INFLUENZA HEMAGGLUTININS OUTSIDE OF THE CONTACT ZONE ARE NECESSARY FOR FUSION PORE EXPANSION

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Abstract. Current models for membrane fusion in diverse biological processes are focused on the local action of fusion proteins present in the contact zone, where the proteins anchored in one membrane might directly interact with the other membrane. Are the fusion proteins outside of the contact zone just bystanders? Here we assess the role of these “outsider” proteins in influenza virus hemagglutinin mediated fusion between red blood cells and either hemagglutinin-expressing cells or viral particles. To selectively inhibit or enhance the actions of hemagglutinin outsiders, antibodies that bind to hemagglutinin and proteases that cleave it were conjugated to polystyrene microspheres too large to enter the contact zone. We also involved hemagglutinin outsiders into interactions with additional red blood cells. We find the hemagglutinin outsiders to be necessary and sufficient for fusion. Interfering with the activity of the hemagglutinin outsiders inhibited fusion. Selective conversion of hemagglutinin outsiders alone into fusion-competent conformation was sufficient to achieve fusion. The discovered functional role of fusion proteins located outside of the contact zone suggests a tempting analogy to mechanisms by which proteins mediate membrane fission from outside of the fission site.
Introduction

The surface of the influenza virus particle is covered with molecules of hemagglutinin (HA), the fusion protein whose low-pH–activated conformation drives fusion between the viral envelope and the membrane of an acidified endosome. This prototypical fusion reaction (1-3) results in delivery of viral RNA into the cytosol of the infected cell. A significant fraction (if not the majority) of HAs at the surface of the viral particle cannot directly interact with the membranes of endosomes and multivesicular bodies (4). Indeed, the inter-membrane contact zone (CZ) usually involves only a fragment of the viral surface, so that some of the HAs, referred here to as ‘insiders’, are situated inside the CZ, while the rest – the ‘outsiders’ – have no access to the target membrane. However, both outsiders and insiders undergo similar restructuring at the pH of fusion (3). In this work we tested the functional role of HA outsiders and found them to be required for driving fusion towards the opening of an expanding fusion pore.

Experimental Procedures

HA-expressing cells (X31 HA-cells, HA300a (5) and Japan HA-cells, HAb2 (6)) were grown as previously described. In many experiments, HAb2 cells were pre-treated with 9-mM sodium butyrate (NaBut) for 24 hours to boost HA expression (7,8). If not stated otherwise, HA0 expressed at the cell surface was cleaved from HA0 to the fusion-competent HA1–HA2 form by trypsin (5 µg/ml, Fluka, Buchs, Switzerland) for 10 min at room temperature as in (9). We labeled human red blood cells (RBCs) with lipid dye PKH26 and with contents dyes 6-carboxyfluorescein (CF) or fluorescent Dextran (FD,10 KD or 70 KD; Molecular Probes, Eugene, OR) using mild hypotonic lysis (9,10). All
fusion experiments were performed at room temperature. HA-cells were incubated with RBCs for 15 min and washed three times with PBS to remove the unbound RBCs. If not stated otherwise, 0–2 RBCs were bound per HA-cell. To measure RBC binding to cells, we selected several areas of the dish and screened at least 200 cells to find the average number of RBC bound to each HA-cell.

HA-cell–RBC pairs were bathed in low-pH solution (PBS titrated with citrate) for a stated time. After the end of a low-pH pulse, fusion extents (lipid and content mixing) were assayed by fluorescence microscopy as the percentage of dye-redistributed cell pairs (9). Restricted hemifusion was detected by chlorpromazine (CPZ) application after the low pH pulse as in (11). For experiments with multiple erythrocytes, we mixed labeled and unlabeled RBCs at a ratio of 1:10, using the same concentration of labeled RBCs as above. Each HA-cell had 0–2 labeled and 10–20 unlabeled RBCs bound to it. Influenza virus (Japan and X31 strains) was purchased from Charles River (Preston, CT) and labeled by membrane dye R18 as described in (12). Fusion of virus with RBC was assayed as R18 dequenching.

In our work we used several antibodies against HA. Rabbit antiserum (FP–AB) against fusion peptide of HA recognizes low pH forms of both X31 and Japan HA (8). Monoclonal antibodies FC125 against neutral pH conformation of Japan HA were a gift from Dr. T. J. Braciale, University of Virginia, Charlottesville, VA. The specific epitope of these antibodies has not been identified. LC89 and HC67 antibodies were both a gift from Dr. J. J. Skehel, NIMR, London, UK. HC67 antibody with an epitope at residue HA1 193 binds only to the neutral pH form of X31 HA (13). The binding site of LC89 antibody includes residue HA2 107 of X31 HA and is exposed only in the low pH form.
of HA (13,14). We also used antibodies against the neuraminidase of influenza virus H3N2 (# 11-227, Clone ST9D2, Argene Inc., Massapequa NY) and fibroblast growth factor receptor (Ab-1, Cat GR21, Oncogene Research Products, La Jolla, CA). Each of these antibodies, as well as thermolysin and trypsin (both proteases from Sigma, St Louis, MO) were conjugated with beads (FluoSpheres carboxylate-modified microspheres: blue-fluorescent microspheres with diameters of 20 nm; 100 nm or 2 µm; Molecular Probes, Eugene, OR) as suggested by the manufacturer. The size of the beads was verified by quasi-elastic light scattering. Bead-conjugated agents were applied to pre-bound membranes either 5 min prior to a low-pH pulse or immediately after it.

In some experiments to evaluate tightness of the CZ we used Quantum dots (Qdot 525 Streptavidin Conjugate, Quantum Dot Corporation, Hayward, CA) and streptavidin Alexa Fluor 488 conjugate (Molecular Probes, Eugene, OR). Surface biotinylation of RBC (4% hematocrit) was performed with 1 mM EZ-link Sulfo-NHS-LC-Biotin (Pierce Chemical Co., product 21335) in PBS for 30 min at 22°C. Biotinylated RBCs were washed twice by 4-min centrifugation at 1500 rpm.

Confocal Microscopy. Live HAb2 cells with bound RBCs were analyzed using Zeiss LSM 510 META confocal scanning microscope (Zeiss, Germany). The microscope settings were as follows: excitation at 458 (in experiments with Qdot 525 Streptavidin Conjugate) or 488 nm (in experiments with streptavidin Alexa Fluor 488 conjugate), emission at 505LP into channel 2, Plan-Apochromat 100x/1.4 Oil DIC lens at an Airy disc setting of 2.0, 5x scan zoom, serial images were taken, spanning a whole RBC with
0.71 µm steps. To obtain linescan profiles images were converted into 16-bit tiff files and further analyzed using ImageJ 1.31v software package (Wayne Rasband, NIH, USA).

**Electron microscopy.** HAb2 cells with bound RBCs were fixed, post-fixed through alcohol-acetone dehydration and epon-araldite infiltration steps, and thin sectioned with an LKB Ultrotome V. After staining with uranyl acetate and lead citrate, the sections were analyzed in a Philips CM-10 transmission electron microscope. The micrographs were analyzed to measure the dimensions of the CZ. In all sections, the diameter of the CZ was more than 90% of the maximum dimension of the RBC. Transmembrane distance, d, was measured uniformly each 40-50 nm apart along at least 5 independent CZs. NaBut pre-treatment of the HAb2 cells and variation of the number of bound RBCs (single or >10/HA-cell) changed neither the area of the CZ nor the mean d (for instance, d=13.5+/-7 nm and 12+/- 3 nm for RBC at either HAb2 or NaBut-treated HAb2 cells).
Results

Experimental Approach. To study the functional role of HA outsiders we have focused on the experimental system of HA-cell –RBC fusion, which is particularly convenient for several reasons. First, the pathway of low pH-triggered fusion can be followed here as the transfer of membrane and aqueous dyes from pre-labeled RBCs to HA-cells (5,9,15). Second, the HA-cell–RBC fusion can be manipulated by anti-HA antibodies (16,17) and by proteases such as trypsin and thermolysin ((9) and references therein). And, finally, as seen in Fig. 1A,B, HA-cells and bound RBCs establish extended CZ with area on the order of tens of square microns (see also (6,7,18)) that are characterized by a relatively constant intermembrane distance of ~13 nm that is close to the height of the HA ectodomain (3). Readily distinguishable pools of insider and outsider HAs in this system facilitate characterization of their relative fusogenic activity.

To differentiate between the fusogenic actions of the insider and the outsider HAs, we subjected the latter to different binding and cleaving agents, while leaving the former intact. To achieve this selective effect, we conjugated different antibodies and proteases to polystyrene beads of diameters significantly exceeding the 13-nm transmembrane distance in the CZ. If not stated otherwise, we used beads of 20 nm diameter. Using fluorescence microscopy we verified that fluorescent 20-nm beads with conjugated HC67 antibody added to X31 HA-cells with pre-bound RBCs are excluded from the CZ (Fig. 1C). HA-cell/RBC pairs were incubated with bead-antibodies for 15 min and then washed to remove unbound beads. While HA-cell was fluorescent due to bound beads, patch of an HA-cell under bound RBC (arrow in the figure) was seen as a non-fluorescent black patch.
Tightness of the CZ and its inaccessibility for macromolecules within 5-min time relevant for our experiments was further confirmed by another experimental approach. We surface biotinylated RBCs and brought them into contact with Japan HA-cells. HA-cell/RBC pairs were then incubated with fluorescent streptavidin. Finally we removed unbound streptavidin by washing and analyzed HA-cell/RBC pairs under confocal microscopy. In this experiment we have chosen to biotinylate and, thus, label, RBC rather than HA-cell because this gives more homogeneous labeling. When HA-cell/ biotinylated RBC pairs were incubated with fluorescent streptavidin for 1-hour most of the CZs demonstrated significant fluorescence (Supplemental data, Fig. 1S). In contrast, after 15-min incubation fluorescence of the CZ was less than 2.5% of that of the RBC membrane outside of the CZ (Fig. 1D,E). Thus, at the times relevant for our experiments (5 min-incubations with bead-agents that give fusion inhibition and promotion described below) tightness of the CZ restricts the entry even for a free 60KD protein.

Using similar approach we also found the CZ inaccessible for the smallest Quantum dots (Qdot 525 Streptavidin Conjugate) (Supplemental data, Fig. 2S). According to the manufacturer these Qdots have the effective average diameter of less than 8 nm. HA-cell/ biotinylated RBC pairs were incubated with the Quantum dots for 30 min. After removal of unbound Qdots we found CZ fluorescence indistinguishable from the background and less than 2.5% of the fluorescence of RBC membrane outside of the CZ. Thus, after relevant incubation times the smallest Qdots and even 60 KD streptavidin cover less than 2.5% of the CZ area indicating that the proteins conjugated to 20 nm beads might directly interact only with a negligible fraction of HA insiders.
To evaluate whether the effects observed after application of bead-conjugated agents were specific for HA of a particular strain of influenza virus or instead reflected more general properties of HA fusion, we used cells expressing HA of two different influenza subtypes, H3 (A/Aichi/2/68:X31 strain) and H2 (A/Japan/305/57 strain), which differ in their patterns of activation (8,19). Since HA0 form of HA expressed on HA-cells is fusion-incompetent, if not stated otherwise, prior to our experiments HA0 was trypsin-cleaved in fusion-competent HA1-HA2 form. As expected in all experiments presented below neither lipid nor content mixing were observed when HA-cells/RBC complexes were not treated with low pH.

*HA outsiders are necessary for fusion.* We interfered with the HA outsiders by incubating pairs of RBCs and HA-cells with bead-conjugated HA-inhibiting agents, applied either before or after low-pH treatment. We used antibodies against low-pH forms of HA (FP–AB that recognizes both X31 and Japan HA, as well as LC89 antibodies against the X31 strain of HA), antibodies against neutral-pH HA (HC67 and FC125 antibodies against the X31 and Japan strains of HA, respectively), and thermolysin, which cleaves only low-pH forms of HA of both the X31 and Japan strains (8).

The bead-antibodies (bead- FP–AB and bead LC89 antibodies) against low-pH forms of HA inhibited the mixing of both lipids and contents of the fusing cells. The specificity of the inhibition corresponded to the specificity of our bead-agents: inhibition was observed only when the agents were applied to low-pH–treated X31 HA-cell–RBC pairs (Fig. 2A). The pairs were incubated with the bead-antibodies immediately after the
low-pH pulse. Fusion inhibition by bead-FP–AB applied after the low pH pulse was observed not only for 20 nm beads but also for 100 nm beads (Fig. 2B). Since by the time the inhibiting agents were added, the fusion had already commenced, the inhibition seen in Fig. 2A,B reflects both the effectiveness of the bead-inhibitors and the rapidness of their action.

Bead-FP–AB similarly inhibited Japan HA-mediated fusion. For Japan HA-cell/RBC pairs treated with 1-min pulse of pH 4.9 medium bead-FP–AB applied immediately after low pH pulse inhibited lipid mixing from 49.8+/-6.4 % to 5.1+/-1.7 % and content mixing assayed as CF redistribution from 19.2+/-2.9 to 0% (n>3).

Bead-antibodies against neutral-pH HA inhibited fusion when added to the cells prior to a low-pH pulse (shown in Fig. 2C,D for bead-FC125 antibody and Japan HA-cells). Similar results were observed for bead-HC67 antibody and X31 HA (Supplemental data, Fig. 3S). Importantly, fusion inhibition by bead-HC67 antibody was not accompanied by any decrease in the number of bound RBCs. After 30 min incubation of X31 HA-cell–RBC pairs with bead-HC67 antibody we found 0.21 bound RBC per HA-cell vs. 0.2 bound RBC/HA-cell in the experiment with no beads added. In another control experiment, application of bead-HC67 antibody to HA-cells prior to adding RBCs inhibited subsequent RBC binding to 0.02 bound RBC/HA-cell.

In Fig. 2C we varied the time of the pre-incubation of the Japan HA-cell–RBC pairs with the bead-FC125 antibody to test whether the inhibition can be explained by gradual extraction of HAs from the CZ driven by HA-antibody interactions. The lack of any increase in the inhibition of the content mixing when the time of the pre-incubation was increased from 5 to 60 min argues against this hypothesis.
The specificity of the inhibition was confirmed by the experiments, in which neither lipid nor content mixing was affected by treatment of RBC–HA-cell pairs with protein-free beads and with beads conjugated with immunoglobulins of the pre-immune serum (for instance, bars 3 in Fig. 2A). Fusion was also unaffected when beads with antibodies to HA were replaced with beads conjugated with antibodies against one of the abundant surface proteins of the HAb2 cells, fibroblast growth factor receptor (Supplemental data, Fig. 4S). To control for the possibility that the free agents released from the beads cause the inhibition, we also verified that treatment with supernatant from the fusion-inhibiting bead-agents did not change the fusion efficiency (for instance, bars 4 and 6 in Fig. 2A).

Different fusion phenotypes have different sensitivity to bead-FC125 antibody (Fig. 2D). Content mixing between Japan HA-cell and RBC was strongly inhibited by the bead-FC125 antibody. Lipid mixing, which can be observed even in the absence of content mixing (the ‘hemifusion’ phenotype (5)), was less sensitive to the bead-agents. Finally, bead-FC125 antibody did not suppress the early fusion phenotype referred to as ‘restricted hemifusion’ and detected by its transformation into complete fusion with agents such as CPZ that break the hemifusion structures (9,11,20). Since the number of low pH activated HAs required to establish given fusion phenotype increases from restricted hemifusion to lipid mixing to content mixing (11), we conclude that more advanced fusion phenotypes are more dependent on HA outsiders. A similar relative potency of the bead-inhibitor on different fusion phenotypes, i.e. strong inhibition for content mixing and no effect on restricted hemifusion, was also observed for X31 HA-mediated fusion (Fig. 2B).
Surface density of HA in the stable cell lines used in the experiments shown in Fig. 2A-D is significantly lower than that in viral particles (7,21). Does fusion at higher densities still depend on HA outsiders? A very high density of HAs (~12x10^3 trimers/µm^2) can be achieved by the pre-incubation of Japan HA-cells with NaBut (7,8). Lipid mixing between these HA-cells and pre-bound RBCs was very robust and almost insensitive to the bead-thermolysin. However, content mixing assayed as transfer of either CF or 10KD FD was strongly inhibited when the bead-thermolysin was applied after, but not before, a low pH pulse (Fig. 3A). Fig. 3B shows similar inhibition of 10KD FD transfer by the bead- FC125 antibody. However, in contrast to the bead-thermolysin, the bead- FC125 antibody inhibited fusion only when applied before low pH pulse. This difference was expected taking into account that while thermolysin interacts with low pH form of HA, FC125 antibody interacts with neutral pH HA.

Fusion inhibition by bead-conjugated FC125 antibody was also observed in fusion of RBC with influenza virus (Japan strain) particles, where HA density is close to the maximum level allowed by the size of HA (~15x10^3 trimers/µm^2). Since leakiness of the fusion of viral particles hinders application of content mixing assays (22), virus/RBC fusion was measured as lipid mixing which, as shown above, is less sensitive to bead-agents than content mixing. Indeed, at pH 4.9, because of an excess of fusion-competent HAs, lipid mixing was very robust and insensitive to inhibiting agents. However, at pH 5.3 we observed statistically significant ~35% inhibition of fusion (Fig. 3C,D). In our experiments on viral particles of X31 strain we observed similar inhibition. We also found that, in contrast to the bead-conjugated antibodies to HA, beads coupled with
antibodies to another glycoprotein of influenza virus envelope, the neuraminidase, had no effect on the rate of lipid mixing (Supplemental data, Fig. 5S).

Relatively small bead-agents can approach the edge of the CZ. In contrast, an RBC cannot possibly get close to the CZ between another RBC and an HA-cell. Additional RBCs bound to the same HA-cell can play the role of extra-large (~6-µm) HA-binding beads. If HA outsiders are indeed functional, fusion between an HA-cell and a particular RBC can be inhibited if additional RBCs are bound to the HAs of the same cell. To test this prediction, we compared fusion between HA-cell and a single labeled RBC, when this labeled RBC was the only RBC bound to the cell, with fusion observed when, in addition to a single labeled RBC, HA-cell was covered with many unlabeled RBCs (Fig. 4). To minimize the possibility that the binding of additional RBCs decreased HA density in the CZ between the labeled RBC and the HA-cell, we used NaBut–pre-treated HA-cells. While crowding RBCs around HA-cells did not affect redistributions of our lipid probe and a small aqueous dye CF, which are very robust at high HA densities, it clearly inhibited fusion assayed as transfer of larger aqueous probes such as FD of 10 KD and especially 70 KD (Fig. 4A). Thus, the larger the pores, the stronger were the inhibiting effects of additional RBCs.

Interestingly, inhibition of fusion pore expansion by the additional RBCs also radically changed the morphology of the fusion product (Fig. 4B). In contrast to fusion with a single bound RBC resulting in a quick flattening of the RBC membrane, additional RBCs stabilized the configuration in which the fused RBCs with redistributed membrane dye remained clearly distinct rather than fully flattened.
In contrast to bead-conjugated agents, additional RBCs not only interact with HA outsiders but also fuse with HA-cell and, thus, provide additional membrane area. To verify that the latter effect is not required for the observed inhibition of fusion pore expansion we replaced additional RBCs with large 2-μm diameter beads. NaBut–pre-treated Japan HA cells with bound RBC (0–2 RBCs per HA-cell) were incubated with 2-μm diameter polystyrene beads with conjugated FC125 antibodies for 10 min, and then treated with a 1-min pulse of pH 4.9. Application of these very large beads with conjugated antibody decreased content mixing (10KD FD) extent to 44.8+/-5.8% vs. 79+/- 7.8% in the control experiment with no beads added and 83.8+/- 6.6% (n>3) in the control experiment were HA-cell/RBC pairs were pre-incubated with the supernatant from the beads rather than with the beads themselves.

The effects of the additional RBCs and FC125 antibody conjugated to very large beads indicate that direct interaction between HA-binding agents and HAs located at the very edge of the CZ is not required for the fusion inhibition.

In summary, fusion inhibition by the agents too large to enter the CZ indicates that HA outsiders are required for the transition from early fusion intermediates to an expanding fusion pore. For bead-conjugated HA-targeting agents and for additional RBCs, fusion inhibition was never complete, suggesting either that fusion depends only partially on the presence of HA outsiders or that, very likely, fusogenic activity of HA outsiders was never fully blocked by our inhibiting agents.

HA outsiders are sufficient for fusion. To test whether HA outsiders are sufficient for fusion, we kept HAs inside the CZ fusion-incompetent. As mentioned above, prior to
low pH application, HA expressed at the surface of HA-cells has to be cleaved by trypsin from the HA precursor HA0, which is competent for receptor-binding but not for fusion, into the fusogenic HA1-HA2 form (3). NaBut–pre-treated cells with uncleaved HA0s were brought into contact with RBCs and then incubated for 2 min with bead-trypsin to selectively bring HA outsiders into the fusion-competent form. Surprisingly, a 5-min and even a 2-min low-pH pulse applied immediately after the bead-trypsin treatment produced robust fusion (Fig. 5). These results indicated that the HA outsiders are sufficient for fusion.

**Discussion**

In many examples of ubiquitous protein-mediated membrane fusion reactions at the time of fusion only a fraction of all fusion proteins is located in the immediate vicinity of the target membrane. In this work we show that prototype fusion reaction, fusion mediated by HA, depends not only on HAs that at the time of low pH application were present in the CZ, but also on HAs located outside of the CZ. Interfering with the activity of HA outsiders by different HA binding- and cleaving- agents (antibodies and proteases conjugated to beads of 20 nm-, 100 nm- and 2 μm- diameters and additional RBCs) that were too large to enter the CZ within relevant times inhibited the transition from early fusion intermediates to an expanding fusion pore in fusion mediated by HAs of two divergent influenza subtypes. Selective conversion of HA outsiders alone into fusion-competent conformation produced fusion. These findings indicate that HA outsiders are required and may be sufficient for fusion.

*Functional role of HA outsiders.*
What may be the mechanism of the bead-agent modulation of fusion? This modulation cannot be explained by non-specific covering of the HA-cell surface with bound beads. Covering the HAb2 cells with the 3-fold more of control beads conjugated with pre-immune serum (assayed as cell-associated fluorescence) did not affect fusion. In addition, the inhibition induced by the covering of the surface would not explain why bead-thermolysin inhibits fusion only when applied after a low-pH pulse, and bead-trypsin promotes fusion.

Another possibility is that fusion modulation by the bead-agents involves changes in the number and/or properties of the HA insiders mediated by an exchange of HAs between the CZ and the surrounding HA-cell membrane. Fusion inhibition by replacing HA insiders with thermolysin-cleaved fusion-incompetent HA outsiders would imply that functional fusion machines are formed by a dynamic assembly of HAs initially spread over very large area of the membrane. However, kinetic considerations argue against this mechanism. The agents, that target HA outsiders, act very fast. For instance, under the experimental conditions in Fig. 3A content mixing reaches final extents within 2 min. Still bead-thermolysin, added already after the first minute at pH 4.9, greatly inhibited content mixing. This leaves rather limited time for the exchange between HA outsiders and insiders, most of which are expected to be bound to the receptors on the RBCs (23,24) and, thus, probably have greatly reduced mobility (25). Low pH application additionally decreases mobility of activated HAs (26).

Fusion inhibition by the bead-antibodies against neutral pH form of HA also cannot be explained by the HA-exchange mechanism suggesting that bead-antibodies shift the equilibrium distribution between the insider and outsider HAs and, in effect, extract the
insiders from the CZ. Since HA mediates binding between HA-cells and receptors at RBC, extraction of the HAs from the CZ should result in decrease of this binding. However we observed no decrease in the number of bound RBCs after 30 min incubation of X31 HA-cell–RBC pairs with bead- HC67 antibody. Furthermore, any extraction of HAs from the CZ to allow them to interact with the antibodies or additional RBCs should increase the surface density of HA outsiders. When the initial HA density cannot be increased, such HA redistribution is impossible. Therefore, our finding that bead-antibodies and additional RBCs inhibit fusion even at the surface densities of HA close to the maximum density allowed by the size of HA argue against HA extraction as a possible basis for the effects of bead-conjugated fusion-inhibiting agents. Finally, the assumption that bead-antibodies inhibit fusion by lowering the number of HA insiders is inconsistent with our finding that longer pre-incubation of HA-cell/RBC pairs with bead-antibodies against neutral pH form of HA, aimed at allowing HA insiders more time to leave the CZ, did not result in stronger inhibition of fusion (Fig. 2 C). This finding indicates that either HA insiders and outsiders do not exchange in the relevant time, or, assuming that mobility of HA insiders is not hindered by HA-receptor interactions, that fusion is insensitive to significant changes in the numbers of insiders (10-fold difference in the number of HA insiders between the experiments with 5- and 60- min long pre-incubations as estimated for the CZ of 3 µm-radius and the lateral mobility of HA in the absence of the target membrane ~3x10^{-10} cm^2/s (7)). Similarly, the lack of the increase in fusion extents upon extension of the time interval between bead-trypsin and low pH applications from 2 to 30 min (Fig. 5) argues against HA-exchange interpretation of the bead-trypsin experiments. If trypsin-cleaved HA outsiders enter CZ by lateral diffusion
and then, upon low pH application, mediate fusion from there, fusion efficiency has to be insensitive to ~9-fold difference in the number of HA insiders.

Since the evidence indicates that the exchange mechanisms cannot explain fusion modulation by the HA outsider-targeting agents, we conclude the fusion protein outsiders directly influence the most energy-intensive stages of fusion leading to opening and expansion of a fusion pore.

The observation that formation of early fusion intermediates such as restricted hemifusion is not affected by the HA outsider-targeting agents might indicate that fusion involves two radically different activities of the same fusion protein HA. First, local action of HA insiders generates local hemifusion. (In this scenario, in the absence of fusion-competent HA insiders, as in the experiment in Fig. 5, local hemifusion can develop only at the rim of the CZ.) Later fusion stages yielding the expanding fusion pore are driven by HAs located outside of the initial fusion site. The achieved fusion phenotype might depend on the total number of activated HAs including both insiders and outsiders. At the highest HA densities, only the most advanced fusion stage, an expansion of fusion pore, depends on HA outsiders and is inhibited by our bead-agents. For lower density of activated HAs, outsiders are required even for lipid mixing.

The functional role of HAs that at the time of the low pH application are located outside of the CZ goes against the accepted view that fusion is a highly localized phenomenon driven only by a few fusion proteins in the CZ that directly interact with the target membrane. At the same time, our results rationalize the interesting findings that the entry of many viruses (27) and exocytotic fusion (28) are inhibited by macromolecules,
which are added to pre-docked membranes and are, apparently, too large to rapidly enter the tight CZ.

Functional role of HA outsiders in our experiments might indicate that as in the case of vacuolar fusion (29) most of the HA-mediated fusion events develop along the circumference rather than in the central region of the CZ. If so, HA outsiders located near the periphery of the CZ can be important for fusion. Alternatively, HA outsiders do not need to be in the immediate proximity to the fusion site and drive opening and expansion of a fusion pore by generating tension in membrane bilayer. We have recently suggested a specific mechanism by which fusion protein outsiders can produce long-range stresses in the membrane (30,31). According to this hypothesis, the activated fusion proteins outside the CZ form a dense interconnected protein coat that bends the coat-covered membrane out of its initial shape. The resulting stresses in the membrane bilayer then drive its fusion with the target membrane within the CZ or along the circumference of the CZ.

While the mechanisms by which fusion proteins outside of the CZ influence fusion remain to be understood, our work suggests an interesting convergence between mechanisms of membrane fusion and fission. Involvement of the outsider fusion proteins in the expansion of a fusion pore can be analogous to that of the proteins that mediate membrane budding in diverse membrane fission reactions from the outside of the future fission site, i.e., without being present between the merging membrane monolayers (32,33).
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**Abbreviations list.**

CPZ, chlorpromazine; CF, carboxyfluorescein; CZ, contact zone; FP–AB, an anti-fusion peptide antiserum, HA, influenza virus hemagglutinin; HA-cell, HA-expressing cell; FD, fluorescent Dextran; NaBut, sodium butyrate; RBC, red blood cells.
Figure Legends.

**Fig. 1.** HA-cell and RBC establish extended and tight contact zone (CZ). A, B Thin-section electron micrographs show an extended and tight CZ between the membranes of an RBC (top) and a HAb2 cell (bottom). Scale bars in A and B, 1 µm and 25 nm, respectively. C. HA300a cell with a bound RBC (shown by an arrow) was incubated for 15 min with blue-fluorescent beads with conjugated HC67 antibody. While HA-cell is fluorescent due to bound beads, the CZ under RBC is not fluorescent indicating its inaccessibility for the beads. Scale bar 10 µm. D, E. DIC (D) and confocal fluorescence microscopy (E) images of the same single RBC attached to an HAb2 cell. HA-cell/biotinylated RBC pairs were incubated for 15 min with fluorescent streptavidin. Images were taken after removal of unbound streptavidin. Fluorescence linescans along Y and X- axes in E are shown at the top and to the right of the image. Very low level of CZ fluorescence indicates that tightness of the CZ severely restricts streptavidin entry.

**Fig. 2.** Fusion between HA-cell and RBC is inhibited by bead-antibodies. A. HA300a cells with bound RBCs were treated with a 1-min pulse of pH 4.9 immediately followed by an application of the bead- FP–AB (bars 2) or bead- LC89 antibody (bars 5). In control experiments, either we did not apply any agents (bars 1), or we applied bead-conjugated pre-bleed serum to control for FP–AB (bars 3), supernatant from the bead-FP–AB (bars 4), or supernatant from the bead- LC89 antibody (bars 6). Open and filled bars represent the final extents of lipid and content (CF) mixing. B. In contrast to the experiment shown in A, FP–AB was conjugated to 100-nm beads rather than to 20-nm beads. HA300a cells with bound RBCs were treated with a 1-min pulse of pH 4.9.
immediately followed by an application of the bead-FP–AB (Bars 2, 4). In control experiments, we applied no bead-conjugated agents (bars 1, 3). Bars 1, 2 show the extents of content (CF) mixing. Bars 3, 4 represent the percentage of cell pairs with restricted hemifusion intermediates assayed as content (CF) mixing after a 1-min application of 0.5-mM CPZ. CPZ was applied 5 min after the end of the low pH pulse. C. RBC/HAb2 pairs were pre-incubated with bead- FC125 antibody for 5, 10, 20 and 60 min and then treated with a 2-min pulse of pH 4.9. In control experiment cell pairs have not been treated with bead- FC125 antibody. Bars represent the final extents of content (CF) mixing. D. HAb2 cells with bound RBCs were treated with a 1-min pulse of pH 4.9. The gray bars represent the percentage of cell pairs with restricted hemifusion intermediates assayed as content mixing after a 1-min application of 0.5-mM CPZ. 5 min prior to a low-pH pulse, RBC/HAb2 pairs were treated with bead- FC125 antibody (bars 2, 4, 6). Bars 1, 3, 5 represent the control experiments (no agent added). Open and filled bars represent the final extents of lipid and content (CF) mixing. Bars in A-D are means ± SE, n>3.

**Fig. 3. Bead-agents inhibit fusion at the highest HA densities.**

**A, B.** Fusion of NaBut–pre-treated HAb2 cells with bound RBCs was triggered by a 1-min pulse of pH 4.9. Fusion was assayed as lipid mixing (open bars) and mixing of aqueous probes (CF, filled bars or 10 kD FD, striped bars). Bars are means ± SE, n>3. **A.** In bars 2 immediately after the low pH pulse cells were treated with bead-thermolysin. In control experiments, either we did not apply any agents (bars 1) or applied bead-thermolysin *before* rather than *after* the low-pH pulse (bars 3). **B.** Fusion was assayed as
10 KD FD transfer. 5 min prior to a low-pH pulse the cell pairs were treated with bead-FC125 antibody (bar 2). In control experiments, either we did not apply any agents (bar 1), or we applied supernatant from the bead- FC125 antibody (bar 3) or applied bead-FC125 antibody after rather than before the low-pH pulse (bar 4).

C, D. Fusion of pre-bound R18-labeled virus (Japan strain) with RBC is inhibited by bead- FC125 antibody applied 5 min prior to application of pH 5.3 medium (curve 2 in C and bar 2 in D) as compared with the control (curve 1, C and bar 1, D), where no agents have been applied. In the representative experiment (C) and in (D), where the final extents of fusion were averaged over 3 experiments, fusion was assayed as R18 dequenching.

**Fig. 4. Crowding of an HA-cell with RBCs inhibits fusion pore expansion. A, B**

Fusion of a NaBut–pre-treated HAb2 cells with bound RBCs was triggered by a 10-min pH 4.9 pulse. A. Fusion of HA-cell with a single RBC loaded with different aqueous dyes (CF, 10 KD FD and 70 KD FD) was assayed as dye transfer for HA-cells with and without multiple unlabeled RBCs (open and closed bars, respectively). Each bar represents the mean ± SE, n=2. Lipid mixing with and without additional RBCs was 99±0.5%. B. Fluorescence microscopy images of an HA-cell with a single PKH26-labeled RBC bound (left) and with a single labeled RBC (indicated by a white arrow) together with multiple (>10) unlabeled RBCs bound (right). Images were taken 20 min after application of a low pH pulse. In the right image, additional RBCs prevent flattening of fused RBCs into the HA-cell membrane.
**Fig. 5.** Fusion observed when only HA outsiders were trypsin-cleaved into the fusion-competent form. Pairs of RBCs and NaBut–pre-treated HAb2 cells expressing fusion-incompetent HA0 were treated for 2 min at room temperature with bead-trypsin to cleave HA0 into a fusion-competent form and then immediately (bars 2 and 6) or 30 min later (bars 3 and 7) exposed to a 5-min (bars 1-5) or 2-min (bars 6 and 7) pulse of pH 4.9. Fusion was assayed as lipid and content (CF) mixing (open and filled bars, respectively). In control experiments, either we did not apply bead-trypsin (bars 1), or we replaced bead-trypsin with the supernatant from these beads (bars 4) or treated HA-cells with bead-trypsin prior to adding RBCs (bars 5). Each bar represents the mean ± SE, n>3.
Fig. 4
Fig. 5
**Supplemental Data.**

Fig. 1S. **Fluorescent streptavidin enters the CZ after 1-hour incubation.** DIC (A) and confocal fluorescence (B) images of the same single RBC attached to HAb2 cell. HA-cell/biotinylated RBC pairs were incubated for 60 min with fluorescent streptavidin. Images were taken after removal of unbound streptavidin. Fluorescence linescans along Y and X axes in B are shown at the top and to the right of the image. 1-hour incubation with streptavidin results in the entry of streptavidin into CZ.

Fig. 2S. **Qdot 525 Streptavidin conjugate is excluded from the CZ.** DIC (A) and confocal fluorescence (B) images of the same single RBC attached to HAb2 cell. HA-cell/biotinylated RBC pairs were incubated for 30 min with Qdot 525 Streptavidin conjugate. Images were taken after removal of unbound QDots. Fluorescence linescans along Y and X axes in B are shown at the top and to the right of the image. Very low level of CZ fluorescence indicates that tightness of the CZ severely restricts entry of Qdot 525 Streptavidin conjugate.

Fig. 3S. **Inhibition of lipid mixing by pre-incubation of X31 HA-cell/RBC pairs with bead-HC67 antibody.** Bar 1 - control experiment, lipid mixing was triggered by a 1-min pulse of pH 4.9 medium at room temperature. Bar 2 – prior to low pH pulse cell pairs were pre-incubated with bead-HC67 antibody for 5 min. Bar 3 - cell pairs were first treated by low pH pulse and then incubated with bead- HC67 antibody for 5 min. Bars are means +/- SE, n>3.
Fig. 4S. **Bead-antibodies to fibroblast growth factor receptor, in contrast to bead-antibodies to HA, do not inhibit fusion between Japan HA-cells and RBCs.** Bars 1 – lipid (open bar) and content (CF) mixing (black bar) in the control experiment, fusion was triggered by a 1-min pulse of pH 4.9 medium at room temperature. Bars 2 – prior to low pH pulse cell pairs were pre-incubated with bead-FC 125 antibody for 5 min. Bars 3 - bead-FC 125 antibody was replaced with bead-antibody to fibroblast growth factor receptor. Bars are means +/- SE, n>3. Beads with both antibodies similarly bound to HA-cells as evidenced by fluorescence microscopy.

Fig. 5S. **Lipid mixing between X31 influenza virus and RBC is inhibited by bead-conjugated antibodies to HA but insensitive to bead conjugated antibodies to viral neuraminidase.** Two independent experiments were performed as described in Methods and in the legend to Fig. 2C. Fusion of pre-bound R18-labeled virus (X31 strain) with RBC is inhibited by bead- HC67 antibody applied 5 min prior to application of pH 5.3 medium (bars 3) as compared with the control (bars 1), where no agents have been applied and with the control (bars 3) where bead-HC67 antibody was replaced with antibody to viral neuraminidase.
Lipid Mixing, %

- Control
- Bead-HC67 antibody/Low pH
- Low pH/ Bead-HC67 antibody

Fig. 3S
Fig. 4S
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