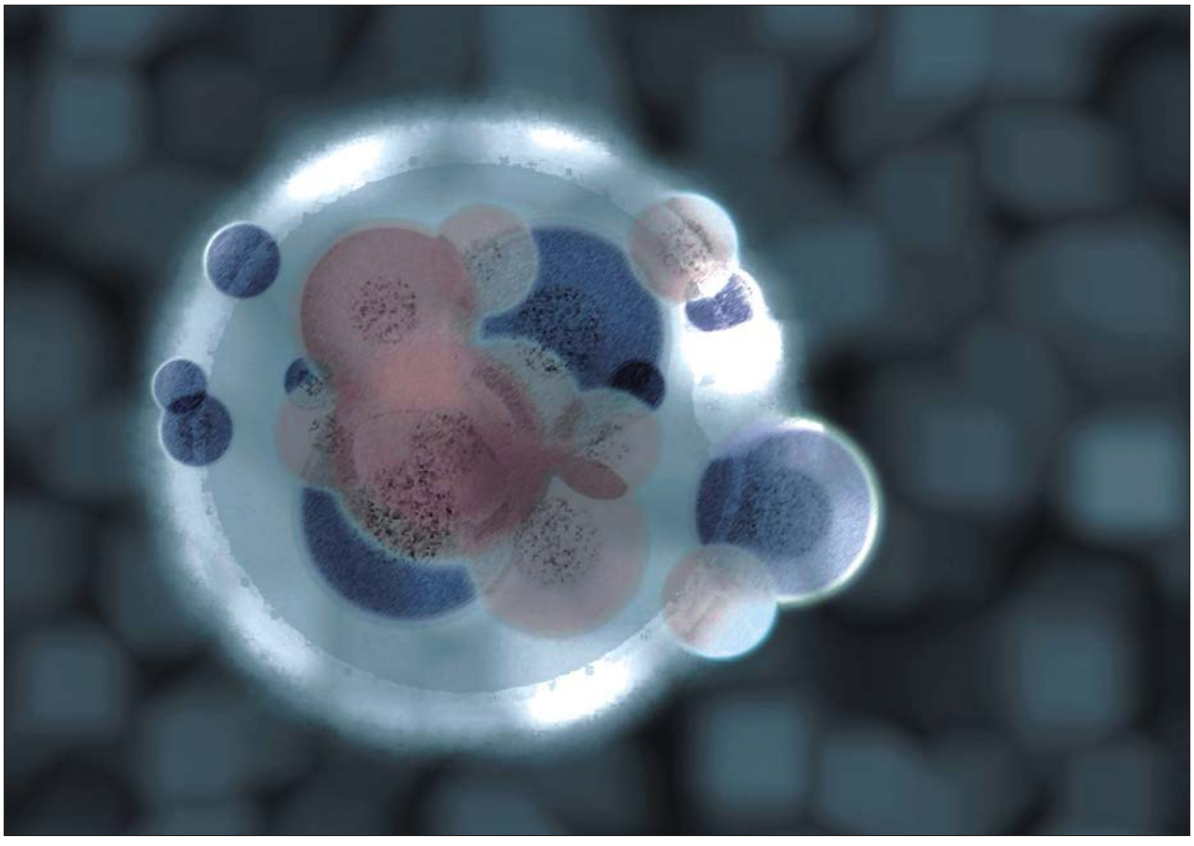

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Precursor Protein but Not Intermediate and Mature Protein of Frataxin Directly Interacts with a 54 kDa Protein of Mitochondrial Processing Peptidase

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Abstract: The aim of the study was to unravel the function of frataxin. The function of frataxin is not known yet and is a mystery. In order to find the function of an unknown protein it is better to find out interacting partner protein. Interacting partner proteins of frataxin were captured by immunoprecipitation and subsequently visualized in the form of band on western blotting. While unraveling the frataxin function by capturing interacting partner proteins through immunoprecipitation, a novel protein of 54 kDa was identified beside previously known 30 kDa and 18 kDa precursor and mature proteins of frataxin respectively. Immunohistochemistry of normal human brains sections revealed the perfect specificity of frataxin protein that we used for the immunoprecipitation. Given that 30 kDa and 18 kDa frataxin proteins previously reported, we report here the presence of a 54 kDa protein which could be an interacting partner protein of frataxin. Further investigations are required to identify type of protein and its relevant role in the appropriate functioning of frataxin.

Key words: Neurodegenerative disease, frataxin, Friedreich's ataxia, MPP-β

INTRODUCTION

FRDA is an autosomal recessive inherited disease with onset within 20 years, absent of lower limb reflexes, dysarthria, Babinski responses, limb weakness, decrease vibration sense, skeletal deformities, cardiomyopathy and diabetes[1]. FRDA is caused by mutation in FRDA gene, which encodes for a mitochondrial protein, frataxin[2-6].

Like many other mitochondrial proteins, frataxin is initially synthesized in the cytoplasm as a larger precursor with N-terminal presequences that targets frataxin to the mitochondria[3,7,8]. Targeted frataxin protein must undergo maturation in order to be imported into the matrix of mitochondria. In yeast, maturation of Yeast frataxin homolog 1 protein (Yfh1p) takes place with Hsp 70 homolog (Ssc2p)[7]. In this way frataxin protein is cleaved off removing leader peptides and hence facilitates the import of nuclear encoded mitochondrial protein into mitochondria[9]. Second maturation of Yfh1p is carried out through Yeast Mitochondrial Intermediate Peptidases (YMIP) yielding a mature frataxin protein[10].

Furthermore western blot of transfected cos-1 cells provided information of a full length 30 kDa precursor protein[11]. Precursor protein is processed by MPP to two smaller products with apparent molecular mass on SDS/PAGE, intermediate frataxin 21 kDa (I-fxn) and mature frataxin 18 kDa (m-fxn)[12]. At steady state, mature form of frataxin is predominantly detected in human tissues whereas intermediate protein is not[5].

Function of frataxin is still not identified. In order to unravel its function, mouse frataxin protein partners were screened through yeast two-hybrid assay. The only protein that made direct interaction with precursor mouse frataxin was Mitochondrial processing peptidase-beta (β MPP)[6]. In this way it was confirmed that both human and yeast frataxin are cleaved off in two sequential steps by MPP[7,8,13].

There is no data showing experimental determination of the molecular weight of a mature chain of human β MPP. Calculated molecular weight is available by Swiss-Prot, which is 54.366 kDa. This molecular weight of the mature chain of human β MPP is calculated as the sum of all amino acids plus the mass of H_2O in the Swiss-Prot entry [http://www.expasy.org/cgi-bin/niceprot.pl?O75439#tools].

Our western blot of immunoprecipitated fibroblast cells samples also gave us three bands on SDS-PAGE with apparent molecular weight of 30 and 18 kDa

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precursor and mature proteins of frataxin along with a 54 kDa of interacting partner protein of β MPP. Considering previous results of Koutinikova in which the only interacting partner protein found was β MPP and the same molecular weight of β MPP which is calculated by swiss prot, we suspect our protein as β MPP. Further investigation must be required to confirm presence of β MPP and its relevant role in proper functioning of frataxin protein.

MATERIALS AND METHODS

Culturing cells: Healthy control (GM00010) and FRDA (GM03816) fibroblasts were purchased from the National Institute of General Medicine and Science (NIGMS). Frozen cells were thawed by gentle agitation in 37°C water bath and revived in MEM Eagle-Earle BSS 1X concentration of essential and non-essential aminoacids with 2 mM L-glutamine and 15% fetal bovine serum (FBS) heat uninactivated. Cells were subcultured in MEME containing 446 mg L^{-1} glutamax, 10% FBS, 2.5 mM sodium pyruvate, followed by trypsinizing with 0.25% (w/v) trypsin-0.03% (w/v) EDTA solution.

Isolation of mitochondria: Cells were isolated by trypsinizaiton and washed once with ice-cold Phosphate Buffer Saline (PBS) at 800 rpm for 10 min. Total number of $5x10^6$ cells was suspended in 300 μL Mitobuffer [20 mM HEPES (pH 7.5), 1 mM EGTA, 1 mM EDTA, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 0.1 mM PMSF, 2 μg mL^{-1} leupeptin, 2 μg mL^{-1} pepstatin and 2 μg mL^{+} aprotinin]. Cells were lysed by homogenizing on ice for 10-15 min and then centrifuged at 800xg for 20 min at 4°C to remove unbroken cells and nuclei. The supernatant was centrifuged at 10,000xg for 20 min at 4°C and pellet was saved. The supernatant was further centrifuged at 16,000xg for 20 min to remove the residual mitochondria. The pellet containing mitochondria was pooled and lysed by incubation for 15 minutes in TNC buffer [10 mM Tris-acetate (pH 8.0), 0.5% NP-40, 5 mM $CaCl_2$, 1 mM DTT, 0.1 mM PMSF, 2 μg mL^{-1} leupeptin, 2 μg mL^{-1} pepstatin and 2 μg mL^{-1} aprotinin].

Immunoprecipitation: One mililiter of both mitochondrial and cytosolic cell fractions were pre-cleared at 12,000 g for 20 sec with 50% slurry of protein G sepharose 4 Fast Flow suspension with gentle mixing for 1 h at 4°C. Supernatant (500 μL) were coupled with 5 μg of antifrataxin antibodies (CHEMICON) with over night gentle mixing at 4°C. Immune complex was precipitated by adding 50 μL of 50% slurry of protein G sepharose 4 Fast Flow suspension and gently mix for 1 h at 4°C. The complex was then centrifuged at 12,000 g for 20 sec and pellet was saved. Saved pellet was washed three times with wash buffer and centrifuged at 12,000 g for 20 sec. Protein was dissociated from beads by suspending the final pellet in 30 μL of sample buffer (50 mM Tris, 10% SDS, 2 mL of glycerol, 1 mL of 2-mercaptoethanol and few crystals of bromophenol blue in total volume of 10 mL). The sample was loaded on 12 or 15% SDS-PAGE and visualized by silver staining.

Western blotting: Whole cell lysate, mitochondrial and cytosolic fractions of FRDA and healthy control cell lines were obtained by the method mentioned above in material and method for the isolation of mitochondria. Protein concentration was determines by BCA protein assay kit (Pierce). Total protein extract (15-30 μg) were loaded in each well and analyzed on 12 or 15% SDS-PAGE followed by staining with commassie blue.

Proteins were transferred to PVDF membrane blocked with 0.2% non-fat milk (blocking reagent) and then incubated with primary monoclonal anti frataxin antibodies (1:150,000) and secondary goat anti rabbit IgG coupled to peroxidase antibodies (1:50,000) overnight at 4°C. Monoclonal antibodies (MoAb) against frataxin (clone IgG2) was purchased from Chemicon International (Temecula, CA)[14]. Bands were observed by the reaction with the ECL kit (Amershan) according to the manufacturer instructions.

SDS-PAGE: For the analysis and western blotting, we used 12% or 15% separating gel overlaid with T= 4% stacking gel (where, T denotes the total concentration of acrylamide and bis-acryl amide), from a stock solution of 40:1.7 acrylamide: bisacrylamide; electrophoresis was started at 180 V, shifted to 240 V after the samples had completely entered the separating gel.

Immunohistochemistry: Brain stem and cerebral hemisphere section of an 87-year-old woman were fixed in block and embedded in histopack. Sections were cut to 5 μm thickness. Histology and immunohistochemistry were performed using standard procedures. Immunostaining was carried out using the following antibodies. Anti frataxin (chemicon) and goat anti rabbit IgG HRP conjugated (sigma chemical Co., St. Louis, Mo). Tissue sections were deparafinized, rehydrated and blocked in PBS containing 1% BSA and 1% goat serum at room temperature for 30 min. Incubation with primary antibody or secondary biotinylated conjugated goat anti rabbit (sigma chemical Co., St. Louis, Mo) was performed at room temperature for 30 min followed by 5 min washes in PBS. Negative control was performed by incubating the

tissue sections with secondary antibody without pretreatment with primary anti body. Finally, all sections were incubated in Di aminobenzidine (DAB) solution till color develops, rinsed with tap water and counter stained with heamatoxylin.

RESULTS AND DISCUSSION

Appearance of 50 and 25 kDa proteins commassie stained gels: To determine the interacting partner proteins of frataxin, cytosolic and mitochondrial fractions of FRDA fibroblast cells were immunoprecipitated. In both fractions two distinct bands with apparent molecular weight of 50 and 25 kDa were observed (Fig. 1) when the gels were stained with either commassie or sliver staining. In this gel we did not observed any band with such a 30 or 18 kDa molecular weight that denotes frataxin. So, this data created doubt if the appeared bands are of light 25 kDa[15] and heavy 50 kDa[15,16] chains of frataxin antibodies that were used to capture interacting proteins at the time of immunoprecipitation.

Appearance of 18, 30 and 54 kDa proteins on western blots: True interacting partner proteins were identified on western blots using strepavidin alkali phosphatase

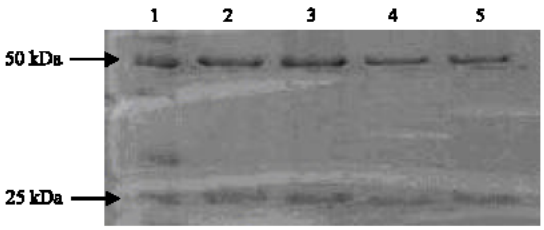

Fig. 1: Commassie staining of gel shows light and heavy chains of frataxin antibodies after immunoprecipitation. Lane 1: Low molecular weight Ladder [10-220 kDa], lane 2: Mitochondrial fraction of control, Lane 3: Mitochondrial fraction of FRDA fibroblast cells, Lane 4: Cytosolic fraction of control, Lane 5: Cytosolic fraction of FRDA fibroblast cells. Fibroblast cells of healthy control and FRDA cells were cultured in MEM Eagle-Earle medium followed by cell lysis with lysis buffer. Mitochondrial (M) and cytosolic © fractions were immunoprecipitated. After several washing with low stringent buffers, fractions were run on 12% SDS-PAGE and stained with commassie blue. Two bands with apparent molecular weight of 50 kDa for heavy chain and 25 kDa for light chain of frataxin antibodies are observable

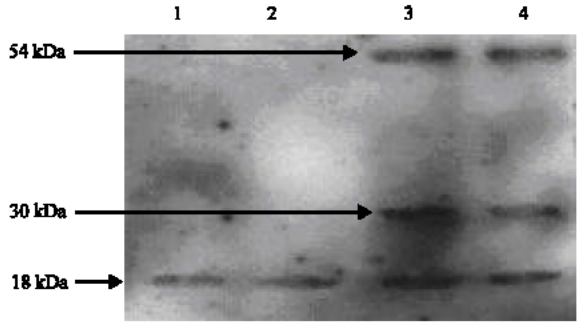

Fig. 2: True interacting partner protein (β MPP, apparent molecular weight of 54 kDa) of frataxin is observable along with precursor (30 kDa) and mature frataxin (18 kDa) in the cytosolic fraction only when immunoprecipitated sample was western blotted. Lane 1: Mitochondrial fraction of control, Lane 2: Mitochondrial fraction of control, Lane 3: Cytosolic fraction of control, Lane 4: Cytosolic fraction of control. Fibroblast cells of healthy control were cultured in MEM Eagle-Earle medium followed by cell lysis with lysis buffer. Mitochondrial (M) and cytosolic © fractions were immunoprecipitated. After several washing with low stringent buffers, fractions were run on 12% SDS-PAGE and western blotted using antifrataxin antibodies. Three bands with apparent molecular weight of 54 kDa (β MPP), 30 kDa (precursor frataxin) and 18 kDa (mature frataxin) are observable in the cytosolic fraction where as only one band of mature frataxin (18 kDa) is observable in mitochondrial fraction. As no further processing of precursor protein is required once mature protein is processed and entered in the matrix of mitochondria, therefore no other bands are visible in this fraction

conjugate (Fig. 2). A blot probed with specific antifraraxin antibodies demonstrated three distinct bands with apparent molecular weight of 18, 30 and 54 kDa. Previous studies reported presence of 30 kDa precursor protein which is processed by MPPs into two smaller products of 21 kDa intermediate and 18 kDa of a mature frataxin protein.

Koutnikova observed true interaction of frataxin with only β MPP in yeast two hybrid system. Likewise, calculated molecular weight of β MPP is 54 kDa in human (swiss prot). These observations suggest that the presence of third band in our experiment is of β MPP. The presence of MPP-β along with frataxin protein suggests a physical interaction of β MPP with the frataxin protein in human.

Three bands were observed in cytosolic fraction whereas only a single band was observed in mitochondrial fraction. β MPP cleaves the precursor protein into two products so that the mature protein is facilitated to enter into mitochondria[7,8]. In this experiment we also found the presence of MPP along with precursor and mature frataxin protein [54, 30 and 18 kDa] in the cytosolic fraction only. As no further cleavage is required so no 54 kDa MPP-β band is seen in the mitochondrial fraction.

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