Fusion Activity of Transmembrane and Cytoplasmic Domain Chimeras of the Influenza Virus Glycoprotein Hemagglutinin

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The role of the sequence of transmembrane and cytoplasmic/intraviral domains of influenza virus hemagglutinin (HA, subtype H7) for HA-mediated membrane fusion was explored. To analyze the influence of the two domains on the fusogenic properties of HA, we designed HA-chimeras in which the cytoplasmic tail and/or transmembrane domain of HA was replaced with the corresponding domains of the fusogenic glycoprotein F of Sendai virus. These chimeras, as well as constructs of HA in which the cytoplasmic tail was replaced by peptides of human neurofibromin type1 (NF1) or c-Raf-1, NF78 (residues 1441 to 1518), and Raf81 (residues 51 to 131), respectively, were expressed in CV-1 cells by using the vaccinia virus-T7 polymerase transient-expression system. Wild-type and chimeric HA were cleaved properly into two subunits and expressed as trimers. Membrane fusion between CV-1 cells and bound human erythrocytes (RBCs) mediated by parental or chimeric HA proteins was studied by a lipid-mixing assay with the lipid-like fluorophore octadecyl rhodamine B chloride (R18). No profound differences in either extent or kinetics could be observed. After the pH was lowered, the above proteins also induced a flow of the aqueous fluorophore calcein from preloaded RBCs into the cytoplasm of the protein-expressing CV-1 cells, indicating that membrane fusion involves both leaflets of the lipid bilayers and leads to formation of an aqueous fusion pore. We conclude that neither HA-specific sequences in the transmembrane and cytoplasmic domains nor their length is crucial for HA-induced membrane fusion activity.

Fusion of influenza virus with its target membrane is mediated by the viral envelope protein HA at low pH. Acidification converts the HA into a fusogenic conformation, thereby exposing the hydrophobic N terminus of the HA2 subunit (36). This fusion peptide, which is highly conserved among different influenza virus strains, is believed to interact with the target membrane, where it causes a transient destabilization of the lipid bilayer (33). While the relevance of this peptide for fusion has been well documented, the role of other HA sequences in fusion is less established. Recent experiments emphasize the relevance of the membrane-spanning (15) and cytoplasmic (12, 32) domains of HA for the formation of a fusion pore and virus infectivity. Replacement of the TMR by a GPI anchor suppressed the formation of an aqueous pore between HA-expressing cells and RBC ghosts, while membrane (hemi)-fusion was not inhibited (15, 17, 19, 25). Furthermore, deletion of the CT of HA has been suggested to affect fusion kinetics (12) and shown to modulate virus infectivity (14, 32). Thus, while the interaction of the fusion sequence with the adjacent membrane is sufficient to trigger membrane fusion, the TMR and possibly the CT are essential principally for formation and widening of the aqueous fusion pore.

It has been shown for several other enveloped viruses that modification of the membrane-spanning domain and the CT of the glycoprotein may affect fusion activity. Alterations of the TMR of the gp41 envelope glycoprotein of human immunodeficiency virus by various point mutations (10, 26) or its substitution by the respective domain of vesicular stomatitis virus G protein (26) decreased or abolished cell fusion. Truncation of the cytoplasmic sequence of gp41 blocked the fusion activity (26) and infectivity (7) of virus. Specific truncation of the cytoplasmic domain of the virus envelope protein of simian immunodeficiency virus enhanced (3, 29) or diminished (34) syncytium formation. Truncation of the COOH-terminal region (i.e., the CT) of the fusion protein F of paramyxovirus SV5 led to a decrease of cytoplasmic content mixing activity, which correlated with the extent of the deletion (2). However, modification of those domains does not necessarily affect fusion activity. For example, while deletion of the entire cytoplasmic domain of the fusion protein of human parainfluenza virus type 3 did eliminate cell fusion activity (37), it was not impaired in a mutant of human parainfluenza virus type 2 with a truncated CT (37).

Whether for influenza virus HA the presence of any transmembrane and cytoplasmic domains per se is sufficient to trigger formation of an aqueous fusion pore or whether the specific HA sequences required for complete fusion have not been addressed systematically. Earlier studies suggested that the parallel replacement of both domains of the wt HA by related domains of another enveloped virus fusion protein does not inhibit fusion activity (6, 30). However, those studies left open whether both domains form a functional entity and whether the replacement of only one domain would affect fusion. Moreover, it remains to be elucidated whether the kinetics of fusion mediated by chimeric constructs with changed transmembrane and/or cytoplasmic domains were altered. Here, we addressed these questions by investigating the fusogenic ability of various chimeric HA. In one set of chimeras, the CT and/or TMR of HA (subtype H7) was substituted by the corresponding domains of the glycoprotein F of Sendai
We expressed wt HA and the various chimeric protein constructs in monkey kidney cells (CV-1) by using the vaccinia virus-T7 polymerase transient-expression system (1, 9, 28). This transient-expression system allowed us to efficiently screen chimeras for differences in their low-pH-induced fusion activity with RBCs as targets. By fluorescence microscopy and spectroscopy, both membrane mixing and the formation of an aqueous fusion pore were assessed by double labeling of RBCs with appropriate fluorophores, the lipid analog R18, and the water-soluble fluorophore calcein. Our results show that neither sequence specificity nor a certain length of the CT is required for HA to induce membrane fusion or pore formation.

### MATERIALS AND METHODS

**Abbreviations used in this paper.** aa, amino acids; BSA, bovine serum albumin; CT, cytoplasmic tail; DMEM, Dulbecco’s modified Eagle’s medium; DMEM+ , DMEM supplemented with 5% FCS; DMEM− , DMEM without FCS; DMEM/met− , methionine-deficient DMEM; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; FDQ, fluorescence dequenching; GPI, glycosylphosphatidylinositol; HA, influenza virus hemagglutinin; PB, phosphate-buffered saline; PBS, pH 7.4 phosphate-buffered saline; RBC, human erythrocytes; R18, octadecyl rhodamine B chloride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TMR, transmembrane region; WGA, wheat germ agglutinin; wt, wild type.

**Materials.** The fluorophores R18 and calcine-AM were purchased from Molecular Probes; and Trans[113]I]-label (>1,000 Ci/mmol) was purchased from ICN Radiochemicals. The enzymes used in molecular cloning were obtained from New England Biolabs. DMEM, trypsin versene, and FCS were purchased from BioWhittaker; DMEM without L-methionine and L-glutamine, L-glutamine, 2YT medium, and Lipofectin reagent were purchased from Life Technologies Inc.; protein A-Sepharose beads were purchased from Pharmacia Biotech. AmpliTaq DNA polymerase was obtained from Perkin-Elmer. The ESite PCR-based site-directed mutagenesis kit was purchased from Stratagene; the Sculptor in vitro mutagenesis system was purchased from Amersham. Oligonucleotide primers used for sequencing and PCR were synthesized by Pharmacia Biotech and Gibco BRL. Neuraminidase (VCNA from Vibrio cholerae) was obtained from Behringwerke AG, trypsin-EDTA solution (0.05% trypsin and 0.02% EDTA in PBS) was purchased from Biochrome KG, and secondary antibody was purchased from Dakopatts. Cell culture dishes were purchased from Nunc (Biochrome KG).

**Recombinant DNA procedures.** All basic DNA procedures were done as described by Sambrook et al. (31). For transient expression in CV-1 cells, the F wt cDNA and the HA wt (subtype H7) cDNA were subcloned into the EcoRI site in the polylinker of the pTM1 vector (22), resulting in pTM/F and pTM/HA, respectively.

The H/FF chimera (see Table 1) was created by removing the sequence between the NdeI site at bp 1670 of the HA gene (followed by treatment with

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### Table 1. Sequences of the TMR and CT of the proteins used in this study

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*a* H/H/H, wt influenza virus HA subtype 7; H, from wt influenza virus HA; F, from the CT mutant of Sendai virus glycoprotein F with cysteine residues replacing Ser-530 and Gly-534 (28); HF, a combination of a large part of the TMR of HA (24 aa, toward the ectodomain) and a short part from the TMR of F (7 aa toward the CT); R, Raf81 (residues 51 to 131; 81 aa) of C-Raf-1, N, NF78 (residues 1441 to 1518, 78 aa) of human neurofibromin type1 (NF1). The table shows the designations for these proteins with the respective domains.

**b** The sequences are as follows: H, KNGNMRCTICI-COOH; F, YRLRRCMLMCNPDERIPDRTOTLEPKRHMOTGNGDFDAMAEKR-COOH; R, PSKTSNTIRFLNPQKRTTVNVRNGLS1HDCLMKALKVGRGLOPECCAVFRLHHEHGKKKDLWNTDAASILGEGELOFDL-COOH; N, RPFNPVKNSFDAA<String omitted>...
Klenow fragment) and the XhoI site at a polylinker downstream of the HA gene in pTM/H/A. The fragment of the F cDNA encoding the C-terminal 54 aa isolated after digestion of pTM/F with SspI and XhoI was then subcloned into the above construct. The H/H/F chimera was made from H/HF/F with the ExSite PCR-based site-directed mutagenesis kit (Stratagene) as specified by the manufacturer. The mutagenesis sense PCR primer was 5'-TAT AGA CTC AGA AGG TGT ATG CTA ATG TGT AAT C-3' and the antisense primer was 5'-CAC TAT AAT GAA CAC CAA GCC CAT TGC-3'.

The H/F/F and H/F/H chimeras (see Table 1) were produced by standard PCR protocols and the overlap extension technique (11). The internal sense PCR primers were 5'-GTG GCT ACA AAG ATG TGA TTA CGA TCA TAG TAG-3' (H/F/F) or 5'-ATC ATC GTG CTT AAG AAC GGA AAC ATG CGG-3' (H/F/H), and the antisense primers were 5'-TGA TCG TAA TCA CAT CTT TGT AGC CAC TAC TC-3' (H/F/F) or 5'-GTT TCC GTT CTT AAG CAC CAT GAT GAT GAT CAC T-3' (H/F/H). The external PCR sense and antisense primers were 5'-ATG AAC ACT CAA ATC CTG GTT TTC-3' and 5'-TGA GCC CTC TCG AGA TGC AG-3', respectively. The H/H/N and H/H/R chimeras were constructed by PCR as described recently (24). The primary sequence of all mutants and chimeras was verified by double-stranded DNA sequencing at the level of the final plasmid.

Cell culture and vaccinia virus-based expression of foreign genes. CV-1 cells were maintained as monolayer cultures in DMEM. Transient expression of HA as well as the chimeric proteins in CV-1 cells was performed as described previously by Fuerst et al. (9). Subconfluent monolayers of cells grown on 35-mm-diameter plastic dishes were infected with recombinant vaccinia virus vTF7-3 (9), which expresses T7 RNA polymerase, at a multiplicity of infection of 10 PFU per cell and incubated at 37°C for 60 min. The virus inoculum was removed, and the cells were washed once with DMEM and then transfected with 3 μg of the respective plasmid DNA, using Lipofectin as the transfecting agent as described by the manufacturer. At 4 h posttransfection, the cells were washed again with DMEM and further treated for metabolic labeling, FACS analysis, or fusion assays.

Isotopic labeling of polypeptides, immunoprecipitation, and SDS-PAGE. Cells expressing the recombinant glycoproteins were incubated in DMEM for 30 min at 4 h posttransfection and labeled with 50 μCi of Tras[35S]label in 600 μl of DMEM. After 3 h, the cells were washed once with cold PBS and lysed in ice-cold RIPA buffer (0.15 M NaCl and 20 mM Tris-HCl [pH 7.4] containing 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, and 10 mM iodoacetamide). Lysates were centrifuged at 14,000 × g for 5 min, and the resulting supernatants were immunoprecipitated.
overnight at 4°C with polyclonal antibodies directed against influenza A virus (subtype H7). Immunoprecipitates were analyzed by SDS-PAGE on 12% acrylamide gels and visualized by fluorography (1 day) with Kodak X-Omat AR films. Quantification of fluorograms was carried out with an Epson GT-9000 scanner and Biometra ScanPack 2.0 software.

**FACS analysis.** After being washed with DMEM + 5 h postinfection, CV-1 cells expressing viral protein were incubated overnight at 32°C in DMEM+. They were then washed three times with ice-cold PBS, removed from the dish by gentle scraping, and fixed with paraformaldehyde (5% in PBS) for 1 h on ice. After three washes with PBS containing 0.5% BSA, the cells were incubated with polyclonal HA antibody (anti-influenza A virus rabbit serum, diluted 1:200 in PBS–0.5% BSA) and then treated with a secondary antibody (fluorescein-conjugated porcine immunoglobulin directed against rabbit immunoglobulins, diluted 1:30). Unbound antibodies were washed out at every step with PBS supplemented with 0.5% BSA. Finally, the antibody-cell complexes were suspended in PBS and analyzed with FACS equipment and Lysys II software (Becton Dickinson, Heidelberg, Germany).

**Labeling of RBCs with R18 and calcein-AM.** Human RBCs were labeled with the lipid probe octadecyl rhodamine B chloride (R18) as described previously (20, 21). A 10-µl sample of R18 (2 mM in ethanol) was added to RBCs (1% hematocrit in 5 ml of PBS) under vortexing. After the addition of 5 ml of PBS and incubation for 30 min at room temperature in the dark, 20 ml of DMEM + was added to the suspension to absorb unbound probe. After further incubation at room temperature in the dark for 20 min, the RBC suspension was washed five times in 40 ml of PBS, and each wash was followed by centrifugation. The last washing step was in PBS containing 1 mM CaCl₂ and 1 mM MgCl₂ (PBS11). The cells were kept in PBS11 at 4°C for up to 48 h. The fluorescence intensity of intact labeled RBCs due to R18 self-quenching was 25 to 30% of that of labeled RBCs treated with 0.5% Triton X-100 (100% fluorescence because of infinite dilution of R18). Double labeling was performed by the method of Morris et al. (21) with slight modifications. Briefly, calcein-AM (1 mM in di-
methyl sulfoxide) was added to R18-labeled RBCs (10% in PBS) to a final concentration of 20 mM. After incubation for 30 min at 37°C in the dark, the RBC suspension was washed and further incubated in PBS for 20 min at 37°C in the dark. The wash in PBS was repeated twice, and doubly labeled RBCs were kept in PBS at room temperature until use within 2 h.

Binding of fluorescently labeled RBCs to cells expressing viral proteins. After being washed with DMEM 2.5 h postinfection, the cells were incubated overnight at 32°C in DMEM, washed once with DMEM (without serum), and incubated for 1 h at 37°C with 100 mL of neuraminidase per mL in DMEM. A 1-mL volume of labeled RBCs (0.2% hematocrit) in PBS was added to the monolayer and incubated for 15 min at room temperature in the dark with occasional gentle agitation. Unbound RBCs were removed by five washes with PBS.

R18 fusion assay. R18 RBC-acceptor cell complexes were suspended by brief treatment (2 min at room temperature) with 100 mL trypsin-EDTA solution (pretempered to 37°C), subjected to repeated washings with PBS at 4°C, and placed on ice until further use.

At the beginning of each measurement, 30 mL of the R18-labeled RBC acceptor cell suspension was placed in a cuvette containing 1.97 mL of prewarmed buffer (37°C) at the desired pH and kept stirred by a magnetic Teflon-coated stirrer. FDQ of R18 was measured continuously by using a spectrofluorimeter (SLM Aminco-Bowman, series 2) with 0.5-s time resolution at excitation and emission wavelengths of 560 and 595 nm, respectively. A 570-nm cutoff filter was placed in the emission optical pathway to reduce scattering. To normalize the data, the percent FDQ (%FDQ) at any time point was calculated from the equation:

\[ \%FDQ = \left( \frac{F(t) - F_0}{F_T - F_0} \right) \times 100 \]

where \( F_0 \) and \( F(t) \) are fluorescence intensities at time 0 and at a given time point \( t \) and \( F_T \) is the fluorescence intensity in the presence of 0.5% Triton X-100, defined as the fluorescence at “infinite” dilution of the probe (20, 21).

Fluorescence microscopy. After binding of doubly labeled RBCs to acceptor cells, complexes on the dish were studied at room temperature. By using the inverted microscope Axiovert 100 (Zeiss) with a 40× objective (LD Achromplan; Zeiss), fusion was observed after PBS++ (pH 7.4) was replaced by NaAc++ (20 mM NaC2H3O2 plus 150 mM NaCl containing 1 mM CaCl2 and 1 mM MgCl2 [pH 5.0]). Calcein fluorescence was monitored with a “blue” filter set (450- to 490-nm excitation filter, long-path 520-nm emission filter). R18 fluorescence was visualized with a “green” filter set (510- to 560-nm excitation filter, long-path 590-nm emission filter). Photomicrographs were taken with an MC 80 microscope camera (Zeiss) on an Ektachrome EPH P1600x color reversal film for push processing (Kodak) at 3,200 ASA.

Binding of RBCs to CV-1 cells by lectin and fusion assay. Control experiments were performed to exclude fusion induction by vaccinia virus proteins as follows. Cells expressing only vaccinia virus proteins or, additionally wt HA were incubated for 5 min at room temperature with 500 mL of WGA (8 mg/mL; Sigma) in PBS, and 500 mL of labeled RBCs (0.2% hematocrit) was added. After further incubation for 15 min at room temperature in the dark under occasional gentle agitation, unbound RBCs were removed by five washes with PBS. Fusion was monitored by fluorescence microscopy as described above.

RESULTS

Expression and characterization of HA and HA chimeras in CV-1 cells. wt HA and chimeric proteins were expressed in CV-1 cells with the vaccinia virus-T7 polymerase transient-expression system.

In Table 1, the sequences of the TMRs and of the CTs of both HA of influenza virus (subtype H7) and glycoprotein F of Sendai virus, as well as the sequences of the peptide fragments
derived from the cytoplasmic proteins neurofibromin (NF78) and c-Raf-1 (Raf81), are shown. These peptides were chimerically combined with HA, replacing its cytoplasmic domain. HF is a combination of the complete transmembrane domain of HA and a short part of the TMR of F (see Materials and Methods). In Table 1, all the studied proteins are shown with their respective chimeric composition and designation.

The results of labeling experiments with [35S]cysteine/methionine indicate that all proteins were expressed at comparable levels to wt HA (H/H/H) except for H/H/F, which was expressed at a slightly lower level, and cleaved into two subunits irrespective of their composition in the TMR or CT (Fig. 1). Further control experiments included a cross-linking study with dithio-bis(succinimidylpropionate), glycosidase treatment, and assessment of surface expression by cytometric analysis. The results demonstrated that all protein species were expressed as trimers, which were processed properly during biosynthetic glycosylation (data not shown). Interestingly all chimeras were palmitoylated, although the acylation efficiency differed between constructs (24, 28). Flow cytometry measurements revealed that the chimeras were expressed with a lower transfection efficiency than was wt HA, except for H/F/H and

FIG. 5. Kinetics of FDQ of R18-labeled RBCs bound to transfected CV-1 cells at different pHs. Typical time courses of the fusion of CV-1 cells expressing H/H/H or the H/F chimeric constructs with R18-labeled human RBCs at 37°C are shown. The cell-RBC suspension was added to the buffer in the cuvette at the respective pHs as indicated, and fusion monitoring started immediately (t = 0). At t = 590 s, Triton X-100 was added for infinite dilution of the fluorophore. The kinetics were normalized as described by Morris et al. (20). The fluorescence intensity at pH 7.4 was set to 0%, and fluorescence intensity after the addition of Triton X-100 was set to 100% FDQ (for details, see Materials and Methods). The results of two independent experiments with wt HA (H/H/H) are presented to show the experimental variability.
found a tendency for slower lipid mixing. The fluorescence observed a smaller fusion extent, and in some preparations we H/H/R, as shown for pH 5.0 in Fig. 6. For both constructs, we observed similar time courses of FDQ for H/H/N and an intermediate pH value (pH 5.8) resulted in a slower increase of R18 fluorescence intensity, reaching a plateau after about 100 s, was observed (Fig. 5). Measurements at steep increase of R18 fluorescence intensity was observed after the cell membrane between different transfections. It has been established that the density of HA molecules in the plasma membrane considerably influences the lag time (time between triggering and onset) as well as the initial rate (highest rate of membrane fusion mediated by influenza virus HA (5, 18)).

**Kinetics of membrane fusion.** Since no difference between the fusogenicity of the various proteins was apparent from these microscopic studies, we also compared the kinetics of membrane fusion induced by chimeras with that of wt HA at various pHs. In Fig. 5, the typical kinetics are shown for wt HA (H/H/H) and the chimeric constructs between HA and F at pH 4.8, 5.8, and 6.7 and 37°C. For all the proteins, no significant increase of R18 fluorescence intensity was observed after the addition of RBC-CV-1 cell complexes to the buffer set to pH 6.7 (Fig. 5). On the other hand, immediately after addition of the RBC-CV-1 cell complexes to the buffer set to pH 4.8, a steep increase of R18 fluorescence intensity, reaching a plateau after about 100 s, was observed (Fig. 5). Measurements at higher immediately pH value (pH 5.8) resulted in a slower increase of the R18 fluorescence for all the studied proteins (Fig. 5). We observed similar time courses of FDQ for H/H/N and H/H/R, as shown for pH 5.0 in Fig. 6. For both constructs, we observed a smaller fusion extent, and in some preparations we found a tendency for slower lipid mixing. The fluorescence increase is caused by the relief of R18 self-quenching due to redistribution of the lipid-like fluorophore between the RBC and CV-1 cell membrane upon fusion of plasma membranes. The normalized data show that in all cases fusion activity was established with about the same kinetics but with some differences in the extents, most probably due to the different expression levels, in particular for H/H/N and H/H/R (Fig. 2; Table 2).

**DISCUSSION**

The use of chimeric constructs offers the opportunity to search for dependencies of protein-induced membrane fusion...
on specific amino acid sequences and properties of the transmembrane and/or cytoplasmic domain of the fusion protein. We have constructed chimeras of the well-characterized fusion protein of influenza virus, HA, with distinct alterations in its membrane-spanning and cytoplasmic domain, and studied the fusion properties after expression. The domains in question were replaced in a combinatorial fashion by the respective parts of the fusion protein of Sendai virus, F. Although the two proteins are related in their functions, the sequences and physical properties of their transmembrane and cytoplasmic domains differ considerably (Table 1). In addition, we studied two HA chimeras which contained peptides derived from totally unrelated soluble cytoplasmic proteins as their cytoplasmic domains of HA. wt HA and all constructs were expressed on the plasma membrane of the transfected CV-1 cells as trimers and showed the appropriate posttranslational modifications. Serious differences were observed only in the degree of palmitoylation. However, reports from other groups and our previous studies had shown that this hydrophobic modification has no major effect on either membrane fusion or formation of an aqueous fusion pore mediated by influenza virus HA (13, 27). Thus, for both of these events, the influenza virus HA requires neither its own specific transmembrane and cytoplasmic domain sequences nor any particular length of its CT. It has been reported that a GPI anchor is not sufficient for promoting the formation of fusion pores (15), which indicates that at least the membrane-spanning domain is required. On the other hand, our data suggest that this is due not to a lack of specific transmembrane and/or cytoplasmic amino acid sequences but probably to the complete deletion of the TMR and CT in GPI-HA. The role of the CT in our system for fusion remains open. Recently, it has been shown for HA of another influenza virus subtype (H3) that this domain is not required for membrane mixing and formation of a small aqueous pore (13).

Recent models of HA-mediated fusion emphasize the role of both domains, in particular of the TMR, for the formation and widening of an aqueous fusion pore (35, 38). One of these models describes a lipidic “stalk” as an intermediate which is formed by merging of the contacting outer monolayers of the fusing membranes, the so-called hemifusion diaphragm (4, 38). Destabilization of this diaphragm is necessary for the formation and widening of an aqueous fusion pore as the successful completion of the fusion event. As suggested by Melikyan et al. (17), it is probably the mechanical coupling of the HA ectodomain to the TMR which causes such a destabilization. The diaphragm can be also disturbed by enhancement of the positive spontaneous curvature of the nonfused inner leaflets (reviewed in reference 4). Whatever the detailed molecular mechanism is, our results imply that this mechanical coupling does not require the specific sequence of TMR and CT of wt HA.

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