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Generation of monoclonal antibodies specific of the postfusion conformation of the Pneumovirinae fusion (F) protein

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A B S T R A C T

Paramyxovirus entry into cells requires fusion of the viral and cell membranes mediated by one of the major virus glycoproteins, the fusion (F) glycoprotein which transits from a metastable pre-fusion conformation to a highly stable post-fusion structure during the membrane fusion process. F protein refolding involves large conformational changes of the protein trimer. One of these changes results in assembly of two heptad repeat sequences (HRA and HRB) from each protomer into a six-helix bundle (6HB) motif. To assist in distinguishing pre- and post-fusion conformations of the Pneumovirinae F proteins, and as extension of previous work (Palomo et al., 2014), a general strategy was designed to obtain polyclonal and particularly monoclonal antibodies specific of the 6HB motif of the Pneumovirinae fusion protein. The antibodies reported here should assist in the characterization of the structural changes that the F protein of human metapneumovirus or respiratory syncytial virus experiences during the process of membrane fusion.

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1. Introduction

Human metapneumovirus (hMPV) and human respiratory syncytial virus (hRSV) are prototypes of the Metapneumovirus and the Pneumovirus genus of the Pneumovirinae subfamily within the Paramyxoviridae family, respectively (for a recent review, Collins and Karron, 2013). hRSV is the most important cause of acute lower respiratory tract infections (ALRI) in infants worldwide (Hall et al., 2009; Nair et al., 2010) and also a common cause of ALRI in the elderly and adults with cardiopulmonary disease or in whom immune responses are impaired or reduced (Whimbey and Ghosh, 2000; Falsey et al., 2005). Although less is known about hMPV, it is also recognized as a pathogen of clinical relevance, only second to hRSV as a cause of ALRI (Schuster and Williams, 2013). Two subtypes of hMPV (A and B) have been identified by comparison of sequences and by antigenic analysis of viral strains (van den Hoogen et al., 2004).

As in other paramyxoviruses, both hMPV and hRSV encode two major glycoproteins that are inserted in the lipid viral envelope (Collins and Melero, 2011). One is the attachment glycoprotein (G), responsible for the initial interaction of the virus with the target cell surface. The other is the fusion (F) glycoprotein that mediates fusion of the viral and cell membrane, facilitating virus entry (Walsh and Hruska, 1982).

The Pneumovirinae F glycoprotein is a homotrimer in which each subunit is synthesized as an inactive precursor that needs to be cleaved to become fusion competent (Gonzalez-Reyes et al., 2001; Zimmer et al., 2001). The hMPV precursor is slightly shorter (539 aa) than its hRSV counterpart (574 aa) due to a variance of their respective proteolytic processing pathways. While hMPV_F, as in other paramyxoviruses is cleaved at a single site placed immediately upstream of a hydrophobic region, called fusion peptide, hRSV_F is cleaved twice at two polybasic sites separated by a 27 amino acid peptide (Gonzalez-Reyes et al., 2001; Zimmer et al., 2001) (Fig. 1). The cleavage site of hRSV_F immediately preceding the hydrophobic fusion peptide is equivalent to the cleavage site of other paramyxovirus F proteins. Once cleavage of hRSV_F is completed, the intervening 27 amino acid peptide is released from the mature protein (Begona Ruiz-Arguello et al., 2002). While hRSV_F is cleaved by furin mainly during transport to the cell membrane, probably in the trans-Golgi network (Collins and Mottet, 1991), hMPV_F is cleaved at the cell surface or in the virus particle by extracellular proteases (Shirogane et al., 2008). In both cases, cleavage generates two chains (F2 end terminal to F1) that remain covalently

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linked by two disulfide bridges. Hence, the mature *Pneumovirinae* F protein is a homotrimer of F1 + F2 subunits.

The F trimer is incorporated into the virus particle in a prefusion metastable conformation. Once the incoming virus is bound to the surface of the target cell, the F protein is activated by still ill-defined mechanisms to initiate a series of conformational changes, including the formation and refolding of a pre-hairpin intermediate by which the viral and cell membranes are brought into proximity. Final refolding of F in a stable postfusion conformation leads to merging of the two membranes and formation of the fusion pore (Lamb and Jardeetzky, 2007). Three-dimensional structures of soluble hRSV, F ectodomains stabilized in either the prefusion (McLellan et al., 2013a, 2013b) or postfusion conformations (McLellan et al., 2011; Swanson et al., 2011) have been recently solved, providing crucial information to understand the conformational changes that the F protein experiences during membrane fusion. One of these changes involves refolding of HRA and HRB sequences of each F1 subunit in a highly stable coiled-coil structure, dubbed the 6-helix bundle motif (6HB).

Conformation specific antibodies would be very useful reagents to monitor the transition of *Pneumovirinae* F from the prefusion to postfusion conformation in structural or functional studies. We have reported previously the generation of rabbit antibodies specific for the 6-HB of hRSV F (Palomo et al., 2014) and the serendipitous isolation of a rabbit monoclonal antibody (MAB) specific of this structural motif. We now describe a systematic approach for the isolation of murine MAbs specific of the 6HB which recognize hMPV F or hRSV F folded in their respective postfusion conformations. We propose that this approach could be extended to isolate postfusion specific MAbs directed against the so-called type I fusion proteins of several virus families.

2. Materials and methods

2.1. Ethics statement

Animal work complied with Spanish and European legislation concerning vivisection and the use of genetically modified organisms. Protocols were approved by the “Comité de Ética de la Investigación y del Bienestar Animal” of “Instituto de Salud Carlos III” (CBA PA 19, 2012).
2.2. Cloning and expression of HRA, HRB and HRA-L-HRB sequences of hMPV-F fused to glutathione-S-transferase

Sequences encoding amino acids 130–176 of hMPV-F (NL/1/00 strain, subtype A1, GenBank accession number AA12968, cloned in the pRB21 plasmid), corresponding to the HRA domain plus a C-terminal linker GSGGSG (see Fig. 2A) were amplified by PCR using the following primers: forward 5′-GCGCACTCCTGAAAGTGAAGTCGACCAACAAACA-3′ (underlined is the Bam HI site used for cloning) and reverse 5′-GCGCACTCCTGAAAGTGAAGTCGACCAACAAACA-3′ (the Sal I site is underlined and the sequence encoding the GSGGSG linker is shown in italics). Similarly, F gene sequences encoding amino acids 479–520 of the HRA domain were amplified using the primers: forward 5′-GCGCACTCCTGAAAGTGAAGTCGACCAACAAACA-3′ (underlined is the Bam HI site) and reverse 5′-GCGCACTCCTGAAAGTGAAGTCGACCAACAAACA-3′ (underlined is the Not I site). The amplified DNAs were digested with the indicated enzymes and cloned into the pGEX-5X-3 plasmid (GE Healthcare). The resulting constructs had the heptad repeat sequences linked through a factor Xa cleavage site and the tripeptide GIL (as described in Methods). The plasmids encoding GST-HRA-L-HRB (see Fig. 2A) were obtained by inserting amplified HRB sequences, flanked by Sal I and Not I sites into pGEX-5X-3-HRA-L.

The different GST-fusion proteins were expressed in E. coli BL21 grown at 37 °C in autoinducible Overnight Express Instant TB Medium (Novagen). Bacteria were collected by sedimentation, resuspended in PBS with 5 mM dithiothreitol (DTT) and lysed by sonication. After clarification, the bacterial extracts were added to Glutathione-Sepharose 4 Fast Flow (GE Healthcare) and incubated overnight at 4 °C in a mixing rotor. After washing with PBS, the bound GST chimeric proteins were eluted with 50 mM Tris–HCl, pH 8.0, 10 mM glutathione.

2.3. Generation of polyclonal anti-six-helix bundle (α-6HB) antibodies

New Zealand white rabbits were inoculated intradermally at multiple sites with 100 μg of purified GST-HRA-L-HRB from hMPV-F, emulsified with an equal volume of Freund’s complete adjuvant. After three weeks, rabbits were bled repeatedly.

To deplete the antibodies able to recognize either GST or HRA-L or HRB, two affinity columns were made with 10 mg each of purified GST-HRA-L or GST-HRB covalently bound to 1 g of CNBr-activated Sepharose beads following the manufacturer’s instructions (GE Healthcare). The beads were washed and equilibrated in PBS. Sera of rabbits inoculated with GST-HRA-L-HRB were loaded first onto the GST-HRA-L-Sepharose column. The unbound material was collected and loaded onto the GST-HRBSepharose column (Fig. 2D). Unbound antibodies (dubbed α-6HB) were collected and stored at −20 °C until used.

2.4. Purification of soluble F proteins

The recombinant vaccinia virus Vac/FIM- which encodes a soluble form of the hRSV-F protein ectodomain (Long strain), generated by the change ile525Stop (ATC to TAA) has been described (Bembridge et al., 1999). Vaccinia viruses expressing the hMPV-F and hRSV-F variants depicted in Fig. 1 were obtained by the method of Blasco and Moss (Blasco and Moss, 1995) as described before.

Fig. 2. Generation of α-6HB polyclonal antisera against hMPV-F: (A) Scheme of the hMPV-F protein primary structure (as shown in Fig. 1A). The HRA (red), HRB (blue) and HRA-L-HRB sequences that were fused to GST are indicated below. Note that HRA is followed by a linker sequence (L, black) encoded by one of the primers used in the amplification. (B) Coomassie blue stained SDS-PAGE gel of the purified proteins in each lane. (C) Serial dilutions of a rabbit serum (abscissa) raised against purified GST-HRA-L-HRB were tested for binding by direct ELISA to either GST or the indicated chimeric proteins. Dotted lines represent preimmune serum binding. (D) Diagram of the depletion protocol used to obtain α-GST-HRA-L-HRB specific antibodies. Rabbit serum was first loaded onto a column of GST-HRA-L bound to Sepharose (red beads). The unbound material was loaded onto a second column of GST-HRIB (blue beads) and unbound antibodies (tentatively named α-6HB) were collected and saved. (E) Direct ELISA binding of α-6HB antibodies to either GST or the indicated proteins. ELISA results of this and other figures are representative of at least three independent assays.
in detail (Brembridge et al., 1998). The different proteins were purified from supernatants of CV1 cells infected for 48 h with the different vaccinia recombinants (0.1 pfu/cell). Culture supernatants were concentrated and buffer exchanged using Vivaflow membranes (Sartorius); then, loaded onto Ni\textsuperscript{2+} columns in 50 mM Na\textsubscript{2}HPO\textsubscript{4}, pH 8.0, 300 mM NaCl, 10 mM Imidazole buffer and, after washing, proteins were eluted with the same buffer containing 250 mM imidazole. Finally, the proteins were concentrated with Vivaspin and exchanged to buffer without imidazole before being loaded onto a HiLoad 16/600 Superdex 200 pg gel filtration column (GE Healthcare) equilibrated and eluted with the same buffer.

### 2.5. Mouse immunization and isolation of α-6HB monoclonal antibodies (MAbs)

Six-eight week old female BALB/c mice were inoculated intramuscularly (im) with 20 μg of either hMPV\textsubscript{F} or hRSV\textsubscript{F}, purified and stabilized as soluble post-fusion proteins, as described in the previous section. Four weeks later, mice were boosted im with 20 μg of purified GST-HRA-L-HRB. Four days later, mice were sacrificed by ISOVA inhalation and their splenocytes fused to Sp2-0 myeloma cells with PEG4000, as described (Sanchez-Fauquier et al., 1987). Hybridomas were selected in Clonacell medium supplemented with HAT (hypoxanthine, aminopterin and thymidine) components and after five days transferred to the same medium supplemented with HT. Antibody production was tested by ELISA of hybridoma supernatants as indicated below. Positive cultures were recloned at least twice by limiting dilution.

### 2.6. Enzyme linked immunosorbent assay

**Direct ELISA:** 96 well plates were coated overnight at 4°C with a predetermined amount of antigen, as indicated in the Figure legends. Non-specific binding was blocked for 1 h with 0.5% serum albumin (BSA) in PBS. Antibody or serum serial dilutions were then added to the plates for 1 h at room temperature and unbound antibodies were removed by washing five times with water. Antibody binding was revealed by incubation with peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (GE Healthcare) and subsequent addition of OPD (Sigma) as substrate as per manufacturer’s instructions.

**Sandwich ELISA:** Wells were coated overnight with purified MAbs and unspecific binding blocked as indicated before. Subsequently, serial dilutions of His-tag proteins were added to the wells. After washing, bound proteins were revealed with an excess of anti-His-Biotin, Streptavidin-horseradish peroxidase antibody and OPD.

### 2.7. Electron microscopy

Purified proteins were applied to glow-discharged carbon-coated grids and negatively stained with 1% aqueous uranyl formate. Micrographs were recorded with a Gatan ERLANGHEN 1000 W CCD camera in a Jeol JEM-1011 electron microscope operated at 100 kV at a nominal magnification of 20,400×.
3. Results

3.1. Purification of soluble F proteins stabilized in different conformations

Fig. 1 shows a diagram of the primary structures of hMPV,F (panel A, line 1) and hRSV,F (panel B, line 1). The different proteins used in this study were produced by using vaccinia virus recombinants, made as described (Blasco and Moss, 1995; Bembridge et al., 1998). A monomeric form of hMPV,F (Fig. 1A, line 2) was obtained by expressing the protein ectodomain followed by a 6-His tag, added for purification purposes. Stabilization of a trimeric postfusion soluble form of the same protein required insertion of the foldon trimerization domain (Meier et al., 2004) before the 6-His tag. In addition, the hMPV,F cleavage site was replaced with the polybasic cleavage site II of hRSV to facilitate proteolytic processing without added trypsin and the first eight amino acids of the fusion peptide were deleted to avoid aggregation of the trimeric postfusion hMPV,F (Fig. 1A, line 3).

The equivalent monomeric form of hRSV,F (Fig. 1B, line 2) was similarly produced, except that cleavage of the monomer was avoided by changing the basic residues of the two furin cleavage sites to Asn (Begona Ruiz-Arguello et al., 2002). However, in the case of hRSV,F and in contrast to hMPV,F, about half of the protein eluted from the gel filtration chromatography as a trimer. The monomeric peak was collected and loaded again onto a second gel filtration column to obtain the profile shown at right of Fig. 1B. To increase the yield of trimeric postfusion hRSV,F, the foldon domain was added at the C-terminus of the ectodomain, before the 6-His tag (Fig. 1B, line 3). Finally, a stabilized prefusion form of hRSV,F (Fig. 1B, line 4) was made by incorporating the mutations described by McLellan et al. for their DS-Cav1 protein (McLellan et al., 2013a). Prefusion hMPV,F could not be made since equivalent stabilizing mutations have not been described yet. All proteins were purified by using Ni²⁺ columns followed by gel filtration chromatography. They were quality checked by SDS-PAGE (Fig. 1) and electron microscopy (Fig. 5) and in the case of hRSV,F by reactivity with conformation specific MAbs (Fig. 1B). MAb D25 has been reported to be specific of the prefusion conformation of hRSV,F (McLellan et al., 2013b) whereas R145 was found to be specific of the postfusion conformation of the same molecule (Palomo et al., 2014).

3.2. Isolation and characterization of α-6HB polyclonal antibodies specific of hMPV,F

Based on a previous report describing cloning and expression in E. coli of hRSV,F heptad repeat sequences fused to GST (Palomo et al., 2014), equivalent constructs were made with sequences of hMPV,F (strain NL/1/00, subtype A1), as shown in Fig. 2A. The corresponding proteins, purified by affinity chromatography showed a major band of the expected size after Coomassie staining of SDS-PAGE gels (Fig. 2B), except GST-HRA-L which contained an additional band co-migrating with GST, probably generated by spontaneous cleavage at the GST-HRA linkage during the purification process.

New Zealand white rabbits were then immunized with purified GST-HRA-L-HRB and the immune serum was tested by ELISA for reactivity with either the immunogen or with its purified moieties;
i.e., GST-HRA-L,GST-HRB or GST. The results obtained (Fig. 2C) demonstrated the presence of antibodies that reacted with each of the antigens included in the ELISA.

To enrich for antibodies that required both heptad repeats for binding, the anti-GST-HRA-L-HRB antiserum was passed successively through Sepharose columns that contained covalently bound either GST-HRA-L or GST-HRB (Fig. 2D). The antibodies that did not bind to either column were collected and tested again in ELISA with the same antigens. Although the antibodies so depleted lost binding to GST, GST-HRA-L or GST-HRB, they still maintained most of the reactivity with GST-HRA-L-HRB (Fig. 2E) and were tentatively dubbed anti-six-helix bundle (α-6HB) antibodies, by analogy to equivalent antibodies previously described for hRSV_F (Palomo et al., 2014).

3.3. Isolation and characterization of murine MAbS specific for the α-6HB motif of hMPV_F

Once it was demonstrated that the GST-HRA-L-HRB protein could induce antibodies that required the two F protein heptad repeats for binding, mice were first immunized with purified hMPV_F stabilized in the postfusion conformation and then with GST-HRA-L-HRB to boost production of antibodies specific for the 6-HB motif. Hybridoma supernatants derived from mouse splenocytes were screened in parallel by ELISA for antibodies binding to both postfusion F and GST-HRA-L-HRB. Three hybridomas that produced such antibodies were selected and cloned twice by limiting dilution. The specificity of the corresponding antibodies was finally confirmed as shown in Fig. 3. Fig. 3A demonstrates that the three MAbs MF1, MF2 and MF3 bound efficiently to GST-HRA-L-HRB but not to any of the individual moieties, GST, GST-HRA-L or GST-HRB. Furthermore, the three MAbS efficiently reacted with postfusion soluble trimers of hMPV_F derived from either NL/1/00 (A1 subtype) or NL/1/99 (B1 subtype) strains but not with the monomeric form of the latest protein. As controls, a polyclonal anti-GST (α-GST) antiserum and an anti-hMPV_F MAb (MF14) were included in Fig. 3 to demonstrate reactivity of GST and monomeric hMPV_F, respectively. MF14 antibody (to be described elsewhere) behaves with hMPV_F as palivizumab (Johnson et al., 1997) does with hRSV_F (see Fig. 4B); i.e., both antibodies recognize epitopes that are shared by their respective monomeric and trimeric proteins.

The results of Fig. 3 hence demonstrate that the MAbS MF1, MF2 and MF3 bind specifically to the HRA-L-HRB motif which is reproduced in the post-fusion F trimer of the two strains tested but not in the protein monomer.

3.4. Isolation and characterization of MAbS specific for the α-6HB motif of hRSV_F

We have previously shown that GST-chimeric proteins with heptad repeat sequences from hRSV_F were capable of inducing rabbit polyclonal antibodies equivalent to those shown in Fig. 2 for hMPV_F (Palomo et al., 2014). Hence, a similar strategy to that described in the previous section was used to obtain murine MAbS that reacted with both post-fusion hRSV_F and GST-HRA-L-HRB. Three hybridomas were thus isolated which produced MAbS 114F, 116F and 117F. These three MAbS specifically bound GST-HRA-L-HRB but not any of its moieties (GST, GST-HRA-L or GST-HRB) (Fig. 4A). Interestingly, MAb 114F was able to bind a previously described double mutant of GST-HRA-L-HRB (SS90/S513C) which could not bind rabbit MAb R145 nor the new murine MAbS 116F and 117F. Therefore, the three murine MAbS described in this study recognize at least two different, although probably highly overlapping epitopes. Additionally, the three MAbS bound soluble hRSV_F stabilized in the post-fusion conformation but they did not react with either the pre-fusion conformation or monomeric F (Fig. 4B). Hence,

![Fig. 5. Electron microscopy of proteins and immune complexes](image)

binding of MAbS 114F, 116F and 117F to hRSV_F is fully dependent on folding of the protein trimer into the postfusion conformation.

3.5. Electron microscopy

To visualize binding of the MAbS described in the two previous sections to their corresponding F protein trimers, they were incubated with either soluble hMPV_F or hRSV_F stabilized in their respective post-fusion conformations and negatively stained before being observed by electron microscopy. Fig. 5 shows representative images of the purified proteins with the characteristic cone shape of post-fusion trimers and selected pictures of protein-antibody complexes. The MAbS bound to the protein stem end (i.e., distal from the protein head) where the 6-HB motif is located, confirming the MAb specificity inferred from the ELISA binding results.

4. Discussion

As reported in our previous publication (Palomo et al., 2014), immunization of rabbits with GST chimeras expressing HRA-L-HRB sequences derived from either hMPV_F or hRSV_F induced high levels of polyclonal antibodies that require both heptad repeats for binding. Although HRA can assemble by itself in a coiled-coil helix trimer, the α-6HB antibodies required incorporation of HRB to the inner core for binding, reflecting the structure acquired when the two heptad repeats are mixed together (Lawless-Delmedico et al., 2000; Matthews et al., 2000). These binding requirements are
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