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A fluorescence polarization assay using an engineered human respiratory syncytial virus F protein as a direct screening platform

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ABSTRACT

Human respiratory syncytial virus (hRSV) typically affects newborns and young children. Even though it can cause severe and, in some cases, lifelong respiratory infections, there are currently no Food and Drug Administration (FDA)-approved therapeutics that control this virus. The hRSV F protein facilitates viral fusion, a critical extracellular event that can be targeted for therapeutic intervention by disrupting the assembly of a postfusion 6-helix bundle (6HB) within the hRSV F protein. Here we report the development of a fluorescence polarization (FP) assay using an engineered hRSV F protein 5-helix bundle (5HB). We generated the 5HB and validated its ability to form a 6HB in an FP assay. To test the potential of 5HB as a screening tool, we then investigated a series of truncated peptides derived from the "missing" sixth helix. Using this FP-based 5HB system, we have successfully demonstrated that short peptides can prevent 6HB formation and serve as potential hRSV fusion inhibitors. We anticipate that this new 5HB system will provide an effective tool to identify and study potential antivirals to control hRSV infection.

Despite attempts to develop safe and cost-effective treatments to control human respiratory syncytial virus (hRSV)[1]-associated illness [1,2], hRSV remains the leading pathogen causing severe lower respiratory tract infections in infants and children. hRSV infections cause more than 120,000 pediatric hospitalizations and 2000 deaths in the United States alone, with costs of $356 million to $585 million annually [3–7]. Most children are infected with hRSV at least once before 2 years of age, and recurrence is common [8]. hRSV has also been an increasingly recognized cause of high morbidity and mortality in the elderly, causing up to 10,000 deaths annually in individuals over 65 years of age [9,10]. Antiviral drug discovery to control hRSV infections has relied mainly on the screening of chemical libraries or natural products using common virology assays and animal models, yielding limited success [2,11]. The only clinically approved antivirals to date are a nucleoside analog, Ribavirin, and a humanized monoclonal antibody, Synagis. Due to efficacy and cost, these drugs are restricted to high-risk children (babies born at less than 36 weeks or who have heart or lung problems) [12]. With treatment options limited to measures such as supportive care, development of safe and specific agents against hRSV is essential.

Viral entry into cells is important to the hRSV infectious cycle. On attachment, hRSV uses the fusion (F) protein to cause membrane fusion with the host cells at neutral pH, similar to other paramyxoviruses. During the process of membrane fusion, F protein refolds to form a stable 6-helix bundle (6HB), structurally characterized by X-ray crystallography [13–16]. In the sequence of the F protein, two hydrophobic heptad repeats, HRA and HRB, are located adjacent to the fusion peptide and transmembrane domain, respectively. HRA and HRB are very far from each other, with approximately 250 residues of intervening sequence between them (Fig. 1A). When the F protein is activated, a hydrophobic fusion peptide is exposed and then inserted into the host cell membrane as an anchor. Subsequently, the protein undergoes a dramatic refolding, bringing HRA and HRB together to form the 6HB. This 6HB formation, linked to F protein refolding, is thought to bring the viral and host cell membranes close together, leading to membrane fusion and viral entry [14,15,17–20]. The F protein-mediated membrane merger occurs at the cell surface, so the process is theoretically accessible to inhibition by antivirals. Therefore,

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[1] *Abbreviations used:* hRSV, human respiratory syncytial virus; F protein, fusion protein; 6HB, 6-helix bundle; 5HB, 5-helix bundle; FP, fluorescence polarization; PCR, polymerase chain reaction; LB, Luria–Bertani; IPTG, isopropyl-β-D-thiogalactopyranoside; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; CV, column volume; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BCA, bisinchoninic acid; CD, circular dichroism; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; TIPS, triisopropylsilane; RP, reverse-phase; ESI, electrospray mass spectrometry; ELISA, enzyme-linked immunosorbent assay; EBV, Epstein–Barr virus; FITC, fluorescein isothiocyanate.

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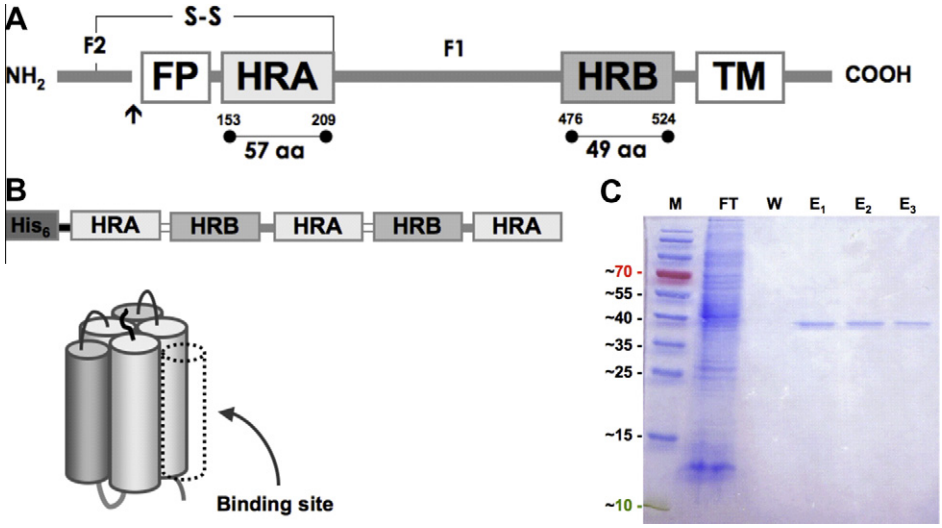

Fig.1. Schematic diagram of hRSV F protein and 5-helix bundle (5HB) with a resulting sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of 5HB purification. (A) F1 and F2 are formed after proteolytic cleavage (arrow) of the precursor protein, F0. The fusion peptide (FP) and transmembrane domain (TM) are indicated. Heptad repeat regions, HRA and HRB, are located adjacent to FP and TM, respectively. (B) An illustration of the designed 5HB and its single-chain polypeptide sequence are shown. A binding site for the missing third HRB and potential fusion inhibitors is shown with the dotted line. (C) Purified 5HB samples were analyzed by SDS–PAGE on a 12% nonreducing gel. M, molecular marker; FT, flow-through; W, wash; E, elution fractions.

F has been recognized as a potential therapeutic target, yielding numerous peptidic or nonpeptidic small molecule fusion inhibitors [1,2,21–31].

To our knowledge, there is no established, simple, non-cell-based method to screen potential antivirals specifically targeting the hRSV F protein. Previously, the 5-helix bundle (5HB) of the HIV-1 fusion protein gp41 was shown to be a viral entry inhibitor [32] as well as a suitable target for screening small molecule libraries in a high-throughput format [33], suggesting that similar approaches would be applicable to other viruses, such as hRSV, that rely on class I viral fusion proteins. We created a 5HB variant hRSV F protein and developed a competitive fluorescence polarization (FP)-based assay using the 5HB as a target protein and using a fluorescently labeled peptide as a tracer. To validate that the competitive FP-based 5HB assay can provide a reliable screening platform, a series of N- and C-terminally truncated peptides derived from the HRB domain of hRSV F protein were synthesized and tested. Thus, we demonstrate that this simple, low-cost, *in vitro* FP assay can be readily expanded to libraries of peptides, peptidomimetics, and small molecules to rapidly screen potential hRSV fusion inhibitors.

Materials and methods

Protein synthesis

5HB cloning

The 5HB DNA construct is composed of three N_{57} and two C_{49} helices, representing residues 126–182 (HRA) and 476–524 (HRB) of the hRSV F protein. Each fragment was amplified by polymerase chain reaction (PCR) using the hRSV strain A2 genome as a template and connected using short linkers; N_{57} was joined using a linker (PPPELGGP) to C_{49} to generate a heterodimer (N_{57}–C_{49}), two heterodimers were connected with a short linker (KGSSK), and the final N_{57} was linked after the second C_{49} via the linker (KGSSK) (Fig. 1B). The engineered gene encoding the 5HB was cloned between the *Nde*I and *Bam*HIII restriction sites of the hexahistidine expression vector pET-15b (Novagen, San Diego, CA, USA). The resulting plasmid carrying the complete 5HB construct was transformed into *Escherichia coli* strain BL21(DE3) for protein expression.

5HB expression and purification

Protein was recombinantly expressed in *E. coli* strain BL21(DE3) grown to an optical density of 0.8 at 600 nm and 37 °C in Luria–Bertani (LB) medium. Protein expression then was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (ITPG), and cells were grown for an additional 20 h at 20 °C to enhance the solubility of protein [34]. The cells were harvested by centrifugation at 4500*g* for 15 min, and the resulting cell pellet was resuspended in 20 mM phosphate-buffered saline (PBS) and stored at −80 °C. Cells were lysed in lysis buffer (CelLytic B cell lysis reagent [cat. no. C8740, Sigma–Aldrich, Milwaukee, WI, USA], 20 mM PBS [pH 7.4], 1 mM phenylmethylsulfonyl fluoride [PMSF], protease inhibitor cocktail [Sigma–Aldrich], 1% Triton X-100, 500 mM NaCl, and 0.2 mg/ml lysozyme) and incubated for 1 h at room temperature. Cell lysate was then clarified by centrifugation at 18,000*g* for 30 min. The soluble fraction was immediately incubated with a nickel-immobilized chelating Sepharose Fast Flow resin (cat. no. 17-0575-02, GE Healthcare, Piscataway, NJ, USA) at room temperature for 30 min with gentle agitation. The protein-bound resin was washed out with more than 10 column volumes (CVs) of a wash buffer (20 mM PBS [pH 7.4], 100 mM imidazole, 1% Triton X-100, and 500 mM NaCl). The 5HB was eluted with an elution buffer (20 mM PBS [pH 7.4], 300 mM imidazole, and 500 mM NaCl). The purity of protein was assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and the protein was used without further purification. Protein concentration was determined by using the bisinchoninic acid (BCA) protein assay (cat. no. 23225, Pierce, Rockford, IL, USA). The final yield of soluble 5HB was approximately 1 mg/L cell culture with batch-to-batch variation.

CD spectroscopy

Circular dichroism (CD) spectra were obtained with a Jasco J-815 spectrophotometer (Jasco, Easton, MD, USA). Sample was prepared in 20 mM PBS (pH 7.4) in a concentration of 35 μM. Data were recorded from 195 to 260 nm with a scanning speed of 20 nm/min and a bandwidth at 1.0 nm in a 0.1-cm path-length quartz cell. Each CD spectrum was an average of three measurements and corrected for buffer blank obtained under identical conditions. The resulting data were converted to per residue molar ellipticity units, $[\Theta]$ (deg cm^2 $dmol^{-1}$ $residue^{-1}$), and the

secondary structure content was analyzed with the DichroWeb software package. The thermal stability of 5HB was monitored by measuring its molar ellipticity between 0 and 85 °C at 222 nm. For this study, 2.5 μM of 5HB was used. The rate of temperature change was 2.5 °C/min with a scanning speed of 50 nm/min and a bandwidth of 1.0 nm.

Peptide synthesis

Peptide synthesis reagents were purchased from Applied Biosystems (Foster City, CA, USA) or Sigma–Aldrich. Resins and Fmoc-protected amino acids were purchased from Novabiochem (San Diego, CA, USA) or Anaspec (San Jose, CA, USA). Solvents for high-performance liquid chromatography (HPLC) were purchased from Fisher Scientific (Pittsburgh, PA, USA). All chemicals were used without additional purification. Fluorescently labeled peptide (Fl–C_{35}) and truncated peptides (C_{30} and C_{35}) of 95% purity were commercially obtained (EZBiolab, Carmel, IN, USA, and Bio Basic, Markham, ON, Canada) and used without further purification. The remaining truncated peptides (C_{20}, C_{17}, and N_{15}) were synthesized in the laboratory using standard Fmoc chemistry on solid support (preloaded Wang resin, Novabiochem) with an ABI 433A automated peptide synthesizer (Applied Biosystems). After synthesis, the peptides were cleaved from the resin and deprotected in trifluoroacetic acid (TFA)/water/triisopropylsilane (TIPS)/thionisole (90:5:2.5:2.5, v/v) for 1.5 h at room temperature. Peptides were purified by preparation reverse-phase (RP)–HPLC on a C18 column using a linear gradient of 5–99% solvent B in solvent A over 60 min (solvent A was 0.1% [v/v] TFA in water, and solvent B was 0.1% [v/v] TFA in acetonitrile). Final purities of synthetic peptides were confirmed to be more than 95% by analytical RP–HPLC, and the molecular weight of the purified product was confirmed by electrospray mass spectrometry (ESI) at the Stanford University Mass Spectrometry (SUMS) facility.

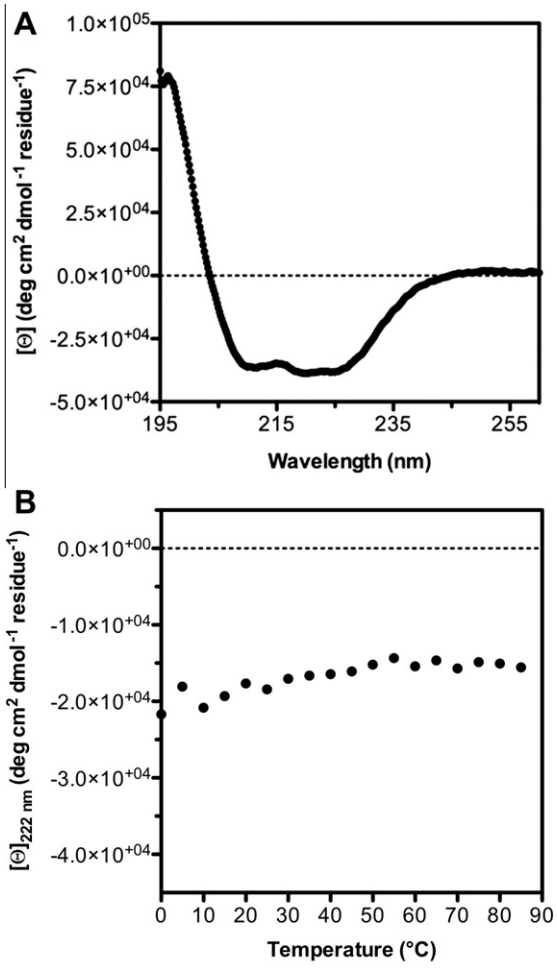

Fig.2. Secondary structure analysis and thermal stability of 5HB by CD spectroscopy. (A) CD spectrum of 5HB in 10 mM PBS at pH 7.4. (B) Melting curve of 5HB obtained from the ellipticity measurements at 222 nm between 0 and 85 °C. [Θ], per residue molar ellipticity measured in deg cm^2 $dmol^{-1}$ $residue^{-1}$.

FP measurements

FP measurements were performed using a Synergy4 (Biotek, Winooski, VT, USA) plate reader with a tungsten lamp as a light

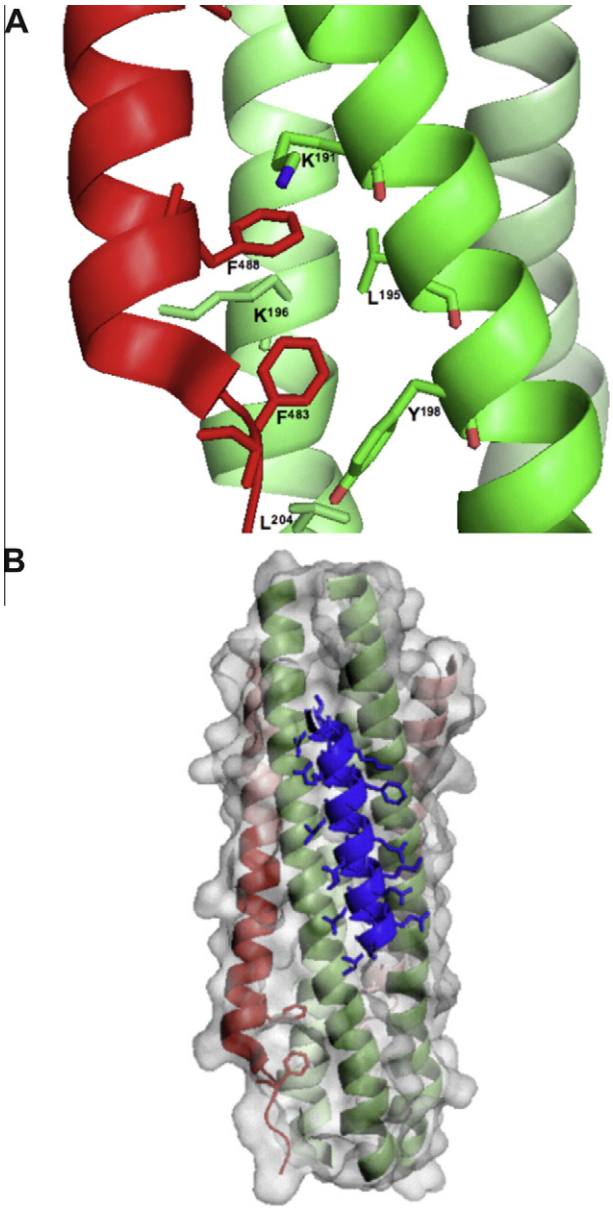

Fig.3. A key interaction between HRA and HRB helices in the 6HB assembly and a three-dimensional model of C_{20}-bound 5HB. (A) Two phenylalanine residues of the hRSV F HRB domain (red) play a key role in the interaction with the hRSV F HRA helices (green) by packing into the hydrophobic pocket formed by HRA helices (adapted from Ref. [13]). (B) The proposed 5HB model with C_{20} peptide (blue) and surface area of the 5HB (light gray) are shown. The hydrophobic groove formed by two neighboring HRA helices will provide a wide binding site for future antiviral development. Figures were generated by PyMOL software [50]. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

source and with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Fluorescently labeled 35-amino-acid peptide, Fl–C_{35}, was chosen as a tracer due to its inhibitory potency (EC_{50} of 0.051 μM) [21]. Lyophilized Fl–C_{35} was dissolved in 20 mM PBS, and subsequent dilutions were done in FP buffer (20 mM PBS [pH 7.4], 500 mM NaCl, 0.01% [v/v] Tween 20, and 0.05 mg/ml bovine gamma globulin). Specific control groups included free Fl–C_{35} (probe), bound Fl–C_{35} (Fl–C_{35} in the presence of 5HB), and FP buffer for every measurement, allowing accurate estimation of specific polarization.

Saturation binding FP assays

The saturation binding experiments of Fl–C_{35} to 5HB were performed under the following conditions. Each well in a black 96-well plate (Corning, Lowell, MA, USA) contained 5 nM Fl–C_{35} tracer peptide and increasing concentrations (0–500 nM) of 5HB in FP buffer in a final volume of 185 μl. The polarization (in millipolarization [mP] units) was measured after 1 h of incubation at room temperature. Data obtained were analyzed using GraphPad Prism 5.0 software (GraphPad, San Diego, CA, USA) to calculate a binding dissociation constant (K_d) by fitting the experimental data using a one-site specific binding model. Experiments were performed in duplicate.

Competitive FP binding assays

Each well in a black 96-well plate (Corning) contained 20 nM 5HB and increasing concentrations (0.001–200 μM) of each truncated peptide in FP buffer in a final volume of 185 μl. After 1 h of incubation at room temperature, 5 nM Fl–C_{35} was added, followed by an additional 30 min of incubation at room temperature. The FP response was measured in duplicate with controls that included free Fl–C_{35}, bound Fl–C_{35}, and FP buffer. All experimental data were plotted using GraphPad Prism 5.0. The percentage of inhibition was calculated using the following equation:

$$\%\text{inhibition} = 100 \times [(mP - mP_f)/(mP_b - mP_f)],$$

where mP is the millipolarization of the bound inhibitor to the 5HB, mP_f is the millipolarization of the free Fl–C_{35} control, and mP_b is the millipolarization of the bound Fl–C_{35} control.

Results and discussion

5HB construct design, expression, and purification

A 5HB construct of HIV-1 fusion protein gp41 has been tested as a fusion inhibitor [32] and used as a target protein for screening small molecule libraries [33]. However, analogous constructs for hRSV F protein have been tested only as fusion inhibitors or vaccine candidates [31,35]. Therefore, we designed a 5HB construct to specifically mimic the 6HB that forms during hRSV infection of the host cell. The 5HB was generated by connecting three HRA and two HRB helices in an alternating sequence using short peptide linkers. The absence of the third HRB in the 5HB would create a

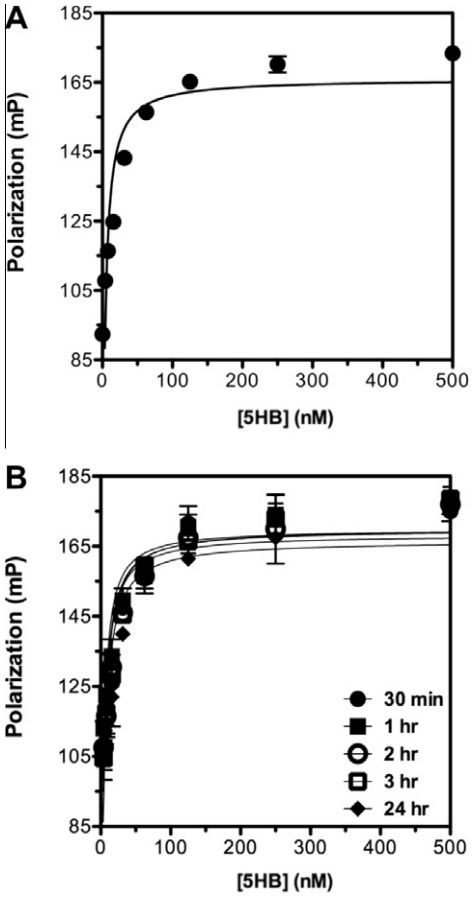

Fig.4. Binding titration curve of Fl–C_{35} and its binding stability to the 5HB. (A) The FP response of Fl–C_{35} binding to the 5HB was monitored as the concentration of the 5HB increased. The experiment was performed using 5 nM Fl–C_{35}, and the 5HB concentration ranged from 0 to 500 nM. (B) The stability of Fl–C_{35} binding to the 5HB was monitored over a 24-h period using 5 nM Fl–C_{35} in the presence of increasing amounts of the 5HB.

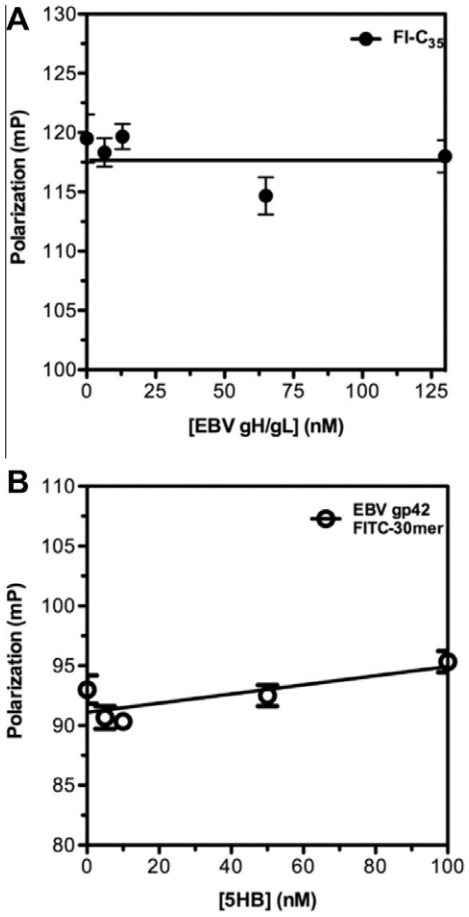

Fig.5. Specificity of Fl–C_{35} to the 5HB. To confirm the specificity of Fl–C_{35} to the 5HB, negative controls were tested. (A) Fl–C_{35} (5 nM) in the presence of increasing amounts of EBV gH/gL, a fusion protein that leads the EBV infection, was tested. (B) EBV gp42 FITC–30mer (5 nM) with a wide range of the 5HB in concentration was monitored.

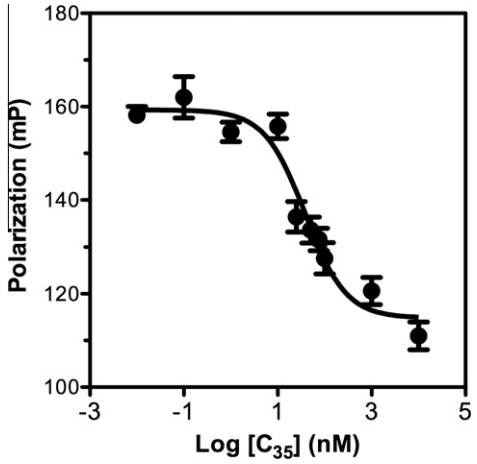

Fig.6. Competitive FP assay using unlabeled C_{35} to compete with the binding of Fl–C_{35} to 5HB. Increasing concentrations of unlabeled C_{35} were tested in the presence of 5 nM Fl–C_{35} and 20 nM 5HB.

large open binding site for potential fusion inhibitors (Fig. 1B). Soluble 5HB was expressed in *E. coli* strain BL21(DE3) cells and purified by metal affinity chromatography (Fig. 1C). The secondary structure of the 5HB was assessed by CD spectroscopy (Fig. 2A), showing approximately 90% α-helicity, as calculated using DichroWeb [36,37]. In the temperature range from 0 to 85 °C, the thermal denaturation of the 5HB was not observed, indicating that the secondary structure of the 5HB is highly stable (Fig. 2B).

Saturation binding FP measurement

To develop a reliable FP assay, the binding affinity and specificity of the probe to the target protein should be high [38]. Previously, it has been shown that a series of overlapping 35-amino-acid peptides from the conserved HRB domain within various paramyxovirus F proteins could block syncytium formation with EC_{50} values in the range of 0.015–0.25 μM [21]. Taking account of this previous study, we decided to use T-108 (35mer: YDPLVFPSDEFDASISQVNEKINQSLAFIRKSDEL) derived from hRSV F protein as a probe for developing an FP assay for the following reasons. First, the low EC_{50} value of 0.051 μM suggests that T-108 should bind tightly to the 5HB. Second, T-108 contains two phenylalanine residues (F^{483} and F^{488}), located at the N terminus of the HRB region, that engage a deep hydrophobic pocket located at the C terminus of the HRA helices known as an antiviral drug target (Fig. 3A, B shows the entire assay system in schematic format) [13,25,26]. This T-108 peptide was labeled with a fluorescein at its N terminus (Fl–C_{35}). To determine the binding affinity of Fl–C_{35}, we used a fixed concentration of 5 nM Fl–C_{35} and monitored the FP response of Fl–C_{35} with increasing concentrations of the 5HB, as shown in Fig. 4A. The FP results were consistent with high affinity binding (K_d = 21 nM). The stability of the FP assay using Fl–C_{35} and the 5HB is also important as potential use for a high-throughput screening format. Therefore, we tested the stability of Fl–C_{35} binding to the 5HB by incubating the plate at room temperature over 24 h. Resulting binding curves show that this assay is highly robust (Fig. 4B). The specific binding of Fl–C_{35} to the 5HB was also confirmed in enzyme-linked immunosorbent assays (ELISAs) (resulting data and detailed protocols are provided in the Supplementary material). To further verify the selectivity and specificity of binding Fl–C_{35} for hRSV F protein, we took advantage of our previous findings of the high-affinity binding of a fluorescein isothiocyanate (FITC)–30mer (30mer: KPNVEVWPVAAPPPVAEQEYGDKEVKLPHW) derived from the Epstein–Barr virus (EBV) gp42 to the EBV gH/gL complex [39,40]. The interaction of the EBV gp42 and the gH/gL complex is known to play key roles in membrane fusion, and we previously reported that the EBV gp42-derived FITC–30mer specifically binds to EBV gH/gL protein with an IC_{50} value of 1.3 nM [40]. To confirm the selectivity and specificity of both the hRSV 5HB and the Fl–C_{35} probe, we cross-tested the EBV gp42-derived FITC–30mer against the hRSV 5HB (Fig. 5A) and cross-tested our hRSV-derived Fl–C_{35} against the EBV gH/gL complex under the same conditions (Fig. 5B). There was no evidence of nonspecific binding in these controls, indicating that the interaction between Fl–C_{35} and the 5HB is specific and selective, and this provided a solid basis for developing a competitive FP-based 5HB assay.

Competitive FP assays

Based on the K_d value observed in the saturation binding FP assay, we established a competitive FP assay to evaluate potential inhibitors based on their ability to displace the Fl–C_{35} (probe) from the 5HB (target). We first tested unlabeled C_{35} against Fl–C_{35} in the presence of the 5HB (Fig. 6). Unlabeled C_{35} peptide blocked the increase in FP with an IC_{50} of 38.52 nM, competing with Fl–C_{35} over the binding site on the 5HB. Manufacturing longer bioactive peptides can be problematic due to high cost, but shorter unstructured peptides can easily lose their efficacy, thereby implying two significant but contradictory criteria of designing peptide therapeutics. Therefore, we prepared a series of truncated peptides derived from the HRB domain of hRSV F protein (Table 1) and investigated their ability to compete against Fl–C_{35} using our competitive FP-based 5HB assay (Fig. 7). Truncated peptides tested in this study do not

Table 1
Peptides derived from the HRB sequence of hRSV F protein and tested in this study.

Peptide	Sequence (amino to carboxy)	% Inhibition (100 μM)	IC_{50} (μM)
HRB[a]	NFYDPLVFPSDEFDASISQVNEKINQSLAFIRKSDELLHNVNAGKSTTN	–	–
T-108	YDPLVFPSDEFDASISQVNEKINQSLAFIRKSDEL		0.051[b]
C_{35}	YDPLVFPSDEFDASISQVNEKINQSLAFIRKSDEL	100	0.038
C_{30}	VFPSDEFDASISQVNEKINQSLAFIRKSDE	100	6.800
C_{20}	ISQVNEKINQSLAFIRKSDE	>90	14.920
C_{17}	VNEKINQSLAFIRKSDE	<50	>100
C_{13}	INQSLAFIRKSDE	NM	>500
C_{10}	SLAFIRKSDE	NM	>500
N_{15}	VFPSDEFDASISQVN	<50	>100

Note: Peptides more than 20 amino acids long were commercially obtained, and the rest of the peptides were synthesized in the laboratory using standard Fmoc chemistry. The purity of peptides is more than 95%, as judged by analytical HPLC. NM, not measurable.
[a] HRB sequence from hRSV F protein.
[b] EC_{50} value from Lambert and coworkers [21]. Crude peptide T-108 was analyzed for its ability to prevent cytopathological effect in infectivity assays with hRSV.

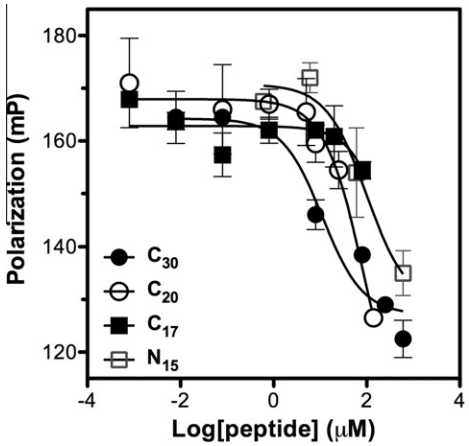

Fig.7. Competitive fluorescence polarization assays of N- and C-terminally truncated peptides. The competitive binding ability of each truncated peptide derived from the hRSV HRB domain was evaluated. Various concentrations of peptides were competed with 5 nM Fl–C_{35} in the presence of 20 nM 5HB.

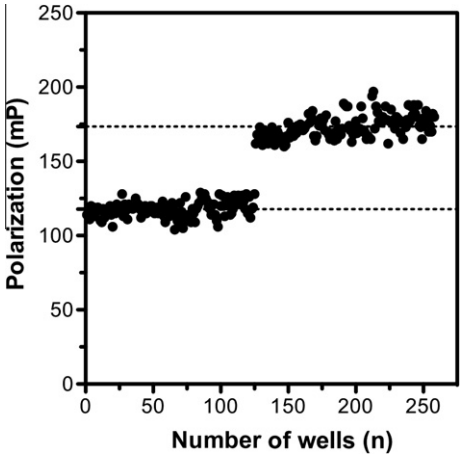

Fig.8. Assay robustness test by FP measurements of free and bound Fl–C_{35} controls. FP response for 5 nM Fl–C_{35} was monitored in the absence and presence of the 5HB construct (20 nM) as negative and positive controls.

have two phenylalanines that bind to the hydrophobic pocket (Fig. 3A) that have been the focus of a small molecule drug discovery effort [1,13,41]. However, in previous work by Lambert and coworkers, the absence of these residues in various 35mer peptides still revealed significant antiviral activity [21]. The results in Fig. 7 and Table 1 summarize our quantitative observations of the binding activity of increasingly truncated peptides, providing important information on the location as well as the length of peptides that mediate inhibition. These data clearly suggest that the entire hydrophobic groove on the C terminus of neighboring HRA helices would be available as a potential drug target for developing hRSV fusion inhibitors (Fig. 3B). Among tested peptides, C_{20} (IC_{50} = 14.92 μM) was further investigated for its antiviral activity, which showed EC_{50} > 12.50 μM (detailed methods are provided in the Supplementary material), denoting that our FP-based assay can be comparable to conventional virological assays. Additionally, as shown in Fig. 8, the reproducibility of measurements for free and bound Fl–C_{35} controls were examined to calculate the Z' factor, a parameter for the quality and robustness of the assay [38], according to the guidelines provided by the National Institutes of Health (NIH) [42]. The resulting Z' factor (Z' > 0.8) suggests that our competitive FP-based assay can be further applied in a high-throughput screening approach.

Conclusions

In this study, we demonstrated that a peptide binding assay can be used as a direct screening platform for identifying potential antivirals against hRSV using FP, which has been widely used for direct, nearly instantaneous measurement of molecular interactions such as protein–protein [43–45], DNA–protein [46], and small molecule–protein [47,48] interactions. Our competitive FP-based assay for the formation of the hRSV 6HB can measure biological activities of short F protein-derived peptides over a wide range of binding affinity, suggesting that this system is sufficiently sensitive to screen weak binders to the 5HB as potential antiviral candidates. Moreover, this assay could be suitable for preparatory high-throughput screening efforts prior to *in vivo* assays to prioritize inhibitor candidates. This 5HB construct from the hRSV F protein may prove to be useful for other viruses within the paramyxovirus family for future drug discovery efforts.

Recently, it has been suggested that a small molecule inhibitor of hRSV F protein entry may act not by blocking 6HB formation but rather by distorting the final 6HB conformation [49]. These studies indicate that the inhibitor (TMC353121) engages both HRA and HRB regions and may thereby enhance 6HB assembly into a non-functional structure. The 5HB system described here may allow quantitative studies of this and other hRSV F protein fusion inhibitors and help to classify the mode of action of these compounds.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2010.10.020.

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