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ADVANCES IN VIRUS RESEARCH, VOL. 33

DOMAINS OF VIRUS GLYCOPROTEINS

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I. Introduction

Replication of a virus in a susceptible host cell begins with its attachment to the cell surface and ends with the assembly of newly formed virus components into organized structures which ultimately are released from the cell. For enveloped viruses, attachment is mediated by glycoproteins which form spikes projecting outward from the virion's surface. The polypeptide backbone of the spike glycoprotein is encoded by virus-specific genes and these proteins are extensively modified after synthesis by host-cell-specific enzymes. Most of the protein mass lies outside the virion's lipid bilayer but a short sequence of hydrophobic amino acids within the protein spans the bilayer, thereby anchoring the spikes to the membrane.

In addition to their role as major determinants of cell tropism these glycoproteins have two other functions in the replication process. They possess a membrane fusion activity which enables the viral nucleoprotein to enter the cell cytoplasm, and they participate in the assembly and budding of new virions. Viral glycoproteins are also important in another context. They are the major determinants to which the immune system responds when an organism is infected with an enveloped virus. The interactions between the components of the immune system and viral glycoproteins are complex and most likely are the determining factor in the outcome of an infection.

In this article, we review current information about the structure and function of virus glycoproteins. We do not intend this to be a comprehensive accounting of the virus glycoprotein literature, and have selected a few virus glycoproteins which we feel provide prototypes for illustrating important relationships between the functions cited above and glycoprotein structure. One of the major advances in our knowledge about virus glycoproteins has come from the application of recombinant DNA technology, which led to a determination of the primary structure of many virus glycoproteins as well as to information about the role of various amino acid sequences in glycoprotein

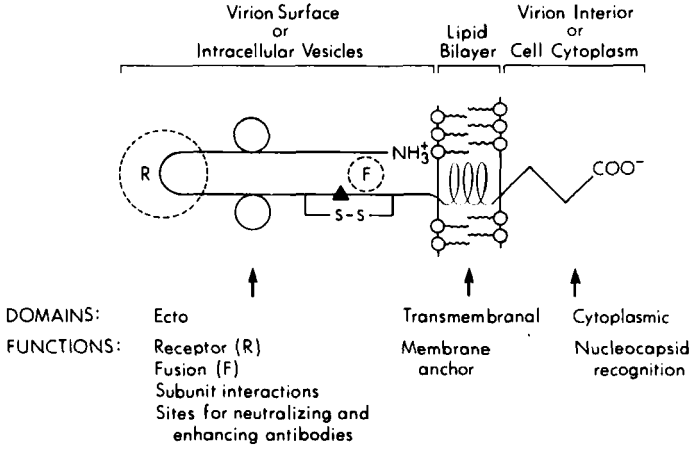

FIG. 1. A typical transmembranal virus glycoprotein. Some proteins have a reversed orientation with their amino terminus inside and the carboxy terminus outside the virion. The distribution of polypeptide sequences between the ecto- and cytoplasmic domain can also vary greatly; however, the transmembranal domain usually consists of about 20 to 25 hydrophobic amino acids. Most proteins have oligosaccharide groups (○) bound only to asparagine but others have glycosyl residues attached to serine and threonine as well. A posttranslational proteolytic cleavage (noted by ▲) converts many virus glycoproteins into disulfide-linked dimers. In addition these polypeptides often exist in their native state as oligomers (dimers, trimers, tetramers), held together by strong nonconvalent protein–protein interactions.

localization and function. The amino acid sequences for those glycoproteins discussed here will not be presented; instead we consider these proteins in terms of their structural domains. Figure 1 illustrates these domains and their assigned functions. One set of domains we shall not discuss in depth are those regions of glycoproteins mapped as antigenic determinants or epitopes. An article in a recent volume of this series describes in detail influenza virus glycoprotein antigenic sites and their variability (Air and Laver, 1986).

II. INFLUENZA VIRUS HEMAGGLUTININ

The influenza A and B viruses contain two genes encoding membrane glycoproteins—one produces a neuraminidase (NA) and the other a hemagglutinin (HA). There is more information about influenza virus hemagglutinin than any other virus membrane protein and its structure–function relationships could well serve as a paradigm for a large number of similarly structured virus and cell transmembrane glycoproteins. Three factors account for the wealth of data about HA:

(1) a determination of the three-dimensional structure of the crystallized, bromelain-cleaved soluble form of HA by Wilson *et al.* (1981), (2) the sequencing of a large number of HA polypeptides with distinct serological types (reviewed in Palese and Kingsbury, 1983), and (3) the cloning of a complete cDNA copy of the HA-RNA gene and the ability to form HA in cultured cells carrying this cDNA (Gething *et al.*, 1980; Gething and Sambrook, 1981). We draw on data based on each of these to describe the domains of the HA protein (see Fig. 2).

The typical HA protein is initially synthesized as a molecule of 566 amino acids; however, the 16 amino acids at the amino terminus constitute a signal peptide and are cleaved shortly after the complete polypeptide is made. Oligosaccharides are added to asparagine residues at six to seven sites along the chain during synthesis; most of these are attached to the amino-terminal portion of the protein and are on the stalk of the HA trimer. They are also in other regions and can influence the receptor-binding and fusion activities. Processing of these glycosyl residues occurs shortly after their attachment and such modifications can profoundly influence the conformation of the protein (see Section IV,A,1). A model for the folding of this large protein is found in the analysis of the X-ray crystal structure (Wilson *et al.*, 1981). Six disulfide bridges form during folding and prior to a critical, single proteolytic cleavage in a highly conserved region of HA at a site

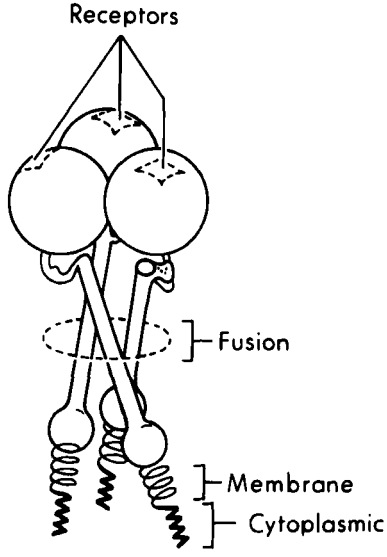

FIG. 2. The trimer structure of influenza virus HA. (Adapted in part from Wilson *et al.*, 1981.)

221 amino acids from the carboxy terminus. A protease in the Golgi stacks converts HA to a disulfide-linked dimer of HA1 with 328 and HA2 with 221 residues. A few additional amino acids are removed from the carboxy terminus of HA1. An important event during processing is the formation of HA1-HA2 trimers which are stabilized through α-helical-coiled coils and salt bridges in the stalk region of the protein. Changes in antigenic properties of HA have been detected during these processing events (Bachi *et al.*, 1985; Nestorowicz *et al.*, 1985).

A. Ectodomain

1. Fusion Activity

HA has a cryptic fusogenic activity but two changes must occur before this protein can fuse membranes. First, there must be a proteolytic cleavage at a lysine (arginine) at position 328, which produces the disulfide-linked HA1 and HA2 chains (Klenk *et al.*, 1975; Lazarowitz and Choppin, 1975). This cleavage is accompanied by conformational changes in the protein's structure since the amino terminus of HA2 and the carboxy terminus of HA1 move some 21 Å apart. In addition, the HA2 amino terminus folds into the interior of the HA trimer. The importance of this cleavage and subsequent fusogenic activity to influenza virulence has recently been noted as a result of examining the HAs from virulent forms of a chicken influenza virus. Although cells infected with the virulent A/chick/Penn/83 (H5N2) could cleave HA to HA1 and HA2, the avirulent form of this virus has an HA that required exogenously added trypsin for cleavage (Kawaoka *et al.*, 1984). At least one epitope is different between the virulent and avirulent types (Robertson *et al.*, 1985). Amino acid sequences are not different between HAs in the region of the connecting HA1-HA2 peptide, but the molecular weight of the virulent HA1 appears lower than the avirulent strain except when glycosylation was blocked by tunicamycin, a result indicating that there is one less oligosaccharide in the virulent HA. Four amino acids are changed between avirulent and virulent types and one of these, at position 13 of HA1 located near the HA1-HA2 connecting peptide, is postulated to alter a glycosylation site such that the HA1-HA2 cleavage site now becomes accessible. The actual cleavage sequence contains dibasic amino acids, which are sites for limited proteolysis in a wide variety of proteins including other enveloped virus glycoproteins, peptide hormones, and neuropeptide precursors. The proteolytic activity is localized to a trans-Golgi vesicle (Morrison *et al.*, 1985) and is possibly the calcium-activated, thiol-type protease described by Steiner *et al.* (1984).

The sequences at the amino terminus of HA2 are the most highly conserved among HA variants. Furthermore, an analogous sequence of very hydrophobic amino acids is found in the fusion proteins of all paramyxoviruses (Spear, 1986), indicating that this site is essential for fusion. This hypothesis is now strongly supported by two separate studies of HA variants that fuse at pH values significantly above that of the wild type. In one series of analyses, variants were selected by growth in chorioantoic membranes treated with amantadine hydrochloride to raise the endosomal pH (Daniels *et al.*, 1985). After two passages and plaquing on chicken embryo fibroblasts in the presence of the drug, a number of isolates hemolyzed red blood cells at pH values 0.2 to 0.8 greater than the wild-type HA. Changes in amino acid sequences of 41 variants were analyzed with regard to their sites in the quaternary structure of the trimer. All changes could be interpreted to affect either the binding of the HA2 amino-terminal sequence to internal regions of the protein or the stability of subunit interactions in the trimer.

The other set of experiments consisted of performing *in vitro* site-directed mutagenesis in regions of the cDNA encoding the HA2 amino terminus (Gething *et al.*, 1986). Three substitutions were made: glutamic acid for glycine at positions 1 and 4 and glycine to glutamic acid at position 11. A glutamic acid at the amino terminus blocked all fusogenic activity, although the HA bound to lipid at lower pH and became protease sensitive. This finding indicates that this HA could still be structurally altered, possibly as a result of dissociation to a monomeric form. Substituting a charged residue at position 4 destabilized HA and led to an increased threshold pH (5.6 compared to 5.3 for normal HA) but a 50% loss in "efficiency" of fusion. The glycine replacement at position 11 extended the hydrophobic character of the HA2 another seven amino acids. This had little effect on HA stability but interfered with the ability of this HA to cause cell–cell fusion. All of these results suggest a dual role for the fusion site. One is coincident with an exposure of the hydrophobic sequence as the trimers "relax" and possibly dissociate; a second is an interaction of the polypeptide with lipid, producing a destabilization and coalescence of bilayers. A two-step kinetic mechanism of HA-mediated fusion has been proposed by Van Meer *et al.* (1985) in which a rapid association of the two membranes is followed by the actual fusion at a rate varying with lipid composition.

It is clear from studies using expressed cDNAs in transfected cultured cells that HA fusion does not require the neuraminidase (White *et al.*, 1982). In other systems employing liposomes containing orthomyxovirus glycoproteins, however, the HA-promulgated fusion is

detected only when an active neuraminadase is present although it does not have to be inserted into the liposome (Huang *et al.*, 1980).

2. Receptor Binding Site

It has long been known that sialic acid is the host cell surface receptor for influenza virus, but the receptor site on HA has only recently been characterized. A tentative identification of this site is described for the three-dimensional structure (Wilson *et al.*, 1981) and consists of a pocket on the surface of HA at the distal end of the spike in the HA1 subunit (see Fig. 2). Assignment of this region is based on the observation that a variety of HA sequences show a strong conservation for five distinctive amino acids located in the same topological position in the HA structure. More recent analyses of HA variants which differ in their recognition of two different sialic acid structures confirm the assignment of the receptor site (reviewed in Air and Laver, 1986). The variants differ in amino acid 226 of HA1, which is in the postulated surface pocket. Other sites at positions adjacent to the pocket also influence receptor structure since variants with changes in these positions show differences in agglutinating red blood cells containing three distinct sialic acids: *N*-acetyl-, *N*-glycolyl-, and *N-O*-diacetylneuraminic acids (Higa *et al.*, 1985).

HA receptor binding is affected by glycosylation at sites on HA1 close to the pocket. For example, influenza B strains harvested after growth on Madin–Darby canine kidney (MDCK) cells differ in their HAs from those strains isolated after adaptation to growth in the chicken embryo allantoic membrane (Robertson *et al.*, 1985). One major difference, a decrease in the oligosaccharide content of the egg-grown virus, arose as a result of an amino acid substitution that removed an HA1 glycosylation site adjacent to the receptor site. A glycosylation site is also lost from HA1 in a variant of influenza A WSN strain that is selected during passage of virus on Madin–Darby bovine kidney (MDBK) cells (Deom *et al.*, 1986). Compared to the initial isolate from chicken embryo fibroblasts, the selected variant shows a stronger binding to MDBK cells and erythrocytes (Crecelius *et al.*, 1984).

B. Transmembrane Domain

The amino acid sequences of a large number of influenza virus HAs show a hydrophobic region of about 25 amino acids close to the carboxy terminus. The evidence that this region of the glycoprotein tethers the molecule to the lipid bilayer comes from *in vitro* constructed deletion mutants that remove the carboxy-terminal hydrophobic sequences

(Gething and Sambrook, 1982). A cDNA containing a partial copy of the RNA gene for HA from influenza A/Japan/305/57 was inserted into an SV40 vector and monolayers of CV-1 cells infected with virus stocks prepared so they contained helper and recombinant viruses. The particular cDNA used for the analysis had altered sequences near the 3′ end of the gene which replaced in the HA the last 38 amino acids at the carboxy terminus with 11 amino acids that were predominantly hydrophilic. When expressed in the monkey cells, the truncated HA was secreted into the culture medium. HA cDNAs have also been inserted into bovine papilloma virus plasmids and these vectors used for isolation of cell lines which constitutively make the HA proteins. The 3′ truncated cDNA encodes a protein that was secreted, but that coding for the normal HA made a glycoprotein that was retained at the surface of the cells (Sambrook *et al.*, 1985). Additional mutants of HA with deletions and insertions in the region containing the anchor sequences have been constructed from cDNAs of HA (Sveda *et al.*, 1982) and SV40 vectors carrying these cDNAs expressed in African monkey kidney cells. Normal HAs were completely cell associated but varying amounts (one-fourth to two-thirds) of mutant HAs were secreted from the cells, and mutant and wild-type HAs were differently glycosylated. Another HA mutant that had a totally different carboxy-terminal sequence beginning near the membrane domain of HA2 was retained inside the cell (Sveda *et al.*, 1984). Clearly, it is possible to affect glycoprotein transport and sorting (see below) by modifying large portions of the membrane domain.

C. Cytoplasmic Domain

The carboxy-terminal 10 amino acids of HA protrude from the cytoplasmic face of the lipid bilayer. The last five amino acids (Lys-Arg-Ile-Cys-Ile) are highly conserved among HAs of different strains of influenza, suggesting a critical role in HA function. This sequence is not essential, however, for synthesis and transport of the glycoprotein from the endoplasmic reticulum (ER) to the cell surface and rather extensive changes can occur in the cytoplasmic domain without seriously altering intracellular transport (Doyle *et al.*, 1985). These results emerged from *in vitro* constructions of the HA cDNA in which the carboxy-terminal sequences were mutated to highly diverse structures. The HA cDNA was inserted into the SV40 genome such that high levels of expression occurred and sufficient HA was produced to measure cellular location, state of processing, and fusogenesis as well as hemagglutinating properties of the surface glycoprotein. The most subtle change of three amino acid substitutions (Arg to Ser, Cys to

Glu, and Ile to Arg) in the carboxy terminus had no effect on synthesis and transport. A more drastic change in sequence and an elongation of six amino acids, as a result of substituting the cytoplasmic tail of Rous sarcoma virus (RSV) glycoprotein cytoplasmic sequence, also had no effect on transport. Some partial effects were noted in those cytoplasmic tails truncated to three amino acids or extended by 22 amino acids with RSV cytoplasmic sequences. However, extension with 16 amino acids from a bacterial plasmid sequence did interfere with transport of HA from the ER. Another random 16-amino acid sequence also blocked transport, but only after the protein reached the Golgi network.

The cytoplasmic domain appears also to be the site for fatty acylation. The human and avian viruses of H0, H1, H2, H3, H7, and H10 serotypes have palmitate linked to the HA2 subunit and the fatty acid is retained in the membrane-bound fragment after bromelain releases HA (Schmidt, 1982). In addition a carboxy-terminal 6-kDa cyanogen bromide peptide isolated from fowl plague virus contains palmitate. Removal of the bound fatty acid from fowl plague virus or isolated HAs by hydroxylamine inhibits HA-induced hemolysis under conditions that do not affect hemagglutination titers or cause gross morphological change in virus structure or HA conformation (Schmidt and Lambrect, 1986). Neuraminidase activity of virions is unaffected by the hydroxylaminolysis which blocks fusogenic function; however, until the deacylated HA can be restored to function by reacylation, the conclusion that acyl groups are required for fusion must be a tentative one.

III. Alphavirus Glycoproteins

Glycoproteins of the two closely related alphaviruses, Sindbis and Semliki Forest (SFV), have been studied in considerable detail (reviewed by Schlesinger and Schlesinger, 1986). The two major glycoproteins (E1 and E2) encoded by these viruses are initially expressed as a polyprotein (Strauss and Strauss, 1986). Nascent proteolytic cleavages produce a precursor of E2 (P62) and E1. The two glycoprotein genes are separated by a sequence encoding a 55-amino acid peptide. The E1 protein has 439 amino acids with two extended regions of hydrophobic amino acids. The P62 has 487 amino acids and 64 of these are removed during the proteolytic conversion to E2. E1 and E2 form a relatively tight, noncovalent heterodimer, and three of these heterodimers are arranged in triangular clusters on the virion's surface (Harrison, 1986). Figure 3 illustrates the E1-E2 spike with four distinct domains.

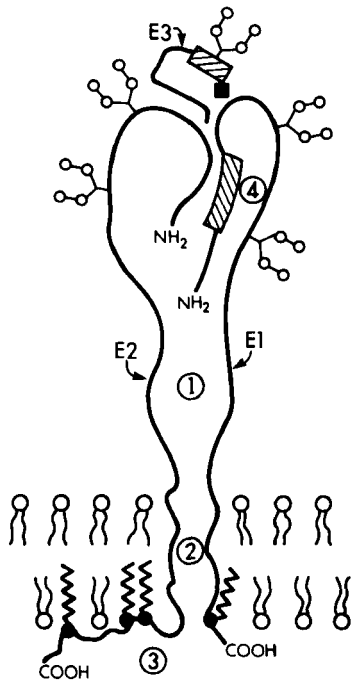

FIG. 3. A model for the E1–E2 heterodimer of Sindbis virus. (From Schlesinger and Schlesinger, 1986.) Domains noted are hydrophilic globular portion (1), membrane-spanning region (2), cytoplasmic fragment (3), and hydrophobic area not in the membrane (4); (⁂) oligosaccharides; (■) acetylated amino terminus; (∿) covalent fatty acids; (▨) hydrophobic regions.

The P62 contains a hydrophobic sequence of 19 amino acids at the amino terminus of the nascent polypeptide, which functions as a signal sequence for insertion and transfer of this protein into the ER lumen. Unlike most signal sequences, however, the P62 sequence is glycosylated at an asparagine residue in the middle of the sequence and there is no release of this sequence by signal peptidase. In addition, a significant fraction of the P62 molecules are acetylated at the amino-terminal residue (Bell *et al.*, 1982). For insertion of the E1 protein through the ER membrane, there is a separate signal sequence which consists of the carboxy-terminal one-third part of a 6-kDa peptide located between the P62 and E1 genes (Melancon and Garoff, 1986). The 6-kDa peptide does not appear in the virion (Welch and Sefton, 1979). Early (immature) forms of P62 and E1 can be distinguished from the E2 and E1 structures on the virion by appropriate monoclonal antibodies (Roehrig *et al.*, 1982; Schmaljohn *et al.*, 1983; Burke *et al.*, 1983). In addition, the weak protein–protein interactions

between P62 and E1 become much stronger when P62 is converted to E2 (Rice and Strauss, 1982; Ziemiecki and Garoff, 1978). Transport of E1 from the ER to the cell surface is facilitated by the presence of P62 (Hashimoto *et al.*, 1981), but P62 can traverse the secretory pathway in the absence of E1 (Garoff *et al.*, 1983).

A. Ectodomain

1. Fusion Activity

The heterodimer spikes of alphaviruses contain a cryptic fusion site which is activated by treating virions or infected cells at pH values of 5.5–6.0 (White and Helenius, 1980). The regions of the glycoprotein assigned to this function are postulated to consist of a conserved, hydrophobic amino acid sequence between positions 45 and 58 of the E1 subunit (Rice and Strauss, 1981). With SFV, in addition to low pH, fusion requires cholesterol or related β-hydroxysterols embedded in the target membrane (Kielian and Helenius, 1985; White and Helenius, 1980). Dependence on cholesterol was noted earlier for the binding of low-pH-treated Sindbis virus to liposomes (Mooney *et al.*, 1975). At low pH, irreversible changes occur in the conformation of both E1 and E2 of SFV (Kielian and Helenius, 1985) and the E2 of Sindbis virus (Edwards *et al.*, 1983). Additional information comes from analysis of water-soluble forms of the E1-E2 complex released from their membrane anchor domains by brief protease treatment (Kielian and Helenius, 1985). The soluble complex has much weaker heterodimer binding and monomeric E2 could be studied. E1 retained a resistance to protease after low pH but only in the presence of membrane cholesterol, suggesting a binding of cholesterol to the E1 hydrophobic sequence near its amino terminus. At low pH, the water-soluble form of E2 becomes somewhat hydrophobic but is still unable to bind to liposomes.

Fusion activity as measured by syncytial formation is detectable at the surface of mammalian cells expressing cDNA copies of SFV genome sequences for E1 and E2 (Kondor-Koch *et al.*, 1983), but low pH is required. In a cDNA with the sequences for the carboxy terminus of E1 altered, no E1 was at the surface and fusion did not occur, a result confirming a role for this protein in fusion. However, absence of E1 also interferes with P62 processing. In Sindbis virus-infected cells, the P62-E1 complex (Mann *et al.*, 1983) has fusion activity. Isolated preparations of E1 from Western equine encephalitis virus reconstituted into lipid bilayers are able to lyse erythrocytes (Yamamoto *et al.*, 1981). Antibodies specific to E1 can inhibit fusion (Chanas *et al.*,

1982), and monoclonal antibodies have been found that recognize an E1 epitope appearing after low pH (Schmaljohn *et al.*, 1983). At least one SFV fusion mutant has been isolated based on its ability to have a lower pH threshold for fusion, but location of the mutation has not yet been determined (Kielian *et al.*, 1984).

2. Host Range Determinants

Alphaviruses have a broad host range; their natural hosts are arthropods and avian species. No specific host cell surface structure has been identified as a receptor and no unique region of the spike has been assigned as a site for receptor. Neutralizing antibodies are mainly directed against the E2 glycoprotein (Roehrig, 1986), but some monoclonal antibodies directed against epitopes on E1 have neutralizing activity (Schmaljohn *et al.*, 1983; Boere *et al.*, 1984). A change in the ionic charge of the surface of Sindbis virus can have a profound effect on the host range of the virus (Symington and Schlesinger, 1975). The mutations that led to changes in the host range and surface charge of the variant were located in both E1 and E2, but the precise place in the primary sequence was not determined (Symington and Schlesinger, 1978). Adaptation of an avirulent Sindbis virus strain to neurovirulence led also to changes in both E1 and E2 (Stanley *et al.*, 1985); however, a selection for Sindbis virus variants based on rapid growth in baby hamster kidney (BHK) cells and avirulence in suckling mice led to a strain with an alteration only in E2 (Olmsted *et al.*, 1984).

B. Transmembrane Domain

In common with most virus transmembranal glycoproteins, the membrane domains for the alphavirus consist of 20–30 hydrophobic amino acids close to the carboxy terminus of the polypeptide and have basic amino acids at the cytoplasmic face of the bilayer. For SFV E2, the membrane domain could be replaced by an analogous region of the VSV G or the fowl plague virus hemagglutinin, yielding chimeric transmembranal proteins which are transported to the cell surface and retain fusogenic capacity (Riedel, 1985). Other site-directed mutations in the P62/E2 cDNA have been constructed to determine effects of inserting a single charged amino acid (i.e., glutamic acid) in the middle of the domain and of removing the positively charged amino acids at the cytoplasmic face of the bilayer (Cutler and Garoff, 1986; Cutler *et al.*, 1986). For the latter, the wild-type sequence of Arg-Ser-Lys was changed to Gly-Ser-Glu or Gly-Ser-Met. When expressed in mammalian cells in constructs that also contained E1 sequences or lacked them, the three mutants were translocated and glycosylated, and

reached the cell surface at efficiencies similar to the wild-type P62/E2, regardless of whether E1 was present or absent. At the surface, they retained fusogenic activity after low pH treatment, like the wild-type protein. They were nondefective in P62 proteolytic processing except in the absence of E1 when, like the wild type, cleavage did not occur but transport of P62 alone to the cell surface was observed. One major difference was detected between the mutants and wild type when membranes were treated at pH 11.5: at this pH, only 10% of the wild-type glycoprotein was released but 40–50% of the mutant proteins were lost from the membranes. These results show that a hydrophobic sequence alone, in the absence of cytoplasmic charged groups, can stop the translocation process and that the basic amino acids contribute to anchorage stability. The lack of effect of the glutamic acid in the middle of the membrane domain is puzzling and raises the possibility that a nonhelical conformation with fewer amino acids can span the lipid bilayer. The alterations tested in these experiments apparently do not affect those P62-E1 interactions that are needed for proteolytic cleavage and E1 transport.

C. Cytoplasmic Domain

Only one of the alphavirus glycoproteins (P62/E2) has a substantial number of amino acids extending into the cytoplasm. The E1 protein has two basic amino acids at this position, whereas E2 has 31 amino acids which include three cysteines and three prolines at sites conserved among three different alphavirus strains (Strauss and Strauss, 1985). Fatty acids are postulated to be bound to some of the cysteines (Magee *et al.*, 1983). The E2 of Sindbis virus contains three palmitic acid groups which are acylated to the glycoprotein after synthesis but before transport to the Golgi (Berger and Schmidt, 1985; Schmidt and Schlesinger, 1980). Sindbis virus E1 has one fatty acyl group postulated to be bound to a hydroxyamino acid (Magee *et al.*, 1983). The cytoplasmic portion of P62 may loop back through the bilayer during polypeptide synthesis since P62 is believed to be released from the nascent polyprotein by a signalase activity located on the lumenal side of the membrane.

It has been proposed that the E2 cytoplasmic domain binds to nucleocapsid during viral assembly at the plasma membrane (Ziemiecki and Garoff, 1978). What prevents this interaction from occurring at intracellular membranes is unclear and, in fact, initiation of the assembly process may well take place intracellularly (Johnson *et al.*, 1981). An inhibition in glycoprotein intracellular transport imposed by the ionophore monensin leads to an accumulation of P62 and E1 in

Golgi membranes and nucleocapsids are found on these membranes (Johnson and Schlesinger, 1980; Kääriäinen *et al.*, 1980). The cytoplasmic domain is not essential for intracellular transport, since cDNAs with deletions in this domain express a P62 which moves to the cell surface (Garoff *et al.*, 1983). The largest deletion left only three amino acids beyond the membrane domain, but deleting both the cytoplasmic and membrane sequences produced a protein which remained in the endoplasmic reticulum, presumably because of misfolding and aggregation.

IV. Vesicular Stomatitis and Rabies Virus Glycoproteins

The spikes covering the surface of the vesicular stomatitis and rabies virions are composed of a single species of glycoprotein, the G protein. The amino acid sequences of these G proteins have been deduced from cDNA sequences, rabies G by Anilionis *et al.* (1981) and VSV G by Rose and Gallione (1981). Both proteins are similar in size; the rabies (ERA) G has 523 amino acids and the VSV (San Juan) has 511 amino acids. A comparison of the two sequences shows only a 20% identity with the introduction of seven gaps. There are, however, several regions including those of the carboxy-terminal glycosylation site that show a stronger homology (Rose *et al.*, 1982). These two proteins contain amino-terminal signal sequences that are removed during synthesis of the polypeptide and they are oriented so their carboxy termini form the cytoplasmic domain. There is little information about the tertiary structure of these glycoproteins but studies using cross-linking reagents indicate that the G on the surface of VSV is a trimer (Dubovi and Wagner, 1977; Mudd and Swanson, 1978). More recent results obtained with VSV-infected cells show the presence of G oligomers (Kreis and Lodish, 1986). A soluble form of G that is monomeric is produced by treatment of VS virions with cathepsin D at pH 5 (Crimmins *et al.*, 1983). Although exposure to low pH could be responsible for dissociation of an oligomer, G protein solubilized from virions with octyl-β-glucoside also appears to be monomeric (Crimmins *et al.*, 1983). The finding that these treatments of VSV produce a monomeric form of G can be reconciled with the data indicating that G is an oligomer if interactions between monomers are weak. Such interactions may be analogous to those that occur between the trimers on the surface of alphavirus virions which are disrupted by nonionic detergents (Harrison, 1986). The subunits of the trimer consist of E1-E2 heterodimers that are stable to nonionic detergents.

VSV has a broad host range, and infection leads to a rapid and

synchronous formation of virus-specific proteins accompanied by inhibition of host cell protein synthesis. As a result, the G protein of VSV has provided a valuable model for many studies on the synthesis and localization of membrane glycoproteins. This section is devoted almost entirely to studies of the G protein of VSV, but there are two areas of research with the rabies G protein that deserve special mention. First, considerable effort has gone into mapping the antigenic domains of this protein. That work has recently been reviewed (Wunner *et al.*, 1985) and will not be discussed further. Second, the nicotinic acetylcholine receptor is thought to be a receptor for rabies virus and the evidence for this proposal is reviewed below.

A. Ectodomain

Most of the mass of the G protein extends outward from the surface of the virion. This domain includes two sites for attachment of oligosaccharides, the region that binds cellular receptors, and the sequences involved in the fusion of the virus membrane with cellular membranes.

1. Oligosaccharide Sites

There are two potential asparagine-linked oligosaccharide sites at residues 178 and 335 in the VSV G protein, based on the deduced amino acid sequence which contains two Asn-X-Ser/Thr stretches (Rose and Gallione, 1981). This is the amino acid configuration that specifies an asparagine-linked glycosylation site (Marshall, 1972, 1974). Both sites contain complex oligosaccharides (Etchison and Holland, 1974; Reading *et al.*, 1978). Glycosylation normally begins with the transfer of the precursor oligosaccharide, $Glc_3Man_9GlcNAc_2$ from the carrier lipid, dolichol phosphate, to nascent polypeptides. The extent to which this precursor is processed determines the final structure of the asparagine-linked oligosaccharides on mature glycoproteins (Kornfeld and Kornfeld, 1985). The first step in processing, the removal of the three glucose residues, occurs shortly after completion of the polypeptide chain while the protein is still in the ER. The synthesis of complex oligosaccharides involves the removal of all but three of the mannose residues and the addition of *N*-acetylglucosamine, galactose, sialic acid, and fucose. These reactions take place in the Golgi vesicles.

Initial studies on the role of glycosylation in the synthesis and localization of the G protein made extensive use of the drug tunicamycin, an antibiotic which inhibits the synthesis of *N*-acetylglucosaminylpyrophosphorylpolyisoprenol and prevents the addition

of any carbohydrate to asparagine residues of potential glycoproteins (Struck and Lennarz, 1980). The effects of this drug on VSV depend on which strain of the Indiana serotype is analyzed (Gibson *et al.*, 1979; Chatis and Morrison, 1981). The two strains, VSV (San Juan) and VSV (Orsay), have related but distinct polypeptides. In the presence of tunicamycin, virus yields for both strains are severely inhibited at 38°C. At 30°C the yield of VSV (San Juan) is still inhibited by tunicamycin but that of VSV (Orsay), containing nonglycosylated G protein, is nearly equal to that in the absence of drug. The retention of VSV (Orsay) production at 30°C correlates with the ability of the nonglycosylated G to fold correctly at the lower temperature (Gibson *et al.*, 1979). Thus, in the absence of carbohydrate, the folding of G protein becomes temperature sensitive, but the sensitivity depends on the amino acid sequence of the protein. A comparison of the deduced amino acid sequences of G (San Juan) and G (Orsay) reveals a difference of nine amino acids in the ectodomain (Gallione and Rose, 1985). Four of these represent an increase in hydrophilic amino acids in G (Orsay). Rose and Gallione pointed out that at position 179, which is six amino acids from the glycosylation site, there is a tyrosine in G (San Juan) and an aspartic acid in G (Orsay). The presence of more hydrophilic amino acids may explain why the oligosaccharide requirement for G (Orsay) is less stringent than that of G (San Juan).

To determine the role of each of the two glycosylation sites on the intracellular transport of G, Machamer *et al.* (1985) site-mutagenized the cloned cDNA of the G protein. Their results show that retention of either oligosaccharide site permits the G protein to reach the cell surface at 37°C, but when both sites are removed, a condition analogous to treatment with tunicamycin, the protein appears in a Golgi-like compartment of the cell and does not reach the cell surface.

The role of oligosaccharides in determining the proper localization and function of the G protein has also been analyzed using cell mutants and drugs that affect the processing pathways. The importance of the structure of the oligosaccharides on G to the formation of VSV was first seen in a mutant cell line which transfers $Glc_3Man_5GlcNAc_2$ instead of the normal precursor to nascent polypeptides (Gibson *et al.*, 1981). The yield of VSV is temperature sensitive in this mutant and VSV (San Juan) is more temperature sensitive than VSV (Orsay). Recently, the drugs 1-deoxynojirimycin and castanospermine were found to inhibit the glucosidases which act at the initial stages of oligosaccharide processing (Saunier *et al.*, 1982; Saul *et al.*, 1983; Pan *et al.*, 1983). To determine if these early processing events and some of the following steps in oligosaccharide processing are critical for certain proteins, the yields of VSV (San Juan) and VSV (Orsay) in the

presence of these drugs and in cell variants altered in the processing pathway were measured (Schlesinger *et al.*, 1984). These studies show that a block in the removal of the glucose residues by 1-deoxynojirimycin, by castanospermine, or by growth in a cell mutant lacking glucosidase II inhibits the yields of VSV (San Juan), but not of VSV (Orsay), at 40°C. Inhibition of later oligosaccharide processing steps has no effect on virus yield. In these experiments, the G protein reaches the cell surface, indicating that the alteration in the G protein is insufficient to prevent migration to the cell surface, but is significant enough to prevent virion formation. The finding that it is only the initial processing of the oligosaccharide that affects the formation of VSV suggests that the structure of the oligosaccharide plays an important role in the folding of the polypeptide chain. By the time the glucose residues have been removed, the polypeptide has achieved a conformation that is no longer influenced by the oligosaccharide structure. The effect of 1-deoxynojirimycin on a number of other glycoproteins is consistent with this conclusion, since some glycoproteins such as the hemagglutinin of influenza virus and IgM are not affected by this drug (Burke *et al.*, 1984; Peyrieras *et al.*, 1983), while other glycoproteins, for example IgD (Peyrieras *et al.*, 1983), acetylcholine receptor (Smith *et al.*, 1986), and α1-proteinase (Gross *et al.*, 1983), are affected.

2. Receptor Binding and Fusogenic Activity

The oligosaccharide chains play little or no role in binding of virus to cells since virions with no carbohydrate on the G protein are infectious (Gibson *et al.*, 1978). Potential sites on G required for attachment to cells have not been identified, although Schlegel *et al.* (1983) suggest that phosphatidylserine is a receptor for VSV.

The G protein itself is a fusogen under appropriate conditions. The cell surface expression of G protein from cloned cDNA is sufficient to cause cell–cell fusion if the cells are subjected to a brief treatment at acid pH (Riedel *et al.*, 1984; Florkiewicz and Rose, 1984). The effect of low pH on the conformation of G has not been examined in detail, but a reversible conformation change in the cathepsin D-treated, solubilized form of G is observed when the protein is acidified to pH 5 (Crimmins *et al.*, 1983). Schlegel and Wade (1984) found that a 25-amino acid peptide corresponding to the amino terminus of the G protein is a pH-dependent hemolysin. Antibodies prepared against this peptide are nonneutralizing, but react with denatured protein. This result suggests that the amino terminus is buried in the native protein and may become exposed upon acidification. Further studies by Schlegel and Wade (1985) with smaller peptides identified the six amino-terminal

amino acids as the hemolytic domain, a result supported by the observation that a single amino acid change of a lysine to glutamic acid at the amino terminus abolished the peptide's hemolytic activity. However, when the amino terminus of the G is changed by site-directed mutagenesis of the G cDNA to give the same amino terminus as the inactive peptide, the intact G protein retains its pH-dependent fusion activity (Woodgett and Rose, 1986). Thus, the domain in intact G responsible for fusion has yet to be identified.

3. Receptor Binding Activity of Rabies Virus

Rabies virus invades neuronal cells as a result of retrograde axonal transport along peripheral nerves to the spinal chord and eventually to the brain. The first suggestions that acetylcholine receptors might act as receptors for rabies virus came from the observation that rabies virus was distributed on mouse diaphragms and cultured chick myotubes coincident with the receptor (Lentz *et al.*, 1982). Furthermore, binding of virus to these tissues was prevented by α-bungarotoxin, an irreversible inhibitor of the nicotinic acetylcholine receptor. Lentz *et al.* (1984) found a significant degree of homology between the sequences of neurotoxins and the rabies glycoprotein. There is a 50% identity between residues 189 to 214 of the glycoprotein and alignment positions 30 to 56 of the neurotoxins. These findings raise the possibility that the neuronal cell tropism and resulting pathogenesis of rabies virus may be due to the affinity of the viral glycoprotein for the acetylcholine receptor. Lentz (1985) pointed out, however, that the acetylcholine receptor is not the only receptor for rabies virus since cells lacking this receptor are susceptible to the virus. The similarity between a domain of the rabies glycoprotein and that of neurotoxins may reflect an evolutionary relatedness between this viral protein and a cellular protein.

B. Transmembrane Domain

Most transmembrane glycoproteins contain a stretch of about 20 hydrophobic amino acids which span the bilayer in the form of an α-helix. The 20 amino acids of the G protein that span the membrane can be identified by inspection of the carboxy-terminal sequence (Kyte and Doolittle, 1982). They have also been defined by several types of experiments. Protease digestion of microsomes prepared from VSV-infected cells removes only 20–30 amino acids from the carboxy terminus, presumably because the rest of the protein is buried inside the microsomes (Katz *et al.*, 1977; Chatis and Morrison, 1979). In contrast, protease treatment of intact virions protects a carboxy-terminal frag-

ment which includes the cytoplasmic tail and the adjacent hydrophobic sequences (Rose *et al.*, 1980). Furthermore, there is a naturally occurring form of G (Gs) lacking both the membrane-spanning and carboxy-terminal regions, which is secreted from infected cells (Kang and Previc, 1970; Little and Huang, 1978). A similar form of G has been constructed by making a specific deletion in the cDNA clone expressing the G protein, and this form of G is also secreted from cells (Rose and Bergmann, 1982).

To determine the actual requirements for spanning the lipid bilayer, Adams and Rose (1985a,b) analyzed two types of alterations in this region of G protein. They generated specific deletions and also changed a specific amino acid. In those examples in which the transmembrane region is shortened, G proteins containing 18, 16, or 14 amino acids are still able to be transported to the cell surface. When this region contains only 12 or 8 amino acids the G protein still spans the bilayer but it is transported only to Golgi-like regions. A mutant in which the transmembrane sequence is deleted is only detected in the cell in the ER and behaves much the same as the form of G protein lacking both the transmembrane and cytoplasmic domains. Secretion of the former, however, has a half-time of about 12 hours which is much slower than that of the latter which is about 2–4 hours (Rose and Bergmann, 1982). Adams and Rose (1985b) also altered the membrane-spanning domain by replacing an isoleucine with either glutamine or arginine. The substitution of glutamine for isoleucine has no effect on membrane anchoring or localization to the cell surface. When arginine is the substituted amino acid, however, the protein still spans the membrane but is transported poorly to the cell surface.

Another approach to examining domains of a protein is to produce chimeric proteins, either exchanging a segment of one protein for another or adding a segment from one protein to another (Riedel, 1985). The exchange of domains between VSV G protein and influenza HA produced polypeptides that are not transported to the cell surface (McQueen *et al.*, 1984), but a hybrid between the HA and a retrovirus glycoprotein is transported (see Section VII). Guan and Rose (1984) fused the membrane-spanning and cytoplasmic domains of the G protein cDNA to the cDNA encoding rat growth hormone, a protein that is normally secreted from cells. The fused protein becomes membrane bound, does not reach the cell surface membrane, and appears to remain in the Golgi. Guan *et al.* (1985) then created, by site-directed mutagenesis, glycosylation sites in this protein, and showed that a single site in either of two positions or glycosylation at both positions allows the protein to be transported to the cell surface. The evidence

that the fused protein reaches the cell surface came from cell surface immunofluorescence and lactoperoxidase-catalyzed cell surface iodination. Although it was not possible to calculate the percentage of the protein that reached the cell surface, the authors did find that iodination of the singly glycosylated protein was at least 10-fold higher and that of the doubly glycosylated protein 34-fold greater than the iodination of the original, nonglycosylated protein.

One explanation for this result is that carbohydrate acts as a signal for transport to the cell surface. This interpretation, however, does not take into account the variety of results obtained by treating cells with tunicamycin to prevent the addition of carbohydrate. Although tunicamycin treatment does affect the transport of many glycoproteins to the cell surface, there are a significant number of membrane proteins that do reach the cell surface in the absence of glycosylation (Gibson *et al.*, 1980). If carbohydrate acts as a specific recognition signal, it can do so only for a subset of glycoproteins. The result described above in which two different VSV G proteins behave differently when they are not glycosylated (Gibson *et al.*, 1979) complicates any attempt to divide membrane glycoproteins into such subsets. The finding that an oligosaccharide chain at different sites on a polypeptide permits cell surface transport is difficult to interpret in the absence of a three-dimensional structure of the protein. The explanation that an oligosaccharide chain can have effects on the folding, conformation, or stability of *some* proteins provides an interpretation that encompasses all of the data so far described.

C. Cytoplasmic Domain

The cytoplasmic domain of the G protein consists of the carboxy-terminal 29 amino acids (Fig. 4). Rose and Bergmann (1983) altered this domain by creating deletion mutants and in some cases constructing plasmids such that a stretch of amino acids derived from SV40 was added to the carboxy-terminal tail. These altered sequences are shown in Fig. 4. Two criteria were used to analyze the effect that these changes had on the ability of the G protein to migrate to the cell surface. The first was the rate at which the protein oligosaccharides become resistant to endoglycosidase H (Endo H). Endo H cleaves high-mannose oligosaccharide chains but not complex oligosaccharides from the polypeptide chain. The acquisition of the complex sugars (*N*-acetylglucosamine and galactose) converts the oligosaccharide to an Endo H-resistant form and this event occurs in the Golgi apparatus; thus, the loss of Endo H sensitivity can be correlated with the move-

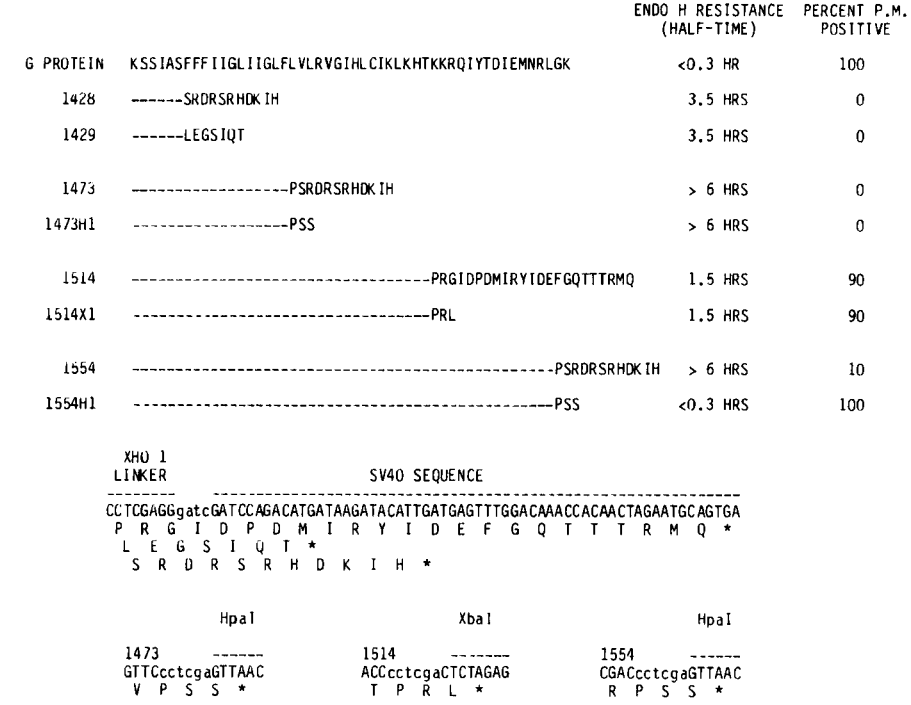

FIG. 4. The amino acid sequences of the carboxy terminus of wild-type G protein and mutationally altered derivatives. The predicted amino acid sequence of the carboxy terminus of the VSV G protein is shown on the top line with the hydrophobic transmembrane segment indicated by the first 21 amino acids. Below this are the predicted amino acid sequences of the G proteins specified by the deletion mutants, in which the dotted line indicates the presence of normal G protein sequence followed by the new amino acid residues encoded by SV40 nucleotide sequences. Deletions are numbered by the nucleotide residue in the G mRNA (Rose and Gallione, 1981) to which the deletion extends. The nucleotide sequence shown at the bottom is the vector sequence to which each of the deletions is joined via the indicated *Xho*I linker sequence. The sequence GATC at the junction is derived from filling in of the *Bam*HI site. The origin of the "extra" sequences in the deleted G proteins is illustrated by the translation of this SV40 sequence in the three possible reading frames. The predicted sequences of the carboxy termini of the deleted G proteins, which should be synthesized after insertion of *Hpa*I and *Xba*I linkers, are shown with the sequences specified by each of the parent plasmids. The nucleotide sequences at the junction of the VSV G sequence with the linker are indicated along with the predicted protein sequences for each junction. The sequence shown in lowercase resulted from filling in of the *Bam*HI site in pSVGL2. The asterisk indicates translation termination sites. The approximate half-times required for the oligosaccharides on each of the proteins to become resistant to endoglycosidase H digestion is given. The percentage of the transfected cells which showed internal G protein labeling and cell surface labeling is indicated as "percent P.M. (plasma membrane) positive." This figure is reproduced with permission of *Cell* (Rose and Bergmann, 1983).

ment of a polypeptide to the Golgi membranes. The second criterion was the percentage of transfected cells showing both internal *and* cell surface immunofluorescence.

All of the mutants require longer times to become Endo H resistant and move from the ER to the Golgi at a much slower rate than the wild-type G protein. The proteins fall into three catagories illustrated in Fig. 4. The first group (numbered 1428, 1429, and 1473), which either lack the carboxy-terminal cytoplasmic domain or have a completely different cytoplasmic domain, acquire Endo H resistance with a half-time about 10-fold longer than the wild-type G protein, and are not detected on the plasma membrane. The second, designated 1514, in which 13 amino acids following the membrane-spanning domain are retained followed by a stretch of amino acids coming from the SV40 vector, is slow to become Endo H resistant, but does reach the plasma membrane. The third category, a protein (1554) with almost all of the correct cytoplasmic domain but with an added 12 amino acids, was a surprise because in most cells it does not become Endo H resistant and appears not to reach the Golgi membranes. A derivative of this mutated protein (1554 H1) was constructed by the introduction of a translation termination codon between the G gene sequences and the SV40 sequences (Fig. 4). This mutated protein now behaves in a manner indistinguishable from the wild-type protein. Similar alterations of the proteins in the first group do not permit recovery of movement.

These studies demonstrate an important role for the cytoplasmic domain of the G protein in the proper localization of this protein. One possibility is that the cytoplasmic domain can affect the oligimerization of the protein and thus the conformation of the G polypeptide. Alternatively, there is increasing evidence that integral membrane proteins are transported between organelles via vesicles. Rose and Bergmann suggested that the cytoplasmic domain could influence the transport of G to sites in ER where membrane vesicles form or that the formation of membrane vesicles is affected by the structure of the cytoplasmic tail.

VSV G, in common with many membrane glycoproteins, contains fatty acids covalently bound to the cytoplasmic domain (see Schlesinger, 1985). Acylation occurs posttranslationally in the ER with transfer from palmitoyl-CoA (Berger and Schmidt, 1985) to a cysteine. The identification of this amino acid as the acceptor site on the polypeptide comes from a series of studies based on both the stability of the acyl–protein's linkage and site-directed mutagenesis experiments. Magee *et al.* (1983) noted that the fatty acids are removed from the protein by treatment with neutral hydroxylamine, a reaction indicative of a labile thioester bond, and the deacylated G forms disul-

fide-linked dimers. Disulfide-linked oligomers of Sindbis virus E1-E2 proteins are detected under similar deacylation conditions. Rose *et al.* (1984) used the cDNA of G to change the cysteine codon in the cytoplasmic domain of VSV (Indiana) to serine and showed that this mutated form of G was not acylated. The nonacylated G is transported to the cell surface membrane; thus, acylation is not essential for intracellular localization. Schlesinger and Malfer (1982) showed that blocking the acylation in VSV-infected cells with the antibiotic cerulenin does not inhibit G movement to the cell surface but does prevent virus budding. These data indicate that acylation has an important function but it is not clear yet what that function is. There are strains of VSV, in particular those of the New Jersey serotype, that replicate perfectly well and their G proteins are not acylated (Kotwal and Gosh, 1984). Among the differences in the sequence between the New Jersey and the Indiana serotypes of VSV G is a substitution of serine for cysteine in the cytoplasmic domain (Gallione and Rose, 1983; Rose *et al.*, 1984). Thus, fatty acids are not essential for the function of some VSV G proteins. A possible role for this modification is to block free -SH groups in the cytoplasmic domain so that aberrant covalent oligomer formation cannot occur.

V. Retrovirus Glycoproteins

Retroviruses have been isolated from a wide variety of species and show a diverse spectrum of disease potentials. The most detailed information about these viruses and their glycoproteins has been obtained for the avian, murine, and, in recent years, human retroviruses. The glycoproteins of retroviruses share many of the characteristics of those viral glycoproteins in which the amino-terminal domain is exposed on the surface (Fig. 1). The synthesis and overall structure of different retrovirus glycoproteins appear similar; the major difference is in the size of the polypeptide. Retrovirus glycoproteins are translated from a spliced subgenomic RNA (Hayward, 1977; Mellon and Duesberg, 1977; Weiss *et al.*, 1977). The primary product, synthesized on membrane-bound polyribosomes, is a glycosylated precursor of large molecular weight, 92,000 for Rous sarcoma virus (RSV) and 90,000 for murine leukemia virus (MuLV) (see Dickson *et al.*, 1982, for review). This precursor is cleaved to produce gp85 and gp37, and gp70 and p15E from the avian and murine protein, respectively. The cleaved polypeptides are covalently bound by disulfide bridges (Leamnson and Halpern, 1976). The larger amino-terminal fragments (gp85 or gp70) are heavily glycosylated; there are 14 potential glycosylation sites on gp85

of Rous sarcoma virus, all of which are thought to be glycosylated (Schwartz *et al.*, 1983; Hunter *et al.*, 1983). The smaller polypeptides (gp37 or p15E) are derived from the carboxy terminus of the precursor and comprise the membrane spanning and cytoplasmic domains of the glycoproteins. Some sequences in p15E are highly conserved among retroviruses, and the isolated p15E is reported to be immunosuppressive (Cianciolo *et al.*, 1984, 1985). It is of considerable interest that a synthetic peptide of 17 amino acids, corresponding to a highly homologous region, is able to mimic this immunosuppressive activity (Cianciolo *et al.*, 1985).

The retrovirus glycoproteins play a role in the life cycle of the virus similar to that of other viral glycoproteins discussed here. They are essential for the adsorption to and penetration of the virus into the host cell, for neutralization of infectious virus, and for the interference specificities of viruses. The major focus of this section is the glycoprotein gp85 of Rous sarcoma virus. We have also included a description of the glycoprotein of the murine spleen focus-forming virus. This glycoprotein is implicated in the pathogenicity of the virus, and the studies identifying the domain associated with the disease potential are relevant to the theme of this article.

A. Ectodomain of the Rous Sarcoma Virus Glycoprotein

A specific region in a protein may be recognized by a biological parameter before it is identified as a specific stretch of amino acids. In this case, to make the correlation between a biological activity and an amino acid sequence it is essential to determine if the "region" is a contiguous stretch of amino acids. An example of a biological property of RSV that is associated with specific regions of amino acids in gp85 is the host range specificity of this virus. Rous sarcoma viruses have been classified into five subgroups based on their ability to infect genetically defined chicken cells (Weiss, 1982). There are at least three autosomal loci in chickens that encode susceptibility determinants for the three RSV subgroups A, B, and C. These loci are thought to encode specific virus receptors.

The conclusion that gp85 determines the host range specificity of RSV stems from the finding that viruses with a defect in the *env* gene assume the host range of the helper virus (Weiss, 1982). T_1-resistant oligonucleotide mapping of the *env* gene initially defined a region of the gene that segregated with a particular subgroup phenotype (Coffin *et al.*, 1978; Joho *et al.*, 1975). Based on this initial finding Doerner *et al.* (1985) determined the sequence of the *env* gene encoding amino acids 8 through 280 of gp85 from a RSV of subgroup B and Rous-

associated virus (subgroup E), and compared the deduced amino acid sequence with those of RSV, subgroup C. Two variable regions termed hr1 and hr2 were defined based on the decreased sequence homology in these regions. The region hr1 consists of 32 amino acids beginning with amino acid 137 and corresponding to nucleotide 5654 in the Prague-RSV-C genome. The hr2 region begins at amino acid 207, corresponding to nucleotide 5846, and extends for a total of 27 amino acids.

A further test of the significance of these variable regions was made by sequencing the relevant region of the *env* gene of NTRE-4, a recombinant virus between Prague-RSV, subgroup B and RAV-0. This recombinant recognizes both the subgroup B receptor on chicken cells and the subgroup E receptor found on turkey cells. The sequence analysis shows that the region hr1 in NTRE-4 comes from the subgroup B genome, but the hr2 region comes from subgroup E. Thus, both regions appear to be involved in the determination of host range.

A further analysis of the sites in gp85 involved in host range determinants was made by Bova *et al.* (1986). They sequenced molecular clones of the *env* gene derived from a subgroup A and a subgroup B virus. Of the four variable regions they describe, two of them, VR-2 and VR-3, correspond to hr1 and hr2, respectively. To establish the role of these variable regions in host range determination Bova *et al.* produced recombinant viruses by substituting a fragment of the gp85 sequence from either RAV-2 (subgroup B) or RAV-0 (subgroup E) for the equivalent fragment in the cDNA clone for the subgroup A genome. These hybrid cDNAs were transfected into susceptible cells to produce virus stocks. These molecularly cloned viruses display the host range expected for the particular cDNA fragment inserted.

B. Cytoplasmic domain of the Rous Sarcoma Virus Glycoprotein

Hunter and colleagues have analyzed the effects of deletions and substitutions in the carboxy-terminal domain on the transport and subcellular localization of the RSV glycoprotein (Wills *et al.*, 1984). The *env* gene of RSV was inserted into an SV40 expression vector and the effects of mutations on the viral glycoprotein were analyzed in CV-1 cells. The rate of transport of the viral glycoprotein to the Golgi cisternae and to the cell surface were not affected by alterations in the five amino acids at the carboxy terminus. Changing the composition of these amino acids and lengthening the tail had no effect. Removal of 15 amino acids from the carboxy terminus and addition of 4 unrelated amino acids did slow the rate of movement to the Golgi apparatus but did not inhibit the ultimate transport to the cell surface. Finally, re-

moval of both the cytoplasmic and transmembrane domains blocked transport and the truncated protein was not secreted.

It may be relevant to these findings that the 22 amino acids of the cytoplasmic domain can be subdivided into an 18-amino acid segment that is highly conserved among strains of RSV and the most carboxy-terminal amino acids which show wide divergence (Hughes, 1982; Hunter *et al.*, 1983). These data with the RSV glycoprotein show a pattern similar to that found for the other viral glycoproteins, namely that only some of the amino acid changes made in this region prevent the glycoprotein from reaching the plasma membrane.

C. Domains of the Glycoprotein of Spleen Focus-Forming Virus

Spleen focus-forming virus (SFFV) is a complex of a competent helper murine leukemia virus and a defective virus. The complex is responsible for causing an erythroleukemia in mice and it is the defective genome that is the causal component of the disease (see Ruscetti and Wolff, 1984, for a review). There have been several independent isolations of the SFFV complex; the first, F-SFFV, was described by Friend in 1957 and the second, R-SFFV, by Rauscher in 1962.

The defective component of the SFFV complexes has been biologically cloned free of helper virus, making it possible to analyze the defective genomes in the absence of helper virus. The defective genomes of F-SFFV and R-SFFV contain different amounts of the retrovirus genome, and code, to different extents, for viral-specific proteins. The critical part of the genome, however, is that region coding for the envelope glycoprotein. The glycoproteins coded by F-SFFV and R-SFFV have apparent M_r values of 54,000 (gp54) and 55,000 (gp55), respectively. These proteins are associated with the disease potential of SFFV.

The *env* gene of the defective SFFV is a recombinant containing sequences from murine leukemia virus and from mink-cell focus-inducing (MCF) virus (Troxler *et al.*, 1977a,b). MCF viruses are able to grow in both mouse and heterologous cells (Teich, 1982). They are, themselves, recombinants between ecotropic and xenotropic murine leukemia virus and can be distinguished by several criteria including the presence of specific antigens (Cloyd *et al.*, 1979).

Studies from several laboratories (reviewed in Ruscetti and Wolff, 1984) on both the defective genomes of SFFV and the glycoprotein coded by the genomes demonstrate that the 3′ terminus (the carboxy-terminal domain of the protein) is derived from the murine leukemia virus genome, whereas the 5′ sequences (the amino-terminal domain) are derived from the MCF genome.

A more detailed and exact comparison between the defective *env* gene of SFFV and the *env* gene of murine leukemia virus followed the cloning and sequencing of these genes (Amanuma *et al.*, 1983; Wolff *et al.*, 1983; Clark and Mak, 1983). The defective genomes have a large deletion spanning the gp70–p15E junction site (Fig. 5). In addition there is a single base insertion in the p15E domain of both gp54 and gp55 that shifts the reading frame leading not only to a different set of terminal amino acids, but also to a shorter carboxy terminus on p15E. Although the exact position of the insertion and the particular base inserted are not always the same, all of the SFFV *env* genes so far examined have this insertion, implying that it may be crucial to the expression of the SFFV phenotype.

The premature termination leads to the lack of any significant cytoplasmic domain. How this affects the transport of this particular glycoprotein is not known, but as discussed earlier, alterations in the cytoplasmic tail can affect the movement of a glycoprotein through the intracellular membrane compartments. Furthermore, only a small

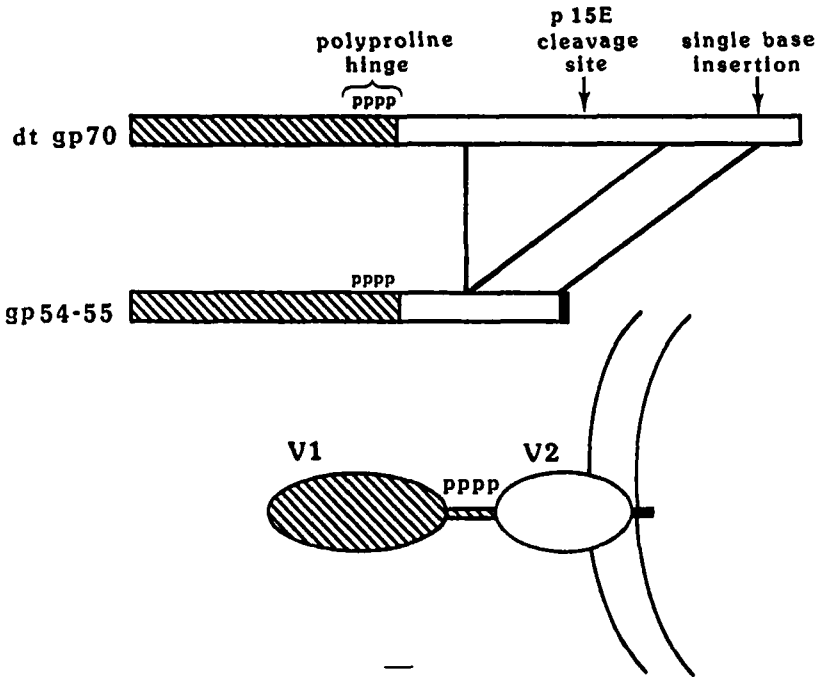

FIG. 5. A model showing the origin and domains of the gp54-55 of SFFV. The dualtropic (dt) gp70 from which the SFFV glycoprotein is derived is shown above. V1 represents xenotropic sequences; V2 is derived from ecotropic sequences. This figure is reproduced by permission of Dr. David Kabat.

fraction of the SFFV defective glycoprotein does reach the cell surface, indicating that there is a defect in intracellular transport (Srinivas and Compans, 1983). Recent studies of Pinter and Honnen (1985) show that a highly processed form of the glycoprotein of F-SFFV, termed gp65, is secreted from cells.

The structure of the glycoprotein of the SFFV proposed by Kabat and colleagues is shown in Fig. 5. Machida *et al.* (1985) presented evidence that there are two independent domains, V-1 and V-2, joined by a proline-rich stretch. Strong support for this model comes from their finding that *Staphylococcus aureus* V8 protease cleaves the SFFV glycoprotein to yield the amino-terminal V-1 fragment and the carboxy-terminal V-2 fragment. The cleavage occurs at Glu-238, located within the proline-rich region. This region also marks the division between the ecotropic sequences (V-2) and the xenotropic sequences (V-1). The position at which the amino acid sequences of the glycoprotein become strongly homologous to murine leukemia viral glycoprotein sequences occurs exactly at the end of the proline-rich stretch. There are several other results supporting the model that V-1 and V-2 are independent domains. Thus, disulfide bonds are only detected within the V-1 domain and are not found between domains; mutations in one domain appear to affect the protease sensitivity of that domain but not of the other domain.

The analyses of nonleukemogenic mutants of SFFV and revertants of these mutants have provided an important tool in developing a model for the structure of the glycoprotein and for establishing the role of the defective glycoprotein in the pathogenesis of SFFV. The *env* genes of two nonleukemogenic mutants were molecularly cloned and sequenced, as was one leukemogenic revertant (Li *et al.*, 1986). One mutant contains three noncontiguous point mutations that cause nonconservative amino acid changes in the V-1 domain. It is not clear if all three changes are required for the loss in pathogenicity. A second mutant contains a point mutation leading to an early termination codon at amino acid residue 304. This mutation occurs immediately after the proposed xenotropic–ecotropic recombination site and therefore eliminates the V-2 domain including the membrane anchor. The leukemogenic revertant regains the wild-type sequence at this site. These results establish that a point mutation in the *env* gene can lead to the loss in leukemogenic potential of SFFV.

env gene mutants were also constructed by the insertion of small in-phase *Hpa*I or *Xho*I linkers into different restriction sites in the cloned F-SFFV proviral DNA (J.-P. Li and D. Kabat, personal communication). Three mutants with insertions in V-1, the xenotropic region, are no longer pathogenic, although one mutant with an insertion in this

region retains activity. Two mutants with insertions in V-2, the ecotropic region, and one mutant with an insertion in the proline-rich region retain their pathogenicity.

Li and Kabat (personal communication) found that glycoproteins coded by pathogenic F-SFFV are expressed on the cell surface but the glycoprotein of nonpathogenic mutants remains intracellular. They also reported a correlation between F-SFFV pathogenicity and the ability to cause a weak interference to superinfection by dualtropic murine leukemia viruses. The latter result suggests that the SFFV glycoprotein which can cause leukemia is also able to interact with viral receptors. These authors suggested that the xenotropic domain of the SFFV membrane glycoprotein binds to these receptors and causes erythroblastosis. They proposed that these receptors normally function as receptors for a hematopoietic growth factor and the SFFV glycoprotein causes its constitutive activation.

VI. Other Virus Glycoproteins

There are of course many more virus glycoproteins than those described above. Most of these have structural and functional domains which are similar to those we have described; however, there are others which are quite distinct in structure and membrane orientation. Some of these are noted below.

A. Influenza Virus Neuraminidase

Four distinct domains can be identified in the subunits of the enzymatically active tetramer of influenza virus neuraminidase (NA). One of these, the cytoplasmic domain, is unusual among transmembrane glycoproteins in that it is the precise amino terminus of the polypeptide chain and consists of only six amino acids (Blok and Air, 1982; Blok *et al.*, 1982). These residues are highly conserved and invariant among nine serologically distinct subtypes. It has been postulated that this domain, in common with other glycoprotein cytoplasmic sequences, interacts with viral components associated with the virus core or with a virus matrix protein.

A second domain consists of a nonconserved sequence of ~28 hydrophobic amino acids which probably serves as both a signal peptide and a membrane anchor. Most of the NA structure extends outward from the virion's surface and is composed of two domains: a short helical stalk near the membrane and a large globular hydrophilic head distal to the surface of the virus. Multiple disulfide bridging occurs within

NA subunits and the typical NA has four sites to which N-linked oligosaccharides are bound. Two glycosylation sites are in the stalk region and are possibly involved in interchain contacts of the tetramer; another site is found near the surface at a region close to a subunit interface. No proteolytic modifications are known for NA.

The catalytic site of this protein is near the surface in the globular head domain. X-Ray analysis of crystals of NA released from membranes by pronase cleavage at amino acid residues 74–77 in the stalk permitted assignments of specific amino acids in the catalytic site (Varghese *et al.*, 1983; Colman *et al.*, 1983). Sialic acid is the product of NA catalysis and binds in a large pocket on the surface. Nine acidic, six basic, and three hydrophobic amino acids surround this pocket and all are conserved in sequence among all NA serotypes. Amino acid substitutions at positions adjacent to several of the invariant residues, however, are found in serologically distinct subtypes arising from antigenic drift. For example, Asp-151 and -152 are invariant but residue 153 varies among subtypes. From the structure, the conserved amino acids face inward toward the pocket and the variable site is oriented outward toward the surface, a geometry in accord with the experimental data. Thus, NA antigenicity, which is known to vary among the virus isolates from different flu epidemics, can be modified in the absence of effects on catalysis.

Studies with cDNAs encoding NA have yielded additional information about the NA domains. Substitution of leucine for tryptophan at position 178 in the catalytic pocket destroyed enzymatic activity (M. R. Lentz and Air, 1986). Deletions in the membrane anchor domain significantly affected the translocation and glycosylation of nascent polypeptide (Markoff *et al.*, 1984).

It is not completely clear what role NA plays in virus replication but its enzymatic activity will remove host cell receptors, thereby allowing elution of progeny virus from infected cells and, as well, preventing self-aggregation of virions. NA allows also for enhanced mobility of virus through mucin encountered in normal routes of infection in nature.

B. Paramyxoviruses

Another interesting variation in virus glycoprotein function is the presence of both a neuraminidase and hemagglutination activity in a single glycoprotein (HN) of the paramyxoviridae family with the fusion activity in a separate glycoprotein (F). In contrast, as noted earlier, the influenza virus hemagglutinin contains the virus fusion activity while the neuraminidase is in a separate glycoprotein. In

common with influenza virus neuraminidase, the Simian virus 5 (Hiebert *et al.*, 1985) and Sendai virus (Blumberg *et al.*, 1985) HNs have amino termini in the cytoplasmic part of the bilayer.

The paramyxovirus F glycoprotein has a fusion region closely resembling that of the influenza HA (see above), and amino acid sequences share high degrees of homology to the hydrophobic amino terminal sequences of the HA2 subunit. The F proteins, however, can act as fusogens in the absence of a low pH environment. All of these F proteins are similar to influenza HA in that they must be proteolytically cleaved in order to activate the fusion site. These F proteins are oriented with their amino terminus on the outside of the virus membrane and carboxy terminus inside. In contrast, the nonfusogenic glycoproteins of these paramyxoviruses (for example, the G of respiratory syncytial virus) have the amino terminus inside and the carboxy terminus outside (Wertz *et al.*, 1985). This distinction in orientation between the fusion protein and the G/HN proteins has been proposed to explain differences in transport rates from the ER to the cell surface, with F proteins (carboxy-terminal anchors) moving much more rapidly than HN/G (amino-terminal anchor) (Blumberg *et al.*, 1985).

The sequence of the major glycoprotein (G) of the respiratory syncytial virus has recently been derived from a cDNA clone (Wertz *et al.*, 1985) and shows a very high content of serine and threonine (30.6% of the total amino acid composition). This is a characteristic of glycoproteins that have carbohydrate linked via *O*-glycosidic bonds, and indirect evidence based on studies with tunicamycin indicates that this protein is extensively glycosylated on hydroxyamino acids. No amino-terminal signal sequence exists nor is there a hydrophobic membrane anchor domain near the carboxy terminus. These results have led to the suggestion that this G protein has its amino terminus, consisting of about 38 amino acids, in the cytoplasm. A hydrophobic sequence from residues 38 to 66 would serve as a signal sequence and membrane-spanning domain, and the balance of the 232 amino acids would constitute an ectodomain. This portion of the polypeptide has 77 of the 91 hydroxyamino acids which are believed to be sites for glycosylation. The protein also has an unusually high content of prolines (10.1%).

C. Coronaviruses

There are two membrane-associated glycoproteins (E1 and E2) in the virions of the Coronaviridae family. One of these, the E1 of mouse hepatitis virus, resembles the G protein of respiratory syncytial virus, discussed above, in that there are a number of O-linked sugars on the

protein (Sturman and Holmes, 1983). Coronavirus E1 appears to be tightly membrane associated and its derived amino acid sequence shows a very hydrophobic region near the amino terminus. A postulated topological distribution of this protein in the membrane shows the amino terminus in the vesicle lumen (outside of the virion), a looping of sequences across the membrane several times, and a long hydrophilic carboxy-terminal region at the cytoplasmic face affixed to the polar groups of the bilayer (Rottier *et al.*, 1986). The other coronavirus membrane glycoprotein, noted E2 or S, is more conventional in its structure; there is an amino-terminal signal sequence and a carboxy-terminal hydrophobic membrane anchor domain. The protein is posttranslationally cleaved from a molecule of 180 kDa to two equivalent-sized subunits of 90 kDa. The carboxy-terminal subunit (90A) has a fusogenic activity that is enhanced after the proteolytic cleavage. Unlike the ortho- and paramyxoviruses, the fusogenic region appears not to be localized to the region of the polypeptide cleavage since the amino acids around this site are not hydrophobic (L. S. Sturman, personal communication). The most likely region for a fusion site is some 200 amino acids from the amino-terminal side of the cleavage site, where there is an extended sequence of hydrophobic amino acids. Another unusual feature of the coronavirus E2 is a clustering of cysteines in the cytoplasmic domain. This sequence contains a tricysteine and two dicysteines; a somewhat similar arrangement is found also in the cytoplasmic tails of the E2 glycoprotein of alphaviruses.

D. Herpesviruses

Relatively little information is currently available about domains of glycoproteins encoded by the herpesviruses, but the genetic analyses of these proteins is now in progress and we can expect considerable more data in the near future. At least four glycoproteins (gB, gC, gD, gE) are encoded in the herpesvirus genome (reviewed by Spear, 1985). One of these, gB, is essential for virion growth and mutations in this protein affect rate of virus entry. This protein appears to have a fusion activity, defined by sites in the genome encoding gB (Bzik *et al.*, 1984). The gC and gE are nonessential for virus growth in tissue culture and do not appear in virions. Mutants which are truncated at the carboxy terminus of gC have been obtained and the altered polypeptide is secreted into the medium, indicating a membrane anchor domain exists at the carboxy terminus of gC (Homa *et al.*, 1986). The herpes gC may have a receptor for the C3b component of complement (Friedman *et al.*, 1984), and gE has a site which binds the Fc portion of the immunoglobulin (Baucke and Spear, 1979; Para *et al.*, 1982). These

proteins are probably made by the virus to thwart the host immune system.

E. Nonenveloped Viruses

The presence of nonessential virus genes coding for glycoproteins is not limited to the herpesviruses. Adenovirus type 2 carries within its genome sequences for a 19-kDa glycoprotein which never appears in the virion and is nonessential for virus growth in tissue culture. This glycoprotein is believed to assist the virus in growth in animals since it is found in a complex with the HLA and β_2-microglobulin in a manner that inhibits transport of the HLA to the cell surface (Burgert and Kvist, 1985). Without sufficient HLA molecules on an infected cell surface, cytotoxic T cells are unable to bind and destroy the infected cell.

Another role for a virus-coded glycoprotein is found in the Reoviridae family whose members do not have lipid bilayers or spikes in the virions. One member of this family, the human rotavirus, produces a glycoprotein which participates in the assembly and secretion of virus into intracellular organelles. Later, the lipid suface is removed from the virion. Expression of the cDNA of this virus glycoprotein has been studied, and deletions of one of its two putative hydrophobic domains altered the location of this glycoprotein in the cell membrane (Poruchynsky *et al.*, 1985). The normal protein remains in the ER where the virus buds and transiently contains a lipid envelope. The protein carrying the deletions was transported from ER to Golgi and secreted. The authors also tested glycoproteins with deletions in the other hydrophobic domain and found no change in location. Thus, one of the hydrophobic domains of the glycoprotein is essential for specifying localization to the ER membrane.

VII. Virus Glycoproteins and Polarized Cells

An important feature of enveloped virus glycoproteins is their apparent ability to determine which cellular membrane is used for virus assembly. We have alluded to this property indirectly in the earlier discussions of the structure and posttranslational processing of these proteins, but there is more direct evidence for this role of the glycoprotein in the virus-infected polarized epithelial cell. Rodriguez-Boulan and Sabatini (1978) first noted a specific distinction in the localization of virus budding between the apical and basal lateral membranes of this kind of cell. They found that influenza virus buds only from the

apical surface whereas VSV is secreted from the basal lateral membrane. More recent experiments confirm this specific sorting of other enveloped viruses: paramyxoviruses are found at the apical surface and retroviruses and herpesviruses move to the basal lateral surface (Roth *et al.*, 1983; Srinivas *et al.*, 1986). It is the glycoproteins of these viruses that determine which membrane of the cell is utilized, and there have been intensive efforts to determine what properties of these proteins specify sorting (reviewed by Simons and Fuller, 1986). Sorting is not dependent on glycosylation pattern or sialic acid, and chimeric recombinant molecules have been constructed and expressed from cDNAs to determine which domains of the protein control sorting. A chimeric molecule composed of an influenza HA as the luminal portion and a VSV G as the transmembrane and cytoplasmic portion localizes according to that part determined by the luminal domain. That is, the chimeric protein moves to the apical surface (McQueen *et al.*, 1986). This result implicates the "ectodomain" as the determinant for sorting. However, a conflicting set of data were found with "recombinant" molecules containing the ectodomain of the Friend mink cell focus-inducing retrovirus but lacking the normal transmembranal and cytoplasmic domains. In this case the protein sorts to both the basal lateral and apical surfaces of the cell where it is secreted (Stephens and Compans, 1986). Thus, the signal for sorting in the polarized cell remains unknown.

VIII. Summary and Perspectives

The primary sequences of many viral membrane glycoproteins are now known. Based on inspection of their sequence most of these proteins can be divided into the three major domains described in Fig. 1. These domains have been defined with respect to their orientation in the lipid bilayer, but a complete description of a domain should also include its quaternary structure and function. This is possible, however, only for the HA and NA of influenza virus. X-Ray crystallographic studies provide us with a picture of what the ectodomain of these proteins looks like and permit a specific function to be correlated with a definitive structure. The ectodomains of viral glycoproteins are responsible for several important functions; receptor binding, fusogenic activity, and disease potential are among those discussed here. Although in some cases these functions can be associated with specific sequences of a protein, the crystallographic data will be essential to complete the picture.

One focus of this article has been the studies involving directed

mutagenesis and construction of chimeric proteins. The effects of altering specific amino acid sequences, of swapping domains, and of adding a new domain to a protein serve to define the functions of a domain and to show that a domain can be independently associated with a specific function. The experiments described have been carried out by inserting the genes of particular viral glycoproteins, as cDNAs, into expression vectors and transcribing the cDNAs from the promoter provided by the expression vector. This approach established that localization and functions such as the fusogenic activity are properties of the viral glycoprotein per se and do not require other viral-coded components. The altered proteins have been analyzed for their ability to reach the appropriate location in the cell and to undergo the expected posttranslational modifications. Viral glycoproteins must also be able to participate in the assembly of infectious virions, and some of the changes, particularly those in the transmembrane and cytoplasmic domains that do not affect localization, may not permit critical interactions needed for the budding of particles. Now that we have accumulated some details about the requirements for transport, future work should be directed toward the requirements for virion assembly.

We have concentrated most of our discussion on those viral glycoproteins that (1) span the lipid bilayer once, (2) are oriented such that the carboxy terminus comprises the cytoplasmic domain and (3) contain asparagine-linked oligosaccharides. An increasing number of viral glycoproteins that don't conform to this description are now undergoing scrutiny. They include proteins such as the E1 of coronavirus that may span the membrane more than once, and those oriented with the amino terminus in the cytoplasmic domain. There are also viral glycoproteins with extensive O-linked glycosylation, some of which have been noted here.

Viral glycoproteins have served as important models for cellular membrane glycoproteins that localize to the outer surface of the plasma membrane. Not all viral glycoproteins move to the cell surface and some remain in internal membranes at sites of virion assembly. These distinctions among viral glycoproteins may reflect the diversity of cellular membrane glycoproteins; therefore, more detailed knowledge of different viral glycoproteins should provide valuable models for the spectrum of cellular glycoproteins. Further analyses of these viral glycoproteins will also surely contribute to our understanding of virion assembly and pathogenesis.

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