Virology **281**, 216–230 (2001)
doi:10.1006/viro.2000.0796, available online at http://www.idealibrary.com on IDEAL®

Membrane-Associated Respiratory Syncytial Virus F Protein Expressed from a Human Rhinovirus Type 14 Vector Is Immunogenic

Günter Dollenmaier,*[,1] Sarah M. Mosier,† Frank Scholle,† Nidhi Sharma,*
Kevin L. McKnight,*[,2] and Stanley M. Lemon*

**Department of Microbiology and Immunology, The University of Texas Medical Branch at Galveston, 301 University Boulevard, 4.112 MRB, Galveston, Texas 77555-1019; and †Department of Microbiology and Immunology, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7030*

Received June 19, 2000; returned to author for revision December 11, 2000; accepted December 13, 2000

Human rhinovirus (HRV) replicons have the potential to serve as respiratory vaccine vectors for mucosal immunization in humans. However, since many vaccine immunogens of interest are glycosylated, an important concern is whether HRV replicons are capable of expressing glycosylated proteins. The human respiratory syncytial virus (RSV) fusion (F) protein was chosen as a model glycoprotein and the HRV replicon ΔP1FVP3 was generated by inserting the F protein-coding sequence in frame and in lieu of the 5′ proximal 1489 nucleotides of the capsid-coding segment in the HRV-14 genome. When transfected into H1-HeLa cells, ΔP1FVP3 replicated and led to the expression of the F protein. Inhibition with guanidine demonstrated that F-protein expression was dependent on ΔP1FVP3 replication and did not result from translation of input RNA. Although most of the F protein remained as an immature, glycosylated precursor (F0), a readily detectable fraction of the protein was processed into the mature glycosylated subunit F1, an event known to occur within the Golgi apparatus. Packaged ΔP1FVP3 replicons were generated in transfected HeLa cells by coexpression of homologous HRV capsid proteins using the vaccinia virus/T7 RNA polymerase hybrid system. Packaged replicon RNAs were capable of infecting fresh cells, leading to accumulation of the F protein as in RNA-transfected cells. Mice immunized with HeLa cell lysates containing F protein expressed from ΔP1FVP3 produced neutralizing antibodies against RSV. These results indicate that an HRV-14 replicon can express a foreign glycosylated protein, providing further support for the potential of HRV replicons as a vaccine delivery system. © 2001 Academic Press

Key Words: rhinovirus replicon; mucosal vaccine vector; respiratory syncytial virus vaccine.

INTRODUCTION

The human rhinoviruses (HRVs) constitute a large group of over 100 antigenically distinct serotypes within the picornavirus family. HRVs replicate within epithelial cells and adjacent lymphoid tissues of the upper respiratory tract of humans, and natural infection with HRV is an effective stimulus for production of both local mucosal IgA and systemic IgG antibodies to the virus (Couch, 1996). We have reported previously on the generation of subgenomic HRV replicon RNAs in which the luciferase reporter gene replaced most of the capsid (P1) coding sequence in HRV-14 (McKnight and Lemon, 1996, 1998). HRV replicons express the viral proteins required for RNA replication and are efficiently amplified following transfection into permissive cells. We have also demonstrated that HRV replicons can be packaged when the P1 protein precursor is provided in *trans* by coinfection with intact helper virus. Taken together, these findings suggest that packaged HRV replicons could function as efficient vectors for the expression of foreign proteins in the mucosal environment of the human airway.

As members of the Picornaviridae, the HRVs are nonenveloped positive-stranded RNA viruses. The approximate 7200-nt HRV RNA genome contains a single open reading frame (ORF). An internal ribosomal entry site (IRES) located in the 5′ nontranslated (NTR) RNA of the genome controls the cap-independent translation of the single polyprotein that is subsequently processed into individual gene products by two virally encoded proteinases, 2A and 3C(D) (see Fig. 1A). Based on the proteolytic processing scheme, the polyprotein can be divided into three segments: P1, P2, and P3. The P1 segment comprises the structural or capsid proteins, while P2 and P3 contain the nonstructural proteins required for replication of the RNA genome (Fig. 1A).

Several research groups have explored the possibility of configuring picornavirus chimeras or replicons as vaccine vectors for expression of foreign proteins (Alexander *et al.,* 1994; Andino *et al.,* 1994; Choi *et al.,* 1991; Lemon *et al.,* 1992). The different systems that have been evaluated include antigen chimeras that incorporate epitopes from different viruses in surface loops of HRV

[1] Present address: Institute of Medical Virology, Zurich, Switzerland.
[2] To whom correspondence and reprint requests should be addressed at present address: Eli Lilly and Co., Indianapolis, IN. Fax: 317-276-1743.

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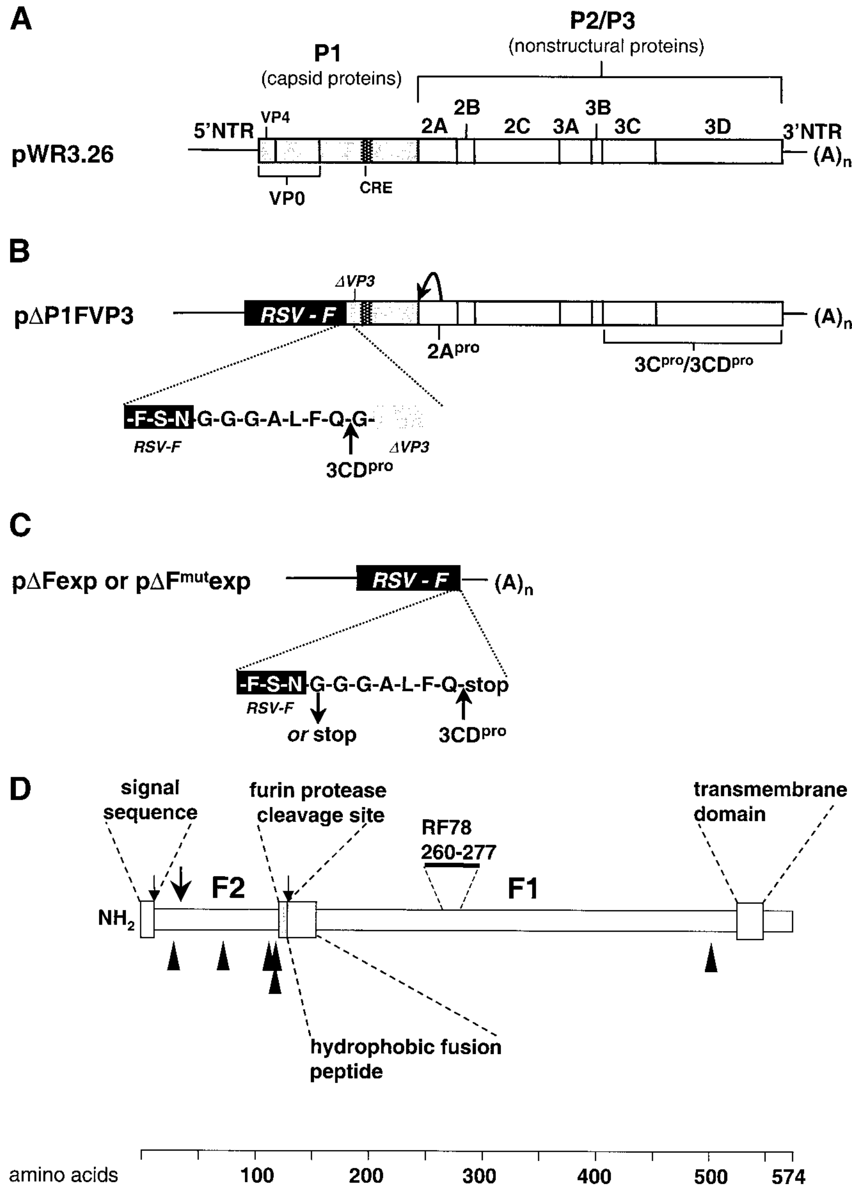

FIG. 1. Structural organization of the HRV-14 genome, HRV-14 replicon ΔP1FVP3, and the RSV F protein. (A) Schematic representation of the infectious cDNA clone of HRV-14, pWR3.26. The open reading frame is divided into precursor protein regions P1, P2, and P3 that are processed by viral proteases 2A and 3C/3CD. The capsid precursor P1 (shaded box) is cleaved into capsid proteins VP4, VP2, VP3, and VP1. The location of the *cis*-acting replication element (*cre*) within the HRV-14 genome is indicated by a striped box. (B) Organization of the HRV-14 derived replicon ΔP1FVP3. The gene for the RSV F protein (solid box) is inserted directly after the 5′ NTR and upstream of the VP3/VP1 junction. A triple glycine hinge, followed by a synthetic viral $3CD^{pro}$ cleavage site, is incorporated at the 3′ terminus of the F gene to facilitate cleavage of the F protein from the remainder of the HRV-14 polyprotein. (C) Schematic representation of the pΔFexp and $p\Delta F^{mut}$exp F-protein expression vectors. Stop codons engineered as described in the text are indicated. (D) Linear structure and domain organization of the RSV F protein (Collins *et al.,* 1996). Shown are the two domains, F1 and F2, as well as the positions of the signal sequence, hydrophobic fusion, and transmembrane domains. Small arrows indicate the sites of cleavage by signal peptidase and furin protease. Large arrowheads indicate the glycosylation sites. In addition, the amino acid position corresponding to the location of the anti-peptide antibody RF78 is shown.

and PV coat proteins (Arnold *et al.,* 1994; Dedieu *et al.,* 1992; Girard *et al.,* 1995; Lemon *et al.,* 1992; Smith *et al.,* 1998). Another approach includes the construction of modified, mono- or dicistronic PV RNA genomes. In the monocistronic configuration, foreign sequences are fused either between the IRES and the P1 segment, or between the P1 and the P2 segments (Altmeyer *et al.,* 1994, 1995; Andino *et al.,* 1994; Crotty *et al.,* 1999; Girard *et al.,* 1995; Tang *et al.,* 1997; Van der Ryst *et al.,* 1998; Yim *et al.,* 1996). These constructs rely on proteolytic processing of the foreign protein by the viral proteinases 2A or 3C(D). In contrast, foreign sequence in the dicistronic configuration is translated under control of the poliovirus (PV) IRES while the PV polyprotein is placed under control of a second downstream IRES; thus there is no requirement for proteolytic processing of the foreign pro-

tein (Alexander *et al.,* 1994; Lu *et al.,* 1995). Although monocistronic PV recombinants were able to induce humoral immune responses against different immunogens (Andino *et al.,* 1994; Crotty *et al.,* 1999; Tang *et al.,* 1997; Yim *et al.,* 1996), this system is limited by the maximal size of the insert and the stability of the modified genome during replication (Mueller and Wimmer, 1998). Another vector construction, used in the present study, utilizes picornavirus subgenomic replicons in which heterologous protein-coding sequence replaces the capsid-coding region, P1, and is fused in frame to the remainder of the viral polyprotein coding sequence (Anderson *et al.,* 1996, 1997; Choi *et al.,* 1991; McKnight and Lemon, 1998). In the replicon configuration, the foreign protein is cleaved from the polyprotein by one of the two viral proteases (for example, see Fig. 1B). Since replicons do not express the structural proteins, they cannot spread from cell to cell. This strategy tends to maximize insert integrity, as genomes that would have lost heterologous sequences are not expanded by second-round infections. Picornavirus replicons can be packaged by providing capsid proteins in *trans* (e.g., by coinfection with helper virus or by using a vaccinia virus-based expression system (Ansardi *et al.,* 1993; Jia *et al.,* 1998; and this study)). Packaged PV replicons have been used in several studies and demonstrate that picornavirus replicons have strong potential as vaccine vectors (Anderson *et al.,* 1996, 1997; Ansardi *et al.,* 1994; Basak *et al.,* 1998; Moldoveanu *et al.,* 1995).

Based on previous work with PV and our own work with HRV replicons (McKnight and Lemon, 1996, 1998), we hypothesized that HRV-14 replicons may serve as unique and efficient vectors for mucosal immunization in humans. There are several potential advantages for using HRV replicons in mucosal immunizations: (i) since HRVs naturally target the upper respiratory tract of humans, HRV-based vaccine vectors are likely to be effective in stimulating protective mucosal immunity, (ii) HRV infections are associated with relatively low pathogenicity, and (iii) current efforts at worldwide eradication of poliomyelitis make the use of PV-based replicons potentially problematic.

Since many medically important immunogens are glycosylated, it is important to determine whether an HRV-based vaccine vector can express glycoproteins. We therefore selected the fusion (F) protein of respiratory syncytial virus (RSV) for the present study. RSV, a member of the paramyxovirus family, is the most common cause of bronchiolitis and pneumonia among children under one year of age in the United States. RSV infection causes about 90,000 hospitalizations and 4500 deaths per year from lower respiratory tract disease (Institute of Medicine, 1985). Candidate vaccines including formalin-inactivated RSV and subunit vaccines have been developed, but do not provide effective protection against RSV infection (Collins *et al.,* 1996). Thus, there is a critical need for an effective and safe vaccine against RSV infection.

The F protein is a well-defined major neutralization antigen of RSV and contains several conformational epitopes that can induce protective immune responses (Collins *et al.,* 1996). Alterations of the tertiary structure of the F protein during vaccine preparation may have been one of the reasons that vaccination studies using formalin-inactivated virus or affinity-purified F protein were not successful (Gotoh *et al.,* 1992). Therefore, it is speculated that the F protein needs to be generated in its mature, processed, and glycosylated form to be able to induce a virus-neutralizing protective immune response (Sakurai *et al.,* 1999). This is supported by the work of Olmstead *et al.* (1986) and Wertz *et al.* (1987), who showed that F protein expressed from a recombinant vaccinia virus that is fully processed and glycosylated is capable of inducing a protective immune response against RSV challenge in a mouse model.

During the RSV replication cycle, the F protein is synthesized as a glycosylated 68- to 70-kDa F0 precursor that is processed during its transport through the Golgi compartment by a cellular proteinase into the mature disulfide linked F1 (49 kDa) and F2 (20 kDa) subunits (see Fig. 1D) (Collins *et al.,* 1984; Collins and Mottet, 1991). The F2 subunit contains an N-terminal signal sequence that is cleaved by signal peptidase (type 1 transmembrane protein), while the C-terminal transmembrane domain within the F1 subunit anchors the protein into the host cell membrane (Collins *et al.,* 1984). Furthermore, the F1 and F2 subunits contain, respectively, one and four potential acceptor sites for N-linked carbohydrate side chains, which are all used for glycosylation (Fig. 1D).

In this report we describe the construction and evaluation of a recombinant HRV-14 replicon ΔP1FVP3 in which the 5′ one-third of the P1 segment has been replaced with the entire RSV F protein coding sequence. The residual, downstream HRV P1 sequence is retained in the replicon as it contains the *cre* (*cis*-acting replication element), a signal necessary for replication of the HRV-14 RNA (McKnight and Lemon, 1996, 1998). We demonstrate that this replicon is replication competent, and characterize the physical attributes and immunogenic activity of the F protein expressed from ΔP1FVP3 RNA in H1-HeLa cells. Our findings support the idea that HRV replicons can be configured to successfully express glycosylated immunogens, and have potential for further development as a vaccine delivery system in the human airway.

RESULTS

Construction of candidate replicon ΔP1FVP3

The candidate replicon ΔP1FVP3 was generated by inserting the cDNA sequence encoding the F protein of

RSV Long strain in lieu of the 5′ one-third of the capsid (P1)-coding segment of the full-length HRV-14 sequence contained in the plasmid pWR3.26 (Fig. 1). The F coding sequence was inserted in pWR3.26 such that the 5′ end of F gene sequence was fused to the authentic AUG initiation codon of the HRV-14 IRES, with the 3′ end fused in frame at nt 2117 of the HRV-14 P1 segment (Fig. 1B). To allow for efficient processing of the F protein, an artificial HRV-14 $3CD^{pro}$ cleavage site (A–x-x-Q/G) was engineered at the 3′ terminus of the F sequence, downstream of a triple glycine hinge that was included to allow flexibility of the peptide chain to optimize cleavage potential (Fig. 1B). The efficient processing of this cleavage site in the context of the luciferase-encoding replicon ΔP1LucVP3 has been described previously (McKnight and Lemon, 1996). This modification resulted in the extension of the cytoplasmic tail of the F protein by seven additional amino acids. To maintain replication competency, it was necessary to retain the HRV-14 *cre* containing sequence in the ΔP1FVP3 construct (McKnight and Lemon, 1992, 1996), which maps to nt 2317 to 2413 of the P1 segment. The RNA transcribed from pΔP1FVP3 is 260 nt (3.5%) longer than the infectious HRV-14 RNA genome.

Analysis of ΔP1FVP3 replication

The extent to which modified picornavirus genomes are able to express foreign glycoproteins that carry a signal sequence is not clear. Previous studies by Lu *et al.* (1995) indicated that dicistronic PV genomes encoding secreted proteins with a signal sequence from the T-cell receptor, CD4, were blocked at the level of RNA replication. Furthermore, inhibition of the cellular secretory pathway by PV proteins 2B and 3A poses further limitations to the ability of the PV system to express secreted proteins (Aldabe *et al.,* 1996; Barco and Carrasco, 1995; Doedens *et al.,* 1997; Doedens and Kirkegaard, 1995), and this may also be the case for HRV. However, the successful expression of a glycosylated envelope surface proteins of simian immunodeficiency virus using monocistronic PV replicons has been reported (Anderson *et al.,* 1996; Tang *et al.,* 1997). The RSV F protein contains a cleaved signal sequence at its N-terminus and a C-terminal transmembrane domain that stops the transfer of the polypeptide chain into the lumen of the ER (Fig. 1D). In the context of the HRV replicon, the stop-transfer activity of the anchorage domain should result in the cytoplasmic localization of the C-terminus of the F protein and the downstream HRV-14 polyprotein. This configuration should allow efficient recognition and processing of the engineered $3CD^{pro}$ cleavage site in ΔP1FVP3 by the viral protease and the release of the HRV-14 polyprotein to the cytosol.

To determine whether the ΔP1FVP3 replicon was able to replicate following transfection in H1-HeLa cells, RNAs representing ΔP1LucVP3, ΔP1Luc, and ΔP1FVP3 were transfected into H1-HeLa cells and cellular lysates were assayed for HRV-14 RNA at the indicated times following transfection by ribonuclease protection assay (Fig. 2). In this experiment, the ΔP1LucVP3 replicon served as a positive control while the nonreplicating ΔP1Luc replicon (which lacks the *cre* sequence) served as a negative control. In order to monitor the cellular RNA content of the lysates, an RNA that corresponds to the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was included as a second probe.

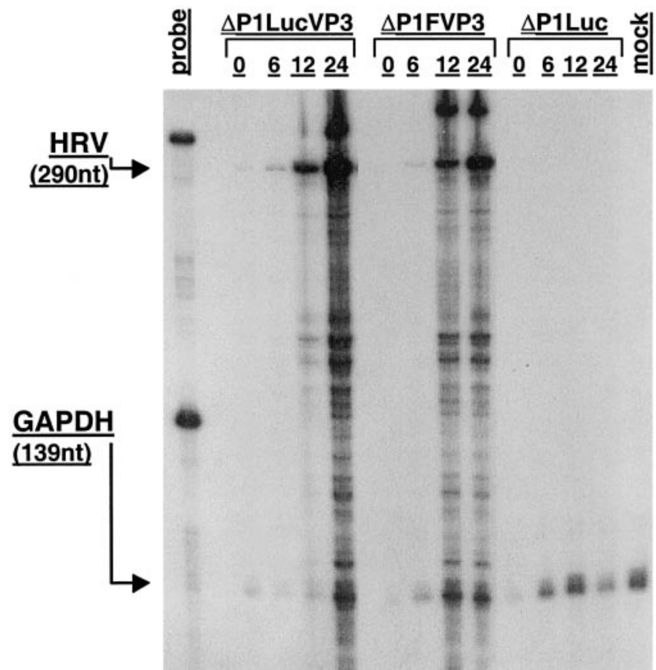

FIG. 2. Ribonucleic protection assay of ΔP1FVP3 RNA replication in H1-HeLa cells. Cell lysates were collected at 0, 6, 12, and 24 h following transfection with ΔP1LucVP3 and ΔP1FVP3 replicon RNAs or from mock-transfected cells. Virus-specific RNA was detected by hybridization with an excess of HRV-14-specific probe (HRV) followed by RNase treatment and denaturing polyacrylamide gel electrophoresis (PAGE). The cellular RNA content of each lysate was monitored by an internal control probe hybridizing to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The left lane contains 1:100 the amount of each probe used in individual hybridization reactions. Protected fragments specific for virus (263 nt) and for GAPDH (100 nt) are indicated with arrows.

As shown in Fig. 2, the amount of HRV-14-specific RNA in lysates of ΔP1LucVP3 and ΔP1FVP3 transfected cells increased during the 24 h following transfection, indicating successful replication of these RNAs. These results contrast sharply with the level of protected RNA observed following transfection with the nonreplicating ΔP1Luc RNA. The presence of probe-specific high-molecular-weight bands at the later time points has been reported previously; these most likely represent protected RNA hybrids that have escaped the denaturation conditions of the gel (McKnight and Lemon, 1996, 1998). When the amount of protected HRV-14 RNA was normalized to the amount of protected GAPDH RNA, the replication of the ΔP1FVP3 RNA was found to be only slightly

reduced when compared to the ΔP1LucVP3 replicon (data not shown). Thus, the HRV-14 replicon ΔP1FVP3 that contains the RSV F glycoprotein sequence is able to replicate to levels equivalent to that of the ΔP1LucVP3 replicon (Fig. 2). This result indicates that the coding sequence from a foreign protein with an N-terminal signal sequence and a C-terminal stop-transfer domain can be inserted into the HRV-14 ORF without disrupting replication of the replicon RNA.

Immunoblot analysis of F-protein expression following transfection of ΔP1FVP3 RNA

We next determined whether the F protein was being expressed from the ΔP1FVP3 replicon and whether post-translational processing of the F protein had occurred. Cellular lysates were prepared from H1-HeLa cells 24 h following transfection with $\Delta P1Luc_{cre}$ (a successfully replicating HRV-14 replicon that expresses luciferase) and ΔP1FVP3 RNAs. Proteins were analyzed by immunoblot using monoclonal antibody RF78, which is specific for amino acids 269–277 of the F1 subunit of the F protein (Fig. 1D). Lysates from RSV-infected or mock-infected HEp-2 cells were used as positive and negative controls, respectively (Fig. 3A, lanes 1 and 2). In lysates from RSV-infected cells, the RF78 antibody specifically detected a major protein of approximately 48 kDa that corresponded to the expected molecular mass of 49 kDa for the glycosylated F1 subunit of the F protein (Fig. 3A, lane 2). A second specific protein of approximately 24 kDa was present at an abundance similar to that of the F1 subunit. This protein may represent a degradation product of the F protein. Because the RSV-infected HEp-2 cells were harvested late in infection (2 days postinfection), the F protein was completely processed and therefore no F0 precursor (69 kDa) could be detected. Analysis of lysates from ΔP1FVP3-transfected H1-HeLa cells revealed two major specific proteins when compared to $\Delta P1Luc_{cre}$ or mock-transfected cells (Fig. 3A, lane 5 vs. lanes 3 and 4). The dominant protein migrated with an apparent molecular mass of 66 kDa, consistent with the expected size of 69 kDa for the glycosylated F0 precursor (Collins *et al.,* 1984). A lesser, faint band of approximately 48 kDa comigrated with the RSV F1 subunit (Fig. 3A, lanes 2 and 5). These results suggest that there is at least partial processing of the F0 precursor into the F1 and F2 subunits in the Golgi apparatus in cells transfected with ΔP1FVP3 RNA.

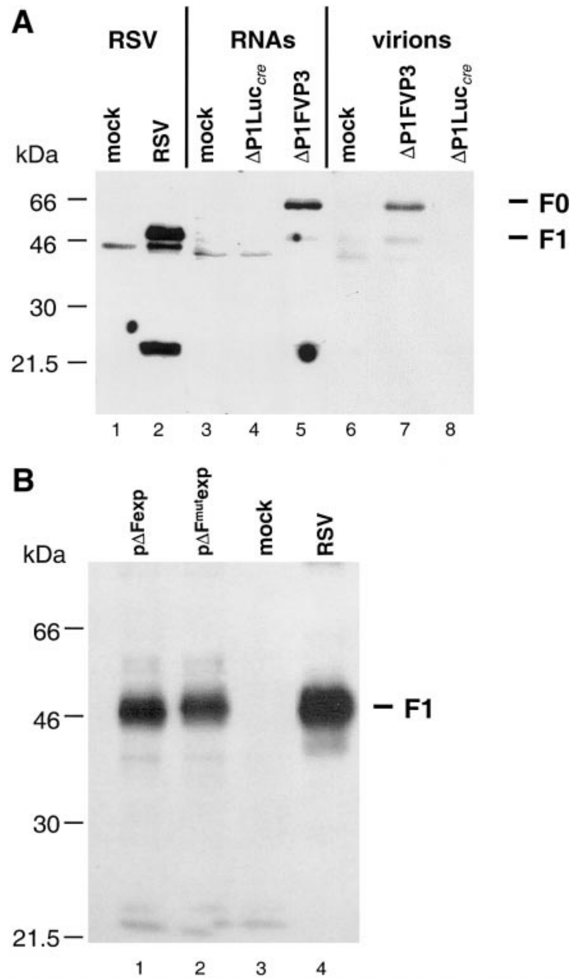

FIG. 3. Expression of F protein in ΔP1FVP3, pΔFexp, or $p\Delta F^{mut}exp$ transfected and ΔP1FVP3 virion infected H1-HeLa cells. (A) H1-HeLa cells were transfected with replicon RNAs (lanes 3 to 5) or infected with 30 μl of a packaged replicon stock (lanes 6 to 8) and cellular lysates were analyzed 24 h later by SDS–12% PAGE and immunoblot as described under Materials and Methods. Lysates from HEp-2 cells collected 48 h after RSV infection served as positive control (lanes 1, 2). The molecular weight marker is indicated on the left and positions of different F-protein species (F0 precursor and F1 subunit) are shown on the right. Mock, lysates from mock-transfected or mock-infected cells. (B) H1-HeLa cells were transfected with pΔFexp (lane 1) or $p\Delta F^{mut}exp$ (lane 2) plasmids and superinfected with 5 m.o.i. of vTF73 and cellular lysates were analyzed 24 h later by SDS–8% PAGE and immunoblot as in Fig. 3A. Lysates from mock-transfected and vTF73-infected cells (lane 3) and lysates from RSV-infected Hep-2 cells (lane 4) served as negative and positive controls, respectively. Note that vaccinia virus has been shown previously not to block F-protein expression and processing (Olmstead *et al.,* 1986; Wertz *et al.,* 1987).

It could be argued that the failure to completely process the ΔP1FVP3-expressed F0 precursor is due to the presence of the additional seven amino acids fused to the C-terminus of the F protein following proteolytic processing by rhinovirus $3CD^{pro}$ (Fig. 1B). Therefore, we constructed two bacteriophage T7 expression vectors that could be used to document the extent in which mutant F protein expressed from the ΔP1FVP3 vector could undergo processing into F1 and F2. Derived from the ΔP1FVP3 replicon, pΔFexp contains the F protein under translational control of the HRV-14 IRES. A stop codon was engineered after the last amino acid (aa) of the F-protein coding sequence, which is immediately followed by the 3′ NTR and poly(A) tract from the vector (Fig. 1C). $p\Delta F^{mut}exp$ was similarly constructed except that it contains the seven additional aa fused to the F

protein following processing of the ΔP1FVP3 polyprotein (Fig. 1C).

H1-HeLa cells were transfected with either pΔFexp or pΔF^{mut}exp plasmid DNA and infected with vaccinia virus expressing the bacteriophage T7 RNA polymerase (vTF73) as described under Materials and Methods. Cell lysates were harvested for immunoblot analysis 24 h following transfection and infection. As shown in Fig. 3B, the F protein produced from both expression vectors was able to undergo complete processing, as indicated by the appearance of only the F1 subunit in lanes 1 and 2 of the immunoblot. Only a very faint amount of the F0 precursor could be seen upon longer exposure of this blot (data not shown). Thus, the additional C-terminal aa fused to the F precursor expressed from ΔP1FVP3 have no effect on its processing into F1 and F2.

Immunoblot analysis of F-protein expression following packaged replicon (vΔP1FVP3) infection

We also assessed the expression of the F protein in cells infected with packaged replicon RNAs. Packaged RNA was generated in a vaccinia virus/bacteriophage T7 RNA polymerase hybrid expression system as described under Materials and Methods and by Jia *et al.* (1998). We initially characterized the efficiency of this packaging system using the luciferase-expressing replicon ΔP1Luc$_{cre}$. Replicon packaging efficiency was documented by following luciferase activity and by *in situ* immunostaining of cells infected with packaged replicon RNA. Luciferase activity could be completely blocked by pretreatment of packaged replicon RNA stocks with antibodies against HRV-14. Average titers of packaged replicon stocks ranging between 0.5 and 2×10^5 luciferase-expressing cells per milliliter could be routinely approximated from cell monolayers immunostained with luciferase antibody (see Materials and Methods; and data not shown).

To verify F-protein expression from packaged replicon RNA, H1-HeLa cells were infected with the packaged ΔP1FVP3 and ΔP1Luc$_{cre}$ RNAs (vΔP1FVP3 and vΔP1Luc$_{cre}$, respectively). Cellular lysates were prepared 24 h following infection and subjected to immunoblot analysis as described above (Fig. 3A, lanes 6–8). Cells that had been infected with vΔP1FVP3 expressed a panel of immunoreactive proteins identical to those in the ΔP1FVP3 RNA-transfected cells. This result indicates that the ΔP1FVP3 RNA had been successfully packaged into virions and that these virions were able to deliver the replicon RNA into the infected cell (Fig. 3A, lane 7). In contrast, control cells infected with packaged lysates from ΔP1Luc$_{cre}$ RNA (vΔP1Luc$_{cre}$) and mock-infected cells did not contain a comparable panel of immunoreactive proteins (lanes 6 and 8).

In situ analysis of vΔP1FVP3-infected cells expressing F protein

To further confirm the expression of the F protein in H1-HeLa cells, indirect immunofluorescence staining was carried out on cells that were infected with vΔP1FVP3. In contrast to cells that were infected with vΔP1Luc$_{cre}$ or that were mock-infected (Figs. 4C and 4D, respectively), a strong fluorescent signal was present in 30–50% of the vΔP1FVP3-infected cells, indicating the successful infection of a substantial proportion of the cells and the resulting expression of the F protein, which was present primarily in the cytoplasm (Figs. 4A and 4B). The fluorescent signal observed from the packaged virions was equivalent to that observed from transfected replicon RNAs (data not shown), indicating that there is little difference in the amount of expressed F protein derived from these two methods of replicon RNA delivery. We also performed confocal fluorescent microscopy on cells transfected with ΔP1FVP3 RNA and found limited immunostaining of F protein on the cell membrane (data not shown).

Time course analysis of ΔP1FVP3 expression of the F protein

We examined the expression of the F0 and F1 proteins in H1-HeLa cells in a time course experiment. Cells were transfected with ΔP1FVP3 RNA, or mock-transfected with PBS only, and lysates were prepared at the time points indicated in Fig. 5. Equal amounts of the lysates were subjected to immunoblot analysis as described for Fig. 3. As shown in Fig. 5A, the F0 precursor and the F1 subunit began to accumulate as early as 9 h post-transfection with F0 as the predominant protein species. Coincidently, this is within the initial time point when replication of the transfected ΔP1FVP3 RNA is being detected (compare between the 6- and 12-h time points in the gel of Fig. 2), suggesting that F-protein expression is linked to RNA replication because at this time point there would be more RNA available for translation. The amounts of both the F1 and the F0 proteins were significantly increased by the 14-h time point; however, the relative proportion of both proteins remained constant during this period (compare lanes 5 and 7). By 24 h, the overall expression of F0 and F1 appeared somewhat reduced but an abundant amount of F1 could still be detected. Densitometric scanning of the F-specific bands in Fig. 5A, as well as immunoblots from additional experiments, indicated that F1 represented 20–35% of the detectable F protein (data not shown), indicating the extent of processing by the furin-like protease located in the trans-Golgi compartment. The overall reduction in F-protein expression at the 24-h time point may be due to shutdown of cellular vesicular transport by this time following transfection. However, we did not observe a consistent amount of the processed F1 protein in every experiment, suggesting that

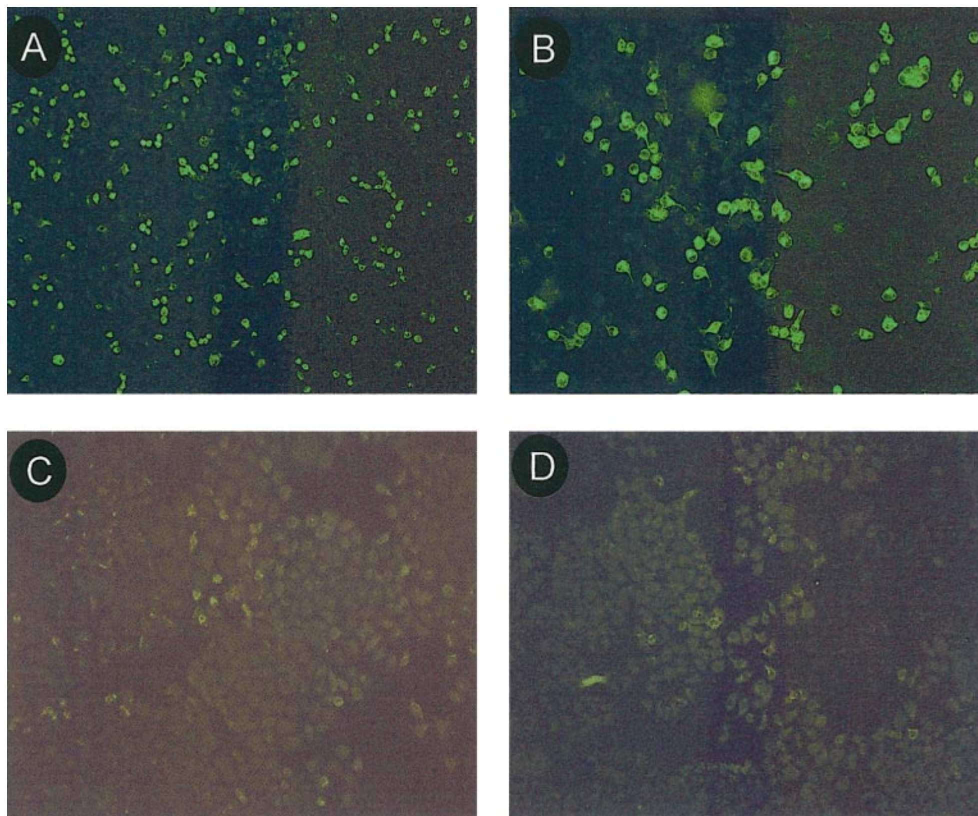

FIG. 4. *In situ* detection of F-protein expression by indirect immunofluorescence. H1-HeLa cells on chamber slides were infected with packaged ΔP1FVP3 virions and 16 h after infection F-protein expression was detected by indirect immunofluorescence as described under Materials and Methods. (A, B) Infection with ΔP1FVP3 virions at magnifications of 10× and 20×, respectively. (C) Infection with packaged $\Delta P1Luc_{cre}$ virions or (D) mock infection. The apparent packaging efficiency of ΔP1FVP3 was equivalent to that observed with the reporter $\Delta P1Luc_{cre}$ replicon (data not shown).

other factors may contribute to the reduced F-protein expression, such as variable transfection efficiencies or cell conditions. Similarly, there could be some inconsistencies in the transfer of proteins onto nitrocellulose in the different blotting experiments.

We also examined the time-dependent expression of F protein following infection with encapsidated ΔP1FVP3 RNA. The results of this experiment are shown in Fig. 5B. The appearance of the F0 precursor was similar to what was observed in the RNA transfection experiment depicted in Fig. 5A (compare lanes 5 and 7 in both). However, the overall expression of the F protein from the packaged virions was consistently lower than that observed from transfection of RNA (compare Figs. 5A to 5B). This is likely due to the fact that the packaged virions were not titered and that a smaller proportion of cells were infected than the proportion undergoing successful transfection of RNA. By comparing *in situ* staining of cells transfected with ΔP1FVP3, we have noted a higher percentage of cells expressing F protein (50–70%, data not shown) than in cells infected with vΔP1FVP3 (30–50%, Figs. 4A and 4B).

Taken together, these data indicate that the F protein is expressed from the ΔP1FVP3 replicon in RNA-transfected or virion-infected cells. At 9, 14, and 23 h following transfection or infection, the uncleaved F0 precursor protein represented the major F-specific protein species, whereas smaller amounts of the mature F1 subunit could also be detected. This indicates that a readily detectable amount of post-translational modification of the F protein was taking place in the Golgi compartment.

Expression of the F protein is dependent on ΔP1FVP3 RNA replication

The results in Figs. 4 and 5 clearly indicate that the ΔP1FVP3 replicon expresses the F protein. However, this expression could be related only to the initial translation of the input RNA only, and could be blocked at later times following transfection when replication of the replicon RNA has induced shutdown of the cellular vesicular transport pathway. To determine whether F-protein expression was dependent on replication of ΔP1FVP3 RNA, we monitored the expression of F protein in transfected cells treated with guanidine hydrochloride (GuHCl). Treatment of cells with millimolar concentrations of GuHCl is known to block the synthesis of picornavirus RNA but does not interfere with picornaviral translation (Barton and Flanegan, 1997; Caliguiri and Tamm, 1968). As shown in Fig. 6, at later times (9, 14, and 24 h) following

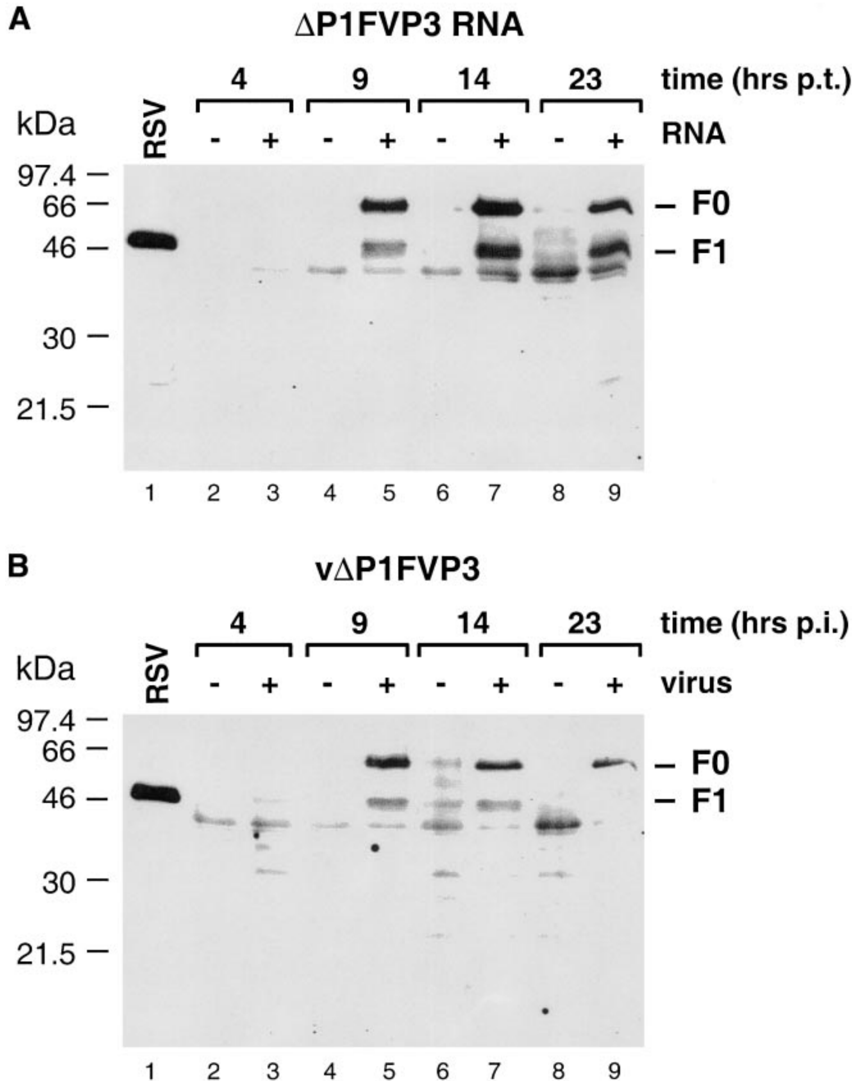

FIG. 5. Time-dependent expression of F protein in ΔP1FVP3 RNA-containing H1-HeLa cells. H1-HeLa cells (A) were transfected with ΔP1FVP3 RNA or (B) infected with packaged ΔP1FVP3 replicon RNA. Cellular lysates were prepared 4, 9, 15, and 24 h following transfection or infection. F-specific proteins were analyzed as described in Fig. 3. The F1 protein present in RSV-infected HEp-2 cell lysates was used as a control. Due to continued cell growth during the 24 h following electroporation, the nonspecific signal to cellular proteins also increases at later times. The molecular weight marker and the different F-protein species (F0 precursor and F1 subunit) are shown as in Fig. 3A. This experiment was repeated twice with similar results. p.t., post-transfection; p.i., postinfection.

transfection of ΔP1FVP3 RNA, when replication of the RNA is otherwise maximal (see Fig. 2), no expression of the F protein was detected in lysates from cells treated with GuHCl (lanes 3, 5, and 7 in Fig. 6). In contrast, in lysates from cells transfected in the absence of GuHCl treatment, expression of the F protein increased during the 24 h following transfection, as observed in previous experiments (compare lanes 2, 4, and 6 of Fig. 6 to the results in Fig. 5). We also observed little or no expression of F protein during earlier times following transfection (3 and 6 h) in the presence of GuHCl, indicating that translation of the F protein from input RNA alone was likely below the level of detection in the immunoblot (compare in Fig. 5; and data not shown). These results confirm that the observed expression of the F protein is dependent on replication of ΔP1FVP3 RNA.

F protein expressed by the ΔP1FVP3 replicon is glycosylated

The apparent electrophoretic mobility of the F0 and F1 proteins expressed by ΔP1FVP3 corresponded to the expected molecular masses of the respective glycosylated proteins (Collins *et al.,* 1984). To determine experimentally whether these proteins were indeed glycosylated, lysates from H1-HeLa cells taken 15 h following transfection with the ΔP1FVP3 replicon and control lysates from RSV-infected cells were subjected to treatment with PNGase F or endoglycosidase H

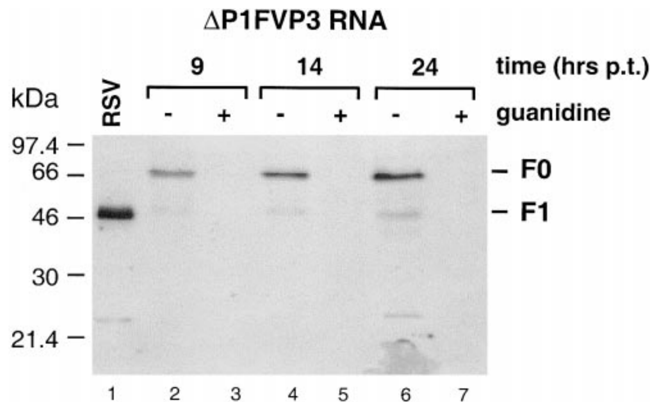

FIG. 6. Expression of the F protein is dependent on replication of ΔP1FVP3 RNA. Immediately following electroporation, H1-HeLa cells were placed in normal media (−) or media containing 2 mM GuHCl (+). At 9, 14, and 24 h following transfection, cell lysates were subjected to SDS–10% PAGE and analyzed by immunoblot for analysis of F-protein expression. p.t., post-transfection.

(endo H) and analyzed by immunoblot (Fig. 7). PNGase F is a glycoamidase with broad specificity that separates almost all N-linked oligosaccharides from glycoproteins. In contrast, endo H has a more limited substrate specificity and prefers high-mannose and hybrid oligosaccharide side chains that are characteristic for the endoplasmic reticulum (Kobata, 1979; Tarentino *et al.,* 1985).

As shown in Fig. 7A, PNGase F treatment of RSV-infected cell lysates caused a shift in electrophoretic mobility of the F1 protein that corresponded to a reduced molecular mass of approximately 2.7 kDa (Fig. 7A, lanes 2 and 3). This observation is consistent with the fact that one carbohydrate chain is predicted to contribute 2.1 kDa to the molecular mass of a glycoprotein and that the F1 subunit only contains one acceptor site for N-linked carbohydrates (Fig. 1D; Collins *et al.,* 1996). Treatment of the lysates with endo H did not alter the mobility of F1 (Fig. 7A, lanes 4 and 5). This observation is in agreement with earlier findings that the maturating F protein acquires resistance to endo H attack during its transport through the *trans*-Golgi compartment and is indicative of proper glycosylation of the protein (Collins and Mottet, 1991).

When lysates from ΔP1FVP3-transfected cells were subjected to PNGase F digestion, the apparent molecular mass of the dominant F0 precursor protein was reduced by approximately 12.5 kDa, indicating that all five carbohydrate side chains were removed by the enzyme (Fig. 7B, lanes 2 and 3). Following treatment with endo H, the F0-specific band was shifted to the same molecular mass as seen after PNGase F treatment (compare lanes 3 and 5 of Fig. 7B). The susceptibility of F0 to the endo H suggested that this protein accumulated in compartments prior to the *trans*-Golgi compartment. In contrast, the minor F1 band was not reduced in mass by endo H digestion, although its mass was reduced approximately 2.5 kDa by treatment with PNGase F. These results indicate that the F1 expressed from the replicon was glycosylated in a fashion similar to the native viral protein shown in Fig. 7A.

The results from Fig. 7 indicate that the ΔP1FVP3 replicon is able to express the F protein in a glycosylated form. The major protein species represented the immature glycosylated F0 precursor protein, whereas the mature F1 subunit that was properly processed and glycosylated was present in much smaller amounts.

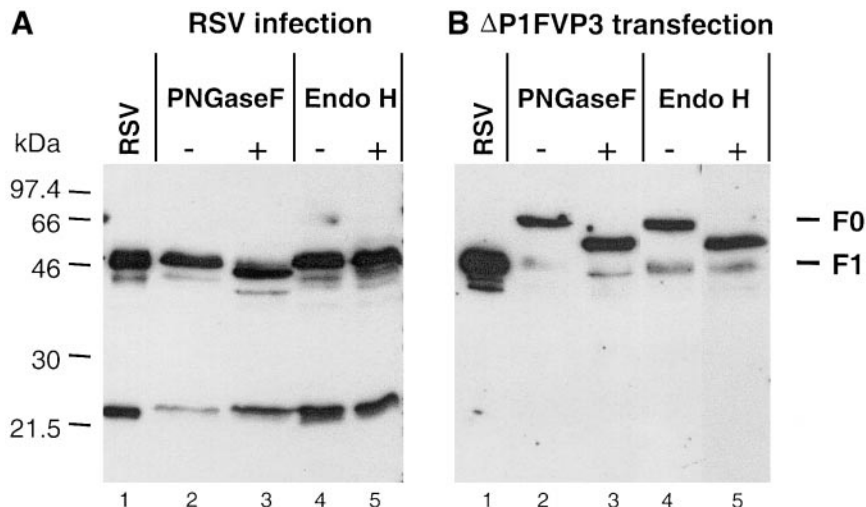

FIG. 7. Enzymatic deglycosylation of F protein. Lysates from H1-HeLa cells, collected 15 h after transfection with the ΔP1FVP3 replicon, or control lysates from RSV-infected HEp-2 cells were subjected to glycosidase treatment (+) or mock-treated (−). After overnight incubation with PNGase F or endo H, the proteins were separated by SDS–12% PAGE and analyzed by immunoblot. (A) Lysates from RSV-infected HEp-2 cells. (B) Lysates from H1-HeLa cells following transfection with ΔP1FVP3 RNA.

TABLE 1

Anti-RSV Neutralizing Antibody Response of Mice Immunized with Lysates from ΔP1FVP3 Replicon-Transfected Cells

Immunogen[a]	Mouse	Reciprocal serum antibody titer to RSV
ΔP1FVP3	1	64
	2	128
	3	64
	4	≥256
$\Delta P1Luc_{cre}$	5	<2
Mock	6	<2

[a] Serum collected from mice that had been immunized intraperitoneally with the indicated replicon RNA was tested for the presence of anti-RSV antibodies as described under Materials and Methods. Four mice were immunized with ΔP1FVP3 lysate, and one each with $\Delta P1Luc_{cre}$ lysate or normal HeLa lysate.

Immunization of mice with HeLa cell lysates transfected with ΔP1FVP3 induces neutralizing antibodies to RSV

The only *in vivo* systems available for testing immunizations with packaged rhinovirus vectors are chimpanzees and humans. We are currently developing a protocol to test our packaged rhinovirus replicons in a primate model. However, in an effort to initially determine whether the F protein produced by ΔP1FVP3 was capable of inducing neutralizing antibodies against RSV, we immunized Balb/c mice with lysates of HeLa cells that had been transfected with ΔP1FVP3. Serum was collected 2 weeks following a booster immunization and tested for the presence of RSV neutralizing antibody as described under Materials and Methods. The results (Table 1) show that the serum from each of four immunized mice produced significant titers of neutralizing antibodies to RSV, ranging from 64 to ≥256. In contrast, serum from mice that had been immunized with lysates of cells transfected with an unrelated HRV replicon ($\Delta P1Luc_{cre}$) or mock-transfected cells did not contain any detectable RSV neutralizing antibodies (titer <2, Table 1). Thus, the F protein produced from ΔP1FVP3 induces an appropriate immune response that is effective in neutralizing RSV.

DISCUSSION

We have shown in ribonuclease protection assays that the HRV-14 replicon ΔP1FVP3, which contains the gene for a membrane glycoprotein (F protein) of RSV, is able to replicate following transfection in H1-HeLa cells. The expression and limited processing of the F protein in ΔP1FVP3 RNA-transfected cells (or cells infected with packaged replicon RNA) were confirmed by immunoblot analysis and indirect immunofluorescence. Expression of the F protein was dependent on replication of the ΔP1FVP3 RNA. Glycosidase digestions indicated that all of the F protein produced was glycosylated, but both the Golgi-specific modification of the N-linked carbohydrate side chains and the proteolysis of the F0 precursor into the mature F1 and F2 subunits were impaired. However, even though most of the expressed product was not completely processed, the F protein produced following transfection with ΔP1FVP3 was capable of inducing neutralizing antibodies to RSV when used to immunize mice.

Earlier work by Lu *et al.* (1995) suggested that in the context of a dicistronic PV expression vector, the presence of the T-cell receptor CD4 signal sequence from HIV-1 gp120 inhibited early steps of viral replication, probably due to the sequestration of the viral polysome in the rough ER. Since the inclusion of the CD4 transmembrane domain did not restore replication, which could only be accomplished by removal of the signal sequence, the authors concluded that PV vectors in general are unable to express glycosylated proteins. Similarly, Ansardi *et al.* (1994) reported that insertion of the sequence for carcinoembryonic antigen (CEA) into PV replicons abolished replication, and that replication could be restored by deletion of the CEA signal sequence. In contrast, more recent studies using PV replicons or monocistronic recombinant PV constructs have suggested that these vectors were indeed able to express foreign proteins that were glycosylated (Anderson *et al.,* 1996, 1997; Tang *et al.,* 1997). However, expression of the RSV F protein by the ΔP1FVP3 replicon provides additional evidence of possible limitations of picornavirus-based expression of glycoproteins: since the F protein is processed in the Golgi compartment by a cellular furin-like protease, we were able to determine that protein modifications occurring past the ER may be restricted. We speculate that this restriction is due to the membrane alterations carried out by the viral 2B or 3A proteins (Aldabe *et al.,* 1996; Barco and Carrasco, 1995; Doedens *et al.,* 1997; Doedens and Kirkegaard, 1995). Nevertheless, the partially processed F protein expressed from ΔP1FVP3 remained immunogenic, which further supports the idea that picornavirus vectors are suitable for expression of medically important foreign glycoproteins as immunogens.

The replication competence of our construct probably derives from the efficient stop-transfer activity of the transmembrane domain of the F protein, which results in the downstream artificial $3C^{pro}$ cleavage site being located in the cytoplasm and accessible to the viral protease. We also constructed similarly configured HRV-14 replicons, ΔP1SEAPVP3 and ΔP1HGHVP3, which contain the genes for secreted alkaline phosphatase (SEAP) and human growth hormone (HGH), respectively. Neither replicon is able to replicate following transfection in H1-HeLa cells (McKnight, 1999; Mosier, 1997). Since the SEAP and HGH glycoproteins do not contain transmembrane domains it may be possible, as suggested by Lu *et al.* (1995), that the engineered $3C^{pro}$ cleavage site at the

C-terminus of SEAP or HGH is translocated into the ER during translation, becoming inaccessible to the viral protease and fixing the unprocessed viral polyprotein in an environment that would not be conducive to viral replication. Alternatively, we have noted from Kyte and Doolittle analysis (Kyte and Doolittle, 1982) that the SEAP and HGH proteins have significantly lower hydropathic indexes when compared to the RSV F protein (−2.85 and −2.69 versus −0.38). Thus, it is interesting to speculate that the more hydrophilic glycoproteins migrate more quickly into the vesicular transport compartment, which would similarly place the viral polyprotein within the ER. Taken together, these data strongly suggest that there are limitations on the foreign sequences that can be successfully inserted into HRV genomes and that the replication capacity of a replicon depends on the specific foreign sequence inserted.

The RSV F protein is synthesized as a glycosylated 68- to 70-kDa F0 precursor that undergoes post-translational maturation in the Golgi compartment. F0 forms an oligomer in the rough ER and is cleaved in the *trans*-Golgi compartment by a cellular endoprotease (furin) to form disulfide-linked F1 and F2 subunits (Collins and Mottet, 1991; Gotoh *et al.,* 1992). The mature F protein is finally expressed at the cell surface forming a viral envelope tetramer spike (Collins *et al.,* 1996). In cells supporting replication of the ΔP1FVP3 RNA replicon, the F protein could be detected as a 66-kDa F0 precursor, indicating that the artificial $3CD^{pro}$ cleavage site at the C-terminus of the protein was efficiently processed. However, the predominance of the F0 form when compared to the expression level of the mature, F1 48-kDa glycoprotein suggested that the furin proteinase processing of the precursor protein was not very efficient. This could be either due to differences in furin activity between HeLa and HEp-2 cells or to impaired transport of F0 to the Golgi compartment. Since both cell types efficiently support RSV replication and therefore probably contain similar levels of furin it appears more likely that there was an inhibition of the secretory transport of the F0 precursor prior to the *trans*-Golgi compartment. The susceptibility of F0 to endo H digestion indicated that this protein was stalled in the ER and had not yet reached the medial Golgi where the N-linked carbohydrate side chains acquire endo H resistance (Collins and Mottet, 1991). This is consistent with the finding that PV protein 3A directly blocks vesicular transport from the ER to the Golgi apparatus, and suggests that HRV-14 is likely to express a protein with a similar activity during viral replication (Doedens *et al.,* 1997; Doedens and Kirkegaard, 1995).

An important issue for generation of replicon-based vaccines is the development of an efficient and safe packaging system to generate quantitative amounts of encapsidated replicons. The PV replicons that have been developed by Morrow and colleagues (Ansardi *et al.,* 1993) have been packaged by supplying capsid proteins *in trans* with a recombinant vaccinia virus. The system we used to package our HRV replicons was modified from that described by Jia *et al.* (1998), and is based on the vaccinia virus/T7 RNA polymerase hybrid expression system. Because this method allowed us to generate only small amounts of packaged replicons, we are currently investigating alternative methods.

In summary, data presented here indicate that HRV-14 replicons are able to express clinically relevant secreted glycoproteins and provide further support for the use of HRV replicons as a vaccine delivery system. It has recently been shown that neutralizing antibodies generated during RSV infection have greater affinity for virion F protein when compared to purified F protein or to that present in lysates of RSV-infected cells (Sakurai *et al.,* 1999). Therefore, it appears that it is critical to deliver the mature oligomeric F protein as an immunogen in order to induce a strong RSV-neutralizing immune response. Our data indicate that the ΔP1FVP3 replicon expresses readily detectable amounts of F protein, and initial studies indicate that the antigen produced is able to stimulate a neutralizing humoral immune response in mice. Further work will be required to determine whether packaged ΔP1FVP3 RNA can be delivered to the upper respiratory tract and elicit similar immune responses in primates.

MATERIALS AND METHODS

Cells, viruses, and antibodies

H1-HeLa and HEp-2 cells were obtained from the American Type Culture Collection (ATCC) and were maintained in 1× minimal essential medium (MEM) supplemented with Earle's salts (GIBCO/BRL), L-glutamine, and 10% fetal calf serum (MEM-10). Dr. Bernard Moss (National Institutes of Health, Bethesda, MD) provided recombinant vaccinia virus vTF7-3 and a stock was produced using previously described techniques (Moss and Earl, 1998). Dr. Roberto Garofalo (University of Texas Medical Branch at Galveston) provided stocks of the A2 strain of RSV. Monoclonal anti-peptide antibody RF78 was a kind gift from Dr. Ping-Chuan Hu, University of North Carolina at Chapel Hill, and was directed against amino acids 269–277 of the F1 subunit of the F protein (Fig. 1D). FITC-conjugated goat anti-mouse antibody was purchased from Sigma (St. Louis, MO). Antibodies against HRV-14 were purchased from ATCC and luciferase antibodies were purchased from Sigma.

Plasmids

The coding sequence of the RSV fusion protein was amplified by PCR from plasmid p213 that contains the F sequence of the RSV Long strain (kindly provided by Dr. Ping-Chuan Hu). The primers used were 5′-ccacaacct-agg*ccatgg*accttgaaacaaagctcctcctccGTTACTAAATGCAAT-

ATTATTTAT-3′ and 5′-caaccc*tgatca***atg**GAGTTGCCAATCC-3′, where lowercase bases represent HRV-14 and 3CD^{pro} recognition sequences, the italicized bases represent unique restriction sites (*Nco*I and *Bcl*I), the capitalized bases represent F-protein sequence, and the authentic HRV-14 AUG initiator codon is indicated in boldface. The resulting PCR product was partially digested with *Nco*I and *Bcl*I and ligated to these sites in the plasmid pSPHRV5 (McKnight and Lemon, 1996), resulting in the fusion of the F-protein sequence to the HRV-14 IRES. An IRES-F protein fragment (*Sal*I–*Nco*I) was removed from this intermediate plasmid and inserted into pSPHRVLuc/VP3 (McKnight and Lemon, 1998) in lieu of the luciferase sequence, creating the pSPHRVF/VP3 plasmid in which the 3′ F-protein sequence is fused in frame to the downstream P1 segment of HRV-14 (Fig. 1). Finally, a *Sal*I–*Avr*II fragment was removed from pSPHRVF/VP3 and ligated to these sites in pWR3.26, creating the final pΔP1FVP3 replicon.

The plasmid pΔP1Fdpp in which the sequence encoding the downstream capsid and nonstructural proteins were removed was utilized in constructing the pΔFexp or pΔF^{mut}exp expression vectors. Using QuikChange mutagenesis (Stratagene, La Jolla, CA), stop codons (UAG) were engineered between the last aa (Asn) of the F protein and the 3′ NTR or between the first position aa (Gln) of the HRV-14 synthetic 3CD^{pro} cleavage site and the 3′ NTR (Fig. 1C). The following primers and their exact complements were used in this mutagenesis reaction: fwt: 5′-**gcatttagtaac**TAG ggaggaggagg-3′ and fmut: 5′-gg*agctttgtttcaa*TAGggtccatggac-3′, where the F-protein sequence is boldfaced, the HRV-14 3CD^{pro} cleavage site sequence is italicized, and the inserted stop codons are capitalized. Successful mutagenesis was confirmed by sequence analysis, and the selected the pΔFexp or pΔF^{mut}exp plasmids were further sequenced to verify no other spurious mutations.

The HRV-14 capsid expression plasmid, pCap14, was constructed by inserting the small *Aat*II-*Eco*RV fragment of pWR3.26 into the pSP64 poly(A) vector (Promega, Madison, WI). The resulting plasmid contains the HRV-14 IRES and capsid coding sequence under control of the T7 promoter and terminating with stop codon after the last amino acid of capsid, and a 30-nt poly(A) tract. The construction of the pΔP1Luc, pΔP1Luc/VP3, and pΔP1Luc$_{cre}$ replicons has been described previously (McKnight and Lemon, 1996, 1998).

In vitro transcription, transfection, and preparation of lysates for immunization

To generate *in vitro* transcripts of the different replicons the respective plasmids were linearized at a unique *Mlu*I site that immediately follows the 3′ terminal poly(A) tail of the virus genome. The linear templates were used in a previously described 100-μl *in vitro* transcription reaction, and the integrity and amount of transcripts were determined by agarose gel analysis (McKnight and Lemon, 1996). H1-HeLa cells (5×10^6) were used for electroporation with 10–20 μg of RNA, as described previously, and transfected cells were plated into two 60-mm plates or four 35-mm plates containing 1× MEM-10, and incubated at 34°C until harvest (McKnight and Lemon, 1996). For the generation of F-protein-containing lysates for mouse immunizations, 5×10^6 cells were plated into one 100-mm plate and harvested 24 h following transfection. Cells were removed with a scraper, washed 1× with PBS, and resuspended in 100 μl 1× PBS. The cell suspension was subjected to three freeze/thaw cycles to break up the cells and F-protein expression was confirmed by immunoblot analysis.

Ribonuclease protection assay

Ribonuclease protection assays were carried using reagents and protocols provided with the Lysate Ribonuclease Protection Kit (U.S. Biochemicals). At 0, 6, 12, and 24 h post-transfection, transfected cell monolayers were washed twice with 1× PBS before the addition of 125 μl of lysis solution containing 4 M guanidine thiocyanate, 25 mM sodium citrate, and 0.5% sarcosyl. Lysates were stored at −70°C until hybridization. Riboprobes for monitoring HRV positive-strand RNA synthesis and cellular glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA were prepared as described previously (McKnight and Lemon, 1996); 1×10^6 cpm of each probe was added to 45 μl of lysate and the hybridization reaction was carried out overnight at 37°C. Subsequently, lysates were digested with RNase and proteinase, and RNA was precipitated with isopropanol. Protected RNA duplexes were separated on 6% polyacrylamide/8 M urea gels and visualized by autoradiography.

Immunoblot analysis

At different time points following transfection or infection, cells in 35-mm wells were removed by scraping, washed with PBS, and pelleted by centrifugation. Cellular pellets were resuspended in 40 μl of NP-40 lysis buffer (150 mM NaCl, 1.0% v/v NP-40, 50 mM Tris–HCl [pH 8.0]) and incubated for 30 min on ice. The lysate was centrifuged at maximum speed for 10 min at 4°C in a microcentrifuge to remove debris. Ten to fifteen microliters of the supernatant was separated by 8 or 10% SDS–PAGE and subsequently transferred to a nitrocellulose membrane. The membrane was blocked with TBS-T (TBS [pH 7.6] with 0.1% v/v Tween-20) containing 5% w/v nonfat dry milk for 1 h at room temperature and subsequently washed in TBS-T. Incubation with monoclonal antibody RF78 (diluted 1:500 in TBS-T) was for 1 h at room temperature followed by three washes. The membrane was incubated for another hour with a secondary, horseradish peroxidase-conjugated sheep anti-mouse antibody (Amersham Pharmacia Biotech, Piscataway, NJ), diluted

1:40,000 in TBS-T. Finally, peroxidase was detected with SuperSignal ULTRA chemiluminescence substrate (Pierce, Rockford, IL), and immunoreactive protein bands were visualized by exposure to X-ray film.

In situ immunostaining

H1-HeLa cells were grown in eight-well tissue culture chamber slides and infected with packaged replicons. Alternatively, cells were transfected with replicon RNA and subsequently seeded on chamber slides. After overnight incubation (14–16 h) at 34°C, cells were fixed for 2 min with cold methanol:acetone (1:1), washed with PBS, and blocked with PBS/3% w/v BSA for 1 h at 4°C. After three washes incubation with RF78 (1:50 diluted in PBS/3% w/w BSA) was performed followed by washing in PBS. A 200-fold dilution of a goat anti-mouse FITC-conjugated antibody (Sigma) was added and incubation was continued for 30 min at 4°C. After a final washing step, cells were mounted using Vectashield (Vector Laboratories, Inc., Burlington, CA), coverslipped, and sealed. The slides were examined by fluorescent microscopy.

Packaging of replicon RNAs

Generation of packaged replicons was carried out essentially as described by Jia *et al.* (1998). H1-HeLa cells (50–75% confluent) in 35- or 60-mm plates were cotransfected with 750 μl of OPTI-MEM (GIBCO/BRL) containing 6 μl Fugene transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN), 2 μg each of plasmid DNA encoding the HRV replicon, a second plasmid, pCap14, which encodes the HRV-14 capsid proteins, and vTF7-3 (5 PFU/cell). Cells were incubated at 34°C for 6 h, washed twice with MEM (without serum), and, following the addition of 1 to 2 ml of MEM containing 10% FBS, incubated at 34°C for 36 to 40 h. After harvesting, the cells were lysed by addition of NP-40 to a final concentration of 0.5% w/v and incubation for 30 min on ice. Cell lysates were cleared by microcentrifugation and supernatants were adjusted to 1% w/v sarcosyl and 0.1% w/v 2-mercaptoethanol and loaded on a 1-ml sucrose cushion (30% sucrose in 1 mM NaCl in 20 mM Tris-acetate [pH 7.4]) and centrifuged for 2 h at 40,000 rpm and 16°C in an SW-41 rotor. The pellets that contained packaged HRV replicons were redissolved in PBS/0.1% w/v BSA and stocks of virions were stored at −80°C. Titers of packaged stocks were estimated from *in situ* immunostaining of cell monolayers infected with aliquots of packaged virions. Multiple fields (4–6) were scored for numbers of fluorescent cells and an average was calculated as luciferase-expressing cells per milliliter.

Enzymatic deglycosylation of F protein

Cellular lysates were adjusted to 50 mM 2-mercaptoethanol and 0.05% w/v SDS and heat-denatured for 4 min at 100°C. After cooling, 10 μl of denatured proteins was mixed with 3 μl 0.5 M sodium citrate (pH 5.5) and 2 to 7 μl of endo H (5 unit/ml, Roche Molecular Biochemicals) in a total volume of 20 μl and incubated overnight at 34°C. Endo H was inactivated by heating for 5 min at 100°C and deglycosylated proteins were subsequently separated on a SDS/12% polyacrylamide gel and detected by immunoblot. For PNGase F digestion, cellular lysates were adjusted to 50 mM 2-mercaptoethanol and 0.25% w/v SDS and heat-denatured as above. Ten microliters of lysate was mixed with 5 μl 1 M Tris–HCl, pH 8.0, 0.8 μl 50% v/v NP-40, and 0.2 to 1 unit of PNGase F (200 unit/ml, Roche Molecular Biochemicals) in a total of 20 μl. Overnight incubation and subsequent analysis of the samples were done as described for the endo H treatment.

Animals

Six- to 8-week-old female Balb/c mice, obtained from Charles River Laboratories (Raleigh, NC), were used for RSV immunization experiments. Each mouse was inoculated intraperitoneally with 100 μl of cell lysate (see above) mixed with an equal volume of complete Freund's adjuvant (Sigma). Mice were boosted with 100 μl of cell lysate mixed with an equal volume of incomplete Freund's adjuvant (Sigma) 2 weeks later. Thus, each mouse was inoculated with material derived from one 100-mm dish, which corresponds to 5×10^6 cells. Mice were bled from the lateral saphenous vein 2 weeks after the booster immunization.

RSV neutralizing antibody assay

Serum RSV neutralizing antibody assays were carried out in duplicate on HEp-2 cell monolayers in 96-well tissue culture plates. Briefly, serial twofold dilutions of heat-inactivated sera (100 μl at 30 min, 56°C) were incubated (1 h, 37°C) with an equal volume containing 100 $TCID_{50}$ RSV (A2 strain) in the presence of 10% complete guinea pig complement (Sigma). The monolayers were inoculated with the virus–serum mixture (200 μl) and incubated at 37°C for 1 h to allow virus adsorption. The inoculum was removed, complete media were added, and the cells were allowed to incubate at 37°C for 4 days. Monolayers were scored for the presence or absence of virus infection by staining with 0.1% crystal violet and microscopic examination.

ACKNOWLEDGMENTS

We thank Ping-Chuan Hu for the gift of RSV F-protein cDNA and RF78 antibody, and Roberto Garofalo for the gift of RSV virus stocks and many helpful discussions. We are also grateful to David Sangar and Annette Martin for critical reviews of the manuscript. This work was supported by grants to S.M.L. from the National Institute of Health (RO1-AI40282) and the Cystic Fibrosis Foundation (S880), and to K.L.M. from the University of Texas Medical Branch Technology Grant Program (98-11).

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