Biochemical mapping of the measles virus H and F envelope glycoprotein protein-protein interface

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The Paramyxoviridae family includes several viruses that are important to human health, including measles virus. The envelope glycoproteins are essential for attachment and entry of the virus into the host cell [1, 2]. To develop novel therapeutics against the virus, detailed knowledge of envelope glycoprotein-protein interaction is important. The goal of this study is to characterize the MeV entry machinery on a molecular level. Interaction of haemaglutinin (H) and fusion (F) protein in pre-fusion form can be biochemically detected with DTSSP. To map the interaction site of H and F protein in pre-fusion form, we have mutated lysine (K) to arginine (R) in the F protein, and examined surface expression. The mutated F was still expressed on the surface and amount of surface expression correlated with fusion activity. The altered F proteins produced in this study will be used to further characterize the H-F interaction.

INDEX WORDS: - Measles, Glycoproteins, haemaglutinin (H) and fusion (F) protein, DTSSP.
BIOCHEMICAL MAPPING OF THE MEASLES VIRUS H AND F ENVELOPE
GLYCOPROTEIN PROTEIN-PROTEIN INTERFACE

by

NEHA ARUN PANCHBHAI.

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of
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BIOCHEMICAL MAPPING OF THE MEASLES VIRUS H AND F ENVELOPE

GLYCOPROTEIN PROTEIN-PROTEIN INTERFACE

by

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May 2014
DEDICATION

This thesis is dedicated to my Mom, Dad, Brother Swapnil and my husband Parag for their endless love, support and encouragement.
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LIST OF ABBREVIATIONS

F protein- Fusion protein
H protein – Haemaglutinin
MeV – Measles virus
MMRV – Measles Mumps Rubella Vaccine
N protein – Nucleocapsid protein
L protein – Large protein
M protein – Matrix protein
PIV5 – Para Influenza Virus 5
HR – Heptad Repeat
DTSSP- 3,3´-Dithiobis(sulfosuccimidylpropionate)
F – Lysine
K – Arginine
hSLAM – human Signaling Lymphocytic Activation Molecule
coIP – co Immune Precipitation
LB medium – Luria-Bertani medium
AMP- Ampicillin
PCR – Polymerase chain reaction
1. INTRODUCTION

The measles is one of the most contagious human disease known to man. Its $R_0$ value is 10-12. [3] After the vaccine for measles is developed (1963) the outbreaks of measles have been reduced significantly and so as the death rate due to measles. As this is completely human disease and the virus have no animal reservoir it is possible to irradiate the disease from the surface of the earth. But due to good vaccination programs less frequent outbreaks of the disease are seen recently and therefore people are forgetting about the impact of the disease on human society. Because of this reason now a-days people are refusing to get vaccinated against the measles. As a result of this now we can see more frequent outbreaks of measles. To avoid complications from the measles and also to avoid big measles outbreak once again, novel therapeutics have to be discover. To develop anti measles inhibitory drug, detailed knowledge of entry mechanism of the measles virus is important.

1.1. Epidemiology of Measles

The disease Measles is caused by the Measles Virus. It is an acute type of highly infectious disease found in humans but not in other animals.[2] In 10th century Persian physician Rhazes described measles as “more dreaded than smallpox.” [2, 4] Figure 1 shows the worldwide measles occurrence in 2013.

‘The basic reproductive number $R_0$ is the average number of individuals directly infected by one infectious case (secondary cases) during the entire infectious period, when the infectious agent has entered a totally susceptible population’ [5]. $R_0$ for the measles is 11-18 [6].
Figure 1 Worldwide map showing cases of measles outbreak in 2013 [7].

1.2. Signs and symptoms of Measles

The measles infection starts at epithelial lining of nose and the lungs. [2] Within 3-4 days it spreads in lymph nodes and rash starts to appear on 10-12th day. [2] The viremia starts to appear on day 3 and spread the infection in retinal tissues. [2, 4] on second viremia stage (day 5-7) the infection spread across the other parts of the body.

Measles virus takes 10-12 days of incubation period from initial infection to prodrome [4], After the 10-12 days of incubation, symptoms of measles starts to appear with high temperature (about 104°C), usually coupled with runny nose, cough and conjunctivitis. [4] Koplik spots starts to appear inside the mouth and chicks. After 1-2 days red rash starts to appear starting from hair lining of the face towards hands and legs which lasts for 5-6 days.[2, 4] After 5-6 days rash starts to fade up starting from face towards the extremities.
Complications from measles can occur in young children under the age of 5, in adult over 20 years old, unborn child if the mother gets measles during pregnancy, immunity compromised people, etc.[2, 4] Blindness encephalitis, diarrhea, pneumonia, ear infection, miscarriage are the few examples of complications. [2, 4] According to the WHO report measles has 1/1000 of the death rate. The risk increases in malnourished children and with poor healthcare facilities.[2]

1.3. Treatments available for Measles

There is no specific treatment for measles.[2] To maintain the body fluid that is lost during diarrhea or other complications from measles can be prevented by proper fluid intake. [2] There are antibiotics to prevent from eye, ear infection or bacterial infection. Vitamin A supplements can prevent eye damage from measles. [2] It has also shown that Vitamin A supplements can decrease the death rate from measles by 50%. [2]

Measles can be prevented from measles’s vaccine. In 1963 first 2 types of measles vaccines were licensed in U.S. First is inactivated vaccine and the other is live attenuated vaccine. [4] The inactivated vaccine was not very successful in preventing measles so it was removed from the market in 1967.[4] The live attenuated Edmonston B vaccine was also giving complications after people getting vaccinated. So in 1968 more attenuated vaccine using Edmonston-Enders strains of measles virus was introduced.[4] Now In the U.S. live more attenuated measles vaccine called as “Morten” is available. It is given in combination with mumps and rubella vaccines and together known as MMRV. This vaccine gives lifelong immunity from measles.[4] The chick embryo’s fibroblast tissues are used to culture measles vaccine.[4]
1.4. Measles vaccine efficacy

Measles vaccine should be given after the first birthday. Vaccinating before the age of 12 months does not create immunity against measles.[4] Two doses of the vaccine separated by 4-6 weeks are necessary and highly recommended to create full immunity against measles. [4]

Failure of the vaccine can be seen in immunologically compromised persons or at old age when immunity starts to decline. The vaccine given before the age of 12 months fail to create full immunity against measles because at that age the child has passive immunity from his mother. Only one dose of the vaccine does not create sufficient antibody tiers in the body, second booster is necessary.

1.5. Measles Virus

Measles virus belongs to the genus Morbillivirus in the family Paramyxoviridae. It is an enveloped virus with a helical shaped capsid. Its genome is a negative sense single-stranded RNA. The envelope contains two glycoproteins, the attachment protein, haemaglutinin (H), and the fusion protein (F).[1] H and F are responsible for attachment of the virus to the host cell and membrane fusion. The matrix protein (M) either surrounds the nucleocapsid or coats the interior membrane. Genome is encapsidated by nucleocapsid proteins (N) and associated with the large (L) and phospho (P) protein [1]

The virus cannot survive in the air for more than 2 hr. It is highly sensitive towards heat, acidic pH, light, ether, Trypsin etc. [2]
Figure 2: Structure of Measles Virus showing different proteins and viral RNA [4]
1.6 Fusion protein of Measles

MeV F has homology model based on the crystal structure of PIV5 F protein (citation). It is a class I membrane fusion protein. It is formed from 3 monomers linked together with hydrophobic interactions to form a homotrimer. It has 2 major domains F1 and F2 linked together with disulfide linkage. F1 is embedded in viral membrane forming transmembrane domain and F2 forms extracellular domain. [8-10] The homology model shows that it has a globular head attached to membrane through helical stalk. The stalk is made from membrane-proximal heptad repeat (HR)-B linker domain. [11] In its pre-fusion form it coexists and is attached with H on the viral membrane.[8] After H binds with the cell receptor it triggers conformational changes in F resulting F in to the post-fusion form.
Figure 3: Structure of Fusion protein MeV. A homology model of MeV F based on the crystal structure of PIV5 F protein. Model is build using Pymol algorithm.
1.7. Folding of F from pre-fusion to post-fusion form during the fusion event

As a classical example of class I membrane fusion protein, in pre-fusion form MeV F exist as a homotrimer. But in the post-fusion form it forms 6 helix bundle [12]. In order to facilitate the membrane fusion, the pre-fusion form of the F has to undergo large conformational changes [12]. When H attaches to the receptor protein it creates conformational changes in the F. The HRA region (different regions of the F shown in Figure 3) extends towards the cell membrane exposing the fusion peptide which is hydrophobic in nature and tucked into the head domain of the F in the pre-fusion form which then get inserted in the hydrophobic region of the host cell membrane attaching the fusion protein to the host cell membrane at N-terminus domain and to the viral cell membrane at the C-terminal domain resulting into formation of the pre-hairpin structure [12]. Then the HRB region folds backward towards the fusion peptide forming 6 helix bundle and resulting into fusion of the viral membrane to the host cell membrane [12]. This create the fusion pore.

![Diagram of fusion pore formation](image)

Figure 4: Formation of the fusion pore. Structural changes in the pre-fusion - post-fusion form of the MeV F[13].
1.8. Mechanism of action

H and F are responsible for attachment of the virus to the host cell and membrane fusion. In order to fuse, F has to transform from a pre-fusion to a post-fusion form. This transformation is triggered by H, which activates F refolding upon receptor binding. H and F are known to form a complex within the infected cell [14-17]. They travel to the plasma membrane together and are incorporated into viral particles [14, 15, 18, 19]. H and F interaction can be monitored through co-immunoprecipitation [20]. This assay utilizes the chemical cross-linker DTSSP to covalently link the two proteins together. DTSSP will bind with amino groups in lysine residues present on the surface of the H and F oligomers. The aim of this study is to characterize the molecular interphase of the H and F hetero-oligomeric pre-fusion complex.

In order to achieve this goal, we identified lysine residues predicted to be located on the surface of the pre-fusion F trimer using the Pymol algorithm. Utilizing a homology model of MeV F based on the crystal structure of PIV5 F protein, I highlighted the lysine residues that were found on the outer surface of the protein structure; residues 111, 244, 248, 292, 364, 396, 410, 474, and 488. These lysine residues could have the potential to interact with DTSSP and mediate covalent cross-links with H. To test the contribution of individual residues to cross-linking, we mutated the lysine residues to arginine. Arginine was chosen because of its biophysical similarity to lysine. However, absence of a primary amino group in the side chain prevents covalent amino-ester formation with DTSSP. I decided to mutate these lysines to arginines stepwise, hypothesizing that arginine will not affect the biological activity of F or protein folding, but prevent H-F crosslinking biochemically.
Figure 5: Structure of Fusion protein of MeV showing all the mutated lysine molecule sites. A homology model of MeV F based on the crystal structure of PIV5 F protein. Model is build using Pymol algorithm.
2. MATERIALS AND METHODS

2.1 Site directed mutagenesis

A site-directed mutagenesis technique was used to make specific and intentional changes in the DNA sequence of a fusion protein of MeV. This is a linear amplification technique, unlike standard PCR where we get exponential amplification of the product.

Figure 6: General procedure for site directed mutagenesis [21].
To change the lysines (K) in positions 111, 292, 244, 248, 364, 396, 410, 474, 488 to arginine (R) primers were designed. Primer sequences for mutagenesis were:

- F-K111R –F – GCTTCAAGTAGGAGACACAGGAGATTGCAGGAGTAGATCC
- F-K292R-F – GACGCTGTCCGAGATTAGGGGGGTGATTGTCACCCAGGC
- F-K396R –F – GTGCATCAATCTTTGCAGTTACACACACAGGAACG
- F-K244-248R–F-
  GGAGGAGACATCAATAGGGTGTTAGAAAGGCTCGGATACAGTGGAG
- F-K364R-F – CTCCGGGGGTCCACTAGGTCTCTGCTGCTCGTACA
- F-K410R-F
  GATCATTAATCAAGACCCTGACAGGATCCTAACATACATGCT
- F-K474-F- GCTAAGTTGGAGATGCGCAGGAATTGTGCTTGAGTC
- F-K488R-F CAGATATTGAGGAGTATGAGAGTATCGAGCAC

5 µl of the site-directed mutagenesis product was run on a 0.8% agarose gel to check whether the bands are in the desired molecular weight range. We saw a band corresponding to our product. To get rid of the parental DNA strand, we conducted DpnI digestion.

DpnI Digest: We added 1 µl of DpnI (20 U/µl, New England Biolabs) to the reaction and incubated at 37°C for overnight.

2.2. Transformation

To get higher quantities of the engineered plasmids, transformation was done. Competent bacterial strain of E. coli was placed in a reaction tube. 2 µl of engineered plasmids (site-directed mutagenesis product) were added to the bacterial culture and incubated on ice for
30 min. Heat shock was given for 30 sec to the bacteria so that their cell membrane becomes permeable to take up the plasmid. For recovery from heat shock, 200 µl of L.B. plain medium was added in each tube and bacteria were incubated in 37°C incubator for 1 hr. and, bacteria were inoculated on LB agar plates. 30 µl of 1000X Ampicillin/plate was used for positive selection. The plates were incubated in 37°C incubator for overnight.

2.3. Mini prep

Colonies from each plate were selected and grown in 3 ml of LB Amp medium, for overnight in 37°C shaker. To get high-purity plasmid DNA from the bacteria, the Qiagen mini prep kit was used and protocol was followed.

To check whether our amplified plasmid was correct or not, DNA was digested with suitable enzyme and incubated at 37°C overnight using Water + DNA 2 µl + Buffer 2 µl +BSA + 0.5 µl of each enzyme (Total 20 µl). Digested according to the guidelines in the enzyme / buffer book.

Agarose gel electrophoresis was carried out to make sure the DNA was the right size. Concentration of DNA was determined using spectrophotometry. These DNAs were sent to Genewiz Company for sequencing.
Figure 7 General procedure for QIAgen mini-prep DNA purification [17]
2.4. Midi Prep

Left over liquid bacterial culture from Mini prep was use to inoculate 200 ml LB Amp medium. It was then incubated overnight in 37°C shaker. To generate more of the purified DNA, Qiagen midi prep kit was used and the protocol was followed. The concentration of the collected DNA was identified µg/µl with the spectrophotometer.

2.5. Cell lines used.

For these experiments we 150 W Vero cell line. These cells are isolated from African Green Monkey’s epithelium cells of kidney [22]. These are immortalized cells used in tissue culture. These cells stably expresses the plasmid encoding hSLAM= (human) signaling lymphocytic activation molecule also called as CD150, which act as a receptor molecule for measles H protein [22-24]. These cells are developed by Dr. Yusuke Yanagi, Kyushu University, Kukuyoka, Japan [22, 24].

The virus do not infect these cells persistently makes these cells easy to maintain in the laboratory conditions [22-24]. These cells need medium containing geneticin to stably express SLAM. This makes these cells little expensive to maintain [22, 24].

2.6. Transfection

Using the concentration of DNA collected in Midi prep, the volume of DNA (H,F and GFP) to be added in each well of plated cells were calculated. 50 µl/well of serum free DMEM and µl/well of Gene juice was mixed well and incubated at RT for 5 min. Then 50 µl of this mixture was added in each tube of DNA and incubated at RT for 10 min. then each tube was added in respective wells drop-wise. Each well was labeled properly and plates were incubated at 37°C for overnight. After 15-18 hr. the plates were observed under the microscope for fusion activity.
2.7. Quantitative cell-to-cell fusion assay

For quantitative cell-to-cell fusion assay Vero 150 W cells were transfected with 0.5 µg of GFP, 0.5 µg of WT H, 0.5 µg of the F constructs. Fusion activity was detected after 12-15 hr. post transfection. The cells were photographed using 200X magnification fusion and by comparing with WT F, cell-to-cell fusion, fusion activity was calculated for each mutant F.

2.8. Surface biotinylation assay

Vero cells 150W were transfected with 4 µg of F constructs and 0.5 µg of GFP. After incubating transfected cells for 36 hr. at 37°C cell surface proteins were biotinylated with 0.5 mg/mL sulfosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate (Thermo Scientific) for 20 min at 4 °C. 700µl of cleared supernatant were collected and allowed to bind with Streptavidin beads in 4°C for 2 hr. Then samples were washed with Washing buffers I and II, 3 times each. Then the bound proteins were denatured with urea buffer buffer [200 mM Tris (pH 6.8), 8 M urea, 5% (wt/vol) SDS, 0.1 mM EDTA, 0.03% bromophenol blue, 1.5% (wt/vol) DTT] for 30 min at 50 °C. The samples were run on 10% SDS/PAGE and blotted on PVD membranes. The blots were blocked with 5% nonfat milk in 1xPBS, then treated with αHA monoclonal primary antibodies for 2 hr. After thorough washing with 1xPBST 3times, 10 min each, the membranes were treated with anti-mouse IgG light-chain conjugate- secondary antibody for 1 hr. Then the membranes were treated with West-Dura buffers and imaged using ChemiDoc XRS digital imaging system (Bio-Rad).[8]
3. RESULTS

3.1. Single lysine to arginine mutations in F have mixed effects on fusion activity.

Fusion activity of the F mutants harboring single lysine to arginine substitutions protein was assayed in Vero-SLAM cells. Upon co-expression of standard H and F in these cells, extensive syncytia formation takes place. Control cells lacking F showed no fusion activity. Taking these as reference, other cells expressing F mutants (F-K111R, F-K364R, F-K474R, and F-K488R) showed strong fusion activity. However, F-K410R, F-K292R and F-K396R returned reduced fusion activity.
Figure 8 Fusion activity results. Each panel showing 150 W cells transfected with MeV H, GFP and pCG EdmF-c3Xflag with MeV F having single mutation. When these cells express MeV’ functional H & F they tend to fuse together forming giant cell with multiple nuclei in it known as syncytia.
3.2. Double mutants F constructs

The single lysine to arginine mutations were then combined in different patterns and the resulting F double mutants tested for fusion activity. Again, Vero-SLAM cells were transfected with these DNA to check the fusion activity of the mutated F proteins. This experiment revealed that F mutants F-K111-292R and F-K244-248R retained strong fusion activity. However, fusion activity was reduced in cells expressing F-K111-364R and F-K292-396R mutations.

Figure 9 Fusion activity results. Each panel showing 150 W cells transfected with MeV H, GFP and pCG EdmF-c3Xflag with MeV F having double mutation.
3.3. Triple K to R mutants have better fusion activity than some of the double mutant

The F-K111-292R mutant displayed high fusion activity and these two residues are naturally separated from the other mutation sites by single-cutting restriction enzyme site. This enabled the convenient generation of triple mutation constructs through a straight-forward A-B cloning technique. In this technique, we cut the specific piece of backbone harboring the FK111-292R mutations with the SpeI and KpnI enzymes. In parallel, we also cut the plasmids having the additional single mutations with the same enzymes, and then ligated the appropriate DNA fragments after purification.

![Figure 10 Schematic representation of A-B cloning technique.](image)

Vero-SLAM cells were co-transfected with H-encoding plasmid DNA and the resulting ligation products to determine the fusion activity of the mutated F constructs. The experiment revealed that all the cells expressing F mutants with triple mutations formed extensive syncytia, indicating high fusion activity of the F triple mutants.
Figure 11: Fusion activity results. Each panel showing 150 W cells transfected with MeV H, GFP and pCG EdmF-c3Xflag with MeV F having triple mutation.
3.4. Different constructs of MeV F with K-R mutations.

The constructs F-K111R, F-K292R, FK 364R, FK396R, FK410R, FK474R, FK488R, FK244-248R, FK111-291R, FK111-396R, and FK292-396R are made using site directed mutagenesis technique. While the construct FK111-292-364R, FK111-292-396R, FK111-292-410R, FK111-292-474R and FK111-292-488R are made using A-B cloning technique. To amplify the DNA, the constructs were transformed into the competent E. coli bacteria. Then purified using QIAgen mini prep kit. The sequences were conformed from the Genewiz sequencing company. After sequence conformation, to get more DNA, leftover bacterial culture was grown in 200 ml of the LB medium in 37°C shaker overnight and then DNA was purified using QIAgen midi-prep kit. The concentrations got in midi-prep purification are shown in the Table 1. Then the fusion activity was checked by Fusion assay. Results for fusion activity as compared to WT are shown in the table 1.

Table 1 Table showing different constructs of MeV F with K-R mutations in it

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<th>SN</th>
<th>MeV F constructs</th>
<th>PCR</th>
<th>Mini prep</th>
<th>sequence confirmed</th>
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<td>+</td>
<td>1.6</td>
<td>3</td>
</tr>
<tr>
<td>15</td>
<td>FK 111-292-474 R</td>
<td>A-B Cloning</td>
<td>+</td>
<td>+</td>
<td>1.6</td>
<td>4</td>
</tr>
<tr>
<td>16</td>
<td>FK 111-292-488 R</td>
<td>A-B Cloning</td>
<td>+</td>
<td>+</td>
<td>2</td>
<td>4</td>
</tr>
</tbody>
</table>
3.5. Grouping of certain mutations

After looking at the results from the previous fusion assays we observed that mutating lysines to arginine affects the fusion activity of the F molecule. To get descent fusion activity but still change the Figure 10.

Figure 12: Fusion protein of MeV showing Different groups of K-to-L mutations
3.6. Fusion assay of different groups of MeV F constructs having K-to-R mutations and HA tag

To be able to coIP F with H with the help of DTSSP, we replaced c3XFlag tag in EdmF with HA tag with A-B cloning method and also inserted K-R mutations so that we can concentrate arginine instead of lysine at certain positions so that fusion activity is retained. Figure 11 and 12 shows 3 field of views of each well of 12 well plate in which fusion assay for different constructs of F in Vero 150W cells was carried out. From the fusion assay we calculated bio activity of F compared with WT F (denoted in red types)

![Image of fusion assay results]

WT F (+4)  FK111-292-244-248R-HA (Top, +2)  FK111-292-396R-HA (Middle, +3)  F-K477-488R-HA (Stalk, +4)
3.7. Different groups of constructs of MeV F with K-R mutations and HA tag in it.

The HA tag was inserted in place of the c-terminal 3x FLAG in the F construct using A-B cloning technique. Different groups of F construct were created by adding mutations in the f construct with site directed mutagenesis, they are as follows,
To amplify the DNA, the constructs were transformed into the competent E. coli bacteria. Then purified using QIAgen mini prep kit. The sequences were confirmed from the Genewiz sequencing company. After sequence conformation, to get more DNA, leftover bacterial culture was grown in 200 ml of the LB medium in 37°C shaker overnight and then DNA was purified using QIAgen midi-prep kit. The concentrations got in midi-prep purification are shown in the Table 2. Then the fusion activity was checked by Fusion assay. Results for fusion activity as compared to WT are shown in the table 2.

Table 2: Table showing different constructs of MeV F with K-R mutations in it.

<table>
<thead>
<tr>
<th>SN</th>
<th>MeV F constructs</th>
<th>PCR</th>
<th>Mini prep</th>
<th>sequence confirmed</th>
<th>Midi prep conc. ug/ul</th>
<th>Fusion activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>FK 111R-HA</td>
<td>A-B Clonig</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>FK292R-HA</td>
<td>A-B Clonig</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>FK 396 R-HA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>1.3</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>FK 364 R-HA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>FK 474 R-HA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>1.1</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>FK 111-292-244-248 R-HA [Top]</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>1.1</td>
<td>2</td>
</tr>
<tr>
<td>7</td>
<td>FK 111-292-396 R-HA [Middle]</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>1.4</td>
<td>3</td>
</tr>
<tr>
<td>8</td>
<td>FK 111-292-396-364 R-HA [mid-Bottom]</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>1.9</td>
<td>2</td>
</tr>
<tr>
<td>9</td>
<td>FK 410-364 R-HA [Bottom]</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>2.2</td>
<td>4</td>
</tr>
<tr>
<td>10</td>
<td>FK 474-488 R-HA [Stalk]</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>1.3</td>
<td>4</td>
</tr>
<tr>
<td>11</td>
<td>FK111-292-396-410R-HA [Middle +410]</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>1.8</td>
<td>2</td>
</tr>
<tr>
<td>12</td>
<td>FK111-292-244-248-410R-HA [Top+410]</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>1.9</td>
<td>1</td>
</tr>
</tbody>
</table>
3.8. Quantification of surface expression of F

To quantify surface expression of the different F on the cell surface we transfected Vero cells 150 W with 4µg of different F constructs and 0.5µg of GFP and incubated in 37°C incubator for 36 hr. Then surface biotinylation assay was performed as described in the procedure. After the western blotting the blots were treated with West-Dura buffers and imaged using ChemiDoc XRS digital imaging system (Bio-Rad). The intensity of the bands was identified and compared with the WT F band. Then the percentage surface expression was plotted against fusion activity. The graph is shown as bellow. Here we can see that In constructs WT, A, C, D, E and G fusion activity is correlated with percent surface expression but in case of B though percentage surface expression is very high, the fusion activity is significantly reduced. Where as in case of construct F surface expression of the protein is very high.

Where

A- Top
B- Top + 410
C- Middle
D- Mid + Bottom
E- Middle + 410
F- Bottom
G- Stalk
Figure 14: Surface biotinylation assay western blot showing Total lysates and Surface biotinylated samples for different F constructs.
Figure 15 Graph plotted percent surface expression Vs Fusion activity of MeV F
4. DISCUSSION

In this study we constructed various F constructs which have K-R mutations at certain positions. These lysines were on the surface of the F molecule. Here we were hypothesizing that when H and F interact, particular surface lysines in F and others in H would be 11-12 Å from one another, enabling DTSSP to cross-link the two proteins. If we can identify pairs of cross-linkable lysines between the two proteins, we can more precisely map the H-F interface.

In the first experiment we inserted single mutations at a time in pCG EdmF c3xflag plasmid to get F-K111R, F-K292R, F-K364R, F-K396R, F-K410R, F-K474R and F-K488R constructs. When we transfected 150W Vero cells with these constructs we observed fusion activity by monitoring the size of the syncytia. The cells with the constructs F-K111R, F-K364R, F-K474R and F-K488R showed large syncytia. But the cells with the constructs F-K292R, F-K396R and F-K410R showed very small syncytia. Thus from these results we can say that changing the lysine to arginine at certain positions changes the fusion activity of the F.

To check if we can regain the fusion activity by combining the two mutations (K-R) we added second mutation in the construct which already has one mutation in it and created sets of F constructs.

In the second experiment we created the sets of F constructs with two K-r mutations as follows, F-K111-292R, FK111-396R, F-K292-396 and F-K244-248R. Here after infecting 150W Vero sells with these new constructs we observed that construct F-K111-292R and F-K244-248R showed large syncytia which means after adding K111R mutation we regain the fusion activity of K292R construct. But in case of the constructs FK111-396R and F-K292-396 the syncytia are very small.
To get the constructs with maximum K-R mutations but at least 70% bioactive we decided to add third mutation on the construct F-K111-292-364R. So we constructed F-K111-292-244-248R, F-K111-292-396R, F-K111-292-410R, F-K111-292-474R and F-K111-292-488R constructs. After infecting 150W Vero cells with these constructs we observed that cells with constructs F-K111-292-364R, F-K111-292-474R and F-K111-292-488R showed large syncytia but the construct F-K111-292-244-248R, F-K111-292-396R and F-K111-292-410R showed small syncytia. By observing these results we cannot say that combining the mutations which had shown good fusion activity on the construct with lower fusion activity does not improves the fusion activity of the construct which had shown poor fusion activity. Therefore for further experiments we decided to change lysine to arginine at certain positions on the F protein.

In the fourth experiment we created groups of mutations which will change the surface lysine to arginine at certain positions. Therefore we created constructs F-K111-244-248-292R (Top), F-K111-44-248-292-410R (Top+410), F-K111-292-396R (Middle), F-K111-292-396-410R (Middle+410), F-K111-292-364-396R (Mid-Bottom), F-K364-410R (Bottom) and F-K474-488R (Stalk) (figure 10). In these constructs we also replaced flag tag with HA tag because Haemaglutinin (H) also has flag tag and this is identified by the antibodies. If we have flag tag in both the H and F then we will not be able to perform the coIP experiment. Here when we infected 150W Vero cells with these constructs we observed large syncytia in F-K111-292-396R (Middle), F-K364-410R (Bottom) and F-K474-488R (Stalk) but small syncytia in F-K111-244-248-292R (Top), F-K111-44-248-292-410R (Top+410), F-K111-292-396-410R (Middle+410) and F-K111-292-364-396R (Mid-Bottom). That means changing lysines to arginine in the bottom and the stalk area of the F does not affect the fusion activity of
the F but when we change the lysines from the top, middle portion of the head domain of the F then the fusion activity is affected.

There are various reasons for the fusion activity of the F to go down. For instance, after inserting mutation/s the protein may not fold properly, the F may not be cleaved properly into F₁ and F₂ from F in the late Golgi apparatus, due to the change/s the F may not make it up to the cell surface, the F may not interact with H in the pre-fusion form, the H cannot make conformational changes in the F after the receptor binding, the F is already in the post-fusion form, etc. If F is not properly folded or not cut properly then there are high chances that it will not be exposed out on the cell surface. So to check whether the mutated F construct can produce the protein which is expressed on the cell surface or not, in the fifth experiment we infected 150W Vero cells with these F constructs and quantify their surface expression with surface biotinylation assay. In these assay we found out that in constructs F-K111-292-396R (Middle), F-K111-292-396-410R (Middle+410), F-K111-292-364-396R (Mid-Bottom), F-K364-410R (Bottom) and F-K474-488R (Stalk) sizes of the syncytia are comparable to the surface expression of the protein. i.e. in the constructs  F-K111-292-396R (Middle), F-K111-292-396-410R (Middle+410), F-K364-410R (Bottom) and F-K474-488R (Stalk) when surface expression goes up the size of the syncytia is large and when the surface expression goes down the size of the syncytia decreases. But in case of the construct F-K111-44-248-292-410R (Top+410) and F-K111-244-248-292R (Top) though they are expressed on the cell surface very efficiently but the size of the syncytia are very small. There could be various reasons for that, the protein is transported on just fine but either it is already in the post fusion form or it cannot interact with H or H cannot induce conformational change in it after binding with the receptor protein.
5. CONCLUSION

From this study we found out that fusion activity of the F gets affected when lysine residue from certain area are changed into arginine. In most of the cases when surface expression of the F protein is higher the fusion activity is higher and when surface expression of the protein is lower then the fusion activity is also lower. But in some constructs though the surface expression of the protein is high but the fusion activity is still low, experiments to find out the reason/s behind these have to be done.

Now we have different F constructs F-K111-244-248-292R (Top), F-K111-44-248-292-410R (Top+410), F-K111-292-396R (Middle), F-K111-292-396-410R (Middle+410), F-K111-292-364-396R (Mid-Bottom), F-K364-410R (Bottom) and F-K474-488R (Stalk) which expresses on the cell surface and which have lysine to arginine mutations at certain positions we can perform further experiment to map the H-F protein - protein interaction in MeV.

The findings of this study will be useful in further study of mapping of H and F interaction in measles virus and eventually development of novel therapeutics against the disease.
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