Definition and functional analysis of the signal/anchor domain of the human respiratory syncytial virus glycoprotein G

Drew L. Lichtenstein,† Sharon R. Roberts, Gail W. Wertz and L. Andrew Ball

The attachment protein G of human respiratory syncytial (RS) virus is a type II transmembrane glycoprotein. A secreted form of the G protein is also produced. To examine the two distinct hydrophobic regions in the N-terminal 63 amino acids of G protein for their role(s) in membrane insertion and anchoring, transport to the cell surface, and secretion, G proteins that contained point mutations or deletions were synthesized by cell-free transcription-translation and in cells by expression from recombinant vaccinia virus vectors. A mutant protein lacking the entire major hydrophobic region (amino acids 38–63) was not glycosylated, not expressed on the cell surface, and not secreted, because it was not inserted into membranes. In contrast, deletion of the minor hydrophobic region (amino acids 23–31) had no detectable effect on membrane insertion or anchoring. These data provided direct evidence that amino acids 38–63 were necessary for membrane insertion and contained the signal/anchor domain of RS virus G protein. Mutant proteins that lacked either the N-terminal or the C-terminal half of this 26 residue hydrophobic region were inserted into membranes and processed to maturity, showing that either half of this region was sufficient for membrane insertion. However, these two mutant proteins were secreted more abundantly than wild-type G protein. We propose that their truncated hydrophobic domains interacted with membranes in a way that mimicked the N-terminal signal sequence of naturally secreted proteins, allowing proteolytic cleavage of the mutant proteins.

Introduction

The G protein of human respiratory syncytial (RS) virus is an extensively glycosylated type II integral membrane protein that differs from other paramyxovirus attachment proteins: it has no amino acid homology with them and it lacks haemagglutinin and neuraminidase activity. In addition, G protein contains a large amount of O-linked carbohydrate as well as some N-linked oligosaccharides (Gruber & Levine, 1985; Lambert, 1988; Wertz et al., 1989) and is predicted to have an unusually high content of proline (10%) and serine and threonine (30%) residues (Satake et al., 1985; Wertz et al., 1985). These features resemble those of the cellular mucinous glycoproteins. Two forms of G protein are produced in infected cells: the 86–90 kDa membrane-bound form and a secreted form that is 6–9 kDa smaller (Hendricks et al., 1987; Roberts et al., 1994).

Two polypeptides have been observed after translation of G mRNA in cell-free systems (Satake et al., 1985; Wertz et al., 1985; Collins & Mottet, 1992; Roberts et al., 1994) and when G protein was expressed in cells that were deficient in N-linked and O-linked glycosylation (Wertz et al., 1989). The larger protein is produced by translation initiation at the first AUG codon in the G protein open reading frame and upon maturation yields the full-length, cell-associated form of G protein (Roberts et al., 1994). The smaller protein is the result of initiation at the second AUG codon, corresponding to methionine-48 of the full-length protein and it is the sole source of secreted G protein (Roberts et al., 1994). Furthermore, the N terminus of secreted G protein purified from the medium of RS-virus-infected cells corresponds to amino acid 66 of the full-length protein and it is the sole source of secreted G protein (Roberts et al., 1994). The deduced amino acid sequence of G protein shows neither a cleavable signal sequence nor a potential membrane anchor near the C terminus. Thus, G protein
is predicted to be a type II membrane protein (intracytoplasmic N terminus and extracellular C terminus) (Satake et al., 1985; Wertz et al., 1985). Type II proteins contain an internal, uncleaved signal/anchor (S/A) domain that serves to direct membrane insertion and anchor the protein in the membrane. The S/A domain of G protein was localized to the N-terminal 63 amino acids by experiments which showed that this portion of G protein could replace the S/A of another type II protein (Collins, 1990). This portion of G protein contains two distinct hydrophobic regions: the major hydrophobic domain that serves to direct membrane insertion and contain an internal, uncleaved signal/anchor (S/A) anchor the protein in the membrane. The S/A domain of penicillin G (50 U/ml), streptomycin (50 μg/ml), and 2 x non-essential amino acids (Gibco BRL). This portion of G protein contains two charged amino terminus and hydrophilic C terminus (amino acids 23-31). The S/A domain of type II proteins has a tripartite structure similar to those of cleavable signal sequences: a central hydrophobic region flanked by a positively charged amino terminus and a hydrophilic C terminus (Haeuptle et al., 1989; Lipp & Dobberstein, 1988). The hydrophobic region is both necessary and sufficient for interaction with signal recognition particles (SRP), membrane insertion and anchoring (Bos et al., 1984; Markoff et al., 1984; Spiess & Lodish, 1986; Zerial et al., 1986). Replacement of the hydrophobic core of the transferrin receptor with an artificial hydrophobic sequence showed that the hydrophobicity of this region and not its exact sequence is important for membrane insertion (Zerial et al., 1987). Experiments in which increasing portions of the hydrophobic region were deleted show that a minimum of 10–16 non-polar amino acids in the hydrophobic core is required for membrane insertion and stable anchoring (Brown et al., 1986; Spiess & Handschin, 1987). Analysis of deletion mutants also suggests that the signal and anchor functions overlap and are redundant within the S/A sequence (Brown et al., 1988; Lipp & Dobberstein, 1988; Spiess & Handschin, 1987). In the work reported here site-specific mutations were introduced into the N-terminal 63 amino acids of the BS virus G protein to precisely define the S/A domain, to investigate potential sub-domains within it, and to examine its role in membrane insertion, anchoring, transport to the cell surface, and secretion.

Methods

**Cells and viruses.** Hep2 and 143 (Tk°) cells were grown as monolayers in minimal essential medium (MEM) and HeLa T4 cells were grown in Dulbecco’s modified Eagle’s medium. Both media were supplemented with 5% (v/v) heat-inactivated fetal bovine serum (Flow Laboratories), penicillin G (50 μg/ml), streptomycin (50 μg/ml), and 2 x non-essential amino acids (Gibco BRL). Recombinant vaccinia virus (VV) was constructed by using the wild-type G protein (vG wt) (Ball et al., 1986; Lichtenstein, 1990). Wild-type (strain WR) and recombinant VVs were grown in HeLa T4 cells and purified by 1, 2-trichloroethanol (Aldrich) extraction of frozen-thawed cells followed by centrifugation through 36% (w/v) sucrose (made in 10 mM-Tris-HCl, pH 9.0). Virus particles were resuspended in 10 mM-Tris-HCl (pH 9.0) and titrated by plaque assay on HeLa T4 cells.

**Site-specific oligonucleotide-directed mutagenesis.** Point and deletion mutants were constructed using the following oligonucleotides: GA85D, 5’ ATTGACATCTATA 3’; GI60K, 5’ GCCATCAAATTTTCTTTTCTGAGA 3’; GA85D 160K, 5’ GACATCAAATTCTCTAATCAATCATA 3’; G25ΔA3, 5’ CTCAATCTTAAATCCTTAATTATACTCATAACC 3’; G38A50, 5’ CTGAATATATTCTCGGCAGAACC 3’; G38A63, 5’ CTGAATATATTCTGGCAAGACC 3’. Oligonucleotides were phosphorylated using unlabelled ATP or [γ-32P]ATP (Amersham) as described (Wallace et al., 1981). A sub-fragment containing the 5’-385 bp of the G cDNA of the A2 strain of human RS virus was the template for the mutagenesis. The procedure of Kunkel (1985) was used to enrich for mutants. Mutants were identified either by sequencing (Sanger et al., 1977; Tabor & Richardson, 1987) or by hybridization to 32P-labelled oligonucleotides (Wallace et al., 1981; Bauer et al., 1985). The entire sub-fragment of the G cDNA was sequenced to ensure that no additional mutations had been introduced into the cDNA, and then a full-length cDNA for each mutant was reconstructed.

A BamHI fragment that contained the entire wild-type or mutant G cDNAs was inserted into the BamHI site of pBAM-3 (Promega) such that mRNA-sense transcripts were synthesized by bacteriophage SP6 RNA polymerase. The mutant genes were also inserted into the BamHI site downstream of the VV 7.5K promoter in the vector pAB191 (Stott et al., 1986) in order to construct recombinant VV vectors.

**Cell-free transcription and translation.** Plasmid templates for cell-free transcription were linearized by digestion with EcoRI and purified by phenol extraction and ethanol precipitation. Cell-free transcription reactions contained: linearized plasmid (100 μg/ml), 0.5 mM-cap analogue [7mG(5)ppp(5)G]; New England Biolabs], RNasin (1 U/ml; Promega), 40 mM-Tris–HCl (pH 7.5), 6 mM-MgCl2, 2 mM-spermidine, 10 mM-dithiothreitol, 0.5 mM each ATP, CTP and UTP, 0.05 mM-GTP, and bacteriophage SP6 RNA polymerase (0.2 U/ml; New England Biolabs) (Krieg & Melton, 1984; Melton et al., 1984). Reactions were incubated at 40°C for 60 min. More SP6 RNA polymerase (0.2 U/ml) was added and the reactions were continued for an additional 60 min at 40°C. The plasmid DNA was digested with RQ1 DNase (1 U/μg of DNA; Promega) for 15 min at 37°C. Transcripts were purified by phenol extraction followed by two ethanol precipitations in the presence of 2 M-ammonium acetate. Transcripts were quantified by measurement of the absorbance at 260 nm and adjusted to a concentration of 344 μg/ml.

Translation reactions contained 70% (v/v) micrococcal nuclease-treated rabbit reticulocyte lysate (Promega), 200 μM of 19 amino acids (minus methionine), RNasin (0.8 U/ml), [35S]methionine (0.8 μCi/ml, > 800 Ci/mmol; New England Nuclear) and RNA (6.9 μg/ml). In some reactions canine pancreatic microsomal membranes (Promega) were present at a concentration of 0.8 equivalents per ml. Reactions were incubated for 1–2 h at 30°C. Samples were immunoprecipitated or analysed directly by SDS-PAGE (Laemmli, 1970). Where indicated, gels were prepared for fluorography using the method of Chamberlain (1979).

**Virus infection and metabolic labelling.** Hep2 or 143 cells were infected with VV vectors at an m.o.i. of 10 p.f.u. per cell (Hruby et al., 1979), then incubated at 37°C for 3.5 h. Cells were washed with serum-free MEM lacking threonine and then starved for threonine in the same medium for 30 min at 37°C. Cells were labelled with [3H]threonine (100 μCi/ml, 3–20 Ci/mmol; Amersham) for 3.5 h. Cytoplasmic lysates were prepared as described previously (Lerch et al., 1989). Where
indicated, glycosylation was inhibited by the addition of tunicamycin (3 μg/ml; Boehringer Mannheim) and monensin (1 μM; Calbiochem) 3 h prior to, and during metabolic labelling.

Immunofluorescence, immunoprecipitation and endoglycosidase H (endo H) digestion. Immunoprecipitation and immunofluorescence assays were performed using the anti-G protein monoclonal antibody L9 (a kind gift of Ed Walsh, University of Rochester School of Medicine, NY, USA) as described previously (Roberts et al., 1994). For endo H treatment, immunoprecipitated samples were heated at 100 °C for 5 min in 10 μl 1% SDS, then 9 μl 150 mM-sodium citrate (pH 5-2) and either 1 μl H2O (mock endo H treatment) or endo H (3 μU/μl; Boehringer Mannheim) were added. Samples were incubated at 37 °C for 16–24 h, then analysed by SDS–PAGE.

Results

Site-specific oligonucleotide-directed mutagenesis

Seven mutants of the human RS virus G protein were constructed by site-specific oligonucleotide-directed mutagenesis in order to examine the contribution of the N-terminal hydrophobic regions to membrane insertion and anchoring, cell surface expression, and secretion. The deduced amino acid sequence of G protein shows that the major hydrophobic region (amino acids 38–63) is preceded by a smaller hydrophobic region (amino acids 23–31) that is present in all known sequences of RS virus G protein, including human, bovine and ovine (Fig. 1). Two deletion mutants that removed either the major (mutant G38A63) or the minor (mutant G23A31) hydrophobic region were constructed in order to examine the contribution of each hydrophobic segment to signal and anchor functions (Fig. 1). Potential sub-domains within the major hydrophobic region that might contribute to signal and/or anchor function were investigated by deletion of the N-terminal half (mutant G38A50) or C-terminal half (mutant G51A63) of this region (Fig. 1). In addition, the role of the most hydrophobic stretch of amino acids within the major hydrophobic region, located between amino acids 55 and 64, was investigated by the introduction of a positively charged residue (mutant GA58D), a negatively charged residue (mutant GI60K), or both (mutant GA58D/ I60K; Fig. 1).

Cell-free synthesis of wild-type and mutant G proteins

RNAs made by cell-free transcription were translated in a rabbit reticulocyte lysate in the absence or presence of canine microsomal membranes. Two major polypeptides were synthesized by wild-type G mRNA in the absence of membranes (Fig. 2a, lane 2) (Satake et al., 1985; Wertz et al., 1985; Collins & Mottet, 1992; Roberts et al., 1994). Previous work has shown that these proteins result from translation initiation at the first and second AUG codons in the G protein open reading frame, respectively (Roberts et al., 1994). In accordance with this, two proteins were also synthesized by translation of the mRNAs for mutants GA58D, GI60K, GA58D/I60K, G23A31 and G51A63 (Fig. 2a, lanes 3, 4, 5, 7 and 9). The faster mobility of the G23A31 and G51A63 proteins reflected the deletions in these mutant proteins. However, only one protein, whose mobility was appropriate for a deleted version of the larger wild-type

<table>
<thead>
<tr>
<th>Wild-type G</th>
<th>GA58D</th>
<th>GI60K</th>
<th>GA58D/I60K</th>
<th>G23A31</th>
<th>G38A50</th>
<th>G51A63</th>
<th>G38A63</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSKNKNQRTAKTLERTWDTLHLLFISLYCLNLKSVQITLSILAMIISTSLIAIIFIASANKVP</td>
<td>MSKNKNQRTAKTLERTWDTLHLLFISLYCLNLKSVQITLSILAMIISTSLIAIIFIASANKVP</td>
<td>MSKNKNQRTAKTLERTWDTLHLLFISLYCLNLKSVQITLSILAMIISTSLIAIIFIASANKVP</td>
<td>MSKNKNQRTAKTLERTWDTLHLLFISLYCLNLKSVQITLSILAMIISTSLIAIIFIASANKVP</td>
<td>MSKNKNQRTAKTLERTWDTLHLLFISLYCLNLKSVQITLSILAMIISTSLIAIIFIASANKVP</td>
<td>MSKNKNQRTAKTLERTWDTLHLLFISLYCLNLKSVQITLSILAMIISTSLIAIIFIASANKVP</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. Predicted amino acid sequence of the first 70 amino acids of the wild-type and site-specific mutants of RS virus G protein. The single-letter amino acid code for the first 70 amino acids of the wild-type and mutant RS virus G proteins is shown. The change(s) present in the single and double point mutants are indicated in reverse-type. Blank spaces are shown in place of the amino acids that were removed in the deletion mutants. The minor and major hydrophobic regions are underlined in the wild-type sequence.
D. L. Lichtenstein and others

(a) 

(b) 

Fig. 2. Cell-free translation of wild-type and mutant RS virus G protein mRNAs in the absence or presence of microsomal membranes. Cell-free translation reactions were carried out as described in Methods. Proteins were separated by electrophoresis on 10–17.5% polyacrylamide–SDS gels. Autoradiographs of the dried gels are shown. Cell-free translation reactions contained no RNA (lane 1), or the following mRNAs: wild-type G (lane 2), GA58D (lane 3), GI60K (lane 4), GA58D/I60K (lane 5), G38A50 (lane 6), G51A63 (lane 7), G38A63 (lane 8), and G23A31 (lane 9). (a) Proteins synthesized in the absence of microsomal membranes. (b) Proteins synthesized in the presence of microsomal membranes. The sizes (kDa) of the protein markers are shown to the left of each gel.

protein, was detected in translation reactions that contained G38A50 or G38A63 mRNAs (Fig. 2a, lanes 6 and 8). Since both deletions removed the AUG codon within the major hydrophobic region, these results confirmed our previous observations that this codon is required for translation initiation of the smaller protein (Roberts et al., 1994). The identity of the approximately 40 kDa band present in some lanes is unknown.

Membrane insertion of the wild-type and mutant proteins was assayed by their glycosylation in cell-free translation reactions supplemented with microsomal membranes. Translation of wild-type G mRNA in the presence of membranes resulted in the appearance of at least three major and one minor protein species (Fig. 2b, lane 2) as reported previously (Collins & Mottet, 1992; Roberts et al., 1994). The presence of N-linked high mannose carbohydrate residues on the new protein species was confirmed by digestion with endo H which restored the proteins to close to their original mobilities (Fig. 3, lane 3). The intensity and migration of the digested proteins showed that both forms of G protein were capable of being glycosylated, and that no polypeptide cleavage event (by signal peptidase, for example) had occurred during membrane insertion in the cell-free system.

Glycosylated forms of all of the mutant proteins, except G38A63 protein, were detected when the mRNAs were translated in the presence of microsomal membranes (Fig. 2b, lanes 3–9). The glycosylation patterns for GA58D, GI60K, GA58D/I60K, G23A31 and G51A63 were similar to those of wild-type G proteins except that the proteins of the latter two migrated slightly faster than the corresponding wild-type proteins due to the deletions in the mutant proteins. Translation of G38A50 transcripts in the presence of microsomal membranes yielded only three glycosylated protein species, perhaps because only one unglycosylated form of this mutant protein was detected (Fig. 2b, lane 6). In contrast to the other mutants, no additional forms of G38A63 protein were made in the presence of microsomal membranes (Fig. 2b, lane 8). Moreover, G38A63 protein was the only one which, when synthesized in the presence of membranes, was not protected from digestion with trypsin (data not shown). These results strongly suggest that the G38A63 protein was not inserted into membranes, whereas all the other mutant proteins were.

Partial deletion of the S/A domain of several other type II proteins results in secretion of the proteins into the lumen of microsomes (Spiess & Handschin, 1987; Lipp & Dobberstein, 1988) or into the cell culture medium (Hogue & Nayak, 1994). It is believed that secretion is the result of signal peptidase digestion at a formerly cryptic cleavage site. Since G38A50 and G51A63 proteins each possessed only half of the major hydrophobic region, the glycosylated proteins were assayed for proteolytic processing by examining their sizes after treatment with endo H. This treatment yielded two deglycosylated forms of G38A50 protein, one of which migrated faster than the sole unglycosylated G38A50 protein made in the absence of microsomal membranes (Fig. 3, compare lanes 4 and 6). This strongly suggested that at least some of the G38A50 protein had been proteolytically cleaved. Endo H treatment of the
glycosylated forms of G51Δ63 protein increased the intensity of the smaller species compared to the corresponding species present in the untreated sample (Fig. 3, compare lanes 8 and 9). The smaller protein made by G51Δ63 would not be expected to interact with membranes because its hydrophobic region was only 3 amino acids long (see Fig. 1). Therefore, we attribute the increase in intensity of the smaller form of G51Δ63 protein after endo H digestion to proteolytic cleavage of the larger species.

**Expression of G protein mutants from vaccinia virus vectors**

In order to examine O-glycosylation, cell surface expression, and secretion, the deletion mutant proteins were expressed by infection of cells with recombinant VV vectors. Synthesis of the intermediate forms and the mature, cell-associated form of wild-type and mutant G proteins was examined by immunoprecipitation of [3H]threonine-labelled proteins from cytoplasmic extracts of recombinant VV-infected cells. As seen previously (Wertz et al., 1989), the mature 90 kDa N- and O-glycosylated form and the immature 45 kDa forms containing N-linked carbohydrates were synthesized in cells infected with the recombinant VV that expressed wild-type G protein (vG wt; Fig. 4, lane 3). The proteins with single point mutations behaved similarly to wild-type G protein (data not shown). The deletion mutant G23Δ31, whose sequence lacked the minor hydrophobic region, accumulated mature and immature proteins at an elevated level (Fig. 4, lane 4). In contrast, no intermediate or mature forms of G protein were detected in vG38Δ63-infected cells (Fig. 4, lane 7). As seen in Fig. 2(b), this mutant produced a single protein whose migration was consistent with that of the unglycosylated form of G38Δ63 protein. Pulse-chase analysis of vG38Δ63 showed that the half-life of G38Δ63 protein in recombinant VV-infected cells was about 60 min and that the protein was neither chased into a higher molecular mass form nor secreted into the medium (data not shown). These data confirm that in vivo, G38Δ63 protein was not inserted into membranes.

The two mutant proteins that each lacked half of the major hydrophobic region (G38Δ50 and G51Δ63) showed similar patterns of protein expression: trace amounts of the mature protein and the immature 45 kDa species (not seen in Fig. 4) and abundant amounts of a protein of about 40 kDa (Fig. 4, lanes 5 and 6). In addition, the mature mutant proteins were smaller than the mature wild-type protein by more than the difference attributable to the deletions. These data and the abundant new protein at 40 kDa suggested that the G38Δ50 and G51Δ63 proteins may have been proteolytically processed in infected cells as they were in the cell-free translation system.
Fig. 4. Wild-type and mutant G proteins present in cytoplasmic extracts of recombinant VV-infected cells. Hep2 cells were mock-infected or infected at an m.o.i. of 10 p.f.u. per cell with wild-type VV or recombinant VVs that expressed the wild-type or mutant G proteins. At 4 h post-infection cells were metabolically labelled for 3-5 h with [3H]threonine. G protein-specific proteins were immunoprecipitated from cytoplasmic lysates with monoclonal antibody L9 and separated by electrophoresis on a 10% polyacrylamide-SDS gel. A fluorograph of the dried gel is shown. Hep2 cells were mock-infected (uninf.) (lane 1), or infected with: wild-type VV (lane 2), vG wt (lane 3), vG23A31 (lane 4), vG38A50 (lane 5), vG51A63 (lane 6) or vG38A63 (lane 7). The sizes (kDa) of the protein markers are shown to the left of the gel.

Immunofluorescence analysis of mutant proteins

In order to examine the sub-cellular localization and cell surface expression of the mutant proteins, recombinant VV-infected cells were examined by indirect immunofluorescence microscopy and FACS analysis. Surface and intracellular staining patterns for both single point mutants were similar to wild-type G protein (data not shown). Although immunofluorescence microscopy showed similar patterns of cell-surface expression for G23A31 and wild-type G proteins (Fig. 5B, C), quantitative FACS analysis showed that cell-surface expression of G23A31 protein was delayed compared to the wild-type G protein, and was 2–3-fold lower throughout infection (data not shown). Conversely, when cytoplasmic expression of G23A31 protein was compared to that of wild-type G protein by immunofluorescence microscopy, the mutant showed a slightly elevated level of perinuclear staining (Fig. 5, compare E and F). These data, combined with pulse-chase analysis of G23A31 protein, which showed no difference in the kinetics of protein glycosylation compared to the wild-type G protein (data not shown), suggested that G23A31 protein may be slightly defective at a late stage of transport to the cell surface. The three remaining deletion mutants (G38A50, G51A63 and G38A63) showed little or no surface fluorescence above the background (Fig. 5A, G, H and I). However, these three mutants showed wild-type levels of cytoplasmic fluorescence, consistent with the detection of these proteins in lysates of infected cells (Fig. 5J, K and L). The diffuse pattern of staining throughout the cytoplasm of cells infected with vG38A63 (Fig. 5L) suggested that this protein remained in the cytoplasm, consistent with the lack of membrane insertion shown by the results presented earlier.

Secretion of mutant proteins

The proteolytic cleavage of G38A50 and G51A63 proteins observed in the cell-free translation analyses raised the possibility that the lack of surface fluorescence for these mutant proteins might be due to increased secretion as was seen previously with S/A deletion mutants of influenza virus neuraminidase (Hogue & Nayak, 1994). Secretion of the wild-type and mutant G proteins was analysed by immunoprecipitation of G protein from the medium of recombinant VV-infected cells. Among the mutants, only G38A63 failed to secrete G protein into the medium (Fig. 6, lane 7). Since removal of the entire S/A domain from this mutant abolishes its insertion into membranes, this result was expected. Low amounts of wild-type and G23A31 proteins were secreted (Fig. 6, lanes 3 and 4), while G38A50 and G51A63 proteins were secreted abundantly (Fig. 6, lanes 5 and 6). Interestingly, G38A50 protein was secreted in greater quantity than wild-type G protein even though the initiation codon from which the secreted wild-type G protein is synthesized was deleted in this mutant. Therefore, secreted G38A50 protein must have been generated by proteolytic cleavage of the larger form of the protein which, in the wild-type, remains uncleaved and stably cell-associated.

Proteolytic cleavage of G38A50 and G51A63

To determine if proteolytic cleavage of G38A50 and G51A63 proteins was responsible for secretion of these proteins from recombinant VV-infected cells, the sizes of the unglycosylated proteins were determined by treatment with tunicamycin (an inhibitor of N-linked
Fig. 5. Surface and internal immunofluorescence microscopy of recombinant VV-infected cells. 143 cells were infected with: wild-type VV (A, D), vG wt (B and E), vG23Δ31 (C, F), vG38Δ50 (G, J), vG51Δ63 (H, K) or vG38Δ63 (I, L). At 5 h post-infection cells were fixed for surface immunofluorescence by treatment with 1% paraformaldehyde (A, B, C, G, H, I) or for internal immunofluorescence by treatment with 95% ethanol/5% acetic acid (D, E, F, J, K, L).

Fig. 6. Analysis of the secreted form of G protein. Hep2 cells were infected and metabolically labelled as described for Fig. 4. Clarified medium from cells infected for 7.5 h was immunoprecipitated with monoclonal antibody L9 and then separated by electrophoresis on a 10% polyacrylamide-SDS gel. A fluorograph of the dried gel is shown. Hep2 cells were mock-infected (uninf.) (lane 1), or infected with: wild-type VV (lane 2), vG wt (lane 3), vG23Δ31 (lane 4), vG38Δ50 (lane 5), vG51Δ63 (lane 6) or vG38Δ63 (lane 7). The sizes (kDa) of the protein markers are shown to the left of the gel.

Fig. 7. Analysis of the G protein synthesized in the presence of tunicamycin (lanes 5-7) and monensin (lanes 6-8). Two unglycosylated species were detected for wild-type G protein (Fig. 7, asterisks in lane 5). These species have been identified previously and were shown to correspond to polypeptides synthesized from translation initiation at methionine-1 (AUG1) and methionine-48 (AUG2) in the G protein open reading frame respectively (Roberts et al., 1994). The major band that migrated slower than the larger wild-type G protein was most likely a partially glycosylated intermediate form of G protein. Two predominant G38Δ50 proteins were detected in the presence of tunicamycin and monensin (Fig. 7, lane 7) which contrasted with the synthesis of only one unglycosylated protein in the cell-free translation system (see lane 6, Fig. 2a, b). The slower migrating species had the mobility expected for the unglycosylated full-length protein, which had a 13 amino acid deletion. We interpret the faster migrating protein as a cleavage product since this mutant lacked the initiation codon for the smaller protein. These results were consistent with the observation that endo H treatment of the glycosylated G38Δ50 proteins synthesized in the presence of microsomal membranes yields two polypeptides (Fig. 3, lane 6). Two predominant G51Δ63 proteins were also detected in the presence of tunicamycin and monensin (Fig. 7, lane 9). The slower migrating species, which co-migrated with that of G38Δ50, probably represented the full-length form of this mutant protein. The faster migrating form of G51Δ63 protein may have arisen by internal initiation (as is the case for wild-type G protein) and/or by proteolytic processing of the full-length species of this glycosylation) and monensin (to prevent O-linked glycosylation). Two unglycosylated species were detected for wild-type G protein (Fig. 7, asterisks in lane 5).
Fig. 7. Detection of unglycosylated forms of wild type and mutant G proteins in cells treated with tunicamycin and monensin. Hep2 cells were mock-infected (lanes 1 and 2) or infected with: wild-type VV (lanes 3 and 4), vG wt (lanes 5 and 6), vG38A50 (lanes 7 and 8), vG51A63 (lanes 9 and 10) or vG38A63 (lane 11). At 1 h post-infection cells remained untreated (lanes 2, 4, 6, 8, 10 and 11) or were treated (lanes 1, 3, 5, 7 and 9) with tunicamycin (3 μg/ml) and monensin (1 μM) for the duration of the experiment. Cells were metabolically labelled and cytoplasmic extracts were immunoprecipitated as described for Fig. 4. Samples were separated by electrophoresis on a 10% polyacrylamide-SDS gel. A fluorograph of the dried gel is shown. The absence (−) or presence (+) of the glycosylation inhibitors is indicated above the gel. The sizes (kDa) of the protein markers are shown to the left of the gel.

protein. It is likely that at least some of the faster migrating form of G51A63 protein was the product of proteolytic cleavage since this protein was shown to be cleaved in the cell-free translation system and substantial amounts of the mature G51A63 protein were secreted from infected cells (Fig. 6, lane 6).

Discussion

Synthesis, processing, transport to the cell surface, and secretion of RS virus G protein mutants were examined by using a combination of cell-free transcription-translation and expression from recombinant VV vectors. Deletion of the major hydrophobic region (mutant G38A63) eliminated insertion of G protein into membranes as judged by the inability to detect glycosylated and secreted forms of the protein and the lack of cell-surface expression. Since deletion of the minor hydrophobic region had no apparent effect on membrane insertion, these data provide direct evidence to map the S/A domain to the major hydrophobic region. The fact that the minor hydrophobic region consists of only 9 non-polar amino acids supports studies with other type II S/A domains which show that a minimum of 10–16 non-polar amino acids is necessary for membrane insertion (Hong & Doyle, 1990; Lipp & Dobberstein, 1988; Spiess & Handschin, 1987).

Our results showed that mutants of G protein in which either the N-terminal (G38A50) or C-terminal (G51A63) half of the major hydrophobic region was deleted, without altering the neighbouring amino acids, were still inserted into membranes (Figs 2, 3 and 4). The amino acids remaining within the S/A domain of these two mutant proteins did not overlap. Thus, each half of the S/A domain of G protein seems to be fully functional for membrane insertion. However, the subsequent behaviour of the G protein when anchored by a truncated S/A domain differed sharply from that of the wild-type anchored protein in that it was abundantly secreted into the cell culture medium (Fig. 6). In fact, G38A50 and G51A63 proteins were more similar to the secreted form than the anchored form of wild-type G protein in that all three proteins contained a shortened S/A domain, were proteolytically processed (see below), and were secreted.

RS virus G protein is unique among paramyxovirus attachment proteins in that a secreted form of the protein is normally found in the medium of infected cells. It was expected that neither G38A50 nor G51A63 would be secreted. The G38A50 cDNA lacked the second AUG codon at which the secreted form of wild-type G protein initiates. Also, the form of G51A63 protein produced by initiation at this AUG codon would contain only 3 non-polar amino acids, an insufficient number to allow membrane insertion. However, both mutant proteins were abundantly secreted into the cell culture medium (Fig. 6, lanes 5 and 6). Secretion of these two mutant proteins was probably due to proteolytic cleavage, which was demonstrated for each protein in the cell-free translation system and in recombinant VV-infected cells (see Figs 3 and 7).

Partial deletion of the S/A domain of other type II proteins converts an uncleaved S/A sequence into a cleaved signal sequence, resulting in secretion of the mutant proteins (Hegner et al., 1992; Hogue & Nayak, 1994; Spiess & Handschin, 1987; Lipp & Dobberstein, 1988). It was proposed that the position of the protein in the membrane is altered, thus allowing cleavage by signal peptidase at a previously inaccessible processing site (Lipp & Dobberstein, 1986). Significantly, recent studies show that the length of the hydrophobic region can affect the position of a protein in the membrane, as assayed by the accessibility to oligosaccharyl transferase of a glycosylation site near the C-terminal end of the hydrophobic region (Nilsson et al., 1994). Moreover,
cleavage at a signal peptidase cleavage site occurred if the hydrophobic region was \(\leq 17\) amino acids in length but was eliminated by increasing it to \(\geq 20\) (Nilsson et al., 1994). Shortening the hydrophobic region of G38A50 and G51A63 proteins from 26 to 13 amino acids may have altered the position of the mutant proteins in the membrane, allowing cleavage by signal peptidase at a site that is cryptic in the wild-type protein. A potential signal peptidase cleavage site is predicted between amino acids 65 and 66 by the \(-1, -3\) rule (small, non-polar amino acids at positions \(-1\) and \(-3\) with respect to the cleavage site) (von Heijne, 1986). That the cleavage was mediated by signal peptidase was supported by the fact that the presence of microsomal membranes was sufficient for the cleavage to occur (Fig. 3). In contrast to G38A50 and G51A63 proteins, cleavage of the wild-type G protein did not occur in the endoplasmic reticulum (Roberts et al., 1994).

Although deletion of the minor hydrophobic region (G23A31) had no effect on membrane insertion, this mutant protein was delayed in its transport to the cell surface (data not shown) and showed increased perinuclear staining suggestive of accumulation in the Golgi apparatus (Fig. 5K). Improper folding of G23A31 may be responsible for the observed delay in transport to the cell surface. However, this is unlikely since most misfolded proteins are normally retained in the endoplasmic reticulum and pulse-chase analysis of this mutant showed it was processed to the mature size at the same rate as wild-type G protein (data not shown). RS virus G protein is palmitoylated and since palmitic acid is often attached to cysteine residues that are proximal to or within the membrane spanning region of proteins, it was suggested that the site for this modification is the cysteine residue at amino acid position 29 (Collins & Mottet, 1992). This is the only cysteine residue in the cytoplasmic domain, and it is conserved in 53 out of 56 known G protein sequences, including human subtypes A and B, bovine, and ovine, suggesting an important function for this amino acid. It is possible that the deletion of cysteine 29 in G23A31 resulted in delayed cell surface expression due to lack of palmitoylation. However, no definitive role for protein palmitoylation in cell surface expression of proteins has been described (for review see Schmidt, 1989). In fact, a mutant of vesicular stomatitis virus G protein from which the palmitoylation site was removed was transported to the cell surface normally (Rose et al., 1984). Direct determination of the acylation state of G23A31 protein and the specific role of cysteine 29 in palmitoylation will need to be investigated.

We thank Keith Berry for expert technical assistance with the FACS analysis and Dr Ronald C. Montelaro for critically reviewing this manuscript. This work was supported by Public Health Service grants R37 AI 18270 to L. A. B. and R37 AI 12464 and AI 20184 to G. W. W.

References


(Received 12 June 1995; Accepted 5 September 1995)