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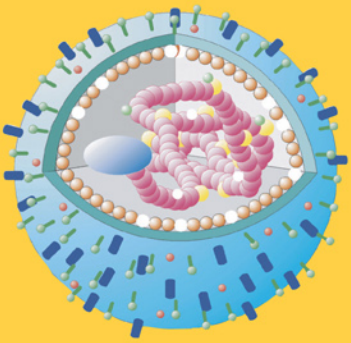
PERSPECTIVES IN MEDICAL VIROLOGY, 14

Patricia Cane, Editor

Respiratory Syncytial Virus



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Volume 14

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Respiratory Syncytial Virus

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Preface

Respiratory syncytial virus (RSV) was first identified half a century ago in 1956. Following its discovery, the virus soon became recognised as a major viral pathogen causing extensive outbreaks of respiratory tract infections in both the very young and in vulnerable adults. It is an unusual virus in that it can cause repeated re-infections throughout life. Our understanding of the molecular biology and immunology of the virus is now very extensive. However, some aspects of its pathology, biology within the community setting, and interactions with other microorganisms remain elusive and a clear understanding of these factors will be necessary for effective control. Vast effort has been devoted over the years by both academia and industry towards the development of an effective and safe vaccine. It seems that goal may finally be almost within reach and it is to be hoped that a vaccine will be available to reduce the burden of disease due to the virus within the next few years.

The topics covered within this volume are wide ranging in scope from the most basic molecular biology of the virus to the clinical picture of RSV in the developing world. The first two chapters provide the background of the molecular biology and immunology of the virus, the next looks at the molecular epidemiology followed by the influence of host genetics. Chapters by Brearey and Smyth, Murata and Falsey, and Nokes examine the clinical picture of RSV in children, the elderly, and in the developing world, respectively. The next two chapters that follow review the current status of interventions against RSV, covering vaccine development and antiviral drugs. Recently, work has resumed on a much neglected surrogate virus, namely pneumonia virus of mice, and the final chapter describes progress with that model.

This volume of Perspectives in Medical Virology has enlisted many of the internationally recognised experts in their particular field of RSV research. The writers were invited not only to review the present state of knowledge, but also to give their perspective on the current situation and to identify the gaps and future requirements for research. It is to be hoped that the views expressed will stimulate new cross-cutting approaches to tackle this major viral pathogen.

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Respiratory Syncytial Virus
Patricia Cane (Editor)

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Molecular Biology of Human Respiratory Syncytial Virus

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Introduction

Human respiratory syncytial virus (HRSV) is the prototype of the *Pneumovirus* genus, which also includes the closely related bovine, ovine and caprine RSV and a more distantly related virus, the pneumonia virus of mice (PVM). The *Pneumovirus* genus is classified within the subfamily *Pneumovirinae* of the family *Paramyxoviridae*. Viruses of this family are grouped, together with those of the *Rhabdoviridae and Filoviridae* families, in the *Mononegavirales* order, which is characterized by having a linear, negative-sense, single-stranded RNA molecule as the genome (Mononegavirales, 2003). Viruses of this order share certain structural and functional features, including: (i) their genomes are tightly associated with the nucleoprotein (N) to form RNase-resistant nucleocapsids, which are the templates for all RNA synthesis, (ii) transcription proceeds in a sequential and polar manner from the 3′-end of the viral RNA (vRNA) by terminating and reinitiating at each of the gene junctions, (iii) replication of the viral genome involves the synthesis of a complementary antigenome (cRNA), (iv) the virus particles are surrounded by a lipid bilayer in which the viral glycoproteins are inserted, and (v) entry of the viral nucleocapsids into the host cells involves membrane fusion. Given these analogies, some details of the HRSV infectious cycle, which have not been addressed directly, are inferred from knowledge acquired from related viruses, particularly paramyxoviruses.

HRSV was first isolated in 1956 from a chimpanzee with coryza (Morris et al., 1956), and a year later from two children with respiratory illness (Chanock et al., 1957). Variability of HRSV isolates was first demonstrated at the antigenic level in a neutralization test performed with hyperimmune serum (Coates et al., 1966). Different panels of monoclonal antibodies were later used to subdivide HRSV isolates into two antigenic groups, A and B (Anderson et al., 1985; Mufson et al., 1985),

which correlate with genetically distinct viruses (Cristina et al., 1990). Further variability among viruses of the same antigenic group has been found, particularly in the attachment (G) glycoprotein. This variability is discussed extensively in another chapter of this book.

The virion

HRSV virions are heterogeneous in size and shape. When observed by electron microscopy (EM), two types of viral particles are identified: (i) round- or kidney-shaped particles ranging in diameter from 150 to 250 nm and (ii) filaments up to 10 μm in length (Bächi and Howe, 1973) (Fig. 1A). These two types of particles can be separated, at least partially, by sucrose gradient centrifugation and both are infectious (Gower et al., 2005).

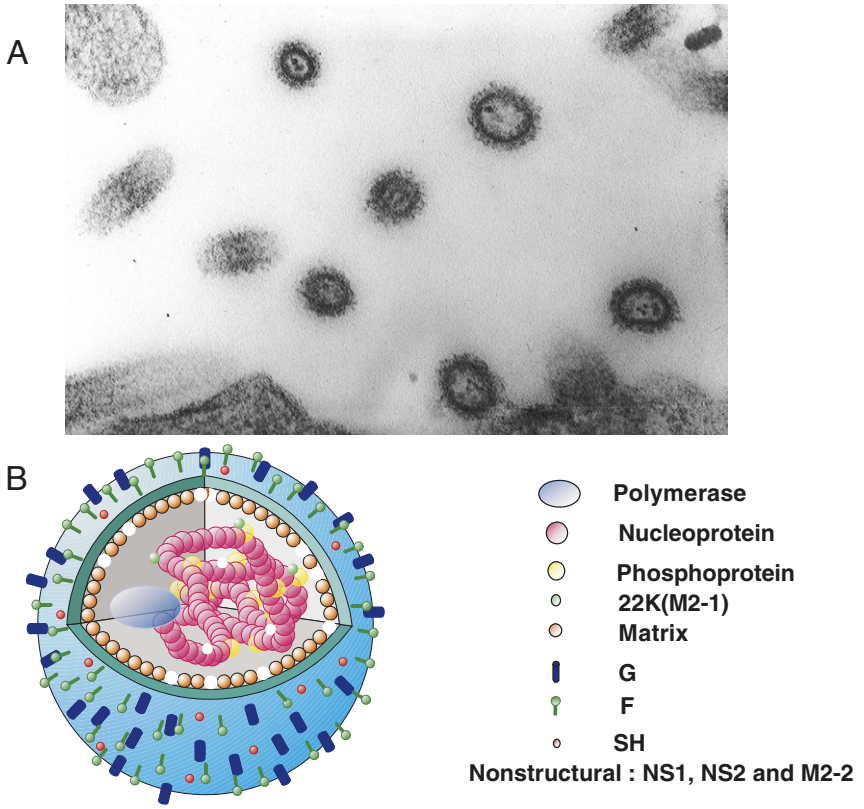

Fig. 1 Electron microscopy (A) and scheme (B) of the HRSV virion. The structural (colour-coded) and non-structural proteins are listed in part B of the figure (for colour version: see colour section on page 323).

Virus particles are surrounded by a lipid bilayer in which the two major surface glycoproteins are inserted (Fig. 1B). These consist of the attachment (G) glycoprotein involved in binding the virus to the cell surface (Levine et al., 1987) and the fusion (F) glycoprotein that mediates fusion of the viral and cell membranes (Walsh and Hruska, 1983). Both G and F form the characteristic spikes of HRSV virions seen by EM. A third small hydrophobic (SH) glycoprotein encoded by HRSV is expressed abundantly at the surface of virus-infected cells but is incorporated only in low amounts in the virus particles (Collins and Mottet, 1993).

The virus nucleocapsid, found inside the virion, consists of the vRNA (the genome) tightly bound to the nucleoprotein (N), forming a helix of "herringbone" morphology when imaged under a transmission electron microscope. The helical nucleocapsids of HRSV are more flexible and appear less well ordered than those of other paramyxoviruses. Pitches range from 68 to 74 Å and the nucleocapsid diameter from 14 to 16 nm, considerably narrower than that of other paramyxoviruses (Bhella et al., 2002). In addition to vRNA and N, other viral proteins are incorporated into the nucleocapsid but they are not observed by EM. These include the RNA-dependent RNA polymerase (or L protein), the phosphoprotein (P) and, probably, the 22k (or M2-1) protein.

HRSV encodes three non-structural proteins (NS1, NS2 and M2-2), which are produced in the infected cell but are not incorporated in the virus particles (Fig. 1B).

The infectious cycle

The different steps of the HRSV infectious cycle are illustrated in Fig. 2. Viral entry into the host cell occurs by the initial binding of virions to cell surface components, followed by activation of the F protein to trigger fusion of the viral and cell membranes. After the internalization of the viral nucleocapsids into the cell cytoplasm, sequential transcription of the viral genome is activated to generate a set of mRNAs that instruct translation of the corresponding gene products by the cell ribosomes. Sometime after infection RNA synthesis changes from transcription to the replication mode, generating a full-length copy of the vRNA of opposite polarity, named cRNA (or antigenome). This antigenome also complexes in nucleocapsids with the N protein, which are the templates for the synthesis of progeny genomes. Eventually, the different HRSV gene products accumulate near the cell membrane where they are assembled into progeny virus particles that are released from the infected cell by budding. The entire infectious cycle of HRSV, which follows the archetype of other paramyxoviruses, can take place in enucleated cells (Follet et al., 1975). However HRSV infection influences the expression of certain nuclear genes, as discussed latter in this chapter and in other chapters of this book.

Experimental systems to study the replicative cycle of HRSV

A wide range of animal species, including mice, cotton rats, mice, ferrets, guinea pigs, marmosets, lams and several non-human primates can be infected by the

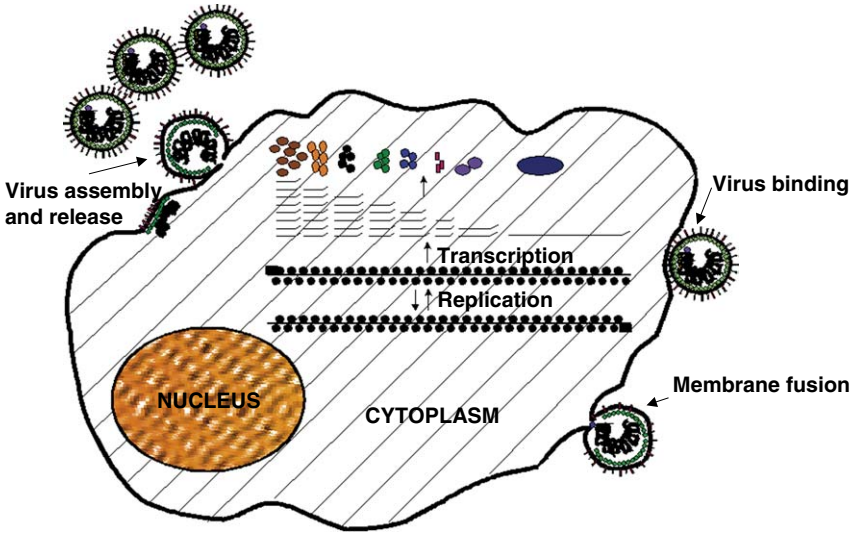

Fig. 2 Diagram of the HRSV infectious cycle (for colour version: see colour section on page 323).

administration of HRSV directly into the respiratory tract (Collins et al., 2001). The chimpanzee alone reproduces faithfully the infection observed in humans. The related bovine RSV infects calves and, perhaps, represents the best model to study RSV infections, since calves are the natural host of this virus (Taylor et al., 2005). However, the high cost of calves and the intensive labour involved in manipulation of calves precludes the routine use of these animals for experimental purposes.

Although animal models are useful to study HRSV infections, the *in vivo* approach is not open to manipulations that allow dissection of the different steps of the replicative cycle. Therefore, infection of established cell lines, or occasionally certain primary cultures, has been employed as the standard system to study the replicative cycle of HRSV. This virus can infect a wide variety of human and animal cells but it should be borne in mind that *in vivo* the epithelial cells of the respiratory tract are the major sites of virus replication.

The reverse genetics approach has revolutionized studies of mononegavirales since the rescue of infectious rabies virus from a full-length cDNA clone was achieved (Schnell et al., 1994). In the case of HRSV, the standard procedure involves cloning of the entire genome in the form of DNA (cDNA), flanked by a promoter of the bacteriophage T7 polymerase and a hammerhead ribozyme followed by T7 terminator(s) (Collins et al., 1995; Jin et al., 1998; Buchholz et al., 1999) (Fig. 3A). When this plasmid is transfected into cells that express the T7 polymerase, transcription by this enzyme generates a positive-stranded copy of the viral RNA (the cRNA or antigenome), which after ribozyme cleavage has the correct ends of the antigenome (although HRSV allows certain flexibility at the RNA ends). If the cells are co-transfected with support plasmids that encode N, P,

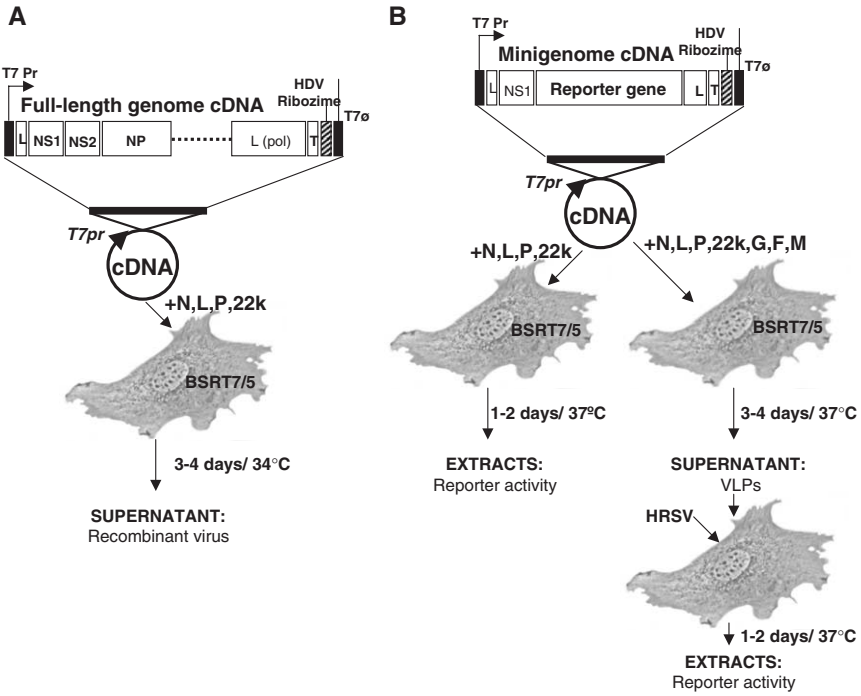

Fig. 3 Reverse genetics systems to study the HRSV replicative cycle. (A) Rescue of recombinant HRSV. BHK cells, expressing constitutively the bacteriophage T7 RNA polymerase (BSR T7/5 cells, Buchholz et al., 1999), are transfected with a plasmid carrying a full-length cDNA copy of the HRSV genome and co-transfected with plasmids encoding N, L, P and 22k proteins under a T7 promoter. Recombinant viruses are generated and released to the culture supernatant from where they can be grown to high titres in new cells. (B) Amplification of minigenomes. BSR T7/5 cells are co-transfected with plasmids carrying a minigenome version of the HRSV vRNA and supporting plasmids encoding N, P, L and 22k proteins under a T7 promoter (left). Extracts are made after 1–2 days in culture to measure reporter activity. If supporting plasmids encoding G, F, and M are also included in the transfection mixture (right), virus like particles (VLPs) carrying the minigenome are generated and released to the culture supernatant. These VLPs are used to infect fresh cultures that are superinfected with helper virus (HRSV). Reporter gene activity, coming from the minigenome encapsidated in the VLPs, is assayed in extracts of the infected cells, made after 1-2 days in culture. See text for details.

L and 22k proteins under the transcriptional control of the T7 promoter, the antigenome is replicated to yield copies of the viral genome that can be transcribed to generate the different gene products thus ensuring virus replication. This system provides the means to introduce predetermined changes into infectious virus via the cDNA intermediate and to test their effects on virus replication and phenotype.

A simplified model system of reverse genetics is based on minireplicons, which are short cDNA versions of the genome or antigenome in which most (or all) of the viral genes have been replaced by a reporter gene (Grosfeld et al., 1995), or shorter RNA analogues (Yu et al., 1995) (Fig. 3B). As with the full-length cDNA clones,

transfection of plasmids carrying the minireplicon into cells expressing the T7 polymerase generates minigenomes or antiminigenomes that can be amplified by superinfection with wild-type helper virus (Grosfeld et al., 1995), or by providing the support plasmids encoding N, P, L and 22k proteins under control of a T7 promoter (Grosfeld et al., 1995; Yu et al., 1995). Transcription and amplification of the minireplicon can be assessed by biochemical tests (Yu et al., 1995) or indirectly by measuring the reporter gene activity (Grosfeld et al., 1995).

When cells transfected with the plasmid carrying the minireplicon are provided with plasmids encoding the proteins required for virus maturation (M, F and G), besides the proteins required for transcription and replication (N, P, L and 22k), virus-like particles (VLPs) are formed that are released into the culture supernatant (Teng and Collins, 1998). The presence of VLPs is detected by passage of the transfected cell supernatants into fresh cells which are superinfected with helper virus. The expression of the reporter gene is assayed in these newly infected cells. The minireplicon systems (Fig. 3B) are widely used as rapid methods to identify *cis*-acting sequences that regulate HRSV transcription and/or replication and to study the effect of certain mutations upon the functional properties of specific gene products.

Besides the reverse genetics systems described above, some *in vitro* assays have been developed that reproduce certain steps of the HRSV replicative cycle in the test tube. For instance, synthesis of viral mRNAs has been reproduced, at least to a certain extent, using crude extracts of HRSV-infected cells (Barik, 1992) or partially purified nucleocapsids (Mason et al., 2004). These *in vitro* assays provide an opportunity to set up high throughput systems to search for inhibitors that may have a prophylactic/therapeutic use (Liuzzi et al., 2005).

Finally, the last system to be mentioned here is the expression of individual proteins in tissue culture cells. In this way, their effects on cell metabolism and/or cell behaviour can be assessed independently of other viral gene products. Examples of this approach include the expression of NS1 and NS2 proteins in transfected cells that decreases Stat2 levels and the consequent downstream interferon-α/β response (Lo et al., 2005), or the expression of the F protein which leads to cell–cell fusion and syncytia formation (González-Reyes et al., 2001).

Here, follows a description of the different steps of the HRSV replicative cycle and the viral products that participate in each step.

Virus entry

Virus entry by enveloped viruses has been a topic of intensive investigation in recent years since this step occurs outside the host cell, and consequently is more accessible to inhibition by antiviral candidates than later steps of the infectious cycle that occur inside the infected cell. Two steps are well differentiated in this process: (i) binding of the virus to certain cell surface components and (ii) fusion of the virus and cell membranes at the cell surface. The HRSV G and F glycoproteins mediate these two steps, respectively, although G is not required for infection of certain cell types in tissue culture (see later).

The G glycoprotein

This protein is produced in two different forms in the infected cell: (i) as a type II transmembrane protein (Gm) that is incorporated into virions and (ii) as a soluble protein (Gs) that is secreted by the infected cells (Hendricks et al., 1987, 1988) (Fig. 4). The Gm polypeptide precursor of about 300 amino acids (depending on the strain) contains a single hydrophobic domain (residues 38–63) that acts as a combined signal and a transmembrane anchor domain. This hydrophobic region targets the nascent chain, as it emerges from the ribosome, to the endoplasmic reticulum (ER) and ensures translocation of the polypeptide chain across the membrane, bringing about the stable anchoring of the protein in the lipid bilayer. Gm has neither sequence nor structural homology with the attachment protein of other paramyxoviruses (Wertz et al., 1985).

The Gm polypeptide precursor is extensively modified by the addition of both N- and O-linked oligosaccharides and is also palmitylated, probably at a single cysteine residue located in the N-terminal cytoplasmic tail (Collins and Mottet, 1992). High-mannose N-linked sugar chains are co-translationally added to the G protein precursor to yield intermediate species of 40–50 kDa (Wertz et al., 1989; Collins and Mottet, 1992). This step is followed by the conversion of the N-linked sugars into the complex type and addition of O-linked sugars in the Golgi compartment before reaching the plasma membrane. These modifications convert the 32 kDa precursor into a mature protein of 80–90 kDa, as estimated by SDS–PAGE. Although this technique does not provide a very accurate estimate of the molecular mass of glycoproteins, due to the abnormal interaction of oligosaccharides with SDS, the large difference in the electrophoretic mobility between the precursor and the mature protein highlights the contribution of carbohydrates to the mass of the mature Gm.

The C-terminal Gm ectodomain has a central region (amino acids 164–176) and four cysteines (residues 173, 176, 182 and 186), which are, conserved in all HRSV isolates (Johnson et al., 1987). Disulfide bridges occur between Cys173 and 186, and between Cys176 and 182, resulting in a cystine noose motif which resembles the structure found in the 55 kDa tumour necrosis factor receptor (Doreleijers et al., 1996; Langedijk et al., 1996, 1998). Flanking this region, there are two protein segments that have a high level of sequence variation among HRSV isolates. While the conserved region is essentially devoid of carbohydrates, the variable regions have several potential sites for N-glycosylation and multiple serines and threonines which are predicted to be O-glycosylated by the NetOglyc software (Hansen et al., 1998). Both, N- and O-linked carbohydrates are found in the mature Gm. The variable regions have overall amino acid composition similar to that of the mucins secreted by epithelial cells, which are also extensively modified by the addition of O-linked sugars (Apostopoulos and McKenzie, 1994; García-Beato et al., 1996).

Fig. 4 shows a model for the three-dimensional (3-D) structure of the Gm molecule, modified from the one proposed by Langedijk et al. (1996). Although illustrated as a dimer, native Gm is probably a homotetramer, as inferred from its sedimentation behaviour in sucrose gradients (Escribano-Romero et al., 2004).

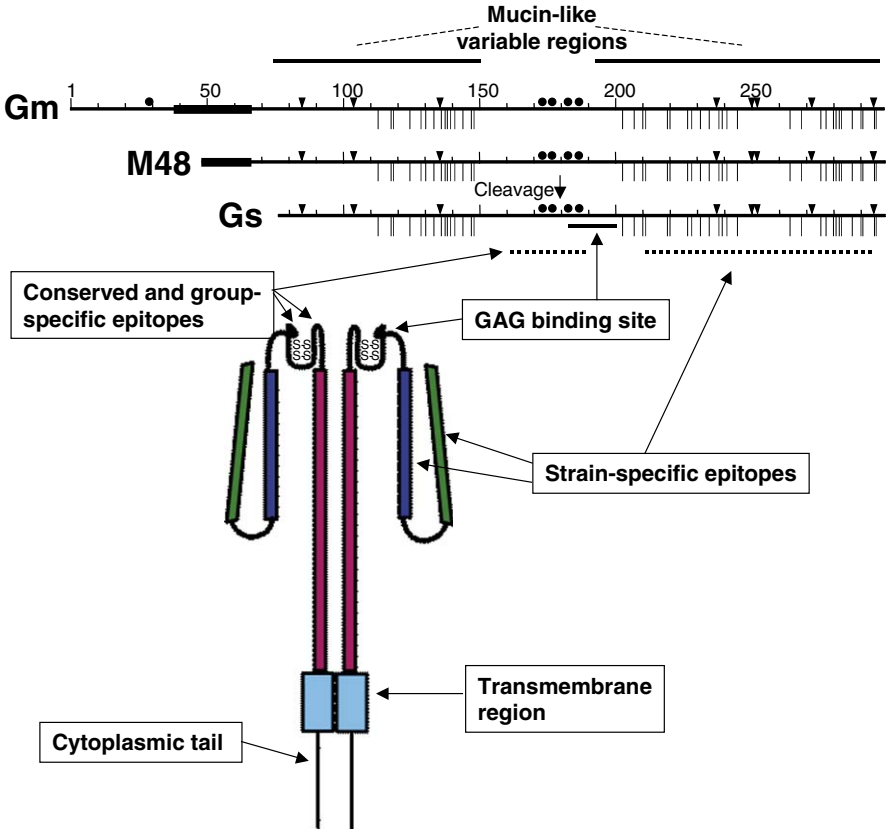

Fig. 4 Scheme of the G protein of HRSV. A straight line of 298 amino acids denotes the Gm polypeptide of the HRSV Long strain. The hydrophobic transmembrane region is indicated by a thick solid line (residues 38–66). The potential N-glycosylation sites (▼), the O-glycosylation sites predicted with the NetOGlyc software (|) (Hansen et al., 1998) and the cysteines (●) are also indicated. Formation of Gs occurs by translation initiation at Met48, and subsequent cleavage after residue 65 (Roberts et al., 1994). A model of the 3-D structure of the mature Gm molecule is depicted in the lower part of the figure. Although Gm is likely a tetramer (Escribano-Romero et al., 2004), it is represented as a homodimer for simplicity. Several structural motifs are denoted in the model and in the primary structure.

Several structural domains can be identified in each protein monomer. The first N-terminal 37 amino acids represent the cytoplasmic tail and the following 26 residues the transmembrane region. The first hypervariable region, preceding the cysteine cluster, probably adopts a rod-like structure due to the presence of multiple, closely spaced, O-linked sugar chains that leads to elongation of the polypeptide backbone (Jentoft, 1990). The cystine noose, made up of the conserved Gm region, including the cluster of four cysteines, follows this region. The 3-D structure of the cystine noose was determined by nuclear magnetic resonance of a 19 amino acid core, within a 32-residue peptide corresponding to amino acids

158–189 (Doreleijers et al., 1996). The cystine noose core structure has a relatively flat surface formed by two short α-helices connected by a type I′ turn. A characteristic hydrophobic pocket, lined by conserved residues, lies at the surface of the cystine noose motif. This hydrophobic pocket was proposed tentatively to act as a receptor-binding site (Johnson et al., 1987), although there is still a lack of experimental evidence to support this hypothesis. However, a short amino acid segment near the cystine noose (residues 184–198) has been implicated in binding of Gm to cell surface glycosaminoglycans (GAGs) (Feldman et al., 1999). This type of structural arrangement places the site of interaction of Gm with cell-surface components (GAGs) at an appropriate distance from the transmembrane region that is inserted in the viral envelope. In agreement with its conserved nature, the central segment of the Gm ectodomain contains epitopes that are either maintained in all HRSV isolates (conserved epitopes) or are shared by viruses of the same antigenic group (group-specific epitopes) (Martínez et al., 1997; Melero et al., 1997).

The second C-terminal hypervariable region of the Gm molecule, which follows the cluster of cysteines, is externally located in the 3-D model of Fig. 4, indicating its accessibility to antibodies that recognize strain-specific epitopes and that map in this region of the molecule. Cell-type specific glycosylations influence the expression of epitopes located in this hypervariable region (Palomo et al., 1991, 2000; García-Beato et al., 1996). Notably, this region, in particular the first half, is partially resistant to protease degradation. In addition, cell-specific glycosylations in the C-terminal half of this region influence the expression of certain epitopes in its N-terminal half, suggesting interactions between the two halves of the C-terminal hypervariable region of Gm (García-Beato et al. 2000), as denoted in Fig. 4.

Interestingly, none of the anti-G monoclonal antibodies described to date are highly neutralizing; however, either polyclonal antiserum raised against purified G protein, pools of anti-G monoclonal antibodies (Martínez and Melero, 1998) or an anti-anti-iditotypic antiserum raised against an anti-G monoclonal antibody (Palomo et al., 1990) exhibit enhanced neutralization. These results suggest that inhibition of HRSV infectivity by anti-G antibodies may be determined by steric hindrance of virus interactions with some component of the cell surface.

The soluble form of the G glycoprotein (Gs) is synthesized by alternative translation initiation by the ribosomes at a second in-frame AUG codon, which lies within the signal/anchor domain of the G protein reading frame (Fig. 4). The N-terminal hydrophobic signal peptide is then proteolytically processed following residue 65 (Roberts et al., 1994). The soluble Gs is transported from the lumen of the ER to the exterior of the cell via the exocytic pathway. Secreted Gs remains monomeric, as assessed by sucrose gradient sedimentation, suggesting that the transmembrane region of Gm is required for oligomerization (Escribano-Romero et al., 2004). Despite the differences between Gs and Gm in the oligomerization state, the former protein is also heavily glycosylated by N- and O-linked oligosaccharides and both forms of G are indistinguishable when tested for reactivity with an extensive panel of monoclonal antibodies. Thus, Gs can be considered the monomeric form of the Gm ectodomain.