FRET Determination

APPLICATION NOTE

Dynamic FRET Imaging

Förster resonance energy transfer[1] (FRET) is a phenomenon in which nonradiative transfer of energy occurs between donor and acceptor molecules in close proximity (2-7 nm). Since FRET efficiency decays as a function of the inverse sixth power of the distance between the donor and acceptor, this phenomenon can be leveraged to provide solid evidence of the proximity between a donor and accepter in a FRET pair. In FRET, the donor molecule is returned to a ground state without fluorescence emission while the acceptor molecule is raised to an excited state. Upon decay of the acceptor's excited state, fluorescence emission may be witnessed. Thus, an increase in FRET between label molecules will result in a decrease in donor emission and a simultaneous increase in acceptor emission. Using methods of FRET detection, interactions between molecules can be monitored in subcellular compartments and tracked as a function of time.

FRET Microscopy

Revealing the Interaction between Cytochrome c and Inositol (1,4,5) Triphosphate Receptors in Apoptosis

In 2003, Darren Boehning *et al.* published a study investigating the role of cytochrome *c* binding to inositol (1,4,5) triphosphate receptors in the regulation of calcium-dependent programmed cell death[2]. The data obtained indicate that cytochrome *c* binding to inositol (1,4,5) triphosphate (InsP3R) receptors, above certain concentrations, abolishes calcium-mediated inhibition of InsP3R-mediated calcium release. It was determined that cytochrome *c* translocates from the mitochondria to endoplasmic-reticulum-associated InsP3R, and that binding of cytochrome *c* to InsP3R is a specific interaction.

Microscopic imaging studies using a low-noise CoolSNAP$_{HQ}$ CCD camera from Photometrics were conducted in order to clarify the temporal evolution of calcium release and the roles played by cytochrome *c* and InsP3R. These studies revealed that large, sustained increases in early apoptosis are concomitant with the association of cytochrome *c* and InsP3R. The intimate interaction between cytochrome *c* and InsP3R was revealed in live cells using FRET microscopy.

The first topic of the study conducted by Boehning *et al.* sought to identify proteins that interact with InsP3R. Through these efforts, it was determined that cytochrome *c* binds to a specific amino acid sequence on the InsP3R protein. The influence of cytochrome *c* binding to InsP3R on InsP3R-mediated calcium release was evaluated and it was discovered that above 1-nM concentration, cytochrome *c* abolishes the calcium-dependent inhibition of InsP3R-mediated calcium release activity.

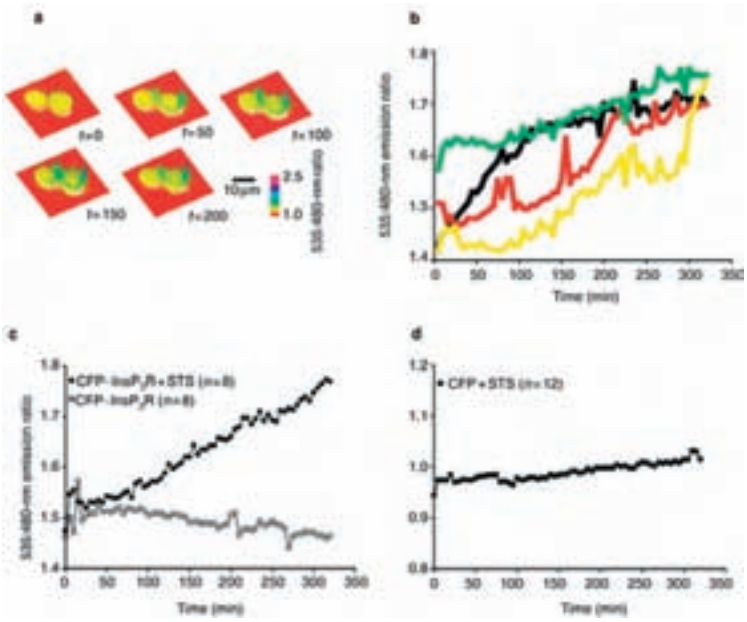

Figure 1. Cytochrome *c*-InsP3R interaction during apoptosis demonstrated by FRET. **(a)** Surface plot display of the 535:480-nm ratio in two PC12 cells stably expressing YFP-cytochrome *c* and co-expressing CFP-InsP3R after induction of apoptosis. **(b)** 535:480-nm ratio of four PC12 cells stably expressing YFP-cytochrome *c* and cotransfected with CFP-InsP3R. This plot demonstrates the heterogeneity of cytochrome *c*-InsP3R interactions observed in response to induction of apoptosis. **(c)** 535:480-nm ratio of YFP-CytC PC12 cells co-expressing CFP-InsP3R in response to induction of apoptosis or vehicle. Only cells in which apoptosis had been induced are positive for FRET activity. **(d)** 535:480-nm ratio of YFP-cytochrome *c* PC12 cells cotransfected with cytosolic CFP in response to induction of apoptosis. No significant change in the 535:480-nm ratio was observed. Data courtesy of Prof. Solomon Snyder, Department of Neuroscience, Johns Hopkins School of Medicine.

Next, the investigators explored the possibility that the cytochrome *c* / InsP3R interaction plays a role in apoptosis. InsP3R in cells is usually associated with the endoplasmic reticulum (ER), whereas cytochrome *c* is generally found in the mitochondria. The hypothesis was formed that cytochrome *c* translocates from the mitochondria to the InsP3R in the ER during apoptosis. To test this hypothesis, groups of cultured cells were induced to undergo apoptosis and then fractionated at different time points. The subcellular fractions were then run on Western blots and probed for InsP3R and cytochrome *c*. It was found that cytochrome *c* immunoreactivity decreased in the mitochondrial fraction and increased in the endoplasmic reticulum fraction several hours after induction of apoptosis. Cytochrome *c* and InsP3R were co-immunoprecipitated from the ER fraction, indicating an association between the two proteins.

These results suggest that cytochrome *c* is released from the mitochondria during the early stages of apoptosis to bind with the InsP3R located in the closely adjacent ER. The end result of this interaction is diminished feedback regulation of InsP3R-mediated calcium release and subsequent mitochondrial and cytosolic Ca^{++} overload. The role of InsP3R in translocation of cytochrome *c* to the ER was further investigated using cells lacking InsP3R. It was found that cytochrome *c* fails to translocate to the ER in such cells, but rather appears in the cytoplasm.

Cell lines cotransfected with EYFP-labeled cytochrome *c* and ECFP-labeled InsP3R were used to evaluate interactions between the two proteins in intact cells. Close association between the two proteins in the living cells results in a FRET interaction between the linked fluorescent protein probes. When apoptosis was induced in the transfected cells, the researchers witnessed a gradual increase in the FRET signal (see **Figure 1**). No FRET signal increase was observed in cells that were not induced to undergo apoptosis or in cells in which CFP not linked to InsP3R was expressed.

Further imaging studies were conducted to ascertain whether there was a link between cytochrome *c* binding to InsP3R. In these investigations, YFP-linked cytochrome *c* and calcium levels were imaged in cells induced to undergo apoptosis. Calcium spiking and cytochrome *c* release were observed to be temporally linked. A peptide competition of cytochrome *c* binding to InsP3R demonstrated that *in vivo* release of cytochrome *c* and altered Ca^{++} regulation is dependent on the cytochrome *c*-InsP3R interaction (see **Figure 2**). Expression of the cytochrome-*c* binding sequence of InsP3R was found to attenuate the calcium release due to induction of apoptosis in living cells. This blocking effect was not witnessed for cytochrome *c*-mediated caspase activation, and caspase inhibitors did not block translocation of cytochrome *c* to the InsP3R. Taken together, these results indicate that cytochrome *c* translocation to the ER and the changes in Ca^{++} regulation leading to cell-wide cytochrome *c* release occur upstream of cytochrome *c*-mediated caspase activation.

Additional Information

To learn more about Prof. Solomon Snyder's research, please visit: *neuroscience.jhu.edu/peopledetail.asp?ID=1*

Citations

1. Förster, V.T. (1948). Zwischenmolekulare Energiewanderung und Fluoreszenz. *Ann. Phys.* **6,** 54-75.

2. Boehning, D., Patterson, R.L., Sedaghat, L., Glebova, N.O., Kurosaki, T., and Snyder, S.H. (2003). Cytochrome *c* binds to inositol (1,4,5) triphosphate receptors, amplifying calcium-dependent apoptosis. *Nat. Cell Biol.* **5,** 12:1051-1061.

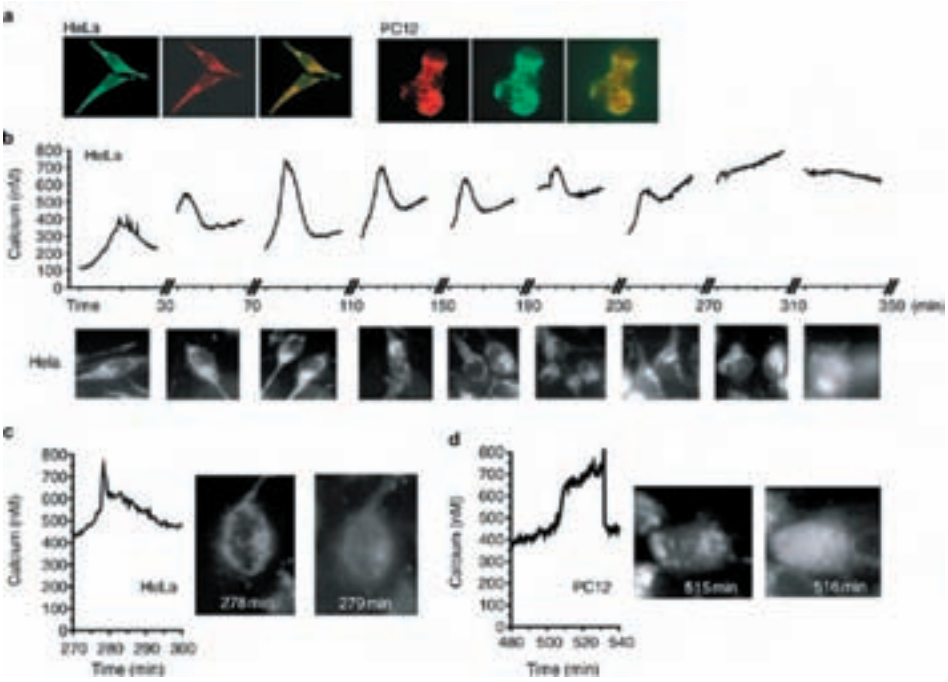

Figure 2. (a) YFP-cytochrome *c* colocalizes precisely with MitoTracker® Red (Molecular Probes), demonstrating that the stably expressed YFP-cytochrome *c* is contained within the mitochondria. **(b)** Fura-2 intracellular calcium measurements in response to induction of apoptosis in HeLa cells stably expressing YFP-cytochrome *c*. Marked oscillations are observed as early as 10 min and continue until approximately 270 min. Cells displayed beneath each 30-min time course manifest the representative distribution of YFP-cytochrome *c* during that time course. **(c)** Fura-2 intracellular calcium measurements of a single HeLa cell in response to induction of apoptosis over the 270-min time course. A large spike of calcium is observed at a time corresponding to the coordinate release of YFP-cytochrome *c* into the cytosol, as depicted in the two adjoining images. **(d)** Fura-2 intracellular calcium measurements of a single PC12 cell in response to induction of apoptosis over the 480-min time course. A large spike of calcium is observed at a time corresponding to the coordinate release of YFP-cytochrome *c* into the cytosol, as depicted in the two adjoining images. Data courtesy of Prof. Solomon Snyder, Department of Neuroscience, Johns Hopkins School of Medicine.