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Structural, antigenic and immunogenic features of respiratory syncytial virus glycoproteins relevant for vaccine development

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Abstract

Extraordinary progress in the structure and immunobiology of the human respiratory syncytial virus glycoproteins has been accomplished during the last few years. Determination of the fusion (F) glycoprotein structure folded in either the prefusion or the postfusion conformation was an inspiring breakthrough not only to understand the structural changes associated with the membrane fusion process but additionally to appreciate the antigenic intricacies of the F molecule. Furthermore, these developments have opened new avenues for structure-based designs of promising hRSV vaccine candidates. Finally, recent advances in our knowledge of the attachment (G) glycoprotein and its interaction with cell-surface receptors have revitalized interest in this molecule as a vaccine, as well as its role in hRSV immunobiology.

Human respiratory syncytial virus (hRSV) was recently classified in the genus *Orthopneumovirus* of the newly created *Pneumoviridae* family within the order *Mononegavirales*, detached from the original *Paramyxoviridae* family [1]. hRSV is an enveloped virus with a genome made of a single-stranded RNA molecule of negative polarity and about 15.2 kb in length. Molecules of nucleoprotein (N) wrap around the entire length of this RNA to form a stable ribonucleoprotein (RNP) complex, which encodes 11 proteins, three of which are membrane-bound glycoproteins (G, F and SH) (for a review, [2]). The G glycoprotein was originally described as the receptor-binding or attachment protein [3]. F was identified by Walsh and Hruska [4] as the fusion protein that fuses the viral and cell membranes enabling the virus RNP to reach the cell cytoplasm. Finally, SH was initially described as a viroporin—a class of small viral proteins that modify membrane permeability [5]—and was later found to form pentameric pore-like structures in the membrane that confer cation-selective channel-like activity, compatible with its initial designation as a viroporin [6].

It is widely accepted that protection against hRSV is conferred mainly by neutralizing antibodies. For instance, high levels of neutralizing antibodies correlate with protection of human adult volunteers to hRSV challenge [7], as well as a lower risk of hRSV infection in

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children [8] and in the elderly [9]. Therefore, the surface glycoproteins, particularly F, have recently received much attention as targets of neutralizing and protective antibodies and as potential antigens to be included in a hRSV vaccine [10]. These aspects of hRSV vaccinology are the topic of this review.

Structure and function of the hRSV G glycoprotein

The G protein is synthesized as a polypeptide precursor of about 300 amino acids (depending on the viral strain) with a single hydrophobic domain (residues 38–63) near the N-terminus that acts as a combined signal and membrane anchor domain [11] (Fig. 1). This hydrophobic region targets the nascent chain, as it emerges from the ribosome, to the endoplasmic reticulum and ensures translocation of the polypeptide chain across the membrane while anchoring the G protein to the lipid bilayer. G has neither sequence nor structural homology with the attachment protein of viruses in the *Paramyxoviridae* family [11].

The G polypeptide precursor is extensively modified by the addition of both *N*- and *O*-linked oligosaccharides and is also palmitoylated at a single cysteine residue in its N-terminal cytoplasmic tail [12]. High-mannose *N*-linked glycans are co-translationally added to the G protein precursor, followed by the conversion of these sugars to the complex type and addition of *O*-linked glycans in the Golgi compartment. These modifications convert the 32 kDa precursor into a mature protein of 80–90 kDa (estimated by SDS-PAGE) in most immortalized cell lines [13], whereas a 180 kDa form has been described in human airway epithelial (HAE) cultures [14] that is postulated to represent either a dimer of the 90 kDa G protein or the 90 kDa form with additional or more extensive *O*-linked carbohydrate chains.

The G protein ectodomain consists of two large heavily glycosylated “mucin-like” domains, rich in serine, threonine and proline residues (characteristic of mucins), connected by a short central region devoid of carbohydrates (Fig. 1) [15]. The sequence of the two mucin-like domains is extremely variable among viral strains [16] but they all have several potential sites for *N*-glycosylation and multiple serines and threonines that are predicted to be *O*-glycosylated by the NetOGlyc software [17]. This sequence variability has been used in numerous studies of molecular epidemiology and evolution of hRSV [18]. Thus, hRSV strains have been classified into two genetic groups, A and B, that correlate with the antigenic groups, initially identified by reactivity with certain monoclonal antibodies (mAbs) [19;20]. Within each group numerous clades or genotypes have been identified. Viruses of different genotypes and even different antigenic groups frequently co-circulate in each yearly outbreak. The dominance of these genotypes changes in successive epidemics and replacement of certain genotypes by others has been noticed at the global level [21].

The central conserved region of hRSV G (aa 163–189) has four cysteines (residues 173, 176, 182 and 186) that are conserved in all viral strains. Within this region there is a stretch of 13 amino acids (164–176) that is strictly maintained in all strains while the remaining sequence of the central region is somewhat group-specific. Disulfide bridges are formed between Cys173 and Cys186, and between Cys176 and Cys182, resulting in a cystine noose motif which resembles the structure found in the 55 kDa tumour necrosis factor receptor [22;23].

The Cys-rich motif is missing in the highly related G protein of human metapneumovirus (hMPV), which otherwise shares the overall amino acid composition and sequence variability of hRSV G [24;25]. The ectodomains of both hRSV and hMPV G are predicted to be disordered (except for the hRSV cystine noose), consistent with the high content of serine, threonine, and proline residues and extensive *O*-glycosylation [26].

The central region of hRSV G contains the CX3C motif (aa 182–186) that can bind to CX3CR1—the specific receptor of the fractalkine chemokine—and hence induce leukocyte chemotaxis [27]. Several authors have reported hRSV binding to differentiated HAE cells by the interaction of the G protein with CX3CR1 in the apical surface of ciliated cells [28–30]. Inhibition of CX3CR1 binding reduces but does not entirely suppress infection of HAE cultures, indicating that CX3CR1is an important but not the only hRSV receptor in these cells. It has been reported that hRSV uses cell surface proteoglycans for attachment to established cell lines [31–33] mainly by interactions of the G protein with glycosaminoglycans (GAGs) [34;35]. Whether proteoglycans may also act as an hRSV G receptor in HAE cells is still controversial [28;30].

In addition to the membrane bound form of hRSV G, infected cells also produce a soluble form of G (sG) [36] by internal initiation of translation at a second AUG codon (Met48) located in the middle of the transmembrane region and subsequent cleavage after residue Asn66 (Fig. 1) [37]. While sG is monomeric, membrane bound G is oligomeric (probably a tetramer) emphasizing the relevance of the transmembrane domain for oligomerization [38]. The actual role of sG in hRSV biology is not known, although it has been postulated to help evade the antibody-mediated restriction of replication by acting as an antigen decoy and through effects on Fc-receptor-bearing leukocytes [39].

Antigenicity and immunogenicity of the hRSV G glycoprotein

Three types of epitopes have been identified in the G protein by murine mAbs: i) ***conserved*** epitopes which are present in all viral strains and that map within the conserved 13 amino acid stretch of the unglycosylated central region, ii) ***group-specific*** epitopes that partially overlap with the conserved epitopes but are shared only by strains of the same antigenic group and iii) ***strain-specific*** epitopes that are present only in certain strains of the same antigenic group and have been mapped in the C-terminal hypervariable region of the G protein ectodomain (Fig. 1) [40]. These variable epitopes are influenced by cell-type-specific glycosylation [13].

The majority of murine mAbs specific for the G glycoprotein have minimal effects on virus infectivity in classical complement-independent neutralization assays performed with immortalized cell lines [41;42]. However, pools of antibodies binding to different epitopes of G showed a synergistic effect on this type of neutralization [43], suggestive of hRSV inhibition by steric hindrance.

Recent studies have demonstrated that 131-2G [41], a murine mAb which binds to an epitope located in the central region of hRSV G and that is conserved in all viral strains tested so far, reduces hRSV binding to CX3CR1 in HAE cell cultures [28;29]. Antibody

131-2G reduces several disease manifestations in hRSV challenged mice, including pulmonary inflammation [44] and mucus production [45]. Mice inoculated with G protein polypeptides or peptides spanning the central conserved region of G elicited antibodies that blocked the interaction of the G protein with CX3CR1 and had reduced pathogenesis mediated by hRSV infection [46]. Likewise, mice vaccinated with recombinant influenza virus carrying a chimeric HA protein containing the conserved domain of hRSV G [47] or nanoparticles carrying the CX3C motif of hRSV G [48] had reduced virus titers and pathology in the lungs after a hRSV challenge. These results extend those previously obtained with a BBG2Na vaccine that comprised residues 130 to 230 of hRSV G fused to the albumin-binding region of the streptococcal protein B [49]. This vaccine was tested in humans but these trials were halted after two individuals in a phase II trial developed type III hypersensitivity, likely attributable to the bacterial component. Nevertheless, the results cited in this paragraph unlock new possibilities for hRSV vaccine development based on the G glycoprotein.

Structure and function of the hRSV F glycoprotein

The F protein is a type I glycoprotein which shares structural motifs with the F proteins of other *Pneumoviridae* (e.g., hMPV) and *Paramyxoviridae* (e.g., parainfluenza virus type 5, PIV5) viruses, despite limited sequence identity, suggesting that they all function through similar mechanisms. The F glycoprotein is synthesized as an inactive precursor (F0) of 574 amino acids that has three hydrophobic peptides (Fig. 2): i) the N-terminal signal peptide (aa 1–21), which directs translocation of the nascent polypeptide to the lumen of the endoplasmic reticulum and is not present in the mature molecule, ii) the transmembrane region (aa 525–550) near the C-terminus, which anchors F to the cell and viral membranes, and iii) the so-called fusion peptide (aa 137–155), which inserts into the target cell membrane during the fusion process. F0 is post-translationally cleaved after two polybasic furin sites at residues 109 (cleavage site I) and 136 (cleavage site II), separated by 27 amino acids (pep27), to become fusion competent [50]. The double proteolytic cleavage is shared with the homologous F protein of bovine RSV but it is a unique feature among the *Pneumoviridae* and *Paramyxoviridae* F proteins, which are cleaved only once. Once cleavage of hRSV F is completed, the intervening pep27 is released from the mature protein [51] and two chains are generated (F2 N-terminal to F1) which remain covalently linked by two disulfide bridges (Cys70–Cys212 and Cys37–Cys439). The newly created N-terminus of the F1 chain contains the fusion peptide. There are two *N*-linked glycosylation sites in F2 and one in F1, and these are conserved in all hRSV strains. The F1 chain has a central cysteine-rich region flanked by two heptad repeats: HRA is located C-terminal to the fusion peptide and HRB precedes the transmembrane region. The mature hRSV F glycoprotein is a homotrimer of F1+F2 subunits.

The main function of hRSV F is to promote fusion of the viral and cell membranes; however, there have been reports of spontaneous deletion mutants [52] and recombinant viruses [53] in which F is the only viral glycoprotein. These mutants replicate in established cell lines but not in HAE cultures [14] and are attenuated in animal models [54]. Therefore, at least in ΔG viruses, F has to assume the virus binding function of the G protein, in addition to its membrane fusion activity. Indeed, F has been found to bind proteoglycans

[55] and other cell-surface molecules, such as nucleolin [56], compatible with its role as a substitute attachment protein. However, whether wild-type virus requires F binding to cells for infectivity is still not entirely clear.

The relevance of the hRSV F double cleavage for membrane fusion is also uncertain. Grafting of the double cleavage site of hRSV F in Sendai virus F resulted in a dramatic increase of cell-cell fusion mediated by the chimeric protein in transfected cells, as well as a decrease in dependence of hemagglutinin-neuraminidase (HN) co-expression for cell-cell fusion [57]. Furthermore, replacement of Sendai virus F by the chimeric protein reduced virus thermostability and decreased dependence on HN binding to sialic acid for infection, mimicking the unique ability of hRSV to fuse and infect cells in the absence of a separate attachment protein [58]. Therefore, the presence of two cleavage sites in hRSV F seems to modulate its membrane fusion activity by still ill-defined mechanisms.

The F trimer is assembled in the virus particle in a metastable conformation, called pre-fusion. During membrane fusion, F experiences a series of conformational changes that result in a highly stable structure, called post-fusion (see later). Important knowledge about these conformational changes has been recently gained by solving the atomic structures of soluble forms of hRSV F folded in either the prefusion or postfusion conformation [59–62], as shown in Fig. 2.

One of the main hurdles in these studies was the stabilization of a soluble form of hRSV F in its prefusion conformation. Initially, it was found that the expression of the F protein ectodomain led to formation of soluble trimers (sF) that retained epitopes recognized by certain neutralizing mAbs [63]. Partial deletion of the fusion peptide prevented aggregation of sF after cleavage [64] and allowed its crystallization in the absence of detergents [59;60]. The X-ray structures determined from these crystals demonstrated that sF was folded in the postfusion conformation, indicating that the F ectodomain assembles spontaneously into the highly stable postfusion form when expressed without the transmembrane region. Of note, the full-length F also refolds into the postfusion form if extracted with detergents from the cell or viral membranes. Therefore, a central challenge was to obtain a soluble hRSV F ectodomain stabilized in the prefusion form, amenable to crystallization. This was initially achieved by co-expression of the hRSV F ectodomain in complex with the Fab fragment of a neutralizing mAb (D25) which did not bind to postfusion F and hence was presumably specific for the prefusion conformation [61]. Indeed, the structure of F in that complex differed substantially from the previously described postfusion hRSV F and resembled the structure of the paramyxovirus PIV5 prefusion F, reported by Yin et al., [65]. Based on that structure, several mutants of the hRSV F ectodomain were made to stabilize it in the prefusion conformation in the absence of antibodies [62]. One of the most stable mutants (DS-Cav1) had two serines (155 and 290) substituted by cysteines to create an intrasubunit disulfide bond, two cavity filling mutations (S190F and V207L) in the F1 chain to help stabilization, and a foldon trimerization domain at the C-terminus [62]. DS-Cav1 has been extensively used in several studies but other mutants, stabilized in the prefusion conformation by alternative strategies (SC-TM), have been obtained with enhanced stability properties [66].

Fig. 2 shows a comparison of the hRSV F prefusion and postfusion structures. Most of the secondary and tertiary structure is preserved in the pre- and postfusion forms. In contrast, the N- and C-termini of the F1 chain undergo substantial conformational changes. During the fusion process, the fusion peptide and the first five secondary-structure elements at the N-terminus of F1 rearrange and fuse with the α5 helix to form an extended helix of >100 Å in length. Near the C-terminus of F1, parallel strand β22 dissociates as the C-terminal helix rearranges to form the outer helix of the postfusion six-helix bundle (6HB). Similar rearrangements had been inferred by comparison of the related prefusion PIV5 and postfusion PIV3 F structures [67,68], suggesting that all these viruses share a similar membrane fusion mechanism.

Fig. 3 illustrates the model of membrane fusion mediated by hRSV F, taken from the initial model proposed for PIV5 [65]. After binding of the incoming virus to the cell surface, prefusion F is activated by an ill-defined process which apparently does not require the attachment G glycoprotein, a major difference with the F protein of the *Paramyxoviridae*. After activation, F initiates a series of conformational changes which probably involve separation of the HRB helices and formation of the long HRA α-helix, as mentioned above. These changes relocate the fusion peptide—which is buried deep inside the prefusion globular head—towards the end of the newly formed helix, which probably assembles into a coiled-coil trimer of HRA sequences. Since the fusion peptide is hydrophobic, it avoids the hydrophilic environment by inserting into the outer layer of the cell membrane, leading to formation of the so-called pre-hairpin intermediate. At this stage, the cell and viral membranes are connected by sequences of the F1 subunit which lie between the fusion peptide and the transmembrane region, respectively. This unstable pre-hairpin intermediate refolds by zipping the HRB helix towards the HRA coiled-coil, bringing the two membranes into proximity. Finally, the fusion peptide and the transmembrane domain of F end up in the same membrane after formation of the 6HB, in which an internal core of three HRA α-helices is surrounded by three antiparallel HRB helices. Completion of the 6HB assembly leads to exchange of lipids between the two membranes, formation of the initial fusion and expansion of this pore to complete membrane fusion.

Antigenicity and immunogenicity of the hRSV F glycoprotein

The first panels of mAbs raised against hRSV F were obtained from immunized mice using the hybridoma technology [41;69;70]. These antibodies identified several epitopes that were eventually mapped in the F protein primary structure by isolation and characterization of escape mutants [69;71;72] or by reactivity of antibodies with peptides or F protein fragments [73;74]. Binding of at least some of these antibodies to F could be competed with human sera, indicating that the matching epitopes were relevant in a natural infection. However, the F proteins used in these studies were likely folded in the postfusion conformation since, as noted before, F folds spontaneously into this structure when it is either expressed as a soluble ectodomain or detergent-extracted from cell membranes. It was thus relatively unsurprising that as reported [75;76] most of the neutralizing activity present in human immunoglobulin preparations could not be depleted by adsorption to immobilized preparations of postfusion F. It was therefore concluded that most natural human neutralizing antibodies recognize epitopes preserved only in the prefusion conformation of

hRSV F. This was further corroborated by the isolation of mAbs from immortalized human lymphocytes [77] that were specific for prefusion F. These mAbs had higher neutralizing potency [61] than those originally described that reacted with both prefusion and postfusion F [76].

Other human neutralizing antibodies have been recently reported that either recognize neutralizing epitopes exclusive to prefusion F, such as AM14 [78], or show preferential binding to prefusion over postfusion F, such as MPE8 [79]. Interestingly, MPE8 cross-neutralizes not only hRSV but additionally three other *Pneumoviridae*: bovine RSV, hMPV and pneumonia virus of mice (PVM). Another mAb, 54G10, raised against hMPV F has also shown cross-neutralization with hRSV [80].

Fig. 4 shows the location of antibody epitopes and antigenic sites identified so far in the prefusion and postfusion conformations of hRSV F. Some of these sites are found only in the prefusion (e.g., site Ø) or postfusion (e.g., site 6HB) conformation, while others are present in both conformations (e.g., site II) since, as mentioned above, an extensive area of the protein surface is shared by the prefusion and postfusion conformations (Fig. 2) [61]. In general, the antibodies that bind preferentially to prefusion F are better neutralizers than those that recognize epitopes shared by prefusion and postfusion F. As expected, antibodies which recognize epitopes specific to postfusion F are non-neutralizing.

It is assumed but not formally demonstrated that neutralizing antibodies bind to prefusion F and block the initiation of the conformational changes that lead to membrane fusion [81]. Concurring with the neutralizing potency shown by the different mAbs, a detailed analysis of the antibodies present in individual human sera demonstrated that the majority of their neutralizing activity was due to prefusion-specific antibodies directed against antigenic site Ø [82].

Several studies have compared the immunogenic and protective efficacy of purified prefusion and postfusion soluble F in animal models. For instance, the prefusion stabilized DS-Cav1 protein was found to induce ten times higher levels of neutralizing antibodies in mice and rhesus macaques than postfusion F [62]. Similarly, the alternatively stabilized prefusion SC-TM protein also elicited 10–20-fold higher levels of neutralizing antibodies than postfusion F in cotton rats that were additionally shown to be protected against a hRSV challenge [66]. A recent comparison of prefusion, postfusion and a monomeric form of F that shares antigenic properties with prefusion F [83] also demonstrated the superiority of prefusion F in inducing neutralizing antibodies and protection against a hRSV challenge in mice without perceptible pathology [84]. However, protection against a hRSV challenge has also been achieved with postfusion F, likely by induction of neutralizing antibodies that recognize epitopes shared with prefusion F [59;85]. It is worth noting that postfusion F is a highly stable molecule, a valuable characteristic from the point-of-view of vaccine production and distribution.

Stabilized full-length prefusion hRSV F has also been incorporated as an extra gene into PIV3 recombinants [86]. These viruses showed an enhanced neutralizing antibody response against hRSV compared with PIV3 recombinants expressing a soluble postfusion form of

hRSV F. Prefusion stabilized hRSV F has also been expressed at the surface of virus-like particles (VLPs) made in cells that expressed the nucleoprotein (NP) and the matrix (M) protein of Newcastle disease virus (NDV). The purified VLPs were inoculated i.m. in either mice [87] or cotton rats [88], and these animals elicited a serum neutralizing antibody response and were protected against an hRSV challenge. Thus, immunization with pre-fusion F, either as a subunit vaccine or incorporated to recombinant viruses or VLPs, seems to be a promising approach for hRSV vaccine development.

Expression of individual F protein epitopes grafted onto different protein scaffolds has also been reported, including the helix-loop-helix motif of antigenic site II [89;90]. Structural analysis of these proteins indicated that they could faithfully reproduce the structural and antigenic features of hRSV F site II. Some of these constructs were able to induce strong neutralizing antibody responses in macaques but only after several immunizations, suggesting that further optimizations of these scaffolds are required before being considered a practical approach to an hRSV vaccine. These scaffolds do, however, represent an interesting alternative for obtaining simplified vaccines with improved production and stability.

In summary, advances in understanding the structure of hRSV glycoproteins, especially the F glycoprotein, have brought new stimulus for development of long-awaited hRSV vaccines which will help to control one of the most important causes of infant hospitalization [91] and one of the leading global causes of infant mortality [92].

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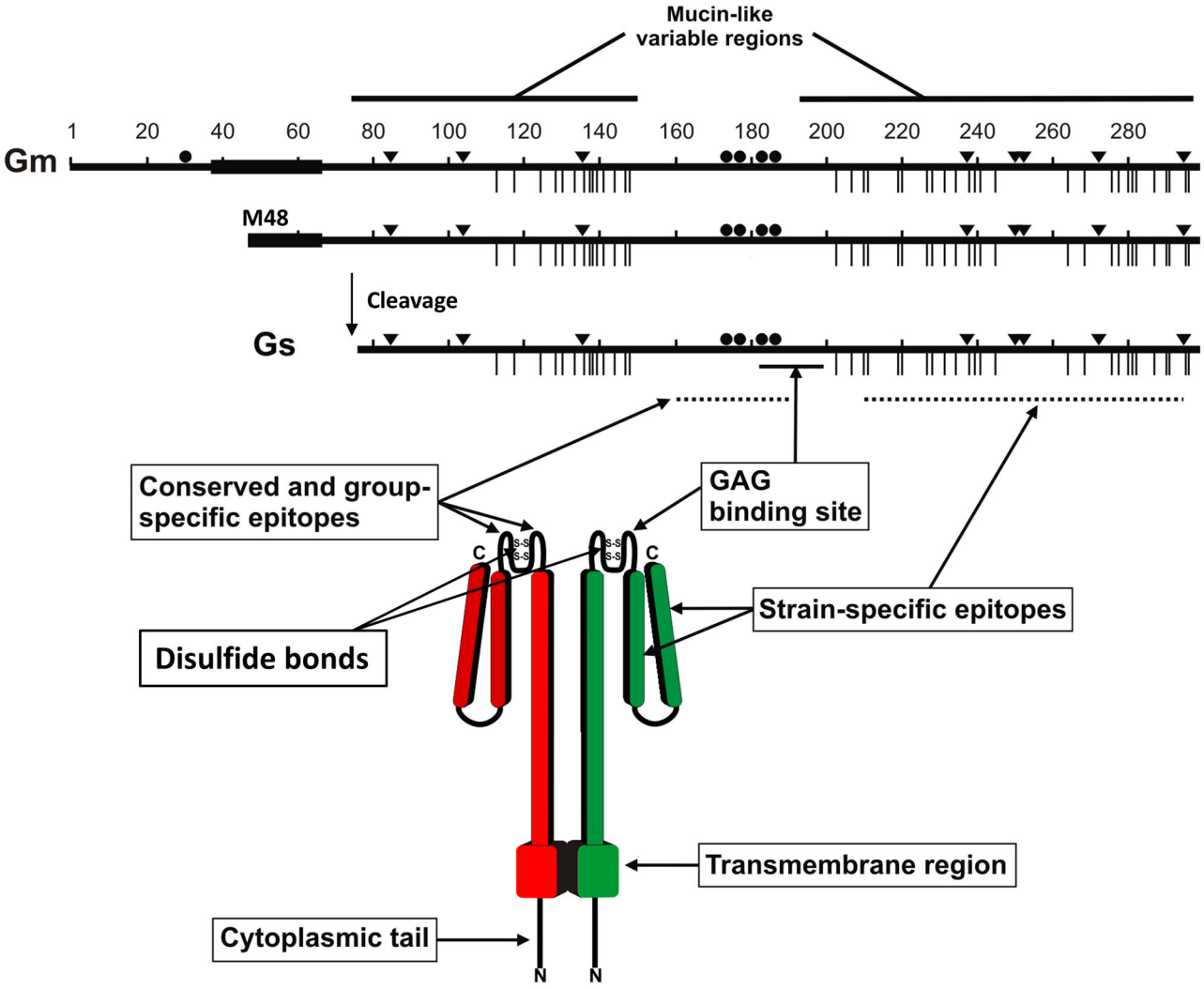

Figure 1. Human respiratory syncytial virus G glycoprotein

The full length, 298 amino acid membrane-anchored G protein (Gm) and the 233 amino acid soluble G protein (Gs) are shown (Long strain). Hydrophobic regions are denoted by thick lines. Gs is formed by alternative translation initiation at M48, followed by cleavage after residue 65. Inverted triangles represent *N*-linked glycosylation sites and vertical lines indicate *O*-linked glycosylation sites. Cysteine residues overlapping the central conserved domain are represented by solid circles. The lower part of the figure depicts a model of the 3-dimensional structure of Gm. While Gm is probably tetrameric [38], a dimer is shown for simplicity. The mucin-like regions are depicted as extended rod-like structures due to the presence of multiple *O*-linked sugars that have a tendency to stretch the polypeptide backbone [93] The second hypervariable region is externally located in the model to denote that it harbors multiple epitopes and it is shown as two halves joined by a protease susceptible site [94]. Antibody epitopes and the glycosaminoglycan (GAG) binding site are indicated by arrows. Figure provided by Alfonsina Trento.

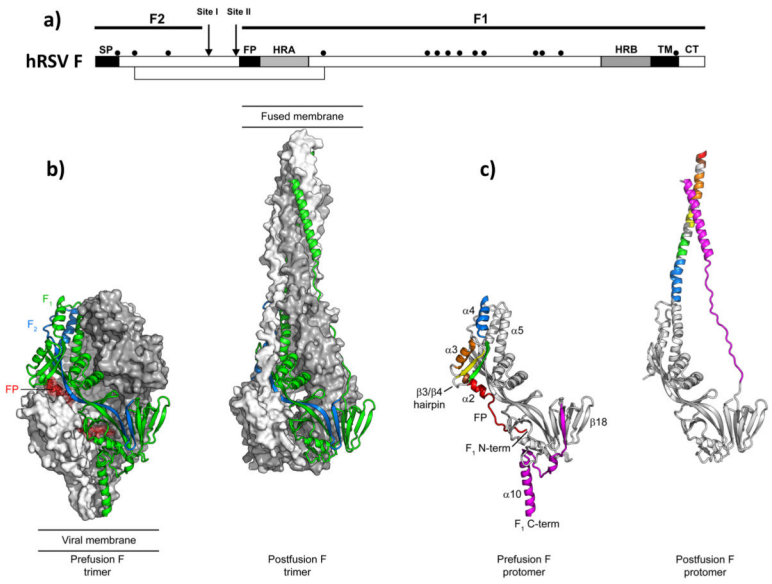

Figure 2. Human respiratory syncytial virus F glycoprotein

(a) Diagram of the F protein precursor denoting the signal peptide (SP), the fusion peptide (FP) and the transmembrane region (TM), as well as cleavage sites I and II and the cysteine residues (black dots). (b) Structure of the F protein trimer folded in the prefusion (left) and postfusion (right) conformation. One protomer is shown as ribbons and colored blue (F2 chain), green (F1 chain) and red (fusion peptide). Molecular surfaces are shown for the other two F protomers, colored grey and white. (c) A single hRSV F protomer is displayed as ribbon, folded in the prefusion (left) and postfusion (right) conformation. The same colors are used for the secondary structure elements (indicated in the prefusion protomer) in the two conformations. Note that the structure colored grey is essentially unchanged in the prefusion and postfusion conformation.

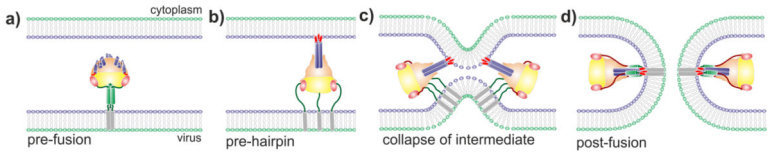

Figure 3. Model of membrane fusion mediated by the hRSV F glycoprotein

(a) A single prefusion F protein trimer is depicted inserted into the viral membrane through a HRB stalk (green). (b) Upon activation, the short α-helices of HRA (blue) refold into a long trimeric coiled-coil (blue) and the fusion peptide of each subunit (red) is inserted into the target membrane, forming the so-called pre-hairpin intermediate. (c) Collapse of this unstable intermediate approaches the two membranes. (d) Assembly of the six-helix-bundle (6-HB), formed by a core of three HRA α-helices surrounded by three antiparallel HRB α-helices, results in formation of the fusion pore.

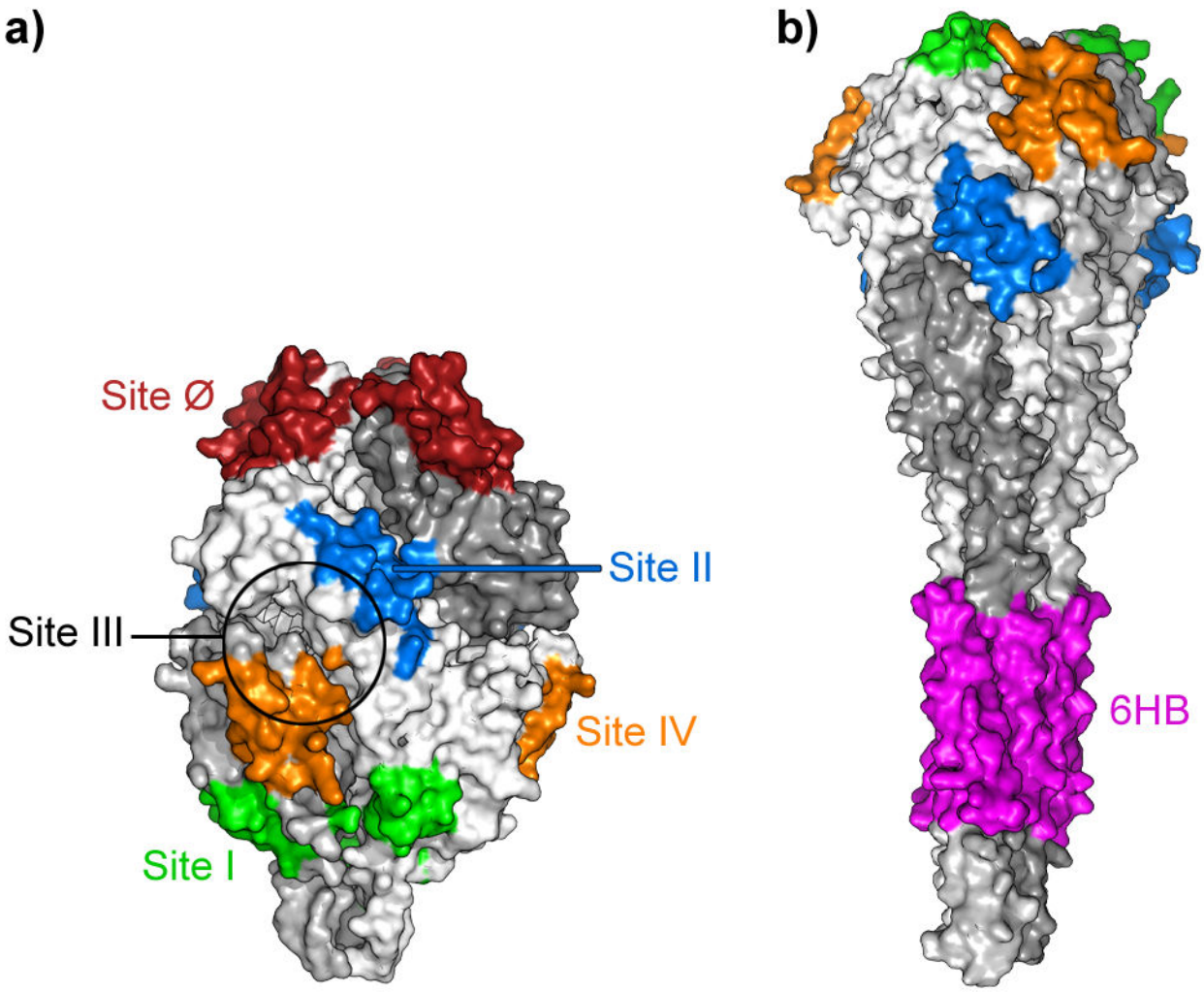

Figure 4. Antigenic sites of hRSV F glycoprotein

The location of the different antigenic sites is shown in both the prefusion (a) and postfusion (b) conformation of hRSV F. Antigenic site III is delineated by a circle which includes residues identified by mutagenesis to be essential for binding of mAb MPE8 [79], since no other structural information is available yet for this site.