PROGETTO DI RICERCA

Detailed biochemical analysis of different pathogenic cytochrome b mutations in the cybrid cell model

Background
Ubiquinol:cytochrome c oxidoreductase (cytochrome bc₁, or respiratory complex III, EC 1.10.2.2) is the central complex of the respiratory chain. In mammals, it comprises eleven subunits among which cytochrome b is the only one encoded by the mitochondrial genome [Vazquez-Acevedo et al. 1993]. Together with the iron-sulfur (Fe/S) protein and cytochrome c₁, subunits, cytochrome b forms the catalytic core of complex III (CIII). In its native form, CIII is dimeric, being closely associated in varying proportions with complexes I and IV to form the respirasome [Schagger et al. 2000]. Respirasome formation impacts reaction kinetics in the respiratory chain and has major physiological consequences [Schagger et al. 2004]. Mutations in cytochrome b, including deletions, frame-shifts, termination and missense mutations, are among the least common abnormalities identified to date in humans. Nevertheless, they exhibit a large spectrum of mitochondrial disease symptoms ranging from pure muscle symptoms, mostly exercise intolerance, to multisystem disorders, including some types of cancer [Benit et al., 2009].

Interestingly, it has been reported that some mutations in cytochrome b are responsible for complex I deficiency and mutations in complex I genes induce CIII deficiency, underlining a close relationship between the two respiratory complexes [Acin-Perez et al. 2004]. Cytochrome b mutations can also increase reactive oxygen species (ROS) production, being indeed complexes III and I the major ROS producers in mitochondria and in turn in cells [Murphy, 2009].

Aim
The overall aim of this project is to investigate the biochemical features of three novel pathogenic cytochrome b mutations. During the first year, the missense homoplasmic mutation m.15579A>G, which substitutes the Tyr 278 with Cys (p.278Y>C), has been deeply characterized in our lab, in collaboration with the group of Prof. Fevzi Daldal, University of Philadelphia, USA, who had previously analysed the effect of the very same mutation in a bacterial model [Lee et al. 2011]. The results of this collaborative study have been recently published [Gelli et al. 2013; Lanciano et al, 2013]. A preliminary analysis has also been carried out in the missense homoplasmic mutation (Q313R) located near the carboxy terminal end of the protein.

Next year we will focus on another novel severe cytochrome b mutation, i.e. the microdeletion (del15643-15660) of six aminoacids (I300 to P305) in the sixth transmembrane helix of the protein, identified in a 41-year-old woman suffering exercise intolerance and fatigability, sensorineural deafness, visual disturbances, postural and gait instability and dysphagia.

To this purpose transmитochondrial cytoplasmic hybrids (or cybrids) have been already developed, by fusing enucleated fibroblasts derived from the patient with osteosarcoma 143B.TK- cells deprived of their own mtDNA (Rho-zero cells) [King and Attardi 1996]. In particular both homoplasmic (100% mtDNA mutated) and heteroplasmic (containing both wild-type and mutated mtDNA) cybrid clones have been generated, in order to investigate the role of the presence of a mixture of both wild type and mutated cytochrome b, which is the phenotype in the patient.

The final goal of the project is to better understand complex III-associated diseases for potential development of pharmacological treatments of affected patients.

Experimental plan

1. Oxidative phosphorylation efficiency of cybrids carrying the pathogenic cyt b del15643-15660 microdeletion
A panel of assays routinely performed our laboratory will be carried out in both homoplasmic and heteroplasmic (60% mutated mtDNA) cybrids, namely:

1.1. Measurement of complex III activity
Enzymatic activity of CIII will be determined in mitochondrial fractions isolated from wild type and mutant cybrids, by assaying for ubiquinol : cytochrome c reductase activity with DBH2 and bovine heart cyt c, as previously described [Fato et al. 1993]. The amounts of cytochrome b, cytochrome c and 2Fe2S cofactors associated with CIII will be estimated using spectroscopic methods.
1.2. Measurement of the rate of mitochondrial ATP production
Oxidative phosphorylation efficiency will be directly determined by measuring the rate of mitochondrial ATP synthesis in digitonin-permeabilized control and mutant cybrids using the luciferin/luciferase assay with a luminometer [Manfredi et al. 2002]. ATP synthesis will be performed in the presence of malate and pyruvate (substrates for complex I), or succinate (substrate for complex II) plus rotenone, or decylbenzoquinol (substrate for complex III) plus rotenone and malonate. The rates of ATP synthesis will be normalized to the citrate synthase activity as an indication of mitochondrial mass content [Trounce et al. 1996]. This assay will allow to determine the overall mitochondrial oxidative phosphorylation capacity of mutant cells compared to controls.

1.3. Determination of viability after metabolic stress
When cells are grown in a glucose-free medium containing galactose (DMEM-galactose, supplemented with 5mM galactose, 5 mM sodium pyruvate, and 5%FBS), the rate of glycolysis is strongly reduced, and cells are forced to rely solely on oxidative phosphorylation to synthesize ATP. Under these conditions, cells bearing defects in the respiratory chain are unable to survive [Robinson et al. 1992; Ghelli et al. 2003]. Cybrids with cytochrome b microdeletion will be incubated in DMEM-galactose, and cell viability determined as a function of time by using the colorimetric sulforhodamine B assay. This assay will provide a first indication of the severity of the mutational effects on oxidative phosphorylation.

1.4. Measurement of ROS production
Given that complexes III is, together with complex I, the major ROS producer in mitochondria and in turn in cells, we will analyse whether cytochrome b microdeletion can induce ROS overproduction. A standardized protocol employing the fluorescent dye dichlorofluorescein will be utilized, associated with determination of cellular content of reduced and oxidized glutathione (GSH and GSSG), as previously described [Porcelli et al. 2008]. In fact, increased GSSG/GSH ratio is considered a good indication of oxidative stress caused by mutation-induced endogenous ROS overproduction.

2. Supramolecular organization of respiratory supercomplexes
The expression levels of representative subunits of CIII as well as of other respiratory complexes will be evaluated by immunoblot using commercially available antibodies. Then the steady-state levels of respiratory complexes and supercomplexes will be examined as cytochrome b mutants might lead to up-regulation of other respiratory complexes (in particular complex I and IV) [Acín-Perez et al. 2004; Schagger et al. 2004; D’Aurelio et al. 2006]. Mitochondria solubilized with DDM or digitonin will be analysed by using BN-PAGE [D’Aurelio et al. 2006; Ghelli et al. 2013].

3. Hypoxia and respiratory supercomplexes analysis
Incubation under hypoxic conditions can alter the supramolecular organization of supercomplexes by inducing transcription of specific assembly factors. We will analyse whether the alteration of supercomplex I-III-IV organization observed in mitochondria bearing the m.15579A>G, p.278Y>C mutation [Ghelli et al. 2013] can be ameliorated by incubation under hypoxic conditions. If necessary, this will be also carried out in cybrids bearing the cytochrome b microdeletion.

4. Lipidomic profile of mitochondria with cytochrome b mutations
Given that the m.15579A>G, p.278Y>C mutation has been shown to alter the supramolecular organization of supercomplexes [Ghelli et al. 2013] and this might also occur in cells bearing the microdeletion (del15643-15660), we are going to compare the mitochondrial lipidic profile of these cybrids with controls. In particular our interest is focused on the content of the anionic phospholipid cardiolipin, mostly localized within the inner mitochondrial membrane, where it associates with the oxidative phosphorylation complexes, modulating their catalytic activities, and providing stability to respiratory chain supercomplexes. On the other hand, cardiolipin biosynthesis was decreased in yeast complexes III, IV and V assembly mutants, suggesting that the two processes are interdependent [Gohil VM et al. JBC 2004]. This part of the project will be carried out in collaboration with the group of prof. Angela Corelli, University of Bari, who has developed an “intact” MALDI–TOF mass spectrometry analysis of the mitochondrial lipid content without solvent extraction [Angelini et al. 2012].
References
PIANO DI ATTIVITA’

Il piano di formazione associato a questo progetto prevede che il titolare dell’assegno approfondisca le conoscenze delle tecniche per la separazione e l’analisi dei lipidi mitocondriali effettuando diretta esperienza della tecnica di spettrometria di massa. A tal fine si gioverà della collaborazione con la Prof. Corcelli, Università di Bari, esperta di analisi lipidomiche.

Specificamente il piano di formazione prevede che il candidato acquisisca esperienze riguardanti:
1. isolamento di mitocondri tramite centrifugazione differenziale;
2. purificazione dei campioni e analisi mediante spettrometria di massa;
3. quantificazione del contenuto proteico dei campioni mitocondriali analizzati sia mediante dosaggio colorimetrico che mediante western blotting con anticorpi contro specifiche proteine delle membrane mitocondriali.

Le competenze per queste tecniche sono disponibili nel dipartimento della Prof. Corcelli a Bari e nel nostro dipartimento.

Il piano di formazione prevede anche che l’assegnista possa tornare per un breve periodo presso il lab del prof. Fevzi Daldal, Università di Pennsylvania, Philadelphia, US, per completare la valutazione mediante spettroscopia EPR della quantità di proteina 2Fe/2S di Riskie, cofattore del complesso III, che potrebbe essere alterato in particolare nella mutazione Y278C nel sito Q₆ dove avviene l’ossidazione del chinolo.

La formazione dell’assegnista prevede inoltre la partecipazione ai seminari del laboratorio e dipartimentali, Journal Club, congressi nazionali ed internazionali e scuole di specializzazione pertinenti e utili per lo svolgimento della ricerca.

Gruppo di Ricerca del Dipartimento di Biologia in cui si inserirà l’assegnista

Prof.ssa Michela Rugolo (Professore Associato Confermato)
Dott.ssa Anna Maria Ghelli (Ricercatore Universitario confermato)
Dott.ssa Anna Maria Porcelli (Ricercatore Universitario confermato)
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1. **National Health Institute-Collaborative Project with Pennsylvania University, Philadelphia, USA, 2011-2014**: Bacterial cytochrome bc1: structure, function and biogenesis, (fonte del finanziamento)
2. **Progetto di Ricerca di Interesse Nazionale (PRIN) 2013-2015**: Mitochondrial mechanisms of cancerogenesis. Co-Investigator in a collaborative national project. (fonte del finanziamento)
3. **RFO 2011**