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Advances in understanding RSV glycoproteins: Insights into viral entry and implications for vaccines and therapeutic development

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Abstract. Respiratory syncytial virus (RSV) causes a significant number of deaths in newborns annually due to respiratory infections. Since its first isolation in 1955, great progress has been made in the development of related vaccines and monoclonal antibodies. Currently, two RSV vaccines and two monoclonal antibodies have been approved for marketing. Understanding the structure, function, and mechanism of RSV glycoproteins' interaction with their receptors involved in its entry is of great significance for the development of vaccines and therapeutic drugs. In this review, we will introduce the substantial progress made in the understanding of the structure of RSV glycoproteins, the receptors they bind with, and the process of RSV entry. Progress made in developing vaccines and neutralizing antibodies which benefit from these studies will also be briefly discussed. Finally, we will outline the unresolved issues and controversies in RSV glycoprotein and entry mechanism research, propose some possible approaches to address these issues, and provide insights into future drug and vaccine development.

Keywords: RSV, Glycoproteins, Receptor, Vaccines, Antibodies.

1. Introduction

Respiratory Syncytial Virus (RSV) is a negative-sense single-stranded RNA enveloped virus belonging to the subfamily Pneumovirinae in the family Paramyxoviridae. Its genome consists of 10 genes encoding 11 proteins. There are nine structure proteins, namely Fusion protein (F), Attachment protein (G), Small hydrophobic protein (SH), Phosphoprotein (P), M2-1, M2-2, Large polymerase protein (L), Matrix protein (M) and Nucleoprotein (N). Among these, F, G, and SH are located on the surface of RSV. The G protein primarily assists RSV in attaching to the host cell membrane, while the F protein is mainly involved in the virus/host cell membrane fusion. Based on the differences in the surface glycoprotein G, RSV can be classified into two subtypes, A and B.

In order to combat RSV infection, the human immune system increases mucus secretion and triggers inflammatory reactions, leading to the narrowing of the respiratory tract and causing bronchiolitis and pneumonia in infants, elderly individuals, and high-risk populations. RSV is the leading cause of hospitalization in infants and the second leading cause of infant mortality after malaria. After 24 months of birth, almost all children have been infected with RSV at least once, with about half of them

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experiencing two or more infections [1]. Globally, RSV is responsible for approximately 59,650 deaths in children under the age of 5 annually [2]. Due to its high infection and severe illness rates, the development of RSV vaccine has been prioritized by the World Health Organization (WHO) [3].

The immune response generated by the human body after RSV infection gradually wanes over time, leading to lifelong recurrent infections. For a long time, Palivizumab has been the sole antibody. However, Palivizumab is expensive and has low neutralization activity, requiring multiple doses. Therefore, it is mostly used for passive immunoprophylaxis in high-risk infants. In October 2022, a more efficient antibody product, called Nirsevimab (Beyfortus), was licensed by EMA, for immunoprophylaxis in newborns. So far, two subunit RSV vaccines have been approved for marketing, namely Arexvy developed by GSK and Abrysvo developed by Pfizer [4]. Currently, more than 10 antibodies with stronger neutralizing activity and longer half-life and over 30 vaccines are undergoing clinical trials.

The G and F proteins are the main protective antigens of RSV. Due to the high conservation of the F protein in both A and B subtypes, it is the primary target for current vaccines and neutralizing antibodies. Therefore, studying the RSV membrane fusion process and the proteins involved in fusion is crucial. This review will introduce the structures of the G and F proteins, their receptors, the process of RSV entry, and briefly discuss the current development of its antibodies and vaccines.

2. F protein

The precursor of the F protein is a peptide called F0, which consists of 574 amino acids. It contains 5~6 N-linked glycosylation sites (differences may exist among subtypes) [5], as well as two multibasic furin cleavage sites, known as FCS-1 and FCS-2. After cleavage, the F1 and F2 subunits are formed. The F1 subunit is composed of several components: the fusion peptide (FP) that inserts into the neighboring cell membrane during infection, a heptad repeat region A (HRA) and region B (HRB) which form a stable six α-helix structure during fusion, functional domains I and II, a transmembrane domain (TM), which is the portion of the F protein that inserts into the viral membrane, and a cytoplasmic domain (CP) that enters the cytoplasm. On the other hand, the F2 subunit primarily consists of the heptad repeat region C (HRC) (Figure 1).

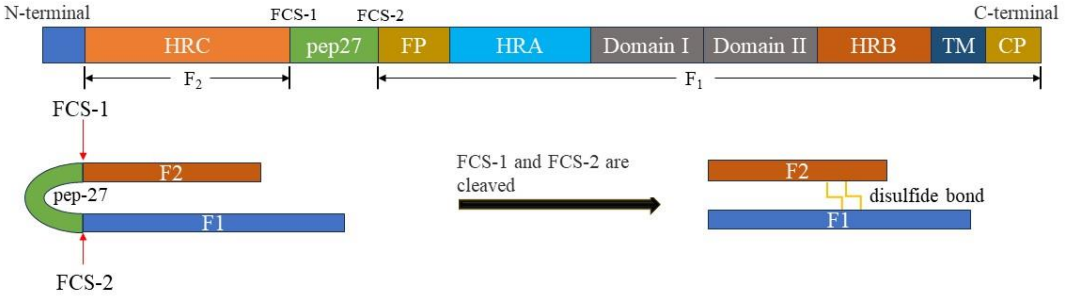

Figure 1. The structure and maturation process of F protein.

After being cleaved by furin-like proteases, F1 and F2 covalently associate through disulfide bonds to form a heterodimer (Figure 1). Subsequently, three heterodimers trimerize to adopt a pre-fusion conformation. Although experimental evidence suggests that F protein can only trimerize after cleavage [6], the exact sequence of cleavage and trimerization has not been definitively confirmed [7]. The pre-fusion conformation of the F protein exhibits a "cone" shape [8] and is unstable. In vitro experiments have shown that higher temperatures [9] and lower osmotic pressure [10] can induce conformational changes in the pre-fusion conformation to form the post-fusion conformation. However, the precise physiological triggers for these conformational changes are still unknown.

During the entry process, the F protein can interact with host cell via the nucleolin (NCL) [11], the insulin-like growth factor receptor-1 (IGF1R) [12], the epidermal growth factor receptor (EGFR) [13],

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the intercellular adhesion molecule-1 (ICAM-1) [14], and the Toll-like receptor 4 (TLR4) [15]. Among these, NCL and IGF1R only assist in virus internalization, while EGFR, ICAM-1 and TLR4 not only facilitate virus entry but also trigger the host's immune response.

NCL contains several RNA-binding domains (RBDs) [16]. It is sparsely distributed on the cell membrane but is mainly found in the nucleolus, nucleoplasm, and cytoplasm. The NCL on the cell membrane has a transport function and serves as a bridge between the cell nucleus and the cell membrane [17]. NCL plays a role in the adhesion and entry processes of various viruses, such as EV71, HIV, H1N1, H3N2, H5N1, H7N9, and PIV3 [18]. In the case of RSV, the RBD1 and RBD2 of NCL act as receptors [18] and mediate RSV internalization through binding with the F protein. This is consistent with the transport properties of NCL on the cell membrane.

Due to the limited presence of NCL on the cell surface, RSV infection efficiency is low if solely relying on NCL located on the membrane. This requires a mechanism to increase the amount of NCL on the cell surface. IGF1R is an α2β2 heterotetramer composed of an extracellular α-subunit and a transmembrane β-subunit and the intracellular portion contains a Tyr kinase domain [19]. Binding of the pre-fusion RSV-F protein to IGF1R activates protein kinase Cζ (PKCζ), promoting the translocation of NCL from the cell nucleus to the cell membrane [12]. The presence of IGF1R increases the amount of NCL on the cell membrane, thereby enhancing viral infection efficiency.

In addition to binding to F protein, some receptors also play a role in promoting virus uptake, triggering host immune responses and inflammation. The interaction between EGFR and the F protein activates downstream effectors such as Cdc42 and PAK1, initiating macropinocytosis for virus uptake [20]. Additionally, EGFR increases the expression of mucins, leading to excessive mucus production in the respiratory tract of patients [13]. Interestingly, the strength of the interaction between EGFR and the F protein varies depending on the RSV strain, the F protein of strains such as 2-20, Line-19, and 2-20-F-N124K are shown to have stronger affinity with the F protein [13]. Binding of ICAM-1 to the F protein significantly upregulates its expression and increases the secretion of chemokine RANTES and endothelin-1 [21], thereby facilitating the adhesion of neutrophils and eosinophils and leading to respiratory inflammation, damage, and obstruction [22]. Interaction between the F protein and TLR4 stimulates the host's innate immune response, inducing pro-inflammatory cytokines, and this induction response depends on the expression of CD14 and TLR4. Compared to normal mice, RSV persists longer in the lungs of TLR4-deficient mice during infection [15]. In vitro, RSV upregulates TLR4 expression in airway epithelial cells [23], and the extent of TLR4 upregulation is closely associated with disease severity. During the acute phase of RSV bronchiolitis, the degree of upregulation in TLR4 expression in individual monocytes is inversely correlated with subsequent infant hypoxia [24].

3. G protein

The G protein of RSV is a single-pass type II integral membrane protein. It has a molecular weight ranging from 84,000 to 90,000 and consists of approximately 300 amino acids. The entire G protein is highly glycosylated, with over 50% of its molecular weight contributed by carbohydrates. Most of the carbohydrates are linked to the G protein through O-glycosylation.

The N-terminus of the G protein is located inside the viral envelope and contains a single cysteine residue. The transmembrane region, Met48, is palmitoylated after translation. The extracellular domain consists of two mucin-like variable domains, with approximately 30-35% of the amino acid residues being Ser/Thr and 8-10% being Pro. These two mucin-like variable domains are characteristic features that distinguish between the A and B subtypes of RSV. Between the two mucin-like domains, there is a central conserved domain and a heparin-binding domain. The central conserved domain consists of 26 amino acids, with 13 amino acids being strictly conserved and partially overlapping with a cysteine-rich noose region. Within the central conserved domain, there are four closely spaced cysteine residues. These four residues are covalently connected in pairs through disulfide bonds(176-182, 173-186), forming the cysteine-rich noose region [25]. The sequence between cysteine residues at positions 182 and 186 forms the CX3C motif. (Figure 2)

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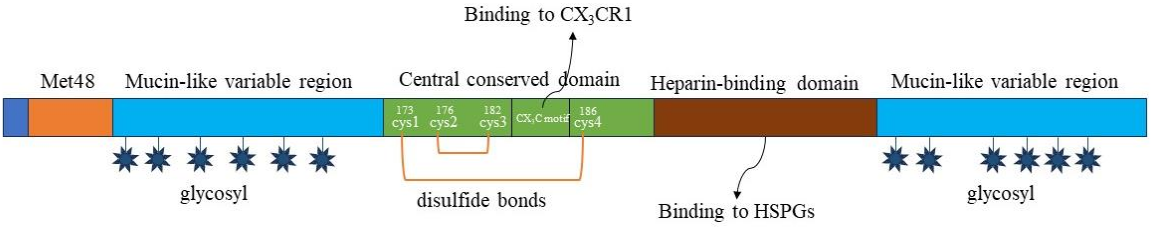

Figure 2. The structure of G protein.

During RSV entry, G protein binds to CX3C Chemokine Receptor 1 (CX3CR1) [26], Heparan Sulfate Proteoglycans (HSPGs) [27], and Annexin II [28]. The G protein enhances cell-to-cell fusion and facilitates the assembly or release of viral particles. However, the G protein is not essential for viral infection, as RSV lacking the G protein can still infect cells *in vitro* but with lower efficiency [29].

4. The viral entry process

First, the G protein binds to the receptors mentioned earlier, allowing the virus to attach to the cell membrane. Subsequently, RSV has two ways to enter the cell. One is to immediately undergo membrane fusion after the attachment process, allowing its RNA to enter the cell. The other is to enter the cell through macropinocytosis first, and then complete membrane fusion within the macropinocytic vesicles. (Figure 3)

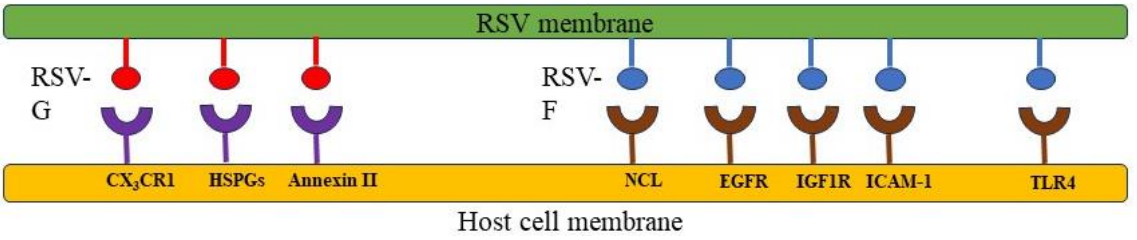

Figure 3. RSV binds with its receptors.

If the first mechanism is adopted (Figure 4), after RSV is attached to the membrane, the F protein binds to its receptor. Subsequently, the F protein undergoes a conformational change, with every three F proteins forming a trimeric HRAα helical bundle structure and exposing its fusion peptide (FP) to insert into the host cell membrane, forming a pre-hairpin conformation. The reason for this conformational change in vivo is not yet understood, but it may be triggered by the binding of the F protein to its receptor. The formation of the pre-hairpin conformation allows HRB to insert into the groove of the HRAα helical bundle, forming a stable HR six-helix bundle. The formation of the six-helix bundle causes the F protein to fold, further bringing the viral membrane closer to the host cell membrane, inducing sufficient distortion and stress to trigger their fusion.

Interestingly, the behavior of the F protein here closely resembles to the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNAREs) involved in vesicular transport in eukaryotic cells. The v-SNAREs on the vesicle and the t-SNAREs on the target cell membrane undergo heterotypic pairing to form SNARE complexes (similar to the HR six-helix bundle mentioned earlier), bringing the vesicle membrane closer to the target membrane. HA, which also belongs to the class I fusion protein like the RSV F and is found on the surface of influenza viruses, exhibits significant structural similarities with SNARE proteins.

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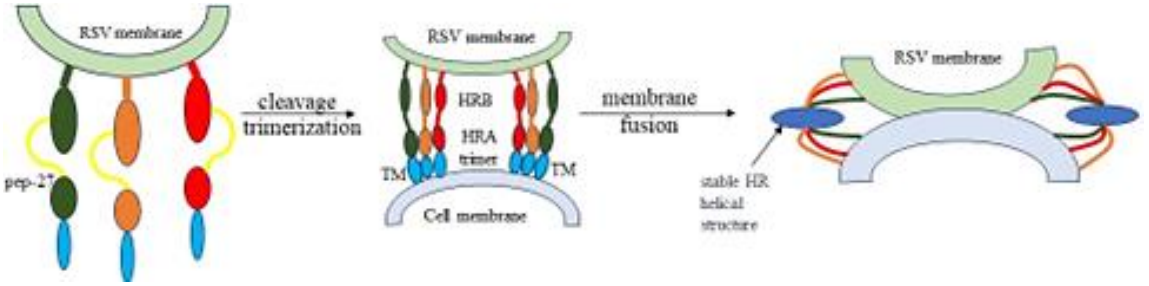

Figure 4. The first entry mechanism.

If the second mechanism (Figure 5) is adopted, RSV first activates the alpha-1 subunit of the Na+-K+ ATPase (ATP1A1). This process involves the participation of G protein, but the exact mechanism by which the G protein activates ATP1A1 is currently unknown. Activation of ATP1A1 leads to phosphorylation of Tyr845, resulting in transactivation of EGFR. Upon activation, EGFR initiates downstream signaling that triggers actin rearrangement and plasma membrane invagination, thereby initiating micropinocytosis [30]. Research has also indicated that RSV can directly bind to EGFR through its F protein, activating phosphatidylinositol 3-kinase (PI3K), p21-activated kinase 1 (PAK1), and downstream effectors through a signaling cascade, ultimately leading to the formation of macropinocytic vesicles [20].

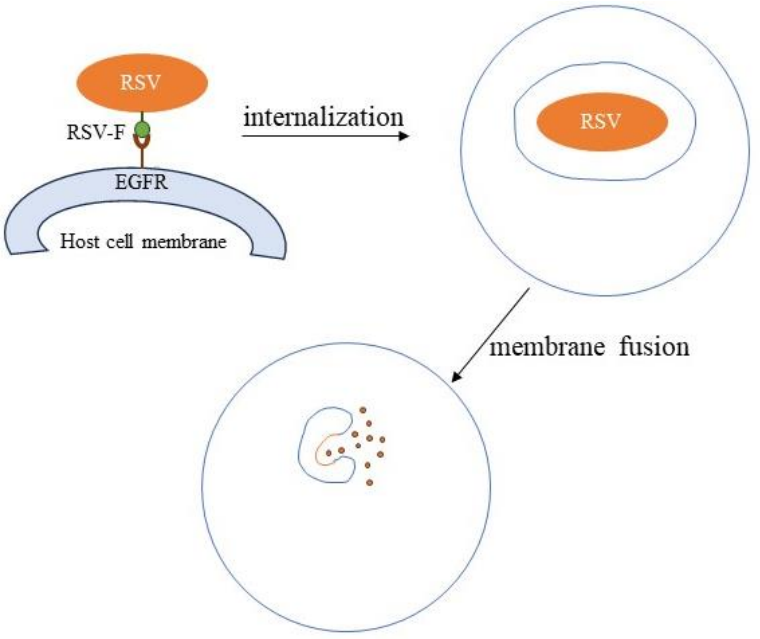

Figure 5. The second entry mechanism.

In the second mechanism, RSV membrane fusion occurs within macropinocytic vesicles, and the process is similar to the first mechanism. However, San-Juan-Vergara et al discovered that in primary NHEB cells, RSV initially contacts cholesterol-rich membrane domains and undergoes hemifusion during the attachment process. This means that only one leaflet of the viral membrane fuses with the target membrane, while the other leaflet remains unfused. Subsequently, RSV is internalized into the cell and completes membrane fusion within macropinocytic vesicles [31].

5. Antibodies and vaccines

Neutralizing antibodies primarily target the F protein, which contains six antigenic sites. The neutralization sensitivity from highest to lowest is Ø, V, III, IV, II and I. Among them, Ø is the

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Nirsevimab's binding site and II is the Palivizumab's binding site. The Ø and V sites are only present in the pre-fusion conformation, and the Ø is the most potent antigenic site [32]. Nirsevimab has much stronger neutralizing activity compared to Palivizumab and requires only a single dose.

RSV vaccine development has primarily focused on four main types: live attenuated vaccines, subunit vaccines, vector-based vaccines, and mRNA vaccines. Arexvy and Abrysvo are designed based on the F prefusion structure. Both vaccines are intended for individuals aged 60 and older. Abrysvo also aims to protect newborns by administering the vaccine to pregnant women through maternal immunization. This treatment plan was approved by the FDA advisory panel on August 21st, 2023.

6. Discussion

Although significant progress has been made in the mechanism of RSV entry and vaccines development, further research is needed to address the current issues and debates. We do not fully understand the maturation process of the F protein and the triggers for its conformational changes during membrane fusion: The stable six-helix bundle (6HB) formed by the F protein during virus-cell fusion is a characteristic feature of the class I fusion proteins. On one hand, studies on other class I viruses, such as HPIV3 and SV5, have indicated the importance of conserved glycine residues in the FP region of the F protein for triggering conformational changes [33]. On the other hand, the binding of gp120 protein, the glycoprotein of another class I virus HIV-1, to its receptor CD4 leads to insertion of the fusion peptide into the host cell membrane. The activation mechanism of RSV F protein may share similarities with these class I fusion proteins. However, it's still uncovered that what is the binding order among RSV receptors and if all of them contribute to the fusion. Additionally, there is controversy regarding whether TLR4 can be classified as the receptor.

Among the five identified receptors for the F protein, NCL appears to be a promising drug target. It is expressed at relatively high levels in target cells during RSV infection, and we also know that IGF1R recruits NCL to the cell membrane. In the future, there may be specific drugs that inhibit this process. Furthermore, there are already several marketed drugs targeting IGF1R and NCL, such as Simcere, an NCL inhibitor used for treating non-small cell lung cancer, and Teprotumumab, an IGF1R antagonist used for treating thyroid-associated eye disease. Since the safety of these drugs has been validated, searching for RSV-specific drugs among these marketed drugs can save a significant amount of time and resources. We believe that in the coming years, there will be more effective drugs to combat RSV and vaccines to prevent its infection, and RSV will consequently no longer be a dangerous virus for newborns.

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