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Individual contributions of the human metapneumovirus F, G, and SH surface glycoproteins to the induction of neutralizing antibodies and protective immunity

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Abstract

We evaluated the individual contributions of the three surface glycoproteins of human metapneumovirus (HMPV), namely the fusion F, attachment G, and small hydrophobic SH proteins, to the induction of serum HMPV-binding antibodies, serum HMPV-neutralizing antibodies, and protective immunity. Using reverse genetics, each HMPV protein was expressed individually from an added gene in recombinant human parainfluenza virus type 1 (rHPIV1) and used to infect hamsters once or twice by the intranasal route. The F protein was highly immunogenic and protective, whereas G and SH were only weakly or negligibly immunogenic and protective, respectively. Thus, in contrast to other paramyxoviruses, the HMPV attachment G protein is not a major neutralization or protective antigen. Also, although the SH protein of HMPV is a virion protein that is much larger than its counterparts in previously studied paramyxoviruses, it does not appear to be a significant neutralization or protective antigen. Published by Elsevier Inc.

Keywords: Human metapneumovirus; Neutralizing antibodies; Protective immunity; Glycoprotein

Introduction

Human metapneumovirus (HMPV), which was first described in 2001 (van den Hoogen et al., 2001), is a member of the Paramyxoviridae family and has been assigned to the *Metapneumovirus* genus of the Pneumovirinae subfamily. This subfamily also includes the *Pneumovirus* genus containing respiratory syncytial virus (RSV). Like RSV, HMPV has been found to infect humans worldwide and is a significant cause of severe respiratory disease in infants and young children and can infect and cause disease in older individuals as well (Boivin et al., 2003; Osterhaus and Fouchier, 2003; Peret et al., 2002). Like all members of the Paramyxovirus family, HMPV is an enveloped, single-stranded, negative-sense RNA virus. The genome is approximately 13 kb in length, and the HMPV 3′ to 5′ gene order is N-P-M-F-M2-1/M2-2-SH-G-L, which is the same as that of the avian members of the *Metapneumovirus* genus (Biacchesi et al., 2003; van den Hoogen et al., 2002).

Sequence analysis of HMPV isolates indicates that there are two major genetic lineages (Bastien et al., 2003; Biacchesi et al., 2003) that are highly related antigenically (Skiadopoulos et al., 2004), and the fusion F surface glycoprotein has been identified as a major cross-protective antigen (MacPhail et al., 2004; Skiadopoulos et al., 2004). In addition to the F protein, all paramyxoviruses also have a separate surface glycoprotein that is involved in attachment and is called the G glycoprotein (subfamily Pneumovirinae), the hemagglutinin H glycoprotein (genus *Morbillivirus*), or the hemagglutinin–neuraminidase HN glycoprotein (all other members of subfamily Paramyxovirinae). For other paramyxoviruses examined to date, the F and G/H/HN surface glycoproteins are the only significant neutralization antigens and are major independent protective antigens (Chanock et al., 2001). HMPV virions appear to have three surface glycoproteins, namely the F (539 amino acids in length for strain CAN83), G (219 amino acids), and small hydrophobic

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SH (179 amino acids) glycoprotein. The HMPV SH protein is much larger than its RSV counterpart (64–65 amino acids). All three glycoproteins are incorporated into HMPV virions (Biacchesi et al., 2004).

In the present study, we examined the relative contribution of each of the three HMPV glycoproteins to inducing HMPV-binding antibodies, HMPV-neutralizing antibodies, and protection against HMPV challenge. Recombinant human parainfluenza virus type 1 (rHPIV1) vectors were constructed that individually express each of the HMPV surface glycoproteins. These were used to immunize hamsters by the intranasal route with either a single inoculation or with an additional booster inoculation. Evaluation of the relative titers of serum antibodies and protection conferred by each of the vectored HMPV glycoproteins showed that the F glycoprotein was the major contributor to induction of serum neutralizing antibodies and protective immunity. Neither the G nor the SH protein induced a detectable level of serum neutralizing antibodies, and the contributions of G and SH to protection were minor or negligible, respectively.

Results

Recovery of rHPIV1s containing an additional gene encoding one of the HMPV surface glycoproteins

The relative contribution of each of the three HMPV glycoproteins to the immunogenicity of HMPV was examined by separately expressing each protein from an additional gene unit inserted into wild-type rHPIV1 by reverse genetics. The full-length antigenomic HPIV1 cDNA was modified by insertion of an additional gene unit consisting of the open reading frame (ORF) for the HMPV strain CAN83 G or SH protein flanked by HPIV1 transcription gene-start and gene-end signal sequences (Fig. 1), as previously described for the F gene insert (Skiadopoulos et al., 2004). cDNA constructs with the G or SH ORF inserted into the pre-N ORF position of the HPIV1 genome were unstable in bacteria; therefore, gene units expressing these ORFs were inserted between the P and M ORFs (Fig. 1), a position that has been shown to also support a high level of expression in recombinant HPIV3 (Durbin et al., 2000; Skiadopoulos et al., 2002). Viruses were readily recovered and grew to high titer in vitro. The growth kinetics of the rHPIV1s carrying each additional gene unit were examined by multi-cycle growth in LLC-MK2 cells (Fig. 2). The rHPIV1 vectors bearing the G or SH gene unit grew to a similar titer and at a similar rate as the rHPIV1 backbone virus. Surprisingly, the rHPIV1-F grew at a moderately slower rate and to a lower peak virus titer than either the backbone virus or the vector carrying the G or SH gene. The basis for the reduced rate of growth for rHPIV1-F is not known but might reflect the insert's position in the genome (von Messling et al., 2004), larger insert size (Skiadopoulos et al., 2000), or the biological activity of the F protein, which is fusogenic on its own without the presence of its cognate attachment protein (Biacchesi et al., 2004).

Expression of the HMPV mRNAs and glycoproteins in vitro

To determine the relative level of mRNA produced by each of the expression vectors in cell culture, Northern blot analysis was performed on total intracellular RNA using dsDNA probes against the HPIV1 HN mRNA (Fig. 3, panel A) and the HMPV F, G, and SH mRNAs (panels B, C, and D). This showed that the rHPIV1-F, -G, and -SH viruses expressed the HPIV1 HN mRNA at approximately comparable levels, although that of the rHPIV1-F virus was somewhat reduced, consistent with its reduced growth efficiency. Each respective HMPV mRNA was expressed at a level that exceeded that present in HMPV-infected cells (Fig. 3).

Expression of the HMPV glycoproteins from the respective rHPIV1 vector was examined by indirect immunofluorescence of infected LLC-MK2 monolayers, and each of the HMPV glycoproteins was found to localize in the cell membrane (data not shown), as expected for these surface glycoproteins. Furthermore, sucrose purified rHPIV1-G was found to contain a high level of HMPV G protein incorporated into the viral particles (data not shown). Incorporation of the HMPV F or SH glycoproteins into rHPIV1 virus particles could not be detected; however, SH protein produced from the rHPIV1-SH expression vector was incorporated into HMPV Δ-SH (an HMPV mutant containing a deletion of the SH ORF; Biacchesi et al., 2004) virus particles in a co-infection of LLC-MK2 cells (data not shown). Western blot analysis was performed to determine the level of expression in cell culture of each HMPV glycoprotein by its respective rHPIV1 vector compared to HMPV (Fig. 4). The HMPV F and G proteins could be detected in total cell lysates using an antiserum raised against HMPV (Fig. 4, panel A), whereas detection of the SH protein required in addition the use of an antipeptide antiserum and even then was inefficient (panel B). Densitometer quantification of the respective F, G, and SH protein bands was performed and, in each case, the relative level of each glycoprotein expressed from either rHPIV1 or HMPV was no more than two-fold different (data not shown). The SH protein was poorly expressed in rHPIV1-SH-infected cells, but this also is the case in HMPV-infected cells. Thus, the level of expression of each HMPV glycoprotein was similar between the rHPIV1 vectors and HMPV (Fig. 4).

Replication in hamsters

To evaluate the level of replication of the rHPIV1 vectors in the upper and lower respiratory tract of hamsters, groups of hamsters were immunized intranasally (IN) with each recombinant, the lungs and nasal turbinates were harvested after 4 days, and the titer of virus present in tissue homogenates was determined. The rHPIV1 vectors expressing the HMPV glycoproteins replicated as efficiently as the rHPIV1 parent virus in the nasal turbinates and only about 10-fold less efficiently in the lungs (Fig. 5). The comparable levels of replication of rHPIV1-F, rHPIV1-G, and rHPIV1-SH in hamsters made it possible to directly compare their immunogenicity and protective efficacy.

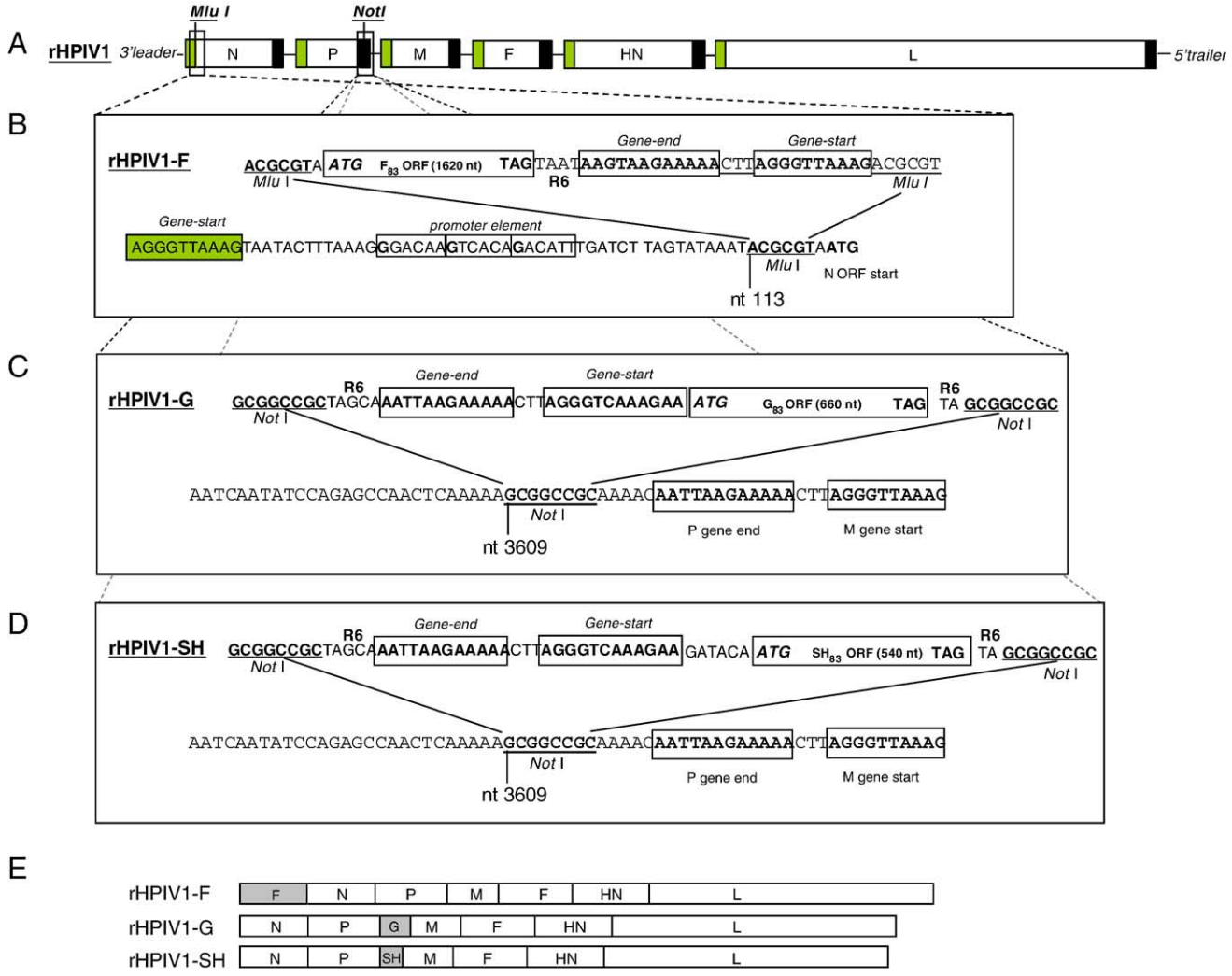

Fig. 1. Construction of rHPIV1 vectors expressing the F, G, or SH protein of HMPV. (A) Wild-type rHPIV1 genome (not to scale) with introduced *Mlu*I and *Not*I restriction sites (underlined). Each of the six rHPIV1 gene units is drawn as an open rectangle flanked by transcription gene start (grey rectangle) and gene end (black rectangle) sequence motifs. (B) Construction of the gene unit expressing the HMPV F protein. The upper diagram shows a PCR-engineered DNA that was inserted into the *Mlu*I site of the rHPIV1 vector (bottom diagram), placing the HMPV F gene unit upstream of the N ORF, as described previously (Skiadopoulos et al., 2004). Four additional nucleotides (R6) were inserted to maintain the polyhexameric length of the genome (Calain and Roux, 1993) and to maintain the phasing of the position of the gene start signals (Kolakofsky et al., 1998), as also was done for the constructs shown in panels C and D. (C) Construction of the gene unit expressing the HMPV G protein. G ORF of HMPV strain CAN83 (GenBank accession number AY297749) (660 nucleotides in length and encoding a 219-amino-acid polypeptide) was engineered by PCR to be preceded by the indicated HPIV1 transcription gene end, intergenic, and gene start signal sequence (top diagram). Insertion of this engineered DNA into the *Not*I site placed the G-encoding gene unit between the HPIV1 P and M genes. (D) Construction of the gene unit expressing the HMPV SH protein. The HMPV strain CAN83 SH ORF (540 nucleotides in length and encoding a 179-amino-acid protein) was engineered by PCR to be preceded by the indicated HPIV1 transcription signals and was inserted into the *Not*I restriction site between the HPIV1 P and M ORFs, as described above for the HMPV G ORF. (E) Schematic representation (not to scale) of the three recombinant HPIV1 each containing an added gene unit encoding the HMPV F, G, or SH glycoprotein ( ).

Immunogenicity in hamsters

To evaluate the immunogenicity of the individual HMPV glycoproteins, groups of hamsters were infected intranasally with rHPIV1-F, rHPIV1-G, rHPIV-SH, HMPV, or rHPIV1 (Table 1). After 30 days, each group of hamsters was either reinfected with a second, higher dose (see Table 1) of the vector carrying the homologous HMPV glycoprotein or with the rHPIV1 vector backbone alone. Serum was collected before the first infection and 26 days after the second infection (day 56). The animals were challenged IN with HMPV 28 days after the second infection (day 58). Thus, this experiment evaluated and compared the immunogenicity and protective efficacy of one or two doses of each rHPIV1-vectored HMPV glycoprotein.

The titers of serum antibodies capable of binding HMPV antigen were determined by ELISA using HMPV-infected LLC-MK2 cells as the solid phase antigen, with binding quantified using an indirect immunoperoxidase assay (Table 1). As a control, the titers of serum antibodies capable of neutralizing HPIV1 were determined; these were found to be similar for the different rHPIV1 vectors, indicating that the immunogenicity of the vectored HMPV F, G, and SH antigens could be compared. Animals receiving one or two doses of the

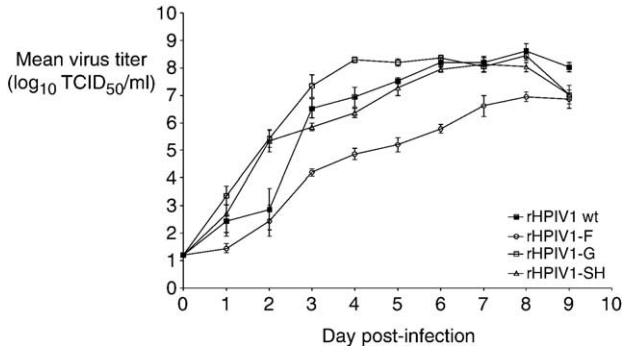

Fig. 2. Replication of rHPIV1-F, -G, or -SH viruses in vitro. LLC-MK2 cell monolayers were infected in triplicate at an m.o.i. of 0.01 with the rHPIV1 parent and HPIV1 vectors, as indicated, and the monolayers were incubated at 32 °C. Aliquots of the medium supernatants were harvested at 24-h intervals, flash-frozen, and subsequently assayed at 32 °C for virus titer. Virus titers are expressed as mean $\log_{10}$ $TCID_{50}$/ml ± standard error.

vector expressing the HMPV F glycoprotein developed a high titer (approximately 1:1000) of HMPV-binding serum antibodies, a titer comparable to that obtained following infection with wild-type HMPV (Table 1). However, two doses of rHPIV1-G resulted in approximately one-tenth the titer of HMPV-binding antibodies compared to that induced by a single immunization with rHPIV1-F (Table 1). Two immunizations with the vector expressing the HMPV-SH protein did not induce detectable HMPV-binding antibodies (Table 1) despite expression of the SH protein by rHPIV1. The binding assay was capable of detecting SH-specific antibodies raised in rabbits against a synthetic peptide of SH, indicating that there was sufficient SH antigen in the infected cell monolayer, and that the lack of detectable anti-SH antibodies in the serum of immunized animals was not due to a limitation of the assay (data not shown). The SH protein of RSV has also been found to be poorly immunogenic (Connors et al., 1991). Thus, the F protein of HMPV is highly immunogenic in hamsters and is significantly more immunogenic than the G protein, whereas the SH protein is very poorly immunogenic.

The ability of rHPIV1-F, -G, or -SH to induce neutralizing antibodies and resistance to HMPV challenge was next determined. One or two immunizations with rHPIV1-F induced a high level of serum neutralizing antibodies to HMPV, confirming previous observations (Skiadopoulos et al., 2004; Tang et al., 2003), and this correlated with a significant level of protection in the upper respiratory tract and complete protection in the lower respiratory tract of immunized animals (Table 1). Two doses of rHPIV1-F induced a greater level of reduction in HMPV virus challenge replication in the nasal turbinates than one dose (2.8 $\log_{10}$ reduction versus 1.8 $\log_{10}$, respectively). However, even two doses of rHPIV1-F did not induce as much protection as infection with a single dose of wild-type HMPV (4.6 $\log_{10}$ reduction). In preliminary experiments, immunization of hamsters with a single dose of rHPIV1-G or rHPIV1-SH followed by challenge with HMPV 30 days post-infection did not confer protection (data not shown). We therefore examined the ability of two doses of the G and SH protein expression vectors to confer neutralizing antibodies or protection against HMPV challenge. Immunization with rHPIV1-SH did not induce detectable neutralizing antibodies and the animals were not protected when challenged with HMPV (protection is defined as being a reduction in challenge HMPV replication of 1.0 $\log_{10}$ or greater) (Table 1). One or two doses of rHPIV1-G also did not induce a detectable level of serum neutralizing antibodies, which was surprising since ELISA binding antibodies were induced. Animals immunized once with rHPIV1-G were not protected from HMPV challenge. However, animals that were immunized twice with the HPIV1-G recombinant were partially protected from HMPV challenge in both the nasal turbinates and the lungs, although reduction in challenge virus replication was statistically significant only in the nasal turbinates (Table 1). Since neutralizing antibodies were not produced even after the second immunization with rHPIV1-G, it is possible that the low level of protection against HMPV challenge observed after two doses might be mediated by some other immune mechanism, such as HMPV-specific $CD8^+$ T-cells.

Discussion

Recombinant HPIV1 was used as an intranasal vector to compare the relative levels of immunogenicity and protective efficacy of the HMPV F, G, and SH proteins. The HMPV F protein had previously been shown to be a neutralization and protective antigen, but the status of G or SH was not known. In particular, while the SH protein of RSV does not appear to be a significant neutralization and protective antigen, the HMPV SH protein is nearly 2.5 times larger and thus should present a significant target on the surface of infected cells and the virus particle. The results confirmed that HMPV F is a major antigen, but surprisingly showed that neither G nor SH induced detectable serum HMPV-neutralizing antibodies even after a

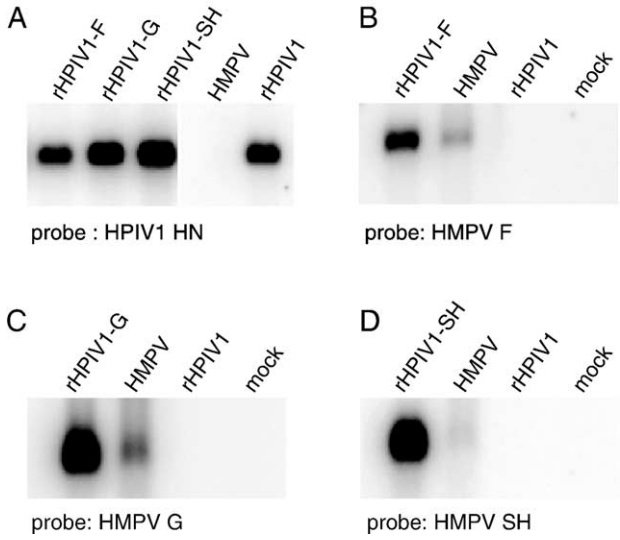

Fig. 3. Northern blot analysis. LLC-MK2 cells were infected at an m.o.i. of 1.0 with the rHPIV1 parent, the rHPIV1 vectors, HMPV, or were mock-infected, as indicated, and harvested after 48 h. 1 μg of purified total RNA for each indicated sample was separated by denaturing agarose gel electrophoresis and analyzed by Northern blot hybridization with the indicated radiolabeled dsDNA probes.

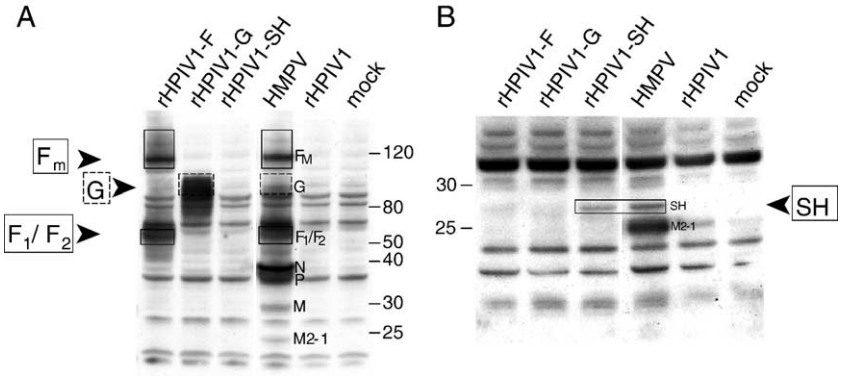

Fig. 4. Western blot analysis. LLC-MK2 cells were infected at an m.o.i. of 1.0 with the rHPIV1 parent, the rHPIV1 vectors, HMPV, or were mock-infected, as indicated, and harvested after 48 h. An equivalent amount of cell lysate for each indicated sample was separated by SDS-PAGE. SDS-PAGE and Western blot analysis were performed using (A) non-reducing conditions and hamster anti-HMPV antibodies or (B) reducing conditions and a mixture of rabbit anti-HMPV and anti-SH peptide antibodies, followed by a peroxidase labeled secondary antibody. The F_m (multimeric form of F) and F_1/F_2 (heterodimeric form of F) and G protein bands are boxed in panel A, and the SH protein bands are boxed in panel B. HMPV protein bands are indicated adjacent to the HMPV lanes.

booster immunization. Furthermore, G induced only a weak protective response against HMPV challenge, while that of SH was insignificant.

The finding that the putative HMPV attachment G protein did not induce a significant response of serum HMPV-neutralizing antibodies and was poorly protective differs from observations obtained with all other paramyxoviruses evaluated to date. The attachment G, H, or HN proteins of other paramyxovirus have been found to stimulate high levels of ELISA antibodies (Paterson et al., 1987; Schmidt et al., 2001). In the case of RSV, the human paramyxovirus most closely related to HMPV, the F and G proteins each induce binding and neutralizing antibodies and protect rodents and non-human primates against RSV challenge after a single dose (Olmsted et al., 1986; Schmidt et al., 2001, 2002). In rodents and non-human primates, the F protein of RSV afforded a higher level of protection in the upper and lower respiratory tract compared to the animals immunized with the G protein indicating that F is the greater protective antigen for RSV seronegative animals (Olmsted et al., 1986; Schmidt et al., 2001, 2002). However, in those studies, the RSV G protein induced a significant level of neutralizing antibodies and was very protective against RSV challenge. In the case of HPIV3, the HN protein, which is the counterpart of the G protein of RSV and HMPV, was found to be the predominant HPIV3 protective antigen, although the HPIV3 F protein also induced neutralizing antibodies (Spriggs et al., 1987). Mice were protected from infection with murine PIV1 (Sendai virus) by prior immunization with a vaccinia virus expressing the Sendai virus F or HN glycoprotein (Takao et al., 1997), but not with other viral proteins (Sakaguchi et al., 1993). For simian virus 5 (SV5), the F protein is more immunogenic than the HN protein, but both the SV5 F and HN proteins expressed by vaccinia recombinants protected equally against homologous challenge (Paterson et al., 1987). Thus, unlike these and other paramyxoviruses characterized to date, HMPV is unique in that only the F protein contributes to the induction of neutralizing antibodies and provides most of the protection against homologous challenge.

The basis for the difference in immunogenicity between the three HMPV surface glycoproteins is not known, but the difference is striking. Both the F and G mRNAs and proteins were expressed well in tissue culture, suggesting that the difference in their immunogenicity is not simply a factor of a difference in their level of expression. The SH mRNA also was

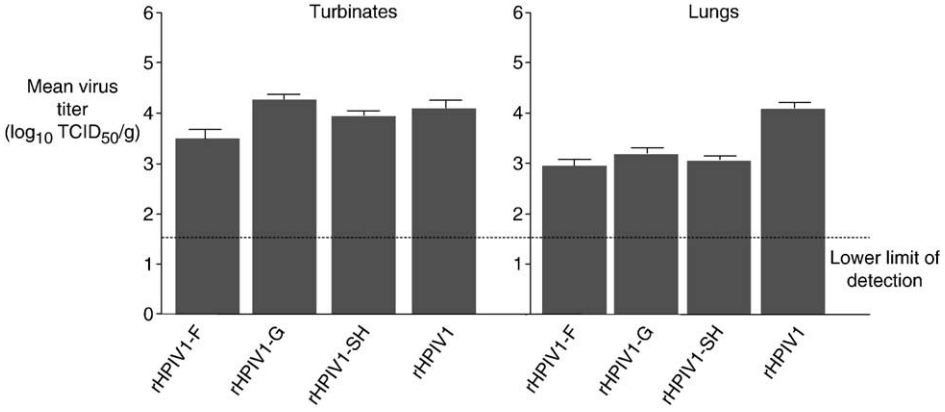

Fig. 5. Replication in hamsters. Hamsters were inoculated i.n. with 10^6 $TCID_{50}$ of the indicated virus. Nasal turbinates and lung tissues from six animals from each group were harvested on day four post-infection. Virus present in tissues was quantified by serial dilution on LLC-MK2 monolayers at 32 °C and is expressed as the mean virus titer (log_{10} $TCID_{50}$/g) for the group.

Table 1
Immunogenicity and protective efficacy of the HMPV F, G, or SH glycoprotein expressed individually by a recombinant HPIV1 vector administered intranasally in one and two doses

No. of animals	Immunization		Serum neutralizing antibody titer (recip. mean $\log_2$ ± SE) on day 56 to the indicated virus[a]		Serum binding antibody titer (recip. mean $\log_2$ ± SE) to HMPV[b] on day 56	Replication of challenge HMPV in the indicated tissues[c]			
	First[d] (day 0)	Second[e] (day 30)				Nasal turbinates		Lungs	
			HPIV1	HMPV		Titer ($\log_{10}$ $TCID_{50}$/g ± SE)	Reduction in titer ($\log_{10}$)[f]	Titer ($\log_{10}$ $TCID_{50}$/g ± SE)	Reduction in titer ($\log_{10}$)[f]
5	HMPV	rHPIV1	3.3 ± 0.3	7.8 ± 0.3	10.3 ± 0.0	≤1.5 ± 0.0[g]	≥4.6	≤1.5 ± 0.0[g]	≥1.9
6	rHPIV1-F	rHPIV1-F	3.9 ± 0.3	8.3 ± 0.4	10.0 ± 0.4	3.3 ± 0.2[g]	2.8	≤1.5 ± 0.0[g]	≥1.9
6	rHPIV1-F	rHPIV1	5.5 ± 0.3	8.3 ± 0.3	9.9 ± 0.4	4.3 ± 0.3[g]	1.8	≤1.5 ± 0.0[g]	≥1.9
6	rHPIV1-G	rHPIV1-G	5.4 ± 0.4	≤2.9 ± 0.0	7.5 ± 0.9	4.6 ± 0.5[h]	1.5	2.4 ± 0.4[i]	1.0
6	rHPIV1-G	rHPIV1	4.7 ± 0.1	≤2.9 ± 0.0	5.9 ± 1.0	5.3 ± 0.3	0.8	2.7 ± 0.4	0.7
6	rHPIV1-SH	rHPIV1-SH	4.8 ± 0.3	≤2.9 ± 0.0	≤2.9 ± 0.0	5.3 ± 0.2	0.8	2.7 ± 0.1	0.7
6	rHPIV1-SH	rHPIV1	5.4 ± 0.3	≤2.9 ± 0.0	≤2.9 ± 0.0	6.0 ± 0.1	0.1	3.1 ± 0.2	0.3
8	rHPIV1	rHPIV1	5.3 ± 0.3	≤2.9 ± 0.0	≤2.9 ± 0.0	6.1 ± 0.2	–	3.4 ± 0.2	–

[a] Sera collected 26 days following the second immunization (day 56) were used in a neutralization assay. The titer is expressed as the mean reciprocal $\log_2$ ± standard error.

[b] Sera collected 26 days following the second immunization (day 56) were tested for the ability to detect HMPV-infected LLC-MK2 cells by indirect immunoperoxidase staining. The mean serum binding antibody titer is the highest dilution of antibody at which the cultures were positive for infection, as determined by immunoperoxidase staining.

[c] Twenty-eight days after the second immunization (day 58), the hamsters were challenged IN with $10^{5.7}$ $TCID_{50}$ of HMPV. Four days after challenge, the nasal turbinates and lungs were harvested and tissue homogenates were prepared. Virus present in the tissue homogenates was quantified by serial terminal dilution on LLC-MK2 monolayers and is expressed as the geometric mean $\log_{10}TCID_{50}$/g for each group ± standard error.

[d] Groups of 5 to 8 HMPV- and HPIV1-seronegative (mean reciprocal serum neutralizing titer ≤1.0 $\log_2$) hamsters were immunized IN with 10^6 (rHPIV1 and rHPIV1 vectors) or 10^5 (HMPV) $TCID_{50}$ of the indicated virus on day 0.

[e] Thirty days following the first immunization, the indicated groups received a second IN immunization with $10^{6.4}$ (rHPIV1-F, rHPIV1-SH, or rHPIV1) or $10^{7.4}$ (rHPIV1-G) $TCID_{50}$ of the indicated virus.

[f] Reduction in titer compared to group that was immunized only with rHPIV1.

[g] Statistically significant difference between this group and animals that received only rHPIV1; $P < 0.001$ (Tukey–Kramer Multiple Comparison Test).

[h] Statistically significant difference between this group and animals that received only rHPIV1; $P < 0.01$ (Tukey–Kramer Multiple Comparison Test).

[i] Statistically significant difference between this group and animals that received only rHPIV1; $P < 0.05$ (Tukey–Kramer Multiple Comparison Test).

present in high abundance, but the level of expression of SH protein appeared to be relatively lower than that of F and G in both rHPIV1-SH- and HMPV-infected cells. However, it is uncertain if the present lack of good antibodies to detect this protein may be a factor in its apparent lower abundance in Western blot analysis. Consequently, one factor for the low level of binding and neutralizing antibodies induced by SH may be its relatively lower abundance in an infection. Another factor could be the relative level of glycosylation of these proteins. The HMPV G and SH proteins are heavily glycosylated compared to the F protein (Biacchesi et al., 2004), and it has been suggested that a high level of glycosylation may affect the immunogenicity of certain proteins (Komada et al., 2000). However, the RSV G glycoprotein is heavily glycosylated yet induces a high level of neutralizing antibodies. The observation in the present study that the moderate level of antibodies against G detected in a binding assay did not neutralize infectivity is remarkable because G is presumed to play an important role in paramyxovirus attachment, and antibodies against attachment proteins generally have neutralizing activity. For RSV, the G protein is essential for replication in vivo (Teng and Collins, 2002; Teng et al., 2001). Recently, it was observed that G and SH could be deleted from recombinant HMPV with minimal or no effect, respectively, on growth in vitro and in vivo (Biacchesi et al., 2004, 2005a). Therefore, these proteins might be weak neutralization and protective antigens because, in addition to being poorly immunogenic, they lack absolutely essential roles in virus infection and replication.

The formal possibility exists that the HMPV F and G proteins might need to be expressed together in order for the G protein to be both immunogenic and protective. However, this would be without precedent among the paramyxoviruses, since all of the F and G/H/HN proteins examined to date have been independent neutralization and protective antigens. Furthermore, sera of wild caught African green monkeys found to be seropositive for HMPV, and sera of monkeys experimentally infected with HMPV, where both the F and G proteins are expressed concurrently, recognize the HMPV F protein exclusively (Western blot analysis of virion proteins, data not shown and Biacchesi et al., 2005b). These observations are consistent with the present findings that the F protein is the major immunogenic surface protein of HMPV whereas G and SH are poorly immunogenic. These observations, together with the finding that HMPV F can confer protection against both genetic lineages of HMPV (Skiadopoulos et al., 2004), have implications for the formulation of a live-attenuated HMPV vaccine. Specifically, they provide support for the strategy of attenuating HMPV by deletion of the G gene (Biacchesi et al., 2004), given the minimal role of the G protein in protective immunity, its loss should not significantly reduce vaccine immunogenicity and efficacy.

In addition, the F protein is clearly the protein of choice for inclusion in a vectored HMPV vaccine, following the

previously-described strategy of using an HPIV vaccine virus as a vector to express protective antigens of additional pathogens such as HMPV or RSV to make bivalent or multivalent pediatric vaccines (Schmidt et al., 2001, 2002). However, it should be noted that, in the present study and in previous studies using other vectored paramyxovirus antigens, the protection afforded by vectors expressing individual proteins was not as complete as that conferred by infection with the wild-type virus. This was true in the present study despite the administration of two doses of rHPIV1-F versus a single dose of HMPV. Incomplete protection occurred in rHPIV1-F-immunized animals despite the induction of a level of HMPV-neutralizing antibodies that was essentially identical to that of the HMPV-immunized animals. In contrast, a single dose of a live-attenuated rHMPV virus with a deletion (Δ) in G or M2-2 coding sequences induced a higher level of resistance to HMPV challenge than did two doses of rHPIV1-F (Biacchesi et al., 2005a). This was so despite the fact that rHPIV1-F replicated to almost wild-type HPIV1 levels in hamsters, which should achieve maximum immunogenicity, whereas the replication of the HMPV ΔG and ΔM2-2 viruses was highly restricted. Previously, we observed a similar phenomenon in animals immunized with HPIV3 vectors expressing the HN protein of HPIV1 and HPIV2 (Skiadopoulos et al., 2002). Vector-immunized animals that were subsequently challenged with HPIV1 or HPIV2 were significantly protected, but the protection was not as effective as that induced by previous infection with the complete homologous virus. In another study, a chimeric bovine-human PIV3 vector expressing the HMPV NL/1/00 strain F protein also did not confer complete protection in HMPV challenged African green monkeys (Tang et al., 2005). These findings indicate that, although vectored vaccines may be useful in inducing neutralizing and protective antibodies, immunization with the whole virus will likely be a more effective strategy. In the present study, since two doses of rHPIV1-F induced a comparable level of neutralizing antibodies to HMPV as infection with wild-type HMPV but a lower level of protection, it is likely that other HMPV proteins also contributed to protection, possibly by inducing HMPV-specific $CD8^+$ T-cells. Previous studies of the contribution of the internal proteins of HPIV3 to protective immunity in hamsters clearly indicated that such antigens play an important role in resistance (Tao et al., 2000), and the findings from the present study suggest that this might be true for HMPV as well.

Materials and methods

Cell lines and viruses

Rhesus monkey kidney cells (LLC-MK2; ATCC CCL 7.1) were maintained in OptiMEM (Invitrogen, Grand Island, NY) supplemented with 5% FBS and gentamicin sulfate (50 μg/ml). HMPV strain CAN97-83, kindly provided by Guy Boivin (Peret et al., 2002), is a clinical isolate that was propagated in LLC-MK2 cells. The recombinant HPIV1 strains Wash/64 and HMPV were grown in LLC-MK2 cells in the presence of 5 μg/ml added porcine derived trypsin (BioWhittaker, Walkersville, MD), as described previously (Biacchesi et al., 2003; Newman et al., 2002; Skiadopoulos et al., 2004). Virus pools were generated, and virus titers were determined at 32 °C in LLC-MK2 cells grown in OptiMEM medium and supplemented with 5 μg/ml added trypsin. Viral titers were quantified by serial 10-fold dilution of virus applied to LLC-MK2 monolayer cultures on 96-well plates (Costar, Corning, NY). Infected cultures were incubated for 7 to 8 days at 32 °C in OptiMEM medium with 5 μg/ml added trypsin. HMPV-infected monolayers were detected by incubation with hamster polyclonal HMPV antiserum (see below) used at a 1:500 dilution, followed by a second antibody (1:250 dilution) consisting of peroxidase-conjugated rabbit anti-Syrian hamster IgG (Jackson Immunochemicals, West Grove PA). Bound antibody–antigen complexes were detected by immunoperoxidase staining achieved with ECL chromogenic substrate (KPL, Gaithersburg, MD). HPIV1-infected cultures were detected by hemadsorption with guinea pig erythrocytes, as described previously (Newman et al., 2002). The virus titers are reported as $\log_{10}TCID_{50}$/ml (50% tissue culture infectious dose/ml).

Generation of recombinant HPIV1s expressing the HMPV G or SH protein

The full-length antigenomic HPIV1 cDNA (Newman et al., 2002) (GenBank accession number AF457102) had been previously modified by nucleotide substitutions to create a unique *Mlu*I site at nucleotides 113–118, which was used as the insertion site for the HMPV F gene unit for the recovery of recombinant HPIV1 expressing the HMPV F protein (rHPIV1-F) (Skiadopoulos et al., 2004). The antigenomic cDNA containing the *Mlu*I site was further modified at nucleotides 3609–3616 (GCTCCCTC) to create a unique cloning site (*Not*I; GCGGCCGC) immediately downstream of the P protein open reading frame (ORF). The HMPV G protein and SH protein ORFs (GenBank accession no. AY297749) were amplified from purified HMPV strain CAN97-83 vRNA and each was modified to contain the flanking HPIV1 *cis*-acting gene start and gene end transcription signals indicated in Fig. 1. The recombinant HPIV1 expressing the G or SH proteins (rHPIV1-G and rHPIV1-SH, respectively) was recovered from transfected HEp-2 cells as described previously (Newman et al., 2002). Recovered viruses were biologically cloned and amplified in LLC-MK2 cells as described previously (Newman et al., 2002).

Growth kinetics of rHPIV1 vectors in vitro

The kinetics of replication of the rHPIV1 vectors was determined by their inoculation, in parallel with the rHPIV1 parent, onto LLC-MK2 monolayers in 6-well plates (Costar; Corning, NY) at a multiplicity of infection (m.o.i.) of 0.01. The cultures were incubated at 32 °C in the presence of 5 μg/ml added trypsin, and 0.5 ml of medium from each well was harvested and replaced with 0.5 ml of fresh OptiMEM medium supplemented with trypsin (5 μg/ml) at 0 h and at 24 h intervals

for up to 8 days post-infection. The aliquots were flash frozen and virus titers were subsequently determined by titration on LLC-MK2 monolayers in 96-well plates as described above.

Northern blot analysis

LLC-MK2 cells were mock-infected or infected with HMPV, rHPIV1, rHPIV1-F, rHPIV1-G, or the rHPIV1-SH virus, each at an input m.o.i. of 1 $TCID_{50}$ per cell, incubated for 2 days at 32 °C in the presence of trypsin (5 μg/ml), and processed to purify total intracellular RNA using an RNeasy kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. Equivalent amounts of total RNA from each sample were electrophoresed in a 1% agarose gel in the presence of 0.44 M formaldehyde, transferred to a charged nylon membrane (Hybond-N; Amersham Biosciences, Bucks, UK), fixed by UV cross-linking, and analyzed by hybridization with denatured double-stranded ^{32}P-labeled DNA probes generated by random priming from HMPV F, SH, or G, or rHPIV1 HN cDNA fragments (Megaprime DNA labeling system; Amersham Biosciences, Piscataway, NJ). Radioactivity was detected by analysis with a Phosphorimager (Molecular Dynamics Inc., Sunnyvale, CA).

Protein electrophoresis and Western blot assay

Aliquots of infected cell lysates were reduced and denatured by heating at 99 °C for 5 min and analyzed on a 4-to-12% polyacrylamide Bis-Tris gel (NuPAGE Novex Bis-Tris; Invitrogen, Carlsbad, CA). The separated proteins were electrotransferred onto a polyvinylidene difluoride membrane (Invitrolon; Invitrogen). The membrane was blocked overnight at 4 °C in Western blocking reagent (Roche, Indianapolis, IN). HMPV F and G were detected under non-reducing conditions, using a hyperimmune serum produced by immunization of hamsters with HMPV (Skiadopoulos et al., 2004). HMPV SH was detected under reducing conditions with a cocktail of rabbit anti-HMPV antibodies and a rabbit antiserum raised against peptide SH 82–96, representing aa 82 to 96 of SH. Bound antibodies were visualized by binding with horseradish peroxidase-conjugated goat anti-rabbit or anti-hamster immunoglobulin G antibodies (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) followed by chemiluminescence (Super-Signal West Pico chemiluminescent substrate; Pierce, Rockford, IL). Western blot autoradiographies were scanned using a densitometer (Personal Densitometer SI; Molecular Dynamics) to quantify HMPV proteins.

Neutralization and binding assays

The susceptibility of HMPV or rHPIV1 to neutralization by serum antibodies from immunized hamsters was determined using an endpoint dilution neutralization assay. For HMPV neutralization assays, 75 μl aliquots of OptiMEM containing approximately 200 $TCID_{50}$ of HMPV were mixed with equal aliquots of OptiMEM containing serial two-fold dilutions of hamster serum that had been heat inactivated at 56 °C for 30 min. The virus–antibody mixtures were incubated at 37 °C for 1 h. 50 μl of each virus–antibody mixture was then transferred to each of two wells of a 96-well plate containing LLC-MK2 cells and incubated for approximately 1 h at 32 °C. The virus–antibody mixture was removed, and the monolayers were washed twice with OptiMEM. 180 μl OptiMEM supplemented with trypsin was added to the cultures, which were incubated at 32 °C for 8 days and supplemented with additional trypsin (250 ng in 50 μl) on day 6. Infected cultures were detected by immunoperoxidase staining using polyclonal hamster anti-HMPV serum, as described above. The neutralization titer is the highest dilution of antibody at which half of the cultures were negative for infection.

The level of serum HPIV1 neutralizing antibodies was determined using a complement-enhanced assay. Two-fold serial dilutions of hamster serum were combined with an equal volume of virus suspension containing approximately $10^{4.9}$ $TCID_{50}$ of rHPIV1 modified to express green fluorescent protein (rHPIV1-GFP) that served as the indicator virus as described previously (Bartlett et al., 2005). The virus and serum mixture was incubated at 37 °C for 1 h in MEM (Gibco-Invitrogen, Inc.) and 10% guinea pig complement (Cambrex Inc., Walkerville, MD) and was transferred to LLC-MK2 monolayers in 96-well plates. After a 1 h adsorption period, the monolayers were washed three times to remove residual serum and complement, and OptiMEM I containing trypsin was added. Infected monolayer cultures were incubated at 32 °C for 7 days, and read for GFP expression on the Typhoon phosphorimager using a Typhoon 8600 scanner (Molecular Dynamics Inc.) control program (settings: fluorescence; filter, 526-SP green fluorescein). The end-point was defined as the highest dilution of the serum/virus mixture that exhibited ≥50% reduction in the level of GFP expression. The neutralization titer is the highest dilution of serum at which 50% of the wells exhibited ≥50% reduction in GFP expression, determined using the IPLab Gel program (Signal Analytics Corp., Vienna, VA).

The HMPV binding assay determined the ability of serial two-fold dilutions of hamster serum antibodies to bind to HMPV-infected LLC-MK2 monolayer cultures. LLC-MK2 monolayers in 96-well cell culture plates were infected with $10^{3.5}$ $TCID_{50}$ HMPV/well. The infected cultures were incubated at 32 °C for 7–8 days. Cells were fixed with 80% methanol for 10 min and were blocked with 5% skim milk powder in PBS for 1 h. Infected cell cultures were identified by indirect immunoperoxidase staining with serial two-fold dilutions of hamster serum followed by peroxidase-conjugated anti-hamster antibodies (Jackson Immunochemicals). The binding titer is the highest dilution of serum at which 50% of the wells were positive for infection by peroxidase staining.

Determination of replication of rHPIV1 vectors and protective efficacy in hamsters

Groups of six 4-week-old Golden Syrian hamsters were inoculated intranasally with 0.1 ml of L15 medium containing 10^6 $TCID_{50}$ of rHPIV1-F, rHPIV1-G, rHPIV1-SH, or rHPIV1. On day 4 post-infection, the lungs and nasal turbinates were

harvested from 6 animals from each group, and the virus titer of individual specimens was quantified by serial dilution of tissue homogenates on LLC-MK2 monolayers, as described above. The mean virus titer was calculated for each group of hamsters and is expressed as $\log_{10}$TCID$_{50}$/gram of tissue.

To determine the level of protective efficacy, hamsters were inoculated IN with 0.1 ml of L15 medium containing 10^6 TCID$_{50}$ of rHPIV1-F, rHPIV1-G, rHPIV1-SH, or rHPIV1 or $10^{5.3}$ HMPV. After 30 days, animals were immunized again with either the empty vector (rHPIV1) or with a second, higher dose of the vector bearing the HMPV glycoprotein (see Table 1). Serum samples were collected before the first infection and 26 days after the second immunization (day 56). On day 58, animals were challenged by IN administration of $10^{5.5}$ TCID$_{50}$ of HMPV in a 0.1 ml inoculum. Nasal turbinates and lungs were harvested 4 days later, and the titer of virus in each tissue homogenate was determined as described above.

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