Membrane Environment Can Enhance the Interaction of Glycan Binding Protein to Cell Surface Glycan Receptors

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ABSTRACT: The binding of lectins to glycan receptors on the host cell surface is a key step contributing to the virulence and species specificity of most viruses. This is exemplified by the viral protein hemagglutinin (HA) of the influenza A virus, whose binding specificity is modulated by the linkage pattern of terminal sialic acids on glycan receptors of host epithelial cells. Such specificity dictates whether transmission is confined to a particular animal species or jumps between species. Here, we show, using H5N1 avian influenza as a model, that the specific binding of recombinant HA to α2-3 linked sialic acids can be enhanced dramatically by interaction with the surface of the lipid membrane. This effect can be quantitatively accounted for by a two-stage process in which weak association of HA with the membrane surface precedes more specific and tighter binding to the glycan receptor. The weak protein–membrane interaction discovered here in the model system may play an important secondary role in the infection and pathogenesis of the influenza A virus.

Pathogen recognition and attachment onto host cells often starts with the binding of glycan binding proteins (GBPs) to glycan receptors on the host cell surface. Here, we probe this initial binding event using a model system related to the influenza A virus. One of the most important questions in influenza research is how a new strain of influenza A virus may emerge to cause human outbreak or pandemic. Unfortunately, there is still no clear answer today, and this has led to the intense research interest and public anxiety on the subject. The demonstrations of genetically altered H5N1 strains to more readily infect mammalian species, and the recent outbreaks in human populations of the H1N1 swine virus and the H7N9 avian virus, all underscore the urgency in finding answers to this question. Past studies of the transmission of influenza A viruses have focused on the specificity of the viral transmembrane protein hemagglutinin (HA) for glycan receptors on host cell surfaces. These glycan receptors contain sialic acid, also known as N-acetylneuraminic acid (NeuSAc), and vary in structure from hosts to hosts. HA exists as trimers in the viral membrane and the weak interaction between each HA and its glycan receptor is strengthened by multivalent binding to each HA trimer and to multiple trimers on the virus surface. The HA proteins of human-adapted influenza A bind preferentially to α2-6 linked sialic acid, whereas those of avian-adapted strains are more specific for α2-3 linked sialic acid, Scheme 1. There is also significant cross reactivity, which depends on the chemical nature of the glycan backbones and functional group modification of the sugar components. Recent studies suggested two general topologies of these glycans with respect to their specificities;
process involving intermediates before the multiple GBP-glycan receptor interaction partners are established. Evidences suggesting the formation of weakly bound intermediates in interfacial interactions can be found in a wide range of chemical and biological processes. For example, the involvement of weakly bound species called "precursors" is believed to play essential roles in the adsorption of small molecules,\textsuperscript{19} polymers\textsuperscript{20,21} and proteins\textsuperscript{22,23} at interfaces. In the formation of protein–protein complex, the process is believed to begin with a solvated encounter complex, followed by one or more weakly bound intermediate states before the final complex is formed.\textsuperscript{24} Zhu et al. demonstrated a similar precursor mechanism in the adhesion of a bacteria cell, \textit{Escherichia coli} (\textit{E. coli}), to mannose receptors on model lipid membrane surfaces: \textit{E. coli} cells can adhere to the membrane surface in both a weakly bound, monovalent state and a strongly bound, multivalent one.\textsuperscript{25}

In the case of GBP-glycan complex formation, the surfaces of both the virus and the host cell are highly heterogeneous, with the binding partners embedded in complex and dynamic environments.\textsuperscript{9,10} We hypothesize that formation of the specific GBP-glycan complex is also preceded by weakly bound precursors that are sensitive to the local physicochemical environment on the cell membrane surface. As a first step toward understanding the mechanism by which the GBP-glycan complexes are formed, we have investigated the binding kinetics of recombinant HA from the H5N1 avian influenza A virus to specific glycan receptors on model cell membrane surfaces. For this purpose, we have used label-free fluidic glycan microarrays. While microarrays of immobilized glycans have been successfully used before for the determination of binding

\begin{scheme}
\centering
\includegraphics[width=\textwidth]{Scheme2.png}
\caption{Structures of Glycolipids and Other Functionalized Lipids Used in the Present Study\textsuperscript{a}}
\end{scheme}

\textsuperscript{a}\textit{ϕ} refers to the hydrophobic dibenzocyclooctyne group for linking azido-functionalized sugar molecules to the lipid anchors using Cu-free click chemistry. The colored symbols represent sugar groups. Neu5Ac: N-acetylneuraminic acid. GlcNAc: N-acetylated glucose. Glc: glucose. Man: mannose. Fuc: fucose. Gal: galactose.
specificity of HAs from various influenza A virus sources, we prefer microarrays of glycans (glycolipids) in the fluidic supported lipid bilayer (SLB) environment to better mimic the dynamic environment of cell membrane. We combined the SLB microarray with surface plasmon resonance (SPR) imaging for label-free and real-time measurement of binding kinetics. SPR has been applied in the past to determine HA binding kinetics with glycan receptors, but not in the array format.

The fluidic SLB format allowed us to easily tune the composition of the model membrane surface by quantitatively mixing functional lipids with matrix lipids for SLB formation. We systematically tuned the environment for HA–glycan complexes and show that the HA–glycan receptor binding rate can be enhanced many folds by changes to the lipid membrane surface, an effect that can be quantitatively accounted for by a precursor-mediated mechanism. We suggest that such a precursor mechanism is of general importance to GBP/glycan receptor interaction on cell membranes.

## RESULTS AND DISCUSSION

The model system we choose is recombinant HA protein from the highly pathogenic H5N1 A/Vietnam/1203/2004 (Viet04). In solution, the recombinant HA proteins are known to be present mostly in the trimeric form as on the virus surface, with a distribution of oligomeric structures. Glycan receptors were prepared as glyco-lipids and incorporated into supported lipid bilayer (SLB), which serve to mimic the cell membrane. We adjusted the chemical environment on the membrane surface by incorporating functionalized lipid molecules in a matrix of egg phosphatidylcholine (eggPC). We fabricated the SLB in an array format on a gold sensor surface for label-free detection via surface plasmon resonance (SPR) imaging, as detailed in Supporting Information (Figure S1). The fluidic nature of the SLB on the array was verified by fluorescence recovery after photobleaching (FRAP), as shown in Supporting Information Figure S2. The spots on each array contain different concentrations of glyco- and functional lipids in the eggPC matrix. We obtained parallel binding kinetics of HA to all spots on the array by recording SPR images (SPRImager II, GWC Technologies Inc., Madison, WI) as a function of time.

The structures of all glycolipids and other functionalized lipids used in the present study are shown in Scheme 2. Trisaccharides possessing α-2-3 linked sialic acid are represented by NeuSAc-α2-3-Gal-β1-4-GlcNAc (33) and NeuSAc-α2-3-Gal-β1-4-Glc (32). These glycolipids are designed to specifically bind HA from avian H5N1. In contrast, trisaccharides possessing α-2-6 linked sialic acid are represented by NeuSAc-α2-6-Gal-β1-4-GlcNAc (36) and NeuSAc-α2-6-Gal-β1-4-Glc (35), and are designed to bind HA from human-adapted influenza A. The sialic acid residues in both sets of trisaccharides contain terminal azide (−N₃) functional groups and were obtained from the Consortium for Functional Glycomics. These trisaccharides were conjugated to one of the two lipid anchors, 16:0 Caproylamine PE, which is short for 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(hexanoylamine), or 16:0 succinyl PE, which is short for 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(succinyl) (Avanti Polar Lipids, Alabaster, AL). The conjugation was done via oligo(ethylene glycol) (OEG) linkers either through Cu-free click chemistry with a dibenzocyclooctyne (ϕ) group, or through an N-hydroxysuccinimide (NHS) coupling reaction to the amine functionality generated by reducing the azides. To change the membrane environments, we used six different functionalities anchored to lipids: the hydrophilic mannose or fucose linked to OEG-lipid via Cu-free click chemistry (Man-ϕ- OEG-lipid or Fuc-ϕ-OEG-lipid), the hydrophobic cyclooctyne group conjugated with or without OEG linker to the lipid molecule (ϕ-OEG-lipid or ϕ-lipid), and the acidic −COOH or basic −NH₂ terminated lipids (HOOC-EG-lipid or H₃N- lipid). The synthesis and characterization of these lipid molecules are detailed in Supporting Information.

We incorporated each of the above glycolipids and the functionalized lipids into the SLB microarray and measured the binding of the recombinant HA protein using SPR. In the experiment, the microarray surface was first equilibrated with the buffer solution, followed by the injection of HA protein solution (marked by a downward arrow) and, after some time, the injection of washing buffer to remove the unbound HA (upward arrow).

We first established the specificity of HA binding to glycan receptors on the model cell membrane. Figure 1A shows typical SPR kinetic profiles for the trisaccharides (in eggPC matrix). Consistent with the known specificity of the avian H5N1 HA protein, binding was only observed to the α-2-3 linked sialic acids (33-ϕ-OEG-lipid, 33-EG-lipid, and 32-ϕ-OEG-lipid, solid curves), with no detectable binding to α-2-6 linked trisaccharides (36-ϕ-OEG-lipid, 36-EG-lipid, and 35-ϕ-OEG-lipid). Figure 1B shows the results of SPR kinetic profiles for the trisaccharides (in eggPC matrix).

**Figure 1.** (A) SPR responses for the binding of recombinant HA protein (400 nM) with α-2-3 linked sialic acids (solid curves: green, 33-ϕ-OEG-lipid; blue, 32-ϕ-OEG-lipid; red, 33-EG-lipid) and with α-2-6 linked sialic acids (dashed curves: green, 35-ϕ-OEG-lipid; red, 36-ϕ-OEG-lipid; blue, 36-PEG-lipid) on a supported lipid bilayer microarray. The density of 33-ϕ-OEG-lipid is 0.8% and those of all others are 4.0%. The downward and upward arrows indicate the times of protein solution and washing buffer injections, respectively. (B) The initial association rates of HA trimers (nm⁻² s⁻¹) as a function of the specific glycolipid concentration in the SLB. The symbols are data points and solid lines are linear fits. Green, 33-ϕ-OEG-lipid; blue, 32-ϕ-OEG-lipid; and red, 33-EG-lipid.
lipid, dashed curves). Furthermore, no measurable binding of HA to any other functionalized lipids was observed (Supporting Information Figure S3). For the three α2-3 trisaccharides, we found that the initial rate of HA binding is proportional to the concentration of glycolipid in the SLB, Figure 1B, as expected from the initial binding kinetics at low surface HA coverage (see kinetic analysis below). For the three glycolipids containing α2-3 linked sialic acid, the association rate of HA with 33ϕ-OEG-lipid is approximately 4× that with either 33ϕ-OEG-lipid or 32ϕ-OEG-lipid. Here, 33ϕ-OEG-lipid and 33ϕ-OEG-lipid possess the same trisaccharide and differ only in the linkage: the bulkier click-linkage (ϕ) to OEG in the former or the peptide linkage to OEG in the latter. The hydrophobic ϕ linking group may increase secondary interaction with hydrophobic domains near the HA binding site,37 leading to the peptide linkage to OEG in the former or the bulkier click-linkage (ϕ) to OEG in the latter. The hydrophobic ϕ linkage: the bulkier click-linkage (ϕ) to OEG in the former or the peptide linkage to OEG in the latter. The hydrophobic ϕ linking group may increase secondary interaction with hydrophobic domains near the HA binding site,37 leading to enhanced interaction with the glycans receptor (33). For 33ϕ-OEG-lipid and 32ϕ-OEG-lipid, the difference is only in the third sugar unit. The sensitivity of HA-glycan complex formation to interactions beyond the linkage pattern of the terminal sialic acid has been observed before.12,16 Note that, for simplicity, we use the density of trimers in the data analysis, as they are the predominant species from recombinant HA. The actual surface HA species are likely a distribution of oligomers,30,31 but the conclusions remain unchanged.

We now turn to the focus of this study: the investigation of the influence that membrane environment has on HA–glycan complex formation. In this experiment, we used a fixed concentration (0.8%) of 33ϕ-OEG-lipid in the membranes in the presence of a second functionalized lipid with a variable concentration (0–4%). None of these secondary lipids alone (in eggPC matrix) showed any binding to HA (see Supporting Information Figure S3). Figure 2A shows SPR responses for HA binding to 33ϕ-OEG-lipid with the hydrophobic ϕ-OEG-lipid as the secondary functionalized lipid. The addition of ϕ-OEG-lipid dramatically increases the rate of HA binding to 33ϕ-OEG-lipid, by as much as 4× when the ϕ-OEG-lipid concentration in the SLB is increased from 0 to 4%. Since ϕ-OEG-lipid alone shows no measurable affinity to HA (Supporting Information Figure S3), we conclude that its effect in the lipid membrane is to assist the binding of HA to 33ϕ-OEG-lipid. An important feature of this assistance effect is that the addition of ϕ-OEG-lipid leads to a systematic increase in the rate of association between HA and 33ϕ-OEG-lipid, but little change to the rate of dissociation of HA-33ϕ-OEG-lipid complex. In addition, the association parts of all the SPR profiles, that is, integrated rate equations (concentration vs time) are superimposable with different multiplication factors (Supporting Information Figure S4).

Similar results were obtained with other secondary functionalized lipids that showed lesser extents of enhancement or inhibition of HA binding with 33ϕ-OEG-lipid, as detailed later in Figure 4. For example, Figure 2B shows SPR responses for HA binding to 33ϕ-OEG-lipid with the hydrophilic H2N-OEG-lipid as the secondary functionalized lipid. Within experimental uncertainty, the addition of H2N-OEG-lipid to the eggPC matrix has no effect on either the association or dissociation of HA with 33ϕ-OEG-lipid. Another interesting comparison to make would be 33ϕ-OEG-lipid versus 33ϕ-lipid; unfortunately, we were not able to carry out the click reaction between 33-azide and ϕ-lipid due to mismatch in solubility between these two molecules.

The observed dependence of complex formation between HA and 33ϕ-OEG-lipid on the nature of the membrane surface environment contradicts the commonly used (but incorrect) model of direct binding of a solution phase protein molecule to glycans receptors on the membrane surface, as the influence of the membrane environment on the kinetics of complex formation is ignored in such a model. Rather, the kinetic results in Figure 2 can be satisfactorily and quantitatively explained by a precursor mechanism. In the simplest form, the precursor mechanism can be represented by

\[
\begin{align*}
(1) & \quad P \overset{k_i}{\underset{k_{-i}}{\rightleftharpoons}} P^* \\
(2) & \quad P^* + G \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} PG \\
(3) & \quad PG + G \overset{k_2}{\underset{k_{-2}}{\rightleftharpoons}} PG_2 \\
(4) & \quad PG_2 + G \overset{k_3}{\underset{k_{-3}}{\rightleftharpoons}} PG_3
\end{align*}
\]

where \( P \) is HA protein (trimer) free in solution; \( P^* \) represents the precursor state, that is, protein molecules transiently adsorbed on the membrane surface before specific binding to the glycans receptor (G) to form bound protein-glycan complexes (PG). The HA trimer can bind up to three glycans receptors,12 as shown by steps (iii) and (iv) in eq 1, but the SPR technique only measures the total amount of protein.
which decreases by 2.4 folds when $\phi$ increased 6 folds as the concentration of surfaces. By increasing the concentration of the hydrophobic summarized in Table 1 and Figure 3A. We observe that constants and the starting concentrations of the protein in the

\[ \alpha \approx \beta [1 - \exp(-\beta t)] \]

The two coefficients are

\[ \alpha = \frac{k_1 k_2}{k_{-1}} \]

\[ \beta = \frac{k_{-2}}{1 + [G]^0 k_2/k_{-1}} \]

where $K_1 (= k_1/k_{-1})$ and $K_2 (= k_2/k_{-2})$ are the equilibrium constants for the two steps in eq 1. In this model, the kinetic profile depends explicitly on the precursor state. The proportionality constant, $\alpha$, is a product of the two equilibrium constants and the starting concentrations of the protein in the solution and the glycan receptor on the membrane surface. A change in the membrane surface environment changes the equilibrium constant of the precursor state ($K_1$) and, thus, proportionally varies the formation rate of protein–glycan complexes.

The red curves in Figure 2A show fits of the initial kinetic profiles to eq 3, with the resulting kinetic parameters summarized in Table 1 and Figure 3A. We observe that $\alpha$ is increased 6 folds as the concentration of $\phi$-OEG-lipid, denoted $\theta$, is increased from 0 to 4%. A smaller change is observed in $\beta$, which decreases by 2.4 folds when $\phi$-OEG-lipid is increased from 0 to 4%. These results can be easily understood from the precursor mechanism. The nonspecific adsorption of protein molecules is known to be more favorable on hydrophobic surfaces. By increasing the concentration of the hydrophobic $\phi$-OEG-lipid, the membrane surface becomes more attractive for the transient adsorption of weakly bound HA proteins and thus increases the equilibrium constant $K_1$ for the formation of the precursor state. Equation 4 predicts that an increase in $K_1$ corresponds proportionally to a larger value of $\alpha$. As shown in eq 4, stronger binding in the precursor state (at higher $\phi$-OEG-lipid concentration) can also be described by a smaller desorption rate constant $k_{-1}$ and, thus, a decreased value of $\beta$ (eq 5).

We now analyze dissociation kinetics from eq 1. For $t \geq 420$ s in Figure 2A, the protein solution is switched to washing buffer and $[P] = 0$. As detailed in the Supporting Information, the kinetic equations cannot be solved analytically and require numerical simulation. However, we can obtain an approximate solution at the short time limit, assuming that the initial dissociation from the membrane surface is dominated by that of the monovalent $PG$, with the concentrations of multivalent $PG_2$ and $PG_3$ remain nearly constant. This assumption is justified as the equilibrium constants of multivalent interactions are many orders of magnitude higher than the corresponding monovalent interaction. We further assume that the membrane surface concentration of free glycan receptor can be approximated as a constant, $[G]^0$, at the short time limit. The initial time profile of the surface bound protein concentration under these approximations is given by

| $\phi$-OEG-lipid (%) | $\alpha$ (nm$^2$) | $\beta$ (s$^{-1}$) | $k_1 = k_2$ (s$^{-1}$) | $k_0$ (s$^{-1}$ M$^{-1}$) | $K_0$ (mM) | $[PG]_0$ (nm$^2$) | $[PG_2]_0$ (nm$^2$) |
|---------------------|-----------------|-----------------|-------------------|-------------------|------------|----------------|------------------|
| 0                   | $1.3 \times 10^{-3}$ | $4.0 \times 10^{-3}$ | $1.0 \times 10^{-2}$ | $1.0 \times 10^0$ | 1.0        | $7.8 \times 10^{-4}$ | $2.0 \times 10^{-4}$ |
| 0.05                | $2.0 \times 10^{-3}$ | $3.4 \times 10^{-3}$ | $7.3 \times 10^{-3}$ | $8.5 \times 10^0$ | 0.86       | $1.1 \times 10^{-3}$ | $3.0 \times 10^{-4}$ |
| 0.1                 | $2.2 \times 10^{-3}$ | $3.6 \times 10^{-3}$ | $7.4 \times 10^{-3}$ | $9.0 \times 10^0$ | 0.82       | $1.1 \times 10^{-3}$ | $3.5 \times 10^{-4}$ |
| 0.3                 | $2.6 \times 10^{-3}$ | $3.1 \times 10^{-3}$ | $7.2 \times 10^{-3}$ | $7.8 \times 10^0$ | 0.94       | $1.3 \times 10^{-3}$ | $3.3 \times 10^{-4}$ |
| 0.5                 | $3.8 \times 10^{-3}$ | $2.5 \times 10^{-3}$ | $7.3 \times 10^{-3}$ | $6.3 \times 10^0$ | 1.4        | $1.6 \times 10^{-3}$ | $3.9 \times 10^{-4}$ |
| 0.8                 | $3.6 \times 10^{-3}$ | $2.9 \times 10^{-3}$ | $7.2 \times 10^{-3}$ | $7.2 \times 10^0$ | 1.0        | $1.9 \times 10^{-3}$ | $3.8 \times 10^{-4}$ |
| 1                   | $5.0 \times 10^{-3}$ | $2.2 \times 10^{-3}$ | $7.5 \times 10^{-3}$ | $5.5 \times 10^0$ | 1.4        | $2.0 \times 10^{-3}$ | $4.0 \times 10^{-4}$ |
| 1.5                 | $5.0 \times 10^{-3}$ | $2.3 \times 10^{-3}$ | $7.3 \times 10^{-3}$ | $5.7 \times 10^0$ | 1.3        | $2.2 \times 10^{-3}$ | $3.9 \times 10^{-4}$ |
| 2                   | $6.6 \times 10^{-3}$ | $1.8 \times 10^{-3}$ | $8.1 \times 10^{-3}$ | $4.4 \times 10^0$ | 1.8        | $2.5 \times 10^{-3}$ | $3.9 \times 10^{-4}$ |
| 3                   | $8.0 \times 10^{-3}$ | $1.5 \times 10^{-3}$ | $7.9 \times 10^{-3}$ | $3.7 \times 10^0$ | 2.1        | $2.5 \times 10^{-3}$ | $4.0 \times 10^{-4}$ |
| 4                   | $8.0 \times 10^{-3}$ | $1.6 \times 10^{-3}$ | $8.0 \times 10^{-3}$ | $4.0 \times 10^0$ | 2.0        | $2.6 \times 10^{-3}$ | $3.9 \times 10^{-4}$ |

*The estimated standard deviation is within ±10% for each parameter.
\[ [P_1] \approx [PG]_0 \exp(-\beta' \cdot t') + [PG_2]_0 + [PG_3]_0 \]  
(6)

where \([PG]_0\) is the bound monovalent HA protein–glycan receptor complex at the time of washing buffer injection \((t' = 0)\). \([PG_2]_0\) and \([PG_3]_0\) are the concentrations of bound multivalent HA protein–glycan receptor complex that are approximated as constants. \(\beta_3\) is given by

\[ \beta = \frac{k_{-2}}{1 + \left[ G \right] k_4 / k_{-1}} \]  
(7)

The green curves in Figure 2A show fits of the initial dissociation profiles to eq 6, with resulting parameters summarized in Table 1 and Figure 3B. With increasing \(\phi\)-OEG-lipid concentration, there is little change to \(\beta_3\) and \([PG]_0\). The most important change is in \([PG_2]_0 = [PG_3]_0 + [PG_3]_0\), which increases by three folds when the \(\phi\)-OEG-lipid concentration is increased from 0 to 4%. We conclude that enhancing the binding of precursor protein molecules on the membrane surface leads predominantly to an increase in the concentration of multivalent \(PG_\alpha\) \((\alpha = 2, 3)\) species.

To further understand the above precursor mechanism, we now compare this to the conventional protein-receptor binding model in which the formation of the complex occurs in a single step:

\[ P + G \rightarrow PG \]  
(8)

where \(k_i\) and \(k_d\) are the association and dissociation constants, respectively. Further steps in the multivalent binding process to form \(PG_2\) and \(PG_3\) are identical to steps iii and iv in eq 1; as discussed earlier, these steps are not resolved in an SPR measurement. At the initial stage of binding, we apply the short time approximation and the time evolution of adsorbed protein signal is given by (see Supporting Information):

\[ [P_1] \approx [G]_0 \left[ 1 - \exp(-k_d [P] t) \right] \]  
(9)

where \([P] = 0.4 \mu M\) is the solution concentration of HA protein molecules; \([G]_0\) is the starting concentration of glycan receptor, 33-\(\phi\)-OEG-lipid, in the supported lipid bilayer. Given the fraction \((0.8\%)\) of 33-\(\phi\)-OEG-lipid in the SLB, and the lipid density \((= 0.5 \text{ nm}^{-2})\) of the SLB, we obtained \([G]_0 = 1.6 \times 10^{-2} \text{ nm}^{-2}\).

Equation 9 is equivalent to eq 3, with \(\alpha = [G]_0\) and \(\beta = k_d [P]\). This model is in clear contradiction to the fitting results in Table 1 for two reasons: (1) For all the SLBs probed in Figure 2A, the receptor (33) concentration is fixed at \([G]_0 = 1.6 \times 10^{-2} \text{ nm}^{-2}\), but the fitting results show \([G]_0\) \((= \alpha)\) increasing from 1.3 \times 10^{-3} \text{ nm}^{-2} to 8.0 \times 10^{-3} \text{ nm}^{-2} when \(\phi\)-OEG-lipid increases from 0 to 3–4%; (2) with increasing secondary lipid \(\phi\)-OEG-lipid) in the SLB, the association constant \(k_d\) \((= \beta/[P])\) actually decreases, in contradiction to the observed increase in HA protein binding.

Further evidence against the direct association/reaction model in eq 8 comes from analysis of dissociation rates and the equilibrium constant, \(K_p = k_d / k_d\). Using the short-time limit approximation, the initial dissociation process from the direct mechanism in eq 8 can also be derived by eq 6, with \(\beta' = k_d\) (see Supporting Information). There is little change to \(k_d\) or \(K_p\) when the secondary \(\phi\)-OEG-lipid concentration is increased from 0 to 3–4% in the SLB with fixed receptor (33-\(\phi\)-OEG-lipid) concentration. Thus, the enhancement of HA protein binding to receptor 33-\(\phi\)-OEG-lipid in the SLB by the addition of \(\phi\)-OEG-lipid, which by itself does not show any binding for HA (Supporting Information Figure S3), cannot be attributed to increased interaction between HA and the glycan receptor 33. Rather, it is a kinetic effect in the precursor-mediated mechanism. Note that, within the inappropriate model of direct binding between solution HA protein and membrane surface glycan, the kinetic parameters \((k_i\) and \(k_d\)) and dissociation equilibrium constant \(K_p\) obtained here (Table 1) are similar to numbers reported by Narla and Sun for the binding of HA from H5N1 to α2-3 linked sialic acids immobilized on the surface of SPR sensors. However, our \(K_p\) values for the specific H5N1 HA/33 interaction are more than 1 order of magnitude higher than that reported by Gaunitz et al. for other HA/α2-3 linked sialic acid combinations, again pointing to the sensitivity of HA binding to the specific glycan linkages.

To understand how the membrane surface environment affects the precursor state and, thus, the specific binding of HA to 33-\(\phi\)-OEG-lipid, we show in Figure 4 SLB microarray results of 33-\(\phi\)-OEG-lipid (0.8%) in the presence of varying concentrations of a second functional lipid (0–4%) in the SLB array. The different symbols represent different functional lipids as shown in the legend. The lines are exponential fits that serve as guides to the eye.
molecules in the precursor state to interact specifically with the glycan receptor.

For other secondary lipids with functional group at similar heights from the membrane surface as the specific glycan receptor (33), we find enhancement or inhibition of HA binding, depending on the nature of the functionality. As the secondary lipid concentration is increased from 0 to 4%, mannose (solid red squares) and fucose (solid green triangles) show enhancements up to 1.6 and 1.8 fold, respectively; these glycosylated lipids are less hydrophobic than ϕ-OEG-lipid and are less effective in promoting the precursor state. The trisaccharide 36 with α2-6 linkage (grey crosses) and the -NH2 group (yellow open squares) exhibit no enhancement, but the −COOH acid group (black open circles) decreases HA binding by up to 32% at 4% functional lipid concentration. These functionalities are hydrophilic and do not appreciably affect precursor binding.

Conclusions. The binding of glycan binding proteins to glycan receptors on cell membranes is fundamentally a kinetic process. Our findings reveal that this kinetic process is determined not only by the specific interaction within the binding pocket (and secondary interactions outside the pocket)12 but also by weak and transient interactions in the precursor states. In the model system probed here, both HAs on virus surfaces and glycan receptors on cell membranes are present in complex and heterogeneous environments. While these local environments may have minimal effect on virus surfaces and glycan receptors on cell membranes are precursors. In the model system probed here, both HAs can drastically affect the precursor state and, thus, the overall binding kinetics. Previous studies on influenza virions, particularly the potential danger of avian influenza becoming transmissible in human, have mainly asked questions at the genetic level, e.g., how antigenic drifts, antigenic shifts, and specific mutation in the laboratory changes the HA binding specificity. A major challenge with the genetic approach is that our knowledge seems to be confined to quantum steps: we know a particular strain becomes transmissible in human only after the fact. We do not know a priori if certain genetic changes are increasing the affinity of a particular influenza A HA toward human host cells, that is, the continuum before the quantum step of human outbreak or pandemic. In view of the findings presented here, we present the following hypotheses: (i) the quantized switching of influenza A virus HA affinity may be assisted by more gradual enhancement in precursor interaction on the host cell membrane surface; (ii) differences in cell surface environment in enhancing or inhibiting the nonspecific precursor interaction may partially account for individual variability to influenza A virus infection; (iii) disruption of the precursor state may be used as a strategy in the development of inhibitor or treatment of influenza A virus infection; (iv) the enhancement of weak interactions in the precursor state may be used as one of the “predictors” or “warning signals” for the potential danger of a particular virus strain. While we are far from being able to verify these hypotheses, we hope the potential importance of these hypotheses will motivate a new direction on influenza A virus research. The precursor mediated mechanism is of general significance to the understanding of a wide range of biological processes on membrane surfaces.

METHODS

See Supporting Information for methods.
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