End-Semester Report (BI4313)

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Title:
Role of the V-domain in regulating Drp1's binding to its adaptor

Aim:
To characterise the role of the V-domain in regulating Drp1's binding to Mitochondrial Fission Factor (Mff)

Background:
Mitochondria is the organelle with the machinery required to produce chemical energy in cells. According to the Endosymbiotic Theory, mitochondria and chloroplast were once aerobic bacteria ingested by another large anaerobic bacteria to eventually give rise to eukaryotic cells as we know them today[1]. Interestingly, mitochondria cannot be formed *de novo*, unlike other organelles typically found in an eukaryotic cell, and have to be generated from pre-existing mitochondria through growth, genome replication and division in order to be inherited by daughter cells formed during mitosis[2]. The process of mitochondrial growth is thereby intrinsically linked to mitochondrial fission.

Detailed studies have shown that specialised protein complexes are responsible for and coordinate the fission-fusion dynamics of mitochondria and consequently enable the organised assortment of this organelle during cell division and merging for repair purposes or as a response to environmental stimuli. Numerous proteins coherently synchronise in their functionalities to produce this phenomenon of mitochondrial fission, and the term 'mitochondrial divisome' has been adopted to describe the entire machinery of proteins[3].

The core components of the mitochondrial divisome are dynamin-family proteins and their corresponding adaptors, which are conserved integral membrane proteins that regulate the divisome assembly at sites of mitochondrial fission[3]. Dynamin-related proteins are a family of large GTPases that manage fusion and fission processes by utilising energy from GTP hydrolysis to modify and remodel mitochondrial membranes[3]. Their functional role depends on whether they are structurally associated with the membrane; Dynamin-Superfamily proteins (DSPs) with a transmembrane domain are involved in membrane fusion, and soluble DSPs are involved in membrane fission. Some examples of these proteins include fusion-regulators like Mitofusin-1 (Mfn1), Mitofusin-2 (Mfn2) and Optic atrophy-1 (Opa1), and fission-regulators like Dynamin-related protein-1 (Drp1)[4].

Drp1 consists of four domains, namely the head (G-domain), neck (Bundle signalling element), trunk (Stalk domain) and foot (Variable domain) of the molecule formed by the polypeptide chain folding back on itself. The G- and stalk domains are essential for GTP hydrolysis and for self-assembly of the complex, respectively. The function of the variable domain of Drp1 is still elusive. The adaptors present on the outer mitochondrial membrane interact with Drp1 at the stalk domain regions.

Drp1 is majorly a cytosolic protein, but a minor fraction is found concentrated near mitochondrial foci, representing future fission sites. Drp1 functions by forming helical oligomers that can induce membrane constriction and sever the mitochondrial membrane. In contrast to archetypal dynamins, which have a pleckstrin homology (PH) domain, the region of the foot in Drp1 is a variable and unstructured 100 residue-long loop at the end of the stalk that is crucial for proper mitochondrial localisation, assembly and activity, and is known as the variable domain (VD)[5]. Previous unpublished data from the lab has shown that the VD indirectly regulates the process of Drp1 catalysed fission by influencing the oligomerization of Drp1. VD-deficient mutants, like Drp1ΔVD, predominantly form lower-order oligomers, potentially inhibiting mitochondrial fission[6]. Conversely, full-length Drp1 with the VD predominantly exists as a tetramer, which is specifically recruited by adaptors for functional fission complex assembly[4,7].

The human Drp1 protein is encoded by the *DNM1L* gene, which is present in chromosome 12 and contains 21 exons. *DNM1L* transcripts undergo alternative splicing in three sites typically (one in exon 3 within the GTPase domain encoding the A-insert, and two in exons 16 and 17 encoding the B-insert within the Variable domain) and give rise to nine different isoforms[8]. Drp1's recruitment to mitochondria depends on the outer mitochondrial membrane's adaptor proteins which include Mff, Mid49, and Mid51. Recent studies show that the mitochondrial fission factor (Mff) is the significant Drp1 receptor[9]. The interaction between Mff and Drp1 is essential for the physiological processes of mitochondrial division, motility, and function both in vitro and in vivo. Studies of functional implications of the B-inserts have shown that the cardiolipin-stimulated GTPase activity of constructs devoid of B-insert has no effect on Mff addition, similar to those with B-insert[6].

Unpublished data from our lab suggests that the variable domain (V-domain) of Drp1 shows differential fission activity on the adaptor-coated membrane nanotubes. To understand this phenomenon, we wanted to test whether the V-domain has any regulatory role on Drp1's binding to these adaptors.

Methods:

Protein expression and purification –
The proteins of interest which are namely MffΔTMD (Trans-membrane domain deleted Mff) which had a 6xHis-tag on the N-terminal and Strep-II tag on the C-terminal, and the Drp1 isoforms (Isoform-1, Isoform-2, Isoform-3 and Isoform-4), which only had a Strep-II tag on the C-terminal, were expressed in NiCo21(DE3) cells in 1L autoinduction cultures (0.5g/L D-(+)-glucose, 2g/L α-lactose) for 36-40 hrs at 18° C. Cell pellets were stored at -40º C.

For proteins with a 6xHis-tag on the N-terminal and Strep-II tag on the C-terminal (Mff-mCherry), the pellets were thawed in 20 mM HEPES (pH 7.6), 500 mM NaCl buffer supplemented with PMSF and sonicated in an ice water bath. The lysates were spun down at 30,000g and the supernatant was incubated with TALON Metal Affinity Resin (Clontech Laboratories Inc.). The resin was then washed with 100 mL of 20 mM HEPES, pH 7.4, 500 mM NaCl buffer, followed by elution in 20 mM HEPES (pH 7.6), 500 mM NaCl, 100 mM EDTA buffer. The elution was loaded onto a Strep-Tactin column (GE Healthcare, Illinois) and washed with 20 mM HEPES (pH 7.6), and 150 mM NaCl buffer. Bound protein was eluted in 20 mM HEPES pH 7.6, 150 mM NaCl buffer containing 2.5 mM biotin (Sigma-Aldrich, St. Louis). For the other proteins, the pellets were resuspended in a buffer of 20 mM HEPES, pH 7.4, with 500 mM NaCl and supplemented with PMSF. Cell lysis was achieved by sonication in an ice water bath, after which the lysate was centrifuged at 30000g for 30 minutes. The resulting supernatant was loaded onto a Strep-Tactin column (GE Healthcare, Illinois) and washed with 20 mM HEPES, pH 7.6, 500 mM NaCl buffer. Bound protein was eluted in 20 mM HEPES pH 7.6, 150 mM NaCl buffer containing 2.5 mM biotin (Sigma-Aldrich, St. Louis). Purified proteins were kept on ice and spun down at 100,000g to remove aggregates before use.

Dot-blot assay for identification of protein-protein interactions –
Dot-blot assay was performed to investigate protein-protein interactions by immobilising target proteins (bait) which were the Drp1 isoforms under consideration on an activated PVDF membrane, followed by incubation with a potential binding partner (prey) which was the Mitochondrial fission factor protein with an mCherry tag on it (Mff-mCherry). After spotting the bait protein on the membrane and allowing it to dry, the PVDF membrane was blocked with blocking buffer (e.g., 5% wt./V solution of BSA in TBS) to minimise non-specific binding. Subsequently, the membrane is incubated with a solution containing the prey protein (Mff-mCherry). Following multiple washes with TBST (0.1% Tween 20 added with 1x TBS) to remove unbound proteins, the membrane was imaged on an iBright 1500 (Thermo Fisher Scientific).

Results and discussion:

To evaluate the role of V-domain on Drp1's affinity towards binding to these adaptors, we recombinantly purified four naturally found Drp1 isoforms having variable length of V-domain (isoforms 1-4) along with fluorescently tagged Mff, ran it on SDS-PAGE gel to check its purity, and performed a dot-blot assay in order to check whether the V-domain has any role in Drp1's affinity to these adaptors.

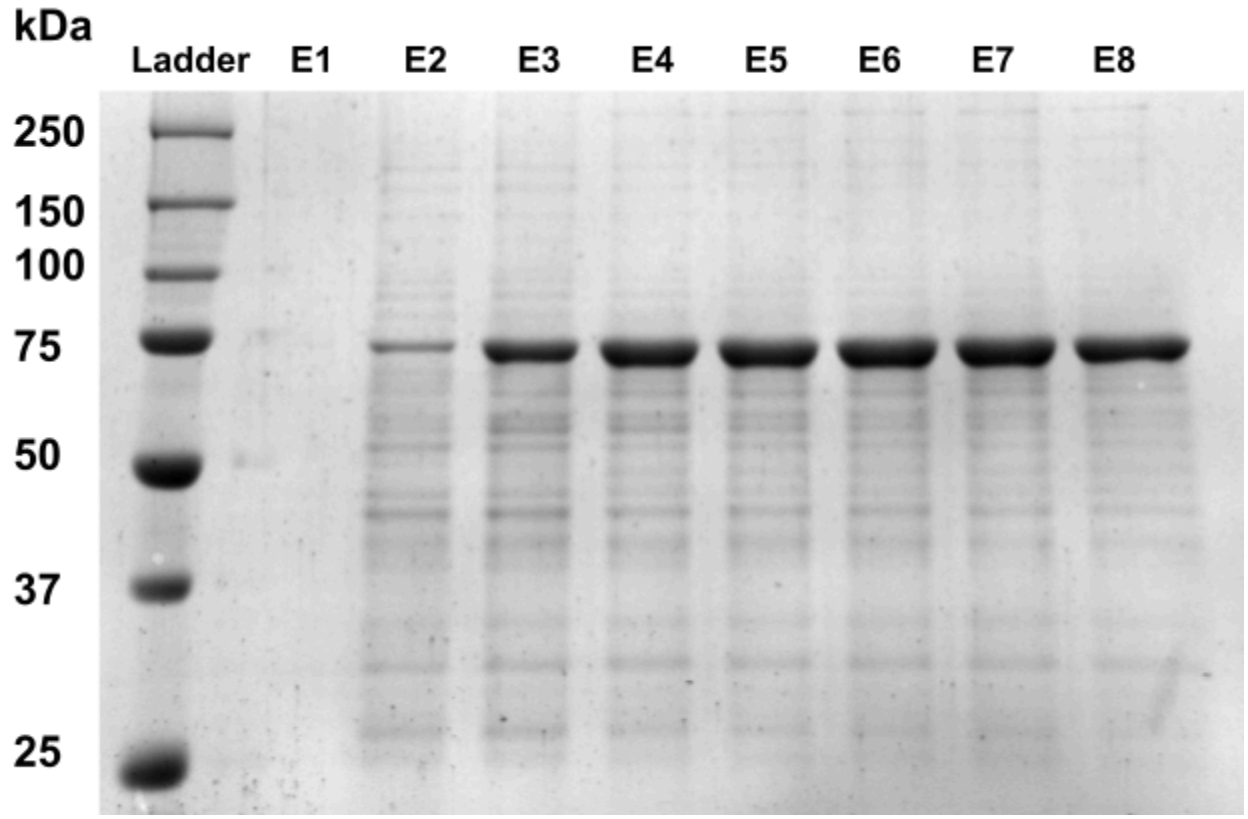

Figure 1. Representative SDS-PAGE gel depicts Drp1 Isoform 3 size to be very close to 75 kDa. Similar gels were made for Mff-mCherry and all other isoforms.

Next, we performed a dot-blot assay to check the interaction between Mff and Drp1 isoforms with variable lengths of the V-domain. To ensure that the quantity of the bait proteins that were loaded on the PVDF membrane were sufficient and equal amongst the different proteins of interest, we utilised Ponceau S dye to visualise the protein on the membrane and confirmed that sufficient protein had been spotted on the PVDF membrane through the visualisation of distinct red bands.

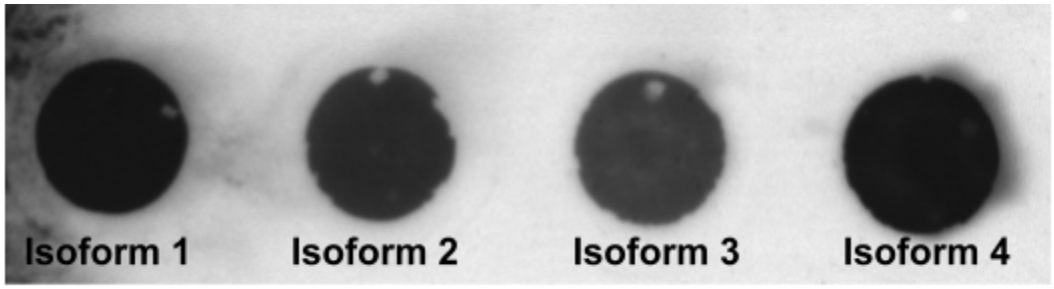

Figure 2. Imaged PVDF membrane after Ponceau S staining to the membrane loaded with the bait proteins (Isoforms 1-4).

On performing the dot-blot assay, as mentioned earlier, we could only see a very faint signal of fluorescence attributable to the binding between the Mff-mCherry (prey) protein and isoforms 1-4 (bait) in the imaged membrane. This indicates that Mff-mCherry by itself is not binding to the Drp1 isoforms. We hypothesise that this is because Drp1 isoforms are incapable of binding to Mff in the absence of cardiolipin, an essential phospholipid that is present on the mitochondrial membrane.

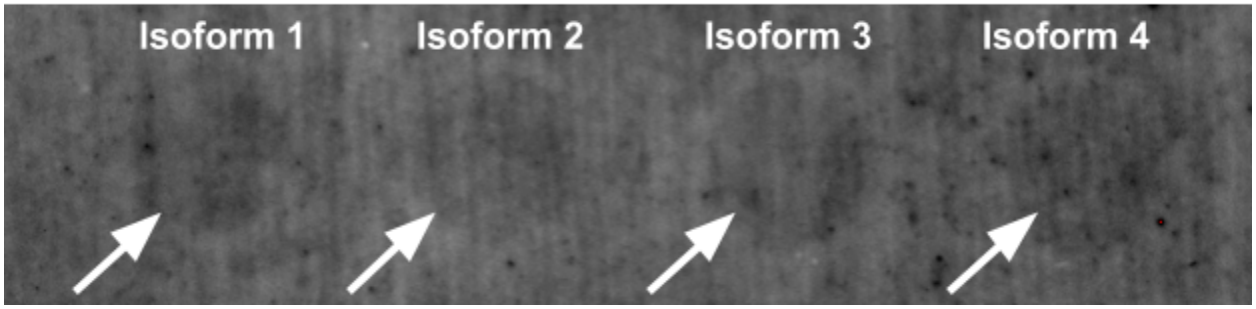

Figure 3. Membrane obtained from performing dot-blot displaying very faint fluorescent signals on the membrane attributable to protein-protein interactions between Drp1 isoforms and Mff-mCherry.

Future directions:
To verify the dependency of Drp1 recruitment to the mitochondrial membrane by Mff, we plan to introduce Drp1 to liposomes having cardiolipin and perform an assay to check the recruitment of Drp1 to Mff.

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