Study assistance for basic research at Tokushima National Hospital

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Abstract

In the clinical research department, to which I belong, study of the molecular mechanism of neurodegenerative diseases including Parkinson's disease is conducted as fundamental research. In recent years we conduct a functional analysis of Parkin. We found that Parkin was located in the mitochondria in the cell growth phase and inhibited apoptosis. We identified Klokin 1 as a new related protein, and reported its molecular mechanism. [1,2].He here report the study that I assist with a main subject.

Introduction

There are three duties that I am engaged in primarily.
1) Real-time PCR; The Parkin mRNA was assayed to examine the transcription control factor of the Parkin gene. 2) The luciferase assay method; A luciferase assay was performed to measure the activity of the transcription control factor of the Parkin. 3) The protein refinement; The Parkin protein was purified in animal cell line and Escherichia coli expression system to conduct a Parkin protein functional analysis.

Materials and Methods

Materials and reagent

Vectors were transfected COS-1 cells using LipofectamineTM LTX Reagent (Life Technologies Corp.). constructed in pcDNA4/HisMax(Invitrogen) were reported our early paper【1,2】. Luciferase Control Vector was used pGV-C2 Vector (WAKO, Japan).

Cell culture

COS-1 (a cell line derived from kidney cells) was cultured at 37°C (5% CO_2) in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-incubated fetal bovine serum (FBS) [1]

Construction of luciferase vector

Human genomic DNA isolated from PBMC was used as the template. The forward and reverse primers used for amplification of promoter region. (Table 1) PCR amplifications were performed in an TaKara PCR Thermal cycler (TaKaRa Inc., Japan) in standard mixtures of 25 μL containing 2× GC buffer I, 10 pmol of each primer, 2.5 nM dNTP, 2.5 U of LA Taq DNA polymerase, The PCR program included one incubation at

95 °C for 5 min and 40 amplification cycles (95 °C for 60 s,53 °C for 60 s and 72 °C for 60 s), followed by one final extension incubation of 7 min at 72 °C. The PCR products were separated on a 1.7% agarose gel, stained with ethidium bromide and visualised on a UV transilluminator. PCR product and the pGL 4.10[luc 2] vector (Promega KK, Japan Tokyo) were digested with Xho I and Bgl II (Takara) and purified(Qiagen), according to the manufactures' conditions. Vector arms were ligated to digested PCR fragments【3,4】. ATF4 Vector (pFN21AB9540) (Kazusa DNA Research Institute, Chiba, Japan) was transfected as control vector.

Realtime PCR

Parkin mRNA was quantified by real time PCR in COS1 cells transfected with several plasmids.

Luciferase Assay

COS-1 cells were plated in 6-well plates at $1x10^5$ cells per well. The next day, the cells were transfected with 4ug pGL4.10-Parkin vector plasmid and pGL4.74 vector. Cells were harvested at 24h transfection. Luciferase activities were measured using PicaGene Luminescence Kit (WAKO Japan) according to the manufucturer's protocol[3.4].

Parkin Protein Purify in Mammalian

COS-1 (a cell line derived from kidney cells) was cultured at 37°C (5% CO2) in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-incubated fetal bovine serum (FBS). COS-1 cells were transfected plasmid including pFN21-Halo Parkin. Cell pellets were lysed with a lysis buffer containing 50mM Tris-HCl(pH 7.6), 150mM NaCl, 0.05% Triton-X100, 20mM MgCl2, 50m, M Arginine,5%glyserol, 1mM DTT, 1mM EDTA, 2mM ATP. After that, Sonication was repeated 10seconds several times adding protease inhibitor cocktail (nacali tesque, Japan Kyoto) diluted to 1:100. And protein was incubated 90minutes RT in order to solve. The sample filutrated filuter paper was applied to Halolink ™ Resin(Promega Japan Tokyo), incubated over night ,and washed. In order to cut Halo tag, the sample was incubated 120minutes 37℃ using Mobi TEV Protease(Mo Bi Tec, Lotzestrasse Germany). The eluent was concentrated. The sample was separated by 4-20% SDS-PAGE, and processed for immunoblotting followed by visualization by the ECL method[2.3.4] and stained Anti-PRK8(SIGMA) diluted to 1:70.

Results

Real-time PCR
As shown in Figure1, the expression 1,000 times or more was found in the lane of the positive control of lane 1 as compared with control (lane C).
Luciferase Assay
As shown in Figure2, 4 times or more of luciferase activity had the positive control (lane 1) as compared with control (lane C).
The protein refinement
The Parkin protein was purified in animal cell line and Escherichia coli expression system to conduct a functional analysis of the Parkin protein.

Discussion

I participated in a technical class positively, and the tech support of the reagent supplier was used frequently. By it, I learned a real-time PCR and luciferase assay technology.
On the other hand, the Parkin protein refinement is very difficult. Expected results were not able to be obtained for the moment. For duties promotion of efficiency, the thing to be able to make was made with oneself, and the account books such as reagents were made. We are going to try for duties improvement in future. It is finally hoped that we lead to pathologic elucidation of Parkinson's disease and development of the Shinji therapy.

References

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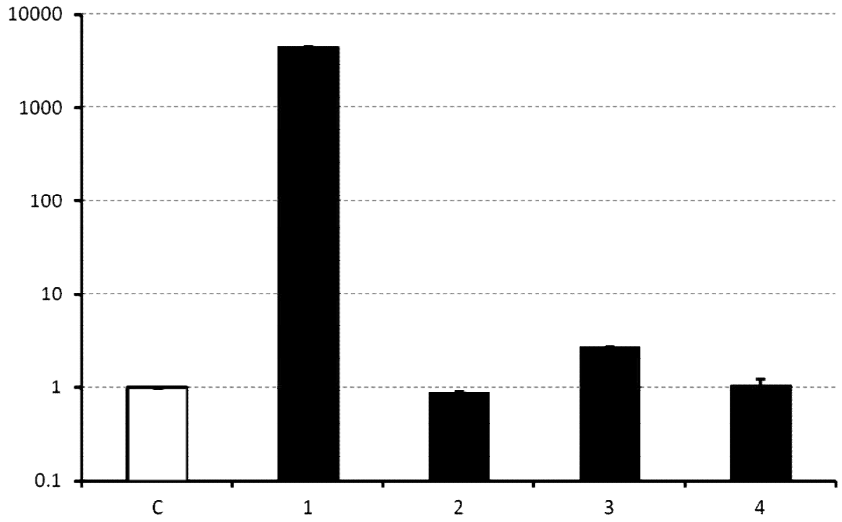

Figure 1. Results of real-time PCR.

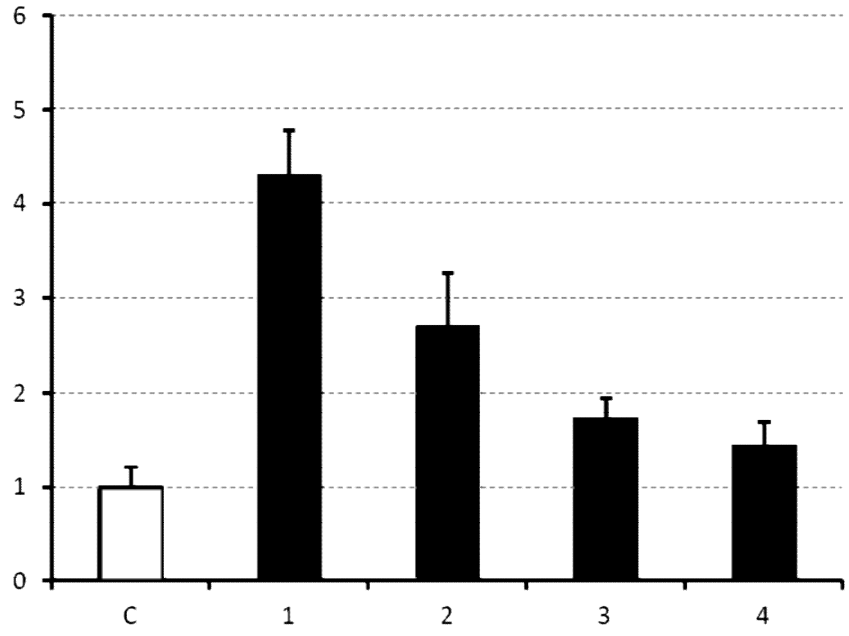

Figure 2. Results of luciferase assay