View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Helsingin yliopiston digitaalinen arkisto

Recombinant structural proteins of rubella virus

Michel Schmidt

Department of Biosciences
Division of Biochemistry
Faculty of Science
University of Helsinki, Finland

ACADEMIC DISSERTATION
To be presented for public criticism, with the permission of the Faculty of Science of the University of Helsinki, in the auditorium 2041, Viikinkaari 5D, Helsinki, on September 29, 2000 at 12 o'clock noon.

Helsinki 2000

ISBN 952-91-2571-2 (nid.)
ISBN 952-91-2572-0 (PDF)
Helsinki 2000
Yliopistopaino

”We busted out of class, had to get away from those fools.
We learned more from a three-minute record, baby,
then we ever learned in school.”

Bruce Springsteen

CONTENTS

ABSTRACT 5
ACKNOWLEDGEMENTS 6
ORIGINAL PUBLICATIONS 7
ABBREVIATIONS 8
1. INTRODUCTION 9
1.1 Togaviridae 9
1.2 Rubella Virus 12
1.3 Medical Significance of Rubella Virus 16
1.4 Diagnosis of Rubella Virus Infection 17
1.5 Expression of Recombinant Rubella Virus Proteins 18
1.6 The Baculovirus Expression Vector System 19
2. AIMS OF THE STUDY 20
3. MATERIALS AND METHODS 21
3.1 Plasmid Constructions 21
3.2 Viruses 22
3.3 Cell Culture and Infection of Cells 22
3.4 Protein Purification 24
3.4.1 immunoabsorbtion 24
3.4.2 Concavalin A Affinity Chromatography 24
3.4.3 Immobilized Metal-Ion Affinity Chromatography 25
3.4.4 Anti-FLAG™ M1 Affinity Chromatography 26
3.5 Protein Analysis Assay 26
3.5.1 Protein Concentration Assay 26
3.5.2 SDS-PAGE and Immunoblot Analysis 26
3.5.3 ELISA 27
3.5.4 Time-Resolved Fluoroimmunoassays 27
3.5.5 Surface Plasmon Resonance 28

4. RESULTS 29
4.1 Recombinant Constructs 29
4.2 Expression in Insect Cells 29
4.3 Protein Purification 31
4.3.1 Immunoabsorbtion 31
4.3.2 Concavalin A Affinity Chromatography 31
4.3.3 Immobilized Metal-Ion Affinity Chromatography 32
4.3.4 Anti-FLAG™ M1 Affinity Chromatography 32
4.4 ELISA 33

4.5 Time-Resolved Fluoroimmunoassays **33**
4.6 Surface Plasmon Resonance **33**

5. DISCUSSION **35**
6. CONCLUSIONS AND FUTURE PERSPECTIVES **40**
REFERENCES **41**
APPENDICES **49**

ABSTRACT

The baculovirus expression vector system (BEVS) was used for the expression of several different constructs encoding the rubella virus (RV) structural proteins. From a construct encoding the structural p110 polyprotein the E1 protein was produced and purified using immunoaffinity chromatography. The purified protein was shown in an ELISA study to have a similar antigenicity to authentic RV. The ability of the purified E1 to react with human RV specific IgM antibodies was compared with authentic RV in a correlation assay, using time-resolved fluorescence (TR-FIA). The TR-FIA analysis showed a positive correlation of r=0,843.

The protein production level was scaled-up into a 10 l bioreactor, and soluble forms of the E1 and E2 proteins, as well as a his_6 tagged form of the capsid protein was produced. The E1 protein was purified to homogeneity using lectin affinity chromatography and the his_6 tagged capsid protein was purified using immobilized metal-ion affinity chromatography (IMAC). The capsid protein was further studied in surface plasmon resonance (SPR) experiments using the BIAcore™ equipment and the interaction between the capsid and a specific antibody could be detected in real-time.

An E1 construct containing the his_6 tag at the C-terminal and an N-terminal FLAG™-tag (Eastman Kodak Co., New Haven, CT) was produced. The E1 construct was purified using IMAC and Anti-FLAG™ M1 Affinity Chromatography. The purified protein was studied using the BIAcore™ equipment in a setup where the Ca^{2+} dependent binding of an antibody specific for the FLAG-tag was used for visualization of the sensitivity of the assay. The impact of the described research for diagnostic application and as providing tools for RV research is discussed.

ACKNOWLEDGEMENTS

This study was mainly carried out at VTT Biotechnology, but it was started at the Department of Biochemistry and Pharmacy at the Åbo Akademi University, and the study was finalized at the Department of Biosciences, Division of Biochemistry in the University of Helsinki. The work was supervised by Professor Christian Oker-Blom. Professor Hans Söderlund at VTT and Professor Carl G. Gahmberg at the University of Helsinki are acknowledged not only for providing excellent working facilities, but also for their friendly and supportive attitude towards me and my work. My sincere thanks also goes to Dr. Kristiina Takkinen and Professor Timo Hyypiä for their comments concerning this thesis, they are also acknowledged for being supportive during my thesis research.

My supervisor Kricke is thanked for getting me interested in science and for many fun moments, too many to mention. All my co-authors and colleagues are thanked for their contributions. A huge thank you goes to my "old" friends at VTT and my "new" friends at the University of Helsinki, I'm sorry I can't name you all, but you now who you are. A special thanks go to my present boss Kari for being so supportive of me writing-up my thesis work and for all the fantastic scientific discussions we have had. The following persons have as colleagues, and/or friends, been absolutely crucial for this work: Robbi, Nina, Arja, Milla, Anja, Annukka, J-P, Taru, Cai, and Sarah. My closest colleague during this time has been David, without whom this could not have been done. Thanks Croc! David is also acknowledged for proofreading this thesis.

Finally I'd like to thank the people who have been the most important for this work, my parents and my wife. Thanks always, without you this wouldn't have been worth anything.

Helsinki, August 31st, 2000

Michel

ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text by their Roman numerals (I-VI).

I Lindqvist, C., Schmidt, M., Heinola, J., Jaatinen, R., Österblad, M., Salmi, A., Keränen, S., Åkerman, K. and Oker-Blom, C. (1994). Immunoaffinity Purification of Baculovirus-Expressed Rubella Virus E1 for Diagnostic Purposes. *J. Clin. Microbiol.* **32:** 2192-2196.

II Oker-Blom, C., Blomster, M., Österblad, M, Schmidt, M., Åkerman, K., and Lindqvist, C. (1995). Synthesis and processing of the rubella virus p110 polyprotein precursor in baculovirus infected *Spodoptera frugiperda* cells. *Virus.Res.* **35:** 71-79.

III Schmidt, M., Lindqvist, C., Salmi, A., and Oker-Blom, C. (1996). Detection of Rubella Virus-Specific Immunoglobulin M Antibodies with a Baculovirus-Expressed E1 Protein. *Clin. Diagn. Lab. Immunol.* **3:** 216-218.

IV Johansson, T., Enestam, A., Kronqvist, R., Schmidt, M., Tuominen, N., Weiss, S.A., and Oker-Blom, C. (1996). Synthesis of soluble rubella virus spike proteins in two lepidopteran insect cell lines: Large scale production of the E1 protein. *J. Biotechnol.* **50:** 171-180.

V Schmidt, M., Tuominen, N., Johansson, T., Weiss, S.A., Keinänen, K., and Oker-Blom, C. (1998). Baculovirus-Mediated Large-Scale Expression and Purification of a Polyhistidine-Tagged Rubella Virus Capsid Protein. *Prot. Exp. Purif.* **12:** 323-330.

VI Schmidt, M., Mottershead, D.G. and Oker-Blom, C. An Affinity-Tagged Rubella Virus E1 Protein for Interaction Analysis. *submitted for publcation.*

ABBREVIATIONS

AP	alkaline phosphatase
BEVS	baculovirus expression vector system
BSA	bovine serum albumin
C	capsid protein
CRS	congenital rubella syndrome
DNA	deoxyribonucleic acid
E1	envelope protein 1
E2	envelope protein 2
ECV	extra-cellular virus
EDTA	ethylene diaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FLAG	eight amino acid affinity epitope tag
HRP	horseradish peroxidase
IgG	immunoglobulin G
IgM	immunoglobulin M
M1	FLAG epitope-specific monoclonal antibody 1
MOI	multiplicity of infection
NSP	nonstructural protein
o/n	over night
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFU	plaque forming units
p.p.	post infection
p.p.	post planting
RNA	ribonucleic acid
RRV	ross river virus
RV	rubella virus
RT	room temperature (approximately 20°C)
SDS	sodium dodecyl sulfate
SFV	semliki forest virus
SIN	sindbis virus
SPR	surface plasmon resonance
TR-FIA	time-resolved fluoroimmunoassay
VLP	virus-like particle

1. INTRODUCTION

Viruses were first discovered by mankind just over 100 years ago. In the latter half of the 19th century many disease causing bacteria were identified. However, several diseases were shown not to have a bacterial origin. This lead to the discovery of a new infectious agent, referred to as virus in the work of Ivanovsky, Beijernick, Loeffler and Froch, between 1892 and 1898. Viruses were defined as small “organisms” capable of replication in the presence of a host cell. In the 1930s viruses were for the first time visualized by Kausche using electron microscopy. The virus particle was shown to consist of either a RNA or a DNA genome encapsidated by several copies of a capsid protein, and for some viruses also a lipid-bilayer containing viral envelope proteins. By 1977 mankind's knowledge of viruses had led to the total eradication of smallpox, being the first "organism" eliminated on purpose by man. Today we have a broad knowledge of the structure and the biology of several viruses. We also have a recombinant virus vaccine (hepatitis B virus) and the process of eliminating several viruses is ongoing. In this century we will also see viruses serve as important tools in gene technology. Only time will tell if viruses, often called the ultimate parasite, will one day serve the purposes of their former hosts (for review see Oldstone and Levine, 2000).

1.1 Togaviridae

The family *Togaviridae* contains two genera, the *Alphaviruses* and the *Rubivirus*. The Alphavirus genus consists of at least 26 members, the most studied being Semliki Forest virus (SFV), Sindbis virus (SIN) and Ross River virus (RRV). Rubella virus (RV) remains the sole member of the *Rubivirus* genus (for classification see; Matthews, 1982) (Fig. 1.). The Alphaviruses are arguably the best characterized enveloped animal viruses today, whereas the molecular biology of RV has not been as thoroughly studied.. Alphavirus crystals have also been obtained (Harrison et al., 1992).

All togaviruses are lipid enveloped small positive stranded RNA viruses with a diameter of approximately 70 nm. The positive stranded genome consists of around 10 000 nucleotides and has one open reading frame (ORF)

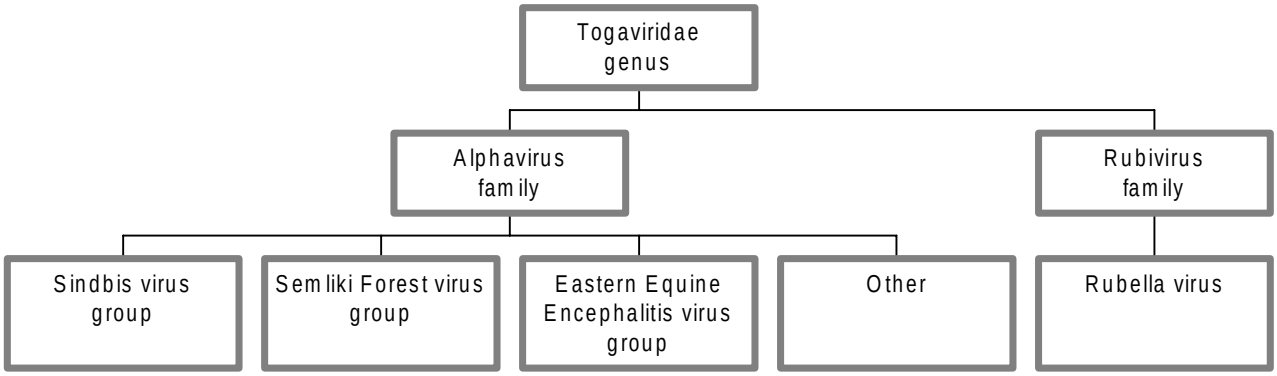


Figure 1. Classification of the Togaviridae genus, based on sequence data. The Alphavirus family consist of 26 or more different viruses, whereas rubella virus is the sole member of the Rubivirus family.

encoding the nonstructural proteins (NSPs) and one ORF for a subgenomic RNA encoding the structural proteins. The NSP-ORF has been shown to encode for two proteins in the case of RV and four proteins in the Alphavirus genus (Fig 2.). Similar transferase, helicase and proteinase regions have been identified in all members of the *Togaviridae*, as well as motifs of unknown function. Despite a similar gene arrangement, sequence analysis has reveled that RV is only distantly related to the Alphaviruses (for review see; Frey 1994; Strauss and Strauss 1994).

The positive stranded RNA genome of the *Togaviridae* viruses is encapsulated by a capsid. The capsid core has a diameter of 30-35 nm in RV and 38-40 nm in Alphaviruses. The RV capsid has been suggested to have a T = 3 icosahedral symmetry, whereas Alphaviruses have a T = 4 symmetry (for review see; Frey 1994; Strauss and Strauss 1994). Residues for interaction between the envelope proteins and the nucleocapsid have also been identified

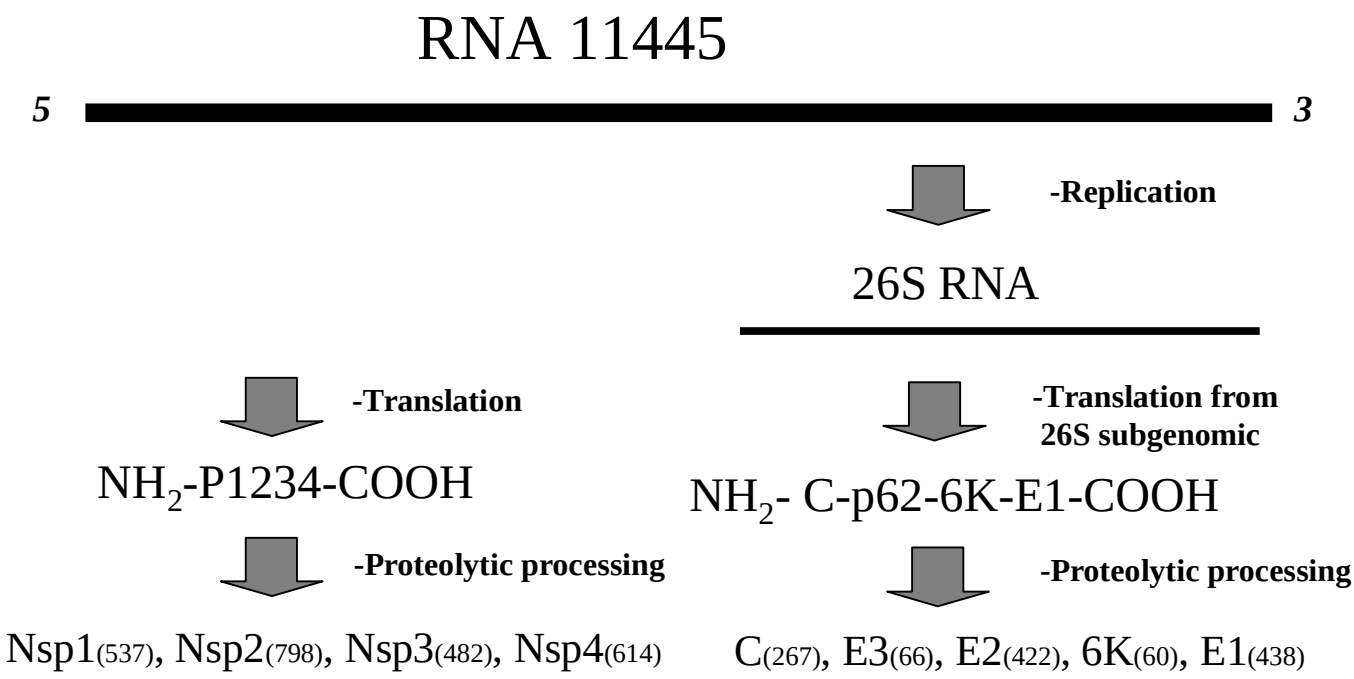


Figure 2. Translation and processing of the two open reading frames of Semliki Forest virus. The size of the proteins are shown as in brackets, as number of amino acids.

in Alphaviruses (Skoging et al., 1996; Owen and Kuhn, 1997), and recently similar studies has been performed for the RV (Yao and Gillam, 1999).

The viral lipid envelope of the *Togaviridae* members is composed of lipids from the host-cell and the virus-specific envelope glycoproteins, E1 and E2. These proteins contain an intra-membrane part, a single membrane spanning region and a large extra-membrane part. Together they form glycosylated spike heteromeres on the virus surface. Alphaviruses may also contain other membrane proteins e.g. E3 and 6K in SFV.

The budding of Alphaviruses and their structure-function relationships have been studied in detail (reviewed in Helenius, 1995; Strauss et al., 1995; and Garoff et al., 1998). Here, a striking difference between the RV and the Alphaviruses has been uncovered. Whereas Alphaviruses bud from the cell membrane, RV matures from intracellular membranes (Baron and Forsell, 1991; Hobman et al., 1993). In addition, the budding process of RV does not, in contrast to Alphaviruses, seam to be driven by interactions between the E1 and the capsid (Garbutt et al., 1999). Several host-cell receptors have been detected for the Alphaviruses whereas similar studies have failed to identify any RV specific receptors (Nath et al., 1989; Wang et al., 1992). Another striking difference is the kinetics of the replication cycle. Cells infected with Alphaviruses reach a maximum virus production 4 – 8 h after infection (Kääriäinen and Söderlund, 1978), whereas RV has a long latent period and reaches a maximum 24 – 48 h after infection. (Hemphill et al., 1988).

Since Alphaviruses have a large host-cell range and are relatively easy and rapid to grow, they have become very useful viral model systems. Many of the viral intracellular mechanisms as well as structural aspects have been first identified among Alphaviruses. Today, Alphaviruses have also found applications as expression vectors and in gene therapy (Garoff and Li 1998a). Many of the basic questions resolved for the Alphaviruses remain to be solved for RV. Clearly, the differences between the Alphaviruses and RV, mean that assumptions cannot be made about RV based on data derived from any of the Alphaviruses. Since RV is a human pathogen many techniques for detecting RV infection have however been developed.

1.2 The Rubella Virus

The RNA genome of RV is 9756 nucleotides long excluding a "cap"-structure and a poly(A) tract (Dominguez et al., 1990). The 5'- two-thirds of the genome encodes the nonstructural proteins (NSPs), and the 3' third encodes the structural proteins. The nonstructural proteins are translated from the genomic RNA as a 200 kDa precursor polyprotein. This polyprotein is cleaved into two NSPs a 150 kDa polypeptide (P150) and a 90 kDa polypeptide (P90) (Forng and Frey, 1995). The order of the NSPs within their open reading frame (ORF) is NH_2 - P150 - P90- COOH and the cleavage is mediated by a protease activity within the P200 (Marr et al., 1994). The two NSPs are thought to form, together with host factors, a complex for RNA replication.

The viral RNA serves as a template for the production of a subgenomic RNA, which when translated gives rise to a polypeptide precursor (p110) for the structural proteins. The p110 is cleaved during translocation into the ER, giving rise to a 33 kDa capsid protein (C) and two envelope glycoproteins, E2 (42-47 kDa) and E1 (58 kDa) (Oker-Blom et al., 1983; Kalkkinen et al., 1984: Oker-Blom 1984) (Fig. 3.). The C protein forms a 30-35 nm core interacting with and encapsulating the virus genome (Liu et al., 1996). The envelope glycoproteins E1 and E2 both have a hydrophobic transmembrane segment anchoring them in the lipid bilayer originating from the host-cell. This lipid bilayer forms a viral envelope of approximately 60 nm in diameter, with E1 and E2 heteromer spike projections of 5-8 nm facing out from the surface (reviewed in Frey, 1994) (Fig. 4.).

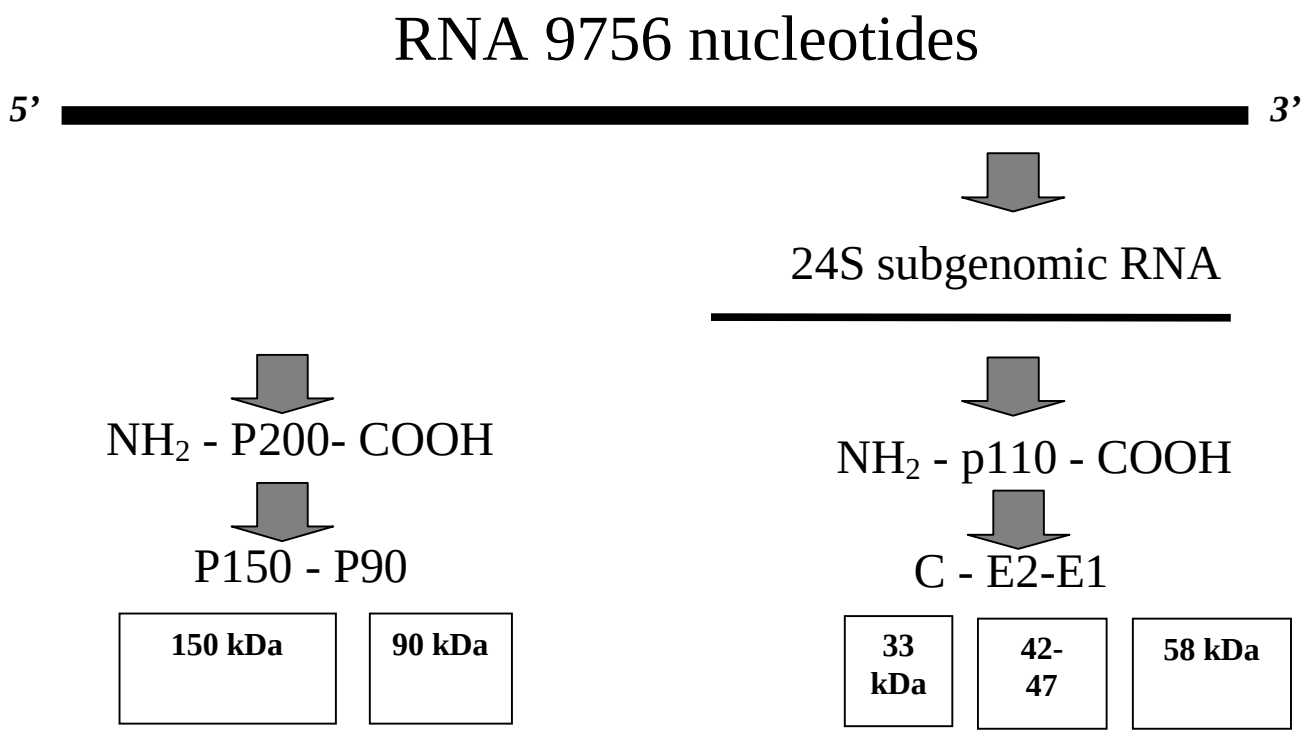


Figure 3. Translation and processing of the two open reading frames of rubella virus.

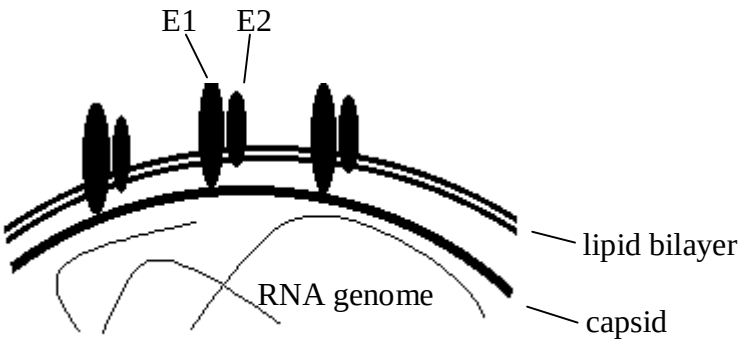


Figure 4. Schematic drawing of a section of RV, showing the characteristics of an enveloped virus.

The C protein has been shown to consist of two subtypes with pI values of 8.8 and 9.5. This could be due to differential phosphorylation (Waxham and Wolinsky, 1985). In the virus capsid, the C protein is present as a disulfide linked dimer (Baron and Forsell, 1991). Within the p110 precursor the carboxy-terminal region of the capsid protein functions as the signal sequence for the E2 protein (Suomalainen et al. 1990).

The E2 protein is heavily glycosylated and 30-40 % of the molecular mass is carbohydrate. This heavy and heterologous glycosylation makes the E2 molecular mass vary between 42 and 47 kDa . However, the major part of the protein seems to be either 42 or 47 kDa in size (Oker-Blom et al., 1983). Both the E1 and the E2 proteins have N-linked glycans, whereas only the E2 protein has O-linked glycans (Qiu et al., 1992). pI values of the different forms of the E2 protein have been reported ranging from 5.0 to 8.0. (Waxham and Wolinsky, 1985). Near the carboxy terminus the E2 protein has a cluster of hydrophobic amino acids anchoring it to the lipidmembrane. The putative transmembrane region is followed by eight amino acids and the signal sequence for the E1 protein.

The E1 protein is thought to have a uniform glycosylation pattern, and to interact with the E2 protein to form heterodimers on the virus surface (Yang et al., 1998). The E1:E2 ratio on the surface of the virus is still not convincingly determined, although a 1:1 ratio seams quite possible. At the carboxy terminus the E1 has a putative transmembrane region followed by a 13 amino acid stretch, thought to be on the interior of the lipid bilayer.

Most enveloped animal viruses enter the cell by receptor mediated endosytosis. For RV no cellular receptor has yet been found, although some evidence for the importance of cellular lipids has been shown (Mastromarino et al., 1990). Although attachment of RV to its host cells is rapid, internalization is slow (Petruzziello et al., 1996).

The NSPs are translated from the 5' region of the viral RNA, and a negative-polarity RNA is synthesized to function solely as a template for synthesis of positive stranded RNA. The regulation of RNA synthesis is based on sequence homology studies thought to function essentially as in the Alphaviruses . P150 is thought to contain methyltransferase and protease activities, and P90 is suggested to contain helicase and RNA polymerase functions (Dominguez et al., 1990; Marr et al., 1994).

The structural proteins are modified following synthesis. The C protein forms dimers and is membrane associated at the time of capsid formation. The C protein is present both in the ER and the Golgi probably due to an association between the C and the E2 protein. The envelope glycoproteins are detectable in the ER, Golgi, intracellular vacuoles and on the cell surface, all of which have been thought to be the site of virus budding. Dimerization between the E1 and the E2 has been shown to be important for protein transport from ER to Golgi, indicating that the assembly of the virus occurs before arrival to the plasma membrane (Baron et al., 1992; Hobman et al., 1994a; Hobman et al., 1995). The E2 protein has been shown to contain a Golgi retention signal within the transmembrane region whereas an ER retention signal has been found in the transmembrane and the cytoplasmic domain of the E1 protein (Hobman et al., 1997).

Studies using virus-like particles (VLPs) containing the RV structural proteins have revealed that the transmembrane and the cytoplasmic parts of E2 and E1 are required for viral assembly. Interactions between the capsid and the cytoplasmic part of E1 does, however, not seem to be important for the viral budding process (Garbutt et. al. 1999). Based on electron microscopic studies the primary site for the viral budding process has been shown to be cytoplasmic membranes. The lipid content of the virion envelope also shows

similarities to intracellular membranes rather than the plasma membrane (reviewed in Frey, 1994).

1.3 Medical Significance of Rubella Virus

Infection caused by RV usually gives rise to a mild self-limited disease called rubella or German measles. The symptoms of rubella are a mild rash, low-grade fever and a sore throat. Often an infection can occur without detectable symptoms. The most prominent feature of the disease is the rash giving rise to the name of the disease. However, if this disease is acquired during the first trimester of pregnancy, severe fetal damage may occur. Infections occurring before the 16th week of pregnancy shows some disease related consequences in 67-85% of the fetuses. The broad range of clinical consequences acquired with fetal infection are called; Congenital Rubella Syndrome (CRS). In most cases (80 %) fetal infection is found to give rise to neuronal damage, sometimes expressed as mental retardation (reviewed in Wolinsky 1990).

Among adult females joint symptoms are reported for more than 50% of patients with rubella. Among children, males and vaccinated patients, the symptoms are more rare. The joint disease is usually of a mild and transient nature. In some patients chronic arthritis has also been reported following RV infection (Ford et al., 1992). RV specific antibodies and in some cases RV RNA (from synovial fluid cells) can also be detected from groups of patients with chronic joint diseases (Bosma et al., 1998). The mechanism and importance of RV in these chronic diseases remains to be resolved.

In cell cultures RV has been shown to induce apoptosis in infected mammalian cells. Apoptosis naturally occurs as a protection system to kill damaged cells, and has been shown to occur during various viral infections. For RV infections apoptosis has not been demonstrated in human cells and the role of apoptosis in severe rubella complications has not been shown (Pugachev and Frey, 1998: Megyeri et al. ,1999).

Vaccination against rubella, with a live attenuated virus, was started in the mid-1970:s in most industrial countries. Several improvements in the vaccine and the vaccination program has led to a near complete elimination of rubella from the Western world. In under developed countries and crisis regions rubella is still seen as a major problem. In Finland we have today more than 95 % of the population vaccinated against rubella and the current opinion

is that our vaccination program should prevent us from outbreaks, thus keeping the annual cases of rubella to fewer than 30 per year (Peltola et al., 1994).

1.4 Diagnosis of Rubella Virus Infection

Since rubella is a mild and sometimes asymptomatic disease a laboratory test is needed for the diagnosis. Since CRS is very serious and linked to the early trimester of pregnancy, rubella diagnosis should be reliable and rapid. The first method to meet this criterion was the heamagglutination-inhibition (HI) test measuring RV specific IgG antibody titers. This was first used for rubella diagnosis in 1967 by Stewart and co-workers (for review see Cradock-Watson, 1991).

Later, enzyme-linked immunoassays (ELISA:s) have added both ease and sensitivity to the antibody titration assays (Voller and Bidwell 1975). Effort have been made on adding sensitivity and specificity to detect IgM antibodies, and to replace the viral antigen by recombinant proteins or peptides (Seppänen et al., 1991; Zrein et al., 1993; Grangeot-Keros and Enders 1997). Further sensitivity to the diagnostic assays was achieved using time-resolved fluoroimmunoassays (Meurman et al., 1982).

Concerning the sensitivity of detection a viral infection based PCR diagnosis is of course superior to all other known methods. In PCR, the detection of just a few copies of the viral RNA genome gives rise to an amplified product (Ho-Terry et al., 1990; Bosma et al., 1995). The risk of contamination and hence a false positive result could, however, be an obstacle in a non-sterile environment.

Since the avidity of immunoglobulins to RV structural proteins seams to reflect the time passed since infection (Mauracher et al., 1992; Hedman et al., 1993), an avidity/affinity measurement of the RV-specific antibodies might reveal the time of virus infection. At present such diagnostic assays are not in general use.

1.5 Expression of Recombinant Rubella Virus Proteins

With the development of gene technology, recombinant constructs containing different regions of the RV genome have been engineered. The very high CG content of the RV genome (67% for the E1 protein) made for some initial problems in the PCR of the RV genome (Cusi et al., 1992), but today different RV constructs have been expressed in several different hosts . Affinity tags have also been engineered to RV constructs, both for purification and detection.

Bacterial (*E.coli*) expression is one of the most used system for production of recombinant proteins. The host-genome is well known and yield and protein trans-location can often be well controlled (for review see Baneyx, 1999). *E.coli* have been used for expression of RV derived constructs. Even though bacteria lack glycosylation capabilities, these RV constructs have been shown to essentially retain their antigenicity (Terry et al., 1989).

Mammalian expression systems have also been used for RV gene expression, thus mimicking as closely as possible the naturally occurring RV proteins. Here the approach has been either to use viral infection or transient transfection of the cells. Both African green monkey (CV-1, COS and Vero) and hamster (Syrian golden hamster, baby kidney cells; BHK) cells have been used for such expression studies(Sanchez and Frey, 1991; Hobman et al., 1994; Qiu et al., 1994). In BHK cells virus-like particles (VLPs) containing the RV structural proteins have been expressed. These VLPs have been helpful not only in immunological studies but also in studying viral morphogenesis (Garbutt et al., 1999).

Several groups have made use of the baculovirus expression vector system (BEVS) for expression of RV constructs, showing processing and antigenicity similar to native RV (Oker-Blom et al., 1989; Seppänen et al., 1991; Cusi et al., 1994). Soluble forms of the envelope proteins as well as affinity tagged constructs have been expressed (Seto et al., 1994). These studies suggest that the BEVS is useful for studying individually the RV proteins.

1.6 The Baculovirus expression vector system

Baculoviruses are a family of insect viruses. Recombinant baculoviruses are widely used for high-level expression of heterologoues genes. The expressed genes are usually placed under the control of the polyhedrin promoter of *Autographa californica* nuclear polyhedrosis virus (AcNPV) or the *Bombyx mori* nuclear polyhedrosis virus (BmNPV). In nature the virus produces high amount of the polyhedrin protein, as a protective shell. The polyhedrin gene can be, in the laboratory environment, replaced by another gene. The corresponding gene product is then, in most cases, processed, modified and properly targeted, thus displaying its expected properties (for review see: O'Reilly et al. 1992; Davis, 1995).

The baculovirus expression vector system (BEVS) combines many of the advantages of other expression systems. With baculovirus mediated expression in insect cells, high expression, sometimes reaching or surpassing yields achieved in bacterial expression, can be obtained. Insect cells are also capable of glycosylation and post-translational modifications similar to higher eukaryotes (for review see Possee 1997). With the introduction of the method of transposon-mediated insertion of the recombinant gene into a baculovirus genome propagated in *E.coli*, construction of recombinant baculoviruses has become relatively straightforward (Luckow et al., 1993).

Many proteins have been expressed utilizing the BEVS, ranging from a single capsid protein to a glycosylated multisubunit complex, such as an ion-channel (Kuusinen et al., 1995). Possibly the most complicated examples of protein production using the BEVS could be the assembly of virus-like particles using triple and quadruple expression vectors (Belyaev and Roy, 1993, Roy and Jones 1996).

Today, the baculovirus expression vector system is still mainly used for expression of heterologous proteins in insect cells. Other uses of BEVS has been as an insecticide (Cory and Bishop 1997) and more recently as a mammalian cell transfer vector (reviewed in: Kost and Condreay 1999) and as eucaryotic display vesicle (Mottershead et al., 2000).

2. AIMS OF THE STUDY

In this study the primary goals were to produce and purify recombinant RV proteins and to compare their antigenicity to authentic RV virus. Protein expression studies were carried out both in small and large scale and different purification schemes evaluated and optimized. The antigenicity of the purified proteins was compared to authentic virus using a collection of patient serum samples. This was initially analyzed in an ELISA type assay, later to be replaced with a time-resolve fluorescence immunoassay.

Towards the end of the study we introduced the surface plasmon resonance (SPR) method into our analysis. The focus of the SPR studies was on establishing a model systems that could be used for diagnostic applications.

3. MATERIALS AND METHODS

3.1 Plasmid Constructions

A plasmid containing the entire coding region of the RV structural genes pGEM2-RV24S (Suomalainen et al., 1990) was used as the starting material in all studies. First, a recombinant baculovirus containing the entire 24S transcription-translation unit of RV was produced. This was performed by removing the 24S cDNA from pGEM2-RV24S and then inserting the 24S cDNA downstream of the polyhedrin gene promoter into a linerized baculovirus transfer vector (pVL1392) using standard cloning procedures (Sambrook et al, 1989). The resultant plasmid was designated pVL1392-RV24S. This construct was used in studies I, II and III.

In study IV the sequences coding for the extra-virion region of E1 and E2 respectively, were amplified from pGEM2-RV24S using PCR (Saiki et al., 1985). The E1 and the E2 coding sequences were first inserted into the *Sma*I site of the plasmid pBluescript ks (Stratagene, La Jolla, CA) and then digested with *Bgl*II/*Xba*I and *Bam*HI/*Eco*RI respectively and ligated into the appropriately cut plasmid pVTBac (Tessier et al., 1991). Thus yielding the transfer plasmids pVTBac-E1 and pVTBac-E2.

In study V and VI the BAC-to-BAC™ Baculovirus Expression System (Gibco-BRL, Grand Island, NY) was used. The sequence coding for the capsid protein and the extra-virion region of the E1 was amplified by PCR from pGEM2-RV24S and cloned into the BglII/XbaI sites of a pBluescript derivative (pK410-1) containing sequences coding for the signal peptide of the rat glutamate receptor GluR-D, followed by a FLAG epitope tag and a carboxy-terminal polyhistidine (His_6) tag (Kuusinen et al., 1995). The resultant E1 sequence containing plasmid designated pK410-1E1sol, encoded a soluble extra-virion form of the rubella virus E1 protein with amino and carboxy terminal epitope tags.

The capsid coding sequence was further subcloned into the NcoI/BamHI site of pK409-1 thereby omitting the signal sequence and the FLAG-tag. pK409-1 is a pFASTBAC1 derivative which was used for the generation of a recombinant baculoviruses via the BAC-to-BAC™ system

(Gibco-BRL). This system is based on the transposon-mediated insertion of foreign genes into the baculovirus genome under transcriptional regulation of the polyhedrin gene promoter (Luckow et al., 1993).The final plasmid product designated pOB504-1 encoded the capsid protein with a carboxy polyhistidine tag. The expression cassette from pK410-1E1sol was directly transferred to the pK409-1 plasmid thus encoding for both the signal sequence and the FLAG-tag as well as the carboxy polyhistidine tag. The resultant plasmids were named pAcRVE1sol and pOB504-1. Sequencing of the constructs was performed according to the protocol of the Sequenace (version 2.0) kit (USB, Cleveland, OH).

3.2 Viruses

The plasmid pVL1392-RV24S was used for cotransfection with wild type *Autographa calfornica* nuclear polyhedrosis virus (*Ac*NPV) DNA into *Spodoptera frugiperda* (Sf9) cells. The recombinant virus (VL1392-RV24S) produced as a result of homologues recombination, was amplified by standard procedures (Summers and Smith, 1987; O'Reilly et al., 1992) and stored at 4°C. This virus was used in study I, II and III.

Production of recombinant baculoviruses from pVTBac-E1 and pVTBac-E2 was performed as mentioned above using cotransfection of the transfer plasmids and wild-type *Ac*NPV DNA into Sf9 cells using a calcium phosphate precipitation.

The expression cassette was transferred from the pBluescript derivatives pFASTBAC1 derivatives pOB504-1 for the capsid protein and pAcRVE1sol which was used for the generation of a recombinant baculoviruses via the BAC-to-BAC^{TM} system (Gibco-BRL). The resultant baculoviruses was designated vOB504-1 for the capsid protein and AcRVE1sol for the E1.

3.3 Cell Culture and Infection of Cells

Lepidopteran *Spodoptera frugiperda* cells (Sf9; ATCC CRL1711, Rockville, MD) were maintained as monolayer and suspension cultures at 27-

28°C. In study IV *Tricoplusia ni* (High Five®; Invitrogen, San Diego, CA) cells were also used. Initially the Sf9 cells were grown in TNM-FH medium (Sigma Chemicals, St. Louis, MO) supplemented with 2 mM L-glutamine, 10 % fetal calf serum and antibiotics (penicillin, streptomycin and fungizone). For study VI only serum-free SF900 II medium (Gibco-BRL) was used.

For amplification of the viruses, cells were first grown either on 25 cm^2 tissue culture flasks or in 250 ml Erlenmeyer flasks, at a density of 1,5 x 10^6 viable cells per ml, and infected with recombinant viruses with a multiplicity of infection (MOI) of 0.5 plaque forming units (PFU) per cell. The extra-cellular virus (ECV) was harvested two days post infection (p.i.). The amplified virus was there after used for protein production. In all studies infections with a MOI of at least 2 PFU were used. Study I, II and III were performed using spinner flasks and study VI 2,8 l Fernbach flasks on orbital shakers (130-135 rpm). Cells were collected at three days p.i. by low-speed centrifugation (1000 x g) and if needed stored at -20°C.

In study IV and V bioreactors were utilized and therefore the viruses were further scaled up by infections of 1000 ml of Sf9 cells, as described above. The cells were first grown in 2,8 l Fernbach flasks on orbital shakers and after reaching a density of 6 x 10^6 to 1 x 10^7 cells/ml, aseptically transferred to a 10 l Biostat ECD bioreactor (B. Braun Biotech, Melsungen, Germany) containing serum-free medium (Weiss et al., 1995a; Weiss et al., 1995b).

The bioreactor had a surface aerator and two large-pitched blade impellers. The speed was initially set to 49 rpm and subsequently raised as the cell density increased. Thus maintaining the oxygen level at 40-45% of air saturation. The pH was kept at 6,2 ± 0,5. The L-lactate and glucose concentrations from the cell culture supernatant were analyzed using a YSI Model 2000 glucose analyzer (Yellow Springs Instruments, Yellow Springs, OH). The osmomolarity of the growth medium was determined by an Advanced Model 3 MO Plus Micro Osmometer (Advanced Instruments Inc., Norwood, MA). The initial cell density was 7-8 x 10^5 cells/ml and cells were grown until a density of 2,4 x 10^6 viable cells/ml was reached. Infection with the recombinant baculoviruses was performed at a MOI of 1-5 PFU per cell. At 48 h (soluble E1 constructs) and 58 h (capsid construct) p.i. cells were harvested, by filtration, using a Sartorius Benchtop Tangential UF system (Sartorius GmbH, Göttingen, Germany) with 0,45-µm cellulose acetate membranes. The recombinant viruses were collected from the supernatant using polyester sulfone 100.000 MWCO membranes (Sartorius). Cells were concentrated by centrifugation 1000 x g for 5 min and stored at -20 °C.

3.4 Protein Purification

3.4.1 Immunoabsorbtion

Cytoplasmic extracts were prepared from cells infected with recombinant baculoviruses containing the RV 24S cDNA (VL1392-RV24S). Cells were resuspended in TNE buffer (10 mM Tris-HCl [pH 7,8], 150 mM NaCl, 1 mM EDTA) containing 1% Triton X-100 and 0,2 mM phenylmetylsulfonyl fluoride (PMSF), to a concentration of 10 x 10^6 cells/ml. The cell suspension was incubated 30 min on ice and clarified by centrifugation 5.000 x g, 10 min.

The immunoabsorbent was prepared by using CNBr activated Sepharose 4B (Pharmacia AB, Uppsala, Sweden) and a protein G purified E1 specific monoclonal antibody 4E10 (I). The CNBr-Sepharose 4B was swollen in 20 volumes of 1 mM HCl for 15 min, washed and equilibrated in coupling buffer (0,2 M $NaHCO_3$, 1,0 M NaCl, pH 8,0). The activated gel was mixed with antibody 4E10 (0,25 mg / ml coupling buffer) at twice the gel volume. Coupling was performed for 2 h, RT, using end-over-end rotation (e-o-e). Unbound antibodies were removed by discarding the supernatant after centrifuging the gel slurry at 200 x g, 5 min. Remaining reactive groups were blocked by incubation with 0,1 M Tris-HCl, pH 8,0, 2 h, RT. Finally, the gel was washed free of reactants with 7 mM Na/K-phosphate buffer (pH 7,3) and three cycles of washes with, first 0,1 M Na-acetate buffer (pH 4,0) with 0,5 M NaCl and then 0,1 M Na_2CO_3 (pH 11,0) with 0,5 M NaCl.

The clarified cell-lysate was incubated with the anti-E1 coupled Sepharose beads o/n, at 4°C, e-o-e. Lysate from 20 x 10^6 cells was incubated per ml of gel. Following the absorption the gel was washed with 7 mM Na/K phosphate buffer (pH 7,3) and absorbed proteins were eluted with 0,01 M Na_2CO_3 with 0,005% CHAPS and 0,1 mM Thimerosal (pH 11,0).

3.4.2 Concavalin A Affinity Chromatography

Cells infected with viruses encoding the soluble E1 construct (VTBac-E1) were harvested and concentrated by centrifugation as mentioned previously and washed with ice cold PBS. Cytoplasmic extracts were prepared by lysing the cells with TNE buffer containing 1% Triton X-100 and 0,2 mM phenylmetylsulfonyl fluoride (PMSF). The soluble extract was sonicated (3 x 5 s) and clarified by centrifugation for 10 min 10 000 x g, at 4°C. The supernatant fractions were thereafter ammonium sulfate precipitated.

Ammonium sulfate saturated solution was added step-wise using 10% salt increasement to the cytoplasmic extract. The protein precipitation was performed at 4°C, for 2 h. The precipitates were collected by centrifugation at 12 000 x g, at 4°C. Precipitate fractions used for Concavalin A affinity chromatography were dissolved in start-buffer (0,5 M NaCl, 20 mM Tris, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7,4) and further dialyzed against this buffer, o/n, 4°C.

The Concavalin A Sepharose 4B column (Pharmacia) was equilibrated with start-buffer containing 0,01% Triton X-100. The recombinant E1 containing extract was exposed to the column by continuos recirculation of the sample through the column (1 ml/min), for 12 h, at 4°C. There after the column was thoroughly washed with the same buffer. Weakly bound glycoproteins were eluted using a gradient of methyl-α-D-mannoside. Elution of E1 from the resin was performed by including 10 mM EDTA in the buffer.

3.4.3 Immobilized Metal-Ion Affinity Chromatography

An agarose gel with Ni^{2+} ions immobilized with nitrilotriacetate (NTA; Qiagen Inc., Chartsworth, CA) was used for purification of the polyhistidine-tagged proteins by immobilized metal-ion affinity chromatography (IMAC; Janknecht et al., 1991; Kuusinen et al., 1995). Initially, frozen cell pellets were disrupted by resuspension in ice-cold lysis buffer (0,5 M NaCl, 10 mM Hepes, 10 % (v/v) glycerol, 0,1 mM EDTA, 5 mM $MgCl_2$, 5 mM imidazole, 75 µg / ml PMSF, 1 % (v/v) Triton X-100, pH 7,8), at 2 x 10^7 cells/ml buffer. The lysis was performed on ice for 1 h with gentle mixing and the crude cell lysate clarified by centrifugation (10 000 x g) for 15 min at 4°C. The supernatant was thereafter incubated with the Ni^{2+}-NTA resin o/n, e-o-e, at 4°C. Prior to the incubation the resin was equilibrated in lysis buffer.

Weakly bound proteins were washed off the resin with lysis buffer (10 x resin volume). More strongly bound proteins were eluted by a step-wise increasing gradient of imidazole (10 and 40 mM) in end-buffer (0,5 M NaCl, 20 mM Hepes, 20 % (v/v) glycerol, 0,1 mM EDTA, 5 mM $MgCl_2$, 75 µg / ml PMSF, 1 % (v/v) Triton X-100, pH 7,8). The recombinant histidine-tagged proteins were eluted with end-buffer containing 250 and 500 mM imidazole.

3.4.4 Anti-FLAG™ M1 Affinity Chromatography

E1 protein samples containing both a polyhistidine and a FLAG-tag were pooled and purified by affinity chromatography (AC) using an anti-Flag™ M1 Affinity Gel (Sigma-Aldrich, St.Louis, MO). Prior to exposure to the affinity matrix E1 fractions pooled from the IMAC were dialyzed against TBS containing 2 mM $CaCl_2$ and 1% Triton X-100. The affinity matrix was also equilibrated in the same buffer. Binding of E1 to the M1 gel was performed o/n, e-o-e, at 4° C. Contaminants were washed off the resin with several column volumes washes of the starting buffer. FLAG-tagged E1 protein was eluted off the column with TBS containing 2 mM EDTA and 0,1 % Triton X-100. Eluted fractions used in surface plasmon resonance studies were dialyzed against TBS containing 0.2% BSA and 0.05% Tween 20.

3.5 Protein Analysis Assays

3.5.1 Protein Concentration Assay

Protein quantity determination were carried out either using the method of Bradford (Bradford 1976) or a bicinchonic acid assay (Pierce, Rockford, IL). The Bradford assay was performed in 1 ml volumes and with BSA as a reference. The Pierce BCA assay was performed in a microtiter plate format, as described by the manufacturer, also utilizing BSA as a standard.

3.5.2 SDS-PAGE and Immunoblot Analysis

All samples were denatured with Laemmli sample buffer (Laemmli, 1970) and boiled for 3 min. The protein samples were separated on 10 % SDS-polyacrylamide slab gels. The total protein content was visualized by Coomassie brilliant blue or silver salt staining according to standard protocols (Sambrook et al., 1979). For immunoblotting proteins were transferred to nitrocellulose membranes (Towbin et al., 1979). The membranes were blocked with 2% milk powder. Detection was performed essentially according to published procedures (Oker-Blom et al, 1989; Seppänen et al., 1991).

The following primary antibodies were used; 1) a rabbit polyclonal RV antiserum, 2) a human convalescent serum, 3) a mouse monoclonal capsid specific antibody (Wolinsky et al, 1991), 4) a mouse monoclonal polyhistidine-tag specific antibody (His-Tag antibody; Dianova GmbH, Hamburg,

Germany), 5) a mouse monoclonal FLAG tag specific antibody (M1 anti-flag™ antibody; Eastman Kodak).

Visualization was performed with the following secondary antibodies according to the manufacturers instructions; a) horseradish peroxidase (HRP)-conjugated goat-anti rabbit antibodies (Caltec Laboratories, San Francisco, CA), b) alkaline phosphatase (AP)-conjugated goat-anti rabbit antibody (Bio-Rad, Richmond, CA), c) alkaline phosphatase (AP)-conjugated goat anti-human IgG + IgM + IgA antibodies (Zymed Inc., San Francisco, CA), d) alkaline phosphatase (AP)-conjugated goat-anti mouse antibodies (Bio-Rad).

Molecular weight markers where from Bio-Rad and Pharmacia. Partially purified rubella virus was also used as a control (Toivonen et al., 1983).

3.5.3 ELISA

Partially purified RV or purified recombinant E1 was diluted in carbonate-bicarbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9,6), to a concentration of 2 µg/ml. Microtiterplates were coated with 50 µl of the antigen solution, o/n, at 4°C. The plates were thereafter washed with PBS – 0,05% Tween 20 and blocked for 1 h, at RT, with PBS -1% BSA. Human sera, of different dilutions, in PBS –1% BSA, were added and incubated for 2 h, at RT. After this incubation the plates were washed four times as above. For detection of human antibodies a HRP conjugated goat anti-human antibody (Caltac Laboratories) was used, diluted 1:3000 in PBS –1%BSA, and incubated 1 h, at RT. The incubation was followed by four washes and detection with chromogen solution (0,66 mg of o-phenylenediamine per ml of 0,1 M citric acid –Na phosphate buffer [pH 5,0] and 0,1 % H_2O_2). The color reaction was stopped after approximately 5 min with 1 M HCl and the optical density measured at 492 nm.

3.5.4 Time-Resolved Fluoroimmunoassays

The diagnostic study (III) analyzing detection of RV-specific IgM antibodies was performed using time-resolved fluoroimmunoassays with the DELFIA® system (Soini and Lövgren, 1987; Wallac Oy, Turku Finland). Microtiterplates were coated with either purified recombinant E1 or RV, at 2 µg/ml in PBS, o/n, at 4°C. The plates were blocked and washed as described for the ELISA. Human serum samples were diluted 1:100 in PBS –1%BSA. Bound IgM antibodies were detected by an Eu^{3+}-labeled anti-human IgM

antibody (anti-IgM clone 7408; Medix Biochemica Oy, Kauniainen, Finland). Labeling of the secondary antibody was performed according to the manufacturers instructions (Hemmilä, 1988; Wallac Oy). Enhancement of the fluorescence and measurement of the signal on a DELFIA® research fluorometer was performed according to the manufacturers protocols (Wallac Oy).

3.5.5 Surface Plasmon Resonance

The BIAcore™ Upgrade biosensor apparatus (Biacore AB, Uppsala, Sweden) was used for the surface plasmon resonance (SPR) experiments. Results were analyzed using the BIA Evaluation v. 2.1 software (Biacore AB). The purified histidine-tagged capsid and E1 protein were immobilized via the His-tag to a NTA sensor chip using essentially conditions suggested by the manufacturer (Biacore AB).

Initially the sensor chip surface was equilibrated with eluent buffer (10mM HEPES, 0,15 M NaCl, 50 µM EDTA, 0.005% Surfactant P20, pH 7,4) with a flow rate set to 5 µl / min. Ni^{2+} was thereafter loaded to the sensor chip surface by injection of 40 µl nickel solution (500 µM $NiCl_2$ in eluent buffer). For immobilization the capsid protein was diluted five-fold and the E1 protein two-fold in ligand buffer (10mM HEPES, 10 mM $CaCl_2$, 10 mM $MgCl_2$, 0,15 M NaCl, 0.005 % Surfactant P20, pH 7.4). The final concentration of the purified proteins was approximately 50 µg/ml.

The protein solution was injected (200 µl volume) over the Ni^{2+} -NTA surface. The capsid specific monoclonal antibody (C-2; Wolinsky et al., 1991) was diluted fourfold in ligand buffer whereas the FLAG-tagged E1 specific M1 anti-flag™ antibody (Sigma) was diluted to a final concentration of 10 µg / ml in ligand buffer.

As one of the controls for the studies with the capsid protein, a nonrelevant (a soluble RV E2 with a polyhistidine tag) protein was expressed, purified and immobilized in a similar manner. The control experiments for the E1 protein were designed to demonstrate the sensitivity of the assay. Therefore we used a ligand buffer without $CaCl_2$. In these experiments washing of the sensor chip surface was also performed in the absence of $CaCl_2$. In this way we were able to study the binding of the M1 antibody to the FLAG-tag both in the presence, and in the absence of, Ca^{2+}. Control experiments with irrelevant immobilized proteins (the capsid protein lacking a FLAG tag) and antibodies (a non-E1 specific monoclonal mouse antibody) using normal ligand binding conditions were also performed.

4. RESULTS

4.1 Recombinant Constructs

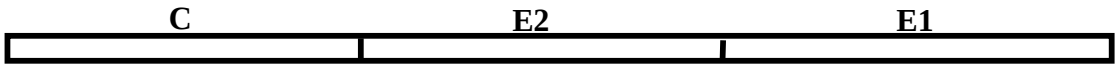
The recombinant viruses produced and used in studies I-IV were formed as a result of homologous recombination between a transfer plasmid and baculoviral DNA. In study V and VI transposon-mediated recombination was used for inserting the RV protein coding sequences into the baculovirus genome. RV sequences were in all cases inserted under the transcriptional regulation of the polyhedrin gene promoter. Made DNA constructs were also sequenced.

Recombinant baculoviruses encoding the following constructs were produced: 1) the p110 polyprotein encoding the RV structural proteins (VL1392-RV24S), 2) a truncated soluble form of the E1 (VTBac-E1), 3) a truncated soluble form of the E2 (VTBac-E2), 4) a polyhistidine tagged capsid protein (vOB504-1), 5) a FLAG and polyhistidine tagged soluble E1 (AcRVE1sol) (Figure 5).

4.2 Expression in Insect Cells

Cells grown in spinner flasks or in Fernbach flasks were collected 2 – 3d p.i. The growth and the morphological state of the cells were visually monitored using phase contrast microscopy. Cells were collected before any prominent occurrence of cell death. Often cells were seen to have a swollen “infected” morphology, before collection. The truncated soluble form of the E1 (VTBac-E1) and the polyhistidine tagged capsid protein (vOB504-1) were expressed in 10 l bioreactors. Growth of the cells was followed both post planting (p.p.) and post infection (p.i.). The speed of the pitched blade impellers (rpm) was adjusted according to the oxygen level, which was maintained at 40 – 42% of air saturation. The cell density was also followed (IV: Table 1; V: Table 1).

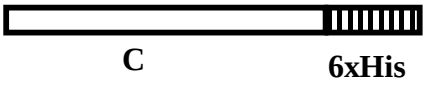
A) the p110



B) the extra viral part of E1, preceded by the melittin signal sequence

C) the extra viral part of E2, preceded by the melittin signal sequence

D) the capsid protein with a polyhistidine tag at the carboxy



E) the extra viral part of E1 preceded by a Flag-tag and with a polyhistidine tag at the carboxy end

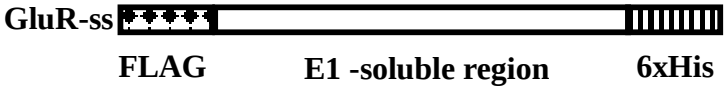


Figure 5. Recombinant RV constructs. Recombinant baculoviruses were obtained using homologues recombination for constructs A) to C). Transposon-mediated insertion was used for constructs D) and E). These two constructs were preceded by the signal sequence for GluR-D glutamate receptor subunit.

Cells were infected 2 d p.p., at a concentration of 2,0 – 2,4 x 10^6 cells per ml, and cell division stopped following infection. At 48 h p.i. (E1) and 58 h p.i. (capsid), prior to the occurrence of cell death, cells were collected. Expression of RV-specific proteins was detected by SDS-PAGE and immunoblot analysis.

4.3 Protein Purification

4.3.1 Immunoabsorbtion

The E1 protein expressed using the RV p110 encoding recombinant baculovirus VL1392-RV24S was purified with Sepharose beads coupled to the E1 specific antibody 4E10. Attached proteins were eluted using alkaline conditions and the purified fractions were pooled and analyzed by Coomassie blue staining of SDS-PAGE gels and immunoblotting. Immunoblotting with a polyclonal rabbit anti-RV serum revealed a RV-specific protein of the expected size of the E1 protein. Coomassie blue staining of these fractions demonstrated the high purity of the E1 protein fractions (I: Figure 1). Quantification of the level of protein using the method of Bradford showed that this final preparation had a concentration of 35 – 50 µg purified protein / ml elution buffer. Giving a total of 600 - 800 µg of purified protein / l of cell culture medium.

4.3.2 Concavalin A Affinity Chromatography

Soluble E1 expressed by infection of insect cells in a 10 l bioreactor with the VTBac-E1 virus was prior to Concavalin A affinity chromatography enriched by ammonium sulfate precipitation. The major portion of the total E1 protein was found to precipitate in 20 % ammonium sulfate. This was determined by SDS-PAGE and Coomassie blue staining as well as immunoblotting using polyclonal rabbit anti-RV serum (IV: Figure 4). The ability of this glycan-specific purification method to purify the E1 protein to homogeneity was shown by immunoblotting (IV: Figure 5). The purified protein was shown to migrate as expected from the calculated molecular weight (52 kDa).

4.3.3 Immobilized Metal-Ion Affinity Chromatography

The polyhistidine tagged capsid protein was expressed in a bioreactor using the recombinant baculovirus vOB504-1. The FLAG and polyhistidine tagged soluble E1 (AcRVE1sol) was expressed in a smaller scale using Fernbach flasks. The immobilized metal-ion affinity chromatography (IMAC) was performed using 5 mM imidazole in the initial binding stage and thereafter a stepwise gradient up to 500 mM. The purification profiles of the recombinant protein were studied by SDS-PAGE and Coomassie brilliant blue staining as well as immunoblotting (V: Figure 3 and 4).

Purification of the capsid construct revealed an elution profile where the majority of the capsid protein eluted at 250 – 500 mM imidazole. Protein quantitation of the different fractions was determined using a bicinchonic acid assay. Protein concentrations of more than 1 mg purified protein / ml elution buffer was achieved using these conditions. A total of 5 mg of purified capsid protein could be obtained from 1 l of cell culture.

The IMAC of the epitope tagged E1 protein resulted in a partially purified product. Protein contaminants and some putative degradation products of the E1 were seen in the 50 mM imidazole fractions. In 250 mM imidazole elution the E1 protein product was shown as a large homogenous band, although some putative degradation were visible (VI: Figure 1).

4.3.4 Anti-FLAG™ M1 Affinity Chromatography

In order to obtain pure epitope tagged E1 protein the IMAC elutants were pooled and further purified by affinity chromatography (AC) using the Anti-FLAG® M1 Affinity Gel. The purification was analyzed by immunoblotting and silver staining of SDS-PAGE gels (VI: Figure 1). The immunoblot shows a large homogenous band corresponding to the expected size of E1. Some higher molecular weight complexes as well as some smaller molecular weight proteins were also visible. These were speculated to be multimeric E1 complexes and breakdown products, respectively, as they are antibody reactive.

The silver stained SDS-PAGE revealed that the contaminating proteins were removed in the flow-through fractions and the eluted fractions showed an E1 protein sample of high purity. The eluted fractions of 1 ml volume usually contained protein at concentrations of 30 – 50 μg / ml buffer, determined by a bicinchonic acid assay. In the experiment shown in study VI the elution was terminated after 6 eluted fractions, giving roughly 200 – 300 μg purified

protein from 1 l cell culture. However the M1 affinity matrix showed a very slow dissociation of the E1 protein after chelating the Ca^{2+} ions present and elutions could sometimes be extended to 10 - 20 fractions with decreasing protein concentrations detectable in each fraction.

4.4 ELISA

The antigenicity of the E1 protein, purified by immunoabsorbtion, was tested with RV-specific IgG from human convalescent sera was studied using an ELISA. In the ELISA the antigenicity of the E1 was compared with authentic RV. Two different RV-negative and two different RV-positive serum samples were tested using a dilution serie (I: Figure 2). The optical density at 492 showed clear differences between the positive and the negative samples. The purified E1 was demonstrated to have an antigenicity similar to the authentic RV.

4.5 Time-Resolved Fluoroimmunoassays

The antigenicity of the purified E1 and authentic RV was analyzed in a correlation study using time-resolved fluoroimmunoassays (TR-FIA) with the DELFIA system. The two different antigens were incubated with 64 human serum samples from patients with recent or past rubella infections. RV-specific IgM antibodies were detected with murine anti-human IgM monoclonal antibodies labeled with Eu^{3+}. The measured fluorescence from the two assays were compared (III: Figure 2). The performed assays were shown to have a positive correlation of r = 0,843, indicating similar antigenicities of the two antigens. The mean fluorescence values were shown to be 9,5 % higher in the TR-FIA where authentic RV was used as the antigen.

4.6 Surface Plasmon Resonance

The IMAC purified histidine tagged capsid protein as well as the IMAC anti-FLAG-AC purified epitope tagged soluble E1 protein were used in surface plasmon resonance (SPR) analysis. Dextran matrix sensor chips containing

covalently coupled NTA were used. Ni^{2+} was bound to the NTA linker and the polyhistidine tagged recombinant proteins were immobilized to the Ni^{2+} charged matrix.

Following immobilization of the capsid protein and subsequent washes, a monoclonal capsid specific antibody (C-2) was injected over the sensor chip surface. Binding of a protein to the sensor chip is detected by an increase in response units, which is relative to the change of mass on the surface . The complete analysis scheme includes the loading on Ni^{2+} to the matrix, immobilization of the capsid protein and binding of the C-2 antibody to the capsid. All steps were followed by washing where the resultant baseline shows the actual change in bound mass and not just the reflective index of the buffers used. The specificity of the C-2 antibody capsid binding is visualized graphically by subtracting the obtained response units from a control experiment were the C-2 antibody was injected over an irrelevant antigen (V: Figure 2).

The epitope-tagged E1 protein was immobilized to the NTA matrix as described above for the capsid protein. Here the Ca^{2+} dependent binding of the M1 antibody to the FLAG-epitope was utilized. The M1 antibody was injected over the E1 immobilized on the sensor chip surface in the presence and in the absence of $CaCl_2$ (VI: Figure 3). Here a clear difference in the binding of the antibody to the E1 is demonstrated.

5. DISCUSSION

The information obtained about the molecular biology of rubella virus (RV) has severely lagged behind the Alphaviruses. This situation has mainly been due to the fact that Alphaviruses has been widely studies as a model virus and to the fact that no good animal model system for rubella infection is available and because some Alphaviruses have also been extensively studied as tools for viral biotechniques (e.g. gene therapy and viral-mediated gene expression systems (Schlesinger and Dubensky, 1999)). Several striking differences in the assembly and budding of the viruses have been found. The RV capsid also lacks the characteristic serin protease domain needed for Alphavirus polyprotein processing (Melancon and Garoff, 1987; Clarke et al., 1988). Both the structural and the non-structural proteins differ in organization, number and some functions between the Alphaviruses and RV.

Alphaviruses have a very broad host range including invertebrates and vertebrates. Several of the different host cell receptors interacting with the viruses have also been identified (for review see Strauss and Strauss, 1994). Studies focusing on the Alphavirus structure have also revealed the role of different sites in the structural proteins (e.g. amino acid residues responsible for interactions between the structural proteins (Lee et al., 1996; Skoging et al., 1996)).

The evident differences between RV and the Alphaviruses shows that assumptions, of structure and function or on the viral biology of RV, cant be made based on information obtained from the Alphaviruses. Since RV is a human pathogen an effort to understand the biology of the virus would not only give information that could be found valuable for other human pathogens, but it would also have significant medical implications.

A good starting point when trying to gain information about a protein is to have a system for producing large amounts of the desired molecule with a high degree of purity and native folding. This has been the main aim throughout this study. We started by expressing the structural polyprotein (p110) in insect cells in spinner flask culture. The E1 protein was purified from the cytoplasmic extract using affinity chromatography with a monoclonal E1 specific antibody (I). The purification process was optimized so that no contaminants were visible in Coomassie blue stained SDS-PAGE gels. One consideration was whether the glycosylation of the E1, in insect cells, would give rise to a native

folded protein. This was partially answered in a earlier correlation study where baculovirus-expressed envelope proteins were compared to the authentic virus (Seppänen et al., 1991). Another concern was whether the E1 protein would retain its antigenicity after purification. This we studied in an ELISA with four serum samples and authentic RV as a reference. In this way we were able to show clear similarities between our assays (I).

Different diagnostic assays for the detection of RV infection have been widely studied (reviewed in Cradock-Watson, 1991). Most serological systems are based on whole authentic RV, but other methods such as peptide antigen and RV-like particle based EIA assays (Bosma et al., 1995; Grangeot-Keros and Enders, 1997) have also been developed. Development of an ELISA-type RV diagnostic kit has from time to time been on the development plan in several clinical companies. To base a diagnostic kit on a recombinant protein would have the obvious advantage of being non-infectious and could be produced and purified in large-scale making it very cost efficient. The diagnostic potential of our purified E1 protein was briefly studied with an ELISA-type assay (I) and more in detail using TR-FIA (III).

The TR-FIA assay comparing the interaction of IgM antibodies with the recombinant E1 or authentic RV was set up for three main reasons. First, we wanted to see how our purified protein would perform in a correlation study compared to authentic RV. Here we were able to detect a strong correlation between the assays, suggesting that the presented scheme could be worth investigating further for its possibility to function as a diagnostic assay for detecting RV infection. Second, we were interested in setting up an IgM detecting assay since IgM is the primary response to an infection. We were able to show that RV specific IgM antibodies could easily be detected. Thirdly, we wanted to see if a TR-FIA in the DELFIA® format would give the assay the needed sensitivity to obtain detectable differences between the different serum samples. In some initial studies with a conventional type EIA assay this had not been achieved. Here, we were able to show clear differences between the serum samples, revealing the sensitivity of the TR-FIA.

Although the initial goals set up for the diagnostic assay were met, several questions concerning the validity of the assay and suggestions for further developments were raised. One obvious difficulty was to set the limit for determination if a recent infection had occurred or not. This difficulty was as clear in our control assay, showing that only one type of assay might not be sufficient for determination of an infection. Another problem was that although we found a strong positive correlation between the assays some individual discrepancies were seen. Whether these were due to cellular protein contaminants in our purified E1 sample giving too high response in some

samples, or the lack of the E2 and the capsid giving too low a response in the recombinant assay, remains to be studied. One shortcoming of the recombinant assay was thought to be the relatively harsh alkaline purification and the partially denaturing process of coating microtiterplates with the E1. Therefore, further studies were designed so that affinity tags in our recombinant constructs would allow a more gentle purification protocol and an affinity-tag mediated immobilization of the recombinant constructs.

As mentioned earlier, one requirement for studying a protein is to obtain large amounts of the molecule in a purified form. Therefore our next aim was to scale-up the baculovirus expression system into a 10 l bioreactor. At this point we had already successfully scaled up our expression from spinner flasks (100 – 600 ml) to Erlenmeyer flasks and 2,8 l Fernbach flasks. When scaling up the cell culturing, we focussed on amplifying our recombinant viruses, as well as keeping the oxygen level in the cell culture sufficient throughout the study. Viruses were in all our studies where proteins are produced, used in excess and the speed of the bioreactor impellers was adjusted to maintain the oxygen level. Following these guidelines we faced no large obstacles in our scaling-up and expression of the recombinant proteins remained at a similar level compared to small-scale cell culturing (IV, V). Therefore we concluded that our expression scheme was favorable and can be widely recommended.

Two affinity epitope tags were introduced in order to provide easy and gentle purifications of the proteins and to obtain a site for immobilization of the proteins in interaction assays. The polyhistidine-tag binds with high affinity to divalent cations. Using this tag we were able to obtain a high affinity complex containing our recombinant histidine-tagged protein and a Ni^{2+}-NTA agarose matrix. The great advantage compared to antibody utilizing affinity chromatography is that not only can the affinity complex be achieved in most buffers, but also disruption of the formed complex can be achieved by competitive elution with imidazole, therefore eluting the recombinant protein using gentle conditions. Another advantage is the high capacity of the Ni^{2+}-NTA affinity matrix, giving a strong concentration effect. Using this scheme we were able to show a simple and gentle one-step purification scheme of the polyhistidine-tagged capsid protein (V).

Using the same one-step IMAC purification scheme we were not able to achieve the same high purity of a histidine-tagged E1. Whether this was due to some general feature of glycoproteins or some E1 specific characteristic, was not further studied. To this E1 construct we had, however, engineered a second affinity tag, the FLAG-tag. The eight amino acid FLAG-epitope has been shown to bind to an antibody (M1) in a calcium-dependent manner.

Utilizing this characteristic feature of the M1 antibody, the E1 could be immobilized to a M1 antibody containing affinity matrix, in the presence of Ca^{2+} ions. Elution of the E1 was performed by chelating the Ca^{2+} ions with EDTA. Using this purification scheme a gentle environment could therefore be obtained during the whole purification protocol (VI).

A surface plasmon resonance (SPR) study of the purified protein using the BIAcore equipment was chosen for several reasons. First, the NTA sensor chips gave us a surface for immobilization of our protein via the affinity tag, therefore avoiding a partial denaturation of the protein which occurs when coating directly to microtiterplates. Secondly, we wanted to introduce the idea of a SPR technique in RV diagnostic studies. By showing some example studies we hope that the SPR technique may be found useful in antibody affinity and avidity studies determining the onset of rubella infection.

With the capsid protein the SPR study was performed simply to show that all critical steps of the assay could be performed. The immobilization of the capsid protein was studied and the mechanism of the interaction between the capsid and a monoclonal antibody was visualized. Here we found that our assay should give a good starting point specific for the capsid, for further studies of interactions between antibodies and viral proteins. Since this study was performed the manufacturer recommends higher flow-rates to be used in kinetic studies. For such studies we therefore also recommend higher flow-rates so that possible rebinding of the antibody could be ruled out (V).

At present SPR studies are performed as single experiments. However, in the future the biosensor technique might have potential for routine diagnostics. This would require more simple experimental schemes, with several tests performed at once. This could possibly be performed with resonance mirror (RM) biosensors which today operate in a cuyvette format instead of a flow cell on a sensor chip.

From our TR-FIA analysis, recognizing RV specific IgM antibodies, we have seen the difficulty to determine between a positive and negative response. Therefore we decided on a new approach to study our recombinant E1 protein with the BIAcore. Instead of just confirming the research analysis scheme obtained for the capsid we tried a more challenging approach. In the E1 SPR study, we relied on the specific characteristics of the M1 anti-FLAG antibody. The M1 antibody binds to the FLAG epitope in the presence of Ca^{2+}. However, it is also generally known, both from immunoblotting and ELISA studies, that when using normal research grade buffers the M1 binds to the FLAG epitope also in the absence of Ca^{2+}. This binding occurs, naturally, to a much lower level. By using a research scheme utilizing the clear positive

binding of M1 in the presence of Ca^{2+} and the more undefined binding of M1 in the absence of Ca^{2+}, we tried to create a system that would mimic the natural occurrence of RV-specific serum antibodies. We were able to show clear differences in the M1 binding, depending on the presence of Ca^{2+}, indicating that the SPR system is sensitive enough for RV antibody interaction studies (VI).

It is evident that biosensors could be useful in diagnostic studies. The equipment today is, however, more suitable for individual experiments than for screening of large amounts of samples. The use of automatic systems with an open detection cuyvette or microtiterplate could however change this. The power of screening for multiple infections in each sample might often be performed using PCR diagnostics, but determination of the onset time of the infection could be followed up by SPR studies. We also see the need for simple ELISA type assays to be used in less well-equipped laboratories and to be produced in a cost efficient matter.

6. CONCLUSSIONS AND FUTURE PERSPECTIVES

In this study we demonstrate that RV structural proteins, expressed by recombinant baculoviruses in insect cells, show similar characteristics compared to the authentic viral proteins. Large-scale expression of recombinant RV proteins could be achieved and proteins purified with or without affinity tags. Since no clear activity assay is available for RV proteins the native state of the proteins was studied using antibody interaction assays and correlation's calculated.

We were able to show how different RV structural protein variants could successfully be expressed and purified. The diagnostic potential of the E1 protein was also analyzed. The SPR analysis technique was also introduced to RV protein analysis.

In the future there are at least three obvious paths to follow from the results presented in this study. First, the protein production and purification schemes presented provide the tools that could be used to study the basic biology of the virus. With large amounts of purified protein structural aspects as well as virus-cell interactions could be studied. A second path is the development of a RV diagnostic kit using recombinant RV proteins. Different concentrations of a mixture of E1, E2 and the capsid protein could be used in the microtiterplate format. Immobilizing the proteins using different linkers or affinity tags could be studied and different conditions for IgG and IgM analysis could be tested. Different visualization assays could also be studied and compared (e.g. ELISA vs. TR-FIA). Finally, the SPR studies should be further developed. A scheme for gathering kinetic data, not only from monoclonal antibody studies, but also from serum samples should be developed.

REFERENCES

Baneyx, F. 1999. Recombinant protein expression in *Escherichia coli*. Curr.Opin.Biotechnol. 10: 411-421.

Baron, M.D., and Forsell, K. 1991. Oligomerisation of the structural proteins of rubella virus. Virology 185: 811-819.

Baron, M.D., Ebel, T., and Suomalainen, M. 1992. Intracellular transport of rubella virus structural proteins expressed from cloned cDNA. J.Gen.Virol. 73: 1073-1086.

Belyaev A.S., and Roy, P. 1993. Development of baculovirus triple and quadruple expression vectors: co-expression of three or four bluetongue virus proteins and the synthesis of bluetongue virus-like particles in insect cells. Nucleic Acids Res. 21: 1219-1223.

Bosma, T.J., Corbett, K.M., O'Shea, S., Banatvala, J.E., and Best, J.M. 1995. PCR for detection of rubella virus RNA in clinical samples. J.Clin.Microbiol. 33(5): 1075-1079.

Bosma, T.J., Etherington, J., O'Shea, S., Corbett, K., Cottam, F., Holt, L., Banatvala, J.E., and Best, J.M. 1998. Rubella Virus and Chronic Joint Disease: Is There an Association? J.Clin.Microbiol. 36(12): 3524-3526.

Bradford, M.M. 1976 A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal.Biochem. 72:248-254.

Clarke, D.M., Loo, T.W., McDonald, H., and Gillam, S. 1988. Expression of rubella virus cDNA coding for the structural proteins. Gene 65: 23-30.

Cory, J.S., and Bishop, D.H. 1997. Use of baculoviruses as biological insecticides. Mol.Biotechnol. 7(3): 303-313.

Cradock-Watson, J.E. 1991. Laboratory diagnosis of rubella: past, present and future. Epidemiol.Infect. 107: 1-17.

Cusi, M.G., Cioe´, L, and Rovera, G. 1992. PCR Amplification of GC-Rich Templates Containing Palindromic Sequences Using Initial Alkali Denaturation. BioTechn. 12(4): 502-504.

Cusi, M.G., Bianchi, Cioe´, L., and Valensin, P.E. 1994. Expression of recombinant E2 and C proteins of rubella virus in insect cells. Arch.Virol. 137: 35-41.

Davis, A.H. 1995. Advances in the use of recombinant baculoviruses for the expression of heterologoues proteins. Curr.Opin.Biotechnol. 6: 543-547.

Dixon, M. 1953. A monogram for ammonium sulphate solutions. Biochem.J. 54: 457-458.

Dominguez, G., Wang, C-.Y., and Frey, T.K. 1990. Sequence of the genome RNA of rubella virus: Evidence for genetic rearrangement during Togavirus evolution. Virology 177: 225-238.

Ford, D.K., Reid, G.D., Tingle, A.J., Mitchell, L.A., and Schulzer, M. 1992. Sequential follow up observations of a patient with rubella associated persistent arthritis. Ann.Rheum.Dis. 51: 407-410.

Forng, R-.Y., and Frey, T.K. 1995. Identification of the Rubella Virus Nonstructural Proteins. Virology 206: 843-853.

Frey, T.K. 1994. Molecular biology of rubella virus. Adv.Virus.Res. 44: 69-160).

Garbutt, M., Law, L.M.J., Chan, H., and Hobman, T.C. 1999. Role of Rubella Virus Glycoprotein Domains in Assembly of Virus-Like Particles. J. Virol. 73(5): 3524-3533.

Garoff, H., and Li, K.-J. 1998a. Recent advances in gene expression using alphavirus vectors. Curr.Opin.Biotechnol. 9: 464-469.

Garoff, H., Hewson, R., Opstelten, D.-J.E. 1998. Virus Maturation by Budding. Microbiol.Mol.Biol.Rew. 62(4): 1171-1190.

Grangeot-Keros, L. and Enders, G. 1997. Evaluation of a new enzyme immunoassay based on recombinant rubella virus-like particles for detection of immunoglobulin M antibodies to rubella virus. J.Clin.Microbiol. 35(2): 398-401.

Harrison, S.C., Schlesinger, S., Schlesinger, M.J., and Strong R.K. 1992. Crystallization of Sindbis virus and its nucleocapsid. J.Mol.Biol. 60: 226: 277-280.

Hedman, K., Lappalainen, M., Söderlund, M., and Hedman, L. 1993 Avidity of IgG in serodiagnosis of infectious diseases. Rew.Med.Microbiol. 4: 123-129.

Helenius, A. 1995. Alphavirus and flavivirus glycoproteins: structures and functions. Cell 81: 651-653.

Hemmilä, I. 1988. Lanthanides as probes for time-resolved fluorometric immunoassays. Scand.J.Clin.Lab.Invest. 48: 389-400.

Hemphill, M.L., Forng, R., Abernathy, E.S., and Frey, T.K. 1988. Time course of virus-specific macromolecular synthesis during rubella virus infection in Vero cells. Virology 162: 65-75.

Ho-Terry, L., Terry, G.M., and Londesborough, P. 1990. Diagnosis of foetal rubella virus infection by polymerase chain reaction. J.Gen.Virol. 71: 1607-1611.

Hobman, T.C., Woodward, L., Farquhar, M.G. 1993. The rubella virus E2 and E1 spike glycoprotein is arrested in a novel post-ER, pre-Golgi compartment. J.Cell Biol. 121: 269-281.

Hobman, T.C., Lundstrom, M.L., Mauracher, C.A., Woodward, L., Gillam, S., and Farquhar, M.G. 1994a. Assembly of Rubella Virus Structural Proteins Into Virus-like Particles in Transfected Cells. Virology 202: 574-585.

Hobman, T.C., Seto, N.O.L., and Gillam, S. 1994. Expression of soluble forms of rubella virus glycoproteins in mammalian cells. Virus Res. 31: 277-289.

Hobman, T.C., Woodward, L., Farquhar, M.G. 1995. Targeting of a Heterodimeric Membrane Protein Complex to the Golgi: Rubella Virus E2 Glycoprotein Contains a Transmembrane Golgi Retention Signal. Mol.Biol.Cell: 6: 7-20.

Hobman., T., Lemon, H., and Jewell, K., 1997. Characterization of an endoplasmic reticulum retention signal in the rubella virus E1 glycoprotein. J. Virol. 71:7670-7680.

Janknecht, R., de Martynoff, G., Lou, J., Hipskind, R.A., Nordheim, A., and Stunnenberg, H.G. 1991 Rapid and efficient purification of native histidine-

tagged protein expressed by recombinant vaccinia virus, Proc.Natl.Acad.Sci.USA 88: 8972-8976.

Kalkkinen, N., Oker-Blom, C., and Pettersson, R.F. 1984. Three genes code for rubella virus structural proteins E1, E2a, E2b and C. J.Gen.Virol. 65: 1549-1557.

Kost, T.A., and Condreay, J.P., 1999. Recombinant baculoviruses as expression vectors for insect and mammalian cells. Curr.Opin.Biotechnol. 10: 428-433.

Kuusinen, A., Arvola, M., Oker-Blom, C., and Keinänen, K. 1995 Purification of recombinant GluR-D glutamate receptor produced in Sf21 insect cells. Eur.J.Biochem. 233: 720-726.

Kääriäinen, L., and Söderlund, H. 1978. Structure and replication of alphaviruses. Curr.Top.Microbiol.Immunol. 82: 15-69.

Laemmli, U.K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227: 680-685.

Lee, S., Owen, K.E., Choi, H-.K., Lee, H., Lu, G., Wengler, G., Brown, D.T., Rossmann, M.G., and Kuhn R.J. 1996. Identification of a protein binding site on the surface of the alphavirus nucleocapsid and its implication in virus assembly. Structure 4:531-541.

Liu, Z., Yang, D., Qiu, Z., Lim, K-.T., Chong, P., and Gillam, S. 1996. Identification of Domains in Rubella Virus Genomic RNA and Capsid Protein Necessary for Specific Interaction. J.Virol. 70(4): 2184-2190.

Luckow, V.A., Lee, S.C., Barry, G.F., and Olins, P.O. 1993. Efficient generation of infectious recombinant baculoviruses by site-specific transposon-mediated insertion of foreign genome propagated in Esterichia coli. J.Virol. 67: 4566-4579.

Marr, L.D., Wang, C-.Y., and Frey, T.K. 1994. Expression of the rubella virus nonstructural protein ORF and demonstration of proteolytic processing. Virology 198: 586-592.

Mastromarino, P., Cioè, L., Rieti, S., and Orsi, N. 1990. Role of membrane phospholipids and glycolipids in the Vero cell surface receptor for rubella virus. Med.Microbiol.Immunol. 179:105-114.

Mathews, R.E.F. 1982. Classification and nomenclature of viruses. Intervirology 17: 1-199.

Mauracher, C.A., Mitchell, L.A., and Tingle, A.J. 1992. Differential IgG Avidity to Rubella Virus Structural Proteins. J. Med. Virol. 36: 202-208.

Megyeri, K., Berencsi, K., Halazonetis, T.D., Prendergast, G.C., Gri, G., Plotkin, S.A., Rovera, G., and Gönczöl. 1999. Involvement of a p53-Dependent Pathway in Rubella Virus-Induced Apoptosis. Virology 259: 74-84.

Melancon, P., and Garoff, H. 1987. Processing of the Semliki Forest virus structural polyprotein: role of the capsid protease. J.Virol. 61: 1301-1309.

Meurman, O.H., Hemmilä, I.A., Lövgren, T.N-.E., and Halonen P.E. 1982. Time-Resolved Fluoroimmunoassay: a New Test for Rubella Antibodies. J.Clin.Microbiol. 16(5): 920-925.

Mottershead, D., Alfthan, K., Ojala, K., Takkinen, K., and Oker-Blom, C. 2000. Baculoviral display of functional scFv and synthetic IgG-binding domains. Biochem.Biophys.Res.Com. 275(1): 84-90.

Nath, A., Slagle, B., and Wolinsky, J.S. 1989. Anti-idiotypic antibodies to rubella virus. Arch.Virol. 107: 159-167.

Oker-Blom, C., Kalkkinen, N., Kääriäinen, N., and Pettersson, R.F. 1983 Rubella virus contains one capsid protein and three envelope glycoproteins, E1, E2a and E2b. J.Virol. 46: 964-973.

Oker-Blom, C. 1984. The gene order for rubella virus structural proteins is: NH2-C-E2-E1-COOH. J.Virol. 51: 354-358.

Oker-Blom, C., Pettersson, R.F., and Summers, M.D. 1989. Baculovirus polyhedrin promoter-directed expression of rubella virus envelope glycoproteins, E1 and E2, in *Spodoptera frugiperda* cells. Virology 172: 82-91.

Oldstone, M.B.A. and Levine, A.J. 2000. Virology in the Next Millennium. Cell 100:139-142.

O'Reilly, D.R., Luckow, V.A., and Miller, L.K. 1992. Baculovirus expression vectors –A laboratory manual. W.H. Freeman, New York.

Owen, K.E., and Kuhn, R.J. 1997. Alphavirus budding is dependent on the interaction between the nucleocapsid and hydrophobic amino acids on the cytoplasmic domain of the E2 envelope glycoprotein. Virology 230: 187-196.

Peltola, H., Heinonen, O.P., Valle, M., Paunio, M., Virtanen, M., Karanko, V., and Cantell, K. 1994. The elimination of indigenous measles, mumps, and rubella from Finland by a 12-year, two-dose vaccination program. N.Eng.J.Med. 331(21): 1397-1402.

Petruzziello, R., Orsi, N., Maccia, S., Rieti, S., Frey, T.K., and Mastromarino, P. 1996. Pathway of rubella virus infectious entry into Vero cells.

Possee, R.D. 1997. Baculoviruses as expression vectors. Curr. Opin. Biotechnol. 8: 569-572.

Pugachev, K.V., and Frey, T.K. 1998. Rubella Virus Induces Apoptosis in Culture Cells. Virology 250: 359-370.

Qiu, Z., Hobman, T.C., McDonald, H.L., Seto, N.O.L., and Gillam, S. 1992. Role of N-linked Oligosaccharides in Processing and Intracellular Transport of E2 Glycoprotein of Rubella Virus.

Qiu, Z., Ou, D., Wu, H., Hobman, T.C., and Gillam, S. 1994. J. Virology 68(6): 4086-4091.

Roy, P., and Jones, I. 1996. Assembly of macromolecular complexes in bacterial and baculovirus expression systems.Curr.Opin.Struct.Biol. 6: 157-161.

Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A., and Arnheim, N. 1985. Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 230: 1350-1354.

Sambrook, J., Fritch, E.F. and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sanchez, A., and Frey, T.K. 1991. Vaccinia-Vectored Expression of the Rubella Virus Structural Proteins and Characterization of the E1 and E2 Glycosidic Linkages. Virology 183: 636-646.

Schlesinger S., and Dubensky Jr, T.W. 1999. Alphavirus vectors for gene expression and vaccines. Curr.Opin.Biotechnol. 10: 434-439.

Seppänen, H., Huhtala, M.-L., Vaheri, A., Summers, M.D., and Oker-Blom, C. 1991. Diagnostic potential of baculovirus expressed rubella virus envelope proteins. J.Clin.Microbiol. 29: 1877-1882.

Seto, N.O.L., and Gillam, S. 1994. Expression and Characterization of a Soluble Rubella Virus E1 Envelope Protein. J.Med.Virol. 44: 192-199.

Skoging, U., Vihinen, M., Nilsson, L., and Liljeström, P. 1996. Aromatic interactions define the binding of the alphavirus spike to its nucleocapsid. 4: 519-529.

Soini, E., and Lövgren, T. 1987. Time-Resolved fluorescence of lanthanide probes and applications in biotechnology. CRC Crit.Rev.Anal.Chem. 18(2): 105-154.

Strauss, J.H., and Strauss, E.G. 1994. The alphaviruses: gene expression, replication, and evolution. Microbiol.Rev. 58: 491-562.

Strauss, J.H., and Strauss, E.G., and Kuhn, R.J. 1995. Budding of alphaviruses. TIM 3(9): 346-350.

Summers, M.D., and Smith G.E. 1987. A manual of methods for baculovirus vectors and insect cell culture procedures. Texas Agricultural Experiment Station Bulletin no. 1555.

Suomalainen, M., Garoff, H. and Baron, M.D. 1990. The E2 signal sequence of rubella virus remains part of the capsid protein and confers membrane association in vitro. J. Virol. 64(11): 5500-5509.

Terry, G.M., Ho-Terry, L., Londesborough, P., and Rees, K.R. 1989. A bio-engineered rubella E1 antigen. Arch.Virol. 104: 63-75.

Tessier, D.C., Thomas, D.Y., Khouri, H.E., Laliberte', F., and Vernet, T. 1991. Enhanced secretion from insect cells of a foreign protein fused to the honeybee melittin signal peptide. Gene 98: 177-183.

Toivonen, V., Vainionpää, R., Salmi, A., and Hyypiä, T. 1983. Glycopolypeptides of rubella virus. Arch.Virol. 77: 91-95.

Towbin, H., Staehelin, T., and Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc.Natl.Acad.Sci.USA 76:4350-4354.

Voller, A., and Bidwell, D.E. 1975. A simple method for detecting antibodies to rubella. Br.J.Exp.Path. 56:338-339.

Waxham, M.N. and Wolinsky, J.S. 1985. A model of the structural organization of rubella virions. Rev.Infect.Dis 7: 133-139.

Wang, K-.S., Kuhn, R.J., Strauss, E.G., Ou, S., and Strauss, J.H. 1992. High-affinity laminin receptor is a receptor for sindbis virus in mammalian cells. J.Virol. 66(8): 4992-5001.

Weiss, S.A., Godwin, G.P., Gorfien, S.F., and Whitford, W.G. 1995b. Insect cell-culture in serum-free media in "Methods *in* Molecular Biology, vol 39 Baculovirus Expression Protocols" (Richardson, C.D., Ed.) pp.79-95, Humana Press, Totowa, N.J.

Weiss, S.A., Whitford, W.G., Gorfien, S.F., and Godwin, G.P. 1995. Insect cell-culture techniques in serum-containing media *in* "Methods in Molecular Biology, vol 39 Baculovirus Expression Protocols" (Richardson, C.D., Ed.) pp.65-78, Humana Press, Totowa, N.J.

Wolinsky, J.S. 1990. Rubella. Virology 2nd ed., Ed: B.N. Fields, D.M., Knipe et al., pp.815-838. Raven Press Ltd., N.Y., N.Y.

Wolinsky, J.S., McCarthy, M., Allen-Cannady, O., Moore, W.T., Jin, R., Cao, S.N., Lovett, A., and Simmons, D. 1991. Monoclonal antibody-defined epitope map of expressed rubella virus protein domains. J.Virol. 65(8): 3986-3994.

Yao, J., and Gillam, S. 1999. Mutational analysis, using a full-length rubella virus cDNA clone, of rubella virus E1 transmembrane and cytoplasmic domains required for virus release. J. Virol. 73(6): 4622-4630.

Zrein, M., Joncas, J.H., Pedneault, L., Robillard, L., Dwyer, R.J., and Lacriox, M. 1993 Comparison of a whole-virus enzyme immunoassay (EIA) with a peptide-based EIA for detecting rubella virus immunoglobulin G antibodies following rubella vaccination. J.Clin.Microbiol. 31(6): 1521-1524.

Yang, D.., Hwang, D., Qiu, Z. and Gillam, S. 1998. Effects of Mutation in the Rubella Virus E1 Glycoprotein on E1-E2 Interaction and Membrane Fusion Activity. J.Virol. 72(11): 8747-8755.