Shallow Boomerang-shaped Influenza Hemagglutinin G13A Mutant Structure Promotes Leaky Membrane Fusion* 

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Our previous studies showed that an angled boomerang-shaped structure of the influenza hemagglutinin (HA) fusion domain is critical for virus entry into host cells by membrane fusion. Because the acute angle of ~105° of the wild-type fusion domain promotes efficient non-leaky membrane fusion, we asked whether different angles would still support fusion and thus facilitate virus entry. Here, we show that the G13A fusion domain mutant produces a new leaky fusion phenotype. The mutant fusion domain structure was solved by NMR spectroscopy in a lipid environment at fusion pH. The mutant adopted a boomerang structure similar to that of wild type but with a shallower kink angle of ~150°. G13A perturbed the structure of model membranes to a lesser degree than wild type but to a greater degree than non-fusogenic fusion domain mutants. The strength of G13A binding to lipid bilayers was also intermediate between that of wild type and non-fusogenic mutants. These membrane interactions provide a clear link between structure and function of influenza fusion domains: an acute angle is required to promote clean non-leaky fusion suitable for virus entry presumably by interaction of the fusion domain with the transmembrane domain deep in the lipid bilayer. A shallower angle perturbs the bilayer of the target membrane so that it becomes leaky and unable to form a clean fusion pore. Mutants with no fixed boomerang angle interacted with bilayers weakly and did not promote any fusion or membrane perturbation.

Influenza virus enters host cells by receptor-mediated endocytosis and membrane fusion as do many other enveloped viruses (1–3). When the pH in the endosome drops to about 5, membrane-anchored hemagglutinin (HA) subunit HA2 undergoes a dramatic conformational change, which ultimately drives membrane fusion (4). In an early and critical step, the fusion domain, i.e. the ~20 most N-terminal residues of HA2, which are often also referred to as the fusion peptide, is exposed from a hydrophobic pocket in the neutral pH HA trimer structure and is propelled toward the virus attachment site where it inserts into the endosomal membrane of the host cell (5). The structure of the endosomal membrane bilayer is perturbed by the action of the fusion domain, and an appropriately controlled perturbation of this bilayer leads to fusion between the viral and cellular membranes.

To better understand the process of viral membrane fusion, it is critical to know the structure of the membrane-inserted fusion domain and to examine its interactions with the surrounding bilayer. The HA fusion domain adopts a kinked “boomerang” structure in detergent micelles and lipid bilayers as revealed by combined nuclear magnetic resonance (NMR) and site-directed spin labeling approaches (6, 7). The subtended angle of the boomerang is about 105° with a kink formed by Glu-11, Asn-12, and Gly-13. The pH 5 structure is quite compact with a hydrophobic pocket formed by several aromatic and aliphatic residues, which are all critical for shaping the boomerang and thus for fusion (8).

To obtain a better understanding of the correlation between structure and function of the kink of the HA fusion domain, it is of interest to know how changes of these residues affect the kink angle of the HA fusion domain and whether such angle changes translate into changes of the fusion activity of HA. Because Glu-11 and Asn-12 were previously shown to cause hemifusion and normal fusion, respectively (9), the most critical residue of the kink appears to be Gly-13. To examine the role of this residue, we expressed HA bearing the G13A mutation in CV-1 cells and studied its ability to mediate fusion with red blood cells. We found a new leaky fusion phenotype. The mutant promoted efficient lipid mixing with very limited content mixing. Rather than transferring contents to the next cell, most spilled into the surrounding medium during fusion. Intrigued by this result, we determined the structure of G13A and found that it exhibited a shallower kink angle than wild type but a more acute angle than non-fusion mutants. The G13A mutant also interacted more weakly with membrane lipids than wild type but more strongly than non-fusion mutants, explaining the unusual leaky fusion phenotype.

MATERIALS AND METHODS

Mutagenesis and Cell Surface Expression of Mutant HA—Mutations were introduced into the pTM1-HA vector with the QuikChange site-directed mutagenesis kit (Stratagene). This vector efficiently expresses full-length HA (X:31) in CV-1 cells where it is targeted to the cell surface. Primers were purchased from Integrated DNA Technologies (Coralville, IA). Mutations were verified by sequencing. Wild type and mutants were

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† The atomic coordinates and structure factors (code 2L4G) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

‡ The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–3 and Tables 1 and 2.

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expressed in CV-1 cells as described previously (9) using 1 μg of DNA for each transfection. Immunoprecipitation and fluorescence-activated cell sorting (FACS) were performed as described previously (10). For FACS analysis, the HA-expressing cells were labeled with an anti-HA site A monoclonal antibody followed by a fluorescein-conjugated secondary antibody. For immunoprecipitation, transfected CV-1 cells were first treated with 5 μg/ml trypsin for 10 min at RT followed by labeling of all surface-expressed proteins with sulfo-NHS-LC-biotin (sulfosuccinimidyl-6-(biotinamido)hexanoate; Pierce). After lysis of the cells with lysis buffer (100 mM Tris, pH 7.4, 1% Nonidet P-40), HAs were immunoprecipitated by an anti-HA monoclonal antibody and protein A-conjugated beads. The bead-bound HA fraction was run on an SDS-polyacrylamide gel, transferred to nitrocellulose filters, and developed with streptavidin-horseradish peroxidase.

Expression and Purification of G13A Mutant Fusion Domain for Structural Studies—A fusion domain construct comprising the N-terminal 20 residues of HA2 (FP) fused at the C terminus with the GCGKKKK host sequence (HS) was cloned into the pET31b (+) vector (Novagen, Madison, WI). The sequence also had a single methionine (M) each at the N and C termini, and the internal Met-17 was mutated to valine. Thus, a construct KSI-M-FP(G13A/M17V)-HS-M-His<sub>6</sub> was expressed in CV-1 cells as described previously (9) using 1 μg of DNA for each transfection. Immunoprecipitation and fluorescence-activated cell sorting (FACS) were performed as described previously (10). For FACS analysis, the HA-expressing cells were labeled with an anti-HA site A monoclonal antibody followed by a fluorescein-conjugated secondary antibody. For immunoprecipitation, transfected CV-1 cells were first treated with 5 μg/ml trypsin for 10 min at RT followed by labeling of all surface-expressed proteins with sulfo-NHS-LC-biotin (sulfosuccinimidyl-6-(biotinamido)hexanoate; Pierce). After lysis of the cells with lysis buffer (100 mM Tris, pH 7.4, 1% Nonidet P-40), HAs were immunoprecipitated by an anti-HA monoclonal antibody and protein A-conjugated beads. The bead-bound HA fraction was run on an SDS-polyacrylamide gel, transferred to nitrocellulose filters, and developed with streptavidin-horseradish peroxidase.

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Purification was performed as described previously for a similar HIV gp41 fusion domain construct (13). After breaking the cells by 10-min sonication, pellets were collected after 30-min centrifugation at 12,000 × g. The pellets were dissolved in binding buffer (5 mM imidazole, 40 mM Tris, 500 mM NaCl, 6 mM guanidinium HCl, pH 7.9). The solution was loaded on a pre-saturated nickel column and incubated for 1 h at room temperature. The column was washed with 20 bed volumes of washing buffer (16 mM imidazole, 40 mM Tris, 500 mM NaCl, 6 mM guanidinium HCl, pH 7.9) and eluted with 5 bed volumes of eluting buffer (300 mM imidazole, 40 mM Tris, 500 mM NaCl, 6 mM guanidinium HCl, pH 7.9). The eluant was dialyzed against water in a 3-kDa-cutoff dialysis bag overnight at RT. The precipitate was collected by centrifugation, and the pellet was resuspended in water. Trifluoroacetic acid was slowly added to the suspension until the pellet was completely dissolved. A 400-fold excess of CNBr over peptide was added to the solution, and the reaction was carried out overnight in the dark under nitrogen and with stirring. This reaction cleaves the protein after methionines and therefore removes ketosteroid isomerase including the methionine that links ketosteroid isomerase to the fusion domain. The reaction mixture was diluted 10-fold with water, frozen in liquid nitrogen, and lyophilized overnight. The powder was dissolved in 30% CH<sub>3</sub> CN, H<sub>2</sub>O and purified by reverse-phase HPLC (Beckman). The correct mass and purity of the expressed fusion domain were determined by mass spectroscopy.

Synthetic Fusion Peptide—Host-guest fusion domains of the same sequence as above but without the M17V replacement and without the His tag were synthesized by solid-phase synthesis and purified by reverse-phase HPLC by the Biomolecular Research Facility at the University of Virginia. Purity and mass were confirmed by mass spectrometry.

Preparation of Small Unilamellar Lipid Vesicles—Four equivalents of 1-palmitoyl-2-oleoyl-3-sn-phosphocholine (POPC) and 1 eq of 1-palmitoyl-2-oleoyl-3-sn-phosphoglycerol (POPG) (Avanti Polar Lipids, Alabaster, AL) were dried overnight under vacuum. Fusion buffer (5 mM HEPES, 10 mM MES, pH 5) was added and vortexed to give a 5 mM lipid dispersion. The dispersion was sonicated in an ice-water bath using a Branson titanium tip ultrasonicator for ~1 h using a 50% duty cycle. Titanium dust was removed by centrifugation from the resulting transparent solution.

Cell Fusion Assay—Fresh human red blood cells (RBCs) were double labeled with octadecyl rhodamine B chloride (R18) and 6-carboxyfluorescein (CF) (Invitrogen) as described (10). HA-expressing CV-1 cells were treated with 0.2 mg/ml neuraminidase and 5 μg/ml trypsin in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum at RT for 10 min. The medium was replaced with double labeled RBCs in pH 7 buffer (100 mM NaCl, 10 mM HEPES, 10 mM MES, 0.2% glucose), and the cells were allowed to bind for 10 min at RT. After this preincubation period, cells were washed three times with pH 7 buffer and superfused with pH 5 fusion buffer (same composition) for 5 min at 37 °C (or as specified) before the buffer was replaced again with pH 7 buffer. Fusion was then observed with a Zeiss Axiovert 200 fluorescence microscope at RT with 20× and 63× objectives (low and high magnification images, respectively) (8). Binding, lipid mixing, and content mixing events were counted, and the results were expressed as (i) the percentage of the number of CV-1 cells that showed lipid mixing to the number of CV-1 cells that bound RBCs (lipid mixing ratio) and (ii) the percentage of the number of CV-1 cells that showed content mixing to the number of CV-1 cells that bound RBCs (content mixing ratio).

Fluorescence Dequenching Assay—Fluorescence dequenching assays were carried out using a Jobin-Yvon Fluorolog 3 spectrofluorometer. To measure lipid mixing, RBCs were labeled with R18. To measure content mixing and leakage, RBCs were labeled with 10 mM 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) and 30 mM p-xylene-bis(pyridinium bromide) using the same procedure as used for CF labeling. Fusion between neuraminidase- and trypsin-treated HA-ex-
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pressing CV-1 cells and RBCs after prebinding in pH 7 buffer (100 mM NaCl, 10 mM HEPES, 10 mM MES, 10 mM succinate, 0.2% glucose) and acidification to pH 5 with a predetermined amount of 1 mM acetic acid was monitored at 37 °C as determined by the program HABAS, dihedral angle constraints derived from 

\[ \text{NOE upper distance constraints derived from NOESY spectra by the program PROCHECK-NMR (20).} \]

\[ \text{Isothermal Titration Calorimetry—All measurements were performed at 25 °C on a high sensitivity MCS-ITC titration calorimeter (MicroCal Inc., Northampton, MA). To determine the enthalpy of binding, 10-μl aliquots of 20 μM protein were injected into the sample cell containing 2 μl of small unilamellar vesicles composed of 5 mM POPC/POPG (4:1). To determine the free energy of binding, 10-μl aliquots of a 5 mM POPC/POPG (4:1) vesicle stock solution were injected into the sample cell containing 2 ml of 1 mM protein. The data were analyzed as described previously (21).} \]

Circular Dichroism Spectroscopy—Desired amounts of protein (0.1 mg/ml) and lipid micelles or lipid vesicles (100-fold molar excess over protein) were mixed in 5 mM HEPES, 10 mM MES, pH 5 and degassed for 5 min at room temperature before each measurement. Spectra were collected at 25 °C on an AVIV Model 215 spectropolarimeter (AVIV Biomedical Inc., Lakewood, NJ), and pure buffer blanks were subtracted from the sample spectra.

Fourier Transform Infrared Spectroscopy—Planar phospholipid bilayers supported on germanium attenuated total reflection (ATR) plates were prepared as described (11). The substrate supported monolayer was dimyristoyl-3-sn-phosphocholine, and the monolayer exposed to the large buffer compartment was POPC/POPG (4:1). Desired amounts of protein in 5 mM HEPES, 10 mM MES, pH 5 fusion buffer were perfused into the holding cell and allowed to incubate for 5 min before excess protein was washed away. All measurements were carried out on a Bruker Optics Vector 22 Fourier transform infrared (FTIR) spectrometer (Bruker Optic Inc., Billerica, MA), and 200 scans were collected for each polarization with 2-cm⁻¹ resolution. Lipid order parameters were calculated from the experimental ATR dichroic ratios as described previously (22).

RESULTS

Characterization of Fusion Phenotype of G13A Mutant by Fluorescence Microscopy—To examine the fusion behavior of the G13A fusion domain mutant, CV-1 cells were transfected with DNA of influenza HA bearing this point mutation. The mutant protein was expressed in cell populations and on the surface of individual cells just as well as wild-type HA as determined by FACS analysis (supplemental Fig. 1A and B). Immunoprecipitation and subsequent Western blot analysis further showed that G13A HA was quantitatively processed by trypsin to HA1 and HA2 on the cell surface (supplemental Fig. 1C). Thus, any potential differences in fusion activity between wild type and mutant are not due to insufficient surface expression or incomplete processing of HA.

R18- and CF-double labeled RBCs were bound to the HA-expressing CV-1 cells, and the pH was adjusted to pH 5 to trigger fusion between the two cells. The transfer of the red dye (R18) indicates mixing of lipids, and the transfer of the green dye (CF) indicates mixing of soluble contents between the two cells. After a 5-min pulse of exposure to pH 5, we observed that G13A induced a reduced level of lipid mixing. Its lipid mixing ratio was only 38% compared with 97% for wild type (Fig. 1). For comparison, the previously well characterized hemifusion mutant E11A had a lipid mixing ratio of 60%, whereas the nonfusion mutant W14A had a negligible lipid mixing ratio of 4%. Prolonged exposure of the G13A mutant to pH 5 indicated that lipid mixing is kinetically restricted in this mutant compared with wild type and other mutants. When the fusion time was increased to 30 min, the lipid mixing ratio increased to 84% (Fig. 1).

G13A also behaved differently compared with wild type and several other mutants when analyzed for content mixing. We consistently observed that after acidification CF leaked into the surrounding environment instead of being transferred to the G13A-expressing CV-1 cells. This is evident from the CF fluorescence images of G13A in which most RBCs have lost their CF dyes after pH 5 exposure (Fig. 1; the remaining few green spots are from RBCs that are not coupled to HA-expressing CV-1 cells). By contrast, contents were transferred into the coupled...
cell in the case of wild-type HA or remained in the RBCs in the case of the hemifusion-inducing E11A and non-fusion mutant HAs (Fig. 1). Although several other HA fusion domain mutants have been studied by this technique, none of them showed a leaky fusion phenotype like G13A does here (8–10, 23). It thus appears that G13A represents a new, not previously described leaky fusion phenotype that is different from normal fusion, hemifusion, or non-fusion phenotypes.

To further demonstrate content leakage, we analyzed the kinetics of CF loss from the CV-1 cell-coupled RBCs. A series of images were recorded at different time points after acidification. To minimize photobleaching, illumination of the sample was gated and turned on only immediately before each image was taken. As shown in Fig. 2, CF was still confined in the RBCs 30 s after low pH triggering. However, many cells lost CF 1 and 2 min after triggering, resulting in a brighter background. Four minutes after triggering, CF-containing RBCs could no longer be observed. The dye leaked into the surrounding environment and did not transfer to coupled CV-1 cells. The R18 lipid fluorescent dye did not visibly transfer even after 5 min in this experiment, indicating that lipid mixing occurs after dye leakage in the G13A mutant. The lipid mixing ratio in this experiment was lower than in Fig. 1 because the pH triggering was performed at room temperature instead of 37 °C.

Typically, about 5–10 RBCs were bound to each CV-1 cell. The percentages of hemifusion and fusion were about the same when calculated on the basis of RBCs rather than CV-1 cells. This indicates that hemifusion and fusion typically occurred with most and not just a subset of RBCs bound to the HA-expressing cells. It should also be noted that hemifusion as measured here must be considered a lower limit of the cells that underwent hemifusion. It is well known that R18 dye spread is sometimes restricted at a hemifused site and therefore underestimates the true level of hemifusion.

**Confirmation of Leaky Fusion Phenotype of G13A Mutant by Fluorescence Spectroscopy**—To confirm the fusion phenotype of G13A in a larger cell population, we measured lipid mixing between prebound R18-labeled RBCs and CV-1 cells by monitoring dequenching of the R18 fluorescence after adjusting the pH to 5 (Fig. 3A). G13A had an initial rate of lipid mixing of 0.41%/s, which is a little lower than 0.48%/s for E11A but significantly lower than 0.66%/s for wild type (Fig. 3B). For com-
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the local conformation of the polypeptide chain, and chemical shift differences between the backbone Hα and HN resonances of the G13A fusion domain and tabulated random coil values are shown in supplemental Fig. 2. Quite large chemical shift differences (>0.25 ppm) were observed in most regions suggesting that the G13A fusion domain in DPC micelles is helical for most parts of the sequence. The Hα and HN chemical shift differences between the G13A and wild-type fusion domains (6) were generally small except for moderate changes of Hα of Glu-15 and HN of Ile-10 and Glu-11 (data not shown).

Because the number of NOEs obtained from the homonuclear experiments was limited and more experimental constraints were required to determine the three-dimensional structure of the G13A fusion domain, we prepared a 15N-labeled sample by expression in E. coli using a new strategy of ketosteroid isomerase fusion protein expression as described under “Materials and Methods.” Because this strategy required CNBr cleavage after methionines, the native Met-17 of the sequence was replaced with a valine. Fig. 1 shows that this substitution did not alter the leaky fusion phenotype of G13A when it was engineered into the full-length HA. CD spectra also show that the double mutant adopted a helical structure in lipid micelles and bilayers similar to that of the G13A mutant (supplemental Fig. 3). Chemical shifts obtained and assigned from 15N HSQC and 15N HSQC-NOESY spectra of the labeled G13A/M17V mutant fusion domain were virtually identical to those of the unlabeled G13A fusion domain except for the substituted residue 17 and the neighboring Ile-18 whose HN resonance showed a small upfield shift in the double mutant.

In total, 168 NOE upper distance constraints were obtained from the NOESY and 15N HSQC-NOESY data. 72 dihedral angle constraints were derived from the chemical shift data, and 20 additional dihedral angle constraints were obtained from 3J_HINHα couplings as described under “Materials and Methods.” The presence of all d_{αα}(i, i + 3) and d_{ββ}(i, i + 3) and several d_{αβ}(i, i + 4) NOEs from Leu-2 to Trp-14 and corresponding 3J_HINHα couplings confirmed the presence of helical secondary structure in this region. The presence of all d_{αα}(i, i + 3) and d_{ββ}(i, i + 3) NOEs and 3J_HINHα couplings between Glu-15 and Asp-19 suggests a second helix in this region.

Using the input from these spatial and angle constraints (supplemental Table 2), 40 structures were calculated, and the 20 lowest energy structures are shown in Fig. 4, A and B, and the mean conformer is shown in Fig. 4C. The structures are well

Comparison, the rate of lipid mixing of the non-fusion mutant W14A of 0.09%/s is negligible. The relative extents of fluorescence dequenching after 7 min also decreased in the order wild type (100%), E11A (65%), G13A (53%), and W14A (42%) (data not shown).

Content mixing and leakage were assayed in bulk by encapsulating the fluorescence-quencher pair ANTS/p-xylene-bis(pyridinium bromide) in RBCs. p-Xylene-bis(pyridinium bromide) quenched the fluorescence of ANTS at the concentrations that were used. However, when the probes diffused to CV-1 cells as a result of content mixing or to bulk solution as a result of content leakage, the fluorescence of ANTS increased. Interestingly, the order of dequenching of E11A and G13A was reversed compared with lipid mixing as shown in Fig. 3C. Wild type and G13A displayed similar extents of fluorescence dequenching after reaching a plateau ~5 min after adjustment of pH to 5. Error bars represent standard deviations obtained from triplicate experiments.

FIGURE 3. Bulk fluorescence dequenching of lipid and content probes after acidification of prebound labeled RBCs and HA-expressing CV-1 cells. A and B, RBCs were labeled with the lipid probe R18. C and D, RBCs were labeled with the soluble probes ANTS and p-xylene-bis(pyridinium bromide). A, kinetics of R18 fluorescence dequenching for wild type (diamond), E11A (square), G13A (triangle), and W14A (cross). B, initial rates of R18 fluorescence dequenching (FDQ) immediately after adjustment of the pH to 5. C, kinetics of ANTS fluorescence dequenching for wild type (diamond), E11A (square), G13A (triangle), and W14A (cross). D, relative extents of fluorescence dequenching after reaching a plateau ~5 min after adjustment of pH to 5. Error bars represent standard deviations obtained from triplicate experiments.

Structure of G13A Fusion Domain in Lipid Micelles—Homonuclear total correlation spectroscopy and NOESY NMR spectra of the synthetic G13A fusion domain in DPC micelles were collected at pH 5. The spectra were well resolved, and backbone and side-chain resonances were completely assigned (supplemental Table 1). Backbone NMR chemical shifts are sensitive to the local conformation of the polypeptide chain, and chemical shift differences between the backbone Hα and HN resonances of the G13A fusion domain and tabulated random coil values are shown in supplemental Fig. 2. Quite large chemical shift differences (>0.25 ppm) were observed in most regions suggesting that the G13A fusion domain in DPC micelles is helical for most parts of the sequence. The Hα and HN chemical shift differences between the G13A and wild-type fusion domains (6) were generally small except for moderate changes of Hα of Glu-15 and HN of Ile-10 and Glu-11 (data not shown).

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Using the input from these spatial and angle constraints (supplemental Table 2), 40 structures were calculated, and the 20 lowest energy structures are shown in Fig. 4, A and B, and the mean conformer is shown in Fig. 4C. The structures are well
ordered with a heavy atom root mean square deviation of 1.40 ± 0.35 Å (supplemental Table 2). Like wild type, the G13A fusion domain has an angled shape, but its angle is less acute than that of wild type (Fig. 5). Numerous differences in observed NOEs are responsible for the change of the fold angle around Asn-12. For example, NOEs between HD(Phe-9) and HA(Trp-14) and between HA(Phe-9) and HN(Trp-14) are present in wild type but not in G13A, and NOEs between HA(Ile-10) and HN(Trp-14) are strong in wild type but weak in G13A. The N-terminal arm of G13A forms an unbroken α-helix from Gly-1 to Ala-13, and the C-terminal arm forms a short helix from Glu-15 to Ile-18. A bend defined by Ala-13 and Trp-14 redirects the C-terminal helix relative to the N-terminal helix with an opening angle of ~150°. Observed hydrogen bonds between HN(Ala-13) and CO(Phe-9), HN(Trp-14) and CO(Ile-10), and HN(Gly-16) and CO(Asn-12) stabilize this bend.

Insertion of G13A Fusion Domain into Lipid Bilayers—To determine whether the different structure of G13A altered its binding and insertion into membranes, we measured interactions of the fusion domain with lipid bilayers by isothermal titration calorimetry. The coefficients $K_{app}$ and $K_0$ of G13A partitioning into lipid bilayers composed of POPC/POPG (4:1) are compared in Table 1 with those of wild type and the hemifusion mutant E11A and non-fusion mutant W14A. The intrinsic partition coefficient $K_0$ corrects the apparent partition coefficient $K_{app}$ for electrostatic contributions using the Gouy-Chapman theory as described previously (12). This takes into account the positive charge of the peptide interacting with a negatively charged membrane and permits one to determine the hydrophobic free energy of insertion $\Delta G$ of the fusion domain into lipid bilayers. It is clear from these data that G13A interacted with lipid bilayers less strongly (~6.1 kcal/mol) than wild type or E11A (both ~7.2 kcal/mol) but more strongly than W14A (~5.6 kcal/mol). These differences were even more pronounced when the enthalpy of insertion was measured ($\Delta H$ in Table 1 and Fig. 6). Wild type has the strongest insertion energy followed by G13A, E11A, and last W14A.

Perturbation of Lipid Bilayer Structure by G13A Fusion Domain—CD experiments showed that G13A has a helical secondary structure in lipid bilayers similar to that in DPC micelles (supplemental Fig. 3). To determine whether the more open

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**TABLE 1**

| Peptide | $K_{app}/10^5$ | $K_0/10^4$ | $\Delta G$ | $\Delta H$ | $\sim T\Delta S$ |
|---------|---------------|------------|------------|-------------|-----------------|
| WT      | 6.3 ± 0.5     | 1.5 ± 0.1  | −7.2 ± 0.4 | −15.8 ± 0.5 | 8.6 ± 0.6       |
| E11A    | 13.3 ± 1.9    | 1.4 ± 0.3  | −7.1 ± 0.7 | −10.2 ± 0.6 | 3.1 ± 0.9       |
| G13A    | 3.7 ± 0.4     | 0.89 ± 0.09| −6.1 ± 0.6 | −12.7 ± 0.8 | 6.6 ± 1.0       |
| W14A    | 0.50 ± 0.04   | 0.12 ± 0.01| −5.6 ± 0.3 | −8.9 ± 0.4  | 3.3 ± 0.5       |

*Data of WT, E11A, and W14A are from Ref. 9.*
angled structure of G13A perturbs the lipid bilayer in a different manner than wild type or mutants with hemifusion or non-fusion phenotypes, we measured by polarized ATR-FTIR spectroscopy the order of the lipid bilayer acyl chains in the presence and absence of respective fusion domains. The lipid order parameter $S_L$ derived from the infrared dichroism of the lipid methylene vibrations was much smaller with G13A and E11A than with wild type but larger than with W14A (Table 2 and Fig. 6). We noted before that the large perturbation by the wild-type fusion domain is due to the replacement of water at the membrane surface (24, 25). Following this argument, we conclude that G13A (as well as E11A), because of its different structure and weaker binding, replaces less water from the membrane interface than does wild type but is more effective in replacing water than W14A. Fig. 6 shows that the lipid mixing activity, membrane insertion energy, and lipid perturbation by the fusion domain correlate with the fusion phenotype. The leaky fusion mutant G13A and the hemifusion mutant E11A exhibited similar intermediate bilayer perturbation, but the leaky fusion mutant bound more strongly to bilayers than did the hemifusion mutant.

**DISCUSSION**

**New Class of Fusion Mutants**—The fusion domains are among the most conserved regions of the sequences of virus envelope glycoproteins including influenza HA. It has been well established that a kinked boomerang structure of the fusion domain in lipid membranes is essential for the proper functioning of the influenza HA fusion domain as an effective fusogen (6). In the present work, we asked why a kinked structure with a fixed angle is so important to confer efficient membrane fusion to influenza virus. Because Asn-12, which forms the apex of the kink, was previously shown to not appreciably affect the fusion activity of HA, we focused on Gly-13, which together with Asn-12 was a likely candidate to define the kink angle of the boomerang structure. Interestingly, mutation of this residue to an alanine resulted in a leaky fusion phenotype when examined by single cell-to-cell or bulk fluorescence fusion assays. Measurements of cell surface expression levels and proteolytic processing of HA0 to HA1 and HA2 showed that the density of mutant HAs on the cell surface and their proteolytic activation were the same as for wild-type HA and could not account for the different observed fusion phenotype.

Hemifusion phenotypes have been observed before for various fusion domain mutants of influenza HA, but the current work reports to our knowledge the first observation of a well characterized leaky fusion mutant. Well characterized influenza hemifusion mutants include those of G1S (23) and E11A (9) HA. Lipid mixing is efficient, but content mixing is completely blocked in both of these mutants. Although cells bearing the G1S mutation were observed to be slightly electrically leaky by patch clamp analysis, efficient leakage of fluorescent dyes into the environment was not observed with this mutant. This is in marked contrast to our current observations with the G13A mutant, which efficiently leaked carboxyfluorescein to the environment when coupled to red blood cells and exposed to pH 5. Therefore, G13A exhibits a new fusion phenotype very different from the typical hemifusion phenotypes represented by the G1S and E11A mutations. However, like G1S and E11A, G13A exhibited a reduced rate and extent of lipid mixing. In further contrast to these intermediate fusion phenotypes, mutations eliminating critical hydrophobic residues further away from the kink of the fusion domain, namely W14A and F9A/110A, completely abolish fusion. Neither lipid mixing nor content mixing or leakage was observed with cells expressing these mutations (8).

It has been reported that fusion is a leaky process even with wild-type influenza HA and that some leakage occurs in the initial stages of fusion before the fusion pore is formed (26–28). However, these leakages were recorded with electrophysiological or other more sensitive assays and are much smaller in magnitude. In none of these other studies was massive leakage of a fluorescent dye observed as we observed here for G13A-in-

**FIGURE 6.** Correlation of fusion activity (black bars), enthalpy of binding to lipid bilayers (gray bars), and lipid perturbation (represented by lipid order parameter $S_L$; white bars) of wild-type and mutant HAs and corresponding fusion domains. The data of wild type, E11A, and W14A are from Ref. 9. Error bars represent standard deviations from approximately 30 injections in the case of $\Delta\Delta$H and duplicate experiments in the case of $S_L$.

**TABLE 2**

ATR dichroic ratios of lipid methylene stretching vibrations and derived order parameters in absence and presence of bound wild-type and mutant fusion domains

|          | $P^\text{ATR}$ | $S_L$  |
|----------|----------------|--------|
|          | 2920 cm$^{-1}$ | 2850 cm$^{-1}$ |
| Lipid only | 1.36 ± 0.03 | 1.34 ± 0.03 | 0.34 ± 0.01 |
| WT       | 1.16 ± 0.06 | 1.12 ± 0.05 | 0.58 ± 0.02 |
| E11A$^a$ | 1.25 ± 0.06 | 1.23 ± 0.05 | 0.47 ± 0.01 |
| G13A     | 1.22 ± 0.05 | 1.21 ± 0.04 | 0.49 ± 0.01 |
| W14A$^a$ | 1.32 ± 0.05 | 1.32 ± 0.05 | 0.38 ± 0.01 |
| G1S      | 1.20 ± 0.07 | 1.18 ± 0.06 | 0.53 ± 0.03 |

$^a$ Data of wild type, E11A, and W14A are from Ref. 9.
duced fusion. Some of the early minor leakage previously observed with wild-type HA was attributed to “bystander” HAs that do not participate in the formation of the fusion pore (29). It is of course possible that our new leaky mutant also exhibits this additional early minor leakage, which, however, is completely dominated by the more massive leakage detected by CF loss from the targeted red blood cells. Apart from bystanders, it is formally possible that only some of the 5–10 RBCs that are bound to each CV-1 cell release their contents into the environment. However, we do not think that this mechanism is likely because we can show that most RBCs bound to CV-1 cells actually lose their contents upon acidification.

Leaky Fusion Mutant Has Shallow Boomerang Structure—The solution structure of the G13A fusion domain in DPC micelles revealed a kinked boomerang structure with a wide opening angle. The kink occurs around residues 12–14 but is by far not as pronounced as in the wild-type fusion domain (Fig. 5). Well defined helices extending from residues 2 to 12 and from residues 15 to 18 define the two arms of the boomerang. The angle subtended by these arms is ∼150°, which is much wider than the ∼105° angle subtended by the two arms of the wild-type fusion domain. There appears to be a good qualitative correlation between this opening angle of the boomerang-shaped fusion domain and the fusion phenotype. The wild-type and mutant F9A structures, which have no fusion impairment, have relatively acute angles (6, 9). The non-fusion mutant G1V and W14A structures are either linear or flexible with no defined boomerang opening angles (8, 30). The new structure of G13A with the leaky fusion phenotype falls in between these two more extreme situations. It has a fixed opening angle leading to lipid mixing, but the angle is too shallow to promote the opening of a clean non-leaky fusion pore. For comparison, the structure of G1S, i.e. the only hemifusion mutant for which its structure has been determined so far, also has an acute opening angle almost like the wild-type and F9A structures (30).

Fusion Phenotype and Fusion Domain Structure Correlate with Strength and Mode of Lipid Interaction—Having established a correlation between the opening angle of the boomerang structure and the fusion phenotype, it is of interest to know whether the changes in structure can be linked to changes in lipid interactions that ultimately must be responsible for the different observed fusion phenotypes. The two lipid interaction parameters that we investigated indeed demonstrate a link between structure and fusion. Perturbation of the lipid order increased as the subtended fusion domain angle decreased, and the fusion activity increased from the non-fusion mutants W14A and G1V to the hemifusion mutants E11A and G1S and the leaky fusion mutant G13A to full-fusion wild-type (Fig. 6 and Table 2). Therefore, although lipid order perturbation distinguishes between non-fusion, hemifusion/leaky fusion, and full-fusion mutants, it does not make the more subtle distinction between the leaky and hemifusion phenotypes at the accuracy of this experiment. However, measurements of the binding free energies of the various fusion domains to lipid bilayer model membranes go further and distinguish not only between fusogenic and non-fusogenic domains but also between hemifusion and leaky fusion domains. The hemifusion domain E11A exhibited a stronger binding energy to membranes than the leaky fusion domain G13A (Table 1). However, the enthalpy of binding was stronger for G13A than for E11A (Fig. 6). This means that the strong energetic component is opposed by a strong entropic component of G13A binding (Table 1). It is likely that this is due to more water being pulled into the membrane and becoming ordered in the hydrophobic membrane environment by the shallow angle G13A leaky fusion domain than by the steep angle full-fusion and hemifusion domains. This excess water in the lipid bilayer makes the membrane more leaky and ultimately more permeable to content dyes like CF.

In conclusion, we have discovered and described the first profoundly leaky fusion mutant of influenza HA. Although not present in a known circulating influenza virus strain, the G13A mutation has been very useful to characterize the molecular structures and lipid interactions that are required to promote efficient non-leaky membrane fusion for the case of influenza virus entry into host cells and indeed probably also other viruses because influenza virus still serves as the best studied model system for understanding the general mechanisms of enveloped virus entry into host cells. Combining the results from the current work with earlier studies on other fusion domain mutants, the steps of membrane interactions that lead to productive membrane fusion become quite clear: the influenza HA fusion domain needs to interact strongly with the lipid bilayer and fold into a boomerang-shaped structure in the membrane with an acute opening angle to produce a productive fusion pore. A shallower angle such as the one observed here for the G13A mutant leads to a weaker membrane interaction and softens the membrane to become permeable to small molecules like carboxyfluorescein. The steep angle by which the wild-type fusion domain inserts allows it to interact with the transmembrane domain of hemagglutinin in the viral membrane and thus fuse the two membranes in a clean non-leaky fashion (28), which cannot happen with the leaky fusion mutant. Therefore, the fusion domain must reach a certain critical angle and depth of membrane insertion to prevent content leakage into the environment during membrane fusion. It is clear from these studies that the structures and hydrophobic properties of fusion domains are fine tuned to strike a delicate balance between leaky and non-leaky fusion without becoming constitutively inserted as non-reactive transmembrane anchors without fusogenic activity.

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