University of California, Santa Cruz

Structure and Binding Kinetics of Monoclonal Antibody 1G1 Support the Inclusion of the Respiratory Syncytial Virus G Protein in Vaccine Development

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By

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Abstract

Respiratory syncytial virus (RSV) is a leading cause of lower respiratory tract infection with significant mortality in infant, immunocompromised, and elderly populations. No approved vaccines or antiviral therapies exist to combat RSV and traditional vaccine development strategies have failed. The G protein on the surface of RSV contributes to the pathogenicity of the virus and is a target of the human adaptive immune response. Previous studies reveal that neutralizing monoclonal antibodies bind to distinct three dimensional epitopes on the G protein. The conformational epitope of another anti-G antibody, 1G1, is explored in this work by x-ray crystallography and binding kinetics assays. The epitope of 1G1 has been determined to lie between residues 157 and 197 of the G protein by binding assays and early x-ray crystallography studies demonstrate the feasibility of determining a precise conformational epitope. Continued efforts in crystallization and kinetic analysis will allow for the construction of a precise atomic resolution structure of 1G1 bound to the RSV G protein and accurate quantification of the affinity of the interaction. Knowledge of how the human immune system neutralizes RSV through the G protein will support innovative efforts to create new vaccines and antiviral therapies.

I. Introduction

Background

Respiratory syncytial virus (RSV) is a top cause of lower respiratory tract infection in both infants and the elderly. In the United States, RSV is responsible for 20% of all hospitalizations of children under five from November to April, which is peak RSV season (1), and incurred a healthcare cost of over one billion dollars between the years of 1997 and 2002 (2). While the economics of RSV infection are more commonly tracked in the United States, the global human toll on life is significant as anywhere between 66,000 and 199,000 children under the age of five died of reported RSV infection in 2005 (3).

Despite a significant impact and demand, there are currently no FDA approved vaccines or antiviral therapies. There is currently one monoclonal antibody (mAb), palivizumab, that is an approved prophylactic. As a prophylactic treatment, the drug must be administered as a preventative measure before infection. This is often impractical as palivizumab costs about \$2133 for each dose (4). The dearth of proper therapies and significant impact of the virus necessitate further research into the biology of the virus and novel approaches to antiviral therapy and vaccine development.

RSV is an enveloped single stranded negative-sense RNA genome virus with 11 encoded genes. There are two surface glycoproteins found on virions which perform the functions of fusion (F protein) of host and viral membranes and attachment to host cells (G protein) (5). Despite the presence of two surface proteins, one protein, the F protein has received the most attention in the literature for a number of reasons. While the F protein is required for the fusion of host and virion membranes, viruses that lack the G protein are capable of infecting and

replicating in cells at lower titers (6). The F protein shows strong sequence conservation, especially compared to the G protein, which suggests that immunity against an F protein antigen would be more broadly protective (5). Finally, anti-F protein antibody titers are over 30-fold higher than anti-G antibody titers, suggesting that anti-F protein antibodies are a stronger correlate of protection in RSV infection (7).

This evidence swayed the focus of therapeutic development in the direction of anti-F antibodies and drugs and RSV vaccines with F protein immunogens. In response to failed attempts at creating a vaccine, including a formalin inactivated virion vaccine that enhanced acute disease in children, new subunit vaccine approaches are being employed. The RSV F protein has been extensively studied and engineered to produce an improved immune response towards the end of creating a subunit vaccine composed of the prefusion stabilized F protein (8). Despite the focus on and development of the F protein, failed vaccine clinical trials indicate a need for new approaches (9).

The G protein, disregarded for discussed reasons, has a significant impact on disease pathology and RSV immunity. Despite global sequence variability, the G protein possesses a central highly conserved cysteine noose motif (5). This conserved domain is bordered by two variable mucin like domains that are subject to heavy O-linked and N-linked glycosylation. Two versions of the G protein exist. The first is embedded in the membrane of the virion by a transmembrane domain. On the surface of the virion, the G protein is responsible for attachment to host cells. A second form of the G protein is produced through an alternate start codon downstream of the transmembrane domain which is then secreted from infected cells shortly after infection (10). Released from cells, this soluble G protein modulates the immune system by

interacting and inducing locomotion of CX3CR1⁺ immune cells (11). While previously the protein was thought to be less immunologically relevant, it has been shown in animal studies that anti-G antibodies reduced viral load and significantly reduce respiratory tract pathogenesis (12, 13).

A recent study reported the isolation of neutralizing anti-G monoclonal antibodies from human survivors (14). These antibodies possess high binding affinities for the G protein in the picomolar range and were even shown to be protective when administered post infection in a mouse model (12). Linear epitope mapping was performed to determine which portion of the G protein was bound by broadly neutralizing monoclonal antibodies isolated from survivors. Most antibodies were found to bind in the central conserved domain. Despite possessing high affinity and neutralization capability, linear epitopes were not determined for a number of antibodies. This finding challenged the notion that the central conserved cysteine motif lacked a significant tertiary or even secondary structure that was important for the binding of and neutralization by antibodies.

Parallel x-ray crystallography studies confirmed the importance of a three dimensional conformation of the central conserved domain (CCD) (15,16). While the linear epitope of one monoclonal antibody, 3D3, was found, crystallographic and binding studies revealed that the antibody did not only bind the linear epitope, but it also bound a more extensive three dimensional surface. Further, no linear epitope could be identified for antibody 2D10, but crystal structures revealed that the antibody bound a conformational epitope in the CCD. While the CCD, a domain lending its tertiary structure to two disulfide bonds, shows strong conformational

identity within the cysteine motif in the four crystal structures published, significant conformational flexibility has been observed in the termini of the CCD.

It is possible that thee conformations of the termini are trapped by antibodies in conformations that are relevant for RSV G function, such as binding to the cellular receptor and modulation of the immune system. Further structural studies of RSV G and antibody complexes may reveal trends in the conformational flexibility of the protein and reveal relevant conformations for biological function.

Problem Statement and Rationale for Experiments

While significant progress has been made in the biophysical characterization of the RSV G protein in complex with neutralizing antibodies, four neutralizing antibodies isolated by Trellis Bioscience remain uncharacterized. Antibodies 1G1 and 6A12 bind the G protein and neutralize the virus, but no linear epitope on the G protein was identified for these antibodies. Further, linear epitopes were determined for antibodies 2B11 and 1G8, but their conformational epitopes may include more residues than previously identified. By investigating the full conformational epitopes of these four antibodies, important information will be learned that will allow for a more informed design of an RSV vaccine.

In order to understand the binding of the four antibodies, x-ray crystallography of the antigen and antibody complex will be performed to create a precise atomic level snapshot depicting the precise residues on the RSV G protein that are involved in antibody binding. In parallel, analysis of the binding kinetics of these antibodies will be performed to quantify the affinity of the antibodies for their antigen.

While the precise epitopes of all four antibodies are desired, this work focuses on antibody 1G1 as no information about its epitope was known and in early experiments, protein constructs of antibody 1G1 were more easily expressed and purified in the laboratory.

Antibody and RSV G Protein Constructs

Antibodies were produced recombinantly for the described experiments. As the full length immunoglobulin G (IgG) constructs are cumbersome in crystallization experiments, smaller constructs are produced that exploit the compact nature of the antigen binding site of an antibody. Only the variable regions of the heavy and light chain of an antibody are required to bind an antigen and these two domains can be combined into one protein construct. A fusion of the heavy chain variable domain and the light chain variable domain is called a single chain variable fragment (SCFV). Using the same logic, full length IgG constructs can be produced that bind the intended antigen with no knowledge of the primary sequence of the constant regions of the native protein. By replacing the variable regions of the heavy and light chains of one IgG with those of another, a full length IgG can be made for any mAb. In this study, the acronym "mAb" will be used when discussing a full length IgG antibody construct.

As was previously mentioned, the RSV G protein can be found in a secreted form that lacks transmembrane and intracellular residues. As membrane bound proteins are difficult to work with, an RSV G protein construct mimicking this secreted version has been produced and was named RSV G ecto. Another construct corresponding to residues 157-197 of the G protein was also produced, This construct, dubbed RSV G 157, contains the cysteine noose motif, the central conserved domain, lacks glycosylation, and has been shown to be as functional as RSV G ecto in binding and cellular assay experiments. Further, this construct allows for crystallography of

complexes of antibodies with the G protein as the full length or G ecto constructs contain large conformationally heterogeneous regions that inhibit crystallization.

II. Expression and Purification of Recombinant 1G1 Antibody Constructs

Antibody Construct Design

In order to study the antibodies isolated by Trellis Bioscience, protein constructs were designed to facilitate their expression and purification in the laboratory setting. To solve the atomic structure of antibodies in a complex with their antigens by x-ray crystallography, the smallest possible antibody constructs are needed. In order to create a protein construct of minimal size that maintains antigen binding, single chain variable fragments (SCFV) of antibodies 1G1, 6A12, 1G8, and 2B11 were designed. The heavy and light chain variable region amino acid sequences were obtained from Trellis Bioscience. A synthetic DNA fragment codon optimized for insect cells was designed with the heavy chain variable amino acid sequence, a fifteen residue linker sequence, and the light chain variable amino acid sequence. These fragments were inserted into an expression vector by Gibson Assembly and the vectors were verified to contain the gene of interest by sanger sequencing. The vector includes a secretion signal upstream of the protein, a thrombin cleavage site, and a twin strep tag downstream of the protein, a puromycin resistance gene, and a metallothionein promoter for the induction of recombinant protein expression.

Figure 2.1 SCFVs are composed of the variable heavy chain (red), linker (black), and variable light chain (blue).



For other experiments, including binding and neutralization assays, it may be preferential to use a full length IgG construct that not only binds an antigen, but may signal to cells through the Fc receptor on the constant region of the heavy chain. The variable heavy and light chains of the four antibodies studied were separately amplified by PCR and inserted into a plasmid containing either a heavy or light chain constant region from the VRC01 IgG. After insertion of the heavy and light chain sequences into the constant backbone by Gibson assembly, the assembled vectors were verified by sanger sequencing. The fusion of light and heavy chains of interest to a constant region backbone allows expression of the 1G1, 6A12, 1G8, and 2B11 antibodies as full length IgG antibodies with the ability to signal to immune cells.

Figure 2.2 IgG antibody constructs were produced by fusing heavy and light chains of interest to a known constant region backbone.



Production of Chemically Inducible S2 Cells for Expression of SCFV Constructs

Schneider 2 (S2) cells, a cell line derived from Drosophila melanogaster embryos, were selected to express the SCFV antibody constructs. S2 cells are capable of high levels of protein production, can be stably transfected with a gene of interest, and do not require the use of fetal bovine serum in their growth media which make them an excellent choice of expression system. Two separate flasks of cells were transfected with each SCFV construct. One of the two were immediately induced by cupric chloride to assay for protein expression and the other was treated with puromycin to select for transfected cells. Media from the transiently transfected and induced cells was collected and assayed for protein by western blot. 1G1, 1G8, and 2B11 SCFVs appeared to be successfully expressed, while the 6A12 SCFV showed little to no expression.

Figure 2.3 Depicted are the results of the anti-strep tag western blot of the transiently transfected cell media. The lanes in order from left to right are, molecular weight standard, dilute 1G1 media, concentrated 1G1 media, dilute 1G8 media, concentrated 1G8 media, dilute 2B11 media, concentrated 2B11 media, dilute 6A12 media, concentrated 6A12 media.



The transfected cells were continually selected for two weeks until cell death was no longer observed in the presence of puromycin. At this time, the cells were weaned off of the rich fetal bovine serum containing media used for transfection and selection onto ESF 921 media, a media designed for protein expression in S2 cells. A portion of the weaned cells were split into T75 sitting flasks at a volume of 15 mL and protein expression was induced using cupric chloride. After seven days, the media was assayed for protein expression by western blot. Once again, 1G1, 1G8, and 2B11 SCFV transfected cells displayed expression of the desired protein, while the 6A12 transfected cells did not show high levels of protein expression. These results indicated that stably transfected S2 cells containing the 1G1, 1G8, and 2B11 SCFVs had been produced which could be used for larger scale expression of these antibodies.

Figure 2.4 Anti-strep tag western blot of the stable cell test expression. The identity of my lanes are 1) molecular weight standards 2) 1G8 media 3) 1G8 column flow through 4) 1G8 elution 5) 6A12 media 6) 6A12 column flow through 7) 6A12 elution 8) 2B11 media 9) 2B11 column flow through 10) 2B11 elution 11) 1G1 media 12) 1G1 column flow through 13) 1G1 elution.



Large Scale Expression of 1G1 SCFV in S2 Cells

S2 cells transfected with the 1G1 SCFV appeared to yield the most protein of any of the stable cell lines. The cells were continuously split and scaled up to 2.5 liters in ESF 921 media. Once this volume had been reached, 1 mM cupric chloride was added to induce protein expression. After six days the cells were harvested and purified on a streptactin column using an Akta FPLC instrument. Overall, 2.6 mg of protein were obtained from the purification.

Figure 2.5 Anti-strep tag western blot of first large scale 1G1 SCFV expression identities of lanes are 1) media sample 2) media after pH adjustment 3) filtrate from concentration 4) concentrated pre-column sample 5) column flow through 6) elution from column.



While enough protein was obtained to attempt crystallization in complex with RSV G 157, it was expected that more protein would be obtained from the expression and that conditions could be improved in order to obtain more protein, which would allow for higher throughput crystallization experiments. One factor that likely lead to poor expression of the protein was the low cell density observed in the 3 L shaking cell culture flasks used in the experiment. While the cell line routinely achieved a density of 20 million cells per mL in smaller format flasks, they were observed at 5 million per mL in the large 3 L flasks used in the induction and expression stages of the experiment.

A second expression of the 1G1 SCFV construct was performed using the stably transfected S2 cells. Frozen aliquots of stable cells adapted to serum free media were thawed and split into a shaking cell culture flask. They were split and scaled up to a total volume of 3 L. Once again, cells reached a density of 5 million cells per mL in the 3 L flasks, despite achieving a density of 20 million cells per mL in 1 L flasks. Cells at a density of 5 million cells per mL were split from a 3 L flask into three separate 1 L flasks. Despite growing in the smaller format flasks, the cells did not achieve a density higher than 5 million cells per mL. The cells were then induced and harvested after six days. 1G1 SCFV was purified using a streptactin column to yield 1.2 mg of protein.

Figure 2.6 Anti-strep tag western blot of second large scale expression of 1G1 SCFV, identities of lanes are 1) molecular weight standards 2) media sample from 3L flask 3) media sample from 1L flask 4) media after centrifugation 5) media after pH adjustment 6) filtrate from concentration 7) empty 8) flow through from column 9) pre-column sample 10) elution from column.



Expression of the 1G1 SCFV was clearly observed, although a low yield suggests that expression was lower than expected. Further, purification of the construct appeared to be successful as no protein was detected in the column flow through.

It was seen from the second expression that the stable cells were reproducibly failing to reach a high density in large format shaking flasks. In addition, the level of protein expression in large format shaking flasks consistently contradicted the high expression observed from the transient transfection and expression and the stable cell test expression. Both large scale expressions were performed using the ESF 921 media, however previous SCFV constructs expressed by previous lab members had been expressed and purified with high yield using Insectexpress, a different serum free media designed for use with S2 cells. Further, notes from other lab members indicated that cells grew to a high density in large volume shaking flasks using the Insectexpress media. Future attempts to express the 1G1 SCFV protein will be made using the Insectexpress media.

Purification of 1G1 mAb Construct from CHO Cell Supernatant

The 1G1 mAb construct was expressed in Chinese Hamster Ovary (CHO) cells by another lab member. As the protein was secreted from cells, the supernatant was collected and purified with a column containing protein A resin. 2.5 mg of protein was obtained from the CHO cell supernatant.

Figure 2.7 SDS-PAGE gel of the 1G1 mAb performed under reducing and non-reducing conditions. The identities of the lanes are 1) molecular weight standards 2) CHO cell media 3) protein A column flow through 4-6) elutions run under reducing conditions 7-8) elutions run under non-reducing conditions, arrows highlight full length IgG, heavy chain, and light chain from top to bottom.



SDS-PAGE of the 1G1 mAb after purification demonstrated that the construct was isolated with high purity. Further, analysis under reducing conditions demonstrated that both the light chain and heavy chain were present as bands were observed at both 25 kDa and 50 kDa respectively. Finally, analysis under non-reducing conditions resulted in a band on the gel

corresponding to a molecular weight of 150 kDa. This indicated that two heavy and two light chains associated as expected to form a full length IgG complex.

III. Structure of 1G1 SCFV RSV G 157 Complex

Purification of RSV G 157 and 1G1 SCFV Complex

In order to solve the three dimensional atomic resolution structure of the 1G1 SCFV in complex with its antigen, the complex was reconstituted in vitro and purified. After expression of the RSV G 157 construct and the 1G1 SCFV antibody, purified fractions of each protein were combined and mixed to allow the two proteins to bind and form a stable complex. 2.5 mg of 1G1 SCFV and 3.5 mg of RSV G 157 were combined. RSV G 157 was added to the mixture in more than ten fold molar excess to saturate antibody, preventing the presence of unbound antibody in the mixture. After the 1G1 SCFV and RSV G 157 mixture was mixed overnight at 4°C, the mixture was concentrated to 250 μ L using a 10 kDa cutoff centrifugal protein concentration unit, which resulted in a final concentration of 10 mg/mL. The complex was then purified using size exclusion chromatography.

The concentrated sample was injected onto a Superdex 75 10/300 size exclusion chromatography column using an Akta FPLC instrument. The results of the purification are displayed in figure 3.1. A single peak in the absorbance at 280 nm spectrum at an elution volume of approximately 12.5 mL was observed. Comparison to previous SCFV antibodies in complex with RSV G constructs suggested that this singular peak was likely due to the successful formation of the 1G1 and RSV G complex. Because excess RSV G 157 was added, a second peak of unbound antigen was expected. No such peak was observed, however there was an increase in absorbance at 260 nm directly after the peak containing the complex. RSV G 157 is known to display significant absorbance at this wavelength, suggesting that this peak is the result of unbound antigen. RSV G 157 is also known to flow through 10 kDa cutoff concentration units, so the excess protein may have been eliminated during sample preparation for chromatography. In order to confirm the identity of the peak, fractions were run on an SDS-PAGE gel.

Figure 3.1 Chromatography trace of the 1G1 SCFV RSV G 157 complex, absorbance at 280 nm is shown in blue, while the absorbance at 260 nm is shown in red.



SDS-PAGE of the size exclusion fractions revealed that both 1G1 SCFV and RSV G 157 were present in the peak observed during chromatography. While it was possible that the two proteins did not bind, but merely eluted from the column in the same fractions, previous studies

of antibody and RSV G complexes suggested that RSV G 157 would elute at a different volume when not bound to an antibody. Further, the two proteins were present with high purity, suggesting that the contaminants present from the difficulty of purifying RSV G 157 were no longer present. The fractions collected from chromatography were then combined and used for crystallization experiments.

Figure 3.2 SDS-PAGE gel of size exclusion chromatography fractions. The lanes, in order from left to right, contain protein standards of known molecular weight (ladder), 1G1 SCFV, RSV G 157, 1G1 SCFV with RSV G 157, and elution fractions in all subsequent lanes.



A second sample was prepared for crystallization following the completion of a second expression of 1G1 SCFV. RSV G 157, and the antibody, 1G1 SCFV, were combined and concentrated, then purified by microscale affinity chromatography using streptactin resin as the

amount of protein was too small to perform size exclusion chromatography with the available materials.

1 mg of 1G1 SCFV was added to 22 mg of RSV G 157. Once again, a vast excess of antigen was used to saturate the antibody. The complex was purified on a 1 mL streptactin column. The elutions were concentrated using a 10 kDa cutoff centrifugal concentrator to a volume of 35 μ L with a concentration of 6.5 mg/mL. Unfortunately, no SDS-PAGE analysis was performed to determine purity as the sample was mistakenly disposed of. Before this incident, the sample was used in crystallization trials.

Crystallization Trials of the 1G1 SCFV RSV G 157 Complex

The size exclusion chromatography fractions of the first sample of the complex were pooled and concentrated using a 10 kD cutoff centrifugal concentrator. A final volume of 60 μ L was reached at a concentration of 11 mg/mL. Using a liquid handling robot, this sample was screened for crystallization using the MCSG1 crystallization screen. While many of the screening conditions have produced phase separation and precipitation, no crystals have been observed in any of the 96 conditions screened.





As this first screen proved unproductive, another crystallization screen, Index HT, was employed. At the time of setting up the screen, the protein sample had been stored at 4°C and pH 8 for multiple weeks. As this pH is quite close to the isoelectric point of the protein, about half of the protein had precipitated from the solution, resulting in a concentration of 6 mg/mL. A liquid handling robot was once again used to set up sitting drops using the Index HT conditions. Three months after the screen was set up, one condition, H1 (0.2M magnesium chloride hexahydrate, 0.1M Tris pH 8.5, 25% w/v PEG 3350), produced a singular protein crystal.

Figure 3.4 Protein crystal produced by the Index HT screen.



This original protein sample had completely crashed out of solution at the time the drop was discovered. The second sample of complex prepared from the second expression of 1G1 SCFV was used to try and replicate the results of the Index HT screen. Four 1 μ L hanging drops were setup using this 6.5 mg/mL protein sample. The buffer and salt concentrations were identical to the original H1 condition and the PEG 3350 concentration was varied for each drop. The drops

contained 23%, 25%, 26%, and 27% PEG 3350. No protein crystals have been observed in these

drops.



Figure 3.5 Two 1G1 SCFV RSV G 157 Index HT H1 hanging drops at PEG 3350 concentrations of 26% (left) and 27% (right).

Additionally, the second sample was used to set up a third crystallization screening tray using the MCSG2 screening conditions. The protein sample was at a concentration of 6.5 mg/mL. No protein crystals have been observed in any of the drops to date.

Figure 3.6 Representative sitting drops of the MCSG2 screen.



A number of variables could be responsible for the lack of crystallization. Issues with expression of 1G1 SCFV have allowed limited choice in the final concentration of the complex. With larger amounts of protein, it would be optimal to attempt crystallization at higher concentrations. Additionally, the twin strep tag on 1G1 SCFV can be removed using the thrombin cleavage site. This could remove a flexible region of the protein complex that inhibits crystallization. Further, the antibody could be screened for binding to even smaller constructs of the antigen. Unbound residues on the flexible C and N termini of RSV G 157 may inhibit crystallization of the complex. The single protein crystal formed was used to collect a diffraction dataset and portions of the structure have been solved, indicating that further attempts to crystallize the protein may have positive results.

X-Ray Diffraction and Structure of the 1G1 SCFV RSV G 157 Complex

The single crystal obtained from the Index HT screen was extracted from the sitting drop of the screening plate and then frozen in liquid nitrogen and transported to beamline 8.3.1 at the Advanced Light Source at the Lawrence Berkeley National Laboratory. Dr. Sarvind Tripathi collected 360 x-ray diffraction images were obtained by rotating the crystal in one degree increments. Through analysis of the diffraction images, a resolution of approximately 3.0 angstroms was obtained and it was found that the crystal had a space group of P 32 2 1. The diffraction images were processed using iMosflm.

The crystal structure of the complex was solved using molecular replacement. The Prediction of Immunoglobulin Structure (PIGS) server was used to find heavy and light chain sequences similar to the 1G1 SCFV sequence for which a structure had already been solved and deposited in the Protein Data Bank (PDB). A model was constructed using the three dimensional coordinates of the most similar light chain and heavy chain sequences. The most similar heavy chain sequence model belonged to PDB structure 5L6Y and the CDR3 of structure 4XML as the

1G1 SCFV sequence possesses a large CDR3. The most similar light chain sequence belonged to PDB structure 3GO1. Molecular replacement was performed using this model and the Phaser program with the reference model split into two separate ensembles corresponding to the heavy and light chains. The molecular replacement model provided phase information that allowed for the solution of the crystal structure of the 1G1 SCFV RSV G 157 complex.

Figure 3.7 Molecular replacement model and sequence alignment of model ("target") to the 1G1 SCFV sequence ("template"). The heavy chain is in dark grey with the light chain in light grey and the CDR loops are colored according to the alignment, two views rotated 180 degrees from each other are shown.

	1	10	20	30		40	50	52	62		74	84	94
	1	1	1	ABCDEFG	GHIJ	1	ABCDEFGH	1	1	ABCDEFGH	1	1	ABCDEFG
target:	FEL	TQPPSVSVSP	GQTARITCSG	DALPK	QYV	YWYQQKPGQAPVLV	IYK	TTERPSG	IPERFSDSSS	5GT	TVTLTISAA	QAEDEADYYCQS	VDSSGT
template:	-SYVL	TOPPSVSVSP	GQTARITCSA	EALSN	0YA	YWYRORPGOAPLLI	IYK	DTKRPSG	IPERFSGSTS	GT	TVTLTISGV	OAFDEADYYCOS	
eavy Chain 1	arget - Te	emplate aligr	iment										
eavy Chain T	arget - Te	emplate aligr	iment 20	30	33	43		55	65	75		92	
leavy Chain T	arget - Te 1	emplate aligr 10 I	iment 20	30 ABCDEF		43 	ABCDEFGH	55 I	65 I	75 1	ABC	92 	ABCDEFGHIJ
leavy Chain T target:	arget - Te 1 QVHLV	emplate aligr 10 QSGVEVKKPG	iment 20 ASVRVSCKAS	30 ABCDEF GYTFAT	33 =G YGITW	43 VRQAPGRGLEWVGW	ABCDEFGH	55 NDRTSYA	65 QIFHGRVTM1	75 ITDTSTNTAYM	ABC ELRSLRSDD	92 DTAMYYCARNHCNI	ABCDEFGHIJ FYHDFWSGL
leavy Chain T target:	arget - Te 1 QVHLV	emplate aligr 10 QSGVEVKKPG	iment 20 I ASVRVSCKAS	30 ABCDEF GYTFAT	33 =G YGITW	43 VRQAPGRGLEWVGW	ABCDEFGH /ITPY	55 NDRTSYA	65 QIFHGRVTM1	75 ITDTSTNTAYM	ABC ELRSLRSDD	92 DTAMYYCARNHCNI	ABCDEFGHIJ FYHDFWSGL



The model obtained by solving the structure gives significant information about the architecture of the 1G1 antibody, however electron density corresponding to the CDR loops of

the antibody and the antigen did not appear in the electron density map and were not modeled in the atomic structure. The noncovalent bonds between the light and heavy chains can be observed and the relative beginning and end points of the CDR loops are clearly defined in the structure. Unfortunately, no information about antigen binding could be learned from these data.

Figure 3.8 a) Molecular model of the 1G1 SCFV, arrows indicate the expected locations of the CDR loops and antigen b) image of the electron density map including one asymmetric unit (yellow) and a neighboring molecule in the crystal lattice (blue) lacking unoccupied density with an arrow pointing to the region that the antigen was expected to be found in.





There are multiple reasons why the crucial CDR loops and the bound antigen may not appear in the electron density map. It is possible, because the crystal formed months after the drop was setup that the protein was damaged by proteolytic agents or enzymes. If this were to occur in the CDR loops, the most conformationally flexible region of the antibody, the antigen could become unbound. Another explanation originates from the observation that the antigen can adopt an array of distinct conformations. If the crystal formed with heterogeneous conformation states of the antigen and CDR loops, accurate diffraction data corresponding to a single structure would not be obtained. While attempts to determine the precise conformational epitope of the 1G1 antibody have not yielded satisfactory results, optimization of crystallization conditions and construct design in conjunction with continued attempts to express the SCFV construct may yield high resolution data allowing for the precise determination of a conformational epitope.

IV. Binding Kinetics of 1G1

1G1 mAb Binds RSV G Ecto and RSV G 157

It was known that antibody 1G1 bound respiratory syncytial virus G protein, but a specific epitope could not be determined. The central conserved domain (CCD) of the G protein, contained in the RSV G 157 construct is the target of five other broadly neutralizing mAbs targeting this protein. 1G1 mAb was tested for binding to this construct using a Fortebio Octet instrument.

1G1 mAb, diluted to a concentration of $15 \mu \text{g/mL}$, was captured by anti-Fc sensor tips. After the antibody had associated, the sensor tips were dipped in solutions containing either RSV G ecto or RSV G 157 at various concentrations. It was found that both constructs associated with the antibody and dissociated with an exceptionally low rate. These data demonstrated that the epitope of the 1G1 antibody is contained in the RSV G 157 construct.



Figure 4.1 Raw data demonstrating the binding of the 1G1 antibody to RSV G constructs. RSV G ecto (top) was diluted to 200 nM, 100 nM, and 50 nM and RSV G 157 (bottom) was diluted to 500 nM, 250 nM, and 25 nM.

The data acquired from this experiment were not analyzed to determine association and dissociation rates for subsequent binding constant (K_D) calculation. Near saturation of both the sensor tips and bound antibody could be seen as the binding curves reached a plateau at the end of the antibody and antigen association steps. In order to determine accurate association and dissociation rates, lower antibody and antigen concentrations would need to be used.

1G1 mAb Binds RSV G Ecto and RSV G 157 With High Affinity

As determination of the dissociation constant (K_D) of this antibody in complex with RSV G would allow comparison to other antibodies, more binding experiments using the Fortebio Octet

instrument were performed. In order to accurately calculate a dissociation constant, lower concentrations of antibody and antigen would need to be used. Saturation of the sensor tip with antibody makes the determination of an antigen association rate difficult as the binding curve will begin to reach a plateau as the complex reaches an equilibrium state. Such heavy amounts of antibody on the sensor tip can also obscure the dissociation rate as antigen can once again become bound shortly after dissociation.

In order to rectify these issues, Octet anti-Fc sensor tips were allowed to associate with 1G1 mAb at a concentration of 2 μ g/mL. The captured antibody was then allowed to bind with RSV G ecto and RSV G 157 at concentrations of 25 nM, 5 nM, and 1 nM each. These conditions resulted in data that could be analyzed to determine the dissociation constant.

Figure 4.2 Binding data of 1G1 mAb binding to RSV G ecto (bottom, blue) and RSV G 157 (top, red), curves used to determine association and dissociation rates are shown.



It was clear that the curves used to calculate rates displayed a poor fit for the RSV G 157 binding experiment while the G ecto experiment resulted in data that was better fit. A strange

increase in signal was observed for both constructs in the beginning of the dissociation step of the experiment. It was expected that after removing the sensor tips from the well containing the antigen, the signal would not increase, as an increase in signal corresponds to an increase in the amount of antigen bound to antibodies. It is possible that this phenomenon was due to non-specific binding of the antigen to the sensor tip and subsequent association to an antibody after the tip had been removed from the antigen containing well.

Regardless of anomalies in data acquisition and high levels of error in precise rates, it was clear that dissociation from the 1G1 antibody was extremely slow for both complexes. This evidence demonstrates that the antibody binds both the full length and the minimal RSV G constructs with high affinity, further confirming that the complete epitope of the 1G1 antibody lies between residues 157 and 197 of the G protein.

Table 4.1 Average K_D and curve fitting statistics determined from binding experiments.

Construct of RSV G	$K_{\rm D} \pm {\rm error} (M)$	\mathbb{R}^2	X^2
RSV G ecto	$<1x10^{-12} \pm 2.722x10^{-12}$	0.9954	0.8643
RSV G 157	$1.181 \times 10^{-12} \pm 1.030 \times 10^{-11}$	0.9556	6.2353

The binding experiments were performed over a wide dilution series of antigen (25 nM to 1 nM), but the data indicate that optimal data could be obtained by using a more narrow dilution series between 25 nM and 5 nM concentrations of antigen. Further, extremely slow dissociation rates necessitate a longer dissociation step in the experiment. An irreversible binding of the antibody to the sensor tip is required to collect data for such a length of time.

Biotinylation of 1G1 mAb and Capture of Antigen by Biotinylated 1G1 mAb

In order to achieve near irreversible binding of the 1G1 mAb construct to the Octet sensor tip for experiments with an hour long dissociation time, the 1G1 mAb was biotinylated for use with a streptavidin sensor tip. The reagent used for biotinylation of the mAb through amine groups was selected for a long polyethylene glycol linker that conferred water solubility and an N-hydroxysuccinimide functional group for reaction with the amine. The reagent was dissolved in pH 7.2 PBS and added to a solution of 1G1 mAb in a molar ratio that would result in most antibodies reacting with 3 to 5 biotin molecules.

Figure 4.3 Structure of NHS-PEG₄-Biotin and biotinylation scheme, biotin molecules are represented by the letter "B" attached to the antibody.



The biotinylated mAb was then used to perform a binding assay using streptavidin sensor tips in the Octet instrument. Antigen concentrations of 25 nM, 12.5 nM, and 6.25 nM were used for RSV G 157-197 and RSV G Ecto and a dissociation time of one hour was selected. The biotinylated mAb at 2 μ g/mL associated with the streptavidin sensor tip, indicating successful biotinylation of the antibody. The baseline step, consisting of dipping the sensor tip in buffer after loading with the antibody, showed no dissociation of the antibody. Unfortunately, no measurable association between the antibody and the antigen occurred. Figure 4.4 Raw data of biotinylated 1G1 mAb Octet assay.



It is likely that the abrogation of binding to antigen was due to steric hindrance caused by the biotinylation of the CDR loops or areas near the CDR loops and subsequent binding to the sensor tip. Further, the binding of multiple biotin molecules elsewhere on the antibody molecule could cause the antibody to bind the sensor tip in an orientation that blocks antigen binding. The biotinylation of the antibody can be optimized to result in more sparse biotinylation of only one biotin molecule per mAb molecule.

Conclusion

Four new antibodies, 1G1, 6A12, 1G8, and 2B11 have been cloned as SCFV and full length mAb constructs and stable cells transfected with the SCFV constructs have been produced. Further, the 1G1 SCFV and mAb constructs have been successfully expressed and purified. While the SCFV has been purified with low yield, sufficient amounts have been produced to pursue a crystal structure of the SCFV in complex with a minimal construct of RSV G. This will allow the determination of three dimensional conformational epitopes of two antibodies with no known linear epitope (1G1 and 6A12) and two antibodies whose epitopes likely encompass residues that lie beyond their determined linear epitopes (1G8 and 2B11). The successful determination of these four epitopes would double the number of anti-G antibody conformational epitopes solved and available to the research community. More information about how RSV G is neutralized by human antibodies may provide valuable information to guide the design of novel immunogens. As novel approaches to vaccine design and the development of antiviral therapeutics increasingly rely on the G protein, accurate information about the targeting of the G protein by the human immune system will become more important.

While a three dimensional atomic resolution structure illuminating the conformational epitope of the 1G1 antibody has not been solved, significant progress has been made. With continued production of the 1G1 SCFV, crystallization in complex with RSV G 157 will continue to be attempted. The complex that has been used has proven to be difficult to crystallize, however slight modifications to the complex may improve success. The thrombin cleavage site N-terminal to the twin strep tag present on the SCFV allows for enzymatic removal of the possibly disordered tag of the SCFV, which may facilitate successful crystallization. Further, the binding

experiments performed on the Octet instrument may inform RSV G construct design for cocrystallization experiments. Excess residues not involved in binding may inhibit crystallization as the termini of the RSV G 157 construct are capable of adopting multiple conformations in solution. Discovering a minimal binding construct of RSV G might facilitate successful cocrystallization and x-ray crystallography of the complex.

Binding experiments using the Octet instrument have demonstrated that the 1G1 antibody, an antibody for which no linear epitope on the G protein could be determined, binds RSV G between residues 157 and 197 with high affinity. Efforts to quantify the affinity of the two proteins will benefit from optimization of biotinylation of the 1G1 mAb construct. A functional biotinylated antibody would allow the for increased dissociation times in binding experiments and more accurate determinations of on and off rates. Upon quantification, differences in the binding of minimal RSV G and full length RSV G constructs can be observed. Further, comparison of different minimal constructs of RSV G could allow for more precise determination of the 1G1 binding epitope lies.

Overall, successful characterization of antibody 1G1 will add to the growing supply of information that can support RSV vaccine design and therapeutic development. It has already been shown that antibody 1G1 binds RSV G in the same location as five other neutralizing antibodies. More specific molecular determinants of RSV G neutralization can be defined by determining the precise epitope of antibody 1G1.

Materials and Methods

Molecular Cloning of Antibody Constructs

Synthetic DNA fragments encoding 1G1, 2B11, 1G8, and 6A12 SCFVs were cloned into a pMt-puro expression vector containing an N-terminal BiP signal sequence and a C-terminal thrombin cleavage sequence followed by a Twin-Strep tag. Full length mAb constructs were produced by amplifying either the light or heavy chain variable regions individually and cloning them into an expression vector containing either the heavy or light chain of mAb VRC01 with an N-terminal CMV promoter.

Production of Stably Transfected S2 Cell Lines for Expression of SCFV Constructs

Schneider 2 cells were transfected with SCFV construct containing plasmids using the Effectene transfection reagent. Cells were grown in the presence of puromycin at a concentration of 5 μ g/mL for two weeks to produce stably transfected cells.

Expression and Purification of 1G1 SCFV

Once S2 cells had been passaged to a sufficient volume, stable cells were induced to express protein using 1 mM cupric chloride. Six days after induction, cells were pelleted by centrifugation and the supernatant was adjusted to the proper pH (8 for first expression, 7.2 for second expression), filtered, and concentrated to a volume of 300 mL using two Sartorius Vivaflow 250R filtration units with 10 kDa cutoffs. Biolock was added to bind to free biotin in the media and the media was loaded onto two 5 mL StrepTrap columns. The columns were washed using strep wash buffer (20 mM Tris pH 8 or 20 mM MOPS pH 7.2, 150 mM NaCl, 1 mM EDTA) and eluted using strep elution buffer (20 mM Tris pH 8 or 20 mM MOPS pH 7.2, 150 mM NaCl, 1 mM EDTA, 2.5 mM desthiobiotin) on an Akta FPLC instrument.

Soluble Expression and Purification of RSV G 157

BL21 *E. coli* were transformed with an expression vector containing the RSV G 157 construct with a C-terminal 6xHis tag. Colonies were used to seed six 30 mL starter cultures which were used to inoculate 6 L of media. Cultures were grown to an optical density of 0.6, induced with 1 mM IPTG, and grown overnight. Cells were harvested by centrifugation, lysed by sonication, and clarified by centrifugation and filtration. Lysate was loaded onto a 5 mL HisTrap Ni column, washed with Ni wash buffer (20 mM Tris pH 8, 150 mM NaCl, 25 mM imidazole) and eluted with Ni elution buffer (20 mM Tris pH 8, 150 mM NaCl, 500 mM imidazole) using an Akta FPLC instrument.

Insoluble Expression and Purification of RSV G 157

BL21 *E. coli* were transformed with an expression vector containing the RSV G 157 construct with a C-terminal 6xHis tag. An individual colony was used to seed one 30 mL starter culture which was used to inoculate 1 L of media. Cultures were grown to an optical density of 0.6, induced with 1 mM IPTG, and grown for three hours. Cells were harvested by centrifugation and lysed by sonication. The lysate was centrifuged and the inclusion bodies were washed with the detergent wash buffer (25 mM potassium phosphate pH 7.5, 1 M urea, 0.25% deoxycholate)

3x100 mL, wash buffer (25 mM potassium phosphate pH 7.5) 3x100mL, and resuspend the pellet in solubilizing buffer (100 mM Tris pH 8, 5 mM DTT, 5 mM EDTA, and 8M guanidine chloride). Solubilized protein was titrated slowly into refolding buffer (100 mM Tris pH 8, 25 mM NaCl, 0.2 mM oxidized glutathione, and 1 mM reduced glutathione). Refolded protein was adjusted to pH 7 and purified using a cation exchange column. Protein was eluted using a salt gradient starting with 25 mM NaCl and finishing at 1M NaCl.

Size Exclusion Chromatography

The 1G1 SCFV and RSV G 157 complex was concentrated to reach a volume of 400 μ L and filtered through a 0.22 μ m filter. The sample was injected onto a Superdex 75 10/300 column and fractions were collected as tris buffered saline (10 mM Tris pH 8, 150 mM NaCl) was pumped through the column at 0.5 mL/min.

Purification of 1G1 mAb by Protein A

CHO cell supernatant was loaded onto a protein A column. The sample was washed with protein A wash buffer (100 mM potassium phosphate pH 7.2, 150 mM NaCl) and eluted with IgG elution buffer (0.1M glycine pH 3). 20 μ L of 2M Tris pH 8 was added to each 1 mL elution to neutralize the acidic buffer.

1G1 Structure Solution and Data Collection

X-ray diffraction data were obtained at the 8.3.1 beamline at the Lawrence Berkeley National Laboratory. Images were acquired by rotating the crystal in one degree intervals and obtaining diffraction images for all 360 degrees around the crystal. Diffraction images were processed in iMosflm. The phase problem was solved by molecular replacement using Phaser in the Phenix suite of programs. Refinement of the molecular model was performed using the Phenix Refine program and Coot was used to manipulate and build the model.

Octet Binding Assay

All Octet binding assays followed the same sequence of steps with different amounts of time for each step. First, the sensor tips were allowed to associate with the 1G1 mAb, then the tip was dipped in buffer as a baseline. The tip was then dipped in either antigen, or buffer as a reference. The tip was then dipped back into buffer to observe dissociation. Data processing was performed using the Fortebio Octet Data Analysis software.

Biotinylation of 1G1 mAb

A 20 mM solution of NHS-PEG₄-Biotin was prepared by dissolving the solid reagent in phosphate buffered saline pH 7.2. 6.5 μ L of this solution was added to 0.5 mL of 1G1 mAb at 1 mg/mL and allowed to react on ice for one hour. After an hour, the sample was desalted using a 2 mL Zeba Spin Desalting Column with a 7 kDa cutoff.

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