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Biologically Active Peptides of the Vesicular Stomatitis Virus Glycoprotein

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A peptide corresponding to the amino-terminal 25 amino acids of the mature vesicular stomatitis virus glycoprotein has recently been shown to be a pH-dependent hemolysin. In the present study, we analyzed smaller constituent peptides and found that the hemolytic domain resides within the six amino-terminal amino acids. Synthesis of variant peptides indicates that the amino-terminal lysine can be replaced by another positively charged amino acid (arginine) but that substitution with glutamic acid results in the total loss of the hemolytic function. Peptide-induced hemolysis was dependent upon buffer conditions and was inhibited when isotonicity was maintained with mannitol, sucrose, or raffinose. In sucrose, all hemolytic peptides were also observed to mediate hemagglutination. The large 25-amino acid peptide is also a pH-dependent cytotoxin for mammalian cells and appears to effect gross changes in cell permeability. Conservation of the amino terminus of vesicular stomatitis virus and rabies virus suggests that the membrane-destabilizing properties of this domain may be important for glycoprotein function.

Vesicular stomatitis virus (VSV) infects host cells via adsorptive endocytosis (3, 4, 6, 10, 12, 15, 17). The first phase of this process, viral attachment, represents the interaction of VSV glycoproteins with a limited number (4,000) of saturable "receptors" as well as nonsaturatable binding sites (16). Rabies virus, a related rhabdovirus, binds to the same saturable cell surface receptor (19), and the specific binding of both VSV and rabies virus appears to reflect interactions with membrane lipids. VSV binds preferentially to the acidic phospholipid phosphatidylserine (13). After binding, VSV is rapidly translocated into plasma membrane "coated pits" and then transferred into intracellular vesicles (endosomes). It is believed that the acidification of these endosomes by an ATP-dependent proton pump is responsible for initiating the fusion of viral and cellular membranes. Several findings indicate that membrane fusion is mediated by the VSV glycoprotein (G protein): (i) mammalian cells can be fused by exogeneous VSV under acidic conditions (18); (ii) cells expressing cloned G protein are fusogenic at low pH (7, 11); and (iii) purified G protein can effect liposome membrane fusion as detected by fluorescence energy transfer and electron microscopy (5). Reflecting its function as a fusogen, G protein (incorporated in liposomes) can hemolyze erythrocytes under acidic conditions (1). Similarly, peptides corresponding to the amino terminus of G protein can also function as pH-dependent hemolysins (14). The purpose of this study was to use additional synthetic peptides to further define the hemolytic domain of G protein and to characterize the biological properties of such peptides.

Hemolysis assays were performed with sheep erythrocytes at a concentration of ca. 7×10^8 erythrocytes per ml. Total hemolysis (in distilled water) produced a spectrophotometric absorbance at 541 nm of 0.50. All hemolysis assays were performed in glass tubes in a volume of 1.0 ml as previously described (14). Due to their inherent tendency to adhere to vessel surfaces, peptides were always added last to the hemolysis assay. Peptides corresponding to the amino terminus of VSV (Indiana, San Juan strain) were synthesized by Peninsula Laboratories in San Carlos, Calif., and subjected to amino acid analysis and high-pressure liquid chromatography to assure correct amino acid composition and peptide homogeneity. Growth conditions for Vero cells have been described previously (16).

Hemolytic activities of peptides corresponding to the amino terminus of G protein. Since the synthetic peptide KFT(25) exhibits hemolytic properties similar to those of VSV and G protein (14), it was speculated that the amino terminus of G protein represents an important domain for membrane destabilization and fusion. To further delineate and define the properties of KFT(25), we synthesized several constituent and variant peptides. The composition of these peptides is shown in Fig. 1. KFT(25) represents the 25-amino acid peptide that was recently shown to be a pH-dependent hemolysin. HNQ(18) represents the carboxy-terminal 18 amino acids, and KFT(6) represents the amino-terminal 6 amino acids of KFT(25). RFT(6) and EFT(6) are modifications of KFT(6) in which the amino-terminal lysine has been changed to either arginine or glutamic acid, respectively. Finally, KFF(6) represents a prototype molecule in which the amino-terminal lysine has been retained but the hydrophobic region consists of a sequence of five phenylalanine molecules. All of the peptides shown in Fig. 1 were tested for their hemolytic activity (Fig. 2). At pH 5.0, KFT(25) showed a half-maximal response at a concentration of 40 μM. Interestingly, the first six amino acids of this peptide, KFT(6), were sufficient to act as a hemolysin. The shorter peptide, however, was more potent than the original KFT(25) and showed half-maximal hemolysis at 3 μM. In contrast to the amino-terminal KFT(6) peptide, the carboxy-terminal peptide, HNQ(18), showed no hemolytic activity.

The potent hemolytic activity of the short peptide, KFT(6), was somewhat surprising and was further characterized by a study of the properties of two additional peptides. RFT(6) and EFT(6) represent variants of KFT(6) in which the amino-terminal lysine is replaced by arginine or glutamic acid. Replacement of lysine with another basic amino acid retains a +2 charge at the amino end and produces a hemolysin which has only slightly decreased potency (half-maximal hemolysis at 20 μM). Replacement of lysine with an

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Consensus Sequence (5 strains):
Lys – – Ile Val Phe Pro – – – – Gly Asx Trp Lys – Val Pro – – Tyr – Tyr Cys

KFT (25) (Indiana San Juan):
Lys Phe Thr Ile Val Phe Pro His Asn Gln Lys Gly Asn Trp Lys Asn Val Pro Ser Asn Tyr His Tyr Cys Pro

HNQ (18)
His Asn Gln Lys Gly Asn Trp Lys Asn Val Pro Ser Asn Tyr His Tyr Cys Pro

KFT (6)
Lys Phe Thr Ile Val Phe

RFT (6)
Arg Phe Thr Ile Val Phe

EFT (6)
Glu Phe Thr Ile Val Phe

KFF (6)
Lys Phe Phe Phe Phe Phe

FIG. 1. Sequences of the synthesized peptides. The consensus sequence for the NH_2 terminus of VSV glycoprotein is shown (9). KFT(25), HNQ(18), and KFT(6) represent peptides of the glycoprotein of VSV (Indiana, San Juan strain). RFT(6), EFT(6), and KFF(6) represent modifications of KFT(6) at its amino terminus or hydrophobic tail. Conserved amino acids are underlined. Asterisks indicate strongly hydrophobic amino acids.

acidic amino acid (glutamic acid) forms a neutral amino terminus and results in the complete loss of hemolytic function. To determine whether hemolysis was a consequence of a short hydrophobic sequence coupled to a positively charged amino acid, we assayed the activity of another peptide, KFF(6), containing an amino-terminal lysine followed by five phenylalanine molecules. This constructed peptide did exhibit some hemolytic activity, but only at relatively high concentrations (half-maximal at 300 μM).

KFT(25) and KFT(6) differ in their pH dependence. Although KFT(6) probably represents the domain of KFT(25) which mediates hemolysis, these two peptides displayed quite different profiles of hemolysis when pH was varied (Fig. 3). As described previously (14), KFT(25) is most active at acidic pH values (less than 6.0). KFT(6), however, shows little variation of activity at pH values ranging from 5.0 to 7.0.

Peptide hemolytic activity is dependent upon buffer conditions. KFT(25) lyses erythrocytes quickly in buffers which are made iso-osmotic with small ionic molecules such as NaCl or RbCl (Fig. 4). However, lysis is delayed when iso-osmotic conditions are achieved with nonionic molecules such as mannitol, sucrose, or raffinose. This inhibition of hemolysis appears to result from a decrease in the rate of hemolysis since the extent of hemolysis is unaffected (data not shown). This would suggest that nonionic buffers are inhibiting the process of hemolysis rather than the binding of peptide to erythrocyte. Studies on the hemagglutination activities of these peptides also indicate that nonionic buffers do not inhibit peptide-erythrocyte binding (see below). Similar findings were also observed with KFT(6).

Hemolytic peptides are also hemagglutinins. When KFT(25) and KFT(6) were added to erythrocytes in sucrose, hemolysis was inhibited but the erythrocytes were observed to agglutinate (Fig. 5B and C). Hemagglutination could also be observed in NaCl buffers, but at pH 5.0 this event was transient and quickly progressed to hemolysis. Nonhemolytic peptides such as HNQ(18) and EFT(6) were nonhemagglutinating under all conditions tested (Fig. 5D and E). Hemagglutination by KFT(25) could be performed in either sucrose or NaCl if the pH was maintained at 7.4. Under such conditions, KFT(25) had a greater hemagglutinating activity in sucrose than in NaCl. KFT caused hemagglutination at 4 μg/ml in sucrose but required 125 μg/ml in NaCl. Hemagglutination per se is not sufficient to initiate pH-dependent hemolysis. For example, the addition of polylysine (500 and 1,000 μg/ml) to the hemolysis assay caused massive hemagglutination at pH 5.0 but no hemolysis.

Protease treatment of erythrocytes does not affect peptide-induced hemolysis. VSV G protein is believed to function as a fusogen via its interaction with membrane phospholipids

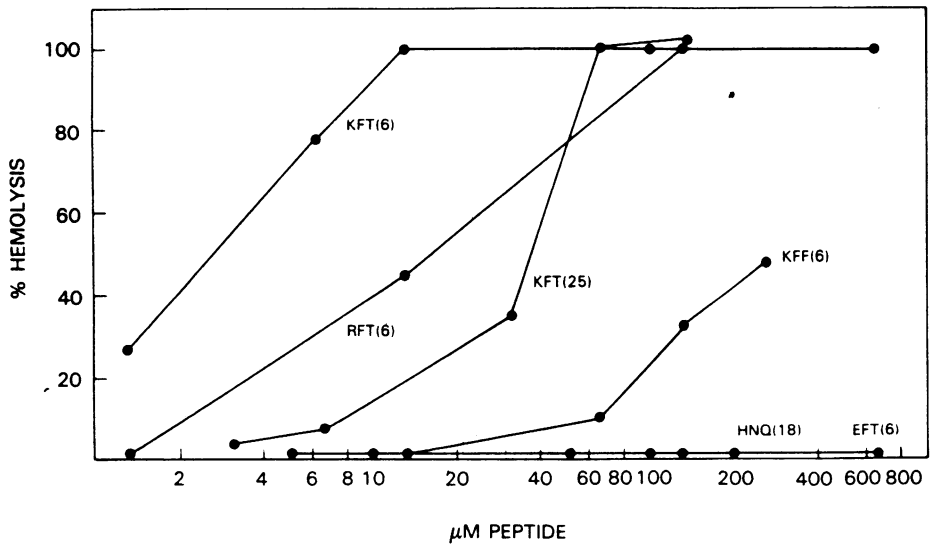

FIG. 2. Comparison of the hemolytic activities of the synthetic peptides. Peptide stock solutions (10 to 100 mM in distilled water) were diluted appropriately into the hemolysis assay, and the release of hemoglobin was measured after 10 min at 37°C. Peptides EFT(6) and KFF(6) were dissolved in dimethyl sulfoxide. Control assays with dimethyl sulfoxide showed no hemolysis. KFT(6) was the most potent hemolysin (half-maximal hemolysis at 3 μM).

(5). Although we believe that the KFT(6) and KFT(25) peptides may be reflecting a membrane-destabilizing function of G protein, there is no direct evidence that these peptides interact with lipids. As a preliminary attempt to discern whether membrane proteins of the sheep erythrocyte might be acting as the binding site for these peptides, we incubated sheep erythrocytes with proteinase K (10 to 200 μg/ml) or trypsin (10 to 200 μg/ml) for 30 min at 37°C. The erythrocytes were washed and used in the standard hemolysis assay. There was no difference in sensitivity to hemolysis between control erythrocytes and those treated with proteases (data not shown).

Hemolytic peptides are cytotoxic. To evaluate whether the hemolytic peptides could also interact with the membranes of nucleated cells, we added KFT(25) to Vero (monkey kidney) cells grown in tissue culture. The pH of the buffer was adjusted to either 7.4 or 5.5, and membrane permeability to trypan blue was assayed at different times of incubation at 37°C (Fig. 6). Cells exposed to KFT(25) at pH 5.5 showed a dose-dependent increase in permeability to trypan blue assayed after 20 min of exposure. Control cells at pH 5.5 showed no staining with trypan blue. In addition, cells exposed to KFT(25) at pH 7.4 also showed no staining. This rapid cytotoxic effect was accompanied by several discernible morphological changes (Fig. 7). Soon after 100 μM KFT(25) was added at pH 5.5, Vero cells swelled and lost their distinct plasma membrane borders. Nuclei became enlarged and cleared and nucleolar structure was obscured (Fig. 7B). However, no evidence of cell fusion was observed. Electron microscopy confirmed gross vacuolization of the cell cytoplasm, nuclear swelling, loss of nuclear matrix, but retention of the plasma membrane bilayer structure (data not shown). Vero cells at pH 5.5 (Fig. 7A) or at pH 7.4 with 100 μM KFT(25) (Fig. 7C) showed essentially normal morphology. We believe these findings are consistent with the KFT(25) peptide causing profound changes in membrane permeability.

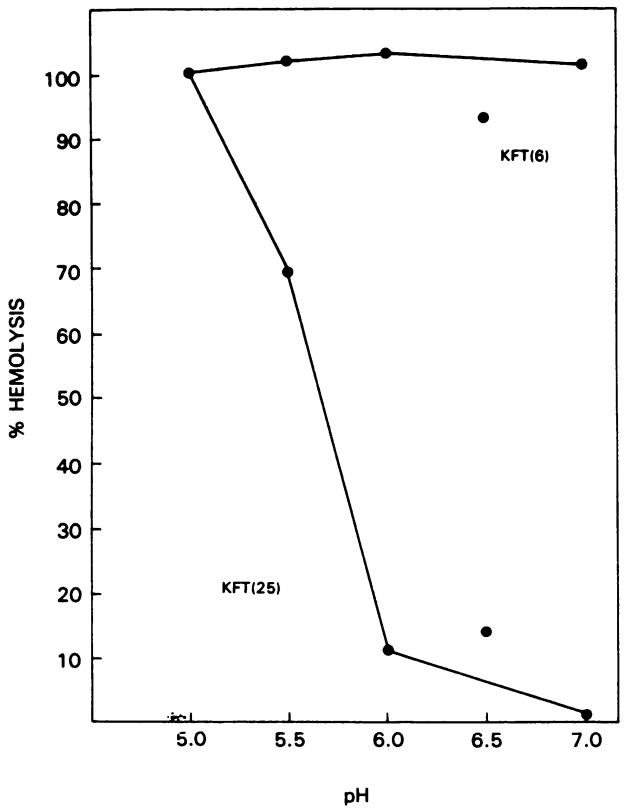

FIG. 3. pH dependence of hemolysis. Hemolysis was quantitated over the pH range 5.0 to 7.0 for the two hemolytic peptides, KFT(6) and KFT(25). Although both peptides correspond to the NH_2 terminus of VSV glycoprotein, the shorter peptide, KFT(6), exhibited little change in activity at the different pH conditions tested.

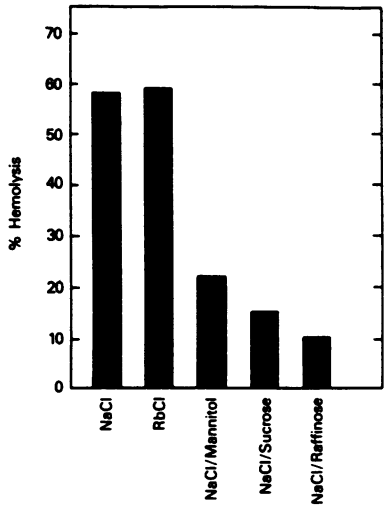

FIG. 4. Dependence of hemolysis upon buffer conditions. Hemolysis assays with half-maximal amounts of KFT(25) were performed in different iso-osmotic buffers at pH 5.0. Buffer compositions were as follows: NaCl (150 mM NaCl, 10 mM morpholineethanesulfonic acid [MES] buffer; pH 5.0); RbCl (150 mM RbCl, 10 mM MES buffer; pH 5.0); NaCl/mannitol (25 mM NaCl, 250 mM mannitol, 10 mM MES buffer; pH 5.0); NaCl/sucrose (25 mM NaCl, 250 mM sucrose, 10 mM MES buffer; pH 5.0); NaCl/raffinose (25 mM NaCl, 250 mM raffinose, 10 mM MES buffer; pH 5.0). NaCl was added to mannitol, sucrose, and raffinose buffers to maintain the ionic environment at 50 mM and to avoid inducing leakage of K^+ from erythrocytes. Identical findings were obtained with 300 mM mannitol, sucrose, and raffinose without NaCl.

The amino terminus of VSV G protein, whether presented as a 6- or a 25-amino acid peptide, can function as a hemolysin. The mechanism by which these peptides mediate hemolysis is unknown. Previous data demonstrating a lag in the initiation of hemolysis (14), coupled with data in this manuscript showing that sucrose or raffinose buffers inhibit hemolysis, are consistent with a colloid-osmotic type of hemolysis. Presumably, such a mechanism would necessitate the formation of membrane defects which would permit only small ions (for example, sodium) to enter the erythrocyte. We are currently evaluating the efflux of rubidium ions and hemoglobulin from the erythrocyte to determine whether there is a pre-lytic leak of small ions. It seems very unlikely that single, six-amino acid peptides could form "ion channels." However, these peptides might be functional in aggregate form. Certainly peptide aggregation could also explain the observed hemagglutinating properties of some of these peptides. Binding studies with the individual peptides have not yet been performed, so it is possible that some of our experimental manipulations (for example, the use of sucrose versus NaCl buffers) might reflect inhibition of peptide-erythrocyte binding rather than interference with the process of hemolysis.

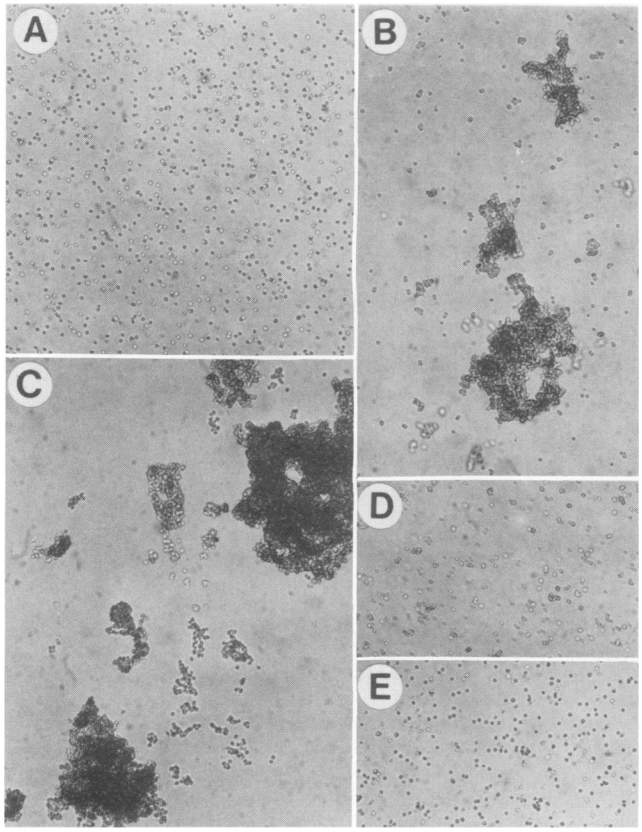

FIG. 5. Hemagglutination of sheep erythrocytes in sucrose buffer at pH 5.0. Sheep erythrocytes were mixed with different peptides according to the standard hemolysis protocol. However, the buffer was changed to NaCl/sucrose as in Fig. 4 (25 mM NaCl, 240 mM sucrose, 10 mM MES buffer; pH 5.0). Two minutes after peptide addition at 37°C, samples were photographed with a phase-contrast microscope. (A) Control erythrocytes; (B) erythrocytes plus 50 μM KFT(25); (C) erythrocytes plus 10 μM KFT(6); (D) erythrocytes plus 200 μM HNQ(18); (E) erythrocytes plus 200 μM EFT(6).

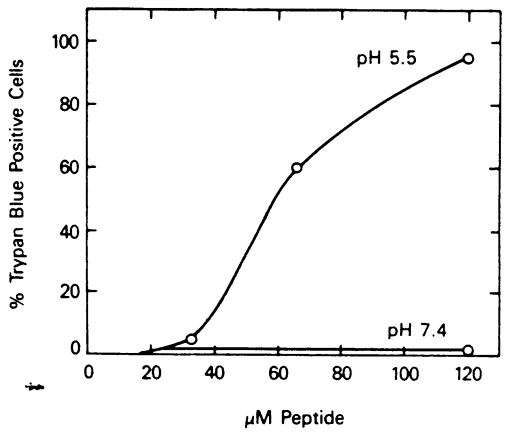

FIG. 6. Staining of mammalian cells by trypan blue after exposure to KFT(25). Vero cells were grown to confluence in 35-mm plastic dishes, washed in phosphate-buffered saline containing Mg^{2+} and Ca^{2+} (GIBCO Laboratories), and exposed to various concentrations of KFT(25) at either pH 5.5 or 7.4 (in 150 mM NaCl). After 20 min at 37°C, the cells were stained with trypan blue and 100 cells (from several high-power fields) were evaluated for staining.

The binding site for the hemolytic peptides does not appear to be protein, since various protease treatments of the cell surface were unable to inhibit hemolysis. Although lipids may serve as the "binding site" for intact VSV and the amino-terminal peptides, it is unlikely that the amino terminus of G protein is participating in the initial binding of VSV to the cell membrane since this region is relatively inaccessible to proteases (2) and antibodies (14) and therefore is probably not exposed on the surface of the G protein. Also, it is unlikely that VSV and hemolytic peptides are interacting with the same type of lipid. VSV shows preferential inter-

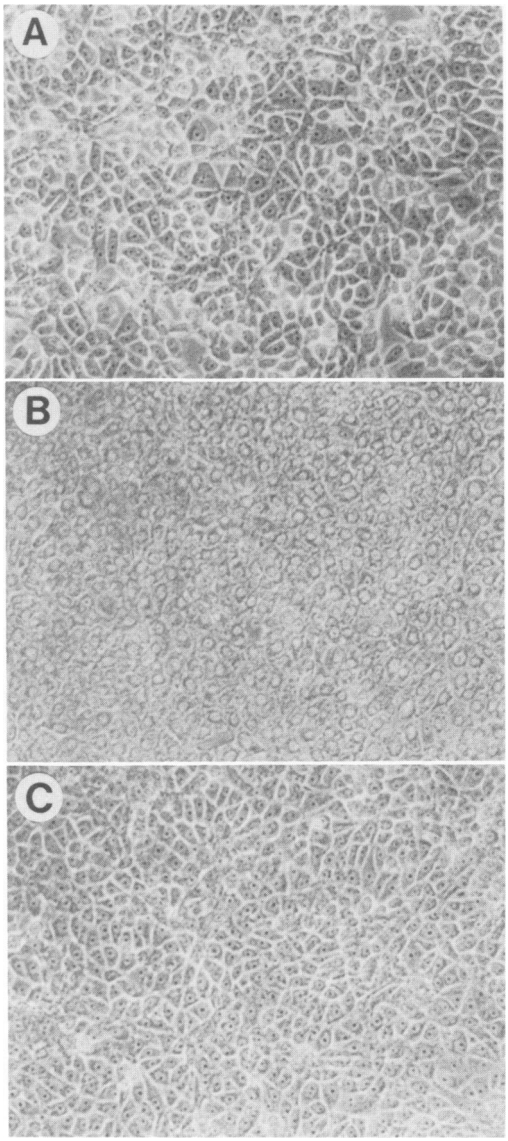

FIG. 7. Morphology of Vero cells after exposure to KFT(25) at pH 5.5 and 7.4. After treatment according to the protocol of Fig. 6, unstained preparations of Vero cells were photographed by phase-contrast microscopy. (A) Control Vero cells (at pH 5.5) revealed typical cobblestone, epithelial morphology with good definition of cell borders, and nuclear and nucleolar detail. (B) Vero cells (at pH 5.5) treated with 100 μM KFT(25). The cell monolayer now shows indistinct cell borders, clear nuclei, and loss of nucleolar structure. (C) Vero cells (at pH 7.4) exposed to 100 μM KFT(25). Monolayers and cell morphology are very similar to those of control cells in A. Some cell rounding (in A) is due to low pH conditions.

actions with phosphatidylserine. Hemolytic peptides interact with erythrocytes which have an asymmetric localization of phosphatidylserine to the internal membrane leaflet. Unless the peptides were able to translocate across the membrane, it is unlikely that they would interact with phosphatidylserine. Interestingly, the KFT(6) peptide does resemble an abbreviated "signal peptide" since it consists of a positively charged amino terminus followed by several hydrophobic amino acids (8).

An initial attempt to produce a prototype hemolysin, KFF(6), was not successful. This peptide exhibits several of the basic features of KFT(6) and RFT(6): a positively charged (+2) amino terminus followed by a short sequence of hydrophobic amino acids. Compared with KFT(6) or RFT(6), however, this peptide had little hemolytic activity. It is possible that the relatively large phenylalanine side groups prohibited a necessary peptide conformation. Whatever the mode of interaction of peptide with membrane, it appears that decreasing the positive charge of the amino terminus to a neutral state with glutamic acid abolishes its activity. Although this might suggest an ionic interaction with a negatively charged molecule, it is still possible that either a positive or a negative charge would suffice at the amino end.

The conservation of the amino terminus of G protein in the various strains of VSV and in rabies virus suggests that this domain may have an important role in glycoprotein function. If the hemolytic activities observed for the synthesized peptides truly reflect the function of the amino terminus of G protein, then one could speculate that the amino terminus functions in membrane destabilization and consequent fusion. It is not unexpected that the hemolytic peptides are nonfusogenic. For G protein to function as a fusogen, it needs to be anchored in the plasma membrane by its hydrophobic carboxy-terminal region (7). The hemolytic peptides lack this membrane anchorage and apparently cannot facilitate the necessary membrane-membrane interaction to effect fusion.

Direct evidence for the participation of the G protein amino terminus in membrane fusion could be obtained from genetic manipulation of cloned G protein. Several plasmid constructs are now available that express G protein at sufficient levels in mammalian cells to initiate pH-dependent membrane fusion (7, 11). Selective deletion of the amino end of the mature VSV G protein should permit one to determine whether or to what extent this domain participates in fusion.

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