_2O. Restriction enzyme analysis was performed. Larger amounts of plasmid DNA were prepared by midipreps. Overnight cultures of 100 ml of LB medium supplemented with 100 µg/ml of ampicillin were used and DNA was purified with Nucleobond plasmid purification kit AX 100 (Macherey-Nagel) according to the manifacturer's instructions.

Flexi I:	*Tris-HCl*	*100 mM*	*[pH 7.5]*
	EDTA	*10 mM*	
	Rnase I	*400 µg /ml*	

Flexi II:	*NaOH*	*200 mM*
	EDTA	*1%*

Flexi III:	*KCH_3COO (aq)*	*300 mM*	*[pH 5.75]*

3.3 Reverse Transcription and PCR conditions

3.3.1 Extraction of RNA from cells and reverse transcription

For reverse transcription (RT) reactions, RNA was isolated from confluent mock and infected cells seeded into 6-well plates using Qiagen Rneasy kit (Qiagen). For IFN-β mRNA isolation cells were infected at MOI of 1 for 16-18 hours. A DNase digest was performed during the RNA extraction process using RNase-free DNase set (Qiagen). RNA was resuspended in 50 µl of DEPC-H_2O and the concentration was determined as described for the DNA concentration. 1 µg of DNase-treated RNA was mixed with 3 µl (30 pmol) of the desired antisense primer and 0.5 µl of Rnasin (Amersham-Pharmacia) in a total volume of 42 µl. After an incubation of 10 min at 65 °C followed by 10 min at 37°C, 2 µl of dNTP mix (25 mM each), 5 µl of 10x RT buffer (provided by the manufacturer) and 1 µl of StrataScript reverse transcriptase were added. The reaction was incubated for 1 hour at 37°C followed by inactivation of enzyme activity at 95 °C for 5 min. 5 µl were used in a PCR reaction.

DNase digested RNA	x µl (1 µg)
Antisense primer	3 µl (30 pmol)
dNTPs (25 mM)	2 µl
10x reaction buffer	5 µl
Rnasin	0.5 µl
reverse transcriptase	1 µl
bidistilled water	up to 50 µl

The following primers were used to amplify:

Human IFN-β

hβ 3' (antisense): 5'-aag atg ttc tgg agc atc tga tag atg-3'

Actin

β-actin 3' (antisense): 5'-ccg cca gac agc act gtg ttg gcg ta-3'

HRSV NS1:

hNS1-EcoRI (antisense): 5'-att gag aat tct tat gga tta aga tca aa-3'

HRSV NS2:

hNS2-EcoRI (antisense): 5'-att gag aat tct tat gga tta aga tca aa-3'

BRSV NS2:

BNS2HA-EcoRI (antisense): 5'-gca ata gaa ttc cta ttt atc gtc atc atc ttt ata atc tgg att taa atc ata ctt ata-3

3.3.2 PCR conditions

Standard PCR reactions were prepared with 100 ng of template DNA, 25 pmol of each sense and antisense primers, 1 μl of dNTPS mix (25 mM of each dATP, dCTP, dGTP and dTTP), 10 μl of DMSO, 10 μl of 10x buffer (supplied by the manufacturer and containing 50mM of $MgSO_4$) and 1 μl of Taq Polymerase in a total volume of 100 μl.

For detection of human IFN-β, the following primers were used:

hβ 5' (sense): 5'-ctc ctc caa att gct ctc ctg ttg tg-3'

hβ 3' (antisense) 5'-aag atg ttc tgg agc atc tga tag atg-3'

To confirm integrity of the DNA and to verify infection, sequences of the β-actin and RSV NS genes were amplified, respectively, using the primers listed below:

β-actin 5' (sense): 5'-ggc atc gtg atg gac tcc-3'

β-actin 3' (antisense): 5'-ccg cca gac agc act gtg ttg gcg ta-3'

hNS1-NcoI (sense): 5'-att gac cat ggg cag caa ttc att-3'

hNS1-EcoRI (antisense): 5'-att gag aat tct tat gga tta aga tca aa-3'

bNS2-BamHI (sense): 5'-aag cgg atc ccc aac cag cca tga gca cc-3'

bNS2FL-EcoRI (antisense): 5'-gca ata gaa ttc cta ttt atc gtc atc atc ttt ata atc tgg att

taa atc ata ctt ata-3’

hNS2-NcoI (sense): 5’-att gac cat gga cac aac cca ca -3’

hNS2-EcoRI (antisense): 5’-gga att cga atc ttg tgt tga aat t-3’

The reactions were prepared on ice in a sterile 0.5 ml tube and contained:

DNA sample	5 µl
Sense primer	2.5 µl (10 pmol)
Antisense primer	2.5 µl (10 pmol)
dNTPs (25mM)	2.0 µl
10x reaction buffer + $MgSO_4$	10 µl
DMSO	10 µl
Taq Polimerase (5U)	1 µl
Bidistilled water	64 µl

PCR reaction was carried out in a Biometra T3 thermocycler including the following steps:

1st step:	denaturation	(95 °C, 5 min)
2nd step:	denaturation	(94 °C, 1 min)
3rd step:	annealing	(x °C, 1min)
4th step:	extension	(72 °C, 1 min 30 sec)
5th step:	extension	(72 °C, 10 min)
6th step:	cooling/pause to 4°C	

Step 2 to 4 were repeated 35 times, before step 5 and 6, for a total number of 36 cycles. The temperature of the annealing step depended on the length and GC-content of the primers: 62°C were used for IFN-β and for β-actin, 58°C for HRSV NS2, 52°C for and 63°C for HRSV and BRSV NS1 gene, respectively. The resulting DNA fragments were mixed with DNA loading buffer and run on a 1.5 % agarose TAE gel with ethidium bromide and visualised with UV light. If required, purification of PCR products was performed by Qiagen PCR purification kit or by QIAquick gel extraction kit (Qiagen).

3.4 Generation of recombinant BRSV viruses expressing HRSV NS1 and NS2 proteins

3.4.1 Construction of rBRSV viruses expressing NS proteins of HRSV strain Long (rBRSVh1/2)

Construction of the recombinant BRSV virus harbouring HRSV Long NS1 and NS2 (rBRSVh1/2) has been previously described (Bossert B. and Conzelmann K.K., 2002). Briefly, cDNA encompassing both HRSV Long NS genes was obtained by RT-PCR of infected Vero cells and subcloned in a plasmid, generating the phNS1hNS2cass. This plasmid was digested with *NotI* and *Acc65I* and the resulting fragment containing the HRSV Long NS genes was inserted in a full length BRSV cDNA, resulting in rBRSVh1/2.

3.4.2 Construction of rBRSV expressing a mutated HRSV NS2 protein (rBRSVh1/2*^{T26I})

3.4.2.1 Quick site-directed mutagenesis of HRSV Long NS2 gene

phNS1hNS2 was used for mutagenesis. Threonine (T) at position 26 of NS2 protein was substituted with an Isoleucine (I) residue, mimicking the situation in HRSV A2 strain. An additional restriction site for the endonuclease *SpeI* was inserted for subsequent restriction digest analysis. Mutagenesis was performed by using a modified Quickchange-Mutagenesis protocol. PCR was done using the following primers:

hNS2*^{T26I} (sense): 5'-ttg tca ctt gag act **att** ata <u>act agt</u> cta acc aga-3'

hNS2*^{T26I} (antisense): 5'-agt ctc aag tga caa tgg tct cat g-3'

The restriction recognition site is underlined and the mutated codon is in bold.

PCR reaction was performed as previously described (3.3.2):

DNA sample	x µl (about 100 ng)
Sense primer	2.5 µl (10pmol)
Antisense primer	2.5 µl (10pmol)
dNTPs (25mM)	1.0 µl
10x reaction buffer	10 µl
Pfu Polimerase	1 µl (2.5 U)
Bidistilled water	up to 100 µl

The following steps were used:

1st step:	denaturation	(95 °C, 30 sec)	
2nd step:	denaturation	(95 °C, 30 sec)	⎫
3rd step:	annealing	(50 °C, 1 min)	⎬ 18 cycles
4th step:	extension	(68 °C, 8 min)	⎭
5th step:	extension	(68 °C, 10 min)	
6th step:	cooling/pause	to 4°C	

PCR product was digested over night at 37°C with *DpnI* (10 U) endonuclease to eliminate residual DNA template and purified by Qiagen PCR purification kit. 5 µl of purified DNA were transformed in competent XL-1 bacteria, which were spread on agarose plates over night at 37°C as described in paragraph 3.2.4. Positive clones were screened by restriction digest, using in combination the following endonucleases: *SpeI* with *NotI* and *NotI* with *Acc65I*. Positive clones were sequenced to confirm the presence of the desired mutation and large scale amount of DNA were prepared at least from two different positive clones by midipreps.

3.4.2.2 Generation of rBRSVh1/2*^{T26I} virus

The plasmid phNS1hNS2*^{T26I} was digested with *NotI* and *Acc65I*. The resulting fragment of 1094 nt was inserted by DNA ligation into the full length BRSV cDNA, previously digested with the same restriction enzymes, resulting in pBRSVhNS1hNS2*^{T26I} (rBRSVh1/2*^{T26I})

3.5 Recovery of recombinant BRSV viruses

Recombinant rBRSVh1/2*^{T26I}, as well as rBRSVh1/2 and BRSV wild-type, were rescued as described before (Buchholz U.J. et al., 1999). BSR T7/5 cells were transfected with the corresponding full length cDNA plasmid after calcium phosphate precipitation (Mammalian transfection kit, Stratagene) as specified by the supplier. 1x 10^6 cells/dish were seeded in 35 mm-diameter dishes in BHK-21 medium supplemented with 10% CS and grown over night. Cells were then washed with serum-free DMEM and 1 ml of the medium was added to each dish. After 1 hour, transfection was performed using a plasmid mixture containing 10 μg of pBRSVhNS1hNS2*^{T26I} (or pBRSVwt or pBRSVhNS1hNS2) and the plasmids pTITB-N (4 μg), pTITB-P (4 μg), pTITB-L (2 μg) and pTITB-M2 (2 μg). Four hours post transfection, the medium was removed and cells were incubated in BHK-21 medium containing 10% FCS. Three days later cells were split (ratio 1:3) and they were maintained in 2.5% FCS BHK-21 medium. Cells were further split every 4-5 days until the formation of syncytia was observed. Virus stocks were obtained as described previously in 3.1.2.

3.6 Generation of recombinant SAD eGFP-P viruses expressing HRSV NS proteins

3.6.1 Generation of recombinant SAD eGFP-P viruses

Recombinant rabies virus (SAD eGFP-P), coding for a eGFP-P fusion protein and carrying RSV NS1 and NS2 genes of different origin were constructed. RSV NS genes were inserted into the intergenic region between G and L genes.

For BRSV NS genes, a full length RV cDNA containing an extra transcriptional stop-restart sequence in the 3' non coding region of G gene and harbouring BRSV NS1 and NS2 genes (pSAD VB NS2FL/NS1HA) was used (Mebatsion T. et al., 1996; Bossert B. and Conzelmann K.K., 2002). The plasmid was digested with *PpMI and MluI* endonucleases and the large fragment of about 5,000 bp was inserted in the full length SAD eGFP-P cDNA resulting in pSAD eGFP-PbNS2flbNS1ha (eGFP-Pb2/1). Similarly, the full length cDNA pSAD eGFP-PhNS2hNS1 (eGFP-Ph2/1) was constructed using in this case a RV cDNA plasmid harbouring HRSV Long NS genes (pSAD VBhNS2hNS1). In the case of the recombinant RV expressing a mutated HRSV NS2 gene (eGFP-Ph2*^{T26I}/1), I used a different strategy as follows. A plasmid containing HRSV-Long NS genes (phNS2hNS1) was mutagenised as

described in 3.4.2.1. The resulting plasmid phNS2*^{T26I}hNS1 was digested with *NotI* and *Acc65I* and treated with K*lenow* polymerase to generate blunt ends. The DNA fragment, which encompassed the NS genes, was inserted into the unique *SmaI* site of pSAD VB resulting in pSAD VBhNS2*^{T26I}hNS1. Positive clones were screened by restriction analysis and sequenced to confirm the presence of the inserted mutation and the correct orientation. At this point the procedure was the same used for the previous two recombinant RVs. pSAD VB hNS2^{T26I}hNS1 was digested with *PpMI and MluI* and the purified DNA fragment was inserted in the full length SAD eGFP-P cDNA resulting in pSAD eGFP-PhNS2*^{T26I}/hNS1 (eGFP-Ph2*^{T26I}/1).

3.6.2 Rescue of recombinant eGFP-P viruses expressing RSV NS proteins

Recovery of recombinant eGFP-Pb2/1, eGFP-Ph2/1, eGFP-Ph2*^{T26I}/1 was performed using the $CaPO_4$-method as previously described for recovery of RSV. In this case plasmids encoding for RV protein N (pTIT-N, 5 μg), P and L (pTIT-P and pTIT-L, 2.5 μg each) with 10 μg the respective cDNA (pSAD eGFP-PbNS2flbNS1ha, pSAD eGFP-PhNS2hNS1 or pSAD eGFP-PhNS2*^{T26I}/hNS1). After transfection, cells were maintained in BHK-21 medium supplemented with 10% CS and cell-culture supernatants were harvested 3 and 6 days post transfection. Fluorescence of the recombinant eGFP-P viruses was monitored to detect virus infection and to determine viral titers of the stocks.

3.7 Growth characteristics of recombinant BRSV and eGFP-P viruses

3.7.1 rBRSV viruses expressing HRSV NS proteins

Recombinant rBRSVh1/2 and rBRSVh1/2*^{T26I} were analysed for their ability to grow in different cells systems and compared to the HRSV prototypes Long and A2. 4x 10^5 Vero (interferon-deficient) and human HEp2 (interferon-active) cells were infected with the recombinant BRSVh1/2 and BRSVh1/2*^{T26I} and with Long and A2 strains at an MOI of 0.3. Infection was performed in suspension, in serum-free DMEM medium for 1-1.5 hours in 15 ml falcon tubes. Cells were kept in suspension by gently shaking each 15 min. Afterwards, cells were transferred into 12-well plate in 1 ml of DMEM containing 2.5% of FCS.

Supernatants were harvested daily for 3 days, centrifuged to remove cell debris and stored at –70°C before virus titers were determined on Vero cells as already described. Cell lysates were also collected to monitor viral protein expression at the different time points and to verify the correct translation of NS proteins in the recombinant BRSVs.

3.7.2 Recombinant rabies eGFP-P viruses expressing RSV NS proteins

Growth characteristics of eGFP-Pb2/1, eGFP-Ph2/1 and eGFP-Ph2*^{T26I}/1 were determined in BRS T7/5 cells, which are defective the type I IFN induction pathway, and in Hep_2 cells. Wild-type SAD L16 and SAD eGFP-P were included as control viruses, since they have been shown to differ in their ability to induce IFN-β.
BSR T7/5 and HEp2 cells, at a concentration of 1x 10^6, were infected with the viruses mentioned above at an MOI of 0.01 and maintained in BHK-21 medium containing 10%CS or DMEM with 5% FCS. Supernatants were collected for 3 days and virus titers were determined on BSR T7/5 as described before.

3.8 IFN treatment of rBRSV viruses

To analyse the effect of IFN type I on the replication of HRSVs and rBRSVs, HEp2 cells were infected at an MOI of 0.3 as described for growth kinetic analysis and IFN-α A/D (PBL Biomedical Laboratories) was added to the culture medium directly after seeding. Concentrations of 500, 1000 and 5000 IU/ml were assayed using a working dilution of 100 IU/µl IFN-α in PBS. Virus titers were determined 2 or 3 days post infection depending on the extent of the observed CPE.

3.9 Cloning of the human TANK-binding kinase 1 (TBK1) and TRAF-binding protein (TANK)

The human kinase TBK1 and TANK genes were amplified by PCR reaction from a human lung cDNA library. PCR reaction was performed as previously described using the following oligonucleotide primers:

hTBK1 BamHI (sense): 5’-atg gat ccc atg cag agc act tct aat cat ctg-3’

hTBK1 NotI (antisense): 5'-ata tgc ggc cgc cta aag aca gtc aac gtt gcg aag gcc-3'

hTANK BamHI (sense): 5'-atg gat ccc atg gat aaa aac att ggc gag-3'

hTANK NotI (antisense): 5'-ata tgc ggc cgc tta agt ctc tcc att gaa gtg-3'

Inserted restriction sites are underlined

Restriction sites for the endonucleases *BamHI* and *NotI* were inserted at the 5' and 3' ends of the amplified ORF respectively to allow subsequent cloning in the expression vector pCR3-Ig.

The reaction was carried out according to the following steps:

1st step:	denaturation	(95 °C, 5 min)	
2nd step:	denaturation	(94 °C, 1 min)	⎫
3rd step:	annealing	(°C, 1min)	⎬ 35 cycles
4th step:	extension	(72 °C, 1 min 30 sec)	⎭
5th step:	extension	(72 °C, 10 min)	
6th step:	cooling/pause	to 4°C	

The obtained DNA fragments were subjected to restriction digest with *BamHI* and *NotI* and purified by gel extraction (QIAquick gel extraction kit, Qiagen). The sequence of DNA fragment obtained by PCR was confirmed by DNA sequencing. To construct the expression vectors expressing the wild type TBK1 and TANK genes, the cDNA fragments were ligated into pCR3-Ig vector plasmid, previously digested with the appropriate restriction enzymes. In this way, N-terminal Ig-tagged TBK1 and TANK fusion proteins were generated (IgTBK1 and IgTANK). Correct expression of the recombinant IgTBK1 and IgTANK were assayed by Western blot and immunofluorescence analysis. Functional activity was tested by gene reporter assay.

3.10 Gene Reporter Assay

3.10.1 Modified Gene Reporter Assay in Vero-p125Luc cell line

Vero cells, stably transfected with the IFN-β gene promoter/enhancer (p125-Luc), were seeded in 24-well-plates, using 2x 10^5 cells/well, and infected with RSV viruses at an MOI of 0.3. After an absorption of 90 min in serum-free DMEM, cells were incubated in DMEM supplemented with 5% FCS. At 48 hours post infection cells were harvested in 200 µl of luciferase lysis buffer [20 mM Tris HCl pH (7.8), 2 mM DTT, 2 mM CDTA, 10% glycerol, 1% triton X-100; incubation 10 min at 37°C]. Samples were than assayed for reporter gene activity in a luciferase assay, that was performed as follows. Aliquots were shortly centrifuged to remove unsoluble parts and 20 µl of each cell lysate were diluted in 80 µl of luciferase lysis buffer. 100 µl of luciferase substrate buffer [20 mM Tricine, 2.67 mM $MgSo_4$, 0.1 mM EDTA, 33.3 mM DTT, 1.07 mM $MgCO_3$ x $Mg(OH)_2$ x 5 H_2O] supplemented with 470 µM AcetylCoA and 530 µM ATP were added to the cell lysates directly through the luminometer (LUMAT LB9501, Perkin Helmer Wallac GmbH). The light emission was measured as relative light units (RLU). Infection was verified by Western blot analysis using the same cell lysates.

3.10.2 Dual luciferase assay and transfection of reporter plasmids

For experiments aimed to verify the biological activity of the non-canonical kinases TBK1 and IKK-ε to induce IFN, BSR T7/5 cells were transfected with a reporter plasmid expressing the firefly luciferase gene under the control of the IFN-ε gene promoter/enhancer (p125Luc) and a plasmid containing a Renilla luciferase gene under the control of the CMV promoter was used as internal control (pCMV-RL). Additionally, plasmids containing wild type human IRF3 (pEF-haIRF3), human TBK1 (pCR3-IgTBK1) or IKK-ε (pFlagIKK-ε) or the kinase inactive form of IKK-ε (pFlagIKK-ε K38A) were cotransfected. BSR T7/5 cells were seeded in a 24-well plate using 1x 10^5 cells/well and grown over night. Transfection was performed with FuGENE 6 reagent (Roche) as indicated by the manifacturer using 0.1 µg of the reporter construct p125Luc, 0.02 µg pCMV-RL, 0.1 µg of the transcription factor IRF3 and 0.1 µg of the indicated kinases. Luciferase activity was measured 48 hours post transfection.

The relative light units (RLU) were standardised based on the Renilla luciferase activity and converted in fold induction.
Similar experiments were also conducted in 293 cells, but in this case cotransfections were carried out using reporter plasmids for IRF3 promoter (pCIB55Luc), pCMV-RL and TBK1 or IKK-ε.

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3.10.3 Transcription factor activation and TBK1 inhibition by RSV infection

To assay the activities of the transcription factors AP-1, NF-kB and IRF3, 2x 10^5 293 cells were transfected with 0.1 μg of the respective reporter construct (pAP-1Luc, pNF-kBLuc or p55CIBLuc) and 0.02 μg pCMV-RL using FuGENE 6. At 24 hours post transfection, cells were infected with the indicated viruses at an MOI of 0.3. At 24 hours post infection, luciferase activity was measured by DL (Dual Luciferase Reporter Assay System, Promega) as indicated by the supplier. To investigate the capacity of the different RSVs to inhibit TBK1-dependent induction of the IFN-β gene promoter, 2x 10^5 293 cells were infected in serum-free DMEM medium with the corresponding viruses at different MOIs. After 90 min of incubation on gently shaking, cells were seeded in 24-well plate and maintained in DMEM containing 10% of FCS. At 24 hours post infection, cells were transfected with 0.1 μg of p125Luc, 0.02 μg of pCMV-RL and 0.1 μg of pCR3-IgTBK1. Viral protein expression was then assessed by Western blot on the remaining cell lysates used to perform the luciferase assay. The same experiment was performed in BSR T7/5 cells with the only difference that infected cells were transfected with the above mention plasmids plus pEF-haIRF3, being the endogenous IRF3 being defective in this cell line.

3.11 Protein expression analysis

3.11.1 Extraction of proteins from cells

Extraction of proteins from cells was performed using an appropriate amount of lysis buffer (500 μl for a 6-well plate, or 250 μl for a 12-well plate). Cell extracts were incubated at 95°C for 5 min, centrifugated at maximum speed (8000 x g) in a Eppendorf table centrifuge for 5 min and equal amounts were loaded onto a sodium dodecyl sulfate-polyacrylamide gel (SDS).

Protein extraction buffer:	*Tris pH 6.8*	*6.25 mM*
	sodium dodecyl sulfate (SDS)	*2%*
	glycerol	*10%*
	urea	*6 M*
	methanol	*5%*
	bromophenol blue	*0.01%*
	bromophenol red	*0.01%*
	2-mercaptoethanol	*0.5%*

3.11.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were separated using the Penguin^{TM} Model P9DS (OWL scientific). The separating gel, containing 10% or alternatively 12% acrylamid, was poured into the gap between the two glass plates of the system and a space of about 3 cm was left to allow the adding of the stacking gel. The gel was overlaid with 2-iso-propanol to ensure a levelled surface and left for polymerisation for about two hours.

Separating gel:	10% gel	12% gel
Polyacrylamide	9.0 ml	10.8 ml
Gel buffer	12.0 ml	12.0 ml
dH_2O	12.9 ml	11.1 ml
glycerol	2.0 ml	2.0 ml
TEMED	0.017 ml	0.017 ml
APS (ammonium persulfate)	0.175 ml	0.175 ml

The indicated amounts of gel mix were sufficient for one 14x 16 cm gel

Gel Buffer:	*Tris*	*364 g*
	SDS	*15 ml of a 20% solution*

After complete polymerisation the 2-iso-propanol was discarded and the stacking gel was prepared and added on the top of the separating gel. The comb was immediately inserted carefully avoiding to trap air bubbles under the teeth.

Stacking gel:	*Polyacrylamide*	1.4 ml
(for two gels)	*Gel buffer*	3.5 ml
	dH_2O	9 ml
	TEMED	0.116 ml
	APS	0.018 ml

After polymerisation (1-2 hours), the comb was removed and the plates mounted in the electrophoresis apparatus, which was filled with anode and cathode SDS-PAGE electrophoresis buffer.

Jagow-Anode buffer:	*Tris*	*242 g*
	dH_2O	*1 l*

The pH was adjusted to a value of 8.9 by HCl addition

Jagow-Cathode buffer:	*Tris*	*121 g*
pH [8.25]	*Tricine*	*179.2 g*
	SDS	*10 g*
	dH_2O	*1 l*

Samples were loaded and the electrophoretic run was performed at 40-60 V until the bromophenol blue left the separating gel at the bottom (about 15-16 hours) depending on the gel percentage. A molecular weight standard (Precision Plus Protein™ Standards, BIO-RAD) was pipetted onto the gel to estimate protein size.

3.11.3 Electroblotting

When the electrophoretic run was completed, gels were removed from the glass plates and incubated in 1x semi-dry buffer for 10-15 min. The separated proteins were transferred to a nitrocellulose membrane in a semidry transfer apparatus (The Panther™ Model HEP-1; OWL Scientific).

Semi-dry transfer buffer (10x):	*Tris*	*58 g*
	Glycine	*29 g*
	SDS	*2.5 ml of a 20% solution*
	dH_2O	*1 l*

For electrophoretic blotting 1x Semi-dry buffer was used; 180 ml of methanol were added to 1 l of fresh 1x solution

One sheet of Whatman blotting paper of the same size as the gel was soaked in transfer buffer and placed on the bottom electrode and pressed to remove air bubbles. The gel and the nitrocellulose membrane, which were also soaked in transfer buffer, were positioned exactly over the bottom paper and covered with another soaked blotting paper. After having placed the upper electrode the system was connected to a power supply (Standard power Pack P25, BIOMETRA) and the transferred was performed at 400 mA for each gel for 120 min.

3.11.4 Western blotting

After the transfer, the membrane containing the separated proteins was incubated at room temperature for 60 min in constant agitation with a blocking solution.

Blocking solution:	*5% instant skimmed milk*
	0.05% Tween 20
	1x PBS

After the blocking, the membrane was washed 3 times for about 15 min at RT with PBS containing 0.05% Tween 20 (PBS-T). Incubation with the primary antibody diluted in PBS-T took place over night at 4°C in constant agitation. The membrane was then washed 3 times for about 15 min at RT with PBS-T and the incubation with peroxide-conjugated secondary antibody diluted 1:10000 in PBS-T occurred for 90 min at RT. Finally the membrane was washed 3 times with PBS-T and once in PBS for 10 min at RT. For detection, the membrane was incubated with 1 ml of ECL Western blotting detection reagent (Perkin Helmer) and exposed to an Hyperfilm ECL (Amersham) for varied times depending on the strength of the signal.

3.12 Immunofluorescence

BSR T7/5 cells were seeded overnight on coverslips in 6-well plate and transfected the day after with FuGENE 6 (Roche). 4 μg of each expressing plasmid where used. At 48 hours post-transfection, cells were washed with phosphate-buffered saline (PBS) and fixed with 3% (w/v) paraformaldehyde in PBS for 30 min followed by treatment with 50 mM NH_4Cl in PBS for 20 min. Cells were permeabilized with 0.5% Triton X-100 for 10 min and blocked for 1 hour at room temperature (RT) with 1% goat serum and 0.5% BSA in PBS. Cells transfected with FlagIKK-ε were incubated for 40 min at RT with the primary antibody anti-mouse Flag (M2, Sigma) diluted 1:1000 in blocking reagent. In the case of Ig-TANK and Ig-TBK1 goat FITC-tagged anti-human Ig were used at dilution 1:50. After primary-antibody incubation, for cells transfected with FlagIKK-ε, a Cy3-conjugated anti-mouse antibody was used at dilution 1:200 in PBS. After 40 min incubation at RT, cells were washed with PBS and nuclei were stained with DAPI (Hoechst) at the concentration of 1 μl/ml. Coverslips were mounted in with Mounting Medium-Vectashield for fluorescence (LINARIS) and examined with a fluorescence microscope equipped for laser-scanning confocal light microscopy (Zeiss, LSM510). Digitalized images were further processed using Adobe Photoshop version 7.0.

3.13 Immunoprecipitation of IgTANK and IgTBK1

BSR T7/5 cells were seeded overnight in 60 mm petri dishes at a concentration of $1x\ 10^6$ cells per dish and transfected the day after with 15 μg of each of the following expression plasmids: pCR3-Ig (empty vector), pCR3-IgTANK and pCR3-IgTBK1 by $CaPO_4$ method (Mammalian Transfection Kit, Stratagene). At 48 hours post transfection, cells were lysed for 30 min on ice with 300 μl of immunoprecipitation buffer (IP buffer). Lysates were centrifuged at 14.000 rpm for 15 min at 4°C to precipitate nuclei and cell debris, followed by transfer of the clean supernatants to fresh 1.5 ml Eppendorf tubes.

IP buffer:	TRIS	50 mM	pH 7.6
	NaCl	150 mM	
	NP-40	1%	
	EDTA	1 mM	
	EGTA	2 mM	
	NaF	20 mM	

Protease inhibitor cocktail (Complete, Roche) was freshly added to the buffer before use

100 µl of Sepharose A beads (Protein A Sepharose^{TM} 4 Fast Flow, Amersham), previously washed 3 times in IP buffer, were added and the lysates were incubated overnight at 4°C in continuous rotation. After incubation, lysates were centrifuged at 8000 x g at 4°C for 15 min to precipitate the beads. The pellets were then washed 3 times in IP buffer. Once the washing step was completed, the beads were pelleted down and resuspended in 100 µl of protein lysing buffer. The lysates were analysed by Western blotting.

3.14 Materials and equipment

3.14.1 Serological reagents

Primary antibodies:

abCam, **Cambridge, UK**	Rabbit α-human IFN-β, polyclonal
BFAV **Insel Reims, Germany**	Rabbit β-RV N and P proteins (S50), polyclonal
Biogenesis, **c/o Quartett GmbH**, **Berlin, Germany**	Goat β-RSV serum, polyclonal
Dianova, **Hamburg,Germany**	Normal goat serum
Santa Cruz Biotec. Inc. **Heidelberg, Germany**	Rabbit β-TBK1, polyclonal Rabbit β-PCNA, polyclonal Rabbit β-IRF-3, polyclonal Rabbit β-HA (Y11), polyclonal

Serotec, **Düsseldorf, Germany**	Mouse α-RSV N protein, monoclonal
Sigma-Aldrich, **Taufkirchen, Germany**	Rabbit α-actin (20-33), polyclonal Mouse α-FLAG (M2), monoclonal
J.A. Melero, **University of Madrid, Spain**	Rabbit α-HRSV NS1/NS2 (α-IC/C), polyclonal
Freiburg, Germany	Mouse α-Mx A, monoclonal

All secondary antibodies (FITC-, Cy3-, peroxidase-conjugated anti-mouse, anti-rabbit and anti-human Igs) were obtained from Dianova ()

3.14.2 Chemicals

Boehringer /Roche **Mannheim, Germany**	DTT (1,4-dithioerythreitol)
Fluka, **Deisenhofen, Germany**	NP40 (nonidet P40) tricine
ICN Biochemical Inc **Cleveland, Ohio-USA**	APS (ammonium persulfate)
Merk, **Darmstadt, Germany**	$CaCl_2$ EDTA Ethidium bromide CH_3COOK KCl $MgSO_4$ $MgCl_2$ CH_3COONa NaCl NH_4Cl Orange G Paraformaldehyde Phenol red Propidium iodide Triton X-100

Amersham-Pharmacia, Freiburg, Germany	Ficoll
Riedel-de-Haen, Deisenhofen, Germany	Ethanol Methanol NaOH
Carl Roth GmbH, Karlsruhe, Germany	Acetone (tech) DMSO (dimethylsulfoxide) Glycerol Glycine HCl Hepes Methanol SDS Tris Tween 20 Urea
Sigma, Taufkirchen, Germany	ATP bromophenol blue CDTA $MgCO_3xMg(OH)_2$ TEMED

3.14.3 Enzymes

MBI Fermentas, St. Leon-Rot, Germany	Klenow polymerase
New England Biolabs, Frankfurt am Main, Germany	Restriction endonucleases T4 DNA ligase endonucleases
Roche Biochemicals, Mannheim, Germany	Shrimp alkaline phosphatase
Stratagene, Amsterdam, Netherland	pfu polymerase Reverse transcriptase

3.14.4 Kits

Machrey & Nagel, Düren, Germany	Nucleobond AX100
Qiagen, Hilden Germany	Rneasy kit QIAquick PCR purification/Gel extraction/Nucleotide removal
Perkin Elmer, Plus **Freiburg, germany**	Western lightning Chemiluminescence Reagent
Promega GmbH, Mannheim, Germany	Dual Luciferase Reporter Assay System
Stratagene, Amsterdam, Holland	Mammalian transfection kit

3.14.5 Miscellaneous

Amersham Bioscience Gmbh, Freiburg, Germany	Hyperfilma ECL Protein A Sepharose^{TM} 4 Fast Flow
BD, Heidelberg, Germany	Bacto yeast extract
BIO-RAD, **Munich, Germany**	Precision Plus Protein^{TM} Standards
Difco, Hamburg, Germany	Bacto Tryptone peptone
Gibco BRL, Karlsruhe, Germany	Agarose Ampicillin DNA 1kb ladder
LINARIS GmbH, **Wertheim, Germany**	Mounting Medium-Vectashield for fluorescence
Merck, Darmstadt, Germany	Acrylamid/Bisacrylamid solution (29:1) Skimmed milk powder

New England Biolabs, Frankfurt am Main, Germany	dNTPs
PBL Biomedical Lab, NJ, USA	IFN-α A/D
Amersham Pharmacia, Freiburg, Germany	Rnasin
Roche Diagnostics GmbH, Mannheim, Germany	FuGENE 6 Complete (protease inhibitor cocktail)
Carl Roth GmbH, Karlsruhe, Germany	Whatman paper
Schleicher & Schuell, Dassel, Germany	Nitrocellulose membrane (0.5 μm)
Sigma, Taufkirchen, Germany	AcetylCoA Luciferin Poly I/C

3.14.6 Tissue culture reagents

Roche Biochemicals, Mannheim, Germany	Foetal calf serum (FCS)
Gibco BRL, Karlsruhe, Germany	BHK-21 medium Dulbecco´s modified medium (DMEM) L-Glutamine 20 mM 100x MEM amino acids (AA) Newborn calf serum (CS) Phosphate-buffered saline (PBS) Trypsin-EDTA 1x Tryptose phosphate broth 50x (TP)
Nunc GmbH, Wiesbaden, Germany	Tissue culture flasks and plates
Sigma, Taufkirchen, Germany	Penicillin/Streptomycin 100x (P/S)

3.14.7 Equipment

Biometra, Goettingen, Germany	T3 Thermocycler Standard Power Pack P25
BIO-RAD, Munich, Germany	Universal Hood II
Eppendorf, Hamburg, Germany	BioPhotometer Centrifuge 5417R Table centrifuge
Olympus, Hamburg, Germany	Microscope, IX71
OWL Scientific/Nunc GmbH, Wiesbaden, Germany	The Panther^{TM} Model HEP-1
Perkinelmer Wallach Gmbh, Freiburg, Germany	Luminometer LUMAT LB9501
Zeiss GmbH, Jena, Germany	Confocal laser microscope, LSM 510

3.14.8 Bacteria and plasmids

E.coli XL1 (blue) were used for preparation of plasmid DNA

All constructs and full-length cDNAs of rabies and BRSV are based on the Bluescript SKII-vector from Pharmacia and subsequently cloned into pTIT vectors controlled by a T7 promoter.

pTIT-N, pTIT-P, pTIT-L	plasmids containing RV N, P or L genes
pTITB-N, pTITB-P, pTITB-M2, **pTITB-L**	plasmids containing BRSV N, P, M2 or L genes

pSAD VB	plasmid harbouring the full-length RV cDNA under T7-promoter control with additional stop-restart sequence between the G and L gene
pSAD VBhNS2hNS1	pSAD VB harbouring HRSV-Long NS1 and NS2 genes between G and L gene.
phNS1hNS2	plasmid harbouring HRSV-Long NS1 and NS2 genes
phNS1hNS2*^{T26I}	plasmid harbouring HRSV-Long NS1 and NS2 genes, where Threonine 26 of NS2 was mutated in an Isoleucin
phNS2*^{T26I} hNS1	plasmid harbouring HRSV-Long NS2 and NS1 genes, where Threonine 26 of NS2 was mutated in an Isoleucin
pSAD VBhNS2*^{T26I}/ hNS1	pSAD VB harbouring HRSV-Long NS1 and NS2* (T26I) genes between G and L gene.
pSAD GFP-P	plasmid harbouring the full-length SAD L16 expressing eGFP fusion P protein
pSAD GFP-PhNS2hNS1	pSAD GFP-P harbouring HRSV-Long NS1 and NS2 genes.
pSAD GFP-PhNS2*^{T26I} /hNS1	pSAD GFP-P harbouring HRSV-Long NS1 and NS2*(T26I) genes
prBRSVhNS1hNS2	plasmid harbouring the full-length BRSV cDNA and HRSV-Long non structural protein genes.
prBRSVhNS1hNS2*^{T26I}	prBRSV hNS1hNS2 where Threonine at position 26 of NS2 was mutated to an Isoleucine
p125Luc	Firefly luciferase gene under the control of the IFN-β gene promoter/enhancer (kindly provided by by T. Fujita, University of Kyoto, Japan)

p55CIBLuc	Luciferase gene under the control of positive regulatory domain (PRDI/IRF3 binding site) of the IFN-β gene promoter/enhancer (kindly provided by by T. Fujita, University of Kyoto, Ja
pCMV-RL	Renilla luciferase gene under the control of the CMV promoter/enhancer
pNF-kBLuc	luciferase gene controlled by a synthetic promoter containing 5 direct repeats of NF-kB binding sites (Stratagene)
pAP-1Luc	luciferase gene controlled by a synthetic promoter containing 7 direct repeats of NF-kB binding sites (Stratagene)
pEF-haIRF3	plasmid encoding human IRF3 gene (kindly provided by T. Fujita, University of Kyoto, Japan)
pFlag IKK-ε	plasmid encoding the N-terminus Flag-tagged kinase IKK-ε gene(kindly provided by Prof. R. Ruckdeschel, Max von Pettenkofer Institute, Munich)
pFlag IKK-ε K38A	plasmid encoding kinase-dead mutant of IKK-ε gene with an amino acid substitution at position 38 (K38→A) (kindly provided by Prof. R. Ruckdeschel, Max von Pettenkofer Institute, Munich)
pIg-TBK1	plasmid encoding an N-terminus Ig-tagged version of the human TANK-binding kinase1 (TBK1)
pIg-TANK	plasmid encoding N-terminus Ig-tagged gene of the human TRAF family member-associated NF-kB activator (TANK)

4 RESULTS

4.1 Nonstructural proteins (NS) of BRSV block activation of interferon regulatory factor 3

We have previously provided evidence that BRSV nonstructural proteins NS1 and NS2 prevent induction of IFN-β by interfering with the activation of interferon regulatory factor (IRF)-3 (Bossert B. et al., 2003). We demonstrated that IFN-β is expressed in HEp2 cells infected with a double deletion mutant (BRSV ΔNS1/2), lacking both the NS proteins, but not in wild-type BRSV-infected cells.

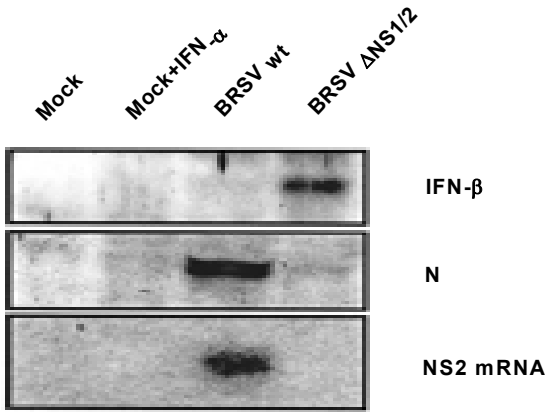

Fig. 1 A recombinant BRSV lacking both NS proteins (BRSV ΔNS1/2) induces interferon synthesis in contrast to wild-type BRSV. HEp2 cells were mock-infected and infected with BRSV wt and BRSV ΔNS1/2. IFN-β and BRSV N protein were detected by Western blotting using a polyclonal anti-human IFN-β and anti-RSV serum, respectively. NS2 mRNA was demonstrated by an NS2-specific cDNA hybridisation probe.

Human respiratory syncytial virus (HRSV) displays a similar capacity to block type I interferon expression. Intriguingly, we have found that HRSV strain Long has lost the capacity to inhibit induction of IFN-α in contrast to HRSV strain A2 (Schlender J. et al., 2005). This finding encouraged me to investigate further these two HRSV strains in the attempt to elucidate the reasons of their different behaviour in IFN induction.

4.2 HRSV prototypes, A2 and Long, differ in their ability to induce type I IFN

4.2.1 Detection of IFN-β induction

To investigate type I IFN induction by different HRSV strains, Vero cells, stably transfected with the IFN-β gene promoter/enhancer (p125-Luc) were infected at an MOI of 0.3 with the subgroup A HRSV prototype viruses: A2 and Long. Reporter gene activity was assayed 24 and 48 hours post infection. Cells infected with the strain Long led to an increase in the IFN-β promoter activity compared to cells infected with A2 virus or mock-infected.

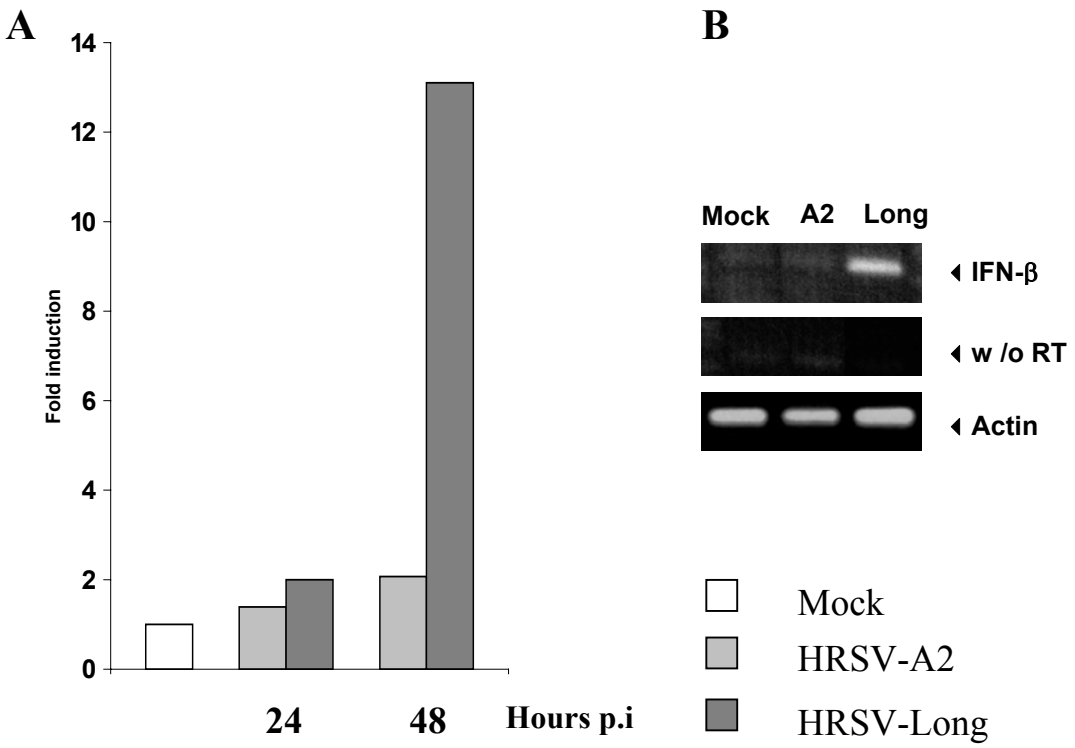

Fig. 2 (A) HRSV Long, but not A2, induces IFNβ-promoter activity. Vero-p125Luc cells were infected with the indicated viruses at an MOI of 0.3. At 24 and 48 hours p.i cells were lysed and a luciferase assay was performed. **(B) IFN-β mRNA expression in cells infected with HRSV A2 and Long**. HEp2 cells were infected with the indicated viruses at an MOI of 1 for 16-18 hours. IFN-β mRNA was detected by RT-PCR, as well as Actin mRNA as control.

To verify the previous findings, RT-PCR was applied to detect IFN-β mRNA in infected cells. HEp2 cells were infected with A2 and Long at an MOI of 1. RNA was extracted 16-18 hours post infection and subjected to RT-PCR analysis. Clearly, strain Long induced IFN-β mRNA expression, while strain A2 did not. Taken together, these data indicate that the two HRSV strains, A2 and Long differ in their ability to induce IFN-β or more correctly, HRSV strain Long lacks the capacity to inhibit IFN-β induction upon infection. The same results were obtained in infected plasmacytoid dendritic cells (PDC). We were able to show that RSV strain A2 but not strain Long was able to shut down IFN-α production in both epithelial cells and PDCs (Schlender J. et al., 2005).

4.2.2 Analysis of activation of IFN-β transcription factors AP-1, NF-kB and IRF3

Investigation of the activation of the transcription factors responsible for IFN-β gene induction (AP-1, NF-kB and IRF3) was performed in HRSV-infected cells.

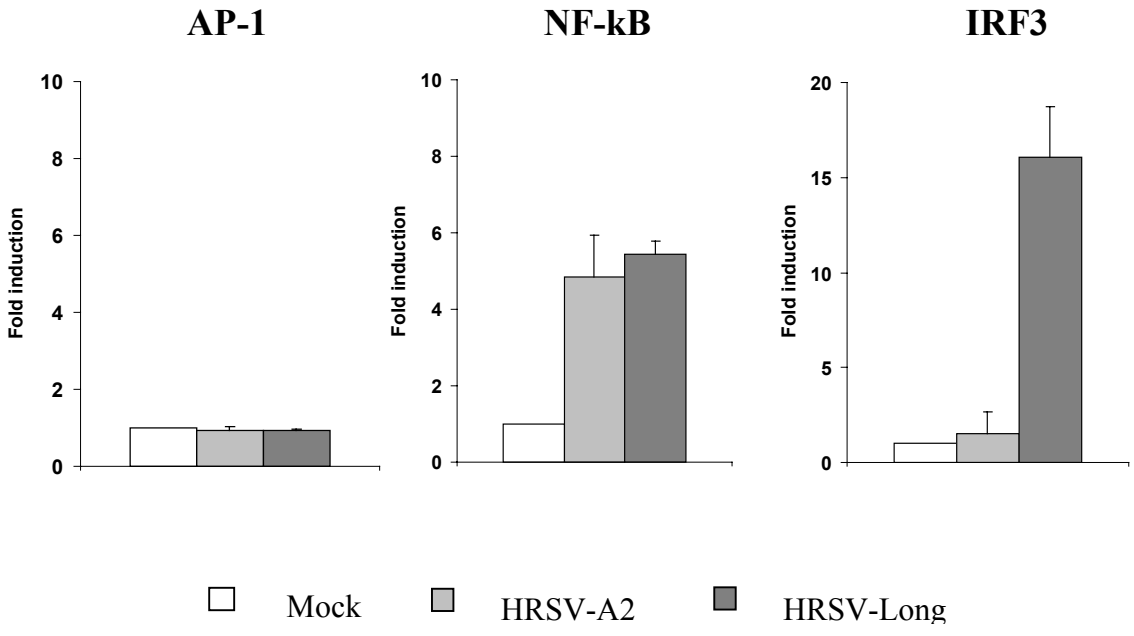

Fig. 3 HRSV-induced activation of IFN-β transcription factors. Luciferase constructs under the control of promoters containing AP-1, NF-kB and IRF3 binding sequences were transfected in 293 cells. After 24 hours cells were infected with the indicated viruses at an MOI of 0.3. At 24 hrs p.i. cells were harvested and luciferase assay was performed. Results show the mean of at least two independent experiments. All the RLU of the firefly luciferase were standardized based on Renilla luciferase activity, which was used as internal control for non-specific transcription ± SD.

Reporter constructs, harbouring the binding sequences for each transcription factors controlling the expression of the downstream firefly luciferase gene were transfected in 293 cells together with the Renilla (pCMV-RL). Renilla gene is under the control of a CMV promoter and it was used as a control for non specific (IRF3-independent) regulation of gene expression in the reporter assays. Twenty four hours later, cells were mock-infected and infected with HRSV A2 and Long at MOI of 0.3. HRSV A2 and Long strains activated NF-kB reporter activity of 4.9-fold and 5.4-fold, respectively, while AP-1 was not significantly induced. Interestingly, infection with HRSV-Long resulted in a 16-fold increase of the luciferase activity in cells transfected with IRF3 reporter construct. In contrast, HRSV A2 caused an 1.5-fold induction. Thus virus-specific activation of IRF3 appeared to be selectively blocked in the presence of HRSV A2, resembling the situation in BRSV wild-type infected-cells previously described (Bossert B. et al., 2003).

4.3 Sequence comparison of RSV NS1 and NS2 proteins

4.3.1 Sequence analysis of HRSV A2 and HRSV Long nonstructural proteins

Since our previous studies identified RSV nonstructural proteins as crucial antagonists of type I IFN induction, nucleotide sequences of the complete NS1 and NS2 genes from HRSV A2 and Long were determined and compared with those of two, out of seven, clinical isolates, namely patients 86 and 112. Clinical isolates were shown to block induction of IFN-α and for this reason they were considered comparable to strain A2 in their inhibitory capacity (Schlender J. et al., 2005). The NS1 protein sequence was highly conserved among the different HRSV viruses analysed. Only two amino acid differences were detected: one in the clinical isolate number 86 (N76S) and the other in HRSV A2 (I115L). None of these changes was considered significant. At the N-terminus of NS2 protein variable residues were identified. At position 7, the clinical isolates displayed a Glycine (G) instead of an Aspartic acid (D). HRSV A2 differed from the other viruses by the presence of an Asparagine (N) and a Lysine (K) at position 8 and 38. The only amino acid which distinguished HRSV Long strain from clinical isolates and HRSV A2 was a Threonine at position 26 instead of an Isoleucine. Threonine is a neutral amino acid like Isoleucine but, differently from the latter, its side chain can undergo O-linked glycosylation and it can become phosphorylated through the action of a threonine kinase. For these peculiar characteristics and its unique presence in

HRSV Long strain, I considered this amino acid change to be potentially involved in the different ability of A2 and Long viruses to inhibit IFN-β production.

A

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CL.ISOL#112    MGSNSLSMIKVRLQNLFDNDEVALLKITCYTDKLIHLTNA     40
CL.ISOL#86     MGSNSLSMIKVRLQNLFDNDEVALLKITCYTDKLIHLTNA     40
HRSV_A2        MGSNSLSMIKVRLQNLFDNDEVALLKITCYTDKLIHLTNA     40
HRSV_Long      MGSNSLSMIKVRLQNLFDNDEVALLKITCYTDKLIHLTNA     40
Consensus      mgsnslsmikvrlqnlfdndevallkitcytdklihltna

CL.ISOL#112    LAKAVIHTIKLNGIVFVHVITSSDICPNNNIVVKSNFTTM     80
CL.ISOL#86     LAKAVIHTIKLNGIVFVHVITSSDICPNNNIVVKSSFTTM     80
HRSV_A2        LAKAVIHTIKLNGIVFVHVITSSDICPNNNIVVKSNFTTM     80
HRSV_Long      LAKAVIHTIKLNGIVFVHVITSSDICPNNNIVVKSNFTTM     80
Consensus      lakavihtiklngivfvhvitssdicpnnnivvksnfttm

CL.ISOL#112    PVLQNGGYIWEMMELTHCSQPNGLIDDNCEIKFSKKLSDS    120
CL.ISOL#86     PVLQNGGYIWEMMELTHCSQPNGLIDDNCEIKFSKKLSDS    120
HRSV_A2        PVLQNGGYIWEMMELTHCSQPNGLLDDNCEIKFSKKLSDS    120
HRSV_Long      PVLQNGGYIWEMMELTHCSQPNGLIDDNCEIKFSKKLSDS    120
Consensus      pvlqnggyiwemmelthcsqpngliddnceikfskklsds

CL.ISOL#112    TMTNYMNQLSELLGFDLNP                         139
CL.ISOL#86     TMTNYMNQLSELLGFDLNP                         139
HRSV_A2        TMTNYMNQLSELLGFDLNP                         139
HRSV_Long      TMTNYMNQLSELLGFDLNP                         139
Consensus      tmtnymnqlsellgfdlnp
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B

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CL.ISOL#112    MDTTHNGTTPQRLMITDMRPLSLETIITSLTRDIITHRFI     40
CL.ISOL#86     MDTTHNGTTPQRLMITDMRPLSLETIITSLTRDIITHRFI     40
HRSV_A2        MDTTHNDNTPQRLMITDMRPLSLETIITSLTRDIITHKFI     40
HRSV_Long      MDTTHNDTTPQRLMITDMRPLSLETTITSLTRDIITHRFI     40
Consensus      mdtthndttpqrlmitdmrplsletiitsltrdiithrfi
                                                  ▲
CL.ISOL#112    YLINHECIVRKLDERQATFTFLVNYEMKLLHKVGSTKYKK     80
CL.ISOL#86     YLINHECIVRKLDERQATFTFLVNYEMKLLHKVGSTKYKK     80
HRSV_A2        YLINHECIVRKLDERQATFTFLVNYEMKLLHKVGSTKYKK     80
HRSV_Long      YLINHECIVRKLDERQATFTFLVNYEMKLLHKVGSTKYKK     80
Consensus      ylinhecivrklderqatftflvnyemkllhkvgstkykk

CL.ISOL#112    YTEYNTKYGTFPMPIFINHDGFLECIGIKPTKHTPIIYKY    120
CL.ISOL#86     YTEYNTKYGTFPMPIFINHDGFLECIGIKPTKHTPIIYKY    120
HRSV_A2        YTEYNTKYGTFPMPIFINHDGFLECIGIKPTKHTPIIYKY    120
HRSV_Long      YTEYNTKYGTFPMPIFINHDGFLECIGIKPTKHTPIIYKY    120
Consensus      yteyntkygtfpmpifinhdgflecigikptkhtpiiyky

CL.ISOL#112    DLNP                                        124
CL.ISOL#86     DLNP                                        124
HRSV_A2        DLNP                                        124
HRSV_Long      DLNP                                        124
Consensus      dlnp
```

Fig. 4 Multiple alignment of NS1 (A) and NS2 (B) amino acid sequences. Identical or similar amino acids are shadowed. Unique amino acids are indicated in white and the arrow indicates an unique amino acid change present in NS2 HRSV Long sequence.

4.4 Chimeric BRSV viruses expressing HRSV NS genes

4.4.1 Construction of recombinant BRSV virus harbouring Long-derived NS1 and a mutated form of NS2

To study the functional implication in IFN-β antagonism by the residue 26 (Threonin vs Isoleucine) of NS2 protein, a recombinant BRSV viruses harbouring Long-derived or A2-like NS proteins were generated. Recombinant BRSV (rBRSVh1/2) possessing NS1 and NS2 genes from HRSV strain Long was previously constructed (Bossert B. and Conzelmann K.K., 2002). In addition, a recombinant BRSV (rBRSVh1/2*^{T26I}) carrying an Isoleucine at position 26 of Long-NS2 protein was created by site-directed mutagenesis as described in Materials and Methods. Chimeric viruses were recovered by cotransfection of BSR T7/5 cells with the rBRSV full length cDNAs and the support plasmids encoding for BRSV N, P, L and M2 genes as described previously. Viruses were viable and comparable in growth.

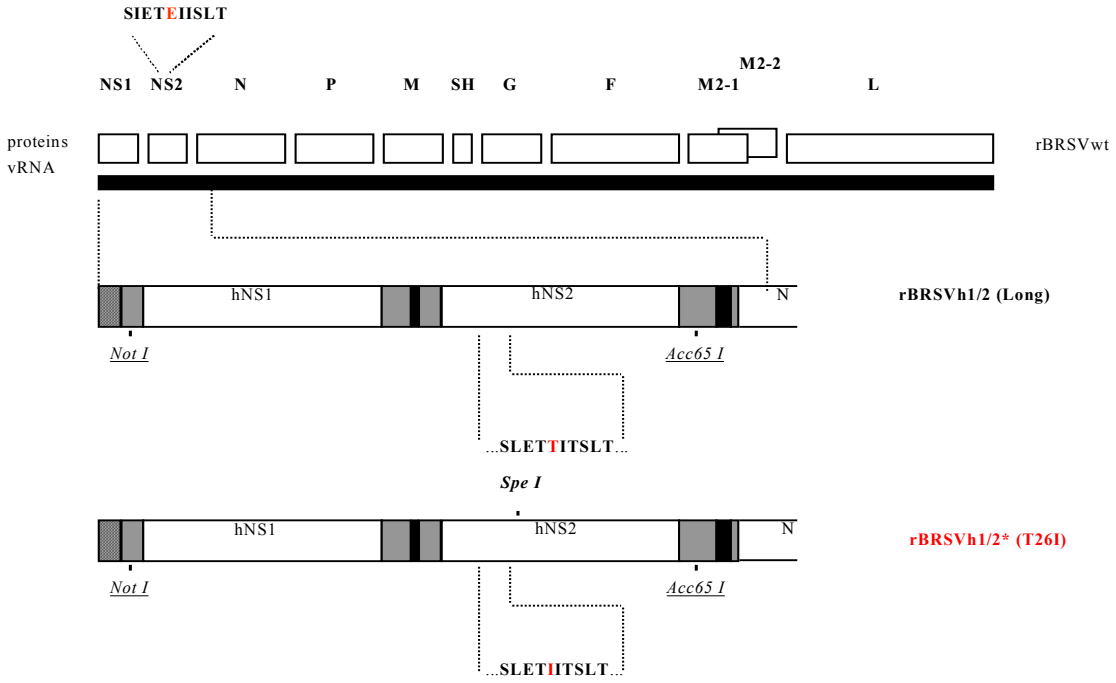

Fig. 5 Chimeric rBRSVs harbouring HRSV NS genes. Viral genome (vRNA) and gene ORF encoded proteins are shown. In the enlargement: BRSV NS proteins were replaced by HRSV Long NS proteins (rBRSVh1/2). An amino acid exchange was subsequently introduced into the NS2 protein at position 26 (T26I) generating the recombinant virus rBRSVh1/2*^{T26I}.

4.4.2 HRSV NS protein expression and cDNA restriction analysis

Western blot analysis was used to confirm the correct translation of NS proteins in Vero cells infected with HRSV prototypes, A2 and Long, and with the chimeric rBRSVs.

Restriction digest analysis was performed on HRSV NS2 cDNA obtained by RT-PCR reaction of virus-infected HEp2 cells. The NS2 ORF of the rBRSVh1/2*^{T26I} contained an additional restriction site for *Spe I* endonuclease, which is absent in the wild-type sequences. After the digest, only NS2 cDNA derived from rBRSVh1/2*^{T26I} showed a lower migrating band.

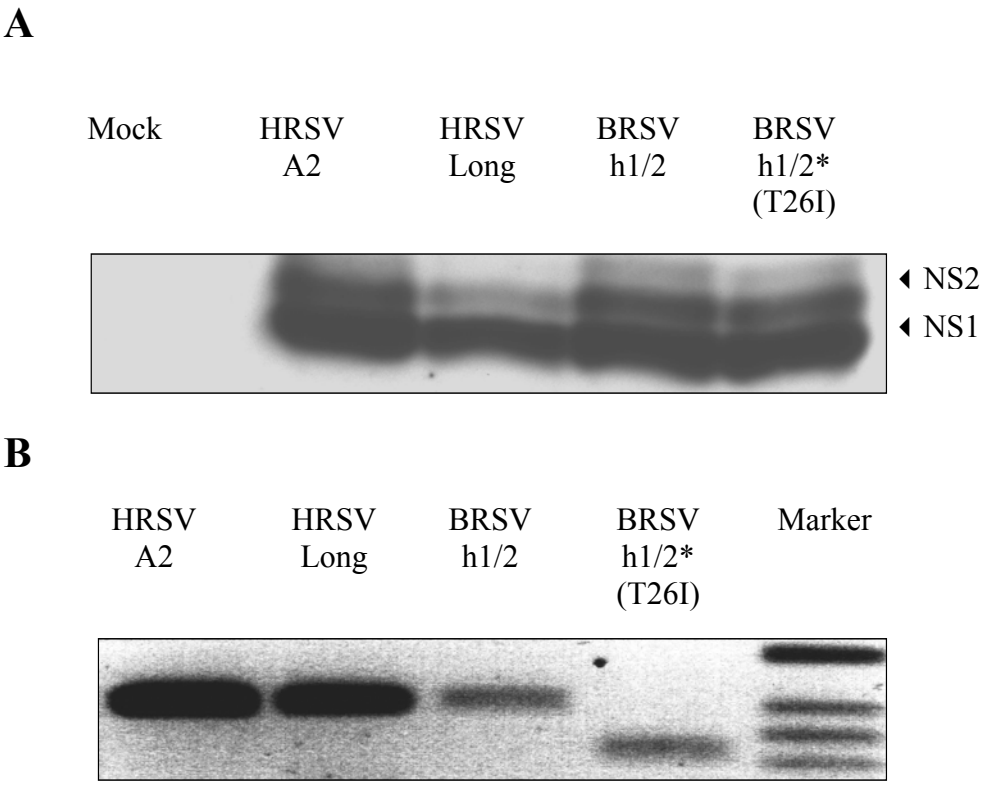

Fig. 6 Analysis of chimeric BRSVs expressing HRSV NS genes. (A) Western blot analysis for NS protein expression in Vero infected cells**.** NS proteins were detected using a polyclonal antibody (α-IC/C) recognizing HRSV NS proteins (kindly provided by J.A. Melero, Spain). (B) Restriction analysis of NS2 cDNA obtained from HEP2 cells infected with HRSV A2, HRSV Long and recombinant BRSVh1/2 and BRSVh1/2^{T26I} .

4.4.3 Growth kinetics of recombinant BRSV viruses

Growth properties of rBRSVh1/2 and rBRSVh1/2*^{T26I} and HRSV strains were analysed in parallel in Vero and HEp2 cells.

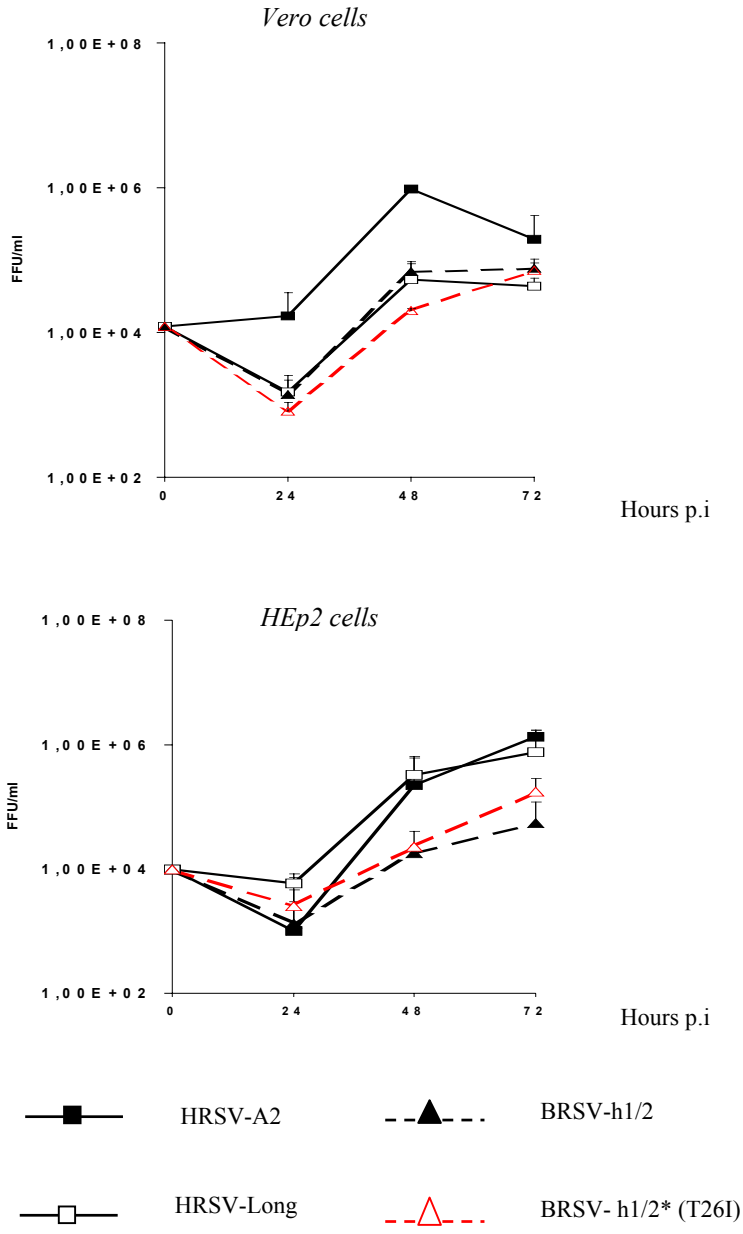

Fig. 7 Analysis of chimeric BRSVs expressing HRSV NS genes. Growth kinetics of chimeric rBRSVh1/2 and rBRSVh1/2*^{T26I}, and HRSV strains A2 and Long in Vero and HEp2 cells. Cells were infected with MOI of 0.3 and viruses were harvested at the indicated time points.

In Vero cells, which do not possess an intact IFN system, the two chimeric rBRSV viruses behaved similarly. After 3 days of infection at a MOI of 0.3, they reached titers of about 7 $x10^4$ FFU, close to those of HRSV A2 and Long. In HEp2 cells, a human cell line competent for type I IFN expression, growth characteristics of rBRSVh1/2 and rBRSVh1/2*^{T26I} were once again comparable. After 2 days of infection, both viruses reached similar titers of about 1.8-2.2 $x10^4$ FFU/ml , respectively. When compared to HRSV strains, which grew to highest titers of about 1 $x10^6$ FFU/ml 3 days post infection, the recombinant BRSV viruses displayed a slightly attenuated phenotype of approximately 1 log. This might be due to the fact that recombinant BRSV viruses are less efficient in the context of a human cell line as a consequence of viral host-specific factors.

4.4.4 Sequence comparison of HRSV NS genes

To verify the identity of recombinant viruses Vero cells were infected with HRSV A2 and Long, rBRSVh1/2 and rBRSVh1/2*^{T26I} viruses and RT-PCR was followed by sequencing of the amplified NS1 and NS2 genes. Amino acid sequence comparison confirmed the presence of an Isoleucin residue at position 26 of the NS2 protein of the chimeric rBRSVh1/2*^{T26I}, while the rest of the protein remained unvaried. Amino acid sequences of the NS1 proteins were also analysed to exclude any unrelated mutation. Western blot analysis was used to confirm correct translation of NS proteins in Vero cells infected with HRSV prototypes, A2 and Long, and with the chimeric rBRSV viruses.

A

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HRSV_A2          MGSNSLSMIKVRLQNLFDNDEVALLKITCYTDKLIHLTNA     40
CL.ISOL#112      MGSNSLSMIKVRLQNLFDNDEVALLKITCYTDKLIHLTNA     40
Cl.ISOL#86       MGSNSLSMIKVRLQNLFDNDEVALLKITCYTDKLIHLTNA     40
BRSVh1/2_T26I    MGSNSLSMIKVRLQNLFDNDEVALLKITCYTDKLIHLTNA     40
BRSVh1/2         MGSNSLSMIKVRLQNLFDNDEVALLKITCYTDKLIHLTNA     40
HRSV_Long        MGSNSLSMIKVRLQNLFDNDEVALLKITCYTDKLIHLTNA     40
Consensus        mgsnslsmikvrlqnlfdndevallkitcytdklihltna

HRSV_A2          LAKAVIHTIKLNGIVFVHVITSSDICPNNNIVVKSNFTTM     80
CL.ISOL#112      LAKAVIHTIKLNGIVFVHVITSSDICPNNNIVVKSNFTTM     80
Cl.ISOL#86       LAKAVIHTIKLNGIVFVHVITSSDICPNNNIVVKSSFTTM     80
BRSVh1/2_T26I    LAKAVIHTIKLNGIVFVHVITSSDICPNNNIVVKSNFTTM     80
BRSVh1/2         LAKAVIHTIKLNGIVFVHVITSSDICPNNNIVVKSNFTTM     80
HRSV_Long        LAKAVIHTIKLNGIVFVHVITSSDICPNNNIVVKSNFTTM     80
Consensus        lakavihtiklngivfvhvitssdicpnnnivvksnfttm

HRSV_A2          PVLQNGGYIWEMMELTHCSQPNGLLDDNCEIKFSKKLSDS    120
CL.ISOL#112      PVLQNGGYIWEMMELTHCSQPNGLIDDNCEIKFSKKLSDS    120
Cl.ISOL#86       PVLQNGGYIWEMMELTHCSQPNGLIDDNCEIKFSKKLSDS    120
BRSVh1/2_T26I    PVLQNGGYIWEMMELTHCSQPNGLIDDNCEIKFSKKLSDS    120
BRSVh1/2         PVLQNGGYIWEMMELTHCSQPNGLIDDNCEIKFSKKLSDS    120
HRSV_Long        PVLQNGGYIWEMMELTHCSQPNGLIDDNCEIKFSKKLSDS    120
Consensus        pvlqnggyiwemmelthcsqpngliddnceikfskklsds

HRSV_A2          TMTNYMNQLSELLGFDLNP                         139
CL.ISOL#112      TMTNYMNQLSELLGFDLNP                         139
Cl.ISOL#86       TMTNYMNQLSELLGFDLNP                         139
BRSVh1/2_T26I    TMTNYMNQLSELLGFDLNP                         139
BRSVh1/2         TMTNYMNQLSELLGFDLNP                         139
HRSV_Long        TMTNYMNQLSELLGFDLNP                         139
Consensus        tmtnymnqlsellgfdlnp
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B

```
HRSV-A2          MDTTHNDNTPQRLMITDMRPLSLETIITSLTRDIITHKFI      40
CL.ISOL#112      MDTTHNGTTPQRLMITDMRPLSLETIITSLTRDIITHRFI      40
CL.ISOL#86       MDTTHNGTTPQRLMITDMRPLSLETIITSLTRDIITHRFI      40
BRSVh1/2_T26I    MDTTHNDTTPQRLMITDMRPLSLETIITSLTRDIITHRFI      40
BRSVh1/2         MDTTHNDTTPQRLMITDMRPLSLETTITSLTRDIITHRFI      40
HRSV-Long        MDTTHNDTTPQRLMITDMRPLSLETTITSLTRDIITHRFI      40
Consensus        mdtthndttpqrlmitdmrplsletiitsltrdiithrfi

HRSV-A2          YLINHECIVRKLDERQATFTFLVNYEMKLLHKVGSTKYKK      80
CL.ISOL#112      YLINHECIVRKLDERQATFTFLVNYEMKLLHKVGSTKYKK      80
CL.ISOL#86       YLINHECIVRKLDERQATFTFLVNYEMKLLHKVGSTKYKK      80
BRSVh1/2_T26I    YLINHECIVRKLDERQATFTFLVNYEMKLLHKVGSTKYKK      80
BRSVh1/2         YLINHECIVRKLDERQATFTFLVNYEMKLLHKVGSTKYKK      80
HRSV-Long        YLINHECIVRKLDERQATFTFLVNYEMKLLHKVGSTKYKK      80
Consensus        ylinhecivrklderqatftflvnyemkllhkvgstkykk

HRSV-A2          YTEYNTKYGTFPMPIFINHDGFLECIGIKPTKHTPIIYKY     120
CL.ISOL#112      YTEYNTKYGTFPMPIFINHDGFLECIGIKPTKHTPIIYKY     120
CL.ISOL#86       YTEYNTKYGTFPMPIFINHDGFLECIGIKPTKHTPIIYKY     120
BRSVh1/2_T26I    YTEYNTKYGTFPMPIFINHDGFLECIGIKPTKHTPIIYKY     120
BRSVh1/2         YTEYNTKYGTFPMPIFINHDGFLECIGIKPTKHTPIIYKY     120
HRSV-Long        YTEYNTKYGTFPMPIFINHDGFLECIGIKPTKHTPIIYKY     120
Consensus        yteyntkygtfpmpifinhdgflecigikptkhtpiiyky

HRSV-A2          DLNP                                         124
CL.ISOL#112      DLNP                                         124
CL.ISOL#86       DLNP                                         124
BRSVh1/2_T26I    DLNP                                         124
BRSVh1/2         DLNP                                         124
HRSV-Long        DLNP                                         124
Consensus        dlnp
```

Fig. 8 Multiple alignment of amino acid sequences of NS1 (A) and NS2 (B) proteins in recombinant BRSV (rBRSVh1/2 and rBRSVh1/2*^{T26I}), HRSV strains A2 and Long and HRSV-clinical isolates. Sequence identity for NS2 and NS1 was 99,19% and 99,82% respectively. The arrow indicates the presence of the inserted mutation in the NS2 sequence of rBRSVh1/2*^{T26I}

4.4.5 rBRSVh1/2*^{T26I} but not rBRSVh1/2 inhibits IFNβ induction

To examine the ability of the two chimeric rBRSVs to induce IFN-β, a modified reporter gene assay was used. Vero cells harbouring the IFN-β gene promoter/enhancer (p125Luc) were mock-infected and infected with wild-type BRSV, rBRSVh1/2, rBRSVh1/2*^{T26I}, HRSV A2 and HRSV Long at an MOI of 0.3. Luciferase activity was measured 24 and 48 hours post infection. An induction of luciferase activity of about 9- and 12- fold was observed in cells infected with HRSV Long and rBRSVh1/2, respectively. A minor induction of the IFN-β gene promoter, compared to mock-infected cells, was detected for the wild-type BRSV, HRSV A2 and the recombinant rBRSVh1/2*^{T26I}.

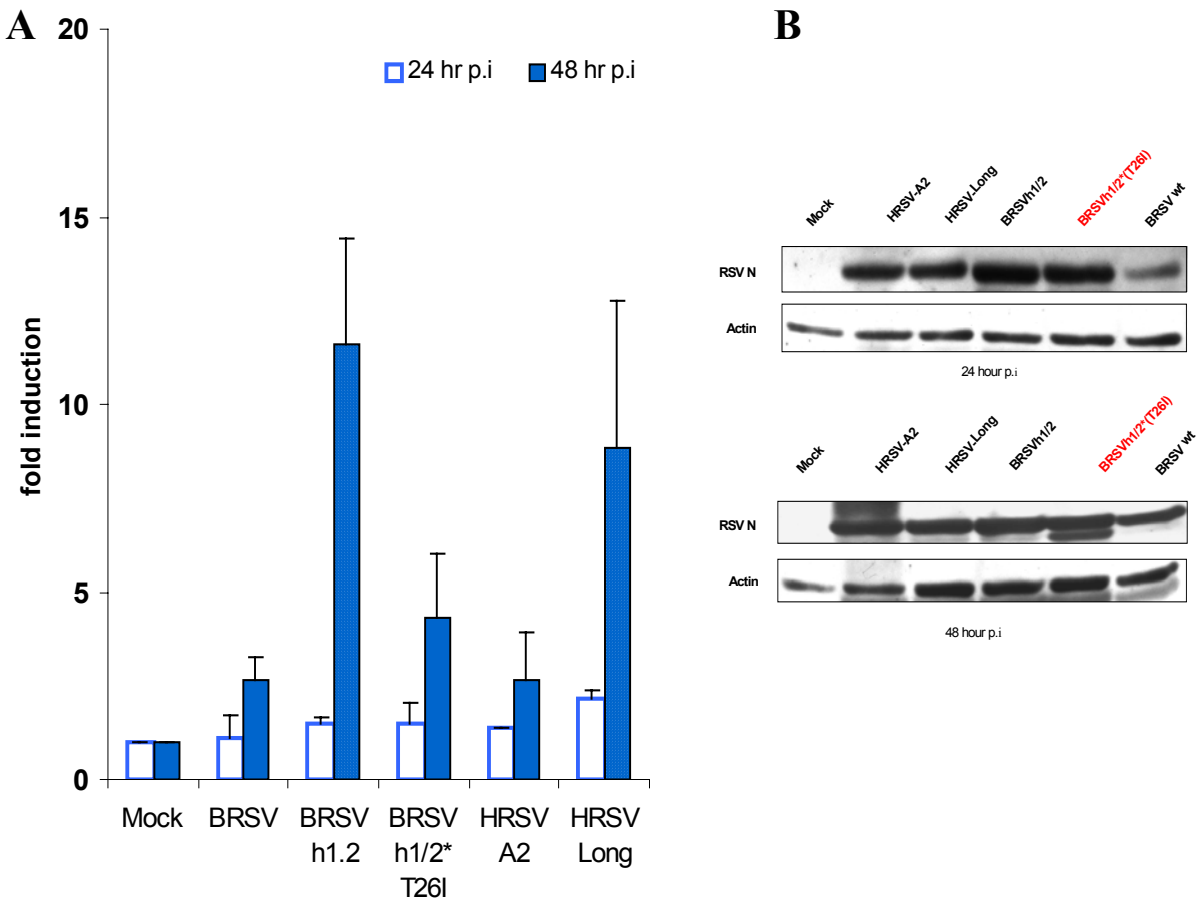

Fig. 9 Virus-induced activation of IFN-β gene promoter. (A) Vero cells expressing the IFN-β gene promoter/enhancer (p125Luc) were infected with the indicated viruses at an MOI of 0.3 24 and 48 hours post infection activity of the luciferase reporter gene was measured and the relative light units were expressed as fold induction relative to the mock-infected control. Results represent the mean value of three independent experiments with error bars indicating standard deviation. (B) Western blot analysis of cell lysates used in the gene reporter assay. Blots were probed with a polyclonal α-RSV serum

These results suggest that the recombinant BRSV (rBRSVh1/2*^{T26I}), expressing the HRSV NS2 protein where the Threonin in position 26 was mutated to an Isoleucine, resembles HRSV-A2 in the capacity to block induction of IFN-β responsive gene. Therefore, rBRSVh1/2*^{T26I} differs from rBRSVh1/2, which instead correlates to HRSV Long. Levels of IFN-β mRNA of infected cells were detected by RT-PCR. Two different human IFN-active cell lines, HEp2 and A549 were infected with wild-type BRSV, rBRSVh1/2, rBRSVh1/2*^{T26I}, HRSV A2 and HRSV Long at an MOI of 1 followed by RNA extraction at 16 hours post-infection.

In A549 cells BRSV Δ1/2, lacking NS genes, was included as a positive control. Amplification of an IFN-β specific fragment was detectable in cells infected with HRSV Long, rBRSVh1/2 and BRSV Δ1/2 but was absent in the case of mock, HRSV A2 and rBRSVh1/2*^{T26I} infected cells. These data were perfectly in line with those obtained in the reporter gene assay. β-actin sequences were also amplified as a demonstration of DNA integrity, while amplification of NS2 gene was performed to evaluate the occurrence of the infection

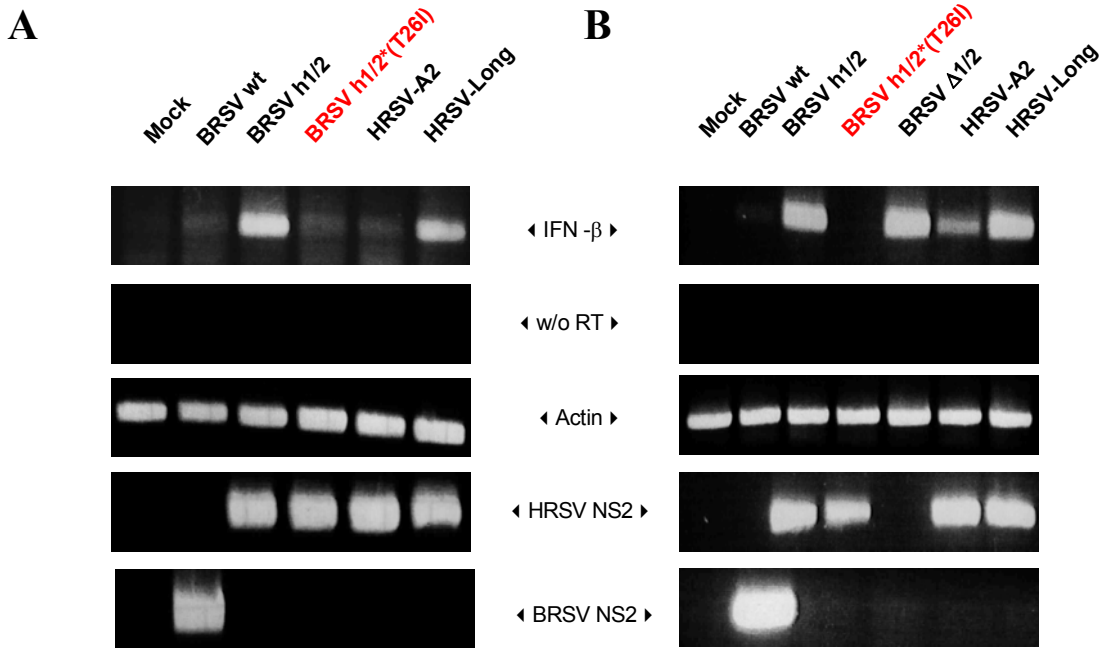

Fig. 10 RT-PCR analysis of IFN-β expression in virus infected cells. Hep2 (A) or A549 (B) were mock- or infected with HRSV A2, HRSV Long, rBRSVh1/2 and rBRSVh1/2*^{T26I} at a MOI of 1. RT-PCR analysis of IFN-β, NS2 and β-actin gene expression was performed 16 hours post infection.

4.4.6 Recombinant BRSVh1/2 *^{T26I} selectively reduces activation of transcriptional factor IRF3

Expression of IFN-α/β genes is strongly induced at the transcriptional level following viral infection. IFN-β promoter contains binding sites for AP-1, NF-kB and IRF3 factors, which form a multiprotein transcriptional-promoting complex. In order to investigate whether the activation of these transcriptional factors is impaired upon RSV infection, 293 cells were first transfected with the corresponding reporter plasmids, as previously described. At 24 hours post transfection, cells were mock-infected and infected at an MOI of 0.3 with HRSV A2 and Long, BRSV wild-type and the recombinant BRSVs: BRSVh1/2 and BRSVh1/2*^{T26I}.

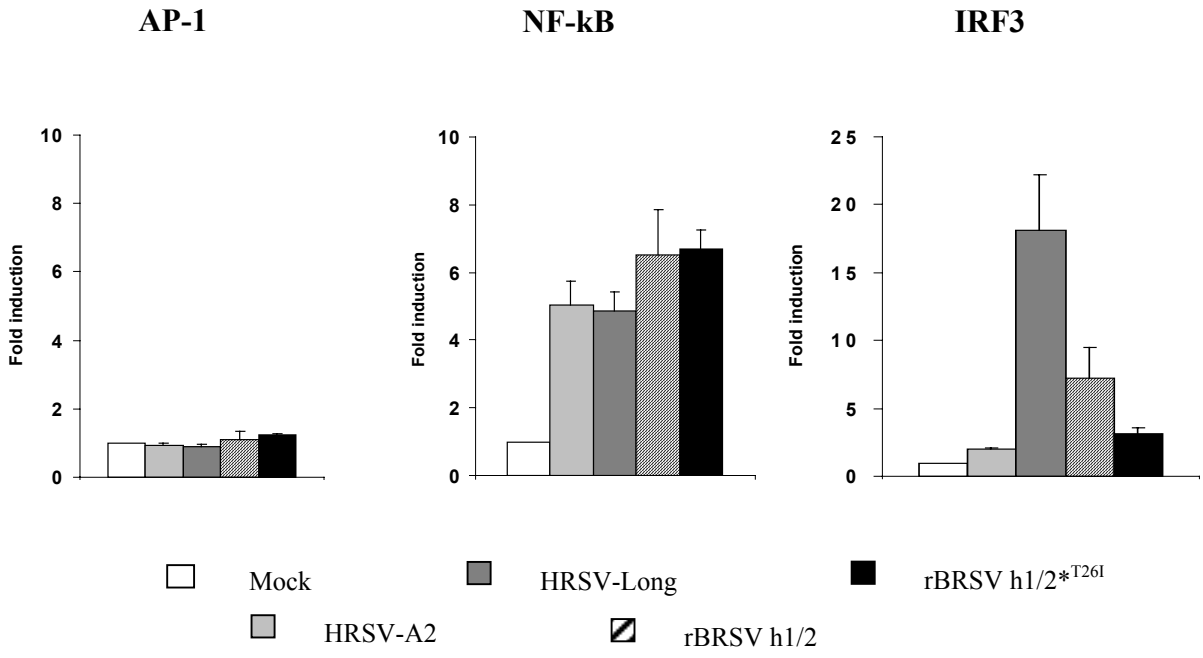

Fig. 11 Induced activation of IFN-β transcription factors. Luciferase constructs under the control of promoters containing AP-1, NF-kB and IRF3 binding sequences were transfected in 293 cells. After 24 hours cells were infected with the indicated viruses at an MOI of 0.3. At 24 hrs p.i. cells were harvested and luciferase assay was performed. Results show the mean of at least two independent experiments

Infection did not result in detectable activation of AP-1 reporter in either case. NF-kB-related luciferase activity showed instead a comparable increase between 4.2 and 6.7-fold in infected cells, with no major differences among the described viruses. Infection with HRSV Long increased 18-fold IRF3-promoter activity, while a very small induction was observed in HRSV A2 and BRSV wt infected cells, as already described. As expected, rBRSVh1/2 promoted IRF3 activation; luciferase activity was lower than in HRSV Long infected cells but still an increase of about 7-fold was observed. When cells were infected with rBRSVh1/2*^{T26I} only a 3.2-fold increase was detected indicating a possible block of IRF3 specific activation in the case NS2 protein contains an Isoleucine at residue 26.

4.4.7 Residue 26 of NS2 protein does not influence interferon resistance of recombinant BRSV viruses

Type I IFNs do not inhibit productive infection of RSV in human epithelial cells suggesting the existence of viral mechanisms for evasion of the airway defence-response.
The behaviour of HRSV strains and recombinant BRSV viruses was analysed in IFN-treated cells. HEp2 cells, infected at an MOI of 0.3 with HRSVs or BRSV chimeras, were treated with increasing amounts of IFN-α A/D and viral titers were determined 3 days post infection. Despite protection was not complete, all four viruses were significantly resistant to IFN-treatment. After application of 5000 IU of IFN, titers of rBRSVh1/2 and rBRSVh1/2*^{T26I} declined by about 1 log. Thus, both chimeric BRSV viruses were only slightly sensitive to IFN and in a comparable way, indicating that the exchange of Threonin 26 with an Isoleucine did not play a role in RSV IFN-resistance.

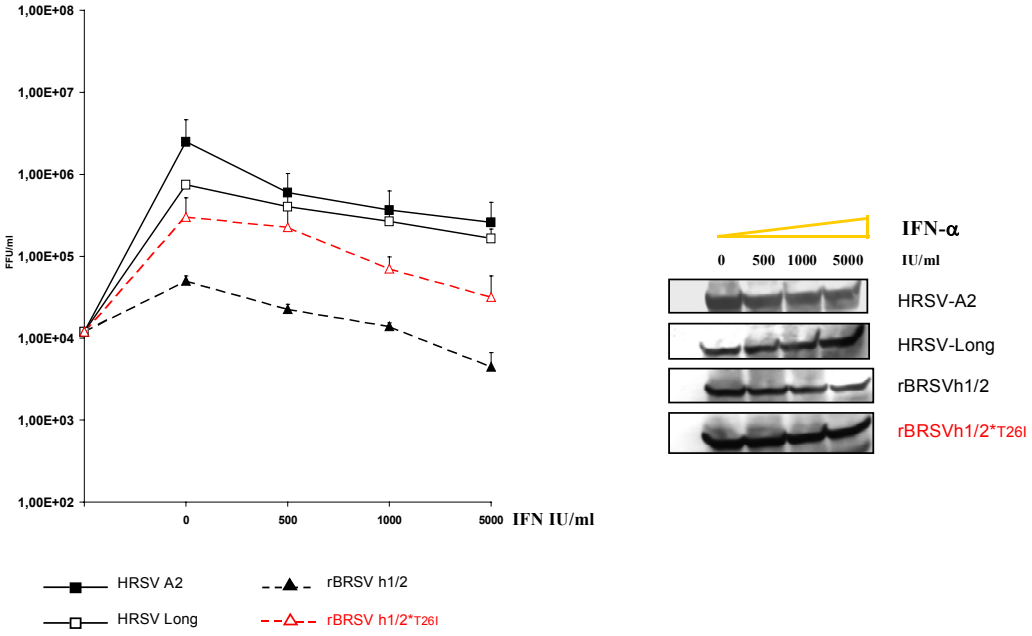

Fig. 12 Recombinant BRSV are only slightly IFN type I sensitive. (A) HEp2 cells infected at an MOI of 0.3 with HRSV A2, HRSV Long, rBRSVh1/2 and rBRSVh1/2*^{T26I} were incubated with the indicated amounts of recombinant IFN-α. Infectious viral titers were determined 3 days post infection. Bars show standard deviation of two independent experiments. (B) Western blot analysis of RSV N protein synthesis in IFN-treated HEp2 cells

4.5 IkappaB kinase-related complex: TBK1, IKK-ε and TANK

4.5.1 Cloning of TANK-Binding Kinase 1 (TBK1) and TRAF family member-associated NF-kappa B activator (TANK)

The inducible IκB kinase (IKK-i or IKK-ε) and the TANK-Binding Kinase 1 (TBK1) act as IRF3 kinases and they are involved in IFN-β expression (see Introduction).

The complete reading frame of TBK1 and TANK (which is a structural component of the kinase complex) were amplified by PCR from a human lung cDNA library. The TBK1 and TANK complete ORFs were then cloned into an expression vector, where they were coupled at their N-termini with a human Ig fragment.

Correct expression of the fusion proteins was monitored in transfected BSR T7/5 cells by Western blot. Specific bands of about 110 kDa and 75 kDa, corresponding respectively to IgTBK1 and IgTANK proteins (80 kDa and 50 kDa plus 24 kDa plus the Ig fragment) respectively, were detected 48 hours post transfection.

IgTBK1 expression was also tested in the same cell line by immunofluorescence and compared with the other two components of the IKK-related complex, IgTANK and FlagIKK-ε (which was kindly provided by Prof. R. Ruckdeschel, MvP-Munich). All three fusion proteins appeared to be strictly confined to the cytoplasma with a diffuse distribution, no structure formation was observed

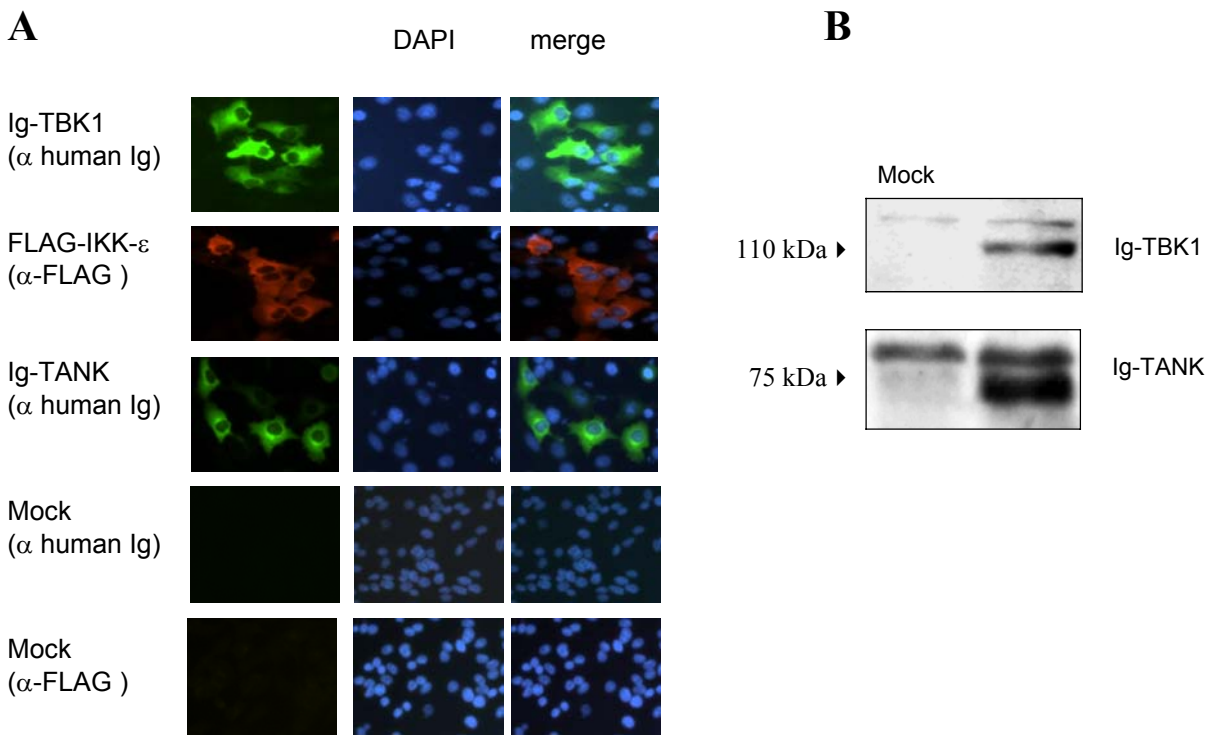

Fig. 13 Expression of the IKK-related complex. (A) BSR T7/5 cells were transiently transfected with the expression vectors for IgTBK1, FlagIKK-ε and IgTANK. Forty-eight hours after transfection, immunostaining was performed using the anti-Ig and anti-flag antibodies. The stained specimens were visualised by confocal microscopy. (B) IgTBK1and IgTANK protein expression in BSR T7/5 cells was confirmed by immunoblotting. Immunoprecipitation was performed using Sepharose-A beads and immunoprecipitates were blotted with the anti-TBK1 and anti-human Ig antibodies.

4.5.2 TBK1 activates IFN-β production

Overexpression of TBK1 and IKK-ε was reported to be sufficient to induce type I IFN production. Therefore, functional analysis of IgTBK1 in this context was carried out by reporter gene assay. Equal amounts of empty vector (pCR3-Ig), IgTBK1, FlagIKK-ε, a kinase dead mutant of IKK-ε (FlagIKK-ε dm), and an analogue of dsRNA (Poly I/C) were transfected in 293 cells together with the IRF3 gene promoter/enhancer (p55CIBLuc).

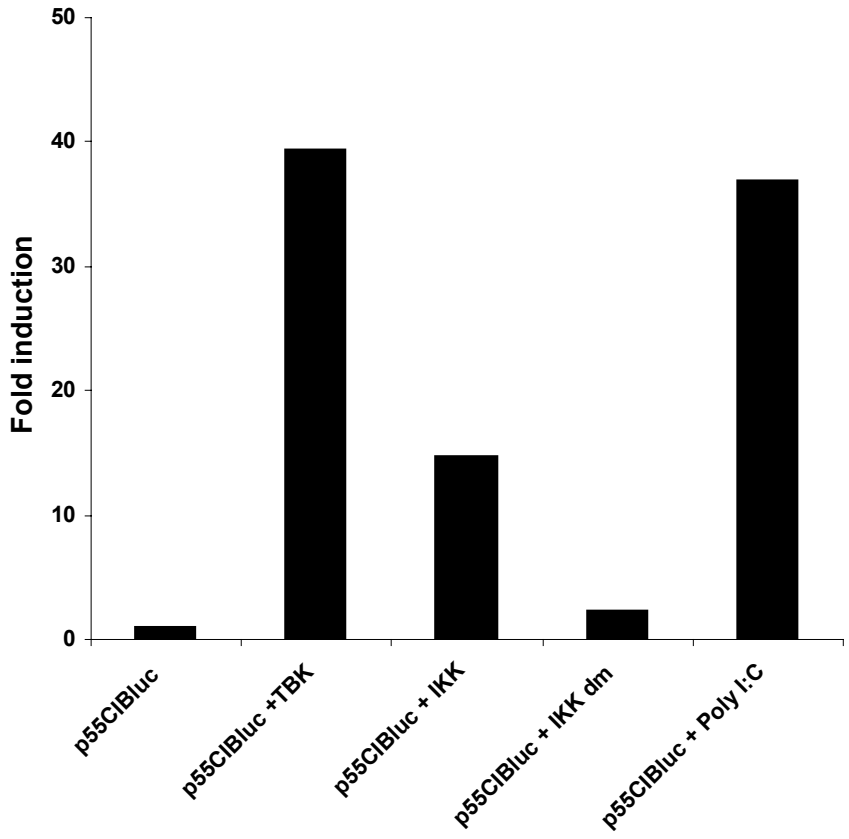

Fig. 14 IFN-β gene promoter activation by expression of IgTBK1 in different cell types. 293 cells were cotransfected with the IRF3 promoter (p55CIBLuc) and expression vectors coding for IgTBK1, FlagIKK-ε, FlagIKK-ε dm or with dsRNA analogue Poly I/C. Cells were harvested 48 hrs post transfections followed by luciferase assay.

Poly I/C is known to trigger IFN induction (Jacobs B.L. and Langland J.O., 1996), for this reason it was included as a positive control. IgTBK1 and FlagIKK-ε mediated activation of the IRF3 reporter expression of 39- and 15-fold, respectively, when expressed from the transfected plasmids. These values were comparable to the PolyI/C-induced luciferase activity, which was about 37-fold.Transfection of IKK-ε dm, an IKK-ε form in which the kinase domain has been disrupted by mutation of Lysin 38 to an Alanin (K38A), did not lead to significant activation as it was expected.

A similar gene reporter assay, using in this case the IFN-β gene promoter/enhancer (p125Luc), was carried out on BSR T7/5 cells.

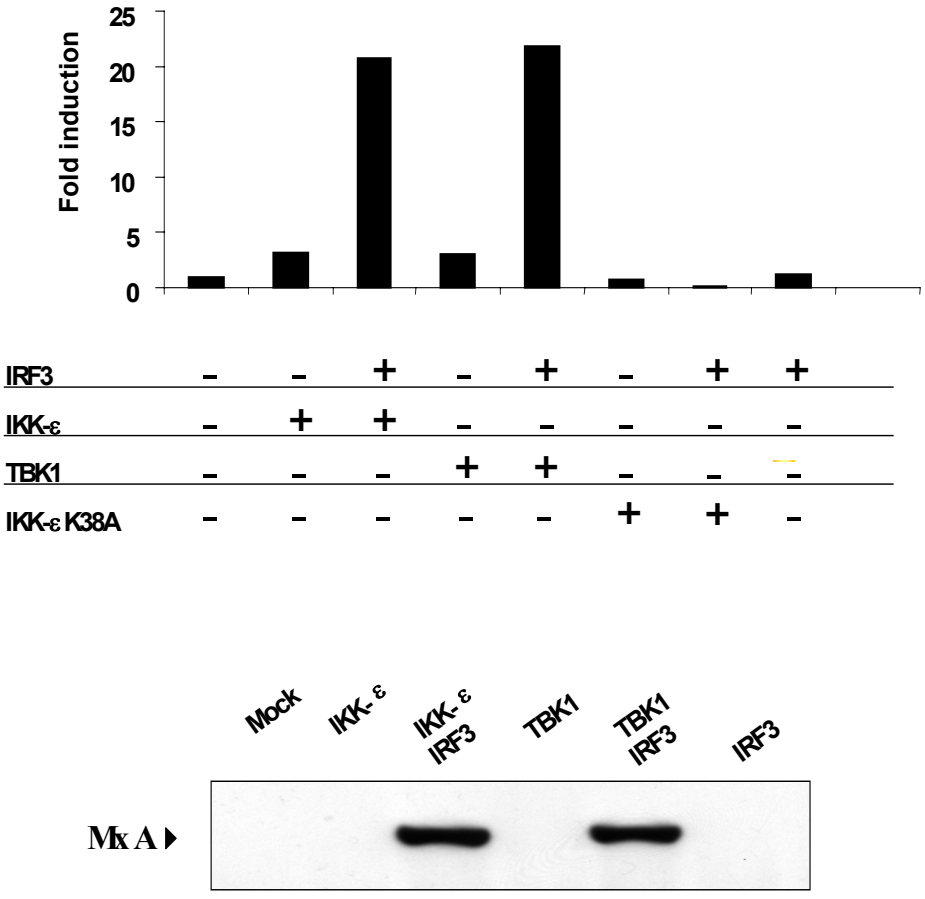

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Fig. 15 IFN-β gene promoter activation by expression of IgTBK1 and IRF3 in BSR T7/5 cells. (A) Gene reporter assay on BSR T7/5 cotransfected with p125Luc and IgTBK1, FlagIKK-ε or FlagIKK-ε dm together with IRF3 as indicated. (B) Western blot analysis of Mx A expression in BSR T7/5 cell lysates obtained in A

This cell type has beenshown to be defective in IFN induction (data not shown). Overexpression of the sole IgTBK1 or FlagIKK-ε did not induce luciferase activation. When IRF3 was coexpressed together with kinases stimulation up to 20-fold was reached 48 hours post transfection. Mx A protein expression, which is an antiviral IFN-induced intracellular protein (Staeheli P. et al., 1986), was detected on the cell lysates obtained in the gene reporter assay by Western blot analysis. Bands of 76 kDa, corresponding to the correct size of Mx A, were present in the samples where IRF3 was cotransfected either with the IgTBK1 or FlagIKK-ε kinase and in which the luciferase activity was abundant.

4.5.3 HRSV strain A2 inhibits TBK1 activation

To examine the effect of HRSV infection on TBK1-dependent IFN-β promoter stimulation, 293 cells were mock-infected and infected with HRSV A2 and Long.

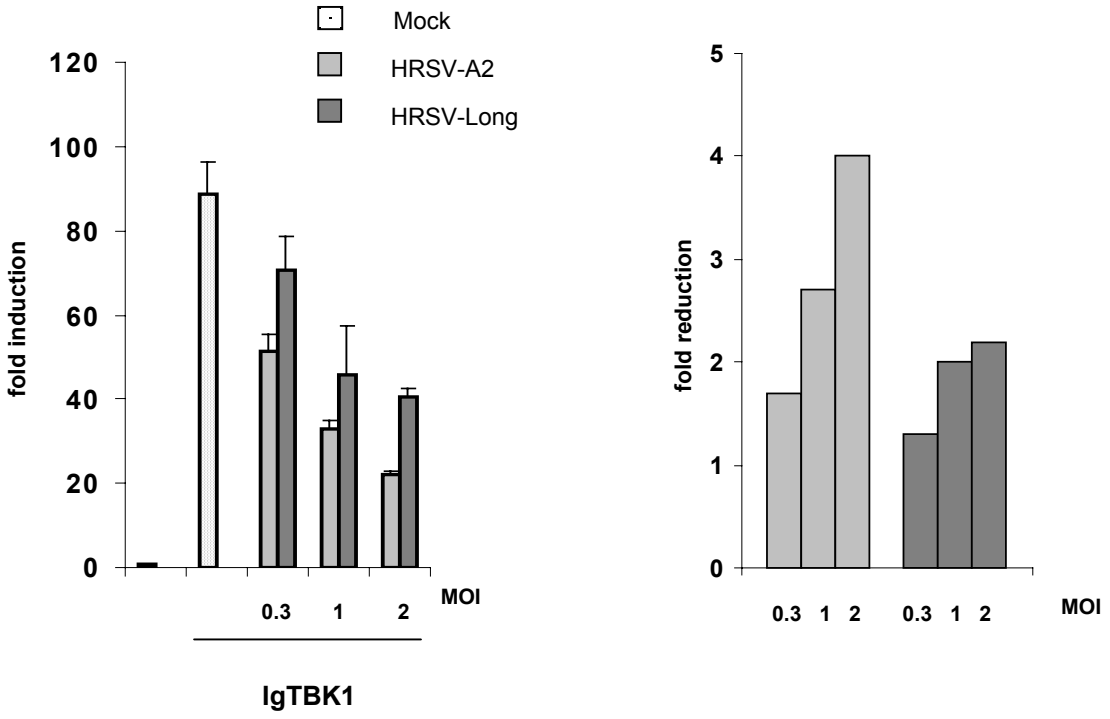

Fig. 16 HRSV inhibits TBK1-mediated expression of IFN-β promoter in an MOI-dependent manner. 293 cells were infected at increasing MOI with HRSV A2 and Long. At 24 hours post infection cells were transfected with the reporter plasmids, p125Luc and pCMV-RL together with IgTBK1. (A) Luciferase activity was assayed 24 hours post transfection and (B) fold reduction was extrapolated. Results show the mean of at least two independent experiments ± SD.

Infection was performed at an MOI of 0.3 or 1 followed, after 24 hours, by cotransfection of the IFN-β reporter construct (p125Luc) with IgTBK1 expression plasmids. IFN-β promoter was responsive to TBK1 expression, resulting in an increase of luciferase activity in mock-infected cells.

p125Luc activation did not decrease in presence of virus infection at an MOI of 0.3 and 1, independently of the strains used. At higher MOI (2), TBK1-induced IFN-β activation resulted to be reduced of 4-fold in HRSV A2 infected cells respect to the 2-fold of Long strain. Inhibition of TBK1 activity by HRSV A2 appeared to be MOI-dependent and more marked than in HRSV Long infected cells.

4.5.4 rBRSVh1/2*^{T26I} inhibits TBK1 activation similarly to HRSV A2

Recombinant BRSV viruses expressing human NS proteins differ in their ability to inhibit IFN-β induction upon infection and this is linked to a specific amino acid residue at position 26 in the NS2 protein. To investigate whether the different capacity of rBRSVh1/2 and rBRSVh1/2*^{T26I} to induce interferon production directly involved TBK1 kinase, like in the corresponding HRSV prototypes Long and A2, I took advantage of the gene reporter assay previously described.

293 cells were virus-infected at an MOI of 1 and 2 and cotransfected with p125Luc and IgTBK1. Luciferase activity was measured and the ability of the different RSV viruses to inhibit the IFN-β gene promoter activation by TBK1 overexpression was evaluated. When cells were infected at an MOI of 1, activation of TBK1-induced IFN-β promoter was slightly reduced: about 3-fold in HRSV infected cells and 1.5-fold in the case of rBRSVs.

At MOI of 2, inhibition of IFN-β promoter induction was more marked and a difference could be seen between rBRSVh1/2 and rBRSVh1/2*^{T26I} as well as between HRSV A2 and Long.

HRSV Long and rBRSVh1/2 caused a reduction in luciferase activity of about 4-fold in contrast to 6.9- and 5.4-fold of HRSV A2 and rBRSVh1/2*^{T26I}, respectively.

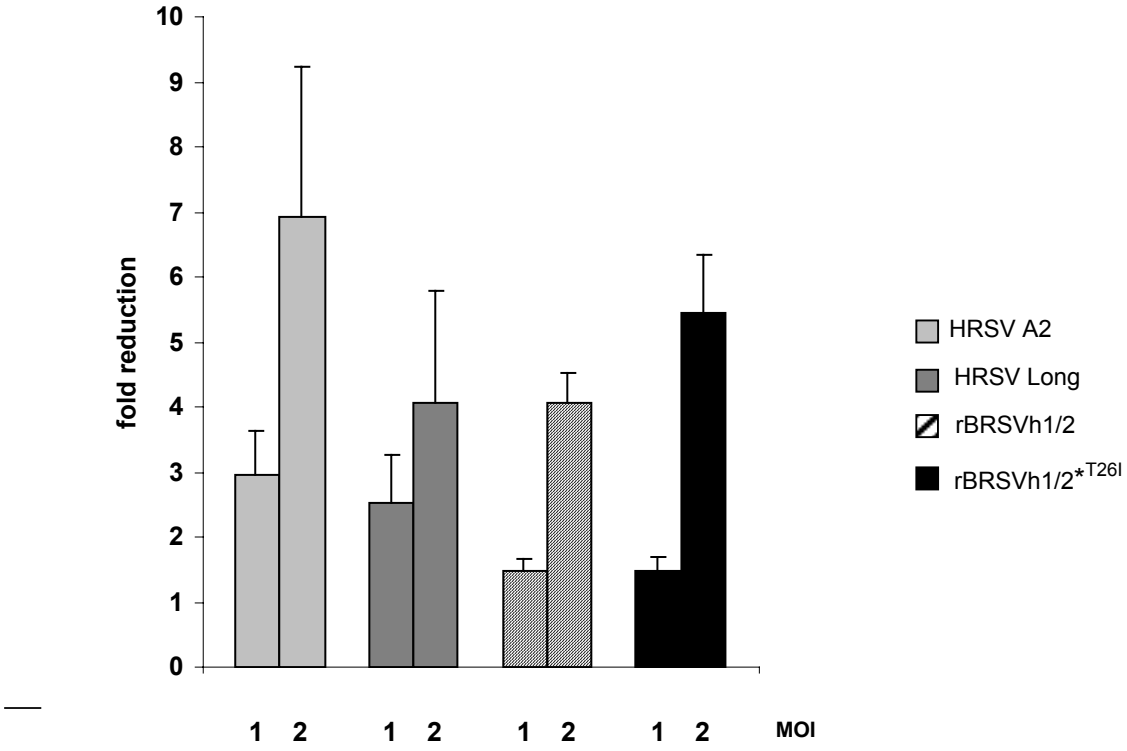

Fig. 17 RSV infection influences TBK1-mediated IFN-β promoter activation. 293 cells were infected at an MOI of 1 and 2 with the indicated viruses. At 24 hours p.i, cells were transfected with reporter plasmids, p125Luc and pCMV-RL together with IgTBK1. Fold reductions were calculated based on the luciferase activity measured 24 hours post transfection. Results show the mean of three independent experiments ± SD

Induction of IFN-β promoter by IgTBK1 transfection in 293 cells is particularly strong and rapid. Since TBK1 self-activation works in a cascade-manner, activity of exogenous TBK1 is likely to be enforced by the presence of endogenous kinase as well as of endogenous IRF3 factor. This might create experimental conditions that are far too different from a natural situation and for this reason viruses might not be able to effectively display their inhibitory ability in such an artificial environment. Moreover viral infection itself has some induction effects. Therefore, I decided to verify TBK1 inhibition by HRSV A2 and rBRSVh1/2*^{T26I} in a different cell system.

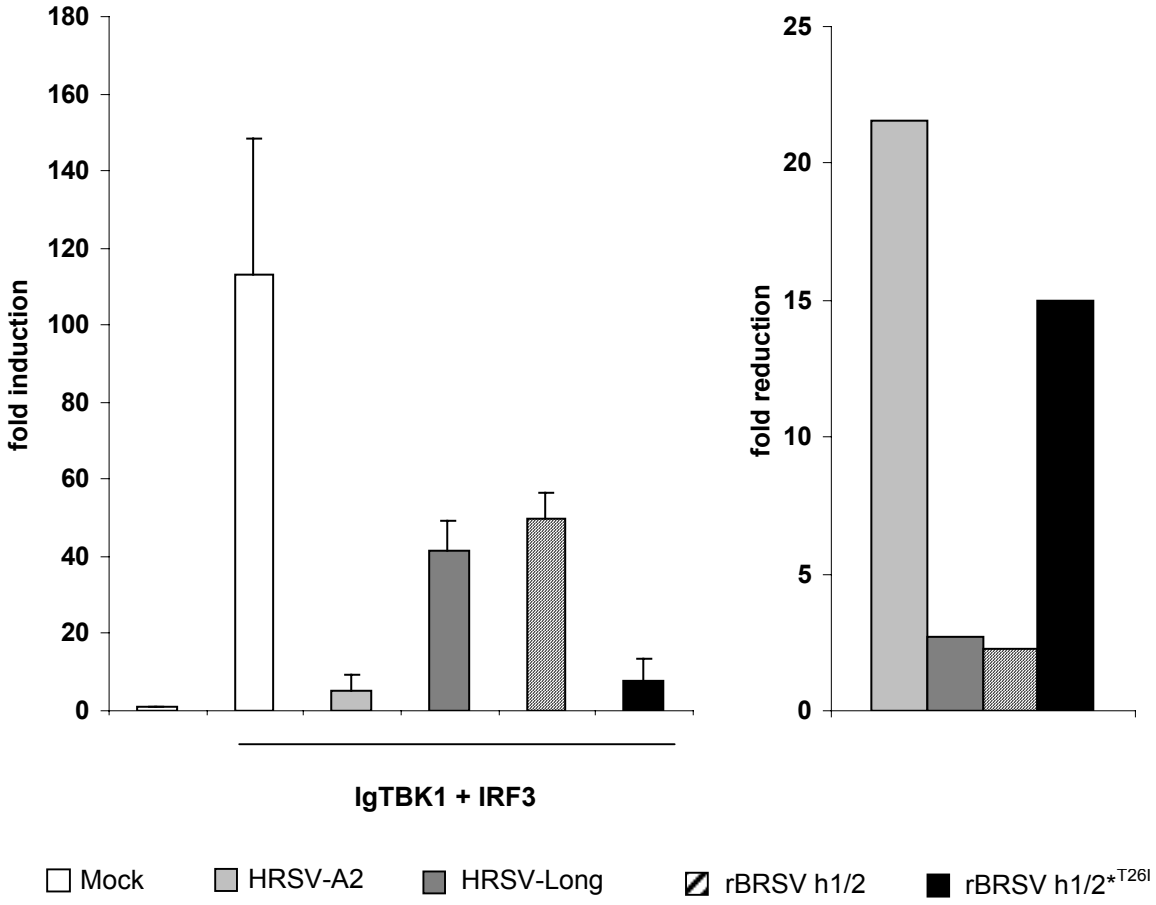

Fig. 18 TBK1-mediated IFN-β promoter activation in BSR T7/5 infected cells. BSR T7/5 cells were infected at an MOI of 1 with HRSV A2, Long, rBRSVh1/2 and rBRSVh1/2*^{T26I}. At 24 hours p.i., cells were transfected with reporter plasmids p125Luc and pCMV-RL together with IgTBK1 and pEF–haIRF3. Fold reductions were calculated based on the luciferase activity of mock-infected cells stimulated with IgTBK1.

I performed the same experiment previously described but in BSR T7/5 cells. This cell line is faulty in IFN-induction signalling pathway and this may be attribute to a defective IRF3 (overexpression of IgTBK1 does in fact not activate p125Luc) and more likely also to TBK1 itself. In this way, I could exclude residual IFN induction by viral infection, which was performed before transfection, and I could induce IFN-β promoter just by exogenous TBK1 and IRF3. BSR T7/5 cells were infected with the indicated viruses at an MOI of 1 followed 24 hours later by transfection with the following plasmids: p125Luc, pCMV-RL, pEF-haIRF3 and pCR3-IgTBK1. Stimulation of mock-infected cells with IgTBK1 and IRF3 led to an increase of luciferase activity of 113-fold. Upon infection with HRSV Long and rBRSVh1/2

a slight reduction of about 2.5-fold was observed In contrast HRSV A2 and rBRSVh1/2*^{T26I} decreased significantly TBK1-dependent activation of IFN-β promoter of 21- and 15- fold, respectively.

4.6 Recombinant RV expressing RSV nonstructural proteins

4.6.1 Construction of recombinant SAD eGFP-P viruses harbouring RSV NS proteins

To determine whether NS proteins were sufficient to block IFN induction by an unrelated virus, I generated RV viruses expressing Long-derived NS1 protein with either NS2 from strain Long (SAD eGFP-Ph2/1) or a mutated form of NS2 where Threonine at position 26 was exchanged to an Isoleucine (SAD eGFP-Ph2*^{T26I}/1).

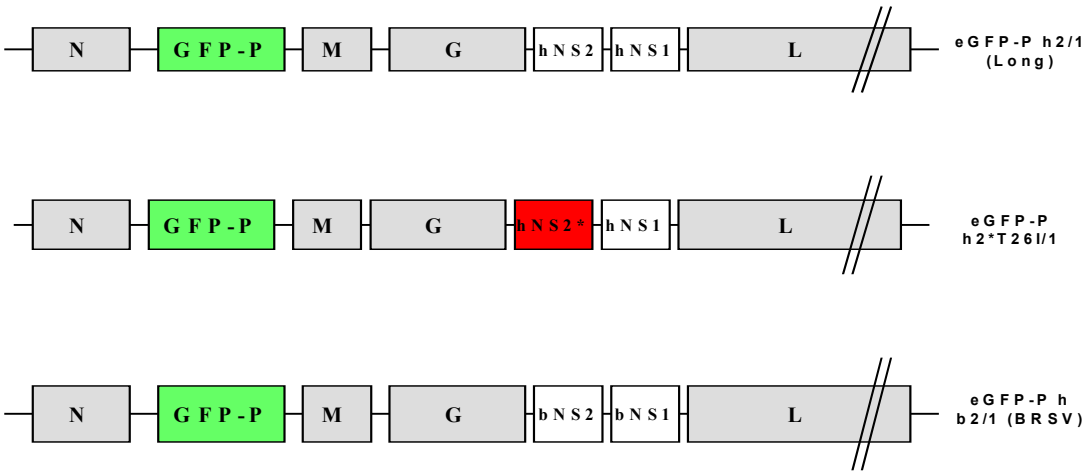

Fig. 19 SAD eGFP-P viruses expressing RSV non structural proteins. Schematic genome organization of eGFP-Ph2/1 expressing NS proteins from HRSV Long; eGFP-Ph2*^{T26I}/1 harbouring the NS1 gene from Long strain and an NS2 gene derived from Long but mutated at position 26 (T26I); eGFP-Pb2/1 with insertion of NS1 and NS2 genes from BRSV wt.

For this purpose, I used a recombinant fluorescent RV in which the fusion of eGFP to P protein (SAD eGFP-P) unabled the virus to block IFN induction upon infection (Brzozka K. et al., 2005). The additional NS genes were introduced between G and L genes of SAD eGFP-P as described in Materials and Methods and recombinant viruses were rescued from cDNA

in BSR T7/5 cells. The growth kinetics of the recombinant eGFP-P viruses in BSR T7/5 cells did not greatly differ from that of SAD eGFP-P virus with the exception of eGFP-Pb2/1. Unexpectedly, the same was observed in IFN-competent HEp2 cells. eGFP-Ph2*^{T26I}/1 mutant was not able to grow productively. Its titers were almost 3 log lower than Sad L16, indicating that the expression of the mutated NS2*^{T26I} protein did not effectively antagonize IFN induction. Addition of RSV nonstructural proteins genes did not conferred IFN resistance to SAD eGFP-P as it was instead reported in the case of recombinant wild-type RV (SAD VBh2/1).

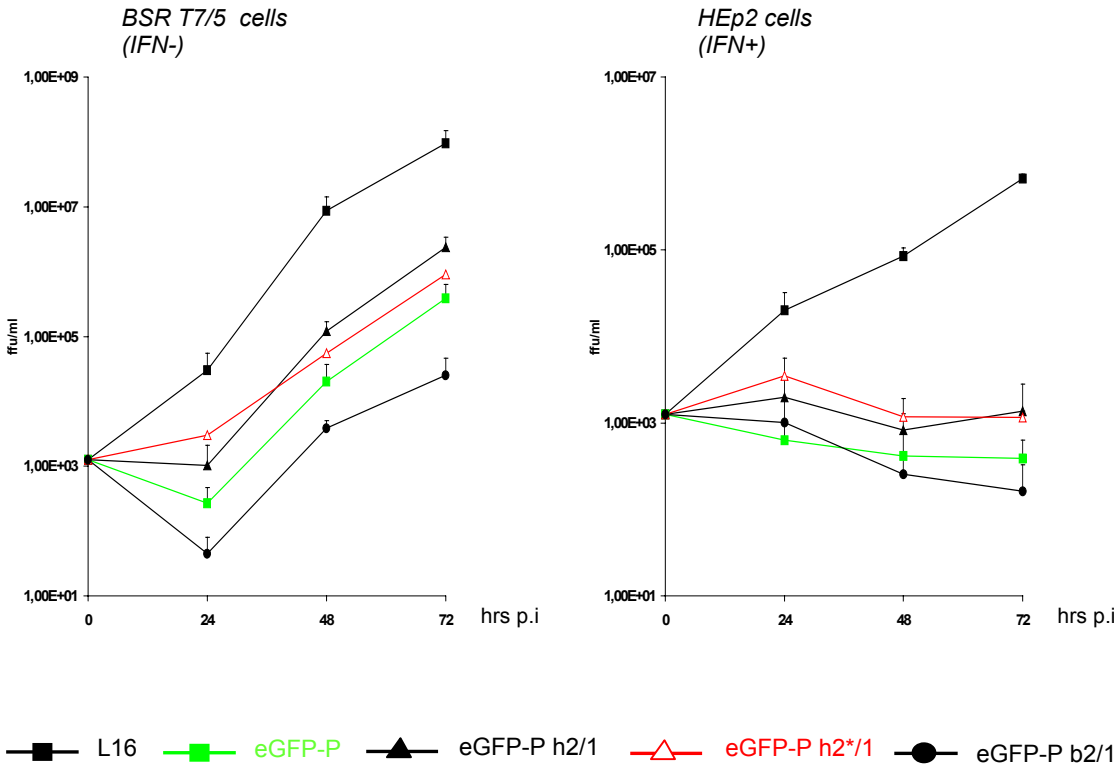

Fig. 20 Growth properties of recombinant SAD eGFP-P viruses. BSR T7/5 and HEp2 cells were infected with the indicated viruses at an MOI of 0.01. Virus titres were determined at different time points from cell supernants. Results show the mean of at least three independent experiments ± SD.

Transcription of IFN-β mRNA was analysed by RT-PCR in infected HEp2 cells. Cells were infected at an MOI of 1 with wild type RV (SAD L16), eGFP-P, recombinants eGFP-Ph2/1, eGFP-Ph2*^{T26I}/1 and eGFP-Pb2/1 (expressing BRSV NS proteins).RNA was isolated 16-18

hours post infection and RT-PCR was performed with specific primer for IFN-β and β-actin, which was used as a loading control. In contrast to mock-infected and wt RV infected cells, IFN-β mRNA expression showed up-regulation in cells infected with eGFP-P and recombinant eGFP-Ps harbouring RSV non structural proteins. The inability of eGFP-P recombinant viruses to inhibit IFN-β gene transcription correlated to their inability to productively grow in HEp2 cells.

Cells extract of BSR T7/5 cells infected with eGFP-P, eGFP-Ph2/1 and eGFP-Ph2^{T26I}/1 at an MOI of 1 were analysed for protein synthesis of RV N and P together with HRSV NS1 and NS2 by Western blotting. The expression of the mutated NS2 protein (NS2*^{T26I}) was significantly lower than the original Long NS2 protein expressed by eGFP-Ph2/1, while levels of NS1 protein were comparable.

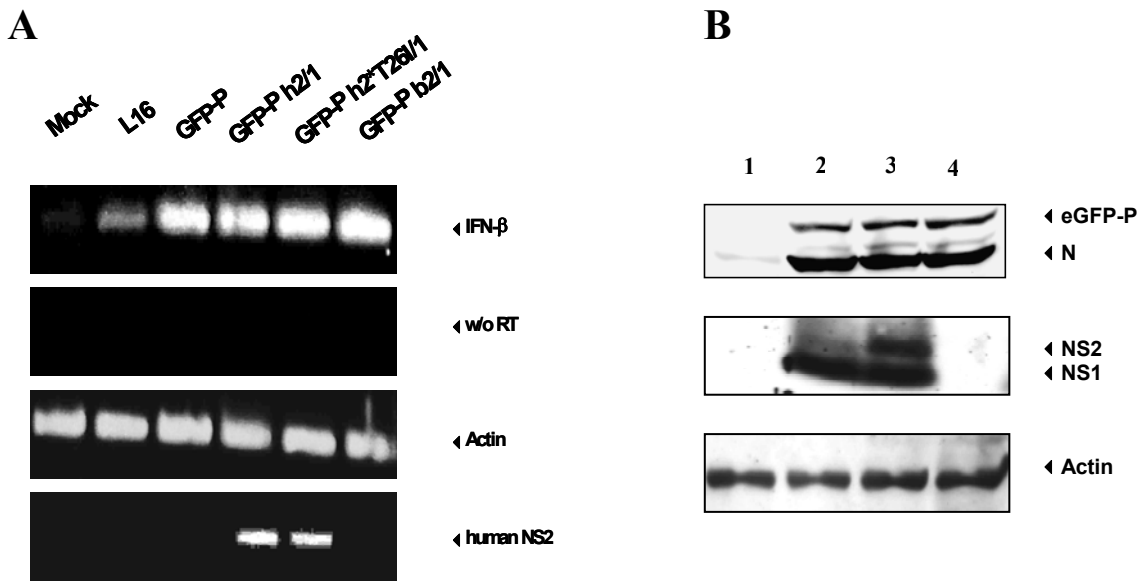

Fig. 21 (A) RT-PCR for IFN-β mRNA expression in rabies infected HEp2 cells. HEp2 cells were mock-infected and infected at an MOI of 1 with the HRSV NS2 mRNA expression. **(B) Western blot analysis for human NS proteins expressed by recombinant eGFP-P viruses.** BSR T7/5 cells were mock-infected (1) and infected with: eGFP-Ph2*^{T26I}/1 (2); eGFP-Ph2/1 (3) and eGFP-P wt (4). Viral protein synthesis was confirmed using the rabbit serum S50 recognizing RV nucleoprotein (N) and eGFP fusion phosphoprotein (eGFP-P). HRSV NS1 and NS2 proteins were detected by the polyclonal rabbit α-NS1 (IC/C) antibody kindly provided by J. Melero, Spain.

5 DISCUSSION

5.1 Human respiratory syncytial virus: strains A2 and Long

5.1.1 Suppression of IFN-β induction differs in HRSV A2 and Long

Human respiratory virus (HRSV) is the major cause of viral bronchiolitis and pneumonia in infants worldwide. Although RSV is commonly known as a paediatric pathogen, it can cause life-threatening lung disease in bone marrow recipients and elderly. The World Health Organization has estimated a global annual infection of something like 64 millions individuals, with an overall claiming of 3-5 millions human lives annually.

RSV isolates can be classified into two subgroups, designated A and B, which exhibit antigenic and genetic differences, with a major divergence in the G glycoprotein. Group A and B can co-circulate during epidemics, which occur during winter and early spring in temperate climates and during the rainy season in tropical areas.

By 2 years of age, essentially 100% of the children get infected with RSV at least once. Moreover re-infections can occur throughout life and are usually symptomatic. Severe RSV infection in infancy is often followed by recurrent childhood wheezing probably leading to asthma exacerbations in older children and adults. RSV-associated disease is in large part due to excessive immune response, and the immunopathology of RSV includes the relatively unique phenomenon of vaccine-enhanced disease or “immunopotentiation”. These particular features of RSV infection, susceptibility to repeat infections and enhanced disease during a recall/memory adaptive immune response to infection after vaccination, indicate that RSV employs several novel mechanisms of immune dysregulation to propagate within the human population. Understanding of RSV effects on the development and expression of both innative and adaptive immune response may provide important information concerning the formulation of effective therapies. IFN-α/β (type I interferons) are inducible cytokines secreted by most eukaryotic cells in response to viral pathogens and they are of particular importance as an early line of innate immune defence. Induction of type I IFN by dsRNA or by virus infection is controlled at the transcriptional level. IFN-β mRNA expression level peaks around 15-18 hours post stimulation and dramatically decreases afterwards due to a negative-feedback loop.

RSV expresses two putative nonstructural proteins, NS1 and NS2. We have previously shown that the NS proteins of the bovine RSV (BRSV) exert an inhibitory activity on the induction of IFN-α/β suppressing interferon-mediated antiviral state (Bossert B. et al., 2003).

Recently, this has been shown also for HRSV, the human counterpart of BRSV (Spam K.M et al., 2004). HRSV strongly inhibits activation of interferon regulatory factor 3, whereas high levels of activation were detected in cells infected with deletion mutants for NS1 and/or NS2 genes (Spann K.M., et al., 2005).

We have demonstrated that BRSV interferes with IRF3 activation by blocking its phosphorylation, which is an essential step for its nuclear import. It is likely that the same mechanism is true for HRSV. We could show that two commonly used HRSV laboratory strains, A2 and Long, differ fin their capacity to interfere with IFN induction. Long has been found to induce IFN-α in epithelial cells and plasmacytoid dendritic cells (PDC) in contrast to A2 and clinical isolates from hospitalised children (Schlender J. et al., 2005). In this study, I have analysed IFN-β induction in epithelial cells infected with the two HRSV prototypes. The first evaluation was performed using a Vero cell-line stably transfected with IFN-β promoter/enhancer controlling the expression of the luciferase gene (p125Luc). While promoter activity increased more than 12-fold after infection with Long, A2 did not elicit a significant increase. Interestingly, the difference in luciferase activity was greatest at 48 hours post infection. This might depend on accumulation of NS proteins in the cell, which is necessary to counteract induction by dsRNA. Spann and colleagues have in fact found that HRSV resembles the NS gene deletion mutants in their ability to activate IRF3 at early stages of infection. Later on, HRSV wt, opposite to the deletion mutants, blocks further activation probably when accumulation of NS1 and NS2 proteins reaches sufficient levels in the cell.

RT-PCR for detection of IFN-β mRNA expression has been performed in HEp2 cells mock-infected and infected with HRSV A2 and Long. Infection of HEp2 cells with HRSV Long strain resulted in a appreciable increase in the expression level of IFN-β mRNA compared to that of mock- or A2-infected cells. Thus, it was possible to confirm the results obtained in the luciferase assay and I concluded that Long strain is a potent IFN-β inducer in contrast to A2.

5.1.2 Activation of IFN-β transcription factors

The IFN-β promoter contains binding sites for three distinct transcriptional factors: ATF-2/c-Jun (AP-1), NF-kB, and IRF3. These factors form a multiprotein complex, called “enhanceosome”, which drives to transcription of the IFN-β gene.

Using reporter constructs expressing the luciferase gene under the control of either AP-1, NF-kB or IRF3 binding sites, activation of all three transcriptional factors by infection with HRSV A2 and Long was assayed.

AP-1 was not significantly activated in response to infection with both strains. The limited responsiveness of AP-1 to RSV infection has been already documented and it has been attributed to sensitivity to the cell culture redox state (Mastronarde J.G. et al., 1998).

NF-kB plays an essential role in early events of innate immune response regulating the expression of inflammatory and immunomodulatory genes. RSV infection has been shown to potently and specifically activate NF-kB *in vivo* (Haeberle H.A. et al., 2002).

Since HRSV-A2 and Long vary considerably in their ability to induce IFN-β *in vitro*, the magnitude of NF-kB activation has been also investigated. The results indicate that the levels of NF-kB-responsive reporter gene activation were essentially the same in the case of both A2 and Long infected cells.

Interferon regulatory factor (IRF)-3 is the critical transcription factor regulating immediate/early alpha-beta IFN genes. Various pathogens activate cell signals resulting in phopshorylation of specific Serine and threonine residues in theIRF3 C-terminal region by recently identified kinases (IKK-ε and TBK1). Several viral proteins have been reported to interfere with IRF3 activation. These proteins are encoded for example by Bunyamwera, Influenza A, Ebola, Bovine respiratory syncytial and Measles virus (Kohl A. et al., 2003; Talon J. et al., 2000; Basler C.F. et al., 2003; Bossert B. et al., 2003). In the present study, I investigated IRF3 activation by HRSV. In contrast to A2, Long correlates to increased activation of IRF3 promoter, therefore indicating a selective block of this factor in A2 infected cells. This clearly explains the reason why IFN-β induction is compromised by infection with A2 virus and not with Long strain.

5.1.3 Sequence analysis of nonstructural NS proteins in HRSV

In an effort to correlate the ability to antagonize IFN-β expression with specific viral factors, I have analysed and compared NS protein amino acid sequences derived from different HRSV strains, which had proved to differ in the achievement of a full inhibitory effect.

In the analysis, HRSV A2 and Long and two representative clinical isolates obtained from hospitalised children were included. Our previous work had shown that the clinical isolates were able to counteract IFN-α production similarly to the laboratory strain A2. Since NS1 and

NS2 proteins have been already described as potent interferon antagonists, I searched for relevant differences in their amino acid sequences.

NS1 protein of BRSV shares 69% identity at amino acid level with the corresponding sequence of HRSV subgroup A NS1. Comparison analysis of NS1 gene of HRSV laboratory strains A2 and Long and the clinical isolates number 86 and 112 revealed amino acid sequences highly similar to each other and no residue changes appeared to be peculiar for one or more strains.

Only a very limited number of differences were apparent in the NS2 protein sequence as well. Four amino acid changes clustered at its N-terminus and among these, I could identify at position 26 the unique presence of a Threonine distinguishing HRSV Long sequence. A2 and the clinical isolates displayed at the same position an Isoleucine residue instead. This mutation was considered particularly interesting because the two residues greatly differ in terms of polarity: Threonine is a polar amino acid while Isoleucine is hydrophobic. In addition the presence of a Threonine results in a potential additional O-glycosylation site but, even more important, represents a potential phosphorylation acceptor. This observation prompted us to formulate the hypothesis by which the different ability to prevent expression of IFN-β by HRSV A2 and Long strains might be specifically linked to the nature of the residue at position 26 of the NS2 protein.

5.2 A mutated Long-derived NS2 protein prevents induction of IFN-β

5.2.1 Recombinant BRSV expressing HRSV nonstructural proteins

BRSV NS1 and NS2 proteins antagonize the induction of IFN-α/β interfering, at the early stages of virus-stimulated IFN pathway, with the activation of IRF3 (Schlender J. et al., 2000; Bossert B. et al., 2003).

To investigate the interferon inhibitory functions of NS2 proteins derived from A2 and Long viruses, I used reverse genetics to generate recombinant BRSV expressing nonstructural protein of HRSV origin. A chimerical BRSV expressing HRSV Long NS proteins (rBRSV h1/2) has been previously generated, indicating that the HRSV nonstructural proteins are able to fulfil the functions of the bovine counterparts with respect to IFN resistance and in supporting viral replication. Notably, rBRSVh1/2 resembled HRSV Long in the ability to induce IFN-β opposite to wild type BRSV. To establish the importance of Threonine at

position 26 of the NS2 protein, I mutated this residue in the context of rBRSV h1/2 to an Isoleucine, resembling the situation in A2 strain and in the clinical isolates. The chimerical BRSV h1/2*^{T26I} was successfully rescued; NS1 and NS2 gene were sequenced to confirm the correctness of the inserted mutation and the expression of NS proteins was assessed in Vero cells. Furthermore, I compared the growth of the recombinant BRSV viruses (rBRSV h1/2 and BRSVh1/2*^{T26I}) to that of HRSV A2 and Long in HEp2 cells, which are competent for the expression of interferon, and in Vero cells, which instead lack the genes of these cytokines. The chimerical BRSVs replicated efficiently and comparably to HRSVs in both cell lines. The virtually identical growth indicated that the mutation in the HRSV NS2 protein (T26→I) did not affect viral replication.

5.2.2 rBRSVh1/2*^{T26I} infection inhibits IFN-β induction by blocking IRF3 activation

Induction of IFN-β promoter activity was analysed making use of a Vero cell line stably transfected with a reporter construct expressing the luciferase gene under the control of the IFN-β promoter/enhancer. A very slight increase in the promoter activity was observed in cells infected with HRSV A2, BRSV wt and rBRSVh1/2*^{T26I} while HRSV Long and rBRSVh1/2 displayed an induction around 10-folds.

IFN-β mRNA expression was also analysed by RT-PCR in HEp2 infected cells. Even in this case, while the rBRSVh1/2 led to an increased of expression readily detectable 15-16 hours post infection, rBRSVh1/2*^{T26I} did not. Taken together these data indicate that the presence of an Isoleucine instead of a Threonine at position 26 of the NS2 amino acid sequence does indeed contribute to the IFN inhibitory functions of the chimeric rBRSVs.

Induction of IFN-β is controlled by three different transcription factors (AP-1, NF-kB and IRF3), which become activated following phosphorylation events in response to viral infection. Therefore I further investigated if the block of IFN-β expression by rBRSV h1/2*^{T26I} was due to impairment of one or more of these transcription factor activities. I used reporter constructs expressing the luciferase gene under the control of either one or the other transcription factor binding sites. IRF3 activation was greatly compromised in rBRSV h1/2*^{T26I} (the same was for HRSV A2 and BRSV wt) but not in rBRSVh1/2 infected cells. This pointed toward a selective block of IRF3 transcription factor activation by rBRSVh1/2*^{T26I} ; no differences were in fact observed in the case of AP-1 or NF-kB.

BRSV and HRSV are known to be resistant to antiviral responses triggered by exogenous IFN and this ability once again is attributable to the nonstructural protein NS1 and NS2. Recombinant BRSV grew identically in HEp2 cells despite in the case of rBRSVh1/2 IFN is produced. To exclude any negative influence on viral IFN-resistance caused by the mutation in the NS2 protein, I tested viral replication of rBRSVh1/2 and rBRSVh1/2*^{T26I} in IFN-treated HEp2 cells. All the viruses tested, including the chimerical BRSVs, were almost completely protected even against high doses of exogenous IFN-α (up to 5000 IU/ml). Therefore, I could exclude that residue 26 of NS2 protein playes a role in the ability of RSV to counteract IFN-induced antiviral immune response.

5.3 The novel IkB-related kinases (IKK): IKK-ε and TBK1

5.3.1 Cloning TANK-binding kinase 1 (TBK1) and TRAF family member–associated NF-kB activator (TANK)

Induction of IFN-β expression by viral infection requires activation of the transcription factors ATF-2/c-Jun (AP1), NF-kB and IRF3. In unstimulated cells, IRF3 is present in the cytoplasm. Activation of IRF3 by viruses or dsRNA occurs via phosphorylation of a cluster of Serine and Threonine residues in its carboxyterminus and particular importance has been attributed to Serine 386 (Mori M. et al., 2003). Phosphorylation leads to dimerization of IRF3 and formation of a complex including CREB-binding protein (CBP) and p300. This complex after translocation to the nucleus, activates IFN gene transcription. The kinases responsible for IRF3 phosphorylation have been recently identified in the inducible IKK-ε and in the constitutively expressed TANK-binding kinase, TBK1 (Sharma S. et al., 2003). TBK1 is an 80-85 kDa protein which exerts kinase activity upon phosphorylation of specific Serine residues in its loop region. Involvement of the cytoplasmic helicases RIG-I and MDA5 has been recently described. RIG-I and MDA5 recognize dsRNA and this leads to downstream activation of IKK-ε/TBK1 and IKKα/β via the adaptor protein IPS-1 (Kawai T. et al., 2005).Viruses, in their attempt to evade the innate immune response, have elaborated sophisticated mechanisms to abrogate the production of type I IFNs. The nonstructural protein NS3/4-A of hepatitis C virus has been described as an inhibitor of RIG-I and MDA5 signalling functions (Yoneyama M. et al., 2005; Breiman A. et al., 2005; Foy E. et al., 2005). On the same line, hepatitis A virus blocks activation of IRF3 by interaction with RIG-I (Fernsterl V. et al., 2005).

Expression of TBK1, as well as IKK-ε alone, is sufficient to trigger activation of IRF3 and therefore to induce IFN expression.

To better understand the role of RSV nonstructural (NS) proteins in perturbing the activation signalling pathway of IRF3, I have cloned and expressed TBK1. Furthermore, I compared its biological functionality in different cell lines. Expression of the Ig-tagged TBK1 was confirmed by Western blotting analysis by a single band at the expected molecular mass of around 110 kDa. Expression of TBK1 is rather weak and difficult to visualize probably due to its rapid turn over, for this reason immunoprecipitation was needed. Expression of TBK1 was also investigated by immunofluorescence. After transfection, TBK1 appeared diffusely distributed into the cytoplasm. Similarly to IKK-ε. (Fig. 22), its expression induced translocation of IRF3 into the nucleus.

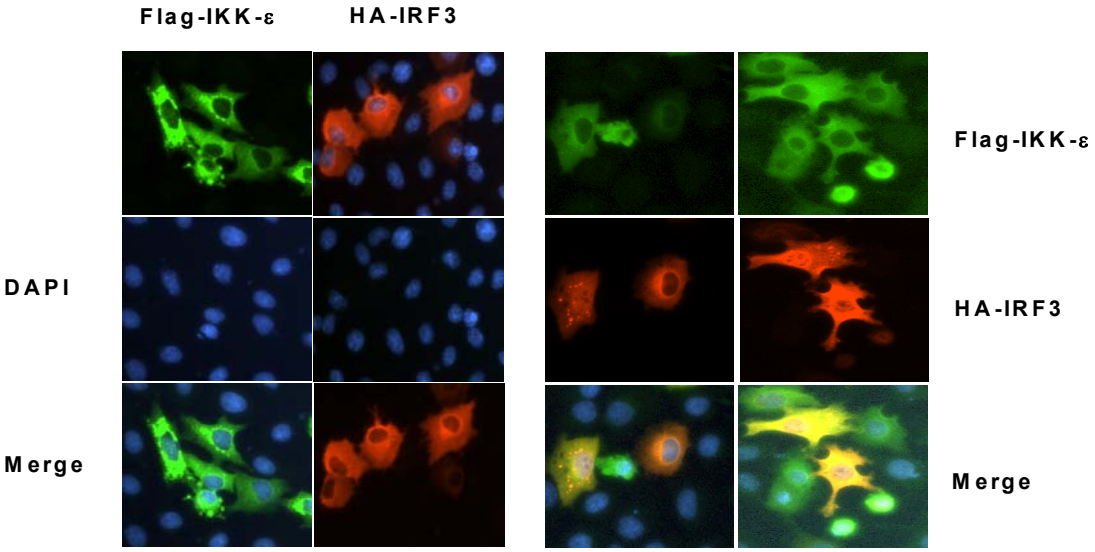

Fig. 22 Nuclear translocation of IRF3 factor upon transfection of IKK-ε. BSR T7/5 cells were transfected with expression plasmids for HA-tagged IRF3 or/and FlagIKK-ε. Cells were stained with the M2 antibody recognizing the Flag epitope while the HA-tag was detected using the antibody Y-11. Nuclei were stained with DAPI (Hoechst).

5.3.2 TBK1-dependent IFN-β induction

As previously described (Fitzgerald K.A. et al., 2003), expression of FlagTBK1 in 293 cells induced activation of IRF3-regulated IFN-β promoter in a luciferase assay.
TBK1 increased luciferase activity several fold in a dose-dependant manner (Marozin S personal observation), comparable to cells transfected with the dsRNA analog Poly I:C.
Furthermore, I was able to reconstitute the IFN-activation signalling pathway in BSR T7/5 cells. This cell line is defective in expressing type I IFN by viral infection and transfection of dsRNA analogues. I have identified the defect in the lack of a functional IRF3 and more likely of TBK1 as well. By cotransfection of TBK1 with IRF3, this function was re-established and provided a useful tool to analyse IFN induction mechanisms in the absence of endogenous interferences. Expression of the IFN-induced MxA protein confirmed IFN production.

5.4 Inhibition of TBK1 by RSV

5.4.1 HRSV A2 blocks TBK1-induced IFN-β expression

To investigate whether infection with HRSV had some adverse effects on TBK1-dependent activation of IFN-β transcription, I assayed TBK1-dependent IFN-β promoter activation in infected cells. My main interest was to identify variations in the inhibitory ability of HRSV strain A2 and Long that could be linked to the different nature of the amino acid residue at position 26 of the NS2 protein.. Initially, 293 cells were infected with the HRSV prototypes at increasing MOI of 0.3, 1 and 2. At 24 hours post infection, cells were transfected with the reporter plasmid for IFN-.β (p125Luc), pCMV-RL (Renilla) and IgTBK1. When infection was performed at MOI of 0.3 and 1 a very poor inhibition of the luciferase activity was observed, in the best case with less than 3-fold reduction for A2 virus. In addition no major discrepancies were detectable between the two strains. At the first sight this indicated that the interference with IRF3 activation was not at the level of the kinases responsible for its phosphorylation but probably the block was lying somewhere upstream. In consideration of the intrinsic characteristics of RSV, such as difficult growth in tissue culture and being prone to losing infectivity, I considered that MOI of 1 might have been not sufficient to target every cells with at least one viral particle. A high cell-infection was a prerequisite for the outcome of the experiment since artificial stimulation of IFN by transfection with TBK1 was probably

more efficient than in natural conditions. Moreover, it seemed that the levels of accumulation of NS proteins into the cytoplasm of infected cells were decisive for the exertion of a full inhibitory effect from RSV. For this reason, I then used an MOI of 2 to perform the same experiment. Unfortunately higher MOI than 2 was no applicable because infected cells after transfection tend to die. In this experimental condition, I was finally able to demonstrate a difference between A2 and Long viruses. HRSV A2 was able to significantly reduce TBK1-induced luciferase activity of p125Luc by about 4-folds. In contrast, strain Long, which itself does not inhibit IFN-α/β expression, had a minor effect at the same MOI (about 2-fold reduction).

5.4.2 Residue 26 of NS2 protein is important for TBK1 inhibition

To better clarify the role of residue 26 (Threonine vs Isoleucine) of HRSV NS2 protein, I performed the same experiment including the recombinant BRSVs expressing HRSV NS1 and either NS2 protein from Long (T 26) or a mutant form of it (I 26) similar to A2 strain. Even in this case, MOI of 2 was necessary to reveal a pronounced difference in TBK1 antagonism by these viruses. An obvious higher reduction in luciferase activity was detectable in HRSV A2 infected cells. rBRSVh1/2*^{T26I} showed a trend to better counteract TBK1 stimulation when compared to rBRSVh1/2. Unfortunately, the difference in folds reduction was not so prominent as in the case of HRSV, just 5.4-folds versus 4 folds, respectively. Therefore, I tried to perform the same experiment in BSR T7/5 cells to better clarify the meaning of the results previously obtained. BSR T7/5 cells were used because they lack at least a functional IRF3 which compromise the endogenous TBK1-dependent IRF3-activation signalling pathway. It was reasonable to assume that in this cell context RSV growth was facilitated by the absence of an endogenous IFN-network and that the following transfection of exogenous TBK1 compromised less the cell viability. In this case, MOI of 1 was sufficient to efficiently block IFN-β promoter stimulation in the case of HRSV A2 and rBRSVh1/2*^{T26I} infection. In view of this data, I concluded that NS2 protein was responsible for blocking TBK1 activity and therefore IFN-β induction. Moreover, it was the nature of residue 26 to play an important role in this function probably being part of a NS2 protein domain responsible for interaction with the cellular kinase TBK1. It is also feasible that this amino acid, or the sequence stretch comprising residue 26, is crucial for interaction between NS1 and NS2. It has been shown that these viral proteins not only form heterodimers but need to

cooperate to efficiently antagonize interferon induction. I could speculate that the presence of a Threonine instead of an Isoleucine may lead to a drastic modification of important recognition motif in NS2 protein and either way, this would explain the loss of inhibitory ability by HRSV Long or the rBRSVh1/2. To unveil the mechanisms behind the identity of this apparently "essential" amino acid in NS2 protein, I tried to immunoprecipitate TBK1 with NS2 protein either from A2 or from Long infected cells. Unfortunately, no co-precipitation was observed in any case. This might indicate that TBK1 inhibition is achieved indirectly by interaction of NS proteins with a third player, so far not known, or simply the results were imputable to technical problems. It must be reminded that expression of TBK1 is quite hard to detect and that expression of NS proteins is also rather difficult to observe. NS1 and NS2 proteins can not be efficiently expressed by plasmid vectors, probably due to their reach content in A/T bases and NS2 in viral infected cells has a turn over of only 30 min. All these critical steps might have compromised the success of the experiment. Therefore, NS2 inhibitory mechanisms remain un-revealed so far.

Intracellular signal transduction pathways are regulated through the action of protein kinases. Protein Ser/Thr-kinases mediate phospho-dependent signalling networks recognizing their substrates by specific Ser/Thr-containing motifs. TBK1 and IKK-ε themselves contain a unique mitogen-activated protein kinase-kinase domains (***E****XXX****S***) within their activation loops functioning as a substrate for upstream kinases (Ehrhardt C. et al., 2004). These IKK-related kinases, TBK1 and IKK-ε, are Ser/Thr-kinases as well and they target ***Ser****XXX****Ser*** sequences in the amino terminal domain of IkBα and in the carboxyterminus of IRF3 and IRF7. Viral infection or treatment with dsRNA or LPS specifically induces phosphorylation of Ser 386 of IRF3 by IKK-ε and TBK1 (Mori M. et al., 2003). Interestingly, the Long NS2 protein also contains a putative Ser/Thr motif. The position of Threonine as the second residue within a potential consensus recognition motif (***Ser****XXX****Ser/Thr***) for phosphorylation in Long NS2 protein is consistent with predicted target motifs for Ser/Thr-kinases. Moreover, the phosphorylation motif in the carboxyterminus of IRF3 is almost identical to the NS2 amino acid stretch containing the Threonine residue.

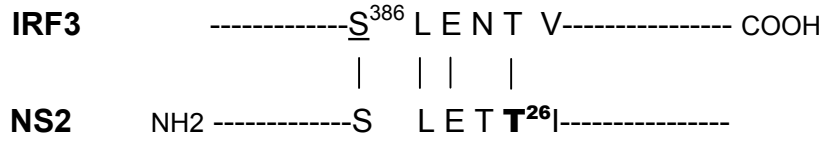

Fig. 23 Amino acid alignment of IRF3 and NS2 proteins potential posphorylation target motifs. Serine 386 of IRF3, which is phosphorylated by TBK1/IKK-ε is underlined. Threonine at position 26 in the NS2 amino-terminus is indicated in bold letter.

The implications of this analogy are not clear. Theoretically, it can be excluded that NS2 protein from Long competes with IRF3 as a substrate for IKK-ε and TBK1 because in this case IFN-β production should be inhibited. Residues S22 and T26 at the amino terminus of NS2 protein may serve as kinase recognition and/or direct phosphoacceptor sites. Phosphorylation by specific cellular kinases might represent the initial step of a multi-step process which would result in loss of IFN inhibitory activity of NS2 protein in HRSV Long strain. I have also considered that phosphorylation at these specific sites might enhance degradation of NS2 protein in Long and rBRSVh1/2 resulting in levels of expression so low to be insufficient to elicit antagonist functions. To rule out this possibility, I tested HRSV NS proteins expression in IFN-competent 293 cells. NS2 protein expression was observed in all the viruses analysed and NS2 levels were comparable, indicating that the presence of a Threonine at position 26 of HRSV Long and rBRSVh1/2 did not induce an appreciable degradation of NS2 protein.

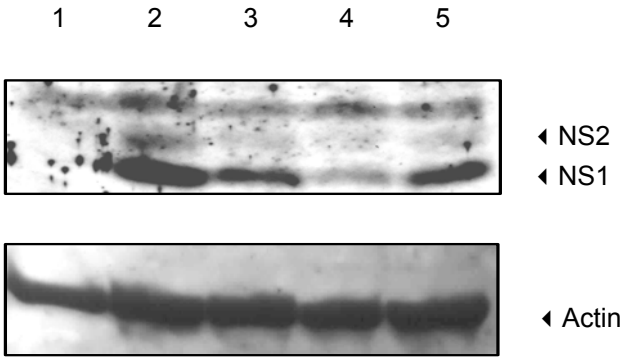

Fig. 24 Western blot analysis of NS2 protein expression. 293 cells were mock-infected (1) and infected at an MOI of 0.2 with HRSV A2 (2), HRSV Long (3), rBRSVh1/2 (4) and rBRSVh1/2*^{T26I} (5). At 48 hours post infection, when the cytopathic effect was apparent, cells were harvested and lysates were immunoblotted with a rabbit polyclonal antibody recognizing NS1 and NS2 proteins (α IC/C, kindly provided by Melero J., Spain) and an anti-actin antibody (Sigma).

5.5 Recombinant Rabies Viruses expressing RSV nonstructural proteins

5.5.1 Generation of SAD eGFP-P viruses harbouring RSV NS1 and NS2 proteins

To obtain more evidence that the RSV NS proteins alone are sufficient to counteract IFN induction, NS proteins were expressed in the context of an unrelated virus. NS1 and NS2 genes derived from BRSV and HRSV Long were introduced into the SAD eGFP-P virus (eGFP-Pb2/1 and eGFP-Ph2/1). A recombinant virus expressing a Long-derived NS2 protein, where a mutation at position 26 was inserted (T26→I), was also generated (eGFP-Ph2*^{T26I}/1). SAD eGFP-P was chosen as a vector because, in contrast to wild-type rabies, it is not able to inhibit interferon induction by blocking TBK1 activity. This characteristic would have enabled to analyse the differential ability of Long NS2 and A2-like NS2 to accomplish IFN antagonist activity in absence of other RSV proteins. Growth kinetics in BSR T7/5 cells showed that recombinant viruses expressing HRSV NS proteins were not significantly attenuated in their viral replication when compared to SAD eGFP-P. Only the chimeric virus harbouring the BRSV NS proteins (eGFP-Pb2/1) was attenuated by about 1 log. Viral titers of SAD eGFP-P itself are lower than of SAD L16 and this is due to a reduction in the amount of phosphoprotein P as a consequence of the fusion with GFP.

5.5.2 Recombinant eGFP-P NS2/NS1 viruses do not counteract IFN production

Growth characteristics of the recombinant eGFP-P viruses were tested in a cell system competent for IFN expression (HEp2). Apparently, insertion of RSV NS genes was not sufficient to rescue the viral replication of SAD eGFP-P. Moreover, also the presence of the HRSV NS2 protein having a mutation to resemble HRSVA2 NS2 protein, did not sustain eGFP-Ph2*^{T26I}/1 growth as expected. IFN-β mRNA expression in HEp2 cells infected with eGFP-Ph2/1, eGFP-Ph2*^{T26I}/1 and eGFP-Pb2/1 was equal to cells challenged with eGFP-P and greatly increased as compared to infection with wild type RV. Clearly, IFN-β was induced in presence of the chimerical eGFP-Ps and this explained a failure in viral growth at least of eGFP-Ph2*^{T26I}/1. Surprisingly, expression of either NS protein combination did not confer IFN resistance to the recombinant viruses. This contrasts with previous observations: wild-type RVs expressing RSV nonstructural proteins had demonstrated a significantly enhanced resistance to IFN challenge (Bossert B. and Conzelmann K.K., 2002). Western blot

analysis of RSV NS proteins expressed by RV has then revealed a problem with the expression of NS2 by the recombinant eGFP-Ph2*^{T26I}/1. While NS1 protein expression was comparable to the one of eGFP-Ph2/1, the NS2 level was significantly reduced. The reason of this low efficiency in expression is not yet clear. RT-PCR sequencing revealed that NS2 gene is correctly inserted in the RV vector and no changes of the sequence were found. Even the lack of detectable IFN resistance by eGFP-Ph2/1, which did express both nonstructural proteins, remains unsolved. I can only speculate that the amount on NS proteins produced is just not enough to protect eGFP-P virus from the IFN-induced antiviral state. In the RSV genome, the NS genes are located at the most upstream position resulting in a high level of expression in infected cells. Recombinant wild-type RVs (e.g RV h1/2), which are IFN resistant, reach very high titres in cell culture, up to 1x 10^8-10^9 ffu/ml, comparable to the parental strain SAD L16 and this is positively influencing the expression levels of the additional genes inserted in their genome. The fact that chimerical RVs are resistant to exogenous IFN already ruled out the need of additional RSV proteins to exert this function. Unfortunately, recombinant RV viruses could not be used for my purposes because wild-type RV itself is able to counteract IFN induction by the activity of the P protein. Therefore discrimination of the role played by RSV NS proteins is impossible. On the contrary, SAD eGFP- P does not block IFN production due to low P levels. The recombinant viruses derived from SAD eGFP-P (eGFP-Ph2/1and eGFP-Ph2*^{T26I}/1) are attenuated in their growth of about 3 logs in BSR T7/5 cells. The inefficient growth of these viruses and the fact that little NS2 protein is expressed from the current constructs precluded the analysis of NS functions.
In the future recombinant RVs should be generated in which high NS levels of expression are achieved by inserting the RSV nonstructural genes in a most proximal position in the rabies genome.

5.6 Final considerations

The results described in this work confirmed thatRSV NS2 protein plays the major role in blocking the IFN-β activation pathway. Hereby, I was able to characterize HRSV evasion of the innate immune response by identifying in the amino acid residue at position 26 of NS2 protein a crucial determinant for this function. Despite the mechanisms by which NS2 protein operates its antagonism remain still to be elucidated, the data provided support to the initial hypothesis that RSV interferes with the very early stages of the IRF3 phosphorylation. RSV have shown to interfere with the activity of the IKK-related kinase TBK1 but no evidence was

found of a direct interaction between this kinase and RSV NS2 protein. When this thesis was in preparation, several publications have revealed in more detail the identity of some of the most upstream components of the IFN-β activation pathway, which determine TBK1/IKK-ε recruitment . This opens the possibility to further elucidate the viral mechanisms involved in the IFN antagonist functions of HRSV NS2 protein.

6 SUMMARY

Interferon Escape of Respiratory Syncytial Virus:
Functional Analysis of Nonstructural Proteins NS1 and NS2

Respiratory syncytial virus (RSV) is recognised as the most frequent cause of severe lung infections in infants and cattle worldwide. Currently, no effective treatments are available and the development of a successful vaccine has been hampered by the fact that natural infection does not provide complete and durable protection. RSV nonstructural proteins, NS1 and NS2, are strong inhibitors of IFN α/β-production by specifically preventing interferon regulatory factor (IRF)-3 phosphorylation. However, the exact mechanisms leading to NS protein-mediated inhibition of IRF3 remain to be unravelled.

One of the objectives of this study was to identify amino acid domains in the human respiratory syncytial virus (HRSV) nonstructural proteins (NS) responsible for their ability to ablate the IFN-β signalling pathway. Furthermore, I wanted to find out at which level of this signalling pathway the NS proteins exert their suppressive activity and which are their major cellular targets. HRSV strains A2 and Long differ in their ability to block interferon type I synthesis. Sequence analysis of their NS proteins revealed the presence of an amino acid residue in the NS2 protein with a potential role for RSV IFN-inhibitory functions.

Two recombinant bovine respiratory syncytial (BRSV) viruses harbouring HRSV NS1 and NS2 genes were generated and tested in their ability to restrict IFN-β synthesis. These recombinant viruses differed only in the identity of the residue at position 26 of the HRSV NS2 protein: rBRSVh1/2 has a Threonine as in the Long strain, while in rBRS h1/2*^{T26I} this amino acid was mutated into an Isoleucin similarly to A2 virus. Sets of *in vitro* tests revealed that IFN-β induction was impaired by rBRSVh1/2*^{T26I} when compared to rBRSV h1/2.
Analysis of the transcriptional factors (AP-1, NF-kB and IRF3) involved in the activation of IFN-β synthesis provided evidence that the inhibitory ability of rBRSVh1/2*^{T26I} was correlated to a selective block of IRF3. The mutation (T26I) in the NS2 protein did neither effect the NF-kB activation pathway nor perturbed the IFN-resistance characteristics of the chimeric viruses.

IRF3 is activated upon phosphorylation mediated by IKK-related kinases (TBK1 and IKK-ε). TBK1 was therefore cloned from a human lung cDNA library and its biological activities regarding the induction of IFN-β were compared in mock-infected and infected cells.
rBRSVh1/2*^{T26I} and HRSV A2 precluded virus-induced IRF3 activation by interfering with TBK1 functions. No direct interaction between TBK1 and NS2 protein was demonstrated indicating that the kinase TBK1 may not be the sole target involved in RSV mechanisms of evasion of the innate immune response.

Recombinant IFN-inducible rabies viruses expressing HRSV Long-derived (rGFP-Ph2/1) or HRSV A2-like (rGFP-Ph2*^{T26I}/1) NS proteins were also generated. The HRSV NS2 protein expressing an Isoleucin (NS2*^{T26I}) at position 26 was not able to suppress IFN-β induction and to rescue the growth of the recombinant eGFP-Ph2*^{T26I}/1 in interferon-competent cells. A low expression of the mutated NS2*^{T26I} protein was probably the reason of this failure.

In summary, these results show that the HRSV NS2 protein possesses an intrinsic IFN-β inhibitory activity, which is achieved throughout a selective inhibition of the IRF3 activation pathway. The block appears to be exerted at the level of IRF3-kinase TBK1. Interferon-antagonist functions of the HRSV NS2 protein are linked to a particular amino acid motif in the N-terminus of the protein. Identification of this amino acid domain and of TBK1 as the cellular target provide a better insight of how the HRSV NS2 protein prevents the establishment of the antiviral innate immune response and therefore it might contribute to the development of an effective vaccine.

7 ZUSAMMENFASSUNG

Unterdrückung der Interferon-vermittelten Immunantwort durch das Respiratorische Synzytial Virus: funktionelle Analyse der Nicht-Strukturproteine NS1 und NS2

Das Respiratorische Synzytial Virus (RSV) zählt weltweit zu den häufigsten Auslösern schwerer Lungenentzündungen bei Kleinkindern und Rindern. Gegenwärtig gibt es dafür keine effektive Behandlungsmethode und die Entwicklung eines erfolgreichen Impfstoffes war nicht möglich, da auch eine natürliche Infektion keinen vollständigen und dauerhaften Schutz gewährleistet.
Die Nicht-Strukturproteine des RSV -NS1 und NS2- wirken stark hemmend auf die alpha/beta-Interferon (α/β–IFN)-Produktion, indem sie die Phosphorylierung des Interferon-Regulierungs-Faktors (IRF)-3 verhindern. Die Mechanismen, die zu einer NS-Protein vermittelten Hemmung des IRF3 führen, sind bisher unbekannt.

Eines der Ziele dieser Arbeit war es, Aminosäurensequenzen der HRSV Nicht-Strukturproteine zu finden, die ausschlaggebend für die Hemmung der ––IFN- Signalkaskade sind. Außerdem sollte geklärt werden, auf welchem Level der Signalkaskade die Nicht-Strukturproteine ihre hemmende Wirkung ausüben und auf welche zellulären Komponenten sie abzielen. Die HSV-Stämme A2 und Long hemmen die Interferon Typ I Synthese auf unterschiedliche Weise. Mittels Sequenzanalysen ihrer Nicht-Strukturproteine wurde ein Aminosäurerest im NS2-Protein identifiziert, der eine mögliche Rolle bei der Hemmung von Interferon durch RSV spielt.

Es wurden zwei rekombinante bovine RS-Viren (BRSV), die Gene für NS1 und NS2 des humanen RS-Virus beinhalteten, generiert und auf ihre Fähigkeiten untersucht, die IFN-–-Synthese zu unterdrücken. Diese rekombinanten Viren unterscheiden sich nur in der Aminosäure an Position 26 des HRSV NS2-Proteins: rBRSVh1/2 hat ein Threonin, wie in dem Long–Stamm, während bei rBRSVh1/2^{*T26I} diese Aminosäure gegen ein Leucin ausgetauscht wurden, wie es in A2 Viren natürlich vorkommt. *In vitro* Untersuchungen ergaben, dass die ––IFN–Induktion durch rBRSVh1/2^{*T26I} im Vergleich mit rBRSVh1/2 abgeschwächt wurde.

Die Analyse der Transkriptionsfaktoren AP-1, NF-kB und IRF3, die an der Aktivierung der β–IFN–Synthese beteiligt sind ergab, dass die inhibitorische Fähigkeit von rBRSVh1/2^{*T26I} mit einer selektiven Blockierung von IRF3 zusammenhängt. Die Mutation T26I im NS2 Protein hatte weder einen Effekt auf den NF-kB-Aktivierungs-Weg, noch störte sie die IFN-Resistenz des Chimären Virus.

IRF3 wird durch Phosphorylierung mittels IKK-verwandten Kinasen (TBK1 und IKK-ε) aktiviert. TBK1 wurde daher aus einer humanen Lungen cDNA-Bibliothek kloniert und seine biologische Aktivität bezüglich der Aktivierung von εεIFN in „mock"-infizierten Zellen und Virus-infizierten Zellen verglichen.

rBRSVh1/2^{*T26I} und HRSV A2 verhinderten Virus-induzierte IRF3-Aktivierung durch Beeinflussung von TBK1-Funktionen. Es wurde gezeigt, dass es keine direkte Interaktion zwischen TBK1 und NS2 gibt. Dies deutet darauf hin, dass die Kinase TBK1 nicht das alleinige Zielprotein der RSV-Mechanismen ist, der angeborenen Immunantwort zu entgehen. Weiterhin wurden rekombinante IFN-induzierbare Rhabdo-Viren generiert, die entweder das NS2 Protein aus dem HRSV Long-Stamm (rGFP-Ph2/1) oder das A2 ähnliche Protein (rGFP-Ph2^{*T26I}/1) exprimieren. Das HRSV NS2-Protein, dass an Position 26 ein Isoleucin besitzt, war nicht fähig die εεIFN -Induktion zu unterdrücken, beziehungsweise das Wachstum von rekombinanten eGFP-Ph2^{*T26I}/1 in IFN-kompetenten Zellen zu ermöglichen. Die lag vermutlich an der geringen Expressionsrate des mutierten NS2^{*T26I}-Proteins.

Zusammenfassend zeigen die Ergebnisse, dass das HRSV NS2-Protein eine intrinsische Aktivität als εεIFN-Inhibitor besitzt, die über eine selektive Hemmung des IRF3 Aktivierungs-Weges erfolgt. Die Blockade wirkt auf der Ebene der IRF3-Kinase TBK1. Die Interferon-antagonistischen Funktionen des HRSV NS2-Proteins begründen sich in einem speziellen Aminosäure-Motiv am N-Terminus des Proteins. Die Identifizierung dieser Aminosäure-Domäne sowie von TBK1 als zelluläres Ziel von NS2 ermöglicht einen besseren Überblick über die Mechanismen, durch die das HRSV NS2-Protein die angeborene antivirale Immunantwort unterdrückt. Dies könnte ein Ansatzpunkt für die Entwicklung eines effektiven Impfungsstoffes sein.

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CURRICULUM VITAE

Personal information:

Name:	Sabrina Marozin
Date of Birth:	31/03/1971
Place of Birth:	Riyadh, Saudi Arabia
Nationality:	Italian

Education:

1985 – 1990	Scientific High School , Liceo „Luigi Cremona“, Milano, Italy
1990 – 1997	**D.V.M**., Veterinary Medicine, University of Milano, Italy
1998 – 1999	Postgraduate Research Grant of the I.Z.L.E. (Brescia, Italy)
2000 – 2001	Research Associate, WHO World Influenza Centre, National Institute for Medical Research, London (GB)
2001 – 2002	Doctoral student at the Medizinische Hochschule/ TIHO Hannover, Germany
since 01/2003	Doctoral student at the Max von Pettenkofer Institute by the Gene Center, Ludwig Maximilian University, Munich

München, den 24/10/05

(Sabrina Marozin)

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