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EFFECTS OF DISULFIDE BRIDGES IN GLYCOPROTEIN E1 ON FUSOGENIC ACTIVITY OF RUBELLA VIRUS

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Summary. – Rubella virus (RUBV) infects cells via an acid-triggered membrane fusion process. RUBV virions contain two cysteine-rich glycoproteins, E2 and E1. The latter is believed to be involved in the membrane fusion. Using a recombinant plasmid containing RUBV E1 and E2, 11 of total 20 cysteines present in the ectodomain of wild type E1 were mutated to test their role in the fusion via the formation of disulfide bridges. The recombinant plasmids containing mutated E1 (Cys2-Cys20) or wild type (wt) E1 were expressed in BHK-21 cells. Their fusogenic and hemadsorption activities in addition to a potential of cell surface expression of E1 and E2 were assayed. The results showed that the fusogenic activity was lost in all tested mutants, while the hemadsorption activity and cell surface expression potential were affected differently in individual mutants. Since only the Cys5 and Cys8 mutations led to a reduction of both hemadsorption and cell surface expression, we assume that these mutations prevented the formation of the disulfide bridge, what led to a misfolding of E1 and consequently to a failure of recognition of E1 by E2. In conclusion, the disulfide bridges disrupted in all the tested mutants appear essential for the cell fusion, while only the disulfide bridge C(5)-C(8) seems to be crucial for the transport of E1 and E2 in the cell.

Keywords: Rubella virus; glycoprotein E1; disulfide bridge; cell fusion

Introduction

RUBV is the only member of the genus *Rubivirus*, the family *Togaviridae*. RUBV is an enveloped virus containing a linear positive sense single-stranded RNA. It is known as the pathogen for the illness rubella or German measles, which is characterized by a low-grade fever and skin rashes in children (Frey, 1994). Primary maternal infection with RUBV during the first trimester of pregnancy can result in frequent malformations of fetus known as congenital rubella syndrome (Cooper, 1985).

The virions contain three structural proteins, e.g. capsid protein that complexes with genomic RNA to form the nucleocapsid and two envelope glycoproteins (E2 and E1) embedded in the host-derived envelope (Oker-Blom *et al.*, 1983; Pettersson *et al*., 1985). In the infected cell, E1 is translocated into the endoplasmic reticulum (ER) with the help of a signal peptide present within the carboxyl terminus of E2 (Hobman *et al.*, 1988). E1 interacts with E2 to form a heterodimer before they are transported to the Golgi complex (Baren and Forsell, 1991; Hobman *et al.*, 1993). Transmembrane and cytoplasmic regions of E1 can act as an ER retention signal (Hobman *et al.*, 1997), so the expression of E1 in the absence of E2 will result in its accumulation in post-ER and pre-Golgi compartment (Hobman *et al.*, 1992). When expressed together, E2 and E1 are efficiently transported to and retained in the Golgi complex with small amounts of the glycoproteins presented on the cell surface (Baron *et al.*, 1992; Hobman *et al.*, 1993). This might be caused by a dimerization of E1 and E2 that could mask the ER retention signal in E1 (Hobman *et al.*, 1997).

RUBV can fuse erythrocytes of several different species and induce hemolysis in an acidic environment (Väänänen

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Abbreviations: ER = endoplasmic reticulum; p.t. = post transfection; RUBV = Rubella virus; wt = wild type

and Kääriäinen, 1980). A brief acid treatment causes an irreversible conformational change in the glycoproteins E1 and E2. This change confers the virions the liposome-binding ability and fusion ability with infected cells (Katow and Sugiura, 1988). Both E2 and E1 are type I membrane proteins (Oker-Blom *et al.*, 1983). E1 is the major target antigen of RUBV and is responsible for neutralization and hemagglutination (Trudel *et al.*, 1985; Ho-Terry *et al.*, 1986; Mitchell *et al.*, 1993). E2 possesses strain-specific epitopes and one neutralizing epitope (Dorsett *et al.*, 1985; Green and Dorsett, 1986). The fact that the removal of E2 from the virions does not affect the binding of the particles to the liposomes indicates that E1 may contain a non-cleavable fusion peptide (Katow and Sugiura, 1988). An internal hydrophobic domain of E1 between amino acids 81–109 has been proved to be involved in the process of membrane fusion (Yang *et al.*, 1998). E1 is rich in cysteine residues and contains a number of disulfide bridges (Frey, 1994). All of the 20 cysteine residues in the ectodomain of E1 are involved in the formation of disulfide bridges. Eight of these disulfide bridges are identified: C(1)-C(2), C(3)-C(15), C(6)-C(7), C(9)-C(10), C(11)-C(12), C(13)-C(14), C(17)-C(18), and C(19)-C(20). The remaining two disulfide bridges formed among C(4), C(5), C(8), and C(16) have not been identified yet (Gros *et al.*, 1997).

To investigate the effects of disulfide bridges in E1 on the fusogenic activity of rubella virus, we carried out site-directed mutagenesis to substitute some of the cysteine residues with other amino acids. The plasmids with mutant E1 including wt E1 were expressed in BHK-21 cells and syncytium formation was observed. Hemadsorption was used to detect the receptor recognition activity of these mutants. FACS and Western blot were applied to evaluate the expression of these mutants on cell surface and within the whole cells.

Materials and Methods

Viruses and cells. Recombinant vaccinia virus vTF7-3 was kindly provided by Dr. Bernard Moss. RUBV strain JR23 was isolated during the epidemic in Jinan and stored at -80°C. BHK-21 cells were obtained from ATCC and maintained in growth medium DMEM (Gibco) supplemented with 10% newborn calf serum (Hyclone), 2 mmol/l glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Plasmid construct. Recombinant plasmid pBSK-SPE2E1 was constructed by subcloning the genes encoding E2 and E1 of RUBV JR23 strain into the vector pBluescript (SK+) (pBSK) between the *Eco*RI and *Xba*I sites downstream of the T7 promoter.

Site-directed mutagenesis. Site-directed mutagenesis was used to substitute some of the cysteines in RUBV E1 with other amino acids individually in the recombinant plasmid pBSK-SPE2E1. PCR was performed to construct the mutants as described before (Dieffenbach and Dveksler, 1995). The desired mutations for cysteines were contained in the oligonucleotide primers. At the same time appropriate restriction enzyme sites were either added to the primers or removed from the primers by introducing silent mutations. These mutants were firstly identified using restriction enzyme analysis. Then all positive recombinant plasmids were further confirmed by DNA sequencing to ensure that the rest of the plasmids remained unchanged. The mutants were named as Cys2, Cys3, Cys4, Cys5, Cys6, Cys8, Cys9, Cys12, Cys13, Cys17, and Cys20 according to the sequence of the mutated cysteines in E1, respectively.

Transfection. Recombinant plasmids were purified from transformed TG1 using EZNA Plasmid Miniprep Kit (Omega Biotek). BHK-21 cells were grown in 24- or 12-well plates to 90–95% confluence and transfection was done using Lipofectamine™ 2000 (Invitrogen) following the instruction of the manufacturer.

Transient expression. The vaccinia-T7 RNA polymerase expression system (Fuerst et al., 1986) was used to express the recombinant plasmids in BHK-21 cells. The cells were infected with recombinant Vaccinia virus vTF7-3 for 1 hr at 37°C before transfection. An equal volume of medium and 10% newborn calf serum were added to the medium to maintain the cell growth 5 hrs post transfection (p.t.).

Cell fusion assay. BHK-21 cells were grown on 24-well plates and transfection was performed with 1 μg of recombinant plasmids. BHK-21 cells were incubated in fusion medium (FM) consisting of DMEM supplemented with 10 mmol/l MES (2-[N-morpholino]-ethanesulfonic acid) 24 hrs p.t. The acid treatment lasted for 20 mins at 37°C with FM adjusted to pH 5.0. After the incubation in FM, transfected cells were maintained in growth medium for the additional 5 hrs to allow the proteins to change the morphology of the cells. Then cells were stained with Giemsa and observed under an inverted microscope. Multinucleated cells containing more than 3 nuclei were considered as fused cells.

Hemadsorption assay. The hemadsorption activity of the recombinant plasmids were measured by the ability of the transfected cells to absorb pigeon erythrocytes. At 24 hrs p.t., the monolayers of BHK-21 cells were washed once with PBS and then incubated with pigeon erythrocytes. After washing of unabsorbed erythrocytes, the cells were processed as described (Wang *et al.*, 2004). To quantify the hemadsorption, the absorbed erythrocytes were lysed with 50 mmol/l NH4Cl at 4°C. After sedimentation of cell debris, A540 was determined in the supernatant. To eliminate the interference of the background, the vector pBSK was used as a negative control.

FACS analysis was used to evaluate the transfection efficiency and to assess the amount of expressed E1 and E2 proteins on the cell surface. At 24 hrs p.t., the transfected cells as monolayers were washed once with PBSA (PBS supplemented with 5% of newborn calf serum) and then incubated for 1 hr with goat anti-RUBV polyclonal antibodies (ViroStat) diluted 1:200 at room temperature followed by FITC-conjugated rabbit anti-goat IgG (Southern Biotech) diluted 1:500. After incubation, an extensive washing was necessary to remove nonspecific staining. The monolayers were then digested and the cells were washed for assay. Cells transfected with the vector pBSK were used as a negative control.

Western blot analysis. BHK-21 cells grown in 12-well plates were transfected with wt plasmid and mutants and 24 hrs later lysed with 100 μl lysis buffer. Total protein extracts were clari-

fied by centrifugation and PAGE with blotting were performed. Nitrocellulose membranes were blocked with 5% skim milk and incubated with mouse anti-E1 monoclonal antibody (Milipore) diluted 1:2,000 at 4°C overnight and then with HRP-conjugated rabbit anti-mouse IgG diluted 1:5,000 at 37°C for 2 hrs. The wt and mutated proteins were detected by DAB (3, 3'-diaminobenzidine) staining. Cells transfected with the vector pBSK were used as a negative control.

Results

Construction of mutants

In order to study the effects of disulfide bridges on membrane fusion, 11 cysteine residues in the ectodomain of E1 were individually mutated by site-directed mutagenesis. The designations of the mutants and the substitute amino acid for each mutated cysteine were shown in Table 1. The construction of all mutants were confirmed first by restriction analysis and then by DNA sequencing. The substitute amino acids were chosen in order to meet the requirements of changing a restriction site in the mutated primers.

Expression of E1 and E2 on the cell surface

Since the fusion of cells was dependent on the glycoprotein expression on the cell surface, FACS was performed to examine the expression of E1 and E2 on the cell surface. The immunofluorescence was used to evaluate the expression of E1 and E2 of the wt plasmid pBSK-SPE2E1 and the mutant plasmids Cys 2-20. In comparison with the expression of wt plasmid, mutants Cys5 and Cys8 showed dramatically lower expression efficiency. The mutants Cys2, Cys6, Cys9, Cys12, Cys17, and Cys20 had a lower efficiency ranging from 21.4 to 74.8%, while mutants Cys3, Cys4, and Cys13 showed 121, 107 and 114%, respectively, cell surface expression efficiency, which was even higher than control wt plasmid (Fig. 1).

Table 1. Cysteine mutations

Mutant	Cysteine substitute
Cys2	Trp
Cys3	Ser
Cys4	Arg
Cys5	Arg
Cys6	Ser
Cys8	Ser
Cys9	Arg
Cys12	Ser
Cys13	Ser
Cys17	Trp
Cys20	Phe

Western blot analysis

The results of FACS revealed different amounts of E1 and E2 proteins on the cell surface of cells transfected with wt plasmid and mutants. Thus, Western blot analysis was

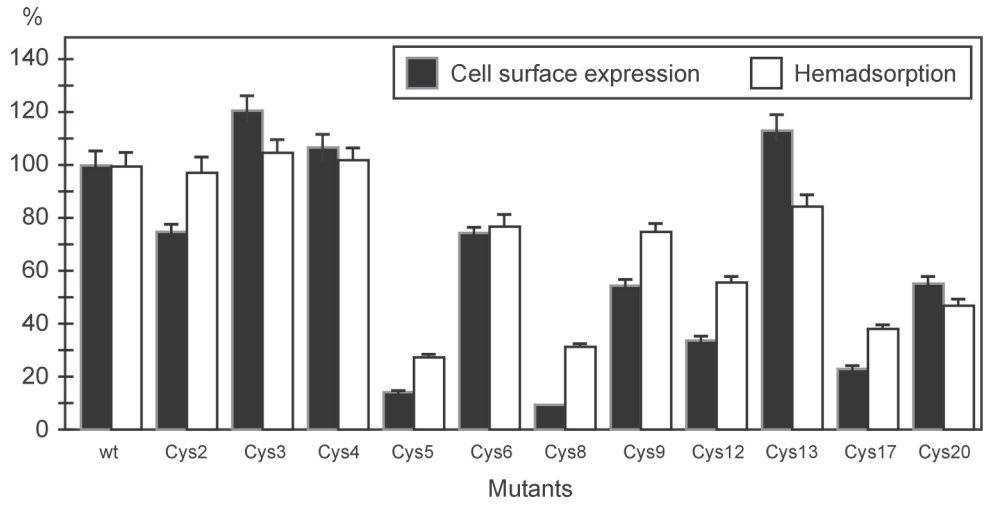

Fig. 1

Cell surface expression of E1, E2 and hemadsorption activity of mutant-transfected BHK-21 cells

The results are expressed as % of wt controls.

Fig. 2

Western blot analysis of E1 expression in mutant-transfected BHK-21 cells

MW markers on the left. Plasmids wt and pBSK served as positive and negative controls, respectively.

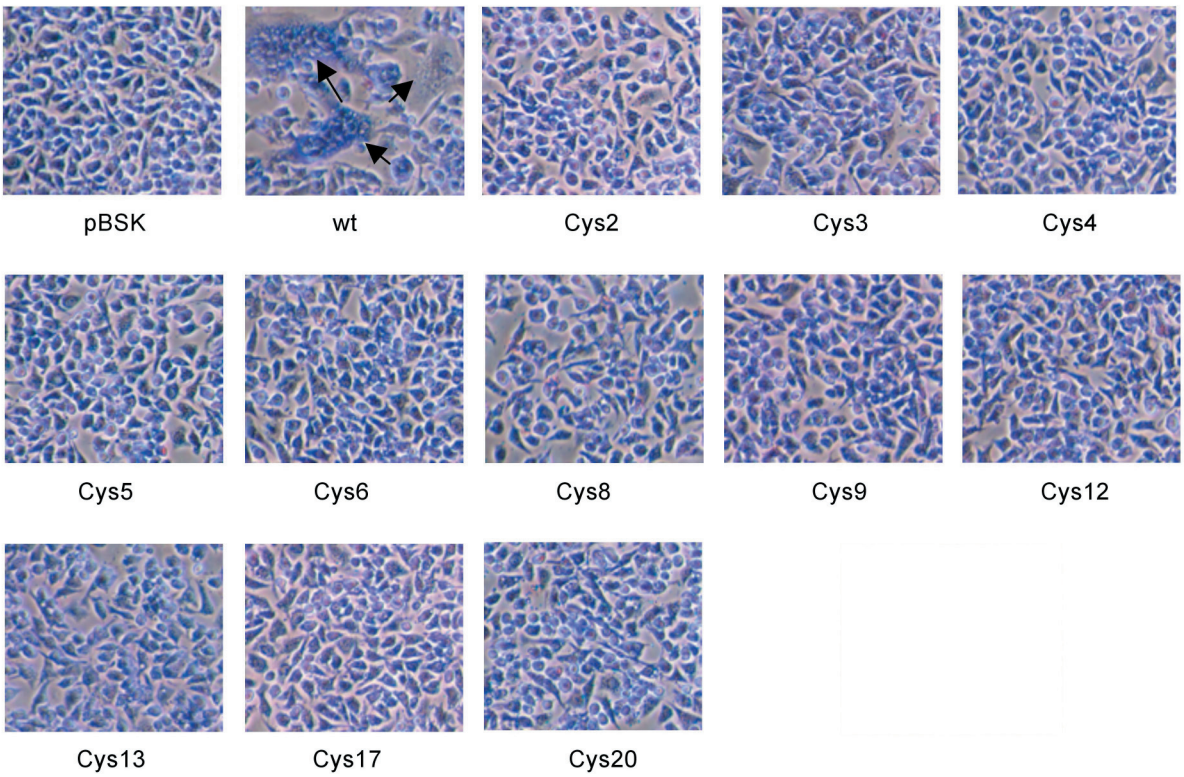

Fig. 3

Syncytia formation in mutant-transfected BHK-21 cells

Staining by Giemsa. Arrows indicate syncytia. Plasmids wt and pBSK served as positive and negative controls, respectively.

carried out to evaluate the total expression of E1 and E2 in the transfected cells. After DAB staining, the specific band about 58 K was found in cell extracts of all mutants including wt plasmid. Visually, the protein content in the bands seemed to be similar in all mutants compared to wt plasmid (Fig. 2). The results of Western blotting indicated that all mutants were successfully expressed in transfected cells in comparable amounts. The differences of cell surface expression of E1 and E2 assayed by FACS were probably due to the changed transport of E1 and E2 in the transfected cells.

Fusogenic activity of mutants

After acidic treatment and Giemsa staining, we observed the formation of the syncytia in the BHK-21 cells under an inverted microscope. The wt plasmid pBSK-SPE2E1 caused extensive formation of syncytia. On the other hand, no syncytia were detected in BHK-21 cells transfected with any of the mutated plasmids Cys 2-20 (Fig. 3). Obtained results indicated that the examined cysteines were indispensable for correct E1 conformation, proper E1-E2 interaction, and resulting cell fusion process.

Hemadsorption activity of mutants

RUBV E1 is the major target antigen functioning also as a hemagglutinin (Pettersson *et al.*, 1985). Therefore, the hemadsorption was performed to determine the receptor recognition activity of the mutant-transfected BHK-21 cells using pigeon erythrocytes. To evaluate this activity, A540 was measured to detect the amount of hemoglobin present in the erythrocytes absorbed to mutant-transfected BHK-21 cells. Mutants Cys 2, Cys3, and Cys 4 showed an enhanced or the same binding ability as the wt plasmid, while mutants Cys5, Cys8, and Cys17 exhibited an substantially decreased ability. Only insignificant drop in the binding ability was observed with remaining mutants. The results of hemadsorption were consistent with that of the cell surface expression efficiency in nearly all mutants (Fig. 1).

Discussion

Studies about individual or co-expression of E2 and E1 indicate that E2 plays an important role in the transport of E1 from ER to the Golgi apparatus and cell surface (Hobman *et al*., 1992; Ojala *et al.*, 2004). To facilitate the expression of E1 on the cell surface, the recombinant plasmid (pBSK-SPE2E1) encoding the genes of E2 and E1 of RUBV JR23 strain has been constructed in our laboratory.

We found that the fusion activity of E1 was completely blocked in all mutants, hence all the disulfide bridges in the ectodomain of E1 were indispensable in the process of cell fusion mediated by the glycoprotein. The loss of the fusogenic activity of E1 might happen due to three reasons: (i) the removal of certain disulfide bridges caused a conformational change of E1 that led to the misfolding of E1. Consequently, the interaction with E2 was incorrect and the expression efficiency of E1 on the cell surface was reduced; (ii) disruption of certain disulfide bridges resulted in the misfolding of E1, what could mask its fusogenic domain; (iii) the disulfide bridges might be located in the putative fusion peptides that affected the fusogenic activity of E1 directly.

The two disulfide bridges that could be formed among C (4), C (5), C (8), and C (16) have not been identified by Gros *et al.* (1997). An interesting observation in our study was that mutants Cys5 and Cys8 had similarly lower cell surface expression efficiency, while the mutant Cys4 showed increased expression efficiency on cell surface than wt plasmid. So, we speculated that two disulfide bridges remaining to be identified might be C (5)-C (8) and C (4)-C (16).

Mutants Cys5 and Cys8 were poorly expressed on the cell surface. Western blot analysis did not detect obvious reduction in the total protein production of any mutants, so the absence of cell surface expression of E1 and E2 assayed by FACS was probably due to the defective transport of the proteins in the transfected cells. We speculated that the disulfide bridge C (5)-C (8) played an important role in the interaction of E1 and E2. The mutation in Cys5 and Cys8 might lead to a conformational change in E1, which could result in the failure of recognition by E2.

Mutants Cys3, Cys4, and Cys13 showed higher cell surface expression than the wt plasmid. Their hemadsorption activities did not demonstrate any significant difference from that of wt E1. So, these mutations didn't affect the transport of the glycoproteins in the cells in these mutants. These mutations probably resulted in the misfolding of E1, which masked its fusogenic region but not the antigenic epitopes. However, there was also a possibility that these disulfide bridges were located directly in the fusion region of E1.

The cell surface expression of Cys2, Cys6, Cys9, Cys12, Cys17, and Cys20 mutants was between 21.4% and 74.8% of wt plasmid. These cysteines also played a critical role in the cell fusion process. However, the proper mechanism remained to be demonstrated.

Our studies showed that all the disulfide bridges in RUBV glycoprotein E1 played an important role in the cell fusion process. Our results provide more detailed information about the glycoprotein E1 membrane fusion activity what can facilitate further research in this field.

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References

Baron MD, Ebel T, Suomalainen M (1992): Intracellular transport of rubella virus structural proteins expressed from cloned cDNA. J. Gen. Virol. 73 (Pt 5), 1073–1086. doi:10.1099/0022-1317-73-5-1073

Baren MD, Forsell K (1991): Oligomerization of the structural proteins of rubella virus. Virology 185, 811–819. doi:10.1016/0042-6822(91)90552-M

Cooper LZ (1985): The history and medical consequence of rubella. Rev. Infect. Dis. 7, S2–10.

Dieffenbach CW, Dveksler GS (1995): PCR Primer: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp. 421–425.

Dorsett PH, Miller DC, Green KY, Byrd FI (1985): Structure and function of the rubella virus proteins. Rev. Infect. Dis. 7, S150–156.

Frey TK (1994): Molecular biology of rubella virus. Adv. Virus Res. 44, 69–160. doi:10.1016/S0065-3527(08)60328-0

Fuerst TR, Niles EG, Studier FW, Moss B (1986): Eukaryotic transient expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. Proc. Natl. Acad. Sci. USA 83, 8122–8126. doi:10.1073/pnas.83.21.8122

Green KY, Dorsett PH (1986): Rubella virus antigens: localization of epitopes involved in hemagglutination and neutralization by using monoclonal antibodies. J. Virol. 57, 893–898.

Gros C, Linder M, Wengler G, Wengler G (1997): Analyses of disulfides present in the rubella virus E1 glycoprotein. Virology 230, 179–186. doi:10.1006/viro.1997.8462

Hobman TC, Lemon HF, Jewell K (1997): Characterization of an endoplasmic reticulum retention signal in the rubella virus E1 glycoprotein. J. Virol. 71, 7670–7680.

Hobman TC, Shukin R, Gillam S (1988): Translocation of rubella virus glycoprotein E1 into the endoplasmic reticulum. J. Virol. 62, 4259–4264.

Hobman TC, Woodward L, Farquhar MG (1992): The rubella virus E1 glycoprotein is arrested in a novel post-ER, pre-Golgi compartment. J. Cell Biol. 118, 795–811. doi:10.1083/jcb.118.4.795

Hobman TC, Woodward L, Farquhar MG (1993): The rubella virus E2 and E1 spike glycoproteins are targeted to the Golgi complex. J. Cell Biol. 121, 269–281. doi:10.1083/jcb.121.2.269

Ho-Terry L, Terry GM, Cohen A, Londesborough P (1986): Immunological characterisation of the rubella E 1 glycoprotein. Brief report. Arch. Virol. 90, 145–152. doi:10.1007/BF01314152

Katow S, Sugiura A (1988): Low pH-induced conformational change of rubella virus envelope glycoproteins. J. Gen. Virol. 69, 2797–2807. doi:10.1099/0022-1317-69-11-2797

Mitchell LA, Décarie D, Tingle AJ, Zrein M, Lacroix M (1993): Identification of immunoreactive regions of rubella virus E1 and E2 envelope proteins by using synthetic peptides. Virus Res. 29, 33–57. doi:10.1016/0168-1702(93)90124-6

Ojala K, Tikka PJ, Kautto L, Käpylä P, Marjomäki V, Oker-Blom C (2004): Expression and trafficking of fluorescent viral membrane proteins in baculovirus-transduced BHK cells. J. Biotechnol. 114, 165–175. doi:10.1016/j.jbiotec.2004.06.015

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

Pettersson RF, Oker-Blom C, Kalkkinen N, Kallio A, Ulmanen I, Kääriäinen L, Partanen P, Vaheri A (1985): Molecular and antigenic characteristics and synthesis of rubella virus structural proteins. Rev. Infect. Dis. 7, S140–149.

Trudel M, Nadon F, Séguin C, Amarouch A, Payment P, Gillam S (1985): E1 glycoprotein of rubella virus carries an epitope that binds a neutralizing antibody. J. Virol. Methods 12, 243–250. doi:10.1016/0166-0934(85)90135-1

Väänänen P, Kääriäinen L (1980): Fusion and haemolysis of erythrocytes caused by three togaviruses: Semliki Forest, Sindbis and rubella. J. Gen. Virol. 46, 467–475. doi:10.1099/0022-1317-46-2-467

Yang D, Hwang D, Qiu Z, Gillam S (1998): Effects of mutations in the rubella virus E1 glycoprotein on E1-E2 interaction and membrane fusion activity. J. Virol. 72, 8747–8755.

Wang Z, Mirza AM, Li J, Mahon PJ, Iorio RM (2004): An oligosaccharide at the C-terminus of the F-specific domain in the stalk of the human parainfluenza virus 3 hemagglutinin–neuraminidase modulates fusion. Virus Res. 99, 177–185. doi:10.1016/j.virusres.2003.11.010