Processing, Surface Expression, and Immunogenicity of Carboxy-Terminally Truncated Mutants of G Protein of Human Respiratory Syncytial Virus

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Posttranslational processing and cell surface expression were examined for three C-terminally truncated mutants of the G protein of respiratory syncytial virus expressed from engineered cDNAs. The truncated mutants, encoded by cDNAs designated G71, G180, and G230, contained the N-terminal 71, 180, and 230 amino acids, respectively, of the 298-amino-acid G protein. To facilitate detection of G71, which reacted inefficiently with G-specific antisera, we constructed a parallel set of cDNAs, designated G71/13, G180/13, and G230/13, to encode the same truncated species with the addition of a C-terminal 13-amino-acid reporter peptide which could be detected efficiently with an antipeptide serum. G71, G180, and G230 were expressed as species of M_r 7,500, 48,000, and 51,000, respectively, compared with 84,000 for parental G protein. The proteins encoded by G180 and G230, like parental G protein, contained both N-linked and O-linked carbohydrate. Also, the protein encoded by G71/13 appeared to be O glycosylated, showing that even this highly truncated form contained the structural information required to target the protein for O glycosylation. As for parental G protein, the estimated M_r s of the proteins encoded by G180 and G230 were approximately twice the calculated molecular weight of the polypeptide chain. Experiments with monensin showed that most of this difference between the calculated and observed M_r was due to posttranslational processing in or beyond the trans-Golgi compartment, presumably owing to the addition of carbohydrate or aggregation into dimers or both. Like parental G protein, all three truncated forms accumulated abundantly at the cell surface, and in each case the C terminus was extracellular. Thus, the N-terminal 71 amino acids of the G protein contained all the structural information required for efficient membrane insertion and cell surface expression, whereas the extracellular domain was dispensable for these activities. Cotton rats were immunized with recombinant vaccinia viruses expressing the G71, G180, G230, or parental G protein to compare their abilities to induce serum antibodies and resistance to challenge virus replication. The G71 and G180 recombinants failed to induce significant levels of G-specific antibodies or resistance to challenge, whereas the immunogenicity of G230 equaled or exceeded that of parental G protein. This suggested that the C-terminal 68 amino acids of the 236-amino-acid ectodomain do not contribute to the major epitope(s) of the G protein that is involved in inducing protective immunity.

The attachment G glycoprotein of human respiratory syncytial virus (RSV) is distinct structurally from the attachment hemagglutinin-neuraminidase (HN) or hemagglutinin (H) glycoproteins of other paramyxoviruses (6, 16, 31, 41). The G protein lacks detectable sequence homology with the HN or H protein (33), is distinct from the others in having O-linked carbohydrate side chains (see below), is substantially shorter in chain length, and lacks the hemagglutinin and neuraminidase activities associated with the HN protein.

The deduced amino acid sequence of the G protein (298 amino acids, calculated polypeptide molecular weight of 32,587, for strain A2) contains a single major hydrophobic region that is located near the N terminus (amino acids 41 to 62) and is thought to serve as the membrane anchor (31, 41). The C-terminal three-fourths of the G protein is thought to

be extracellular, and this membrane orientation is one major structural feature that the G and HN proteins share.

The G protein contains both N- and O-linked carbohydrates (9, 12, 19, 31, 41), but the number and locations of the side chains are unknown. The deduced amino acid sequences for the G proteins of three RSV strains (A2, Long, and 18537) contain between three and eight potential acceptor sites (Asn-X-Ser/Thr, where X usually is not Pro or Asp) for N-linked side chains and more than 70 potential acceptor sites (Ser or Thr) for O-linked sugar (16, 31, 41). The molecular mass of the total (N-linked and O-linked) carbohydrate was recently estimated by direct methods to be 12,000 to 24,000 (P. Paradiso, B. Hu, and S. Hildreth, Abstr. VII Int. Cong. Virol. 1987, R25.21, p. 189), a value that is considerably less than that of 51,500 to 57,500 suggested by experiments utilizing glycotransferase inhibitors in conjunction with gel electrophoresis and by comparison of the electrophoretic mobility of the mature protein and the calculated molecular weight of the polypeptide moiety (9, 12, 31, 41).

The G protein isolated from infected cells consists of two electrophoretically distinct species with apparent molecular weights estimated by gel electrophoresis (M_r) of 46,000 (46K protein) and 84,000 (84K protein) (9, 12, 13). In pulse-chase

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FIG. 1. Primary structure of the RSV G protein and locations of the truncated C termini of G71, G180, and G230. The amino acid sequence of the G protein of strain A2 was deduced in previous work (31, 41) from cDNA sequencing. Amino acid sequence positions are indicated for the C termini of G71, G180, and G230 (▲); for potential acceptor sites for O-linked carbohydrate (|); for potential acceptor sites for N-linked carbohydrate (N); for cysteine residues (C); and for the hydrophobic region that is proposed to serve as the signal sequence and membrane anchor (□).

experiments, the 46K species appeared to be converted into the 84K species (9, 13). This apparent conversion was inhibited (9, 12) by monensin, an ionophore that inhibits transport from the medial to the trans (late) compartment of the Golgi complex (5, 11, 37). Consistent with this, the N-linked carbohydrate chains of the 46K species were sensitive to endoglycosidase H, whereas those of the mature 84K species were fully resistant (9, 12). Thus, the 46K species appears to be a precursor that is converted to the 84K species in or beyond a trans-Golgi compartment. It was previously assumed that, by analogy to glycoproteins of herpes simplex virus and coronavirus (15, 23), monensin completely blocked the addition of O-linked carbohydrates to the G protein (9, 12, 31). More recently, however, the 46K and 84K species were shown to each contain both N-linked and O-linked sugars (Paradiso et al., Abstr. VII Int. Cong. Virol. 1987). Thus, at least some components of the O-linked sugars of the G protein are added before the trans-Golgi compartment, as has also been demonstrated for numerous cellular proteins (5, 8, 17, 25, 28, 29, 32, 35, and references therein). The large difference in M_r between the 46K and 84K species might be due to the elongation and addition of additional O-linked side chains in the trans-Golgi compartment. Consistent with this possibility, the elongation of O-linked sugar side chains of other proteins has been shown to have disproportionate effects on electrophoretic mobility (for example, reference 2). Alternatively, the 84K species might result from dimerization of the 46K species (10; Paradiso et al., Abstr. VII Int. Cong. Virol. 1987). An example that might be analogous is glycophorin A, which aggregates into dissociable dimers when analyzed by gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) (22, 38).

Interestingly, the G protein was found to be highly divergent between the two RSV antigenic subgroups, with the G proteins of strain A2 (subgroup A) and strain 18537 (subgroup B) sharing only 53% amino acid identity compared with 72 to 96% identity for eight other RSV proteins (16; unpublished data). Most of the amino acid sequence divergence occurred in the proposed C-terminal extracellular domain (44% identity between strains A2 and 18537), although a single circumscribed conserved region was identified that might have functional significance. In contrast, the proposed N-terminal cytoplasmic and transmembrane regions were relatively highly conserved (81 and 86% identity, respectively), suggesting that these regions contain conserved sequences or structures important in G protein transport, processing, or function.

In this report, we describe the processing, transport, and immunogenicity of three C-terminally truncated forms of the G protein expressed by cDNAs engineered by site-directed mutagenesis. These were examined as a first step in identifying regions of the G protein that are involved in membrane insertion, O glycosylation, transport to the cell surface, and induction of RSV-neutralizing antibodies and protective immunity.

MATERIALS AND METHODS

Construction of G protein truncation mutants G71, G180, and G230. Site-directed mutagenesis was used to construct three cDNAs that encode G proteins with truncated C termini (39). The predicted structures of the truncated G proteins encoded by these cDNAs are summarized in Fig. 1 and 2. In each construction, a codon pair in the open reading frame of the G gene (CCAACA, nucleotides 226 to 231 encoding Pro-71 and Thr-72; CCAACC, nucleotides 553 to 558 encoding Pro-180 and Thr-181; CCCACA, nucleotides

FIG. 2. Amino acid sequences of the C termini of the G protein truncation and chimeric mutants predicted from the nucleotide sequences of the engineered cDNAs. For G71, G180, and G230, the C-terminal six amino acids are shown; as described in Materials and Methods, G71, G180, and G230 consist of, respectively, the N-terminal 71, 180, and 230 amino acids of the G protein (amino acids 71, 180, and 230 are indicated above) followed by a heterologous Gly residue (G, underlined). For G71/13, G180/13, and G230/13, the C-terminal 18 amino acids are shown; in these constructs (Materials and Methods), the heterologous G residues of G71, G180, and G230 were replaced by a 13-amino-acid reporter sequence (CKTFELPRARVNT, underlined).

703 to 708 encoding Pro-230) was modified to be an in-frame *Sma*I site (CCCGGG, consisting of a Pro codon, CCC, and a Gly codon, GGG) followed immediately by an in-frame translational termination codon and a *Bam*HI site. Thus, the three truncated G proteins encoded by these cDNAs (designated G71, G180, and G230) contained, respectively, the N-terminal 71, 180, and 230 amino acids of parental G protein followed in each case by a single heterologous Gly residue at the C terminus.

Addition of a C-terminal reporter peptide to G71, G180, and G230. The *Bam*HI restriction fragments encoding G71, G180, and G230 were isolated on low-melting-point agarose gels and inserted individually into the unique *Bam*HI site of the simian virus 40 (SV40) shuttle vector SV2330 (21) described below. The ligation products were cloned in *Escherichia coli* RR1. Recombinants bearing inserts in the correct orientation were identified by standard procedures of colony hybridization and restriction site analysis. To add a reporter peptide to the C terminus of the protein encoded by each of the three recombinants, plasmids were digested with *Sma*I (which cuts each plasmid once, within the cDNA insert site introduced by the mutagenesis described above), dephosphorylated, and ligated to the following phosphorylated synthetic double-stranded DNA insert: 5'-AAGAC CTTTGAATTACCCAGAGCTAGAGTGAACACATAG (mRNA sense). The ligation products were redigested with *Sma*I to linearize and reduce the transformation efficiency of plasmids that had recircularized without an insert. Following transformation of *E. coli*, recombinants were identified by colony hybridization with 5'-end-labeled oligonucleotide, and the orientation and sequence of the synthetic DNA insert was determined by dideoxynucleotide sequencing. When inserted into the *Sma*I site of each truncated G cDNA, the synthetic DNA insert replaced the terminal Gly codon and extended the translational reading frame by 13 codons (CKTFELPRARVNT) followed by a termination codon. G71, G180, and G230 containing the reporter peptide were designated G71/13, G180/13, and G230/13, respectively.

Preparation of SV40 recombinant viruses. The G cDNAs were inserted individually into the *Bam*HI site of the SV40 shuttle vector pSV2330. As described previously (21), this hybrid vector consists of an SV40 genome that has been inserted via an *Xba*I site constructed from the *Taq*I site at SV40 position 4739 into a pBR322 derivative which has an *Xba*I site in the place of the deleted 375-base-pair *Eco*RI-*Bam*HI fragment. In the SV40 moiety, base pairs 335 to 521 have been deleted and replaced by the sequence CTGA; this deletion removes the initiation codon for the agnoprotein gene. Also, base pairs 832 to 2538, containing most of the late region coding sequences, have been replaced by the sequence CCGGATCC, containing a *Bam*HI site (underlined). Foreign cDNAs inserted into this site are under the control of the SV40 late promoter. Packaging of the recombinant SV2330 DNAs was obtained by cotransfection with SVrIns-7, which is an SV40 genome, cloned in the *Eco*RI site of pBR322, which contains a defective early region owing to a deletion of SV40 DNA and insertion of rat preproinsulin DNA (20). The SV40 moieties of pSV2330 bearing G cDNA and of SVrIns-7 were excised from the plasmids with *Xba*I and *Eco*RI, respectively, circularized with T4 DNA ligase, extracted with phenol, and precipitated with ethanol. Recombinant SV40 DNA (5 µg) was mixed with 5 µg of SVrIns-7 DNA and used to transfect secondary cultures of African green monkey kidney (AGMK) cells (purchased as primary cultures from Microbiological Associates Bioproducts, Bethesda, Md.) in 25-cm² flasks by the

DEAE-dextran method (21). Total cell lysis occurred after 10 to 14 days of incubation, and the supernatants were collected and diluted fivefold for use as virus stocks.

Expression of SV40-G recombinant viruses in AGMK cells. Secondary AGMK cells were infected with the SV40 recombinant viruses, incubated for 4 days, and labeled by incubation for 6 to 12 h with 100 µCi of [³H]glucosamine, [³H]leucine, or a mixture of [³H]leucine and [³⁵S]cysteine per ml in minimal essential medium containing one-half the normal concentration of glucose of 1/10th the normal concentration of leucine or leucine and cysteine, as appropriate. In all experiments, the infected cells were incubated in minimal essential medium deficient in glucose, leucine, or leucine and cysteine for 2 h prior to the labeling period. To examine the effect of glycosylation inhibitors on expression, monensin (2 µM) or tunicamycin (2 µg/ml) was present beginning 2 h before the labeling period. The preparation of cell lysates and radioimmunoprecipitation of G-specific polypeptides was performed as previously described (24). Samples were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) on 10, 12.5, or 15% polyacrylamide gels.

Indirect immunofluorescence. Secondary AGMK or CV-1 cells were seeded onto glass cover slips and infected with the SV40 recombinant viruses, incubated for 3 to 4 days for AGMK cells and 6 days for CV-1 cells, fixed for cell surface labeling in phosphate-buffered saline containing 5% Formalin for 10 min at room temperature, and analyzed by indirect immunofluorescence (24).

Immunologic response to immunization and protective efficacy. Young adult cotton rats (*Sigmodon hispidus*) were immunized by intranasal (i.n.) inoculation with RSV (10^{5.5} PFU/0.1 ml) or the appropriate recombinant vaccinia virus administered i.n. (10⁵ PFU/0.005 ml) or intradermally (i.d.) (10⁷ PFU/0.1 ml). The recombinant vaccinia viruses were constructed in previous work (7, 39) and contained the parental or truncated G cDNA under the control of the early-late P7.5 vaccinia virus promoter. At 21 days postimmunization, animals were bled and challenged with RSV (10^{5.5} PFU/0.1 ml) administered i.n. G protein-specific and vaccinia virus-specific antibody responses were measured by enzyme-linked immunosorbent assay with immunoaffinity-purified G protein (the generous gift of Peter Paradiso and Steven Hildreth of Praxis Biologics, Rochester, N.Y.) or gradient-purified vaccinia virus as the solid-phase antigen (24; R. A. Olmsted, R. M. Buller, P. L. Collins, W. T. London, J. A. Beeler, G. A. Prince, R. M. Chanock, and B. R. Murphy, Vaccine, in press). RSV serum neutralizing antibody titers were determined by a 60% plaque reduction assay. RSV titers in nasal turbinates and lung homogenates collected 4 days postchallenge were measured by plaque titration.

RESULTS

Expression of G71, G180, and G230. Recombinant SV40 viruses were constructed to express cDNAs encoding the parental 298-amino-acid G protein or truncated forms containing the N-terminal 71, 180, or 230 amino acids (designated G71, G180, and G230, respectively). An SV40 expression system was used in place of that of vaccinia virus because the rapid and extensive cytopathic effect of the latter might have complicated analysis of intracellular transport and cell surface expression. AGMK cells were infected with the recombinant viruses and analyzed for the expression of G-specific proteins by radioimmunoprecipitation and indirect immunofluorescence. Assays for the detection of G

FIG. 3. Comparison of the polypeptides synthesized in secondary AGMK cells infected with SV40 recombinant viruses expressing the G71, G180, G230, and parental G cDNAs. Polypeptides were immunoprecipitated from infected-cell lysates with a G-specific antiserum (lanes a to d, f, and g) or preimmune serum (lane e) and analyzed by SDS-PAGE. The expressed cDNAs and radiolabels were as follows: lane a, G180, [^3H]glucosamine; lane b, G230, [^3H]glucosamine; lane c, parental G, [^3H]glucosamine; lane d, parental G inserted in the reverse orientation in the recombinant vector, [^3H]glucosamine; lane e, G71, [^3H]leucine; lane f, G71, [^3H]leucine; lane g, parental G in the reverse orientation, [^3H]leucine. The immunoprecipitated proteins were separated on 10% (lanes a to d) or 15% (lanes e to g) polyacrylamide gels and visualized by fluorography. Some of the major G-specific polypeptides described in the text are identified to the right of each gel; 84K, 51K, 48K, and 7.5K are the major species encoded by parental G, G230, G180, and G71 cDNAs, respectively. The positions of molecular size markers (designated in kilodaltons) are marked at the left.

proteins were performed with a G-specific polyclonal rabbit antiserum (the generous gift of Steven Hildreth and Peter Paradiso of Praxis Biologics) that had been raised against immunoaffinity-purified G protein extracted from RSV-infected cells (40).

Radioimmunoprecipitation and gel electrophoresis showed that G180 and G230 were expressed as truncated G proteins of M_r 48,000 (48K protein) and 51,000 (51K protein), respectively, compared with M_r 84,000 for full-length parental G protein (Fig. 3). Like parental G protein, the major forms of G180 and G230 each migrated as a broad band that was labeled heavily by the incorporation of radiolabeled carbohydrate precursors such as [^3H]glucosamine. In some experiments, additional species of lower M_r also were observed, most notably a glycosylated species of M_r 27,000 (27K protein) for G180 and a glycosylated species of M_r 46,000 (46K protein) for parental G protein (this latter species is more evident in Fig. 4). As described below, the smaller species appear to be precursors to the larger mature species.

G71 was expressed as a single species, M_r 7500 (7.5K protein), that appeared to be much less abundant than G180, G230, or parental G protein. The 7.5K species could be detected by the incorporation of radiolabeled amino acids (Fig. 3) but was not labeled detectably with [^3H]glucosamine (see Fig. 5 below). The preparation and analysis of additional, independent virus stocks did not result in increased expression, and intracellular degradation alone did not appear to account for the difficulty in detecting the 7.5K polypeptide (unpublished data). An alternative possibility was that the G-specific antiserum raised against full-length G protein might not efficiently recognize the highly truncated,

relatively hydrophobic 7.5K protein. This interpretation is consistent with results described later.

The identification of the major forms of G180 and G230 (and parental G protein), but not G71, as extensively glycosylated species was generally consistent with the availability of potential acceptor sites in their predicted amino acid sequences. The predicted extracellular domains of G71, G180, G230, and parental G protein contain, respectively, 0, 2, 2, and 4 potential acceptor sites for N-linked carbohydrate and 2, 37, 50, and 78 potential acceptor sites for O-linked carbohydrate (Fig. 1). The glycosylation of these species is examined further below.

Synthesis of G180 and G230 in the presence of tunicamycin and monensin. The glycosylation of G180 and G230 was examined with the antibiotic tunicamycin, which inhibits N-linked glycosylation by depletion of the dolichol phosphate-oligosaccharide donor (18), and monensin, which inhibits transport between the medial and trans-Golgi compartments and thereby indirectly inhibits the addition or elongation or both of O-linked sugars within the trans-Golgi compartment (11, 37). AGMK cells were infected with the SV40 recombinant viruses, preincubated with tunicamycin or monensin, labeled in the presence of the inhibitor with [^3H]glucosamine (Fig. 4A) or a mixture of [^3S]cysteine and [^3H]leucine (Fig. 4B), and analyzed by radioimmunoprecipitation and gel electrophoresis.

Consistent with previous work (9, 12, 19, 31, 41), parental G protein synthesized in the presence of tunicamycin migrated as a major species of estimated M_r 74,000 (Fig. 4A and B). The deduced estimated content of N-linked carbohydrates, 10,000 daltons, suggested that most or all of the four potential acceptor sites were utilized in the absence of the drug. In the presence of tunicamycin, the major, largest forms of G230 and G180 were reduced in M_r by approximately 4,000 to 5,000, indicating that at least one and probably both potential acceptor sites for N-linked carbohydrate were utilized in the absence of the drug. The observation that G180, G230, and parental G protein were labeled with [^3H]glucosamine in the presence of tunicamycin was indirect evidence of O glycosylation (Fig. 4A). Incidentally, in the presence of tunicamycin, the minor 27K form of G180 was not detected. Its disappearance coincided with the appearance of a nonglycosylated species with an M_r of approximately 19,000 (19K protein) that presumably is the unglycosylated form of the 27K protein (Fig. 4B). This suggested that in the absence of N glycosylation some G180 molecules also did not acquire O-linked carbohydrate. One possibility is that these molecules were not exposed to O glycotransferases because they were not transported beyond the rough endoplasmic reticulum.

Consistent with previous observations (9, 12, 31), parental G protein synthesized in the presence of monensin migrated as a series of bands in two size classes of M_r 60,000 to 84,000 and 40,000 to 46,000 (Fig. 4A). The latter size class presumably consisted of forms of the early Golgi 46K precursor protein that might have slight differences in glycosylation. In the presence of monensin, G230 accumulated as a series of glycosylated species of M_r 29,000 to 51,000 with a major species of 29,000 (29K protein) (Fig. 4A and B). Under the monensin treatment, G180 accumulated as a heterogeneous series of glycosylated species of M_r 22,000 to 48,000, with the most abundant species being the 27K and 35K glycosylated polypeptides (Fig. 4A and 4B). Thus, the substantial reductions in M_r observed for G180 and G230 synthesized in the presence of monensin indicated that, like parental G protein, both G180 and G230 undergo extensive posttrans-

FIG. 4. Comparison of the effects of tunicamycin or monensin on the synthesis of the polypeptides encoded by the G180, G230, or parental G cDNAs or by parental G cDNA in the reverse orientation (G rev). AGMK cells were infected with the recombinant SV40 viruses and incubated with [³H]glucosamine (A) or a mixture of [³⁵S]cysteine and [³H]leucine (B) in the presence of monensin (M), tunicamycin (T), or no drug (U). The radiolabeled polypeptides were immunoprecipitated with a G-specific antiserum, separated by SDS-PAGE on 12.5% gels, and visualized by fluorography. Some of the major G-specific polypeptides described in the text are identified at the right, and the positions of molecular size markers (designated in kilodaltons) are marked at the left.

lational processing in or beyond the trans-Golgi compartment.

Cell surface expression of G180 and G230. In a separate study, G71, G180, and G230 were modified by C-terminal addition to encode four copies (totaling 16 amino acids) of the immunodominant repeating epitope (NANP) of the circumsporozoite protein of *Plasmodium falciparum*, and these cDNAs were inserted into vaccinia virus for in vitro and in vivo expression (39). Indirect immunofluorescence with a monoclonal antibody specific for the circumsporozoite epitope showed that in vaccinia virus-recombinant-infected cells, the malarial antigen was expressed on the cell surface by the G180 and G230 hybrid proteins but not by the G71 hybrid protein (39). It was possible that G71 lacked the structural features required for transport to the cell surface or that the addition of the heterologous sequence to G71 interfered with transport. Alternatively, the G71 hybrid protein may not have been detected at the cell surface owing to poor recognition of the repeating epitope when displayed in this context.

Similarly, abundant expression of unmodified G180, G230, and parental G protein could be demonstrated in the cytoplasm and at the infected cell surface by indirect immunofluorescence with G-specific antiserum (data not shown). In contrast, expression of G71 intracellularly or at the cell surface was not demonstrated unequivocally, although faint staining at both locations was observed (data not shown). As described above, this difficulty in detection might be due to inefficient reactivity of the highly truncated polypeptide with the G-specific antiserum.

Expression of G71, G180, and G230 containing a reporter sequence. In an effort to enhance and standardize detection, the G71, G180, and G230 cDNAs were modified by insertional mutagenesis (Materials and Methods) such that the

encoded polypeptide chain was extended at the C terminus by the 13-amino-acid reporter peptide CKTFELPRARVNT. This sequence consists of a Cys residue followed by the C-terminal 12 amino acids of the RSV 1A protein (1). In work to be described elsewhere (R. Olmsted and P. Collins, unpublished data), a synthetic peptide of this sequence had been used to prepare peptide-specific rabbit antiserum. G71, G180, and G230 bearing the C-terminal reporter peptide were designated G71/13, G180/13, and G230/13, respectively.

As anticipated, the antipeptide serum efficiently detected the chimeric proteins in radioimmunoprecipitation and indirect immunofluorescence assays. Apart from increasing the sensitivity of detection, the presence of the reporter peptide on G180/13 and G230/13 had no demonstrable effect on their synthesis and processing as determined by radiolabeling and SDS-PAGE (data not shown).

In contrast to the poor detection of G71 with anti-G serum, G71/13 was detected efficiently by radioimmunoprecipitation with the antipeptide serum. G71/13 migrated as a major band with an M_r of approximately 9,500 (Fig. 5), similar to the predicted polypeptide molecular weight of 8,200 calculated from the sequence. In some experiments (Fig. 5, lane b), but not others (lane h), a second, less abundant band of M_r 8,500 also was detected; its significance is unknown. Attempts to label G71 with [³H]glucosamine were unsuccessful (Fig. 5, lane c), suggesting that this species contains little or no carbohydrate. In contrast, the 9.5K species of G71/13 was labeled detectably with [³H]glucosamine (Fig. 5, lane g), showing that it is a glycoprotein. The 8.5K species might be a nonglycosylated form, although this was not determined directly.

Cell surface expression of G71/13. CV-1 cells were infected in parallel with SV40 recombinants expressing G71/13, G180/

FIG. 5. SDS-PAGE of polypeptides synthesized in AGMK cells infected with SV40 recombinant viruses expressing the G71 and G71/13 cDNAs. Immunoprecipitation of radiolabeled intracellular polypeptides was done with G-specific antiserum, reporter peptide-specific antiserum, or preimmune serum. The expressed cDNA, radiolabel, and antiserum used for immunoprecipitation were as follows: lane a, G71/13, [3 H]leucine, preimmune serum; lane b, G71/13, [3 H]leucine, anti-peptide serum; lane c, G/71, [3 H]glucosamine, anti-G serum; lane d, G71, [3 H]leucine, anti-G serum; lane e, G/71, [3 H]leucine, preimmune serum; lane f, uninfected, [3 H]glucosamine, anti-peptide serum; lane g, G71/13, [3 H]glucosamine, anti-peptide serum; lane h, G71/13, [3 H]leucine, anti-peptide serum; and lane i, uninfected, [3 H]leucine, anti-peptide serum. The immunoprecipitated proteins were separated on 15% gels and visualized by fluorography. The major G-specific proteins are identified: 9.5K is a major, glycosylated species encoded by G71/13, 8.5K is a second G71/13-specific polypeptide, and 7.5K is a major, unglycosylated species encoded by G71. The positions of molecular size markers (designated in kilodaltons) are marked.

13, or G230/13 and analyzed by indirect immunofluorescence with the anti-peptide serum as the primary antibody. This demonstrated abundant expression of G71/13, G180/13, and G230/13 both in the cytoplasm (data not shown) and at the surface (Fig. 6) of infected cells. The fluorescent staining observed with the anti-peptide serum was peptide specific since no reactivity was detected on the surface of cells infected with SV40 recombinant expressing parental G (Fig. 6D). Assuming that each chimeric species was recognized with equal efficiency by the anti-peptide serum, the similar levels of expression observed with the recombinants suggested that G71/13, G180/13, and G230/13 were expressed at the cell surface with similar efficiencies.

Serum antibodies and protective immunity induced by truncated G proteins. Cotton rats were immunized by i.n. or i.d. infection with recombinant vaccinia viruses expressing G71, G180, G230, or parental G protein to compare the abilities of these proteins to induce serum antibodies and resistance to subsequent RSV infection of the respiratory tract. At 21 days postimmunization, serum samples were collected and assayed for RSV-specific antibodies by ELISA with purified G protein as the antigen and by *in vitro* neutralization of RSV infectivity (Table 1). In addition, the titers of vaccinia virus-specific serum antibodies were measured by ELISA (Table 1), indirectly confirming that the vaccinia virus recombinants replicated efficiently in all the groups (16, 24).

Consistent with previous work (16, 24), when administered i.d. or i.n. (the latter at a 100-fold-lower dose than that administered i.d.), vaccinia virus-G induced titers of G-specific ELISA antibodies that equaled or exceeded those induced by i.n. infection with RSV (Table 1). On the other hand, the polyvalent serum antibodies induced by RSV infection were somewhat more efficient in neutralizing RSV infectivity *in vitro*. Also, as noted previously (24), immunization with vaccinia virus-G by either route induced significant but incomplete resistance to subsequent RSV infection, whereas immunization with RSV i.n. induced complete resistance to RSV challenge (Table 1).

FIG. 6. Detection of cell surface expression of G71/13, G180/13, and G230/13 by indirect immunofluorescence. CV-1 cells were infected with SV40 recombinant viruses, fixed with Formalin for cell surface staining, and incubated sequentially with rabbit anti-peptide serum and fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G. The patterns of cell surface staining of G71/13 (A), G180/13 (B), G230/13 (C), and parental G (D) with the anti-peptide serum are shown. The exposure time for panel D was adjusted manually to equal those in panels A through C.

TABLE 1. Immunogenicity and protective efficacy in cotton rats of vaccinia virus recombinants that express the G71, G180, G230, or parental G glycoprotein

Expt	Primary immunization (route) ^a	PFU	No. of animals	Response to immunization: serum antibody titers (reciprocal mean log ₂ ± SE) ^b			Response to challenge ^c : peak RSV titer (mean log ₁₀ PFU/g of tissue ± SE)	
				ELISA-G ^d	ELISA-vaccinia virus ^e	RSV-neutralizing antibodies ^f	Lung homogenates	Nasal turbinates
1	VSC-14 (i.n.)	10 ⁵	6	6.0 ± 1.8	19.8 ± 1.3	≤3.3	4.9 ± 0.2	3.7 ± 0.6
1	RSV (i.n.)	10 ^{5.5}	6	14.6 ± 0.4	9.3 ± 0	10.6 ± 0.5	<1.7	<1.7
1	Vac-G (i.n.)	10 ⁵	7	16.9 ± 0.8	20.9 ± 0.3	7.3 ± 0.8	1.9 ± 0.1	2.9 ± 0.5
1	Vac-G71 (i.n.)	10 ⁵	8	5.8 ± 0.3	17.4 ± 1.8	≤3.4	4.7 ± 0.4	4.7 ± 0.2
1	Vac-G180 (i.n.)	10 ⁵	7	5.9 ± 0.4	17.2 ± 2.0	≤3.4	5.0 ± 0.2	4.8 ± 0.2
1	Vac-G230 (i.n.)	10 ⁵	6	18.5 ± 0.4	20.8 ± 0.3	10.9 ± 1.1	<1.7 ^g	2.5 ± 0.4 ^g
2	Vac-G (i.n.)	10 ⁵	6	11.3 ± 0.5		8.3 ± 1.0		
2	Vac-G230 (i.n.)	10 ⁵	7	13.0 ± 0.5		8.9 ± 1.2		
3	Vac-G (i.d.)	10 ⁷	10	15 ± 0.8				
3	Vac-G230 (i.d.)	10 ⁷	9	16.5 ± 0.5				

^a vac, Vaccinia virus.^b Sera were collected 21 days postimmunization.^c Animals were challenged on day 21 with 10^{5.5} 50% tissue culture infective doses of RSV (0.1 ml i.n.), and their lungs and nasal turbinates were removed for virus titration 4 days later, at the peak of challenge virus replication.^d Immunoaffinity-purified G protein was used as the solid-phase antigen. The reciprocal mean preimmunization titer was 5.3.^e Gradient-purified vaccinia virus was used as the solid-phase antigen. The reciprocal mean preimmunization titer was 8.4.^f Determined by a 60% plaque reduction assay. Preimmunization titers (day 0) were ≤3.3.^g *P* < 0.001 compared with G180 by Student's *t* test.

Both vaccinia virus-G71 and vaccinia virus-G180 were ineffective in stimulating G-specific serum antibodies or resistance to challenge virus replication; the antibody titers and levels of challenge virus replication observed were approximately the same as for animals immunized with a recombinant (VSC-14) expressing the hepatitis B surface antigen.

In contrast, the immunogenicity and protective efficacy of vaccinia virus-G230 was comparable to that of vaccinia virus-G (Table 1). In experiment 1 (Table 1), vaccinia virus-G230 induced titers of ELISA-G antibodies and RSV-neutralizing antibodies that were significantly (approximately fourfold) higher than those induced by vaccinia virus-G. However, this difference was not confirmed in repeat experiments utilizing either the i.n. (experiment 2) or i.d. (experiment 3) route of immunization. This indicated that G230 was approximately equivalent to parental G protein in the ability to induce G-binding antibodies, RSV-neutralizing antibodies, and protective immunity in cotton rats.

DISCUSSION

The expression of C-terminal truncation mutants of the G protein was examined to identify polypeptide regions that are involved in posttranslational processing, cell surface expression, and the induction of host immunity. Mutants G180 and G230 each accumulated at the cell surface in an abundance that was similar to that of parental G protein, showing that all the structural features required for transport of the G protein to the cell surface are contained within the N-terminal 180 amino acids. Cell surface expression of G71 was not demonstrated unequivocally, but this might have been due to inefficient recognition of this highly truncated form (containing a proposed extracellular domain of only 10 amino acids) by the antiserum raised against full-length G protein. Consistent with this, the 71-, 180-, and 230-amino-acid species bearing an additional 13-amino-acid reporter

peptide were all efficiently transported to the cell surface. Also, cell surface expression was demonstrated with the G-specific antiserum for a fourth G protein truncation mutant consisting of the N-terminal 95 amino acids of G followed by a single heterologous Gly residue (unpublished data). The formal possibility exists that G71 (lacking the reporter peptide) was not competent for cell surface transport and that the addition of the reporter peptide made cell surface expression possible. However, we suggest that the simpler, more likely interpretation is that the N-terminal 71 amino acids of the G protein contain all the structural features required for efficient membrane insertion and cell surface transport of full-length G protein.

It is generally assumed that the G protein is anchored in the membrane by the single major hydrophobic region near the N terminus (amino acids 41 to 62), with the C terminus oriented externally. However, this proposed membrane orientation was based on the deduced amino acid sequence alone and had not been tested experimentally. The results described here provided two lines of support for this model. First, removal of most of the C-terminal three-fourths of the G protein had no demonstrable effect on membrane insertion and cell surface expression, suggesting that the structural features involved in membrane insertion, anchoring, and transport are contained in the N-terminal one-fourth of the molecule, colocalized with the major hydrophobic region. Second, indirect surface immunofluorescence showed that the C-terminal reporter peptide of each chimeric protein was exposed on the exterior of the infected cells, consistent with the proposed extracellular orientation of the C terminus of parental G protein.

In the presence of monensin, G180 and G230 accumulated intracellularly as a heterogeneous series of bands with major species of *M_r* 27,000 and 29,000, respectively. These species, which sometimes could be detected in untreated infected cells, appear to be intermediates in posttranslational processing analogous to the 46K precursor described for parental

G protein (9, 12). Like the 46K species of parental G protein, the 27K species (for G180) and the 29K and 32K species (for G230) underwent an approximate doubling in M_r during transit through and beyond the trans-Golgi compartment. As suggested previously for parental G, this apparent doubling in size might be due to dimerization (9; Paradiso et al., Abstr. VII Int. Cong. Virol. 1987). If so, then G180 and G230 apparently contain the structural features necessary for dimerization, whereas G71 does not.

Alternatively, the increase in M_r conferred in the trans-Golgi compartment might be due to the elongation of existing or the addition of additional O-linked sugar side chains with a disproportionate effect on M_r (2). This was supported by the observation that, in the presence of monensin (Fig. 4), G230 accumulated both as the 29K precursor and as a heterogeneous series of larger species intermediate in size to the mature 51K species. This suggested that additional multistep glycosylation, rather than dimerization, is responsible for the size difference between the 29K precursor and the 51K mature form. If the effect of monensin on the G protein is to inhibit the completion of O glycosylation, then by the criteria of monensin sensitivity, G180 and G230 each acquire a substantial content of O-linked sugars in the trans-Golgi compartment that, based on estimated M_r , is approximately half that of parental G protein. One interpretation is that the O-linked sugars of parental G protein are located such that approximately equal amounts of carbohydrate are contained on either side of the cysteine-rich, serine- and threonine-deficient conserved region located at the midpoint of the extracellular domain (Fig. 1). G180 and G230 would contain the N-proximal but not the C-proximal cluster of side chains. However, it also is possible that the C-terminal truncations altered the pattern of O glycosylation in the extracellular domain, which would preclude mapping of the sites of O-linked side chains by this approach.

The structural features that might target a protein for O glycosylation have not been identified. The presence of Ser and Thr residues cannot be the sole determinant because most membrane proteins such as the HN protein of human parainfluenza virus type 3 (6) contain these residues but are not O glycosylated. Some other feature of protein structure might serve as a signal for O glycosylation, such as a short specific amino acid sequence, or a circumscribed feature of higher order structure, or a more generalized, less specific feature such as a high proline content which might contribute to a secondary structure that is a target for O glycosylation. G180 and G230 were O glycosylated, indicating that an approximate signal is contained within the N-terminal 180 amino acids of the G protein. G71, which contained only two potential sites for O glycosylation and none for N glycosylation, was not detectably glycosylated. However, given the general difficulty in detecting G71, it was possible that a lightly labeled glycosylated form escaped detection. With the use of the reporter peptide and antipeptide serum, G71/13 (containing four potential sites for O glycosylation and none for N glycosylation) was shown to be glycosylated. Thus, this 84-amino-acid polypeptide contains all the structural information needed to target it for O glycosylation. One possibility for the apparent difference in glycosylation between G71 and G71/13 is that the two potential acceptor sites in G71 are not utilized, perhaps due to their close proximity to the membrane. The subsequent addition of the reporter peptide would have provided two additional sites that might fortuitously have been utilized. These experiments have narrowed the possibilities for structural features involved in O glycosylation of the G protein. For example, there appears

to be no requirement for most of the C-terminal three-fourths of the molecule. Experiments are in progress with additional truncated and chimeric proteins that might further define the structural features involved in O glycosylation.

In many studies, the introduction of mutations into integral membrane proteins often resulted in inefficient transport of the mutant proteins from the rough endoplasmic reticulum or through the Golgi complex (3, 4, 10, 14, 27, 30, and references therein). One unifying hypothesis is that these mutant proteins were transported inefficiently because they failed to fold properly (10, 30). In this regard, it was surprising that the G protein was O glycosylated and transported efficiently to the cell surface even when large deletions were introduced into the ectodomain. The ectodomain of G was shown previously to be highly divergent between the RSV antigenic subgroups with regard to both the amino acid sequence and the positions of potential acceptor sites for N- and O-linked carbohydrate (16). Also, inhibition of the addition of N-linked side chains did not interfere with transport to the cell surface (31). Taken together, these observations indicate that either (i) the folding status of the G ectodomain has little effect on transport or (ii) the ectodomain has a relatively simple higher order structure that is tolerant of substitutions and deletions.

The inability of vaccinia virus-G71 to induce significant levels of G-specific serum antibodies was not surprising because the highly truncated G71 protein would contain only 4% of the ectodomain of the parental G protein. Vaccinia virus-G180 also was poorly immunogenic even though G180 would contain 50% of the parental ectodomain. One possibility was that the conformation of G180 was highly aberrant and induced antibodies that did not recognize parental G. However, this would seem to be inconsistent with the observation that G180 expressed in tissue culture was bound efficiently in fluorescent antibody staining (data not shown) and radioimmunoprecipitation (Fig. 3 and 4) studies by antibodies induced in cotton rats by RSV infection and in rabbits by immunization with purified G protein. The conformational status of the G180 protein could be investigated in future work by comparing the binding of G180 and parental G proteins by G180-specific antibodies, parental G-specific antibodies, and conformationally specific polyclonal and monoclonal antibodies induced by native and denatured G protein.

Vaccinia virus-G230 equaled or exceeded vaccinia virus-G in the abilities to induce protein-binding antibodies, virus-neutralizing antibodies, and resistance to challenge virus replication. The G230 protein would contain 71% of the parental ectodomain. The components of host immunity that are involved in protective immunity induced by G have not been fully defined, although the finding that the G protein did not induce significant levels of murine cytotoxic T cells (26; R. R. Gupta, J. Bennink, J. Yewdell, R. Olmsted, B. Murphy, B. Moss, and P. Collins, unpublished data) suggested that antibodies have a predominant role. In any case, the results described here indicate that the major protective epitope(s) of the G protein is contained within the N-terminal 230 amino acids. Also, the C-terminal 68 amino acids presumably are not required for the G protein to assume an antigenically authentic conformation. It should be noted that G230, but not G180 or G71, would contain the highly conserved cysteine-rich region (residues 164 to 186) of the ectodomain that was noted previously (16) (see Introduction) and is a candidate to be important in G protein structure, function, and antigenicity.

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