High-throughput Screening Assay Development for Small Molecule Inhibitor(s) of PIAS1

Vipul Madahar, Vicente Nunez, Yang Song, and Jiayu Liao
Department of Bioengineering, University of California Riverside, Riverside, California, USA

Abstract- The STAT (signal transducers and activators of transcription) family of proteins is composed of signal dependent transcription activators for cytokine signal transduction in vivo. The function of these signal transduction pathways is to maintain human health, such as the interferon pathway in which case STATs serve as key mediators of anti-viral responses in the human body. During the signal transduction process, STAT1 activation occurs in response to IFN-γ binding to IFN-γ receptor at cell surface as phosphorylation of a STAT1 tyrosine residue. PIAS1 inhibits the function of STAT1 by binding to the activated STAT1 dimer and mediate sumoylation. The purpose of this project is to develop a high throughput screening assay for small molecule inhibitor(s) of PIAS1 hence interrupting the PIAS1-SUMO1 interaction. To do so we built two DNA constructs, one that encodes for YPet-PIAS1 fusion protein and a second construct that encodes for the SUMO1-CyPet fusion protein. The two constructed proteins, YPet-PIAS1 and SUMO1-CyPet, generate a FRET (Fluorescence Resonance Energy Transfer) signal with the interaction of Ypet and CyPet fluorescent proteins. This result demonstrated the direct interaction of SUMO1 and PIAS1 in vivo, and therefore will serve as high throughput screening platform.

Introduction

Understanding the protein-protein interactions of the JAK-STAT pathway is of great importance to develop a strategy to optimize an organism’s immune response. Inhibiting the activity of PIAS1 as an inhibitor of activated STAT1 has been shown to
boost mice immune response to viral infections\textsuperscript{2}. The development of a high throughput screening assay for inhibitors of PIAS1-STAT1 can potentially lead to a way to treat immune deficient patients by enhancing the immune system.

The mammalian JAK family of proteins includes JAK1, JAK2, JAK3 and tyrosine kinase2, each with a common kinase domain. The STAT mammalian family of proteins contains seven members STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6, each with homologous Src homology domains for creation of STAT dimers, which are a critical for the JAK-STAT pathway.\textsuperscript{3} The JAK-STAT pathway is activated by a cytokine such as IFN-\(\gamma\). The binding of IFN-\(\gamma\) to its cell-surface receptors initiates the dimerization of the receptor. The dimerization of the cytokine receptor activates the associated JAK protein, which in turn phosphorylates a tyrosine residue on the receptor. This phosphorylated tyrosine recruits STAT proteins that dock onto the phosphorylated receptor.\textsuperscript{1,3} The JAK kinases associated with receptors then phosphorylates a tyrosine residue of the docked STAT protein. The phosphorylated STATs form dimers through intramolecular SH2-phosphorytyrosine interaction.\textsuperscript{1,3} This STAT-STAT dimer is able to cross the nuclear membrane and activate transcription of genes for immune response.\textsuperscript{1,3} However, the family of proteins PIAS (Proteins that Inhibit Activated STATs), consisting of five members, have been proven to interact with phosphorylated STAT and mediate sumoylation of STAT to inhibit STAT’s activity and as a result inhibit the immune response.\textsuperscript{1} Studies have shown that PIAS1 and PIAS3 inhibit DNA-binding activity of STAT1 and STAT3, respectively.\textsuperscript{4} Furthermore, it has been shown that PIAS1 promotes the SUMOylation of STAT1 \textit{in vivo}\textsuperscript{4}, showing that PIAS has affinity for SUMO. The overlapping interplay between these proteins triggers us to study their interactions of
PIAS-STAT and/or PIAS-SUMO. The final goal is to develop a screening method for inhibitors of PIAS1 (Figure 1).

Figure 1: Schematic of protein-protein interaction.

Figure 1 above shows the schematic of FRET between the fluorescent protein pair. It also shows the way small molecules can interrupt the FRET signal from the interactions of the SUMO1 and PIAS1 proteins. The energy of one fluorophore can be transferred to another when the fluorophores are at close proximity to each other. So when PIAS1 and SUMO1 are interacting the FRET signal can be observed. Our hypothesis is that, when a small molecular inhibitor of PIAS1 is found, the FRET signal will not be observed due to the disassociation of the two proteins induced by the inhibitor. The relationship of efficiency of FRET to the distance between the two proteins can be calculated using the following equations:

\[ R_0 = \frac{9000\ln(10)\Phi_0 k^2}{128\pi^6 n^4 N_A} J \]  

Equation 1: The half distance equation. See Appendix (A) for variables.

\[ J = \frac{\int_{0}^{\infty} F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda}{\int_{0}^{\infty} F_D(\lambda) d\lambda} = \frac{\int_{0}^{\infty} F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda}{\int_{0}^{\infty} F_D(\lambda) d\lambda} \]  

Equation 2
Equation 2: Integral for the overlapping of excitation and emission of donor and acceptor. See appendix (A) for variables.

\[ f_{\text{FRET}} = 1 - \frac{\Phi_D}{\Phi_D^0} = \frac{\tau_D^0}{\tau_D} = \frac{R_0^6}{R_0^6 + R_{\text{DA}}^6} \]  

EQ (3)

Equation 3: Efficiency Calculations. See Appendix (A) for variables.

The FRET efficiency equation shown in equation 3, relates the distance between our donor and acceptor to the efficiency of our FRET signal. Thus, our constructs will allow us to monitor the interaction between the STAT1 and PIAS1 proteins, as well as other protein-protein interactions, through FRET signal.

It is essential to have a balance between “too much” anti-viral response, since this can lead to auto-immune diseases, and “not enough” response. It is thought that with full control of this balance, health issues related to deficient immune response can be developed.

Results

Using molecular cloning techniques we were able to make a set of protein chimeras consisted of fluorescent protein CyPet/YPet and proteins involved in JAK/STAT pathway including STAT1, STAT3, PIAS1, PIAS3 and SUMO1 as well as the N-terminal domain of STAT1 and C-terminal domain of PIAS1. CyPet and YPet are tagged to either N- or C-terminal of these proteins.

Following the DNA cloning, we transfected the above constructs into mammalian cells either individually or in combination. We then detected the emission spectrum of each individual fusion proteins (Figure 2 and 3) or FRET between these two fusion proteins (Figure 4).
We determined emission spectrum of cells transfected with the CyPet-SUMO1 plasmid shown in Figure 2. The graph shows the high emission peak at close to 475nm of close to $2.20 \times 10^5$ RFUs, when the CyPet-SUMO fusion proteins where excited with light of 414nm. This response confirmed the successful excitation of CyPet-SUMO1. YPET-
PIAS1 didn’t generate fluorescence signal under excitation at 414nm as the fluorescence intensity of YPet-PIAS1 transfected 293 cells was similar to that of background cells of untransfected 293 cells (Figure 3).

FRET was observed in cells that were co-transfected with both the CyPet-SUMO1 and YPet-PIAS1 plasmids where these two fusion proteins were expressed (Figure 4). The cells were excited again with light of 414nm wavelength.

![Emission spectrum of CyPet-SUMO1/YPet-PIAS1 cotransfected 293 cells (open circle) versus untransfected 293 cells (open square).](image)

Figure 4. Emission spectrum of CyPet-SUMO1/YPet-PIAS1 cotransfected 293 cells (open circle) versus untransfected 293 cells (open square).

In this experiment, we can observe the emission of CyPet-SUMO at 475nm and the emission of YPet-PIAS at 525nm, indicating an energy transfer from the donor CyPet to the acceptor YPet.
Discussion

The JAK-STAT pathway plays an important role in our immune system, as it allows protection against virus infection. Our project aims to utilize robust techniques, such as FRET, in efficient analysis of this pathway, and thereafter development of high throughput screening platform for chemical inhibitor discovery. The FRET system will allow us to efficiently find possible candidate chemicals for inhibition of PIAS1 protein, which could serve as both chemical probes and future drug discovery. This unique technique will allow assay in the mammalian cells, as this *in vivo* technology is a large advancement in high-throughput screening.

We have successfully identified engineered fluorescent proteins, Cypet and Ypet, which generate much higher fluorescence intensity in comparison with classical fluorescent proteins. We therefore fused this fluorescent protein FRET pair with two putative interactive proteins, SUMO1 and PIAS1. The results showed FRET between the donor CyPet-SUMO1 and acceptor YPet-PIAS1 proteins when they are excited at Cypet excitation wavelength. This demonstrates this study’s feasibility to observe protein-protein interactions in a robust manner. The stable cell line expressing this FRET pair is underway. This technique has therefore been demonstrated feasible in dissecting SUMO network *in vivo*.

Materials and Methods

Molecular Cloning of Fluorophores and proteins

The cDNA sequences of different proteins were amplified from human cDNA library using PCR Supermix (Invitrogen) with primers containing respective restrictions
sites. The PCR product was then ligated into pCRII vector using TOPO TA Cloning kit (Invitrogen). The chimera constructs were made by digestion and ligation in pCRII vector and they were then transferred to mammalian expression vector pcDNA3.1 to allow expression in mammalian cells.

**Transfection into 293 mammalian embryonic kidney cells**

2 μg plasmid was transfected into HEK293 cells on 6-well plates using Fugene-6 (Roche). The cells were allowed to grow for 48 hours and to a specific confluency for accurate measurements.

**Measurement of chimeric protein fluorescence**

The 293 cells were stripped off in PBS buffer and fluorescence spectrum was measured in 96-well plate by FlexstationII<sup>384</sup> (Molecular Devices).
## Appendix A

<table>
<thead>
<tr>
<th>Constants</th>
<th>Units</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$R_{DA}$</td>
<td>Distance</td>
<td>Distance between fluorophores</td>
</tr>
<tr>
<td>$\text{R}_0^6$</td>
<td>Distance</td>
<td>Förster Distance, depends of spectral properties of donor and acceptor</td>
</tr>
<tr>
<td>$K^2$</td>
<td></td>
<td>Describes the orientation in space of the transition dipoles of the donor and acceptor. Usually assumed to be $2/3$.</td>
</tr>
<tr>
<td>$N_A$</td>
<td>Mole$^{-1}$</td>
<td>Avogadro's Number</td>
</tr>
<tr>
<td>$T_0$</td>
<td>(K)</td>
<td>Temperature</td>
</tr>
<tr>
<td>$N$</td>
<td></td>
<td>Refractive Index</td>
</tr>
<tr>
<td>$\Phi_0$</td>
<td>%</td>
<td>Quantum Yield w/o presence of acceptor</td>
</tr>
<tr>
<td>$\Phi_D^0$</td>
<td>%</td>
<td>Quantum Yield w presence of acceptor</td>
</tr>
<tr>
<td>$\tau^0_D$</td>
<td>Sec$^{-1}$</td>
<td>Time rate it takes the donor to excitation w presence of acceptor</td>
</tr>
<tr>
<td>$\tau^0$</td>
<td>Sec$^{-1}$</td>
<td>Time rate it takes the donor to excitation w/o presence of acceptor</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Functions</th>
<th>Units</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$FD(\lambda)$</td>
<td>M-1cm$^{-1}$</td>
<td>Corrected fluorescence intensity of the donor in wavelength $\lambda + \Delta \lambda$ with total intensity normalized to unity.</td>
</tr>
<tr>
<td>$\varepsilon_D(\lambda)$</td>
<td>M-1cm$^{-1}$</td>
<td>Extinction Coefficient of acceptor at $\lambda$</td>
</tr>
<tr>
<td>$\kappa T(r)$</td>
<td>Sec$^{-1}$</td>
<td>The rate of transfer for donor and acceptor between a distance of $r$.</td>
</tr>
</tbody>
</table>
References