Probing the functions of the paramyxovirus glycoproteins F and HN

with a panel of synthetic antibodies (sABs)

Brett D. Welch\textsuperscript{1,2,†}, Marcin Paduch\textsuperscript{3,†}, George P. Leser\textsuperscript{1,2}, Zachary Bergman\textsuperscript{1}, Christopher A. Kors\textsuperscript{1,2}, Reay G. Paterson\textsuperscript{1}, Theodore S. Jardetzky\textsuperscript{4}, Anthony A. Kossiakoff\textsuperscript{3,*} and Robert A. Lamb\textsuperscript{1,2,*}

\textsuperscript{1}Department of Molecular Biosciences and \textsuperscript{2}Howard Hughes Medical Institute, Northwestern University, Evanston, IL 60208-3500, \textsuperscript{3}Department of Biochemistry and Molecular Biology and the Institute for Biophysical Dynamics, The University of Chicago, 900 E. 57th Street, Chicago, IL, 60637, and \textsuperscript{4}Department of Structural Biology, Stanford University School of Medicine, Stanford, CA 94305

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† These two authors contributed equally to the work

*To whom correspondence should be addressed: RAL - Dept. of Molecular Biosciences, Northwestern University, 2205 Tech Drive, Evanston, IL 60208-3500 Telephone: 847-491-5433; Fax: 847-491-2467; e-mail: ralamb@northwestern.edu; or AAK – Dept. of Biochemistry and Molecular Biology, The University of Chicago, 900 E. 57th Street, Chicago, Illinois, 60637; Telephone: 773-702-9257; Fax: 773-702-0439; e-mail: koss@bsd.uchicago.edu
Paramyxoviruses are enveloped negative-strand RNA viruses that are significant human and animal pathogens. Most paramyxoviruses infect host cells via the concerted action of a tetrameric attachment protein (variously called HN, H, or G) that either binds sialic acid or protein receptors on target cells and a trimeric fusion protein (F) that merges the viral envelope with the plasma membrane at neutral pH. F initially folds to a metastable prefusion conformation that becomes activated via a cleavage event during cellular trafficking. Upon receptor binding, the attachment protein, which consists of a globular head anchored to the membrane via a helical tetrameric stalk, triggers a major conformation change in F which results in fusion of virus and host cell membranes. We recently proposed a model for F activation in which the attachment protein head domains move following receptor binding to expose HN-stalk residues critical for triggering F. To test the model in the context of wild type viral glycoproteins, we used a restricted diversity combinatorial Fab library and phage display to rapidly generate synthetic antibodies (sABs) against multiple domains of the paramyxovirus parainfluenza 5 (PIV5) pre- and postfusion F and HN. As predicted by the model, sABs that bind to the critical F-triggering region of the HN stalk do not disrupt receptor binding or NA activity, but are potent inhibitors of fusion. An inhibitory prefusion F specific sAB recognized a quaternary antigenic site and may inhibit fusion by preventing F refolding or blocking the F-HN interaction.
The paramyxovirus family of negative strand RNA virus cause significant disease in humans and animals. The viruses bind to cells via their receptor binding protein and then enter cells by fusion of their envelope with the host cell plasma membrane, a process mediated by a metastable viral fusion (F) protein. To understand the steps in viral membrane fusion a library of synthetic antibodies to F protein and the receptor binding protein was generated in bacteriophage. These antibodies bound to different regions of the F protein and the receptor binding protein and the location of antibody binding affected different processes in viral entry into cells.
Paramyxoviruses are enveloped, non-segmented, negative stranded RNA viruses that infect host cells by fusing their membrane with the cells plasma membrane at neutral pH (1). The family *Paramyxoviridae* includes many major clinically and economically important pathogens of humans and animals including parainfluenza viruses 1-5 (PIV1-5), mumps virus (MuV), Newcastle disease virus (NDV), Sendai virus, measles virus (MeV), canine distemper virus (CDV), Nipah virus (NiV), Hendra virus (HeV), respiratory syncytial virus (RSV), and human metapneumovirus (hMPV).

Paramyxoviruses mediate membrane fusion and cell entry by the concerted action of two viral glycoproteins – the attachment protein (HN, H or G) and the fusion protein (F). The attachment protein binds cellular surface receptors and interacts with F. This interaction triggers a conformational change in F to induce membrane fusion, thereby releasing the viral ribonucleoprotein complex into the host cell cytoplasm.

Atomic structures of the attachment proteins (HN, H or G) reveal a globular head, harboring a typical sialidase domain created by a six-bladed β-propeller fold (2-11). PIV1-5, MuV and NDV have HN-type receptor binding proteins possessing both hemagglutinating and neuraminidase activities, and HN binds sialic acid as receptor through a central binding site within the β-propeller fold. In contrast H proteins of MeV and CDV and G proteins of HeV and NiV bind cell surface-expressed protein receptors through specific sites on the globular head.

The attachment proteins exist as a dimer-of-dimers, with dimerization occurring through covalent and non-covalent interactions primarily within a stalk domain that connects the globular heads to the transmembrane domain (8, 12-17). Recently obtained atomic structures of HN stalk domains from NDV HN (12) and PIV5 HN (18) showed the stalks to be 4-helix bundles.
A large body of data suggests that F interacts with the attachment protein through the stalk domains (19-30).

Paramyxovirus F protein is a type I viral fusion protein with mechanistic features common to the fusion proteins of several other viruses including human immunodeficiency virus (HIV) Env, influenza virus hemagglutinin (HA), and Ebola virus (GP) (31). F initially folds to a metastable trimeric precursor (F0) that is proteolytically cleaved into the covalently associated F1 and F2 subunits. Atomic structures of F trimers in the prefusion form have been determined for PIV5 and RSV (32-34). An interesting structure of prefusion MPV F monomers bound to an inhibitory Ab has also been reported (35). Prefusion F has a short C-terminal cytoplasmic tail, a single transmembrane domain, a helical stalk, and a globular head domain. Atomic structures of NDV, hPIV3, and RSV F in the postfusion form reveal that a large refolding event occurs to convert prefusion F to postfusion F in which part of the globular head domain rearranges to form a six-helix bundle (36-39). These structures, along with peptide inhibitory data, suggest a model for F-mediated membrane fusion where, upon activation, F1/F2 rearranges to insert a hydrophobic fusion peptide from the N-terminus of F1 into the target cell membrane forming a pre-hairpin intermediate (40). This relatively extended structure tethers the virus to the cell membrane and collapses to form the stable six-helix bundle of the postfusion structure. The transition from the metastable prefusion, to the prehairpin intermediate, to the postfusion conformation proceeds down an energy gradient with the postfusion form representing the most stable state, and the energy released during F refolding is coupled with membrane fusion.

HN, H or G receptor binding is linked to F activation to ensure the correct timing of the F fusion peptide insertion into the target cell membrane. Upon binding receptor, the attachment protein globular heads, are thought to initiate a rearrangement and this movement is believed to be responsible for F activation during the F-HN/H/G interaction (1, 41-44).
The mechanistic details of how attachment proteins trigger F have been elusive. Monoclonal antibodies (mAbs) have proven to be powerful tools for revealing such details in many systems including virus entry into cells (45, 46). For PIV5, two previously described mAbs, F1a and 6-7 have been used with success in several studies (47, 48). mAb 6-7 is specific for postfusion F, while F1a recognizes cleaved F in the prefusion conformation better than uncleaved F (cleavage-specific). Whereas F1a binds cleaved prefusion F more readily than cleaved postfusion F, it is not completely prefusion specific. Therefore, it would be useful to have PIV5 prefusion F-specific Abs as well as Abs that recognize additional unique antigenic sites on both F and HN. However, mAb discovery can be time consuming and expensive. Furthermore, the mAb screening process offers limited control over screening conditions (e.g., pH, halide concentrations, etc.), including the ability to counter screen, which can be especially important for antigens with closely related subtypes or antigens that adopt multiple conformation states.

Antibody engineering using combinatorial libraries and phage display selection strategies can overcome many of the limitations associated with traditional mAb discovery. Phage display is an extraordinarily high-throughput and versatile screening process that isolates target molecule binders from highly-diverse libraries (often >10^{10} unique members). However, phage display libraries that encode all 20 amino acids at each position can only comprehensively cover the sequence diversity resulting from randomizing a limited number of residues (full randomization of eight residues results in 2.5 X 10^{10} possible sequences). Importantly, structural and bioinformatics studies have revealed significant bias for a subset of amino acids in protein interfaces (reviewed in (49)). Combinatorial libraries with minimal sequence diversity (e.g., restricted to Tyr or Ser) based on various scaffolds have demonstrated that conformational diversity is more important than sequence diversity for generating specific and high-affinity protein interfaces (50, 51). Fellouse and coworkers (52) described previously a high-diversity
phage library displaying engineered antibodies comprised of two disulfide-linked Fab fragments with extensive conformational diversity but minimal chemical diversity in their complementarity determining regions (CDRs). They screened this library against a wide variety of antigenic targets and successfully produced high-affinity synthetic Fabs (sABs) against all of them (52).

Recently, Koellhoffer and coworkers (53) used an advanced variant of this library, "Library F", to produce Fabs that bind and neutralize proteolytically cleaved and uncleaved forms of the Ebola virus envelope glycoprotein (53).

Here, we used synthetic antibody technology to generate novel sABs, which were used to test a model for paramyxovirus F-HN/H/G interaction. In total, 53 unique sAB sequences were identified and shown to be specific for pre- and postfusion PIV5 F-GCNet (32) and HN.

Surprisingly, despite strong affinity, only a single anti-prefusion F sAB inhibited fusion whereas none of the anti-postfusion F specific sABs inhibited fusion. The inhibitory prefusion specific sAB maps to a novel antigenic site compared to the well-characterized neutralizing sites on RSV F.

Conversely, each of the anti-HN sABs inhibited fusion; however, we show that the inhibitory mechanisms differ depending on the location of the antigenic site. sABs that bind to the HN stalk likely inhibit by preventing association with F, and we discuss this inhibitory mechanism in the context of a recently proposed model for paramyxovirus F triggering by HN/H/G.
MATERIALS AND METHODS

Cells, plasmids, and antibodies. CV-1, MDBK, and 293T cells were maintained in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). CHO-K1 cells were maintained in DMEM/F12 media containing 10% GlutaMax (Gibco), 10% FBS, and 1% Pen Strep (Invitrogen). Hi5 insect cells were maintained in Express 5 serum free medium (Gibco) supplemented with 10% GlutaMax. S2 cells were maintained in Schneider’s Drosophila Medium containing 10% heat-inactivated FBS and 1% Pen Strep.

pCAGGS-F, and pCAGGS-HN are mammalian expression vectors expressing PIV5 F and HN proteins, respectively. pCAGGS-MCS (multiple cloning site) is a negative control lacking insert. pT7 luciferase (Promega) and T7 RNA polymerase plasmids express firefly luciferase under T7 polymerase control and T7 RNA polymerase, respectively.

Antibodies specific for F included pAbs #245 and R9716, raised in rabbits against purified F-GCNt. Antibodies specific for HN include anti-SDS-HN and R471, raised in rabbits against purified HN ectodomain. F-GCNt and HN ectodomain constructs (residues 56-565) were expressed by a recombinant baculovirus in insect cells.

Target protein expression, purification, and Western blot verification. Hi5 insect cells were infected (moi = 2) with a recombinant baculovirus stock containing the PIV5-HN<sub>57-565</sub> construct and the supernatant was harvested 65 h post infection (8). Protein was purified by affinity chromatography using Ni-NTA agarose (Qiagen) as described previously (18). Protein was then concentrated and further purified by SEC on a Superdex 200 column using 50 mM sodium phosphate pH 7.4, 150 mM NaCl as the running buffer.
F-GCNt was expressed from *Drosophila* S2 cells, supernatant was dialyzed into column loading buffer (50 mM phosphate pH 7.0, 300 mM NaCl, 1 mM imidazole) and purified using Ni-NTA as described previously (18). The sample was further purified by SEC as described above. Following purification, both HN<sub>37-565</sub> and F-GCNt protein samples were > 90% pure by SDS-PAGE and silver staining analysis. A portion of the F-GCNt was heated to 60 °C for 10 min to convert it to the postfusion form as previously described (48).

Western blots were performed using the anti-F #245 and anti-SDS-HN (bleed 10) pAb at a 1:1000 dilution as the primary Abs for F-GCNt and HN<sub>37-565</sub> detection, respectively. A goat anti-rabbit IRDye 6800RD (LI-COR) at a 1:10,000 dilution was used as the secondary Ab. Blots were imaged with an Odyssey Infrared Imaging System (LI-COR).

**Target protein biotinylation.** Pre- and postfusion PIV5 F-GCNt and HN<sub>37-565</sub> targets were biotinylated using NHS-PEO-Biotin as described previously (52, 54-56). The extent of biotinylation was quantified using the EZ-link biotin quantification kit (Pierce) according to the manufacturer's protocol. Biotinylation reactions were optimized such that each target molecule contained 2-3 biotins/molecule on average.

**Phage library, screening, and phage ELISA.** A high-diversity M13 phage library expressing synthetic Fab fragments (sABs) as p3 phage coat protein fusions was used for screening. Library generation methods have been described previously (52, 54-56). The library used employs limited amino acid diversity (Tyr or Ser) at most positions in CDRs H1 and H2, while H3 and L1 are enriched for Tyr, Ser, Gly, and Trp and have variable length.

Phage display selection and phage ELISA (including soluble-target competition ELISA) validation of selected clones was performed as described previously (54).
sAB reformatting, expression, and purification. ELISA-validated phage clones were reformatted for sAB protein expression using Kunkel mutagenesis to insert a stop codon between the sAB heavy chain and gene 3. Details of this method and sAB expression and purification have been described previously (54).

Antigenic site binning assay. 100 nM sABs in HBS buffer were labeled with 1 mM Cy3-NHS or Cy5-NHS for 1 hr and quenched with an equal volume of 100 mM Tris pH 8.0 for 1 hr.

HisSorb 96 well plates (Qiagen) were blocked for 30 min with TBS + BSA (100 mM Tris pH 8.0, 150 mM NaCl, 0.5 % BSA) and wells were coated with 50 μL of 30 nM His-tagged pre- or postfusion F-GCNt or HN37-565 diluted in TBS for 60 min. Wells were washed 5X with TBS + 0.05 % Tween-20, and 50 μL of Cy3-labeled sABs were added to appropriate wells (1 sAB per column). After 30 min, 50 μL of 50 nM Cy5-labeled sABs were added (1 sAB per row), and the plate was sealed and incubated in the dark at 4°C for 24 h. Plates were then washed 5X with TBST and 100 μL of TBS was added to each well. Fluorescence was quantified on a Tecan plate reader using excitation and emission wavelengths for Cy3 signal detection of 540-550 nm and 565-600 nm, respectively, and 640-650 nm and 665-700 nm, respectively, for Cy5.

Surface plasmon resonance. Binding kinetics were determined using a BIAcore-2000 with His-tagged pre- or postfusion F-GCNt or HN37-565 immobilized on a Ni-NTA chip (GE Healthcare). The purified sABs were dialyzed into HBS running buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% Tween 20) and serial dilutions were injected (50 μL each) at a flow rate of 25 μL/min. Binding responses were corrected by subtraction of the response on a blank flow cell. A 1:1 Langmuir model using global fitting of kinetic parameters was used, and K_D values were determined from the k_on and k_off values.
Immunoprecipitation (IP) and SDS-PAGE. 293T cell monolayers in 6 well plates transfected with 1 μg pCAGGS-MCS, 1 μg pCAGGS-F, or 2 μg pCAGGS-HN were starved in DMEM deficient in cysteine and methionine for 30 min, followed by labeling with 67 μCi \( ^{\text{35}}S \)-Label (Promix, GE Healthcare) in the same medium for 90 min. The cells were subsequently lysed in cold DH buffer (50 mM HEPES pH 7.4, 10 mM lauryl maltoside, 150 mM NaCl). The lysate was then clarified by centrifugation at 16,000 rcf for 10 min at 4°C. Clarified lysates were incubated with a suitable antibody for 1 hr at 4°C, followed which protein A Sepharose beads were added and the samples and further incubated for 30 min at 4°C. Antibody-antigen complexes were washed twice with DH buffer and once with 50 mM HEPES pH 7.4, 150 mM NaCl. The proteins were eluted from the beads by boiling for 3 min in protein lysis buffer (containing 100 mM dithiothreitol and 1% SDS) and separated on a 15% acrylamide gel. Radioactivity was detected using a Fuji FLA-5100 image reader with Multi Gauge v3.0 software (Fuji Medical Systems, Stamford, CT).

Electron microscopy. Solutions of F-GCNI or HN(57-565) alone or with sABs at a total protein concentration of approximately 5 mg/ml were absorbed onto 300 mesh copper grids covered with a carbon film that had been freshly glow discharged. Grids were stained with a 1% aqueous solution of freshly prepared and filtered uranyl formate. Grids were observed in a JEOL 1230 electron microscope operated at 100 kV, and images were acquired with a Gatan 831 CCD camera at the Biological Imaging Facility, Northwestern University, Evanston, IL.

Viral inhibition assay. Serial dilutions of sAB were prepared in media containing recombinant PIV5 virus expressing a GFP reporter gene. Virus and sAB were incubated for 30 min at 4°C before adding 100 μL to CV1-E cell monolayers in black walled 96 well plates (Costar) at an m.o.i. = 1.3. Following a 12-16 h incubation at 37°C, cells were washed and 100 μL of PBS was
added. GFP signal was quantified via a SpectraMax M5 plate reader (Molecular Devices) using excitation and emission wavelengths of 488 nm and 510 nm, respectively.

Luciferase reporter cell-cell fusion inhibition assay. CHO-K1 effector cell monolayers in 96 well plates were transfected with 0.1 μg each of pCAGGS-F, pCAGGS-HN, and pT7-luciferase plasmids. CHO-K1 target cell monolayers in 6 well plates were similarly transfected with 2 μg of the plasmid encoding T7 RNA polymerase. After 2h, effector cells were washed with PBS and 50 μL of a 1:1 solution of PBS and OptiMem containing 2X final sAB concentration was added to cells. Target cells were washed with versene (530 μM EDTA in PBS) and incubated with 150 μL of versene for 10 min at room temperature with shaking. 1.6 mL per well of OptiMEM was then added and 50 μL of suspended target cells were overlaid on the effector cells. Following a 12-16 hr incubation at 37°C, cells were lysed by incubation with 50 μL of lysis buffer (Promega) per well for 10 min at RT with vigorous shaking. Subsequently, 50 μL of luciferase assay substrate (Promega) was mixed with the cell lysate. 90 μL of the lysate/substrate solution was transferred to a white opaque 96-well plate and luciferase activity was determined using a SpectraMax M5 plate reader (Molecular Devices).

Neuraminidase activity (NA) inhibition assay. Serial dilutions of sAB at 4X final concentration were prepared in 2X final concentration NA assay buffer (200 mM Tris pH 7.0, 10 mM CaCl2) and added to an equal volume of 40 nM (4X stock) purified PIV5 HN57-565 in 2X NA assay buffer in a black walled 96 well plate. Following a 30 min incubation at RT, an equal volume of 2 mM methylumbelliferyl N-acetylneuraminic acid (MUNANA) in H2O was added and the plate and incubated for 30 min at 37°C. The reaction was quenched by adding 20 mM sodium carbonate pH 10.4 to a final concentration of 37.5%. Fluorescence was quantified via a SpectraMax M5 plate reader (Molecular Devices) using excitation and emission wavelengths of 356 nm and 450 nm, respectively.
Hemagglutination activity (HA) inhibition assay. Serial dilutions of sAB at 4X final concentration were prepared in PBS and added to an equal volume of PIV5 virus diluted 1:8 (4X final concentration) in PBS in a 96 well plate. Following a 30 min incubation at 4°C, an equal volume of 2% fresh chicken RBCs in Alsever's solution was added to each well and incubated for 60 min at 4°C and plates were photographed. The highest dilution of virus for which HA activity was observable in the absence of inhibitor was 1:128 (4-fold more dilute than the virus dilution used in this assay).

Selection for resistance mutations. Resistance mutations to selected inhibitory sABs and mAbs F1a and 4b were generated by serial passage of PIV5 infected MDBK cells in the presence of increasing concentrations of inhibitor. An amount of virus equivalent to an m.o.i. = 0.2 was pre-incubated for 30 min at 4°C in the presence of inhibitor at a concentration that inhibited ~75% of the virus (IC$_{75}$) in DMEM containing 1% BSA. The solution was then transferred to washed 90% confluent MDBK cell monolayers in a T25 flask and incubated at 4°C for 60 min. 4 mL of DMEM containing 2% FBS and the same inhibitor concentration as above was then added and the infected cells were incubated 3-4 days at 37°C. HA assays were performed on cell supernatant on days 3-4 to assess the viral titer (HA titer had been previously correlated with plaque assay titer for wt virus). Based on the HA titer, inhibitor concentrations were adjusted in order to increase selective pressure while maintaining sufficient viral titer. The HA titer was also used to know how much supernatant was required to infect a fresh monolayer at m.o.i. = 0.2. After five passages, viral titers were adequate in the presence of inhibitor at $\geq$10X the original IC$_{75}$ concentration. Supernatants potentially containing a pool of multiple viral clones resistant to each inhibitor were harvested.
Isolation of resistant virus clones and verification of resistance. Individual sAb and mAb resistant viral clones were isolated from supernatant containing resistant virus by standard plaque assay. BHK-21F cells were infected with dilutions of passaged MDBK cell supernatants for 1 h at 4°C. Following infection, monolayers were washed with PBS and overlaid with 1% low melting point agarose in DMEM containing 4% FBS and 2% tryptose phosphate broth. Upon solidification, plates were incubated for 96 h at 37°C and second overlay was added (same as above but with 0.01% neutral red). After an additional 16-24 h 37°C incubation, plaques were picked using a sterile Pasteur pipette. The agar plug was transferred to 1 mL of DMEM containing 1% BSA and used to infect MDBK cell monolayers as described above. In some cases, this procedure was repeated using the amplified supernatant from the original plaque to ensure a clonal population of virus.

Plaque reduction assays were performed on viral clones to verify resistance. Following infection of BHK-21F cells in the presence of inhibitor, these assays were performed as described above, except agarose overlays were substituted with a 1:1 mix of 2.4% Avicel and DMEM containing 8% FBS and 4% tryptose phosphate broth and a 2X final concentration of inhibitor, cells were incubated for 72 h following Avicel overlay, and cells were stained with Napthalene Blue-Black for 60 min at RT.

Viral sequencing. Viral RNA was isolated from the cell supernatant using the QIAamp Viral RNA Mini Kit (Qiagen). Viral RNA was reverse transcribed and amplified using the SuperScript III One-Step PCR System (Invitrogen) using F- or HN-specific primers. Resulting amplicons were sequenced using an Applied Biosystems 3100-Avant automated DNA sequencer.
RESULTS AND DISCUSSION

Target preparation and phage display. A prefusion stabilized version of the PIV5 F ectodomain (F-GCNt) and the HN ectodomain (residues 37-565, HN_{37-565}) were expressed using recombinant baculovirus-infected insect cells (8, 32). The proteins were purified by Ni-NTA affinity chromatography and reducing SDS-PAGE and silver staining indicated the purity of each protein was >90%. Western blotting using PIV5 F and HN specific antibodies confirmed the identity of each protein (Fig. 1 A-B). Subsequent size exclusion chromatography (SEC) indicated that F-GCNt and HN_{37-565} were trimeric and tetrameric, respectively. SEC also removed a significant amount of aggregates, especially from the F-GCNt sample (Fig. 1 C-E). A portion of the purified F-GCNt was heated to convert it to the postfusion form as previously described (48), and a portion of the pre- and postfusion F-GCNt and HN_{37-565} were lightly biotinylated to allow subsequent capture on streptavidin (SA) coated beads during phage display. Electron microscopy (EM) was used to estimate the homogeneity of the pre- and postfusion F-GCNt samples. The prefusion F-GCNt sample was an ~70/30 mixture of the pre- and postfusion conformations, respectively, whereas the postfusion F sample was nearly homogenous (data not shown). The EM data also confirmed that biotinylation did not cause gross misfolding or aggregation of the purified proteins.

Phage display was performed essentially as described by Paduch and coworkers (54). M13 phage bound to biotinylated targets were captured from solution using magnetic streptavidin coated beads, the beads were washed, and the specifically bound phage were eluted and used in subsequent rounds of panning to enrich for phage with the highest target affinity. In addition to simple target screens, counter screens were employed. For example, non-biotinylated postfusion F-GCNt was added to the non-homogenous biotinylated prefusion F-GCNt sample to prevent capture of anti-postfusion phage. Following the selections, clones were amplified and a
phage ELISA was performed to verify target binding. The ELISA was repeated in the presence of soluble competitor (non-biotinylated version of the same target) for initial specificity assessment. The tightest binding (highest ELISA signal) and most specific (lowest competition ratio) phage (upper left quadrant Fig. 1 F-G) were then sequenced to identify unique clones (Table S1). All together, 24 unique anti-prefusion F, 17 unique anti-postfusion F (one sAB recognized both pre- and postfusion F), and 12 unique anti-HN sABs were generated.

**Antigenic site binning and affinity and specificity measurements of sABs.** To characterize the sABs outside the context of phage, DNA from each clone was reformatted for protein expression in *E. coli* by inserting a stop codon after the last residue of the heavy chain (before gene III of the phage). sABs were then expressed and purified in a 96-well micro scale format as previously described (54).

To sort sABs into bins recognizing distinct antigenic sites we performed a two color fluorescence competition assay. sABs were labeled with Cy3-NHS or Cy5-NHS ester and a limiting amount of the relevant His-tagged target was captured onto Ni-NTA plates. Each Cy3-labeled sAB competed with the Cy5-labeled version of itself or each of the other sABs (at equal concentration) for target binding (and vice versa). Assays were normalized to each sABs signal in the absence of competition. Each sAB competed with itself resulting in ~50% signal from each color (e.g., Fig. 2, 3C:3C). sABs that did not compete for the same antigenic site gave ~100% signal for each color for each sAB (e.g., Fig. 2, 3E:3C/3C:3E). sABs that competed for the same antigenic site either gave ~50% signal for each color (if they had similar affinities), or the signal from the higher affinity sAB dominated (e.g., Fig 2, 3D:3C/3C:3D). We determined the anti-prefusion F sABs bound to 5 distinct antigenic sites and all but one of the anti-postfusion F sABs bound to the same antigenic site. Additionally, all the anti-HN sABs bound to a single antigenic site with one exception.
Surface plasmon resonance (SPR) was performed to assess the kinetics and affinity of each sAB. His-tagged pre- and postfusion F-GCNt and HN<sub>57-565</sub> targets were captured onto a four-channel Ni-NTA chip (one channel was used as a reference surface) and purified sABs were individually flowed over each surface. This experimental design also served to validate target specificity. Because the prefusion F-GCNt target was somewhat contaminated with the postfusion form, postfusion-specific sABs bound to that surface but with significantly reduced response. Binding of sAB 3D was unique in that it gave a significant signal for both pre- and postfusion F-GCNt surfaces. All of the sABs to F and HN bound with affinities ranging from ~125 nM to sub nM (the vast majority have a K<sub>D</sub> in the low nM to pM range). The highest affinity sABs recognizing unique antigenic sites were chosen for further characterization based on these results (Fig. 3, Table 1).

We then demonstrated the ability of selected sABs to bind purified targets by immunoprecipitating (IP) soluble pre- or postfusion F-GCNt or HN<sub>59-565</sub> using protein A coated beads (Fig. 4 A-B). Anti-prefusion F-GCNt sABs were unable to IP postfusion F-GCNt (Fig. 4 A) and vice versa (data not shown). sAB 03D was uniquely able to IP pre- and postfusion F-GCNt. Furthermore, selected sABs were able to IP wild-type F or HN from transfected cells (Fig. 4 C-D). These data demonstrate our success in producing conformation specific sABs that bind to PIV5 F and HN and which serve as novel tools for studying PIV5 membrane fusion.

**Functional assays.** The ability of sABs representing each unique antigenic site on F and HN to neutralize PIV5 entry and replication was tested (Fig. 5 A, Table 1). Surprisingly, only a single anti-prefusion F sAB, 5D, blocked virus replication or syncytia formation despite the high affinity (<10 nM K<sub>D</sub>) of each of these prefusion-specific sABs. 5D likely inhibits fusion either by stabilizing F in the prefusion form, thus preventing it from refolding to the postfusion...
conformation or, alternately, by preventing the interaction with HN that triggers F to refold. None
of the postfusion F specific sABs inhibited membrane fusion (data not shown), and this may
explain why many neutralizing antibodies are directed to the attachment protein.

Conversely to the anti-F sABs, each of the anti-HN sABs was inhibitory, albeit F4, the anti-HN
sAB recognizing an antigenic site distinct from the others, was markedly less potent in
neutralization (Fig. 5 A). The ability of anti-HN sABs to inhibit NA (Fig. 5B, Table 1) and HA
(Table 1) activity was also assessed to determine their inhibitory mechanism(s). Interestingly,
F4 uniquely inhibited HA and NA activity and at concentrations similar to its IC₅₀ in neutralization
and inhibition assays. We discuss these results below in light of antigenic site location and a
recently proposed model for F activation by HN.

Inhibition of syncytia formation by the sABs mirrored the ability of the antibodies to neutralize
infectivity with 5D, 1H, 4E and F4 all inhibiting fusion activity (Figs. 5A and C), sAB 1H, as an
example of an HN stalk-specific antibody, was tested for its ability to block activation of F
syncytia formation by binding to the HN stalk 1-117 (41). As shown in Fig. 5D sAB 1H addition
to cells expressing either F and HN or F and HN1-117 stalk, were blocked in syncytia formation.

**Antigenic site mapping by EM and mutagenesis.** We visualized sAB/target complexes by
single molecule EM and negative staining to determine the approximate location of each unique
antigenic site (Table 2). Fig. 6 shows at least one target-bound sAB representing each unique
antigenic site identified by the two-color competition assay for each of the targets. F4, the
unique sAB that inhibited both NA and HA activity bound to the HN NA domain (Fig. 6, top row,
right). Interestingly, the sABs that did not inhibit HA and NA function, represented by 1H and 4E,
but potentely neutralized viral entry/replication and inhibited syncytia formation clearly bound to
the HN stalk (Fig. 6, top row, left). Cartoons of the EM images are shown to help orientate the reader, with F or HN in black and the sAB in blue.

It is surprising that the stalk antigenic site of HN was dominant in our selection given that the antigenic response to viral particles in vivo is mostly against the HN NA domain (57-60). The unique HN NA-domain binder, F4, was identified in an alternate phage display selection in which BT-NTA was used to non-covalently attach biotin to the N-terminal His tag of HN37-565 verses random biotinylation of exposed Lys residues (the biotin labeling strategy otherwise used). Steric constraints may have precluded phage bound to the NA domain from being captured onto the SA surface with random Lys biotinylation, but this would not be expected when biotin is located at the N-terminus of HN’s stalk domain. Alternatively, the dense packing of F and HN on the surface of a virion could sterically obscure the stalk-domain antigenic site when Abs are produced against whole virions. Additionally, the variance could be attributed to differences in how the immune system ‘selects’ antigenic sites in vivo compared to phage display.

sABs representing three of the five different anti-prefusion F antigenic sites identified appeared to bind to the F stalk (Fig. 6, row 2). While it is difficult to distinguish the antigenic sites based on EM alone, 1B appeared to bind to the top of the stalk just below the globular head, 1G appeared to bind to the bottom of the stalk (possibly the GCNt trimerization domain), and 3C appeared to bind to the middle region of the stalk. sAB 3D, competed with 3C for binding to prefusion F-GCNt (Fig. 2), and these sABs both appeared to bind in a similar location in the upper stalk region as observed by EM indicating they have overlapping antigenic sites. The two categories of anti-prefusion F head-binding sABs also appeared distinct by EM. 3E appeared to bind an antigenic site near the top of the globular head while 5D-like sABs bound to an antigenic site on the side of the head (Fig. 6, row 3, left).
Each of the postfusion-specific sABs, represented by 1D, recognized the same antigenic site near the widest part of the 'golf-tee shaped' postfusion F protein. Conversely, 3D recognized an antigenic site at the narrow end (Fig. 6, row 3, right). Given the location of the 3D antigenic sites in pre- and postfusion F-GCNt, it is likely that it recognizes a single antigenic site in HRB with conserved secondary structure between the two forms of F.

In addition to mapping antigenic sites by EM, we selected for resistance mutations of representative inhibitory sABs to determine more precisely their antigenic sites on F and HN. Selected sABs included 1H and 4E (anti-HN stalk), F4 (anti-HN NA domain), and 5D (anti-prefusion F, side of head). Neutralizing mAbs 4b (anti-HN) and F1a (anti-F) (61) were also included in the resistance selections for comparison. To select for mutations, PIV5-infected MDCK cells were passaged with increasing concentrations of inhibitory antibody and viral titers were monitored at each passage via HA titer assay. The concentration of inhibitor was adjusted as seemingly appropriate based on the HA titers at each passage. After five rounds of passaging, resistant virus grew in the presence of inhibitor at a concentration at least 10-fold above the initial ~IC_{75}. Resistant strains from cell supernatants were plaque purified, amplified, and the F and HN gene nucleotide sequence obtained to identify resistant mutations (Table 1). Plaque reduction assays were also performed on clonal resistant virus stocks to ensure resistance compared to wild-type virus (data not shown).

Subtle structural changes at the cleavage site occur in prefusion F upon protease cleavage of PIV5 F0 into F1/F2 (33) and mAb F1a reactivity is sensitive to these changes. In accordance with this observation, the identified F1a resistant mutation, E132G, is a surface exposed residue in the HRA helix adjacent to the cleavage site in the PIV5 cleaved and uncleaved prefusion atomic structures. Also, in the postfusion F atomic structure of hPIV3, a closely related...
paramyxovirus, HRA is an extended helix (36), and this conservation of secondary structure for
the F1a antigenic site likely explains why F1a retains some reactivity for PIV5 postfusion F (Fig.
7 A-B)

Multiple resistance mutations, N34D, E85G, and F372S, were found in separate clones from the
anti-F inhibitory sAB 5D resistance selection. These residues map to a noncontiguous antigenic
site comprised of two adjacent protomers that is distinct from that of F1a or the PIV5 equivalent
antigenic sites (based on multiple sequence alignment) of well-characterized antigenic sites in
RSV (34). The movement of protomers relative to each other upon transition from the pre- to
postfusion conformation disrupts the 5D antigenic site as shown by mapping the identified
residues onto the PIV5 prefusion and hPIV3 postfusion structures (Fig. 7 A-B).

**F-HN interaction and fusion triggering mechanism.**

We recently proposed a simple and potentially general model of paramyxovirus F protein
activation in which the head domains of the attachment protein move from a down position to an
up position following receptor binding (Fig. 8). This movement exposes critical residues in the
stalk domain that interact with and trigger F to initiate membrane fusion (41, 62, 63). This model
is based on the following observations: a) mutations in HN that only affect fusion activity map to
the stalk region of the attachment protein of several paramyxoviruses (12, 18, 42, 43, 64, 65); b)
headless PIV5 and NDV HN, MeV H and NiV G is sufficient to trigger fusion (41, 63, 66, 67); c)
the head domains of HN have been observed by EM to be in various orientations relative to the
stalk for NDV (12) and PIV5 (41); d) a crystal structure of the tetrameric NDV HN ectodomain
reveals the heads in the down position forming an interface with the stalk that overlaps with the
critical fusion promoting region ("4-heads-down" conformation) (12); e) different crystal
structures of PIV5 HN tetramers have revealed the heads can adopt a "4-heads-up"
conformation or a hybrid 2-heads-up/2-heads-down conformation (8, 30); f) there is an energetic requirement for the movement of the heads likely indicating receptor binding is the trigger (41).

A key tenant of this model (Fig. 8) is that when the heads are in the down position, access to the fusion-promoting region of the attachment protein stalk is sterically blocked, but that when the heads move to the up position (or are absent) the block surrounding this critical region is removed allowing access of this region to F. The model predicts that antibodies (or other inhibitors) that bind to the fusion-promoting region of attachment protein stalks would inhibit fusion. sAB 1H, an example of an HN stalk-specific antibody, inhibits headless HN 1-117 stalk from activating F and blocks syncytia formation (Fig. 5D). By EM, sABs 1H and 4E bind to the middle region of the HN stalk, and their resistant mutations map to surface exposed residues A82 and Q86, respectively, which comprise a portion of the head/stalk interface as observed in the 2-heads-up/2-heads-down crystal structure (Fig. 7 C-D). Interestingly, we recently showed that these residues could tolerate a variety of mutations while maintaining the ability to trigger F, but that directly adjacent hydrophobic residues V81 and L85 critical for triggering PIV5 F were much less tolerant to substitution (63). This may partially explain why A82 and Q86 were identified when selecting for viable sAB resistant mutations. Additionally, as the anti-HN stalk-binding sABs do not disrupt NA or HA activity, they further establish the fusion-promoting region of the HN stalk mutation data. The generality of this inhibitory mechanism is supported by previously identified neutralizing mAbs against the hPIV2 HN stalk (68).

The model (Fig. 8) also predicts that Abs binding to the HN interaction site on F would prevent fusion by blocking interaction with the HN stalk. 5D recognizes a quaternary antigenic site and may neutralize by stabilizing F-trimers in the prefusion form or by blocking an association with HN. Interestingly, one of the 5D resistant mutations, F372, is directly adjacent to one of the PIV5 equivalent residues (D373, based on sequence alignment) identified in MeV F as being
important for interaction with MeV H. However, we recently identified a role for several hydrophobic residues in the immunoglobulin-like domain within F that mediate the PIV5 F-HN interaction, and these residues are not close to the 5D antigenic site (60).

CONCLUSIONS

We used synthetic antibody technology and phage display to rapidly generate a large number of unique sABs to various antigenic sites on pre- and postfusion F and HN including conformation-specific sABs. Our results show the feasibility of using synthetic antibody technology and phage display to generate a wide variety of specific and custom antibodies that can be used to test specific models. The inhibitory activity of the anti-HN stalk sABs further establishes the emerging model for paramyxovirus F-HN interaction and fusion promotion independently of attachment protein mutations (including chimeric and “headless” constructs) and high-resolution structural information.
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23173 (to R.A.L.) and U01GM094588 (to A.A.K.). B.D.W. was an Associate and R.A.L. is an
Investigator of the Howard Hughes Medical Institute.
REFERENCES


**FIGURE LEGENDS**

**Figure 1: Target preparation and phage ELISA results.**

A) Silver-stained SDS-PAGE analysis of Ni-NTA purified F-GCNt used for phage display. A corresponding Western blot with anti-PIV5 F Ab confirms protein identity. B) A similar analysis as in A of the HN\textsubscript{37-565} protein used for phage display. C) SEC verified the F-GCNt protein was trimeric and removed aggregates and/or high molecular weight (MW) contaminants. D) SEC of HN\textsubscript{37-565} tetramers. E) Profile of MW markers using the same conditions as for F-GCNt and HN\textsubscript{37-565}. Aggregates in each sample elute in the void volume peak at ~20 min. F) Phage ELISA results of individual clones selected against the biotin-labeled prefusion form of F-GCNt. The Y-axis shows the magnitude of the phage ELISA signal while the X-axis shows the ratio of the ELISA signal in the presence (numerator) and absence (denominator) of non-biotinylated F-GCNt used as a soluble competitor at 20 nM. Clones in the upper left quadrant represent the highest affinity clones with target specificity. Arbitrary cutoffs of 0.3 (A450 nm) and 0.5 (competition ratio) were used to select phage for subsequent analysis. G) Similar plot as in F of the ELISA and solution competition ELISA results for HN\textsubscript{37-565}. Non-biotinylated HN\textsubscript{37-565} was used at 10 nM in the competition ELISA and an arbitrary cutoff of 0.6 was used for both parameters to select clones for subsequent analysis.

**Figure 2: Binning of sABs by unique antigenic site.**

A) Representative data from a two-color solution competition ELISA is shown. Each sAB was labeled with Cy3 and Cy5 fluorescent dye and competitive binding to immobilized target was assessed for pairwise combinations. sABs competing with themselves resulted in half maximal binding for each color label compared to the signal in the absence of competition (e.g., 3C:3C). Different sABs that compete for the same antigenic site that have the same affinity also resulted in half maximal binding. However, the higher affinity sAB dominated when sABs with different affinity competed for binding to the same
antigenic site (e.g., 3D:3C/3C:3D). When sABs do not compete for binding, both sABs bind at similar levels as in the absence of competition (e.g., 3E:3C:3E and 3E:3D/3D:3E).

Figure 3: Surface plasmon resonance (SPR) of various sABs. SPR sensorgrams of representative sABs binding to targets. His-tagged pre- and postfusion forms of F-GCNt and HN37-565 were captured onto separate channels of a 4-channel Ni-NTA surface, and sABs were flowed over each surface at multiple concentrations. sAB 3D uniquely bound significantly to both conformations of F-GCNt (right). Raw data (black lines) and curve fits (red lines) are shown. Approximate K_D values are indicated.

Figure 4: Immunoprecipitation (IP) of various sABs A) IP followed by reducing SDS-PAGE and Comassie staining of the purified prefusion forms of F-GCNt using prefusion-conformation-specific sABs (lanes 3-5). These sABs did not IP F-GCNt in the postfusion conformation (lanes 6-8). B) IP followed by reducing SDS-PAGE and Comassie staining of HN56-565 using anti-HN specific sABs (lanes 3-4), and IP of the postfusion form of F-GCNt using anti-postfusion-specific sABs (lanes 6-7). 3D was able to IP both forms of F-GCNt (lanes 6, 8). C) IP and SDS-PAGE of radiolabeled PIV5 F from transfected cells using an anti F-pAb (lane 3), anti-prefusion-specific sABs (lanes 4-5), and an anti-postfusion-specific sAB (lane 6). D) IP and SDS-PAGE of radiolabeled PIV5 HN from transfected cells using an anti-HN-specific pAb (lane 3) and an anti-HN-specific sAB.

Figure 5: Virus-cell fusion neutralization, cell-cell fusion inhibition, and neuraminidase (NA) inhibition data for representative sABs. A) Virus-cell fusion neutralization data. sABs were preincubated for 30 min with replication-competent, GFP-labeled virus prior to infection at an m.o.i. = 1.3. The anti-prefusion-specific sAB, 5D, was uniquely inhibitory among the anti-F-GCNT sABs (dashed lines). All of the anti-HN sABs (solid black lines) were inhibitory. B) NA
inhibition data generated from incubating soluble purified HN
56-565 and anti-HN sABs with
F and HN were expressed in CHO-K1 cells and at 15 h post-tranfection were overlaid with
CHO-K1 cells expressing T7 RNA polymerase. Fusion was quantified using a luciferase
reported assay.  D) BHK cells expressing F and HN or F and HN stalk 1-117 (41) at 15 hr p.i.
were incubated with 200 nM of sAB 1H for 4 hr and photographed.

**Figure 6:** Single-molecule electron microscopy of pre- and postfusion forms of F-GCNt and
HN56-565 alone and with representative sABs from each unique antigenic site bound. These
images reveal the approximate locations of the different antigenic sites on the target molecules
(i.e., stalk vs. NA domain for HN; and stalk, side of head, and top of head for F). sABs have
been artificially colored to make them more visible. First row: anti-HN stalk sABs (1H and 4E)
and anti-HN head sAB (F4). Second row: anti-prefusion F stalk sABs (1B, 1G, 3C, and 3D).
Third row: anti-prefusion F head sABs (3E and 5D) and anti-postfusion F sABs (1D and 3D).
Fourth row: unbound HN, prefusion F (Fpre), postfusion F (Fpost), and sAB alone. Cartoons of
the EM images are shown to help orientate the reader, with F or HN in black and the sAB in
blue.

**Figure 7:** Ab resistance mutations in F and HN. A) Cartoon representation of the prefusion
cleaved F-GCNt crystal structure (PDB ID: 4GIP). Anti-PIV5 F sAB 5D and mAb F1a resistance
mutations are shown as red and orange spheres, respectively, and mutated PIV5 residue
numbers are indicated. B) Cartoon representation of the postfusion hPIV3 F crystal structure
(PDB ID: 1ZTM) showing the location of the same antigenic sites (based on primary sequence
alignment). For clarity, only a single antigenic site is shown for each Ab within the F trimers in A
and B.  C) Cartoon representation of the PIV5 HN56-565 ectodomain 2-heads-up/2-heads-down
structure (PDB ID: 4JF7) with resistance mutations of anti-PIV5 HN sABs 1H, 4E, F4, and mAb
4b (61) shown as cyan, green, magenta, and blue spheres, respectively. The PIV5 residue numbers corresponding to the resistant mutations are indicated. A sulfate ion in the active site of each NA domain is shown as orange and red spheres. The resistant mutations in all four monomers are shown. D) A zoomed-in view of the stalk region of HN reveals that the identified stalk-binding sAB resistance mutations (shown as sticks) are surface exposed.

Figure 8: Model of PIV5 membrane fusion. A) F trimers (yellow, red, magenta, and blue corresponding to domains I, II, III, and HRB, respectively) and HN tetramers (monomers shown as light blue, hot pink, orange, and green) reside on the surface of the virus membrane (gray). Structural data is lacking for the membrane proximal portion of the HN stalk and this region, the transmembrane domain (TM), and the cytoplasmic tails (CT) are depicted as colored lines. Prior to receptor (light and dark brown representing a glycan chain terminating with sialic acid) engagement on a host cell membrane (light orange), HN is in a 4-heads-down conformation in which the heads sterically block interaction between the membrane distal portion of the HN stalk and the head domain of F. B) HN head domains transition to a 4-heads-up position upon receptor engagement allowing the F/HN interaction. HN is rotated ~45° clockwise in B-C compared to panel A. C) Antibodies (Fab, violet) that bind to the membrane distal portion of the HN stalk inhibit membrane fusion by blocking the interaction with F. D) The F/HN interaction results in a major conformation change in F such that domain III refolds into an extended trimeric coiled coil (HRA) and inserts a hydrophobic fusion peptide (purple) into the target cell membrane (for simplicity HN and receptor is not shown in panels D-F). Structural data is lacking for this “prehairpin intermediate” conformation, therefore, it is depicted exclusively as a cartoon. E) HRB interacts with HRA forming a thermostable 6-helix bundle domain in the postfusion
conformation, which juxtaposes the virus and target cell membranes leading to F) membrane fusion. To make this figure, Sculptor (2.1.1_r1, http://sculptor.biomachina.org) (PMID: 21078392) was used to morph high-resolution structures of HN, F, and the humanized anti-HER2 Fab, 4D5 (which the sAB phage library was based on), to 15 Å surface representations. PDB ID’s 3T1E and 3TSI were overlaid and used for the HN 4-heads down model. Similarly PDB ID’s 1Z4X, 3TSI, and 4JF7 were used for the model of the HN 4-heads up model. PDB ID’s 2B9B and 1ZTM were used for the pre- and postfusion representations of F, respectively. The GCN-t trimerization domain was used as a surrogate to represent the TM and CT domains of prefusion F. PDB ID: 1FVD was used for the Fab.
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<th>NA/HA inhibition $\sim IC_{50}$ (nM)</th>
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<th>Resistance mutation(s)</th>
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*Pre- and postfusion F specific, N.A. = Not applicable, N.T. = Not tested
Table 2: Location of sAb antigenic sites as determined by competition ELISA and electron microscopy

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<th>4- prefusion F stalk (middle)</th>
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* Dual specificity

- 2A, 5A, 5H, 6A, 6B, and 6C bind to the prefusion F stalk but epitopes were not further delineated
- The epitopes for 3G and 4D were not characterized