Molecular Interactions of Sarcolipin and Phospholamban Using Fluorescence Resonance Energy Transfer (FRET) in Live Cells

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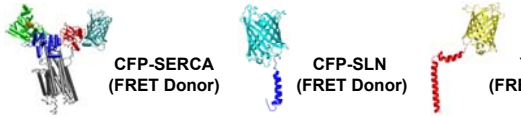
ABSTRACT

We have used fluorescence resonance energy transfer (FRET) microscopy to measure the binding affinities of four protein interactions in muscle to elucidate the binding events that occur during the formation of the SERCA super-inhibitory complex. Calcium is transported into the sarcoplasmic reticulum (SR) during muscle relaxation by the sarcoplasmic reticulum Ca-ATPase (SERCA), which is separately regulated by two transmembrane proteins, sarcolipin (SLN) and phospholamban (PLB). It has been proposed that when SERCA, SLN, and PLB are all expressed in the same muscle cell, the three proteins bind together in a super-inhibitory ternary complex, which decreases SERCA calcium transport by 50%. A key intermediate to this proposed ternary complex is the SLN:PLB heterocomplex. In my project, FRET microscopy was used to confirm the presence of the SLN:PLB heterocomplex and to directly quantitate the degree physical interaction between the two proteins in live cells. For comparison, FRET microscopy was also used to quantify SLN:SLN, SLN:SERCA, and PLB:SERCA interactions. Average FRET was directly calculated for each protein:protein interaction on a cell-to-cell basis. In addition, a Michaelis-Menten binding model and non-linear Hill fitting were used to calculate the dissociation constant for each protein interaction and the intrinsic distance between fluorescent probes. FRET results indicated that SLN and PLB form a low affinity heterodimer in cells with a distance of 4.7 nm between subunits. FRET results also show that SLN:SLN has the highest binding affinity of the four interactions while SERCA:SLN and SERCA:PLB have medium binding affinities relative to SLN:PLB and SLN:SLN. We propose that SLN and PLB first bind independently to SERCA and then bind to each other to induce the super-inhibitory SERCA ternary complex.

Figure 1 – Structural Models of the Fluorescent Fusion Proteins



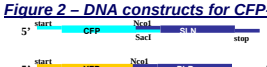
Cyan fluorescent protein (CFP) was fused to the initiator methionine (^{1}Met) of SLN and SERCA. Yellow fluorescent protein (YFP) was fused to ^{1}Met of PLB.

METHODS

Engineering of Fluorescent Fusion Proteins – cDNAs encoding SERCA and SLN were cloned from rabbit fast-twitch muscle using reverse transcription-polymerase chain reaction (RT-PCR). cDNAs encoding enhanced cyan fluorescence protein (CFP) and enhanced yellow fluorescent protein (YFP) were purchased from Clontech. CFP and YFP were fused to the N-terminus of SERCA and SLN using 'sticky-end' DNA ligation of engineered restriction sites (CFP-SLN, YFP-SLN, CFP-SERCA, YFP-SERCA). Mutations were created in SLN fluorescent fusion proteins using the QuikChange Mutagenesis Kit from Strategene. All DNA constructs were verified by DNA sequencing.

Baculovirus/Insect Cell Expression System – Spodoptera frugiperda (Sf21) insect cells were grown in suspension cultures seeded at 0.5×10^6 cells/mL. Sf21 cells were subcultured every 48 hrs. Recombinant baculoviruses expressing SERCA and SLN fluorescent fusion proteins were made using homologous recombination. Then, cells were placed into a 25 mL square flask, seeded at 0.4×10^6 cells/mL. Separately, DNA plasmids with the desired fluorescent fusion protein was added to serum free media (SFM). This mixture was then added dropwise over the insect cells in flasks, and the flasks were incubated for five days. Following the incubation period, the proteins were extracted and homogenized through centrifugation, and the supernatant was saved and used as virus for further amplifications.

Figure 2 – DNA constructs for CFP-SLN and YFP-PLB

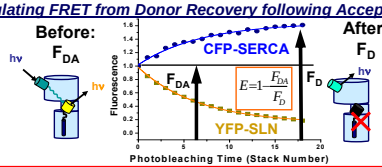


DNA encoding CFP and YFP were spliced to SLN and PLB at the NcoI/SacI fusion site

Fluorescent Microscopy – Insect cells were added to microscope plates in 2.0 mL aliquots, seeded at 3.0×10^5 cells/mL. 30 µL of virus was added to the plates for each virus used in the experiment. The plates were incubated for 48 hours before microscopy experiments were performed. Using a Xenon lamp, the cells were photobleached using a yellow fluorescent filter (514 nm). Using a screen to adjust light intensities, the cells were selectively photobleached in twelve, 2 min intervals for 24 minutes with a 20x objective. In between photobleaching intervals, neutral density filters were added to excitation filters to limit light intensities, and donor and acceptor fluorescence intensity measurements were taken by photograph.

FRET Analysis – Donor and acceptor intensities for each cell observed were identified and recorded for each experiment. Then, the background intensity was subtracted from the observed intensities, and each corrected intensity value was normalized relative to its initial corrected fluorescence intensity. Normalized CFP values were plotted as a function of normalized YFP values for each experiment. Data points within and between days were averaged, and a linear regression fit was added for each experiment. The y-intercept of each graph serves as the average FRET value for each experiment.

Figure 3 – Calculating FRET from Donor Recovery following Acceptor Photobleaching

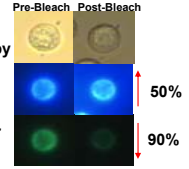


During selective acceptor photobleaching, YFP stops receiving the fluorescence energy transfer from the CFP donor, resulting in an increase in cyan fluorescence and a decrease in yellow fluorescence, relative to initial fluorescence values (F_o).

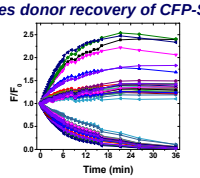
Figure 4 – Fluorescence Microscopy of Protein:Protein Interactions.

(A) Fluorescence Microscope

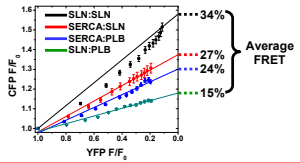
(B) Live cell imaging pre- and post-acceptor bleach.



(C) Acceptor photobleaching of YFP-SLN gives donor recovery of CFP-SLN.



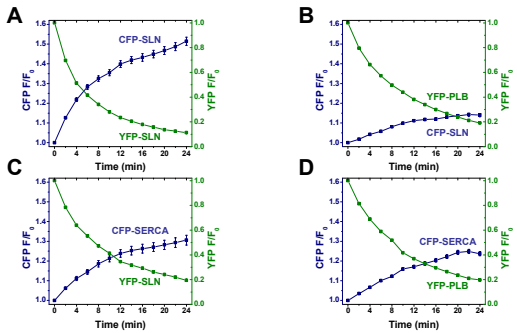
(D) Linear Fits of Acceptor Photobleaches



(A) Nikon microscope with CCD camera is used for FRET quantification. (B) FRET occurs between fluorescent fusion proteins. (C) YFP fluorescence decreases exponentially in each cell as CFP fluorescence increases simultaneously. (D) SLN:SLN forms high-order oligomers (photobleach curve), but SLN:SERCA, PLB:SERCA and SLN:PLB all form dimers (linear fit).

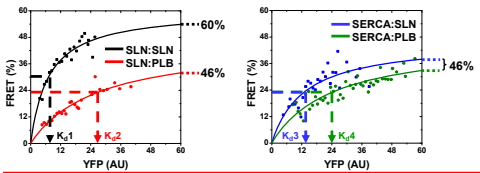
Figure 5 – Acceptor-Selective Photobleaching to Detect FRET Between Fusion Proteins.

(A) SLN:SLN (B) SLN:PLB (C) SERCA:SLN (D) SERCA:PLB



1. Acceptor-selective photobleaching of YFP-SLN gives donor fluorescence recovery for both CFP-SLN and CFP-SERCA *(A, C)*, indicating that SLN binds to itself and to SERCA.
2. Acceptor-selective photobleaching of YFP-PLB gives donor fluorescence recovery for both CFP-SLN and CFP-SERCA *(B, D)*, indicating that PLB binds to SLN and to SERCA.
3. SLN:SLN interactions yield the highest FRET while SLN:PLB has the lowest FRET *(A vs B)*.

Figure 6 – Calculation of Binding Affinities and Förster Distances



Förster Distance Equation:

$$R = R_0\left(\frac{1}{E}-1\right)^{1/6}$$

1. SLN favors self-association (K_d1) over PLB binding (K_d2) by 3.8-fold *(panel A)*.
2. SERCA favors SLN binding (K_d3) over PLB binding (K_d4) by 1.9-fold *(panel B)*.
3. The binding affinity of SLN:SLN (K_d1) is 2.3-fold greater than SLN:SERCA (K_d3), indicating that SLN favors self-association over regulatory complex formation *(panels A vs B)*.

CONCLUSIONS

1. SLN, PLB, and SERCA were tagged with naturally fluorescent proteins (CFP, YFP) and expressed in Sf21 insect cells using the baculovirus system.
2. Fluorescence microscopy was used to detect FRET between SLN and PLB subunits, providing novel evidence for SLN:PLB association. Acceptor photobleaching experiments indicated that SLN and PLB form dimers.
3. Fluorescence microscopy was also used to detect FRET between SLN and SERCA as well as PLB and SERCA. Acceptor photobleaching experiments showed SLN binding to SERCA is favored 1.9-fold over PLB binding to SERCA.
4. It has been proposed that SLN:PLB interactions cause increased binding to SERCA to induce super-inhibition (Asahi et al, 2003). Our FRET data indicate that the separate binding affinities of SERCA:SLN and SERCA:PLB are greater than the binding affinity of SLN:PLB. Thus, we propose that SLN and PLB first bind independently to SERCA, then bind to each other in the ternary complex to induce super-inhibition.

Figure 7 – Cartoon Model of SERCA Regulatory Interactions

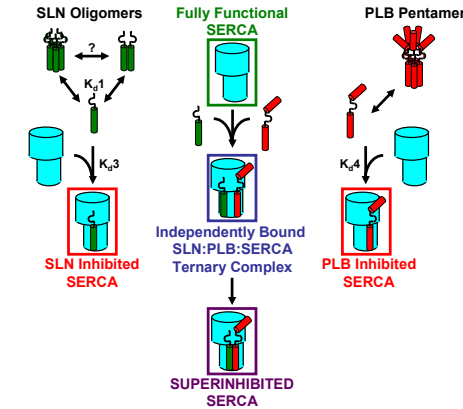


Table 1 –Binding Constants and FRET Parameters

Experimental Parameter	SLN:PLB	SLN:SLN	SLN:SERCA	PLB:SERCA
Dissociation Constant (K_d)	27.0 ± 5.8	7.20 ± 1.0	13.0 ± 4.2	24.0 ± 4.9
Average FRET (%)	15 ± 1	34 ± 1	27 ± 1	24 ± 1
Maximum FRET (%)	46 ± 6	60 ± 3	46 ± 6	46 ± 4
Distance (nm)	5.1 ± 0.2	4.7 ± 0.1	5.1 ± 0.2	5.1 ± 0.1
No. Cells	114	278	195	191
X^2 of Fit	4.5	7.1	20.7	10.7

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For further information

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