Dottorato di Ricerca in Medicina Molecolare e dello Sviluppo - XXXII ciclo

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Al collegio docenti del Dipartimento di Medicina Molecolare e dello Sviluppo

Introduction

One of the central mechanism underlying functional and structural organization of the cells is represented by a wide network of protein-protein interactions (PPI) (e.g. the actin-myosin network during cell contraction). Little is known about the molecular interactions among the many proteins in different cell types, therefore there is the necessity to know and deeply understand them. In particular, we are focusing on the muscle cell organization.

A novel method to investigate PPIs in living cells is the enzyme-mediated labelling technique. This technique takes advantage of the ability of an enzyme that covalently label interacting proteins.

In this first year of my PhD I focused on Proximity-dependent biotin identification (BioID) approach. From papers published by Roux et al. we know that BioID is based on the ability of a mutant biotin ligase (BirA*) (35kDa), derived from Escherichia coli, that covalently attaches biotin to each protein that interact with the bait protein in a range of ~10 nm. Biotinylation is a two steps reaction: in presence of ATP, BirA* catalyzes the conversion of biotin in biotinoyl-5'-AMP that, in a second moment, binds specific lysine residues on the target proteins releasing AMP. BirA* takes the R118G mutation on the active site that causes the early release of biotinoyl-5'-AMP. In this way biotinoyl-5'-AMP can interact not only with the Lys residues of target protein but also with other available Lys residues of proteins in close proximity, that will result biotinylated. Since biotinylation is a covalent protein modification, the reaction can occur in stringent condition. BirA* is fused to a protein of interest (bait) and transfected into the cells, following supplementation of the culture medium with biotin, the reaction take place and biotinylated proteins (preys) can be selectively isolated, by avidin or streptavidin bond, and identified by immunoblot.

BioID2 arises from the necessity to reduce BioID size, a protein of 322 a.a. and to improve efficiency of labelling proximity proteins. BioID2 derives from Aquifex aeolicus, a bacterium

humanized and mutated in the catalytic domain (R40G) in order to allows the promiscuous biotinylation. BioID and BioID2 have the same enzymatic activity, but BioID2 shows an higher efficiency with lower concentration of biotin, 3.2μM instead of 50μM, making the method more easily applicable. It was shown that the optimal temperature for BioID2 is 50°C, compared to 37°C of BioID. Finally, another benefit of BioID2 is that the biotinylation range can be anhanced using a flexible linkers.

I used thiis approach with the purpose of studying JPH1 and its protein interactors in muscle cells. Junctophilins (JPHs) is a family of proteins that stabilize the interaction between plasmalemma/sarcolemma and ER/SR, working like a molecular bridge. JPH1 is the major JPH family member and it plays a key role in the assembly of skeletal muscle triadic junction, this is supported by mutant mice experiments: lacking of JPH1 leads to reduced number and altered shape of triads. In mammals, there are four types of JPH: JPH1 and JPH2, both expressed in skeletal muscle and only JPH2 in cardiac tissue, JPH3 and JPH4 localized in neuronal tissue, where mediate the connection between neurotransmettitor receptors and Ca^{2+} channels. All isoforms share a common structure: the N-terminal region contains eight hydrophobic repeats of 14 amino acids, highly conserved across the isoforms and across the species, named membrane occupation and recognition nexus (MORN) motifs which bind the plasma membrane. The C-terminal region mediates the protein joint into the SR membraneJPH1 is also involved in calcium release complex formation and in the regulation of Ca^{2+} handling at the triads. In addition to a structural role, multiple interactors of JPH1 have been reported, including RyR1, DHPR, triadin, CASQ1, Cav3, STIM1 and Orai1.

Aim

The purpose of my work is to identify any new JPH1 interactors, adapting the novel Proximity-dependent labeling with BioID2 approach, previously used in other biological contexts, to detect protein-protein interactions in C2C12 cell line.

Methods

We generated the 3xmycBioID2JPH1 plasmic vector. Experiments were performed on C2C12 cell line. All cells were cultured at 37 °C in humidified 5% CO2 atmosphere and were grown for a day before transfection.

C2C12 transfection occurs with Lipofectamine PlusTM Reagent (Life Technologies), immunofluorescence (IF) were performed after 8 days of differentiation, it is important step to avoid getting false-positive and to be sure that the fusion protein has the correct localization into the triad. Theese following primary antibodies were used: α-JPH1 1:250 (Invitrogen), α-Myc 1:500

(Clontech) and α-RyR 1:500 (34C) and for immunofluorescence detection Cy3 (indocarbocyanine) or Cy2 (carbocyanine)-conjugated anti-mouse or anti-rabbit secondary antibodies (Jackson Laboratories) were used. After 20 hours of adding 3.2 μM of biotin, Western Blot analysis was carried out. Proteins were separated by a polyacrylamide gel, in according to the protocol of Roux (Roux et al., 2012). Biotinylated proteins were detected by Pierce Horseradish Peroxidasi (HPR)-coniugated streptavidin (1:4000) treatment and after that filters were incubated with α-Myc 1:2000 (Clontech) primary antibody.

Preliminary results and future perspectives

By IF analysis, on C2C12 cell line, we demonstrate that 3xmycBioID2JPH1 fusion protein was localized into the triad, where normally JPH1 localize. We are performing Western Blot analysis to detect, by streptavidin bound, biotinylated proteins in our samples. The following step will be the characterization of these proteins by specific primary antibodies.
In addition there is the necessity to improve the transfection protocol to increase the percentage of transfected C2C12 cells.

Partecipazione a Seminari e Corsi di Formazione

- "iiM – Myology Meeting" 13-16 Ottobre 2016
- "XIX Scientific Convention" Fondazione Telethon Riva del Garda 13-15 Marzo 2017

Il Tuto

La Dottoranda: