The efficiency of cell-cell fusion mediated by heterologously expressed vesicular stomatitis virus G-protein has previously been shown to be affected by mutating its transmembrane segment. Here, we show that a synthetic peptide modeled after this transmembrane segment drives liposome-liposome fusion. Addition of millimolar Ca\(^{2+}\) concentrations strongly potentiated the effect of the peptides suggesting that Ca\(^{2+}\)-mediated liposome aggregation supports the activity of the peptide. Peptide-driven fusion was suppressed by lysolipid, an established inhibitor of natural membrane fusion, and involved inner and outer leaflets of the liposomal bilayer. Thus, transmembrane segment peptide-driven liposome fusion exhibits important hallmarks characteristic of natural membrane fusion. Importantly, the mutations previously shown to attenuate the function of full-length G-protein in cell-cell fusion also attenuated the fusogenicity of the peptide, albeit in a less pronounced fashion. Therefore, the function of the peptide mimic is dependent on its primary structure, similar to full-length G-protein. Together, our data suggest that the G-protein transmembrane segment is an autonomous functional domain. We propose that it acts at a late step in membrane fusion elicited by vesicular stomatitis virus.

Biological membrane fusion involves a restructuring of lipid bilayers brought into close proximity by membrane-anchored fusion proteins. To date, the most thoroughly characterized fusion proteins are those which mediate fusion of viral envelopes with cellular membranes (1). These viral fusion proteins consist of an ectodomain harboring an amphipathic fusion peptide, a single transmembrane segment (TMS), and a cytoplasmic domain. All these domains appear to cooperate in fusion protein function (2, 3). In the case of influenza hemagglutinin (HA), a pH-driven global conformational change of the ectodomain is thought to eject the fusion peptide toward the target bilayer to establish initial contact between both membranes (4). Synthetic versions of many of these soluble fusion peptides drive liposome-liposome fusion in vitro. Furthermore, the fusogenic function of some synthetic fusion peptides is sensitive to point mutations in a similar fashion as the corresponding full-length fusion proteins (2, 5, 6). Therefore, they appear to be partially independent functional domains whose interaction with target membranes is an early event initiating fusion.

A late role in fusion protein function has been ascribed to the TMSs. For example, upon replacement of the TMS by a glycosylphosphatidylinositol membrane anchor (7, 8) or by mutating its TMS, it was initially reported that influenza HA loses its ability to mediate complete bilayer fusion but retains hemifusion, i.e. lipid mixing of the contacting monolayers (7). More recently, lipid-anchored HA was found to induce an aqueous continuity between fusion compartments which, however, formed less efficiently and did not enlarge substantially compared with wild-type (wt) HA. Consequently, the HA TMS was suggested to favor pore formation and growth (10). Similar results were obtained with a point mutant (11). In another study, shortening the HA TMS reduced its ability to support the hemifusion to fusion transition whereas point mutations were without effect (12). Furthermore, the function of other fusion proteins derived from the human immunodeficiency virus type 1 envelope glycoprotein (13) or the Moloney murine leukemia virus (14) was compromised by mutations within the TMSs.

The trimeric vesicular stomatitis virus (VSV) G-protein is a fusion protein that is functionally and structurally related to influenza HA. Replacement of the VSV G-protein TMS by a glycosylphosphatidylinositol anchor rendered this protein non-fusogenic, suggesting that its function requires membrane anchoring by a hydrophobic peptide sequence (15). Similarly, a 6-residue deletion or point mutations within the TMS attenuated the fusogenicity of VSV G-protein as indicated by reduced transfer of cytoplasmic contents between fusing cells. However, the mutant proteins were shown to retain hemifusion activity since lipid probes within the external leaflets of the reacting membranes mixed (16). Similar to the case of influenza HA, the TMS of VSV G-protein is therefore thought to act at a late step in membrane fusion.

Here, we demonstrate that incorporation of a synthetic peptide corresponding to the VSV G-protein TMS into liposomal membranes dramatically increases their ability to fuse. Interestingly, those mutations previously shown to affect full-length VSV G-protein function in HeLa cells also attenuated TMS-peptide-induced liposome-liposome fusion. Thus, the TMS-peptide exhibits sequence-specific fusogenicity in the absence of the ectodomains and thus appears to be an independent functional domain.
EXPERIMENTAL PROCEDURES

Peptide Synthesis—Peptides were synthesized by the standard Fmoc (N-(9-fluorenyl)methoxycarbonyl) solid-phase method on an Abimed AMS422 multiple peptide synthesizer. The peptide-resin conjugates were cleaved with trifluoroacetic acid. Upon dilution in formic acid and 10-fold dilution with water, peptides were purified by high-pressure liquid chromatography on a YMC ODS-H50 reverse phase column using a 40–95% (v/v) gradient of 5% (v/v) acetonitrile including 1% (v/v) trifluoroacetic acid for elution. The identities of the peptides were confirmed by mass spectrometry using a Finnigan-MAT Vision 2000. Peptide concentrations were determined spectrophotometrically via tryptophan absorbance at 280 nm in a 1:1 (v/v) mixture of trifluoroethanol and dimethyl sulfoxide using an extinction coefficient of 5600 M⁻¹ cm⁻¹.

Preparation of Small Unilamellar Liposomes—Liposomes were prepared from mixtures of egg PC/brain PE/brain PS (Sigma or Avanti Polar Lipids) at a ratio of 6:2:2 (w/w/w) with or without 0.8% (w/w) of LPC (Sigma) dissolved at 1.1 mg/ml in fusion buffer was slowly pipetted from proteoliposomes by density gradient centrifugation. 330 µM sodium dithionite on ice for 30 min and excess sodium dithionite was routinely determined in parallel and subtracted from the values obtained with peptide-containing liposomes. The mean P/L ratio was 0.018 unless specified otherwise.

RESULTS

A Synthetic VSV G-protein TMS Drives Liposome-Liposome Fusion—Synthetic peptides harboring the central 15 residues from the predicted TMS of VSV G-protein and a number of mutant sequences were synthesized by solid-phase chemistry. The hydrophobic residues are bordered by 3 lysine residues at both termini to enhance solubility and correct membrane integration (20, 21). A tryptophan residue was included for photometric quantitation of fusion (Fig. 1). The peptides were successfully incorporated into liposomal membranes by sonication as previously demonstrated for a number of other hydrophobic peptides (21, 22). Routinely, a mixture of egg phosphatidylcholine (PC), brain phosphatidylethanolamine (PE), and brain phosphatidylinositol (PS) (at a 6:2:2 weight ratio) was used. This lipid composition had previously been found to optimally support the fusogenicity of TMS-peptides derived from SNAKE (soluble NSF (N-ethylmaleimide-sensitive factor) proteins while exhibiting low spontaneous fusion in the absence of the peptides. To determine the actual P/L ratios present in the fusion experiments, peptide-liposome complexes were separated from the free peptides, which are more dense, by sucrose density gradient centrifugation. The amounts of co-fractionating peptides and lipids were determined and the P/L ratios calculated (see “Experimental Procedures”). Integration of the peptides into membranes is supported by the fluorescence of the marker tryptophan residue. Depending on the identity of the peptide, fluorescence emission maxima ranged from 335 to 340 nm in the relatively hydrophilic solvent dimethyl sulfoxide and from 317 to 326 nm in peptide-liposome complexes (data not shown). These blue shifts (14 to 22 nm) indicate that the marker tryptophan residues of the peptides, and by implication, their hydrophobic sequence, are embedded within the hydrocarbon phase of the membranes (23).

The ability of the liposomes to fuse to each other was examined upon rapidly shifting the temperature to 37 °C by a standard fluorescence dequenching assay (18). This assay is based upon fluorescence resonance energy transfer from N-(7-nitro-2,1,3-benzoxadiazol-4-yl)dioleoylphosphatidylethanolamine (NBD-PE) to N-(lissamine rhodamin B sulfonyl)dioleoyl phosphatidylethanolamine (Rh-PE) being present at quenching concentrations in donor liposomes. Upon fusion of donor liposomes to unlabeled acceptor liposomes, the average distance between the lipid-bound fluorophores increases; this results in an increase of NBD-fluorescence over time which is taken as a measure of lipid mixing. As shown in Fig. 2, incorporation of the VSV wt TMS-peptide

Fig. 1. Amino acid sequences of the peptides used in this study. Dots represent wt residues. The residue numbering system used by Cleverley and Lenard (16) was adopted.

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much less efficiently. Spontaneous fusion of pure control liposomes (pure/pure) proceeds liposomes fuse to peptide-containing acceptor liposomes (VSV/pure). tor liposomes (VSV/VSV) fuse with similar efficiency as pure donor typical fusion kinetics reveal that peptide-containing donor and accep- tor liposomes (VSV/VSV) or only in acceptor liposomes. Both parameters were corrected for spontaneous fusion.

strongly increased the ability of liposomal membranes to fuse as compared with spontaneous background fusion observed with pure liposomes. Extents of fusion, seen after 1 h, and initial fusion rates were similar regardless of whether donor and acceptor liposomes contained peptide (VSV/VSV) or whether peptide-containing acceptor liposomes were fused to pure donor liposomes (VSV/pure).

**TMS-Peptide-mediated Fusion Is Sensitive to Lysolipid and Involves Both Membrane Leaflets**—Biological membrane fusion is thought to proceed through a state termed hemifusion, an intermediate where the outer, but not inner, membrane leaflet mix to form a stalk structure (1, 2, 24, 25). Hemifusion is associated with negative curvature of the outer membrane leaflet. Hence, it is disfavored upon integration of lysolipids into the outer leaflet since lysolipids stabilize positive curvature due to their inverted cone shape (24). Accordingly, lysolipids have been established as potent and reversible inhibitors of different types of natural fusion reactions at a step preceding membrane merger (25). Here, we assessed whether VSV G-protein TMS-peptide-mediated fusion is inhibited by adding LPC micelles to the preformed liposomes. Indeed, LPC efficiently inhibited TMS-peptide-mediated liposome fusion depending on the applied ratio of diacyl-lipids to LPC (Fig. 3A). This is consistent with transient formation of a hemifusion intermediate in the absence of LPC.

To show that fusion involved both membrane leaflets rather than being arrested at hemifusion, we extinguished the fluorescence of the NBD moiety present in the outer leaflet by dithionite treatment (19). Application of 20 mM dithionite elic-
enhanced background fusion as determined in parallel using pure liposomes, millimolar concentrations of Ca\(^{2+}\) ions were indeed found to strongly increase both initial rate and extent of peptide-induced fusion in a concentration-dependent manner (Fig. 4). Therefore, juxtaposition of the membranes, mediated here by random collisions of the liposomes and potentiated by their Ca\(^{2+}\)-mediated aggregation, appears to be a rate-limiting step in liposome-liposome fusion driven by VSV G-protein TMS-peptide.

**Mutations Attenuate the Fusogenicity of the TMS-Peptide—** Based on studies using eukaryotic cells, it was previously found that point mutations in the TMS affect fusogenicity of the expressed full-length VSV G-protein. Specifically, mutating the glycine residues individually to alanine reduced cytoplasmic contents mixing by \(-40\%\) to \(50\%\), whereas double mutations to alanine or leucine resulted in reductions of \(-90\%\) (16).

Here, we tested whether mutant forms of the TMS peptides mimic this sequence specificity of G-protein function. Thus, we compared the fusogenicity of the wt peptide to that of mutant sequences where both glycines were either singly exchanged to alanine or doubled exchanged to alanine, leucine, or valine. Fig. 5A displays the dependence of the mean extent of fusion, as seen after 1 h, on the experimentally determined P/L ratios. In order to better visualize the differences determined at the lower P/L ratios, the mean values obtained for the mutant forms were normalized to the values seen with the wt peptide (=100%). With the single mutations (G6A, G10A) the mean extent of fusion appeared to be only moderately reduced (by 21 to 35%) and only at the lower P/L ratios. For the double mutants (G6A,G10A; G6L,G10L; G6V,G10V) the extent of fusion was decreased by \(-50\%\) at the lower P/L ratios and for 13 to 37% at the highest P/L ratios. We also tested mutants where residues other than the glycines were exchanged. Fusion elicited by peptide A3, where 3 residues in the vicinity of the glycines were mutated to alanine (see: Fig. 1), was reduced by 13 to 30%, depending on the P/L ratio. Strong reduction (71 to 91%) was observed for the L5 peptide where 5 exchanges to leucine had been made. A similarly weak fusogenicity was found for the oligoceamic (L16(W)) peptide which was previously shown to function as an artificial TMS (28). The degree of fluorescence quenching elicited by the mutant peptides was similar with or without prior bleeding of the NBD chromophore of the outer membrane leaflet with dithionite. Thus, fusion elicited by the mutant peptides did not result from hemifusion but involved both membrane leaflets as seen for the wt peptide (see above).

Taken together, the glycine mutations affected the fusogenicity of the TMS-peptides in a fashion similar to that of full-length VSV G-protein (16), albeit less strongly. Thus, our results indicate that the TMS-peptides, at least partially, reproduce the sequence specificity of VSV G-protein function.

**DISCUSSION**

Our results demonstrate that incorporation of synthetic peptides modeled after the TMS of VSV G-protein into the membranes of synthetic liposomes strongly increases their ability to fuse. These peptides represent the central hydrophobic part of the predicted TMS which is located between lysine 462 and arginine 483. Since the hydrophobicity of our peptides is rather
symmetrically distributed, we assume that they fully insert as α-helices into liposomal membranes. This is supported by previous studies where the ability of other lysine-bordered hydrophobic peptides to incorporate into lipid bilayers was tested. Huschilt et al. (20) found that a KKKGL₁₆KK peptide inserted as an α-helix at a perpendicular angle into dipalmitoyl phosphatidylcholine membranes. Furthermore, Webb et al. (29) established that a hydrophobic peptide core of 17 residues fully incorporated into a membrane composed of lipids with C₁₈ acyl chains. The efficiency of these relatively short peptides to integrate into membranes is ascribed to the flexibility of the hydrophobic part of the bordering lysine side chains. This flexibility allows the charged termini to “snorkel up” toward the lipid head group regions. In a previous model study, the lysine termini bordering 17 hydrophobic residues adopting an α-helical conformation have been calculated to extend up to 1.9 nm from the bilayer center (22). By analogy to these model systems, our TMS peptides containing 16 hydrophobic amino acids are likely to span the hydrophobic core of liposomal membranes that are composed of natural lipids which contain mostly C₁₈ lipid acyl chains.

This in vitro fusion system consisting only of lipids and TMS-peptides appears to display characteristic hallmarks of biological membrane fusion. First, its sensitivity to lysolipid is consistent with the transient existence of a hemifusion state, which is thought to precede complete bilayer merger (2, 24). Second, TMS-peptide-driven fusion involves both bilayer leaflets as shown upon bleaching the NBD fluorophore of the outer leaflet by dithionite. Third, both extent and initial rate of fusion are enhanced by Ca²⁺-mediated liposome aggregation, thus suggesting that fusion promotion by the peptides is potentiated by membrane proximity.

Importantly, mutating the glycine residues within the TMS-peptide attenuated its fusogenicity to various degrees depending on the number of exchanges made, the type of introduced residue, and the P/L ratio of the proteoliposomes. Since glycine mutations had previously been shown to affect cell-cell fusion mediated by full-length VSVG-protein (16), our liposome-based in vitro approach appears to reflect, at least in part, the sequence specificity of full-length fusion protein function.

In analogy to results obtained with influenza HA TMS mutants (9), the VSVG-protein TMS has previously been shown to function late in the fusion process (16). This late step appears to correspond to the conversion of the hemifusion intermediate to fully fused bilayers and is thought to be facilitated by the TMS. It should be noted that the extent of fluorescence de-quenching seen here with pure liposomes, i.e., without added peptide, was reduced to an average of 60% upon outer leaflet bleaching. This suggests that a substantial fraction of peptide-independent spontaneous liposome fusion does not proceed beyond hemifusion. We speculate, therefore, that the hemifusion diaphragm may form spontaneously from randomly colliding liposomes. Its transition to full fusion, however, appears to be facilitated by the TMS-peptides.

What is the characteristic feature of the TMS which drives this transition and which is required for optimal fusion protein function? The TMSs of fusion proteins are expected to be inserted as α-helices at orientations close to the membrane normal as demonstrated for the influenza HA TMS (30). A comparison of the amino acid composition of fusogenic and non-fusogenic viral membrane protein TMSs revealed that glycine residues are strongly over-represented in the former (16). It was therefore speculated that the functionally relevant glycines of the VSVG-protein might allow for bending of the TMS helices during the fusion process (16). Indeed, glycine residues have previously been shown to destabilize α-helical hydrophobic peptides in membrane-mimetic environments (31). On the other hand, isoleucine was the single other residue type that was also significantly overrepresented in fusogenic TMSs (16). Interestingly, in our A3 or L5 peptide mutants, two or four isoleucines were exchanged for alanine or leucine, respectively, and this resulted in a marked decrease of fusogenicity despite the continued presence of both glycine residues. Isoleucine ranks among those residues with the highest propensities to form β-sheet structures (23, 34). It is tempting to speculate that not only glycine, but also isoleucine or other residues with β-branched side chains, impart structural flexibility to fusogenic TMS helices. Thus, local deformation or even transient unfolding of these helices may facilitate the restructuring of the lipid bilayer in fusion. It might be relevant in this context that the α-helical synthetic peptide corresponding to the influenza HA TMS increased the acyl chain order of a lipid bilayer (30).

On the other hand, it should be noted that the fusogenic function of VSVG-protein (15), influenza HA (9), or the human immunodeficiency virus type 1 envelope glycoprotein (35) was maintained upon replacement of the respective TMS by unrelated TMSs (9, 15). This is an apparent contradiction to the effects of TMS point mutations as discussed above. The data may be reconciled, however, if one assumes that the structural feature(s) rendering a TMS compatible with fusion protein function are shared by certain unrelated TMSs which would be inactive in their normal contexts (2, 14).

The fusogenic capacity of synthetic TMSs appears not to be restricted to the VSVG-protein. In another study, we found that peptides modeled after the TMSs of SNARE proteins also drive liposome-liposome fusion in a sequence-specific fashion. As SNARE proteins are essential for membrane fusion in the secretory pathway of eukaryotic cells (36), functional autonomy of TMSs may be a phenomenon relevant for both viral and cellular fusion proteins.

Acknowledgments—We thank Drs. A. Herrmann and J. Trotter for critical reading of the manuscript, W. B. Huttner for continuous support, and M. Koch for expert help with peptide synthesis.

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Fusogenic VSV G-protein Transmembrane Segment

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