Recombinant Respiratory Syncytial Viruses Lacking the C-Terminal Third of the Attachment (G) Protein Are Immunogenic and Attenuated In Vivo and In Vitro

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The design of attenuated vaccines for respiratory syncytial virus (RSV) historically focused on viruses made sensitive to physiologic temperature through point mutations in the genome. These prototype vaccines were not suitable for human infants primarily because of insufficient attenuation, genetic instability, and reversion to a less-attenuated phenotype. We therefore sought to construct novel attenuated viruses with less potential for reversion through genetic alteration of the attachment G protein. Complete deletion of G protein was previously shown to result in RSV strains overly attenuated for replication in mice. Using reverse genetics, recombinant RSV (rRSV) strains were engineered with truncations at amino acid 118, 174, 193, or 213 and respectively designated rA2cpG118, rA2cpG174, rA2cpG193, and rA2cpG213. All rA2cpG strains were attenuated for growth in vitro and in the respiratory tracts of BALB/c mice but not restricted for growth at 37°C. The mutations did not significantly affect nascent genome synthesis in human lung epithelial (A549) cells, but infectious rA2cpG virus shed into the culture medium was dramatically diminished. Hence, the data suggested that a site within the C-terminal 85 amino acids of G protein is important for efficient genome packaging or budding of RSV from the infected cell. Vaccination with the rA2cpG strains also generated efficacious immune responses in mice that were similar to those elicited by the temperature-sensitive cpts248/404 strain previously tested in human infants. Collectively, the data indicate that the rA2cpG strains are immunogenic, not likely to revert to the less-attenuated phenotype, and thus candidates for further development as vaccines against RSV.

Respiratory syncytial virus (RSV) is an enveloped, nonsegmented negative-sense RNA virus that belongs to the family Paramyxovirinae, subfamily Pneumovirinae (5, 28). RSV is the major cause of acute bronchiolitis and pneumonia in infants less than 2 years of age. RSV is highly infectious, and aged adults and patients with underlying disease or immunological abnormalities also suffer disease. It is estimated that 70% of infants become infected in the first year of life, with virtually all individuals infected by age 2 (15). Respiratory tract disease caused by RSV imposes a significant economic burden on health care worldwide. In the United States alone, the annual cost for hospitalization of children with RSV-mediated pneumonia is estimated to approach 300 million dollars (27). This economic burden is even more significant, when RSV-related illnesses in all age groups are considered. Thus, there is great need for prophylactic and/or therapeutic vaccines and medicines to prevent disease caused by RSV. Currently, passive immunization with anti-RSV antibodies is the only prophylactic medicine for at-risk infants (17). In addition, one pharmaceutical agent (ribavirin) is presently licensed to treat acute respiratory tract disease caused by RSV. Ribavirin, however, is a teratogen, and efficacy following treatment is controversial (19, 34).

For several decades, significant efforts have been made on the development of live attenuated vaccines for RSV (33). The major challenges for this endeavor are finding the appropriate level of attenuation without sacrificing immunogenicity. Important for safety, the vaccines must be genetically stable and not revert to a less-attenuated phenotype. In the past, the primary means to achieve attenuation was repeated passage at low temperature. A temperature-sensitive (ts) phenotype typically resulted from 1 to 2 nucleotide changes in the RSV genome (70). Thus, identification of genomic mutations that attenuate virus replication, do not compromise immunogenicity, and have low potential for reversion to a less-attenuated phenotype would significantly improve development of future vaccines against RSV.

The genome of RSV contains 15,222 nucleotides organized...
into 10 genes encoding 11 proteins (5, 28). Several efforts have been made using the aforementioned mutagenesis and passage as well as more advanced technologies (e.g., reverse genetics, as reviewed in references 6, 7, and 41) to successfully identify sites within the RSV genome that when altered conferred attenuation. Most notable were RSV strains restricted for replication because of mutations in genes encoding the M2-1 and L proteins. For example, a single nucleotide substitution in the transcription start signal of M2-1 conferred the ts and attenuated (att) phenotype to cpts248/404 (70). Additionally, cpts530/1030 respectively contained missense mutations at nucleotide positions 10,060 and 12,458 of the L open reading frame (ORF) (71). Using reverse genetics, RSV strains were shown to be restricted for replication following total deletion of genes encoding NS1 and/or NS2 (29, 52, 62), M2-2 (62), or M2-2 and NS-2 (29) proteins. However, of the attenuated RSV strains tested in clinical trials to date, none were acceptable for infants in the first few weeks of life (16, 32, 71, 73).

An alternative approach towards attenuated RSV vaccine development is genetic modification of G protein. G protein is a heavily glycosylated 90-kDa type II transmembrane protein that is synthesized in both secreted and membrane-bound forms and has an important role in attachment of RSV to the host cell (26, 35, 50, 63, 68). Recently it was shown that RSV strains with G protein completely deleted were highly restricted for growth in vivo (31, 63). Thus, the data implied the existence of an attenuation site in G protein that may be manipulated for vaccine development. In the study presented herein, we sought to identify this site. The extracellular portion of membrane-bound G protein consists of two heavily glycosylated domains of variable sequence flanking a highly conserved central nonglycosylated domain. Recombinant RSV with deletion of a 26-amino-acid portion of the central domain that included a highly conserved cysteine noose and flanking residues did not affect the growth of RSV in vitro or in vivo (60). We therefore focused on the N- and C-terminal hyper-variable regions as putative sites where alteration might impair effective replication. Herein we demonstrate for the first time that the C-terminal domain of G protein is required for efficient replication in both murine and human cells. Furthermore, the data suggest that the C-terminal ectodomain plays a critical role in packaging and/or budding of RSV from the cell. Importantly, truncation at the C terminus did not appear to impair the immunogenic properties of G protein. This new information is important for design of future attenuated vaccines for RSV.

MATERIALS AND METHODS

Cells and viruses. AS49 (CCL-185; American Type Culture Collection [ATCC], Rockville, Md.), HEp-2 (CCL-23; ATCC), and Vero (CCL-81; ATCC) cells were cultured (37°C, 5% CO2) in six-well plates for the indicated times (105 cells/ml). AS49 cells were cultured in Hams F-12K medium (Sigma, St. Louis, Mo.) supplemented with heat-inactivated 10% fetal bovine serum (FBS; HyClone, Logan, Utah), 2 mM l-glutamine, and 1.5-g/liter sodium bicarbonate (Invitrogen, Carlsbad, Calif.). HEp-2 and Vero cells were cultured in Dulbecco’s minimal essential medium (DMEM) supplemented with 5% FBS, 2 mM l-glutamine, and 1% penicillin-streptomycin (Invitrogen), and the conditions were as follows: 48°C for 45 min; 95°C for 1 min; 40 cycles of 30 s at 94°C, 30 s at 58°C, and 6 min at 68°C; completed by a final extension step of 68°C for 7 min. The amplified fragments were purified with a QiAquick PCR purification kit (Qiagen, Valencia, Calif.), and cycle sequencing was performed on 50 to 100 ng of purified fragment by using the Big Dye Terminator v3.0 ready reaction cycle sequencing kit (Applied Biosystems). Unincorporated dyes were removed with the DyeEx-96 kit (Qiagen), and automated sequence analysis was carried out on the 3100 Genetic analyzer (Applied Biosystems). Sequence data were aligned by using Sequencer v4.0.5 (Gene Codes, Ann Arbor, Mich.).

Virus preparation. Vero cells were infected with the wild-type A2 strain or mutant RSV at a multiplicity of infection (MOI) of 0.1. Cells were grown in complete DMEM supplemented with 5% FBS, 2 mM l-glutamine, and 2% penicillin-streptomycin, and infection was allowed to proceed until a 75% cytopathic effect was observed. The viruses were prepared by freeze-thaw and low-speed centrifugation at 200 g, 15 min to remove cellular debris. Viral preparations utilized for quantitative PCR studies were further purified by precipitation with polyethylene glycol (PEG) and passage over a discontinuous sorbitol density gradient. In brief, clarified supernatants were added to a 50% PEG-15 M NaCl, 0.05 M triethanolamine-0.5 M EDTA to yield a final concentration of 10% (vol/vol) PEG–NTE to culture supernatant. After being stirred for 2 h (4°C), the precipitate was pelleted at 8,500 rpm (30 min, 4°C) with a Sorvall RC-5B Superspeed centrifuge with a GSA rotor. The resulting pellet was resuspended in 20% (wt/wt) sorbitol–NTE buffer and placed over the discontinuous sorbitol gradient. Purified virus was collected at the interface between 60 and 35% sorbitol–NTE and stored at −70°C. Each virus preparation was tested for endotoxin contamination by using a Limulus amebocyte lysate assay kit (Charles River Endosafe, Charleston, S.C.) and found to contain ≤1.0 endotoxin unit per ml of virus. All viruses were stored at −70°C.

RSV infections. RSV infections were performed as described previously (43). Briefly, RSV aliquots were rapidly thawed, diluted in F-12K medium, and added immediately to AS49 monolayers (approximately 90% confluent) at an MOI of 0.09 (0.04 ml of diluted virus/cm2). The cultures were then placed on a rocker for 2 h at the indicated temperature (5% CO2). Thereafter, unabsorbed virus was removed and replaced with fresh medium (0.16 ml/cm2) and the plates were further incubated for the indicated time points.

Virus plaque assay. Plaque assays were performed as previously described (23, 24). Briefly, HEp-2 cell monolayers were seeded (5 × 104 cells/well) in 48-well plates (Costar, VWR, West Chester, Pa.) and incubated overnight. For characterization and temperature sensitivity studies, purified virus preparations were used (MOI of 0.09). Infectious virus titers in the supernatants of AS49 monolayers were determined after infection of HEp-2 cell monolayers at various temperatures (32 to 40°C, 5% CO2). In brief, after serial dilution in DMEM (5% FBS) and infection (2 h), the culture medium was replaced with 0.5 ml of 1% (vol/vol) Sephadex G-75 beads (Pharmacia, Uppsala, Sweden) in DMEM (5% FBS). After 3 to 4 days of incubation, the gel overlay was removed and mono-

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layers were fixed (30 min, room temperature) in 80% methanol. The plaques were visualized following incubation with L4 monoclonal antibody (MAb) directed against F protein (45, 67) or MAbs previously identified to react with specific regions of G protein (amino acids 1 to 174 [131-2G], 174 to 214 [L9], or 214 to 298 [130-2G] (56). Bound antibody was detected with a goat anti-mouse immunoglobulin G (IgG) conjugated to horseradish peroxidase (Kirkegaard & Perry Laboratories, Gaithersburg, Md.). PFU are expressed per milliliter of culture supernatant. Infectious virus titers in murine lungs and noses were also assessed in plaque assays as described previously (22–24) and expressed as PFU per gram of tissue.

Real-time qPCR. Viral genome copy number in RSV-infected A549 monolayers was determined by real-time quantitative RT-PCR (qPCR). RNA was extracted from cells with an RNeasy Mini kit (Qiagen) and QIAshredders (Qiagen) according to the manufacturer’s specifications and measured on a CytoFluor 4000 fluorescence plate reader (Applied Biosystems) after addition of Ribogreen RNA Quantitation Reagent (Molecular Probes, Eugene, Oreg.). All RNA preparations were resuspended in RNase-free water and stored at −70°C. One microgram of total cellular RNA and a TaqMan reverse transcriptase reagent kit (Applied Biosystems) were used according to manufacturer’s specifications. The resulting cDNAs were assayed for the presence of negative-strand cDNA. The PCR primer-probe set (Syntellix, LLC, Houston, Tex.) complementary to a region of the L gene that is 100% conserved in all RSV strains used in this study. The following primers and probes were used in the amplification reactions: RSVAF forward primer (5’-AGACAAAGCTAAAATTA CTAGCGGAATCA-3’), RSVAP FAM/TAMRA probe (5’-TAGACTGCCAG TTACAGAGGT-3’), and RSVAR reverse primer (5’-GTTGTCGACTTTG GAGAATATTTG-3’). PCR products were cloned into a pT7Blue3-vector (Novagen, Milwaukee, Wis.) and serially diluted for use as standards. The following PCR cycling conditions were used: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. TaqMan Universal PCR master mix (Applied Biosystems), 0.5 μM primers, and 0.25 μM probe were used in each reaction. To verify equal loading of cDNA between samples, a TaqMan rRNA control reaction kit (Applied Biosystems) was used to amplify human 18S cDNA according to the manufacturer’s protocol. To determine copy numbers, standard curves were generated with 8 RSVΔ2 L protein or ribosomal DNA standards of known copy numbers per ml. Three independent experiments were performed, with each sample run in triplicate. PCR, fluorescence detection, and data analysis were performed on an ABI Prism 7700 sequence detector (Perkin-Elmer, Pittsburgh, Pa.).

### Experimental infections.
Age-matched (6 to 8 weeks old) female BALB/c mice were used in all experiments and housed in a facility accredited by the American Association for Accreditation of Laboratory Animal Care. The mice were experimentally infected (0.05 ml) on week 0 with 106 PFU of the A2, cp-RSV, or rRSV strain. Control mice received an equal volume of PBS. All infections were administered under injected anesthesia as previously described (24). Lung and nose tissues were harvested 4 and 7 days after infection, weighed, homogenized, snap frozen, and stored at −70°C. To measure efficacy, mice were challenged (106 PFU of RSV A2) 4 weeks after primary infection and 4 days thereafter tissues were similarly collected for infectious virus titer. There were five mice per group.

### Serum antibody determinations.
The geometric mean serum IgG titers were determined as previously described (23, 24) by endpoint enzyme-linked immunosorbent assay. All samples were analyzed on a multiwell plate reader (Molecular Devices, Sunnyvale, Calif.). The neutralization titers were determined by the plaque reduction neutralization test against the A2 strain of RSV in the presence or absence of 5% (vol/vol) guinea pig serum (BioWhittaker, Walkersville, Md.) as a source of complement. The neutralization titers were calculated as the reciprocal of the serum dilution that showed 60% reduction (relative to the virus control) in the number of foci per well.

### Statistical analyses.
Significant differences (P < 0.05) were determined after log transformation by Tukey-Kramer highly significant differences multiple comparison or Student’s t test using JMP statistical discovery software (SAS Institute, Inc., Cary, N.C.). The data are expressed ± 1 standard deviation. All data were confirmed in separate studies.

## RESULTS

### Construction of rRSV strains with altered G protein.
To identify the region important for efficient viral replication, reverse genetics was used to create four RSV strains with unique truncation sites in the gene encoding the G protein. To that end, four cDNA fragments of RSV genome were cloned into one expression vector to create a complete, full-length cDNA. The first cDNA fragment, le-P, encompassed nucleotides 1 through 3009 of the genome, had a T7 RNA polymerase

<table>
<thead>
<tr>
<th>Fragment no.</th>
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<th>Sequence (5’→3’)</th>
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<tr>
<td>1</td>
<td>1–2520</td>
<td>ACAGGAAAAATGCGTACAAACAACTTGGCATACACCTTCGTTATATACCTGGTCCTTTTGGTTACTTCTCATATATC</td>
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<td>2</td>
<td>2416–4871</td>
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<tr>
<td>3</td>
<td>4718–7045</td>
<td>TAAGAGACATTAGAAGAGCCCTGGGACCTTCAATCCTGACTGCTTACATAATATAATGTTGTACCTCACAGAC</td>
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<tr>
<td>4</td>
<td>6902–9326</td>
<td>TGTCATGCTATGGGAAAACCTAAATGTCAGATCCATATTCCTTCAAGATATAGGTGAGT</td>
</tr>
<tr>
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<td>9111–11450</td>
<td>ATACATGGTGGTTAAAATATATACCAAAATTAACACATACATGGTAAATGCTGCTG</td>
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<td>11282–13412</td>
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<tr>
<td>7</td>
<td>13204–15223</td>
<td>TAATACGACCTAGTTATGGGAAATCTATGCTAAGGAGCAGAAAAAGTGGTCAAAACCTAAATATGCTGTAAGA</td>
</tr>
</tbody>
</table>

| Table 1. Primer pairs used in sequence analysis of rA2cpΔG truncation mutants

**Experimental infections.** Age-matched (6 to 8 weeks old) female BALB/c mice were used in all experiments and housed in a facility accredited by the American Association for Accreditation of Laboratory Animal Care. The mice were experimentally infected (0.05 ml) on week 0 with 106 PFU of the A2, cp-RSV, or rRSV strain. Control mice received an equal volume of PBS. All infections were administered under injected anesthesia as previously described (24). Lung and nose tissues were harvested 4 and 7 days after infection, weighed, homogenized, snap frozen, and stored at −70°C. To measure efficacy, mice were challenged (106 PFU of RSV A2) 4 weeks after primary infection and 4 days thereafter tissues were similarly collected for infectious virus titer. There were five mice per group.

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**Statistical analyses.** Significant differences (P < 0.05) were determined after log transformation by Tukey-Kramer highly significant differences multiple comparison or Student’s t test using JMP statistical discovery software (SAS Institute, Inc., Cary, N.C.). The data are expressed ± 1 standard deviation. All data were confirmed in separate studies.
promoter sequence located immediately preceding nucleotide 1, a NotI restriction enzyme (RE) site upstream of the promoter sequence, and an AatII RE site within the ORF of the P gene at nucleotides 3004 to 3009. Notably, the AatII RE sequence did not result in an amino acid coding change from the biologically derived P protein sequence of \textit{cp}-RSV. The second fragment, M-M2, encompassed nucleotides 3004 through 8506, overlapped the le-P fragment at the AatII RE site and contained a naturally occurring BamHI RE site at nucleotides 8501 to 8506. The third fragment, L, encompassed nucleotides 8501 through 14996, overlapped the M-M2 fragment at the BamHI RE site and had engineered BsmBI and PstI RE sites positioned at the end of the L gene ORF. A fourth fragment, tr, corresponded to nucleotides 14997 through 15223 and contained engineered BspMI, BamHI, and BsmBI RE sites. All RSV gene fragments (except L) were obtained by RT-PCR amplification of RNA extracted from Vero cells infected with the virus \textit{cp}ts248/404 (9) and cloned into individual vectors. The L fragment, originally cloned from RSV A2, was obtained by PCR amplification using the expression vector pTM-L (18). Site-directed mutagenesis was used to alter the sequence of the \textit{cp}ts248/404-derived M2 gene and the RSV A2-derived L gene to match the biologically derived \textit{cp}-RSV (8, 10, 13, 70). Hence all rRSV strains described in this study are based on the genome encoding \textit{cp}-RSV.

Truncation of the G protein gene was accomplished by using two DNA primers complementary to genomic sequences immediately flanking the nucleotides to be deleted in the M-M2 RSV genome fragment. Each of these primers contained a BsaI RE site. Using a cloned copy of the M-M2 fragment, extension from these primers occurred in opposite directions such that the region to be deleted was excluded from PCR amplification and plasmid circularization occurred after complete extension and digestion of the PCR product with BsaI. In this way, a total of four separate deletions were made in the ORF of the G gene. These deletions were engineered to encode G proteins C-terminally truncated at amino acid 118, 174, 193, or 213 (Fig. 1). The specific deletions were then incorporated into full-length cDNA by exchanging the M-M2 fragment from the full-length cDNA clone with the altered M-M2 fragment containing the truncation in the G gene.

The vector used for cloning full-length cDNAs (pFL) was based on the previously described measles virus (MV) mini-genome vector, p107MVCat (54). After removal of measles-specific sequences, pFL contained only the ribozyme and two copies of the T7 RNA polymerase transcription terminator sequence downstream of the multiple cloning site. The BsmBI RE site was positioned to allow a precise alignment of the end of the RSV genome with the ribozyme cleavage site. The tr, Le-P, M-M2, and L RSV cDNA fragments were then systematically ligated into the vector with the appropriate restriction enzymes. Following transfection and rescue, the rRSV strain truncated at amino acid 118 of G protein and with amino acids 119 to 298 deleted was designated rA2cpΔG118. Using a similar nomenclature, rRSV strains rA2cpΔG174, rA2cpΔG193, and rA2cpΔG213 were constructed with G protein respectively truncated at amino acids 174, 193, and 213.

![FIG. 1. Construction of rRSV antigenomic cDNA with altered G protein. Large cDNA fragments of total RSV genomic RNA (denoted le-P, M-M2, L, and tr) were sequentially cloned into plasmid pFL. A clone of the M-M2 fragment, containing the M, SH, G, F, and M2 genes of RSV, was genetically altered by PCR mutagenesis such that G protein gene sequences encoding amino acids C-terminal of amino acid (aa) 118, 174, 193, or 213 were excluded from PCR amplification. These truncations are denoted below the schematic representation of G protein. The mutated M-M2 fragment was used to replace the corresponding fragment in the full-length cDNA clone and along with helper RSV plasmids was transfected into Vero cells for virus rescue and purification. CT, cytoplasmic tail; TM, transmembrane region; P, proline residues; C, cystine residues; stalks with circles, potential O-linked carbohydrate acceptor sites; N, potential N-linked carbohydrate acceptor sites.](#)
Characterization of rRSV strains encoding truncated G proteins. The identity of each rRSV strain was confirmed by DNA sequencing (Tables 1 and 2) and mapping with MAb reactive within the region spanned by amino acids 1 to 174 (131-2G), 174 to 215 (L9), or 215 to 298 (130-2G) of G protein (Fig. 2). The sequencing data indicated that in some cases spontaneous mutations occurred independent of the planned truncations (Table 2). The insertion of two AA residues in the L gene of rA2cp/H9004G118 and the A insertion in rA2cp/H9004G193 occurred in noncoding regions of the genome. The A-to-G mutation in the L ORF of rA2cp/H9004G174 was a silent mutation. The only mutation that caused an amino acid change was an A-to-G mutation in ORF1 of the M2 gene, which resulted in a coding change from Ile to Met. No spontaneous mutations were detected in rA2cp/H9004G213. The results following MAb mapping also demonstrated that the truncations were correct (Fig. 2). The rA2cpG193 and rA2cpG213 rRSV strains had positive reactivity with 131-2G and L9 but were negative for 130-2G. The rA2cpG118 and rA2cpG174 rRSV strains reacted with 131-2G, but not with L9 or 130-2G. The parent cp-RSV virus was reactive with all three MAbs. Thus, the rRSV strains expressed the appropriate truncations. Furthermore, reactivity with MAb indicated that the truncated G proteins were similar in situ to full-length protein from cp-RSV. Positive reactivity of rA2cpG193 with L9 MAb also localized the epitope between amino acids 174 and 193 of G protein (Fig. 2).

Replication of rRSV strains in human lung epithelial cell lines. We next compared replication of the rRSV strains to that of A2, cp-RSV, and temperature-sensitive cpts248/404 (16, 73) strains of RSV in human lung epithelial cells (A549). The replication rates were determined by using real-time qPCR that measured nascent negative-genomic-strand synthesis. The resultant infectious virus shed into the culture medium was ascertained by plaque assay using HEp-2 cell monolayers. The data indicated that the rRSV strains with truncated G protein were restricted for replication at 37°C. Compared to the parent cp-RSV strain, virus titers in the culture medium 72 h after infection were diminished 500- to 5,000-fold (Table 3). The

### Table 2. Sequence analysis of nucleotide changes in rA2cpΔG truncation mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>Gene</th>
<th>Nucleotide position</th>
<th>Nucleotide change</th>
<th>Amino acid change</th>
</tr>
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<tbody>
<tr>
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<td>L</td>
<td>15020-15022</td>
<td>AA insertion</td>
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<tr>
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<td>7786</td>
<td>A to G</td>
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<td></td>
<td>L ORF</td>
<td>9698</td>
<td>A to G</td>
<td>Silent</td>
</tr>
<tr>
<td>rA2cpΔG193</td>
<td>N</td>
<td>2322-2327</td>
<td>A insertion</td>
<td>Noncoding region</td>
</tr>
<tr>
<td>rA2cpΔG213</td>
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<td></td>
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</tr>
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</table>

*a Nucleotide changes are shown in positive sense and do not include intended truncations of the G protein gene.

*b Amino acid position in the M2-1 protein.

*c The A insertion occurred in a polyadenine region of the gene for N protein.

FIG. 2. MAb mapping of rRSV strains with altered G protein. Correct truncations were confirmed by using MAbs 131-2G, L9, and 130-2G, which are reactive with epitopes within regions respectively spanned by amino acids (aa) 1 to 118, 174 to 213, and 213 to 298 of G protein. Antibody binding was detected by plaque assay as described in Materials and Methods. 100% denotes amino acids 164 to 176 conserved among all RSV strains. Vertical bars, potential O-linked carbohydrate acceptor sites; “bells,” potential N-linked carbohydrate acceptor sites.
rRSV genome copy numbers in contrast were diminished only 1.1- to 12.0-fold between 24 and 72 h of infection (Table 3). The data therefore suggested that synthesis of the nascent RSV genomes was only modestly affected in comparison to the 2.5- to 5-log10 decrease in viral titer imparted by truncation of G protein. Thus, truncation appeared to inhibit the emergence of infectious rRSV from A549 monolayers. Genome copy number following infection with the temperature-sensitive G protein. Thus, truncation appeared to inhibit the emergence of infectious rRSV from A549 monolayers. Genome copy number following infection with the temperature-sensitive rRSV strain, A2, was able to replicate at temperatures near 40°C. Fig-3. The data confirmed that cpts248/404 RSV and A2 were able to replicate at temperatures near 40°C. When the monolayers were infected with the cpts248/404 RSV strain, plaques were visible at 32°C, but not at 37°C or greater, verifying the 36°C shutoff temperature (9). Following infection with rRSV strains with truncated G protein, plaques were observed in all HEp-2 cell cultures and at all temperatures tested. At the highest temperatures (most notably 39°C and above), the plaques were pinpoint in size and marginally smaller than those observed following infection with cp-RSV. Thus, the shutoff temperatures for all rRSV strains were greater than 40°C. Restricted replication following truncation of G protein was therefore not sensitive to physiological (37°C) temperature.

The effects of G protein truncation on RSV genome replication and productive infection. The results from a previous study (59) suggested that complete deletion of G protein impaired replication because of a deficiency in “packaging” genome and/or budding of RSV from the membranes of infected cells. We therefore sought to investigate the nature of the deficit following truncation of G protein. To that end, the ratios of total RSV genome copy number in A549 cell monolayers to total infectious rRSV strain shed into the corresponding culture medium were compared after culture at 37°C. Figure 4A depicts the ratios of RSV genome copy number to infectious virus for each mutant. The ratios following infection with rA2cpΔG213 or rA2cpΔG193 were, respectively, 100- and 1,000-fold greater than that of cp-RSV. A similar defect also appeared to occur after infection with cpts248/404 expressing full-length G protein. The ratio was nearly 10,000 times greater than that of cp-RSV. Thus, a packaging defect might not be specifically related to G protein. Therefore, the ratio of RSV genome copy number to infectious RSV shed following infection with cpts248/404 was compared to the ratio following infection with rA2cp248/404ΔSH with the gene encoding SH protein deleted (69). Figure 4B demonstrated that a disparity in ratios was not observed when cpts248/404 and rA2cpts248/404ΔSH were cultured at permissive temperature (32°C). Taken together, the data suggest that the C-terminal end of G protein is important in viral budding and/or packaging genome. Deletion resulted in attenuation but not sensitivity to physiological temperature.

Attenuation, immunogenicity, and efficacy in BALB/c mice immunized with rRSV. To assess attenuation in vivo, BALB/c mice were infected with rRSV, A2, cp-RSV, cpts248/404, or rA2cpts248/404ΔSH and 4 and 7 days thereafter replication was measured in the upper and lower respiratory tracts. As depicted in Fig. 5A, the nasal and lung tissues of all mice administered rRSV contained significantly less virus 4 days after infection than mice given A2 and cp-RSV strains. Viral titers were below the lower limit of detection for groups infected with rA2cpΔG193, rA2cpΔG213, rA2cpts248/404ΔSH, and cpts248/404. By day 7, viral titers in the lungs of all mice infected with rRSV were below detectable levels, while infectious cp-RSV (2.5 log10 PFU/g of tissue) was still observed (Fig. 5B). Thus, rRSV strains with truncated G protein were highly attenuated for replication in the respiratory tract of BALB/c mice.

Truncation of G protein might result in the loss of epitopes important in the generation of neutralizing antibodies. We therefore investigated immunogenicity in BALB/c mice following primary experimental infection with rRSV (Table 4). The results demonstrated that rA2cpΔ118, rA2cpΔ174, rA2cpΔ193, or rA2cpΔ213 generated significant serum complement-assisted neutralization titers 4 weeks after infection. The titers were comparable to those elicited following immunization with cpts248/404 and 0.4 to 0.7 log10 less than the neutralization titers generated following infection with cp-RSV. Truncation of G protein, in addition, did not significantly affect the induction of anti-F protein IgG titers. Most importantly, immunization with the rRSV strains elicited efficacious immune re-
responses. Four days after challenge with the A2 strain, there was a 2.5- to 3-log$_{10}$ reduction in infectious virus titers relative to unvaccinated mice. Hence, the efficacy following immunization with the rRSV strains was similar to that of cp-RSV or cpts248/404.

**DISCUSSION**

The majority of live, attenuated RSV vaccines tested to date have proven to be unacceptable for use in human infants. The difficulties primarily resulted from underattenuation yielding unsatisfactory congestion in the respiratory tract (46). In addition, reversion to a less-attenuated phenotype was also of concern because point mutations in the genome associated with the ts and att phenotypes were too often unstable (73). The cp-52 deletion mutant, lacking genes encoding both G and SH proteins, circumvented stability issues associated with point mutations but was overly attenuated when administered to seronegative infants and children (31). Through reverse genetics, the SH gene was subsequently shown to be dispensable for replication in vitro and in vivo (2, 59, 61, 69). Deletion of G protein alone, on the other hand, resulted in rRSV strains that were highly attenuated for replication in the respiratory tract of mice (63). Herein, we used reverse genetics to map the region of G protein necessary for efficient replication. The resultant data demonstrate for the first time the existence of a site within the C-terminal 85 amino acids (residues 214 to 298) of G protein that, when absent, confers attenuation both in vitro and in vivo. Thus, the findings identify a region of G protein that contributes to the efficient replication of RSV. It is noteworthy that the rRSV strains were not sensitive to physiological temperature, and only minor reductions in the replication rate were observed.

The function of the C-terminal third of G protein in RSV replication is not entirely clear. It is generally accepted that G

![FIG. 3. Plaque morphology of rRSV strains with altered G protein. Monolayers of HEp-2 cells were infected with the indicated virus and cultured for 3 to 4 days at the denoted temperature. The plaques were visualized by immunostaining for F protein as described in Materials and Methods.](image-url)
protein participates in attachment of RSV to airway epithelial cells. This reportedly occurs through interactions between positively charged amino acids (184 to 198) in the heparin-binding domain (HBD) and negatively charged glycosaminoglycan (GAG) on airway epithelial cells (12, 20, 21). Thus, deletion or mutation of the HBD could lead to attenuation through decreased attachment of rRSV to target cells. The lack of detectable viral protein synthesis and plaques in HEp-2 monolayers infected with RSV lacking G protein (63) appeared to support this hypothesis. However for the rRSV strains presented herein, this scenario seems unlikely. The HBD was retained in attenuated rsummary/2cp24/404/G213 and when deleted from other rRSV strains (60) did not confer attenuation. Plaques (although reduced in size at higher temperatures) were readily detected following infection of cp-RSV, rA2cpΔG213, cps248/404, or rA2cpΔG213/404ΔSH and cultured at 32°C.

FIG. 4. Ratios of total RSV genome copy number to total shed virus. A549 cell monolayers were infected with RSV (MOI of 0.09), and 72 h thereafter, genome copy numbers were determined by qPCR. Infectious virus titers in the A549 culture supernatants were ascertained on HEp-2 cell monolayers by plaque assay. (A) RSV genome copy per PFU in cells infected with A2, cp-RSV, rRSV, or cps248/404 and cultured at 37°C. (B) RSV genome copy per PFU in cells infected with cp-RSV, rA2cpΔG213, cps248/404, or rA2cpΔG213/404ΔSH and cultured at 32°C.

FIG. 5. Replication of rRSV strains in the respiratory tract of BALB/c mice. Naive BALB/c mice were infected (10^6 PFU) with the indicated viruses. Lung and nasal tissues were collected 4 (A) and 7 (B) days thereafter for the determination of infectious virus titer (log_{10} per gram of tissue) by plaque assay. Infectious virus was not detected in nasal tissues on day 7 and so is not shown. There were five mice per group. The lower limit of detection for both data sets was approximately 1.5 log_{10}.

The C-terminal ectodomain of G protein is highly glycosylated. Thus, reduced glycosylation following truncation might have contributed to attenuation by interfering with the positioning of G protein in the cell membrane. This scenario also appears unlikely. It was recently reported that the signal sequence for posttranslational modification and trafficking of G protein to the cell surface is located between amino acids 23 and 31 (36). All rRSV strains described herein possessed the signal sequence. Moreover, G protein truncated at amino acid 71 and expressed in recombinant simian virus 40 viruses was shown to be O glycosylated with the C-terminal end exposed on the surface of infected CV-1 cells (42). The results from a recent study revealed that RSV assembly involved interaction with numerous host cell proteins and occurred in specific lipid rafts of the cell membrane (40). Several RSV proteins, including N, P, and G proteins, were found almost exclusively in these microdomains. Thus, the C-terminal end of G protein may be involved in lipid-raft localization and truncation may...
TABLE 4. Immunogenicity and efficacy in BALB/c mice infected with rA2cpΔG strains

<table>
<thead>
<tr>
<th>Virus</th>
<th>PFU of RSV&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Antibody titer (log&lt;sub&gt;10&lt;/sub&gt;)</th>
<th>Neutralizing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>F protein IgG</td>
<td>+ complement</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cp-RSV</td>
<td>&lt;1.7 ± 0.03</td>
<td>4.5 ± 0.3</td>
<td>1.69 ± 0.2</td>
</tr>
<tr>
<td>rA2cpΔG118</td>
<td>&lt;1.7 ± 0.04</td>
<td>4.3 ± 0.2</td>
<td>1.3 ± 0.22</td>
</tr>
<tr>
<td>rA2cpΔG174</td>
<td>&lt;1.7 ± 0.04</td>
<td>4.4 ± 0.2</td>
<td>1.3 ± 0.14</td>
</tr>
<tr>
<td>rA2cpΔG193</td>
<td>2.6 ± 0.5</td>
<td>3.3 ± 0.2</td>
<td>1.04 ± 0.0</td>
</tr>
<tr>
<td>rA2cpΔG213</td>
<td>2.3 ± 0.7</td>
<td>3.6 ± 0.2</td>
<td>1.01 ± 0.0</td>
</tr>
<tr>
<td>cpts248/404</td>
<td>&lt;1.7 ± 0.7</td>
<td>3.7 ± 0.1</td>
<td>1.2 ± 0.13</td>
</tr>
<tr>
<td>rA2cpts248/404ΔH</td>
<td>&lt;1.7 ± 0.1</td>
<td>4.0 ± 0.2</td>
<td>1.13 ± 0.09</td>
</tr>
<tr>
<td>PBS</td>
<td>5.09 ± 0.15&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.4 ± 0.0</td>
<td>&lt;1.0</td>
</tr>
</tbody>
</table>

<sup>a</sup>BALB/c mice were experimentally infected on week 0 with 10<sup>6</sup> PFU of the indicated strain of RSV. Control mice received an equal volume of PBS. All infections were intranasal. There were five mice per group.

<sup>b</sup>The numbers are geometric mean log<sub>10</sub> PFU per gram of tissue ± 1 standard deviation.

<sup>c</sup>The titers are geometric means ± 1 standard deviation and determined on serum samples collected 4 weeks after primary experimental infection. Neutralization titers were ascertained in the presence (+) or absence (−) of 5% serum as a source of complement.

<sup>d</sup>P < 0.05 for all comparisons.

inhibit important interactions with glycosylated host cell proteins or with glycosylated F and/or SH proteins (11).

The amino acid sequences within the C-terminal ectodomain of G proteins are highly variable among clinical isolates of RSV (3, 30, 39, 51, 66). One study found 28% sequence variability within the same subgroup (A strain) (53). G protein sequences from A strain viruses are approximately 53% homologous to B strain viruses. Thus, research efforts on RSV sequences from A strain viruses are warranted. G proteins are highly variable among clinical isolates (3, 30, 39, 51, 66). One study found 28% sequence variability within the same subgroup (A strain) (53).

Conserved epitopes are located between amino acids 65 and 298 of the central domain of G protein. T-cell epitopes are located between amino acids 65 and 298 of the central domain of G protein. B-cell epitopes are located between amino acids 65 and 298 of the central domain of G protein. While heparin-like structures on G protein are important for infectivity (1), their role in viral budding/packaging is questionable. Several groups described T-cell epitopes in or near the central, nonglycosylated domain of G protein (25, 47–49, 55, 58, 65). Unless adjuvanted properly, subunit vaccine formulations based on this region were associated with undesirable immunopathology in rodents. Nonetheless, we sought to define the attenuation site with the smallest number of residues deleted to minimize disruption of epitopes that may be important in immunity. The results presented herein suggested that indeed rA2cpΔG213 has potential as an attenuated vaccine. Neutralization titers and efficacy following immunization of mice were comparable to those achieved after vaccination with cpts248/404, a strain tested in human infants (Table 4) (73). Moreover, the possibility of reversion to underattenuated cp-RSV by random mutation is essentially nil, making, e.g., rA2cpΔG213 a safer alternative to previous candidates derived from point mutations.

In conclusion, we identified a novel site between amino acids 214 and 298 of G protein that when absent conferred attenuation without sensitivity to physiological temperature. Using reverse genetics, the site may be further localized within the C-terminal ectodomain in the future. This information, when combined with the rapidly growing library of RSV mutations, will further elucidate the correct combination of mutations.

NOVEL ATTENUATION DETERMINANT IN RSV G PROTEIN

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necessary to genetically construct live attenuated vaccines that elicit efficacious immune responses in human infants with greatly reduced concerns for safety.

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REFERENCES


