# 盘基网柄菌DJ-1蛋白的亚细胞定位研究

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**摘要** *DJ-1*基因的突变或缺失导致帕金森病相关症状, 但其在帕金森病中的作用以及其亚细胞位置尚存在争议。盘基网柄菌是研究神经退化性疾病的模式生物, 通过绿色荧光蛋白(GFP)标记DJ-1蛋白, 利用荧光蛋白技术及DJ-1与GFP共定位技术在正常和氧化情况下研究其在盘基网柄菌的亚细胞定位, 可以为探索DJ-1蛋白亚细胞位置与致病机制之间的联系奠定基础。研究结果表明, 正常情况下盘基网柄菌DJ-1蛋白位于细胞质内, 一旦受到氧化应激, DJ-1蛋白则转移至线粒体, 这个亚细胞位置转移与DJ-1蛋白C117位点的氧化相关。该研究为探索DJ-1蛋白如何在氧化应激条件下完成对细胞的保护提供了实验依据。

**关键词** 盘基网柄菌; DJ-1; 帕金森病; GFP标记; 亚细胞定位; 免疫检测

# The Subcellular Localization of DJ-1 Protein in *Dictyostelium discoideum*

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**Abstract** The loss and mutations of *DJ-1* were reported to cause the symptoms related to Parkinson's disease (PD), but the role of DJ-1 in PD and its subcellular localization still remained controversial. *Dictyostelium discoideum* is one of the recognized models and can be used to study the neurodegenerative disease. DJ-1 protein in *D. discoideum* was labelled by green fluorescence protein (GFP) and its subcellular localization under basal and oxidative stressed conditions was observed using immunofluorescence and colocalization of DJ-1 and GFP in order to discover the possible links between DJ-1 subcellular localization and its mechanisms related to PD. The results showed that DJ-1 was predominantly cytosol under basal condition in *D.discoideum*, but translocated to the mitochondria with hydrogen peroxide. This translocation of DJ-1 could possibly be due to the oxidation of site C117, which provides a crucial clue in studying how DJ-1 protects the cells under oxidative stressed condition.

**Keywords** *Dictyostelium discoideum*; DJ-1; Parkinson's disease; green fluorescence protein; subcellular localization; immunofluorescence detection

帕金森病是仅次于阿尔茨海默症的第二大神经退化性疾病, 65~69岁人群患帕金森病的机率为6.0%, 当年龄增至85~89岁, 患病机率则上升至26%[1]。据统计, 全球目前60岁以上人群中1.8%患有帕金森病, 中国内地帕金森病患者已逾200万, 而且每年还以新增10万病例的速度上升[2]。

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*DJ-1*位于基因位点PARK7, 由23 629个碱基对组成, 含有8个外显子, 其开放阅读框为570 bp, 编码长189个氨基酸的蛋白质[3]。*DJ-1*缺失或者突变导致先天性或零散发作帕金森病, 其突变率在先天性帕金森病基因中占1%~2%, 仅次于*Parkin*[4-7]。就*DJ-1*在帕金森病中的作用, Hao等[8]发现, 提高果蝇*DJ-1*表达水平可以降低因*PINK1*突变引起的功能损伤, 且此作用取决于残基C106。Thomas等[9]研究结果表明, *Parkin*可能作为*DJ-1*下游基因保护人类多巴胺能神经细胞免受因*DJ-1*缺失引起的损伤。Xiong等[10]则认为, DJ-1可能和PINK1、Parkin形成200 kDa复合物(PINK1-Parkin-DJ-1, PPD), 该复合物的结构域相互协作, 促使细胞内未折叠或折叠错误的蛋白质通过泛素蛋白酶复合体系进行降解。也有学者提出, *DJ-1*可能作为转录因子、抗氧化剂或与其它帕金森病相关基因互作等在帕金森疾病中起保护作用[11-13]。就DJ-1蛋白在细胞内的亚细胞定位, Canet-Avles等[14]认为与线粒体无关, 但Zhang等[15]则发现DJ-1蛋白位于线粒体的基质和内膜空间。

关于DJ-1蛋白的亚细胞定位及其在帕金森病中的功能和作用机制争议很多, 需要进一步探讨。盘基网柄菌(*Dictyostelium discoideum*)是美国国立卫生研究院公布的10种模式生物之一, 其全基因组和线粒体基因组序列均已被破译[16-17]。在生长过程中, 盘基网柄菌因食物的多寡表现出单细胞和多细胞两种形态, 从而展现出多种不同表现型, 如单细胞盘基网柄菌的生长和分裂、环腺苷酸化学趋向性、趋光性和趋热性、多细胞子实体的形态特征、细菌吞噬作用与吞噬率、胞饮作用效率等[18-20]。基因型的操作和改变通过上述盘基网柄菌多种表现型现出来, 从而提供可数、可读和可靠的基因型–表现型相关性。除此外, Fisher等[21]建立了盘基网柄菌的线粒体疾病细胞信号传导通路。其研究发现, 在盘基网柄菌细胞内, 线粒体功能一旦受到影响, ATP生成下降, 则AMP升高, 刺激AMPK大量生成, 从而诱导进入AMPK依赖型细胞信号通路, 通过TORC1引起盘基网柄菌一系列表现型变化来降低细胞ATP消耗[22]。大量研究又表明, 线粒体功能紊乱是引起帕金森病的主要机制之一[23-24], 因此这为利用盘基网柄菌作为模型研究帕金森病提供了依据。最后, 盘基网柄菌*DJ-1*基因已被克隆, 其生物信息学分析及基因序列测定结果表明: 盘基网柄菌全基因组存在与人类*DJ-1*同源基因, 全长618 bp, 无内含子, 编码全长为205个氨基酸的蛋白质[25]。

为了研究DJ-1蛋白的亚细胞定位及其在帕金森疾病中的作用, 本研究用绿色荧光蛋白(green fluorescence protein, GFP)标记盘基网柄菌DJ-1蛋白, 再采用两步克隆法(two-step cloning strategy)构建其大肠杆菌和盘基网柄菌表达载体, 并对相应转化株进行筛选, 在正常和氧化应激条件下研究DJ-1蛋白在盘基网柄菌的亚细胞定位, 旨在为后期建立帕金森病的盘基网柄菌模型奠定基础。

## 1 材料与方法

### 1.1 材料

实验所用材料为大肠杆菌和盘基网柄菌, 其菌株名称、基因型等信息见表1。

### 1.2 试剂与仪器

Geneticin(G418)、T4DNA连接酶、限制性内切酶(*Eco*R I和*Cla* I等)购自Promega公司; Taq聚合酶、Mitotracker Red、DAPI、Alexa-Fluor$^{R}$-647抗体、PureLink$^{TM}$ HiPure Plasmid Maxiprep Kit购自Invitrogen公司; HL-5培养基购自Formedium公司; 蛋白酶抑制剂(25×, cocktail tablet)购自Roche; Anti-PARK7/DJ-1抗体MJF-R16(66-5)购自Abcam公司; $H_2O_2$购自Univar公司; 其他常见试剂购自Sigma或Ajax公司。

实验仪器包括: 美国Olympus BX61TRF荧光显微镜和Olympus U-CMAD3、公司Storm 860™

**表1 实验所用菌株名称及基因型**

**Table 1 The name of strains and their genotype**

| 菌株名称<br>Strain name | 基因型<br>Genotype | 参考文献<br>Reference |
|---|---|---|
| *E. coli* DH5α | $F^{-}$, φ80dlacZΔM15, Δ(lacZYA-argF)$_{U169}$, deoR, recA1, endA1, hsdR17(rk$^{-}$,mk$^{+}$) phoA, supE44, thi-1, gyrA96, relA1 | [26] |
| *D.discoideum* AX2 | axeA1, axeB1, axeC1 | [27] |

Fluoroimager(Amersham Biosciences)、Trans-Blot Turbo™ Western blot(Bio-Rad)、Gene-pulser电穿孔仪(Bio-Rad)、Sub-Cell GT Cell 电泳仪(Bio-Rad)、Sorvall RC 5C离心机(Thermo Fisher)、Catalogue培养箱(Contherm)和PTC-150 minicycler PCR仪(Bio-Rad)。

### 1.3 盘基网柄菌DGFP的引物设计、合成与PCR扩增

参照盘基网柄菌*DJ-1*的基因序列, 利用Primerquest软件设计上、下游引物扩增除终止密码子外的*DJ-1*基因片段(*DGFP*)(表2)。引物由Geneworks公司进行合成。以盘基网柄菌总基因组DNA为模板, 利用表2设计的引物和Taq聚合酶对*DGFP*进行PCR扩增。

### 1.4 pPROF683原核表达载体的构建、大肠杆菌转化株的获取与筛选及pPROF683的提取、纯化与测序

利用EcoR I对DGFP和pUC18进行限制性酶切, 凝胶电泳检测后提取DGFP和pUC18并以7:1的比例16 °C过夜进行连接反应, 得到产物pUC18-DGFP(pPROF683)。将pPROF683和电感受态*E.coli* DH5α细胞混合物以25 μF电容、2.5 kV电伏和200 Ω电阻电穿孔后, 37 °C将此细胞混合液振荡培养1 h, 接种到含100 μg/mL阿莫西林的LB培养基上37 °C过夜培养, 利用蓝–白斑法筛选转化株。将筛选的转化株利用碱裂解法提取pPROF683, 经限制性内切酶酶切和凝胶电泳确认后, 再利用PureLink™ HiPure Plasmid Filter Maxiprep Kit的使用方法进行pPROF683大规模提取和纯化。pPROF683载体中DGFP片段测序由Australian Genome Research Facility(AGRF)公司完成。

### 1.5 pPROF693真核表达载体的构建及盘基网柄菌转化株的获取、培养和筛选

利用*Cla* I对pPROF683和盘基网柄菌真核表达质粒pA15GFP进行限制性酶切, 纯化酶切片段DGFP和线性质粒pA15GFP并以7:1的比例16 °C过夜连接生成pPROF693。利用磷酸钙共沉淀法[28]将pPROF693转入盘基网柄菌野生株AX2细胞, 21 °C培养细胞15~18 h, 然后将细胞接种至覆盖着3~4日龄腾黄微球菌(*Micrococcus luteus*)、含20 μg/mL Geneticin 418的标准培养基上, 充分混匀, 无菌干燥后置于21 °C培养2~4周, 直到盘基网柄菌菌落清晰可见。选取约50个不同大小的菌落, 转接到含20 μg/mL Geneticin 418, 并且覆盖有克雷伯氏菌(*K.aerogenes*)的标准培养基上, 利用稀释划线法(Streak-dilution)转接2~3次得到纯化的盘基网柄菌转化株。

### 1.6 盘基网柄菌DJ-1蛋白亚细胞定位的免疫荧光检测

将盘基网柄菌野生株AX2和含pPROF693的转化株同时在HL-5液体培养基中生长至浓度为$1\times10^6$~$2\times10^6$细胞/mL, 取3 mL细胞悬浮液于消毒的培养皿中静置30 min, 用荧光度低的培养液Lo-Flo-HL-5替换掉原培养液HL-5并静置1 h进行平衡。加入1 mL Mitotracker Red(100 nmol/L)并在黑暗中静置45 min进行线粒体染色。PBS洗涤细胞3次, 加入1 mL 3.7%甲醛静置30 min进行细胞固定。去掉甲醛, 加入100%预冷甲醇并放置5 min使细胞通透性增强。PBS洗涤细胞2次, 加入缓冲溶液后振荡处理1 h。去除缓冲溶液, 以1:500比例加入Alexa-Fluor$^R$647抗体并置于4 °C过夜反应。PBS洗涤细胞3次, 加入0.1 μg DAPI 5 min进行细胞核染色, PBS和双蒸水洗涤去掉多余染色液。将染色后的细胞自然晾干, 利用Olympus BX 61 TRF显微镜进行镜检, 并用Olympus U-CMAD3相机拍摄图片。为了检测氧化应激条件下盘基网柄菌DJ-1蛋白的亚细胞定

**表2 扩增DGFP的上、下游引物名称和序列**

**Table 2 Primer sequences for amplification of DGFP**

| 引物名称<br>Primer name | 引物序列(5′→3′)<br>Primer sequence (5′→3′) |
|---|---|
| DGFPF | GC**G AAT TCA TCG AT**A TGA CCA AAA AAA TAT TAT TAT TAT TAT GTA AAG G |
| DGFPR | GC**G AAT TCA TCG AT**A AAA CCC ATT AAA GTT TTT ACT TTT TTA GC |

DGFPF: 扩增DGFP的上游引物, DGFPR: 扩增DGFP的下游引物, 其中*DJ-1*基因的终止密码子TAA被去掉。5′-端添加GC为了提高限制性内切酶内切效率。引物序列中加粗部分为限制性内切酶的识别序列, 分别为: *Eco*R I(GAATTC)和*Cla* I(ATCGAT)。

DGFPF: DJ-1, GFP forward, the forward prime used for amplification of DGFP, DGFPR: DJ-1, GFP reverse, the reverse prime used for amplification of DGFP which removes the stop codon of *DJ-1* for ligation of *DJ-1* and GFP in vector pA15GFP. The direction of primers is 5′-3′ and the addition of extra GC at 5′ end is to increase the efficiency of endonuclease digestion. The bold letters in primer sequences are the recognization sequences of *Eco*R I (GAATTC) and *Cla* I (ATCGAT).

位, 在利用HL5培养AX2和含pPROF693真核表达载体的盘基网柄菌转化株时, 加入150 μmol/Lol·L过氧化氢(30%) 21 °C培养24 h, 然后再进行如上所述的免疫荧光检测。

### 1.7 盘基网柄菌DJ-1蛋白的Western blot检测

将盘基网柄菌野生株AX2和含pPROF693的转化株同时生长至细胞液浓度为$1\times10^6$ ~$2\times10^6$细胞/mL, 收集大约$5\times10^6$个细胞, 2 500 r/min离心2 min, 添加15 μL 1×Laemmli缓冲液后在冰上裂解10 min。加入1 μL蛋白酶抑制剂(25×, cocktail tablet, Roche)以阻止蛋白质降解。将样品煮沸10 min, 再在冰上迅速冷却。将15 μL粗制蛋白质样品加载至12% SDS-PAGE凝胶200 V电泳40 min。利用Trans-Blot Turbo™ blotting转膜设备将蛋白质转移至PVDF膜。对PVDF膜进行洗涤、封闭, 加入Anti-PARK7/DJ1抗体, 室温放置1 h。洗涤PVDF膜, 加入24 μL ECF液室温放置5~10 min。用Whatman 3 MM过滤纸滤干PVDF膜, 利用Storm 860™ Fluoroimager进行扫描和记录。

### 1.8 盘基网柄菌DJ-1蛋白的亚细胞定位预测和氨基酸序列BLAST分析

利用MitoProt II(https://omictools.com/mitoprot-tool)和Helical Wheel Plot(http://www.biology.wustl.edu/gcg/helicalwheel.html)软件对盘基网柄菌DJ-1蛋白的亚细胞定位进行预测。利用人类帕金森病相关DJ-1蛋白的氨基酸序列在dictyBase(the international dictyostelid genomics resource at http://dictybase.org)进行BLAST比对分析[29]。

## 2 结果

### 2.1 DGFP原核及真核表达载体的构建及其限制性酶切结果

盘基网柄菌*DJ-1*基因富含腺嘌呤(A)和胸腺嘧啶(T), 其真核表达质粒pA15GFP(6 600 bp)相对较长, 而且也富含A和T碱基, 这增加了PCR扩增、目的基因与真核质粒连接、真核表达载体直接在盘基网柄菌体内表达、提取、纯化和序列鉴定的困难程度。因此本实验采取两步克隆法先将DGFP克隆至大肠杆菌质粒载体pUC18(2 686 bp), 然后再将其通过限制性酶切克隆至盘基网柄菌质粒载体pA15GFP。

利用DGFPF和DGFPR作为上、下游引物对除去终止密码子TAA的*DJ-1*基因(*DGFP*)进行扩增, 再进行*Eco*R I酶切(digest with *Eco*R I)、基因片段和质粒纯化(purification)、质粒去磷酸化(dephosphorylation of vector)和连接反应(ligation), 将*DGFP*克隆至pUC18, 得到盘基网柄菌DJ-1蛋白原核表达载体pPROF683(图1A)。利用*Cla* I对原核表达载体pPROF683和真核质粒pA15GFP进行限制性酶切, 再进行质粒和原核表达载体纯化、pA15GFP质粒去磷酸化和连接, 将DGFP转移至盘基网柄菌pA15GFP质粒, 生成盘基网柄菌DJ-1:GFP融合蛋白的真核表达载体PPROF693(图1B)。

### 2.2 盘基网柄菌DJ-1蛋白亚细胞定位的预测

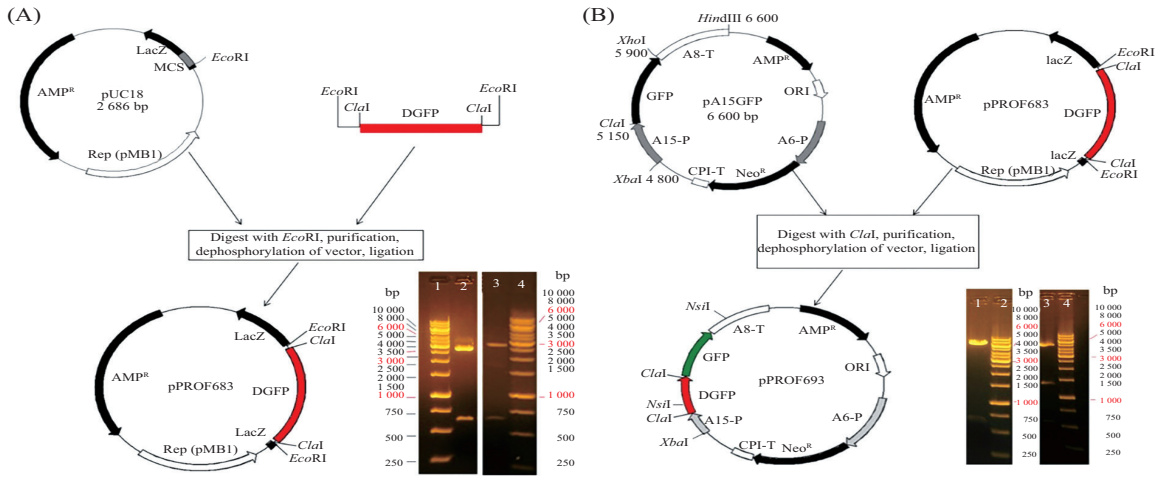
研究认为, DJ-1蛋白的亚细胞位置与线粒体有关[15]。本研究利用MitoProt II和Helical Wheel Plot软件对盘基网柄菌DJ-1蛋白的氨基酸序列进行了分析, 通过其亲水性、疏水性和正负电荷与线粒体蛋白特征之间的相关性判断其是否属于线粒体蛋白。MitoProt II软件分析了盘基网柄菌DJ-1蛋白的205个氨基酸序列, 其N-端13个残基被用于估测是否具有线粒体靶向序列。结果发现, 其净电荷数为–3, 碱性氨基酸残基数为3, 酸性氨基酸残基数为0, 综上认为: DJ-1蛋白位于线粒体的机率仅为15.53%, 这说明, DJ-1的亚细胞位置不太可能是线粒体。 分析所用蛋白质疏水规格见表3。 线粒体蛋白N-端通常有线粒体靶向序列, 这些序列带有正电荷, 而且形成一个两亲的α螺旋, 螺旋的一侧所有氨基酸残基都带正电荷, 呈极性, 而另一侧则呈非极性。经Helical Wheel Plot软件预测分析发现: DJ-1蛋白N-端不具有这些线粒体蛋白靶向序列特征(图2)。

### 2.3 正常和氧化应激条件下盘基网柄菌DJ-1蛋白的免疫荧光检测

从含Geneticin418的标准培养基上筛选出50株含pPROF693的盘基网柄菌转化株后, 先对照盘基网柄菌野生菌株检测其GFP荧光强度, 并筛选出12株荧光强度高的菌株进行免疫荧光检测。研究结果显示: 正常情况下, 盘基网柄菌DJ-1蛋白位于细胞质内, 但受到过氧化氢氧化应激后, DJ-1蛋白转移至线粒体(图3)。

### 2.4 人类和盘基网柄菌DJ-1蛋白氨基酸序列BLAST对比分析

利用人类DJ-1蛋白的氨基酸序列在盘基网柄菌蛋白质组进行查询和在线BLAST对比分析发




A: 第1和4样品列条带均为1 Kb DNA marker, 2和3条带分别为重组载体pPROF683经*Eco*R I和*Cla* I酶切片段(615 bp和2 686 bp)。2最上面的条带系限制性酶切不完全造成。B: 第2和4样品列中的条带均为1 Kb DNA marker, 第1列条带为重组载体pPROF693经*Cla* I酶切片段(615 bp和6 600 bp), 第3列条带为重组载体pPROF693经*Nsi* I和*Xba* I酶切片段(584 bp、1 431 bp和5 200 bp)。

A: the bands in lane 1 and 4 of electrophoresis gel are both 1 Kb DNA marker. The bands in lane 2 and 3 are the fragments of pPROF683 after digestion by *Eco*R I an *Cla* I (615 bp and 2 686 bp). The top band in lane 2 is due to partial digestion of the construct. B: the bands in lane 2 and 4 of electrophoresis gel are both 1 Kb DNA marker. The bands in lane 1 are the fragments of pPROF693 after digestion by *Cla* I (615 bp and 6 600 bp). The bands in lane 3 are the fragments of pPROF693 after digestion by *Nsi* I and *Xba* I (584 bp, 1 431 bp and 5 200 bp).

**图1 pPROF683及pPROF693的构建和限制性酶切**

**Fig.1 Generation and restriction endonuclease digestion of *E. coli* construct pPROF683 and *D. discoideum* construct pPROF693**

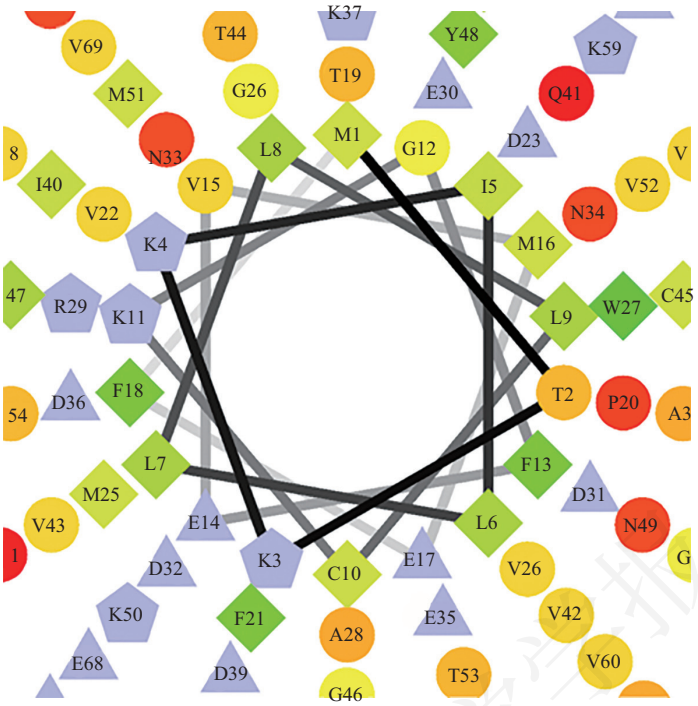
**表3 MitoProt II估测盘基网柄菌DJ-1蛋白亚细胞定位的疏水性规格**

**Table 3 The hydrophobic scales used to predict the subcellular localization of D. discoideum DJ-1 protein using MitoProt II**

| 计算蛋白质疏水性规格 Hydrophobic scale used | 氨基酸残基特性规格 Scales for analysis of amino acid residues | | | |
|---|---|---|---|---|
| | GES | KD | GvH1 | ECS |
| H17 | 0.835 | 1.359 | 0.027 | 0.529 |
| MesoH | –0.973 | 0.319 | –0.456 | 0.203 |
| MμHδ _075 | 14.947 | 10.979 | 5.993 | 1.857 |
| MμHδ _095 | 26.037 | 12.051 | 6.547 | 5.040 |
| MμHδ _100 | 34.806 | 16.396 | 8.945 | 5.995 |
| MμHδ _105 | 37.341 | 18.333 | 9.998 | 6.460 |
| Hmax_075 | 6.650 | 15.633 | 2.117 | 4.422 |
| Hmax_095 | 10.675 | 13.213 | 1.803 | 5.093 |
| Hmax_100 | 19.100 | 19.700 | 3.370 | 6.640 |
| Hmax_105 | 18.000 | 17.100 | 3.151 | 5.870 |
| Hmax_105 | 18.000 | 17.100 | 3.151 | 5.870 |

表格左侧为计算蛋白质疏水性的规格。H17: 序列中疏水性高的17个残基, 其数值越高, 则蛋白质进入线粒体的可能性越低; MesoH: 通过其延伸序列长度可以判断蛋白质的平均最大疏水性; MμHδ: 18个残基在4个不同时刻的最大Eisenberg疏水矩, 4个时刻分别为75"、95"、100"和105"; Hmax: 螺旋结构中每一个疏水面的最大疏水性, 一般取决于最大的MμHδ值。为了减小偏差, 计算由基于不同氨基酸残基特性的4个不同规格和尺度完成, 分别是GES(Goldman, Engelman和Steitz)、GvH1(Gunnar von Heijne 1)、KD(Kyte和Doolittle)和ECS(Eisenberg's consensus scale)。

The column on the left of table lists the scales used to calculate the hydrophobicity of the protein. H17: the 17-residue segment of higher hydrophobicity in the sequence. Increasing the value diminishes the possibility of importing a protein; MesoH: the average of the maximal hydrophobicity of a protein over an extended sequence length; MμHδ: the maximal Eisenberg's hydrophobic moment with 6 angles of 75", 95", 100" and 105", with a scanning window of 18 residues; Hmax: the maximal hydrophobicity of each hydrophobic face in a helical structure. It is calculated between the 18 residues determined by a maximal MμHδ. To minimize the bias introduced by the scale, calculations have been made with up to four scales based on different amino acid residue properties. GES (Goldman, Engelman and Steitz scale), GvH1 (Gunnar von Heijne scale 1), KD (Kyte and Doolittle scale) and ECS (Eisenberg's consensus scale).

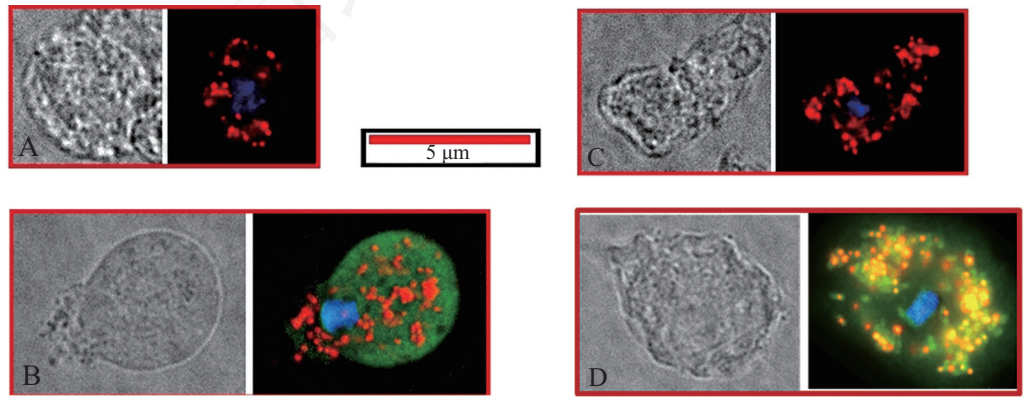



图中亲水性和电荷用不同形状表示: 圆形表示亲水性残基; 钻石形表示疏水性残基; 三角形表示负电荷残基; 五角形表示正电荷残基。疏水性大小用不同颜色表示: 疏水性最强的残基用深绿色表示; 绿色的递减表示疏水性的递减; 黄色代表疏水性为零。亲水性用红色表示, 红色越浅, 亲水性越低。带正电荷或者负电荷的残基用淡蓝色表示。

The hydrophilic residues are shown as circles, hydrophobic residues as diamonds, potentially negatively charged residues as triangles, and potentially positively charged as pentagons. Hydrophobicity is color coded: the most hydrophobic residue is green, and the amount of green decreasing proportionally to the hydrophobicity, with zero hydrophobicity coded as yellow. Hydrophilic residues are coded red with pure red being the most hydrophilic (uncharged) residue, and the amount of red decreasing proportionally to the hydrophilicity. The potentially positively or negatively charged residues are light blue.

**图2 Helical Wheel Plot估测盘基网柄菌DJ-1蛋白的亚细胞定位**

**Fig.2 Prediction of the subcellular localization of *D. discoideum* DJ-1 protein using Helical Wheel Plot**




A: 正常条件下盘基网柄菌野生菌株$AX_2$; B: 含pPRO693(pA15GFP-DGFP)真核表达载体、编号为HPF1246的转化株; C: 氧化应激条件下的AX2; D: 氧化应激条件下的 HPF1246转化株。红色荧光: Mitotracker Red染色的线粒体; 绿色荧光: GFP标记的DJ-1蛋白所在位置; 蓝色荧光: DAPI染色的细胞核。

A: the wild *D. discoideum* $AX_2$ strain under basal condition; B: the *D. discoideum* transformant named HPF1246 containing the construct pPRO693 under the basal condition; C: the AX2 strain under oxidative stressed condition; D: the HPF1246 transformant under oxidative stressed condition. The red fluorescence: the mitochondria stained by Mitotracker Red; the green fluorescence: DJ-1 labelled by GFP; the blue fluorescence: the nuclei of the *D. discoideum* cells stained by DAPI.

**图3 盘基网柄菌DJ-1蛋白的亚细胞定位**

**Fig.3 The subcellular localization of *D. discoideum* DJ-1 protein**

现: 盘基网柄菌蛋白质组中存在人类DJ-1蛋白的同源序列, 二者的完全一致性达到26%(52/203), 可能相似性达到44%(91/203), 其差异性达到10%(21/203)。与人类DJ-1蛋白C106是其活跃且保守位点不同[30], 盘基网柄菌DJ-1蛋白相应的保守位点为C117(图4)。

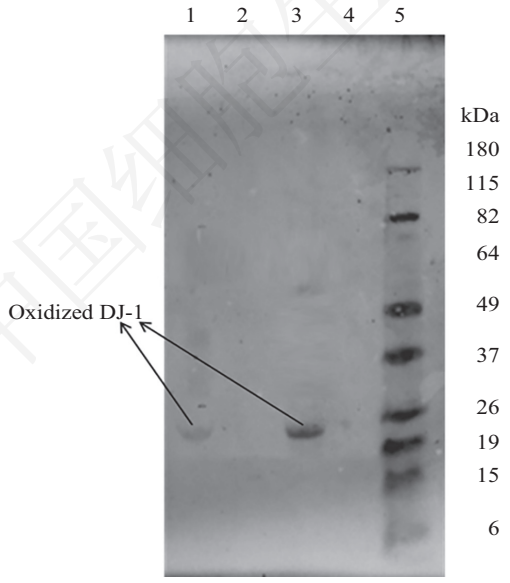
### 2.5 盘基网柄菌DJ-1蛋白的特异抗体检测

在正常和氧化应激条件下, 利用C106位点被氧化的人类特异性Anti-PARK7/DJ-1抗体对盘基网柄菌DJ-1蛋白进行检测。检测结果如图5所示: 当盘基网柄菌细胞受到$H_2O_2$氧化应激时, 其DJ-1蛋白C117位点发生类似人类DJ-1蛋白C106位点的氧化

```
Query   3    SKRALVILAKGAEEMETVIPVDVM----------RRAGIKVTVAGLAGKDPVQCSRDVVI  52
             +K+ L++L KG E ME    VDVM           +A I+V   GL  K  V  +  V +
Sbjct   2    TKKILLLLCKGFEVMEFTPFVDVMGWAREDDNNEDKADIQVVTCGLYNK-MVTSTFGVKV  60

Query   53   CPDASL-EDAKKEGPYDVVVLPGG--NLGAQNLSESAAVKEILKEQENRKGLIAAICAGP  109
               D  L E  K    +D + +PGG  N      + S  V +++++ +++   IA++C
Sbjct   61   QVDVLLGEVVKSLDEFDALAIPGGFENYSFYEEAYSEDVSQLIRDFDSKGKHIASVCVAA  120

Query   110  TALLAHEIGFGSKVTT-------HPLAKDKMMNGGHYTYSENRVEKDGLILTSRGPGTSF  162
              AL    I  G   TT       H + + ++ + G    ++  +  D  ++TS  P T+
Sbjct   121  LALGKSGILKGRNATTYRNSLREHSVRQQQLRDFGANVIADQSIVIDKNVITSYNPQTAP  180

Query   163  EFALAIVEALNGKEVAAQVKAPL  185
               A  ++  L+ +  A +VK  +
Sbjct   181  YVAFELLSRLSDENKAKKVKTLM  203
```

**图4 人类和盘基网柄菌DJ-1蛋白质氨基酸序列BlAST对比**

**Fig.4 BLAST sequence alignment using the canonical *H. sapiens* DJ-1 amino acid sequence as the query to search the predicted *D. discoideum* proteome**




图中5为预染蛋白质marker标记(Benchmark™), 1和2为同一株含*DJ-1*过表达质粒载体(pPROF690, 结果尚未发表)的盘基网柄菌转化株HPF1209, 其*DJ-1*拷贝数为108, 1菌株在培养过程中加入150 μmol/L $H_2O_2$培养超过24 h; 2为未加入任何氧化剂的对照; 3和4为同一株含pPROF690的盘基网柄菌转化株HPF1206, 其*DJ-1*拷贝数为440, 3在细胞培养过程中加入150 μmol/L $H_2O_2$, 4未加任何氧化剂。每一个样品槽中蛋白质的添加量为300 μg(Bradford法测定所得)。

The lane 5 in this figure is Prestained protein ladder (Benchmark™). The strain used in lane 1 and 2 is the same one named HPF1209 containing *DJ-1* overexpression construct pPROF690 (data unpublished) and 108 copies of *DJ-1*. However, during culture of the strain in lane 1, 150 μmol/L $H_2O_2$ was added more than 24 h, but no $H_2O_2$ for the strain in lane 2. The strain used in lane 3 and 4 is the same one named HPF1206 containing the construct pPROF690 and 440 copies of *DJ-1*. 150 μmol/L $H_2O_2$ was added during the growth of HPF1206 and lasted for more than 24 h in lane 3, but no $H_2O_2$ for strain HPF1206 in lane 4. The amount of protein loaded in each well was 300 μg (Bradford assay).

**图5 盘基网柄菌DJ-1蛋白C117氧化形式抗体检测**

**Fig.5 The detection of DJ-1 protein in *D. discoideum* using C106 oxidized form of *H. sapiens* DJ-1**

改变。

## 3 讨论

*DJ-1*最初被认为是与肿瘤发生相关的原癌基因[31], 后来有学者提出*DJ-1*在精子成熟和受精方面也起重要作用[32]。2001年Van Duijn等[33]在帕金森病人家族中发现, *DJ-1*基因1-5外显子缺失, G310A、G293A、A1208G和G192C等突变, 从而认为, *DJ-1*与帕金森病相关, *DJ-1*被命名为PARK7。2003年, Bonifati等[3]绘制了人类*DJ-1*基因结构图, 此后陆续有学者研究了DJ-1蛋白的空间结构、遗传模式、突变位点、患病概率和可能的致病机理等[3,30,34-35]。迄今为止, 已经发现超过15个*DJ-1*基因位点突变导致早发型或零散型帕金森病。这些缺失或者突变导致DJ-1蛋白功能丧失、不稳定或者缩短, 但并未表现出地区差异性[36]。

*DJ-1*属于loss-of-function基因, 对正常细胞执行生理功能起保护作用[25,37]。有学者提出, *DJ-1*可能作为抗氧化剂在氧化应激条件下通过C106位点氧化成亚磺酸来阻止α-synuclein纤维化[38]。Olzmann等[39]认为, DJ-1属于半胱氨酸蛋白酶PfpI家族, 通过蛋白水解作用去除细胞内异常蛋白质保护细胞。Chen等[40]则发现, DJ-1的蛋白水解酶活性只有在氧化应激条件下, 去掉C-端15个氨基酸才可实现。

DJ-1在帕金森病中的功能与细胞是否处于应激状态有关。Bonifati等[3]通过转染Cos和PC12细胞发现, DJ-1蛋白位于胞质和细胞核。Zhang等[15]则认为, DJ-1位于小白鼠和人类神经母细胞瘤细胞的线粒体基质和内膜空间。本研究利用过氧化氢使盘基网柄菌处于应激状态, 对照观察正常和应激条件下DJ-1的亚细胞定位发现: DJ-1蛋白在正常情况下, 存在于细胞质中(图3), 与MitProtII和Helical Wheel Plot软件预测结果一致: DJ-1蛋白无线粒体信号肽序列, 也未显示线粒体蛋白结构、极性和电荷方面的特性, 因此不属于线粒体蛋白(表3和图2)。但研究结果还发现: 当盘基网柄菌细胞受到过氧化氢刺激后, DJ-1蛋白从细胞质转移至线粒体(图3)。这进一步解释了Zhang等[15]的研究结果, DJ-1蛋白只有在细胞处于应激状态时才会转移至线粒体。

DJ-1蛋白缺乏线粒体信号肽序列, 却在氧化应激条件下从胞质到线粒体进行转移的机制值得探讨。Canet-Avles等[14]发现, 人类DJ-1蛋白的C106位点受到百草枯氧化剂刺激后变成更酸性的C-$SO_3$, 因而出现在线粒体内。将C106突变为A106, 则DJ-1蛋白即便受到氧化应激其亚细胞定位也不发生改变。本研究证实C106(盘基网柄菌的相应位点为C117)是DJ-1蛋白保守位点(图4), 且DJ-1蛋白通过该位点氧化进行亚细胞定位转移(图5)。C106位点的氧化是否会导致DJ-1结构变化或者基因序列改变有待进一步研究。另外, 研究表明, 线粒体蛋白质通常含有一个能被线粒体加工肽酶和线粒体中间肽酶识别的精氨酸残基, 这些线粒体酶能去除蛋白质末端肽序列, 使之成为成熟的线粒体蛋白, 从而完成线粒体跨膜运输[41-42]。盘基网柄菌DJ-1蛋白质N端有一个R29精氨酸残基, 这个残基是否能被线粒体酶识别, 然后通过去除DJ-1蛋白部分N-端肽序列进行线粒体转移有待后续研究和验证。

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