CHARACTERIZING A REDOX SENSITIVE GREEN FLUORESCENT PROTEIN FOR DYNAMIC MONITORING OF ENDOPLASMIC RETICULUM REDOX STATE

By

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ABSTRACT

The ability of a protein to fold into its native conformation depends upon the physiological conditions in which folding occurs. Secreted and cell surface proteins undergo oxidative folding in endoplasmic reticulum (ER) and as a result the regulation of reduction-oxidation (redox) state homeostasis within the ER is a crucial cellular process. Oxidized roGFP1-iL, a green fluorescent protein developed by the Remington research group, contains a destabilized disulfide bond and a midpoint reduction potential within the realm of the ER, making it a possible redox sensor for use as a quantitative marker (13). Prior to these studies, the roGFP1-iL had yet to be characterized for use as a redox sensor in the ER of mammalian cells, yet understanding the technical properties of this protein was necessary to diagnose its utility as an *in vivo* visualization tool. The cDNA encoding roGFP-iL was modified to contain two insertions that will result in expression of a signal peptide at the N-terminus and the ER-retaining KDEL sequence at the C-terminus. Here we show that the modified roGFP-iL localized to ER, and did not cause ER stress in 3T3-L1 cells. RoGFP1-iL *in vitro* behaved characteristically *in vitro*, with an excitation peak that varied with oxidation at 390nm and a second peak that varied with reduction at 450nm. Unexpectedly, non-reducing SDS-PAGE analysis showed roGFP1-iL formed dimers when treated with relatively oxidizing conditions of -160mV. It is not fully understood if the dimerization of roGFP1-iL compromises its ability as a quantitative redox marker. Mutagenesis is one possibility to reduce the amount of roGFP1-iL dimers.

INTRODUCTION

Diabetes mellitus affects roughly 26 million Americans, a startling 8.3% of adults over the age of 20. Another discomforting fact is that type II diabetes encompasses between 90 and 95% of all cases of diabetes in the United States. (3). Diabetes mellitus is characterized by elevated blood glucose levels, which can lead to blindness, heart failure, and decreased blood circulation over the course of a lifetime. Insulin is released when blood glucose levels are raised, which then binds to insulin receptors, effectively increasing the rate of uptake of glucose from the blood (19). In particular, Type II diabetes is generally characterized by the body's gradual loss of the ability to synthesize and process insulin caused by defects in the activation of insulin signaling pathways (20). One hypothesis identifies this defect as "cell stress." A form of cellular stress is misfolded proteins (21). Proper protein folding depends on the redox state of the endoplasmic reticulum, the epicenter for protein folding within the cell (15). For this reason, the redox state of the ER may be directly linked to type II diabetes.

The endoplasmic reticulum is a cellular organelle located near the nucleus, which functions as the epicenter for protein processing and lipid biosynthesis (22). The rough ER is characterized by ribosomes which attach to the surface of the ER and translate proteins specifically for transmembrane insertion and extracellular secretion. Defects in the physiological conditions of the ER have been shown to be tightly linked to the protein folding pathway (22). As the ER accumulates misfolded proteins and becomes stressed, the cell responds by initiating the unfolded protein response (UPR). The UPR is a compensatory mechanism which upregulates a variety of genes via enzymes such as PKR-like Endoplasmic Reticulum Kinase (PERK) (21). PERK, along with IRE1 and

Activating Transcription Factor 6 (ATF6), serve as stress sensors which can increase the production of enzymes that are prevalent in ER function (22). Another player in the unfolded protein response is a chaperone protein known as Binding Immunoglobin Protein (BiP) which, under normal conditions, is thought to bind and inhibit PERK, IRE1, and (ATF6). In the case of accumulation of unfolded proteins, BiP binds to these and therefore dissociates from the sensor proteins leading to a cascade of events that help in the survival of the cell and increase in ER functioning (21).

There are a variety of physiological conditions that have the ability to affect protein folding, one being the alteration of reduction-oxidation (redox) state homeostasis within the ER (15, 17). Fundamentally, oxidation and reduction applies to any chemical reaction that involves the transfer of electrons from an ion, atom, or molecule. Reduction is defined as the gain in electrons and therefore a decrease in the oxidation state while oxidation is the loss of electrons and therefore an increase in the oxidation state (23).

In proteins, reduction-oxidation reactions play a major role in protein folding as a result of disulfide bond formations between cysteine amino acid residues (11). Disulfide bond formations are essential for proteins to adopt their functional conformation (8). The chemistry of disulfide bond formation is a two-electron reaction that involves transfer between the thiols of a cysteine residue which requires an oxidizing/ reducing agent (15). The most common process for the formation of a disulfide bond *in vivo* is the result of a thiol-disulfide exchange reaction in which a protein interacts with a sulfhydryl containing molecule (15). In eukaryotic organisms, such small molecules are

represented by an assortment of electron donors/acceptors including the antioxidant glutathione (GSH) (4).

Glutathione is a reducing agent that can become oxidized to form oxidized glutathione (GSSG) which can be used as an oxidizing agent and accept electrons from other molecules. It has been demonstrated that glutathione is the major small molecule redox buffer in the ER (10). In the ER, there is a relatively high concentration of GSSG, indicating that the interlumenal conditions are oxidizing compared to the cytosol of the cell, a condition which favors the formation of disulfide bonds (1). However, glutathione is only a small portion of the oxidative protein folding pathway. It is speculated that glutathione does not directly impact protein folding, but rather its capacity to act as a redox buffer creates ideal folding conditions and may also reverse the effects of oxidative stress (4). By measuring the amounts of glutathione present, studies have shown that the reduction potential of the ER is around -180mV (10).

In the ER lumen of eukaryotic organisms, oxidative protein folding is chaperoned by a variety of proteins including ER oxidoreductin (Ero1) and protein disulfide isomerase (PDI) (7, 8). PDI and Ero1 are required for the net formation of disulfide bonds, via thiol-disulfide exchange reactions, within the ER of a cell and are therefore necessary for oxidative protein folding (15). Studies have shown that Ero1 acts as an oxidizing agent, passing its disulfide to PDI. PDI then acts on the secreted protein functioning as an oxidizing agent, passing on its disulfide allowing for protein folding (7). A majority of proteins are therefore impacted by the redox environment of the ER.

Considering the impact of the redox state of the ER on protein folding and hence cellular function, it is valuable to have the ability to monitor such a characteristic *in vivo*. In fact, redox sensitive markers have been successfully designed for this purpose. Traditional examples of such indicators include enzyme reagents such as dichlorodihydrofluorescein, which only has the capacity to react in a single oxidation reaction (9). More recently, quantitatively accurate reduction-oxidation sensitive green fluorescent proteins have been developed but have previously been limited by the thermodynamics of the protein and its location within the cell, considering the relatively oxidizing conditions of the ER (13).

This family of green fluorescent proteins appear promising as redox sensitive indicators in the oxidizing conditions of the ER lumen, our focus being roGFP1-iL. RoGFP1-iL, a 27kDa protein of the Lohman GFP family, exhibits an isosbestic point with two excitation peaks (13). This duality creates the possibility of roGFP1-iL to behave ratiometric by excitation, allowing one to know the concentration of the GFP independent of oxidation and the quantitative redox state based on fluorescence readings. To achieve such qualities, wild type GFP or GFP-S65T was initially mutated C48S, S147C, Q204C which lead to roGFP1 (5). Further mutation was conducted including: H148S, F64L, F99S, M153T, V163A, I167T. A unique quality of RoGFP1-iL was the insertion of a leucine residue hence the determination between the family of redox markers roGFP1-iX and our unique marker roGFP1-iL (13). As a formality, after the construction and insertion in 3T3-L1 cells, the roGFP1-iL protein was redefined as eroGFP1-iL. Therefore, roGFP1-iL is a reference to the purified protein while eroGFP1-iL is the 3T3-L1 expressed protein. Mutations in roGFP were introduced to achieve two

goals; to allow the GFP to fold more efficiently at biological relevant temperatures and to destabilize the reduction-oxidation sensitive disulfide bridge via the single amino acid insertion (13). With such mutations it appears that roGFP1-iL will have a useful range between -195mV to 265mV, which makes its efficiency in the ~180mV conditions of the ER questionable (13).

In the roGFP1-iX family, cys147 and cys204 were added to form an exterior disulfide bridge, which when oxidized, allows for the activity of the neutral chromophore while the neutral chromophore activity is significantly decreased (13). The chromophore of GFP consists of Phe64, Ser65, Tyr66, Gly67, Val68, Gln69. In the deprotonated and hence anionic state, there is an extra electron in comparison to the protonated form, represented in Figure 1 (2). For this reason, the anionic form is more easily depotonated and hence requires a lower energy photon for the emission of a photon at 505nm. This explains why the roGFP1-iL reduced excitation peak is at ~457nm (lower energy photon) while the oxidized peak is at ~395nm (higher energy photon) (13). It is suspected that formation of the disulfide bridge between cys147 and cys204 exerts a strain upon the backbone of the polypeptide chain, see Figure 2, leading to variation in chromophore activities (2).

RoGFP1-iL has yet to be characterized for use as a redox sensor in the endoplasmic reticulum of any mammalian cell type, including 3T3-L1 adipocytes. Conformation of its function as a redox sensor is an essential first step for testing our hypothesis, that eroGFP1-iL is capable of quantitatively indicating the redox state within the ER. In this study, eroGFP1-iL is analyzed for properties that are essential for its use as a redox sensitive indicator within the ER including: a control to verify co-localization

to the ER of the cell, a control to detect if the eroGFP1-iL (*in vivo*) protein causes inherent cellular stress to an otherwise unstressed cell, and experimentation to determine if the roGFP1-iL (*in vitro*) marker behaves ratiometrically by analyzing excitation spectra at various reduction potentials.

MATERIALS AND METHODS

Plasmid Construction of pBABE-eroGFP1-iL

The following sequence was designed for insertion of the modified eroGFP1-iL sequence**:** GC-BamHI-Kozak-Acrp30 signal peptide-roGFP-MYC-NSE-HDEL-stop-SAlI-GGCG.

Each portion of the sequence has a specific function. CG is over hang, BamH1 is a restriction enzyme site, the kozak sequence is important for the initiation of translation, the signal peptide directs the DNA sequence to the ER for modification, the roGFP1-iL sequence is homologous to portions of the PCR roGFP1-iL template, the MYC-tag is a polypeptide tag that aids in protein purification via affinity chromatography, the HDEL is a common protein sequence that allows for an enzyme to be retained in the ER, and SalI is the second restriction enzyme site. The first primer was constructed to include the sequences upstream of the roGFP1-iL sequence element and consisted of the following DNA sequence: GCGGATCCGCCACCATGCTACTGTTGCAAGCTCTCCT GTTCCTCTTAATCCTGCCCAGTCATGCCGAAGATAGTAAAGGAGAAGAACTTTTCA CTGG. The second primer was constructed to include the sequences downstream of RoGFP1-iL sequence element and consisted of the following DNA sequence: ACACAT GGCATGGATGAACTATACAAAGAACAAAAACTCATCTCAGAAGAGGATCTGAATTC GGAACATGATGAGCTCTAATAGTCGACCGCC.

Next, primers were reacted with template DNA for roGFP1-iL (13) and PFU turbo polymerase (Strategene) in a PCR reaction on the Perkin Elmer Minicycler. BamHI (BioLabs) and SalI (BioLabs) were used in the digest the PCR product and pBABE-puro (14), which were then ligated together using T4 DNA Ligase. Plasmid with ligated insert

was transformed into JM109 competent cells and selected with ampicilin. DNA was isolated and verified via sequencing. The pBABE_eroGFP1-iL DNA and pcl-ECO DNA was then co-transfected into HEK 293T cells to generate viral particles. The collected viral titers, were spun down, filtered, and stored in -80°C. The 3T3-L1 pre-adipocyte were then incubated with cells viral titers for 24 hours and then selected with 6-8µg/mL puromycin for 4 days.

Tissue Culturing Protocol

3T3-L1 cells were grown in 1x DMEM (VWR) solution containing 1mM sodium pyruvate, 2.5g/L Sodium Bicarbonate, 10% Fetal Calf Serum, 2mM L-glutamine, 10µg/mL penicillin-streptomycin, 1mM sodium pyruvate, and 50mg/L nystatin.

For cultivation of a cell line, pre-adipocyte cells were plated at roughly 10% confluency and allowed to grow. Media changes were conducted at least every other day. When the cells reached between 20%-30% confluency, cells were lifted using 3mL of warmed .25% tripsin-EDTA per T-175 flask. Tripsin was incubated on the 3T3-L1 cells for 1minute and then diluted 4:1 media to tripsin ratio. Using a pipet, the cells were removed from the flask and placed in 50mL conical tube, leaving only ~1mL to seat for next freeze down. The cells in the conical tube were then spun in the centrifuge at 600rpm at 4°C for 10 minutes. Next, cells were frozen down in a solution of fetal bovine serum (FBS) containing 10% dimethyl sulfoxide using a cryo-safe isopropanol cooler which achieves a 1°C/min rate of cooling.

For differentiation, cells were plated allowed to reach 100% confluency over a 3-4 day period. At this point, cells were treated with a differentiation medium #1 (DM1) which consists of the standard medium for cell growth described above, with fetal

bovine serum substituted for calf serum and as well as .5mM 3-isobutyl-1-methylxanthine (IBMX), 1μM dexamethasone (DEX), and .01mg/mL insulin. After three days in these conditions, cells are then treated with differentiation medium #2 (DM2) for a period of 2 days, which contains .005mg/mL insulin.

Expression and Purification of roGFP1-iL

RoGFP1-iL_PQE30 DNA (13) was transformed into BL21 (DE3) pLyS competent cells (Stratagene). Transformation protocol was conducted strictly following the Stratagene general use protocol. Bacteria grown in the SOC medium are then plated on ager 10cm plates, which use 100μg/mL ampicillin as a selection agent, and allowed to grow overnight. Several colonies were selected from the plate for induction of protein synthesis using 1mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Protein was then analyzed using coomassie stained gel.

Transformed cells were placed in 250mL of Lysogeny Broth (LB) medium with 100μg/mL ampicillin at 37°C to shake. After overnight growth, a larger 2L inoculation is conducted until the optical density at 600nm is between .4-.9 indicating that there is sufficient bacterial growth for optimal protein production. Protein induction is conducted by adding 1mM IPTG to the 2L culture and allowing it to incubate overnight.

The culture is pelleted by centrifuging at 4000rpm for 20 minutes. The pellet is weighed, quickly frozen in liquid N_2, and then placed at -80°C overnight. The following day, Hi-Trap chelating column equilibration buffer is used for a medium in which to resuspend and sonicate the bacteria. Sonication takes place on ice, using a 6-minute program using a 15 second on, 30 second off, and then off for a minute after completion

of a minute cycle. Once sonication is complete, the sample is centrifuged and the supernatant is extracted for further filtration.

The 6xHis Tag of the roGFP1-iL protein is exploited for protein purification on the 5mL Hi-Trap chelating column. Hi-Trap chelating column protocol from GE Healthcare was used as the procedure. The stripping buffer consisted of binding buffer and 50mM EDTA, the binding buffer consisted of 20mM sodium phosphate, 0.5 M NaCl, 5–40 mM imidazole, pH 7.4, and the elution buffer consisted of 20mM sodium phosphate, 0.5 M NaCl, 0.5 M imidazole, pH 7.4. The column was stripped, washed with distilled water, charged, washed again with distilled water, and then the sample was loaded. After a wash with binding buffer, the protein was eluted using a step gradient of increasing imidazole concentration between 5-40mM.

Typhoon Fluorescence Imaging

Using the *Nernst equation, the concentrations of the oxidized and reduced forms of lipoic acid were manipulated to induce reductive conditions from most oxidized to most reduced: 0mV, -80mV, -180mV, and -280mV.

$$*E = E_0 - \left(\frac{RT}{zF}\right) \ln\left(\frac{[red]}{[ox]}\right)$$

*Nernst Equation: Where E is the potential measured in volts, E_0 is the standard reversible potential, R is the gas constant, T is temperature, z is the number of electrons, in this case 2, involved in the redox reaction, F is Faradays constant, [red] is the concentration of reduced lipoic acid, and [ox] is the concentration of oxidized lipoic acid.

The roGFP1-iL protein was allowed to react in this environment for roughly 4 hours. RoGFP1-iL at an equivalent concentration was used but was not treated with

lipoic acid. A control was in the same buffer as the reactions (20mM HEPES, 140mMNaCl, and 1mM EDTA). Reactions scanned using 457nm (blue) laser on Typhoon fluorescence imager using an emission filter of 520nm, PMT setting of 600, and at high sensitivity.

Co-localization of the RoGFP1-iL in the Endoplasmic Reticulum

Fixed cell image probed for Myc-tag GFP and PDI, a protein known to be found in the ER. Also conducted was live cell imaging of co-transfected eroGFP1-iL and mRuby. mRuby is a construct that contains KDEL sequence for ER retention. The cells were then imaged on Nikon C1si confocal microscope.

ER Stress Assay

3T3-L1 eroGFP1-iL top 10% fluorescent cells, 10-20% fluorescent cells, and ATCC control cells were lysed using a buffer containing 1 lysis mini tablet (Rosche), deoxycholate (.5%), SDS (.1%), sodium orthovanidate (1mM), sodium fluoride (50mM), sodium pyrophosphate (5mM), micro cysteine (1μM), in a 1x RIPA Buffer. Previously, positive control cells were incubated with 1μM Thapsigargin. After sonication lysis, lysates were treated with 20mM NEM and ran on a 10% SDS, 1.5mm, 10-well Tris-HCl gel using tris-glycine running buffer.

After gel electrophoresis, a western blot transfer to a nitrocellulose membrane was conducted using an overnight wet-transfer procedure. The membrane was blocked using Odyssey Blocking Buffer, treated with 1:300 rabbit primary BiP1 antibody (Cell Signaling) washed with PBST for 3, 5 minute intervals, and then treated with 1:300 goat

anti-rabbit HRP secondary (Jackson ImmunoResearch). A ponceau stain was conducted as a load control to indicate overall amount of protein loaded.

Fluorimeter Excitation Spectroscopy

All buffers were degassed, bubbled with nitrogen and allowed to equilibrate in the glove box for 24 hours prior to experimentation. Using the Nernst equation, the concentrations of the oxidized and reduced forms of lipoic acid were manipulated to induce reductive conditions from most oxidized to most reduced: -140mV, -180mV, -220mV, and -280mV.

$$*E = E_0 - \left(\frac{RT}{zF}\right) \ln\left(\frac{[red]}{[ox]}\right)$$

*Nernst Equation: Where E is the potential measured in volts, E_0 is the standard reversible potential, R is the gas constant, T is temperature, z is the number of electrons, in this case 2, involved in the redox reaction, F is Faradays constant, [red] is the concentration of reduced lipoic acid, and [ox] is the concentration of oxidized lipoic acid.

Expressed and purified roGFP1-iL protein was incubated for 4 hours in specific ratio of oxidized and reduced lipoic acid within the anaerobic conditions of the glove box in a buffer of 20mM HEPES pH 7, 150mM NaCl, and 1mM NaCl. Immediately following incubation of the reactions their spectra was observed using the Aminco SLM 8000 fluorimeter. An excitation spectrum was obtained by scanning from 350nm-500nm measuring emission at 505nm. The excitation polarizer was 0, emission polarizer was 0, and all of the emission/excitation slits were set to 4. The EML was set at 700V with a 10x gain. The reference was set to 400V at a 1x gain. Immediately after the samples

were observed on the fluorimeter, NEM (10mM) was added and samples were ran on a NuPAGE Bis-Tris 4-12% 1.5mm gel, using a page ruler plus protein ladder and was then coomassie stained.

RESULTS

Typhoon Fluorescence Imaging

The typhoon fluorescence experiment was conducted as a preliminary analysis of the ability of roGFP1-iL to vary emission intensity at 520nm as the reduction potential of the roGFP1-iL environment is varied, when excited at 457nm. In this particular experiment, the relative fluorescence was important as a preliminary analysis of the fluorescence peak at 457nm as evidenced in Figure 3. It was noted that 0mV, -80mV, and -180mV were relatively similar in fluorescence with a signal greater than the control, non-reduced roGFP1-iL, while the -280mV well demonstrated a significantly higher signal in comparison to the other potentials, using the 520nm wavelength filter. The blank and buffer control lacked a signal.

Co-localization of the RoGFP1-iL in the Endoplasmic Reticulum

Considering eroGFP1-iL is to be used as an indicator of the redox state of the ER, it must be located within the ER sub cellular environment in order to function properly. The co-localization experiment uses imaging techniques to determine the location of the eroGFP1-iL with respect to the ER of the 3T3-L1 cells. As demonstrated in Figure 4, cells were imaged using live-imaging and paraforaldahyde-fixing techniques both exhibiting similar results. The transmitted light cell, can be used as a reference for the other images in the row, which are using various filters for imaging the ER. For all of the images of the paraformaldahyde-fixed cells, the anti-PDI antibody is red and appears to be concentrated to the area immediately surrounding the nucleus of the cell. The anti-myc tagged eroGFP1-iL is also localized to the area immediately surrounding the nucleus. The overlay demonstrates that the eroGFP1-iL and the PDI are located

within the same region of the cell, in the area surrounding the cell's nucleus. The same result was obtained using live-imaging techniques, where mRUBY and eroGFP1-iL are co-localized to the same region surrounding the nucleus.

ER Stress Control Experiment

The 3T3-L1 adipocytes are not stressed when grown in the proper conditions; however, it is unknown whether the cells production of the eroGFP1-iL protein will perturb the cells homeostasis. It is important that the production of eroGFP1-iL is not stressing the cells considering the primary goal for eroGFP1-iL protein: to recognize redox state, a factor that may influence the cell to experience cellular stress. The western blot of untreated top 10% fluorescing eroGFP1-iL expressing 3T3-L1cells, untreated 10-20% expressing 3T3-L1cells, and untreated pBABE-puro vector control 3T3-L1 cells, and thapsigargin treated 3T3-L1 cells were probed for BiP, using BiP1 antibody exhibited in Figure 5. Thapsigargin is known to inhibit the cells' ability to pump calcium into the ER of the cell, therefore increasing calcium concentration in the cytosol and decreasing calcium concentration in ER resulting in ER stress characterized by induction of unfolded protein response (23). Thapsigargin is therefore a positive control for ER stress and hence the presence of cellular stress markers like BiP. A dark band was present at roughly 80kDa for the thapsigargin treated cell lysates but not for the untreated top 10% fluorescing eroGFP1-iL expressing 3T3-L1cells, untreated 10-20% eroGFP1-iL expressing 3T3-L1cells, or untreated pBABE-puro vector control 3T3-L1 cells.

In the blot probing for eroGFP1-iL, where the bands are located at roughly 27kDa, a higher signal is present for the top 10% fluorescing eroGFP1-iL expressing 3T3-L1cells, in comparison to the 10-20% eroGFP1-iL expressing 3T3-L1cells which in turn have a higher signal than the unsorted eroGFP1-iL. The pBABE-puro control lacks a signal for eroGFP1-iL.

The ponceau stain on the lower left side of Figure 5 shows that untreated top 10% fluorescing eroGFP1-iL expressing 3T3-L1cells, untreated 10-20% expressing 3T3-L1cells, untreated pBABE-puro vector control 3T3-L1 cells, have a qualitatively equivalent amount of lysate protein loaded and thapsigargin treated 3T3-L1 cells have qualitatively less.

Fluorimeter Excitation Spectroscopy

The fluorimeter excitation spectrum gives deeper insight into the fluorescent properties of roGFP1-iL in comparison to the typhoon spectroscopy, including the isosbestic point and peak fluorescence with respect to varying excitation wavelengths. Finally, it indicates if quantitative ratiometric calculation is possible using roGFP1-iL. The spectrum of reactions of roGFP1-iL was observed using the Aminco SLM 8000 fluorimeter. An excitation spectrum was obtained by scanning the lipoic acid reactions ranging from -140mV, -180mV, -220mV, to -280mV. The excitation spectra ranged from 350nm-500nm measuring emission at 505nm as evidenced in Figure 6. The isosbestic point of roGFP1-iL was located at roughly 430nm. There are two peaks, one located at 390nm and the other at 450nm. Both peaks vary their relative intensity levels as the redox potential varies. The peak at 390nm is lowest at -280mV, and gets progressively

higher in intensity as the conditions become more oxidizing. The peak at 450nm is lowest at -140mV, and gets progressively higher in intensity as the conditions become more reducing.

Immediately after the samples were observed on the fluorimeter they were ran on a NuPAGE Bis-Tris 4-12% 1.5mm gel and coomassie stained exhibited in Figure 7. Two bands were observed on the gel, on at 27kDa and the other was roughly twice that size at roughly 54kDa. For -140mV, -180mV, and -220mV reducing potentials, the bands at 27kDa and 54kDa have relatively equivalent signals. For the -280mV reducing potential the signal at 54kDa is significantly less than the signal at 27kDa.

Size Exclusion Isolation of Monomer and Dimer

Dimers have been noticed and may exhibit variable fluorescence compared to the monomer form of the protein. To determine spectral properties of the dimer form of roGFP1-iL, the monomer and dimer were isolated and analyzed using with the fluorimeter. Once incubation at -160mV was complete, the sample was loaded onto the Superdex 75 size exclusion column. The elution spectra revealed three peaks elution indicating the presence of monomers and dimers which were isolated and ran on the Aminco SLM 8000 fluorimeter to produce the spectrum in Figure 8. The spectra for a monomer exhibited a broad peak at roughly 390nm with another much lower peak at 450nm. The dimer spectra exhibited a broad, steadily increasing peak between 370nm and 410nm with a defined peak at 450nm.

Immediately after the samples were observed on the fluorimeter they were ran on a NuPAGE Bis-Tris 4-12% 1.5mm gel and odyssey scanned evidenced in the top right

corner of Figure 8. The monomer produced a strong signal at roughly 27kDa. The pooled dimer had a relatively weak signal at about 54kDa and an even weaker signal at 27kDa.

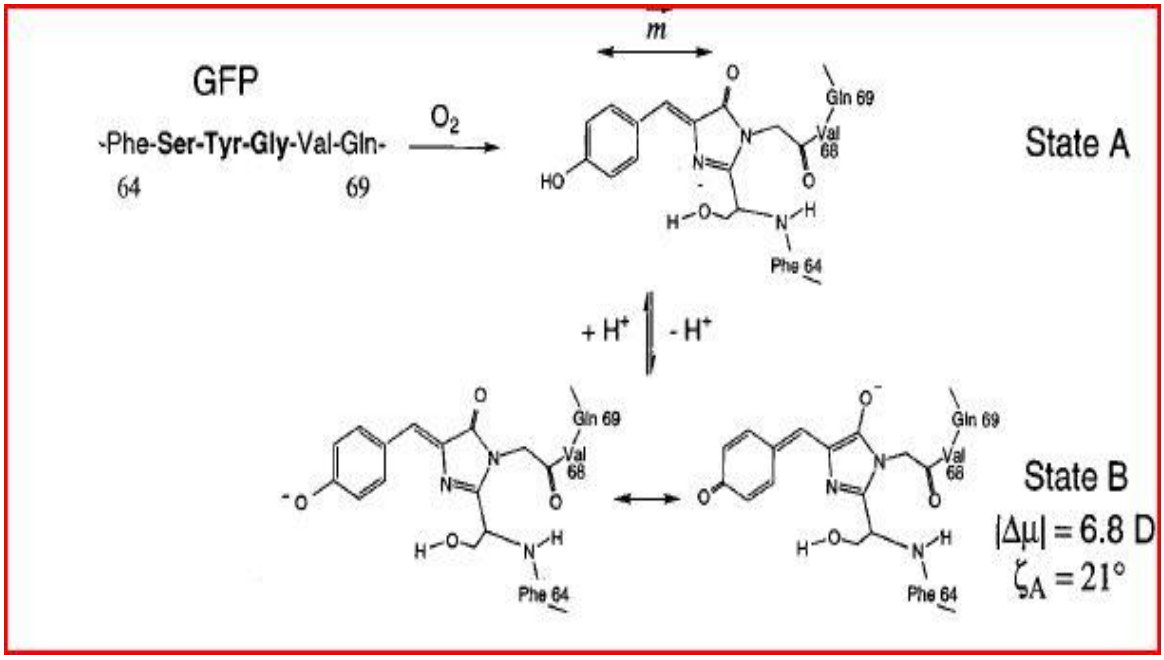

Figure 1: Chemistry of the GFP chromophore represented in neutral and anionic states depending on protonation/deprotonation of the hydroxyl group of Tyr66. Figure is courtesy of (2).

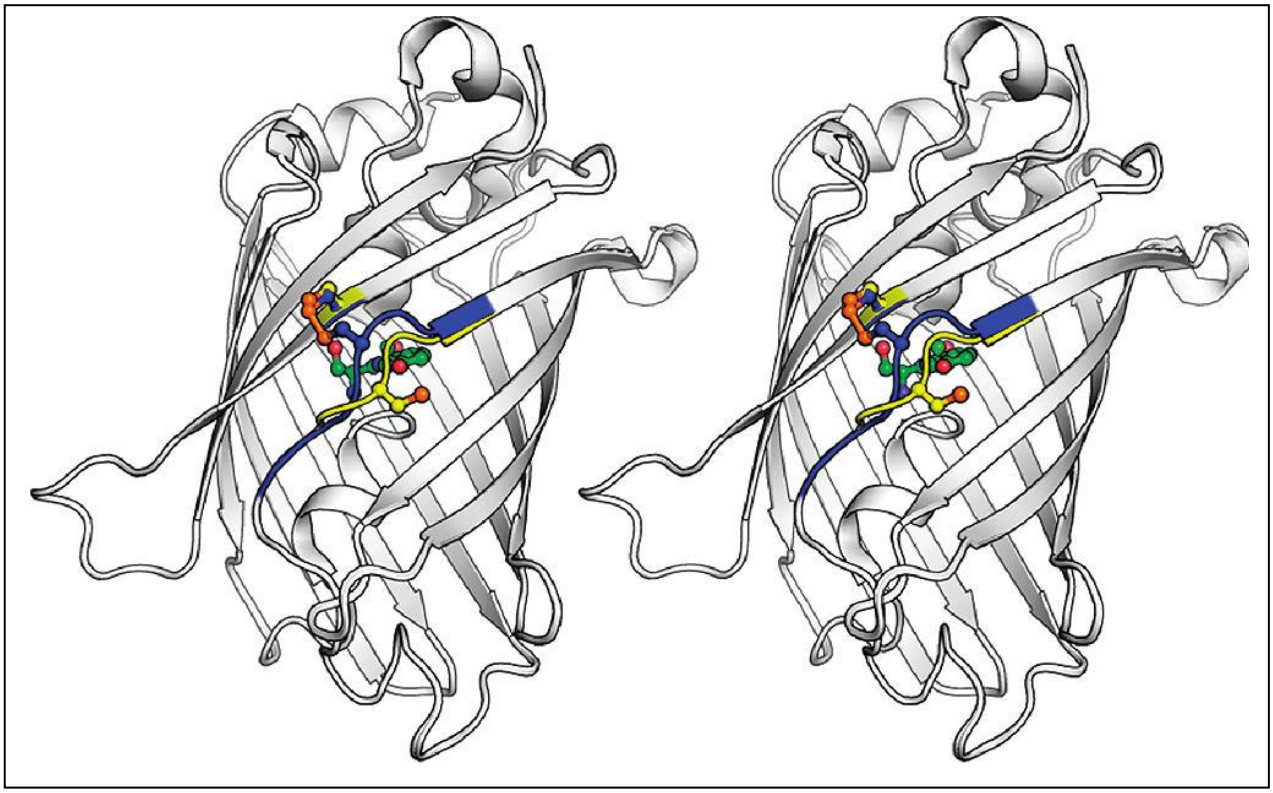

Figure 2: Ribbon diagram of RoGFP1-iX family as determined from the crystal tructures of oxidized and reduced RoGFP1-iR. It is depicting the oxidized (blue) disulfide linkage between cys147 and cys204 (orange ball and stick) and reduced (yellow) disulfide linkage between cys147 and cys204. The GFP chromophore is within the barrel and colored green. Figure is courtesy of (13).

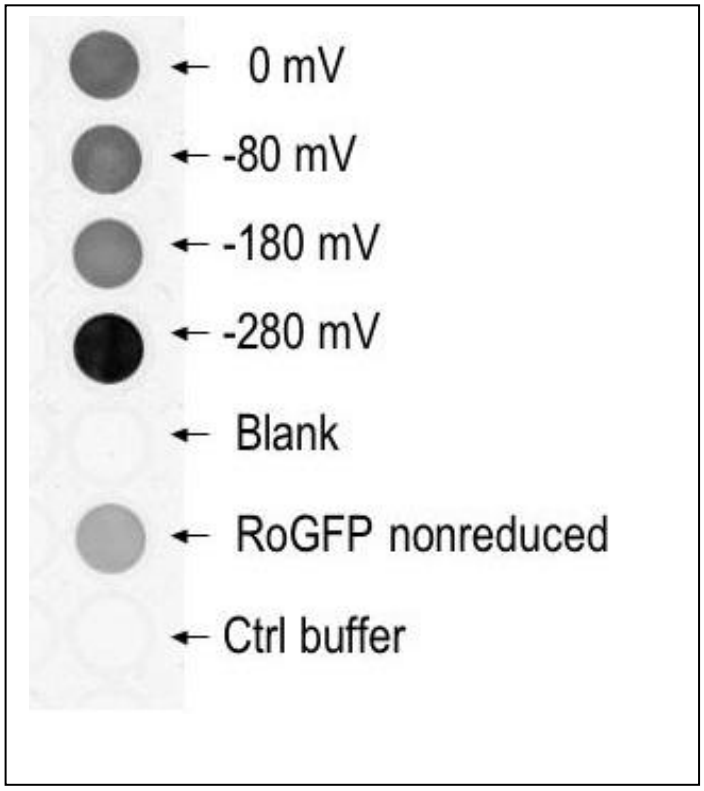

Figure 3: RoGFP1-iL in Lipoic acid from most oxidized to most reduced: 0mV, -80mV, -180mV, and -280mV in buffer (20mM HEPES, 140mMNaCl, and 1mM EDTA). Reactions scanned using 457nm (blue) laser on Typhoon 9400 fluorescence imager using an emission filter of 520nm, PMT setting of 600, and high sensitivity.

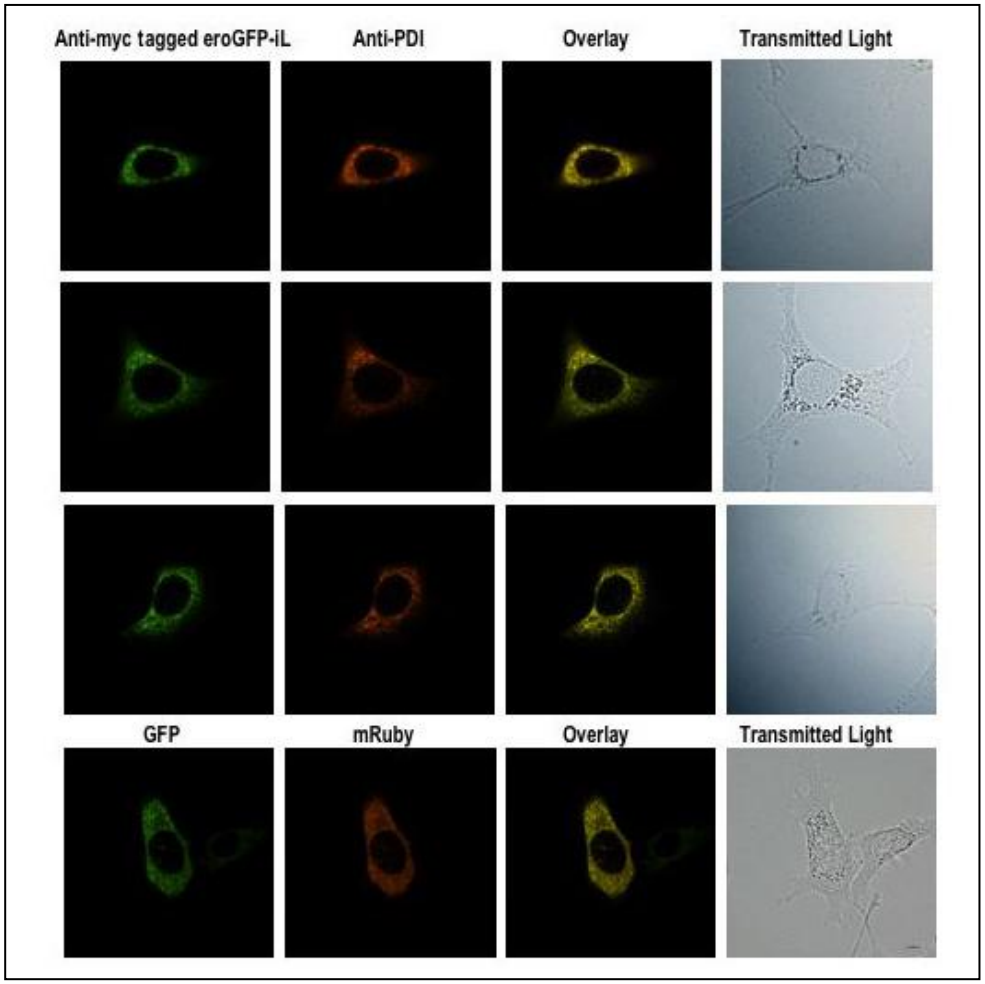

Figure 4: Immunofluorescence of paraformaldehyde fixed 3T3-L1 fibroblasts co-stained for myc-tagged eroGFP1-iL and PDI (Sigma) imaged on Nikon C1si confocal microscope. Live cell imaging of mRuby and eroGFP1-iL co-transfected 3T3-L1 fibroblasts imaged on Nikon C1si confocal microscope.

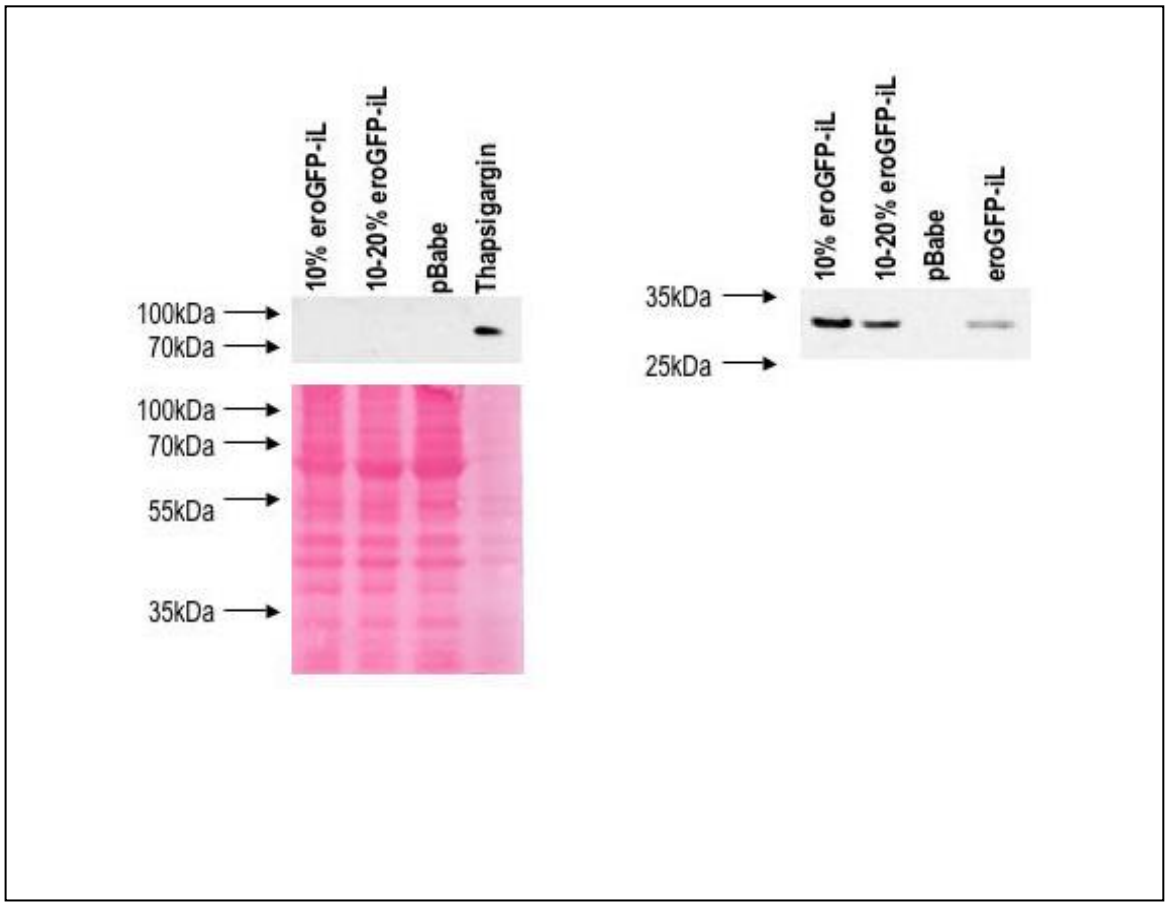

Figure 5: (Top Left) Western blot analysis of FACS sorted, lysed cells using BiP1 antibody scanned using the odyssey scanner observed between 70-100kDa looking for presence of 78kDa protein BiP (Top Right) Same western blot but observed between 25-35kDa looking for presence of 27kDa protein eroGFP1-iL (Bottom Left) Ponceau stain of the same western blot as a protein load control.

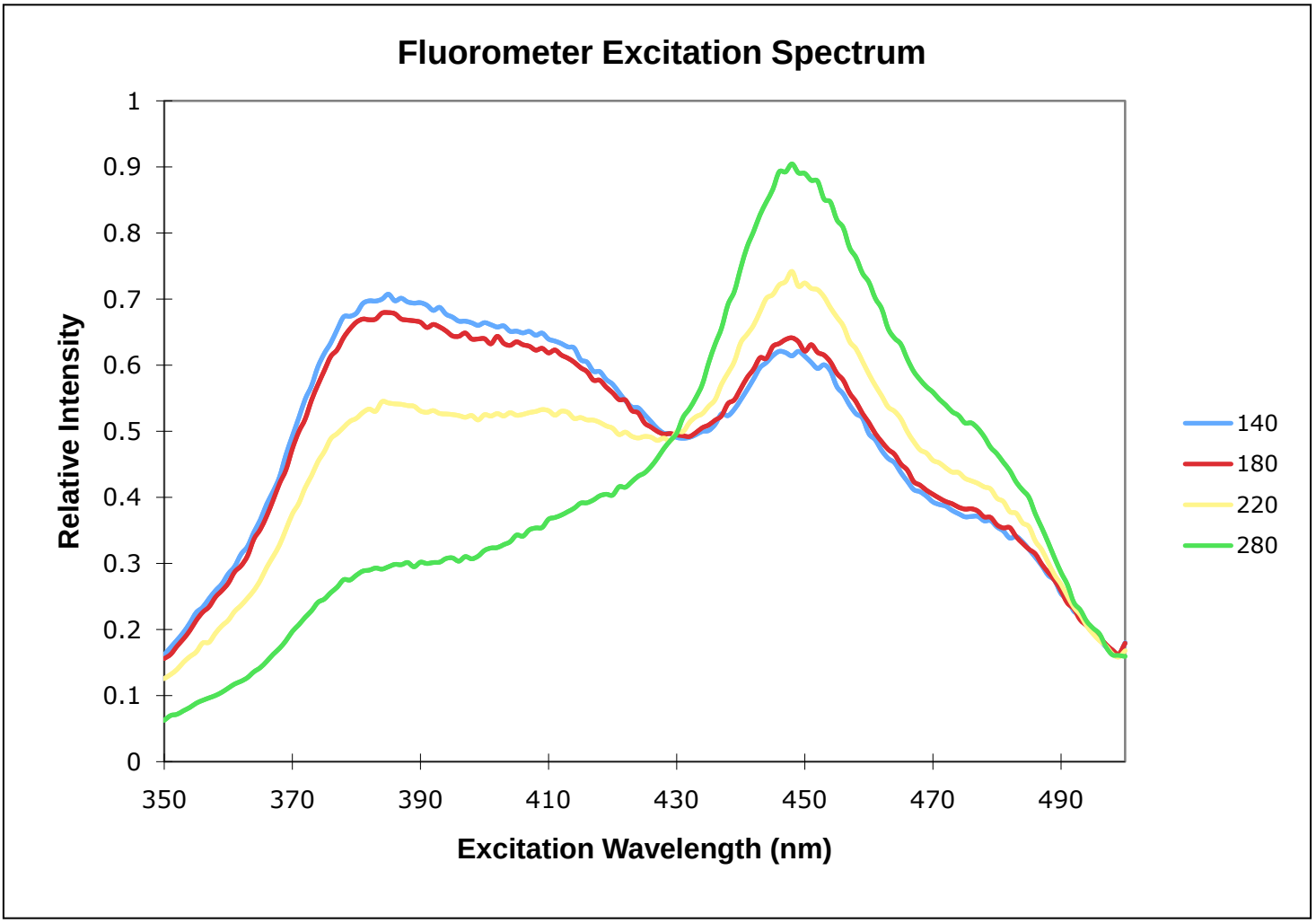

Figure 6: Fluorimeter spectrum of reactions of roGFP1-iL in Lipoic acid (conducted in anaerobic glove box) in buffer (20mM HEPES, 140mMNaCl, and 1mM EDTA) from most oxidized to most reduced: -140mV, -180mV, -220mV, and -280mV.

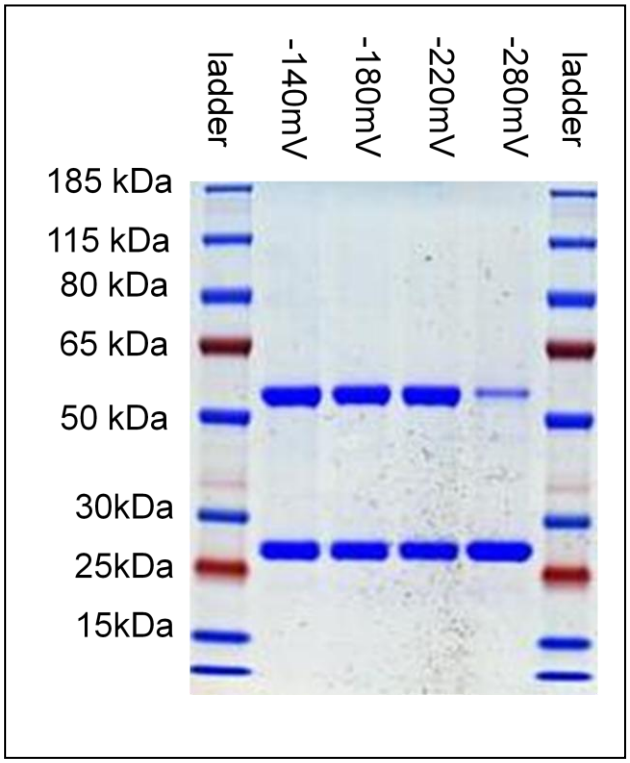

Figure 7: SDS-PAGE gel using the samples from the midpoint potential determination experiment treated with NEM (10mM) on a NuPAGE Bis-Tris 4-12% 1.5mm gel at 150V until complete and coomassie stained gel using a page ruler plus protein ladder.

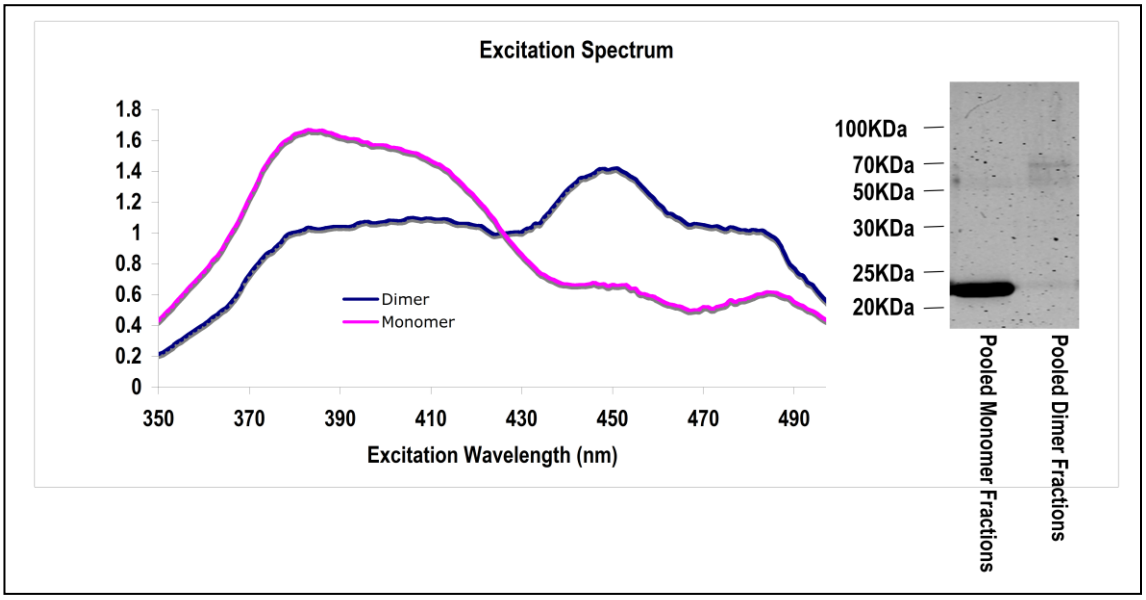

Figure 8: Fluorimeter Excitation Spectrum of monomer and dimer fractions isolated using GE isocratic size exclusion Superdex 75 column and post column, odyssey scanned, NuPAGE Bis-Tris 4-12% 1.5mm gel, using an unstained protein ladder, with a focus on the RoGFP1-iL region of ~27kDa.

DISCUSSION:

RoGFP1-iL has yet to be characterized for use as a redox sensor in the ER of mammalian cells, yet understanding the technical properties of this protein is necessary to diagnose its utility as an *in vivo* visualization tool. The characterization techniques were first aimed at confirming that the 3T3-L1 eroGFP1-iL expressed roGFP1-iL is localized in the ER by the HDEL sequence engineered into the plasmid.

In the co-localization experiment using confocal microscopy, as demonstrated in Figure 6, live-imaging and paraforaldahyde-fixing techniques both exhibited similar results. In the paraformaldahyde fixed cells, the anti-PDI antibody is red and appears to be concentrated to the ER in the area immediately surrounding the nucleus. The anti-myc tagged eroGFP1-iL is also localized to the area immediately surrounding the nucleus. The overlay demonstrates that the eroGFP1-iL and the PDI are located within the same region of the cell. The same result was obtained using live-imaging techniques, where mRUBY and eroGFP1-iL are co-localized to the same region surrounding the nucleus. Hence it can be deduced that the HDEL sequence of the eroGFP1-iL protein is indeed allowing eroGFP1-iL to target the ER.

Next, a control was conducted to determine whether or not the eroGFP1-iL protein was somehow inherently stressing the 3T3-L1 cells. As previously stated, if such stress were present, it would undoubtedly lead to the unfolded protein response which is characterized by the presence of BiP. To test this, eroGFP1-iL expressing 3T3-L1 cells previously sorted using fluorescence activated cell sorting were lysed along with pBABE-puro control cells and thapsigargin treated 3T3-L1 cells. Thapsigargin is known to inhibit calcium channels within the ER of the cells (6) which causes cellular stress,

the unfolded protein response, and hence the expression of BiP. Thapsigargin was therefore used as a positive control. Considering the blot of the lysed cells exhibited in Figure 5, one can deduce that the cells with the eroGFP1-iL insert are not inherently stressed. This is evidenced by the presence of BiP in the stressed thapsigargin cells while every other cell type lacks a BiP signal. The ponceau stain indicated that protein load amounts were held constant among eroGFP1-iL and pBABE-puro and that the absence of a signal was not merely due to low protein concentration.

The following characterization technique was aimed at confirming that the RoGFP1-iL fluoresces *in vitro*. Also, it was important to determine that roGFP1-iL had the quantitative two-state behaviors of other roGFP proteins. It has been noted that roGFP proteins behave ratiometrically, meaning that it exhibits two peaks; one reduced and on oxidized peaks, typically at 390nm and 475nm on the excitation spectrum (13).

The Typhoon 9400 fluorescence imager is a technique for measuring global fluorescence by exciting using a laser of a particular wavelength and observing the emitted fluorescence using a particular filter. In this experiment, the relative fluorescence was measured at the peak at 457nm, correlating to the peak which varies as the GFP is reduced. As evidenced in Figure 2, reactions at 0mV, -80mV, and -180mV reduction potentials were relatively similar in fluorescence with a signal greater than the control non-reduced roGFP1-iL. The reaction correlating to -280mV reduction potential demonstrated a significantly higher fluorescence signal in comparison to the other potentials, using the 520nm wavelength filter. The reactions correlating to 0mV, -80mV, and -180mV are relatively oxidizing environments compared to the -280mV environment. Since the Typhoon imager is exciting at 457nm, it is hypothesized that the

-280mV reduction potential reaction would have the greatest emission at 520nm when excited at this wavelength, if the protein behaves like a characteristic roGFP. Indeed, the most reduced reaction condition, -280mV, has the greatest fluorescence signal, indicating “ratiometric-like” behavior. This test was preliminary and demonstrated that the RoGFP1iL does indeed fluoresce at 520nm, however, required further quantitative in-vitro testing to corroborate this test.

The development of a fluorescent spectrum over a variety of excitation wavelengths using a fluorimeter is a more quantitatively accurate method to characterize fluorescence. An excitation spectrum was obtained by scanning the lipoic acid reactions ranging from -140mV, -180mV, -220mV, to -280mV. The spectrum produced, exhibited in Figure 3, is characteristic of a ratiometric roGFP protein as described previously, since it has two excitation peaks which vary in fluorescence as the redox conditions vary. The peak at 390nm is lowest at -280mV, and gets progressively higher in intensity as the conditions become more oxidizing. The peak at 450nm is lowest at -140mV, and gets progressively higher in intensity as the conditions become more reducing. A notable quality in the spectrum is the very slight difference between the peaks for -140mV and -180mV. The isosbestic point is present at 430nm, meaning overall protein concentration could theoretically be calculated independent of the oxidation state of the protein (13).Such a spectrum corroborates with the Typhoon preliminary test; however, SDS-PAGE protein analysis of the reactions indicated a significant issue, complicating the sensors utility.

The protein gel electrophoresis showed the presence of a dimer as evidenced by the band at 27 kDa and a second band at 54kDa when the conditions were relatively

oxidizing. At -140mV, -180mV, and -220mV reducing potentials, according to Figure 4, it appeared that there was an equivalent monomer and dimer concentration. As conditions became more reducing, hence -280mV, there was higher conversion of dimer to monomer forms of the protein. A dimer is caused by the formation of a disulfide bond linkage between monomers of a protein. Also, presence of dimerization is indicative that a free exposed thiol from other proteins, namely glutathione, could produce mixed disulfides with the RoGFP1-iL. The quantitative ratiometric behavior of RoGFP1-iL is founded upon the idea that the GFP will be found in two states: reduced or oxidized. Thus, we can use the fluorescence spectrum to relate the relative intensities of the reduced peak and oxidized peak to the isosbestic point. Formation of dimers and mixed disulfides introduces an intermediate linkage that inhibits the ability to quantitatively calculate the redox state of the cellular environment. For this reason, it was recognized that understanding the fluorescence properties of the dimerized protein may contribute a better understanding of how the excitation spectra is qualitatively affected.

It was demonstrated previously that -160mV was the ideal dimerization reduction potential, which is our rationale for incubating the RoGFP1-iL at -160mV prior to running the reaction through the Superdex 75 size exclusion column. The spectra for a monomer exhibited a broad peak at roughly 390nm with another much lower peak at 450nm, characterized in Figure 8. The dimer spectra exhibited a broad, steadily increasing peak between 370nm and 410nm with a defined peak at 450nm. In qualitative observation, it is apparent that the dimer has a significantly different fluorescent spectrum in comparison to the monomer. The variability between the dimer

and the monomer does not necessarily indicate that the eroGFP1-iL is inadequate as a sensor however.

The major findings of the research conducted with eroGFP1-iL demonstrate that it is fluorescing, localized to the ER, and is not inherently inducing cellular stress to the 3T3-L1 cells. RoGFP1-iL *in vitro* is behaving as expected, with an excitation peak that varies with oxidation at 390nm and another peak which varies with reduction at 450nm. A presence of a dimer was demonstrated in SDS-PAGE analysis of the protein when treated with relatively oxidizing conditions of -160mV. One possible explanation for the dimerization is the hydrophobic protein surface of the roGFP1-iL with residues such as Phe223 near the external cys147 and cys204, The ability to form a dimer leads to the possibility that roGFP1-iL cannot be used as a quantitative sensor in the ER as it is currently composed. Mutagenesis is currently underway to prevent dimerization of RoGFP1-iL by reducing the hydrophobic interaction of the residues near the disulfide bridge. A significant limitation faced when dealing with roGFP1-iL is that it requires particular excitiation wavelengths near 395nm and 457nm. Very few machines have both of these lasers, and typically if they do have such capability, they are not sensitive enough nor are they compatible with the 3T3-L1 cells and its minute eroGFP1-iL signal.

In future studies, if the formation of dimers can be overcome, we hope to apply eroGFP1-iL to a variety of conditions and quantitatively calculate the redox state in such situations. If it cannot be overcome, it may be necessary to pursue other markers as indicators of the ER redox state.

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