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Overexpression of MIP2, a novel WD-repeat protein, promotes proliferation of H9c2 cells

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ABSTRACT

WD40 repeat proteins have a wide range of diverse biological functions including signal transduction, cell cycle regulation, RNA splicing, and transcription. Myocardial ischemic preconditioning up-regulated protein 2 (MIP2) is a novel member of the WD40 repeat proteins superfamily that contains five WD40 repeats. Little is known about its biological role, and the purpose of this study was to determine the role of MIP2 in regulating cellular proliferation. Transfection and constitutive expression of MIP2 in the rat cardiomyoblast cell line H9c2 results in enhanced growth of those cells as measured by cell number and is proportional to the amount of MIP2 expressed. Overexpression of MIP2 results in a shorter cell cycle, as measured by flow cytometry. Collectively, these data suggest that MIP2 may participate in the progression of cell proliferation in H9c2 cells.

Introduction

WD40 repeats (also known as WD or β-transducin repeats) are short ~40 amino acid motifs, often terminating in a Trp–Asp (WD) dipeptide. WD-containing proteins have 4–16 repeating units, all of which are thought to form a circularised β-propeller structure [1–3]. The best characterized WD-repeat protein is the β-subunit of the G-proteins, which contains seven WD40 repeats [4]. Each WD40 repeat consists of two sites: a poorly conserved site A with a pair of glycine and histidine (GH), and a well-conserved site B with a pair of tryptophan and aspartate (WD) [5]. Proteins containing WD40 repeats have a wide range of diverse biological functions including signal transduction, cell cycle regulation [6–8], RNA splicing, and transcription as described in earlier reviews [5,9,10].

MIP2 is a novel gene cloned in our laboratory as a 498-aa protein that is up-regulated in ischemic preconditioned rat heart and we named it myocardial ischemic preconditioning up-regulated protein 2 (GenBank Accession Number: AY221751). A search of the rat genome sequence (NCBI) indicates MIP2 is composed of 14 exons and 13 introns and maps to chromosome 1q42.11. There is one CTLH domain at the N terminal of the hypothetical protein and five WD40 repeats at the C terminal of the protein. Our previous study indicates that MIP2 is expressed abundantly in heart and skeleton muscle. The aim of the present study was to characterize the role of MIP2 in the growth regulation of rat cardiomyoblast cell line H9c2. We demonstrate that overexpression of MIP2 promotes the growth of H9c2 cells.

Materials and methods

Plasmid construction. The open reading frame (ORF) of MIP2 was amplified by polymerase chain reaction (PCR) with Pyrobest (Takara) using human cDNA as a template and using the following primers: forward, 5′-ATGCAAGAGTCAGGATGTCG-3′; and reverse, 5′-TCAACTATCCATGCTACTGC-3′. The PCR products were cloned into the pGEM-T vector (Promega, Mannheim, Germany) and subsequently released by *Xho*I and *Kpn*I double digestion and ligated into pEGFP-N1 vector (Clontech). The putative MIP2 expression constructs GFP-MIP2 were confirmed by automated DNA sequencing.

Cell culture, stable transfection, and proliferation assay. The protein coding ORF of MIP2 was inserted into the expression vector pEGFP-N1 and termed GFP-MIP2. Vector alone (pEGFP-N1) was used as a negative control. H9c2 cells were transfected with pEGFP-N1 plasmid alone or with GFP-MIP2 with the use of 20 μl/ml lipofectin reagent (Life Technologies) and mixed with 8 μg/ml of either plasmid. After antibiotic selection (1 mg/ml G418) to eliminate untransfected cells, transfectants were pooled and expanded in 500 μg/ml G418, and MIP2 content was determined by Western blot. For proliferation assays, equal numbers of stable transfectants were seeded into 12-well plates at a density of 6000 cells per milliliter. After 1, 4, and 7 days, cells were trypsinized and counted by

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using a standard hemocytometer. After antibiotic selection, individual colonies were selected and expanded in 500 μg/ml G418, and MIP2 content was determined by Western blot.

Western blotting. Cell lysates were incubated on ice 20 min, sonicated for 10 s, then centrifuged at 13,000*g* for 10 min at 4 °C, and stored at −20 °C. Equal protein concentrations of cell extracts were determined by Bradford assay, and were electrophoresed through an 10% polyacrylamide gel under reducing conditions and transferred to a nitrocellulose membrane. To ensure quality of the transfer, the membranes were stained with Ponceau Red and then washed with deionized water. Membranes were blocked with 5% nonfat powdered milk in TBST buffer (0.1 mol/l Tris–HCl, pH 8.0, containing 1.5 mol/l NaCl and 0.5% Triton X-100). Membranes were incubated with a 1:500 dilution of anti-human MIP2 antibody or anti-GFP antibody, and a 1:2000 dilution of goat anti-rabbit secondary antibody. The membrane was washed with TBST, and reactive proteins were visualized by using DAB (Boster Biological Technology) according to the manufacturer's instructions.

Flow cytometry. Cell cycle was analyzed by flow cytometry. Cells were seeded into 6-well plates and incubated with medium containing 10% fetal bovine serum (FBS) or 0.3% FBS for 24 h, and then were harvested by trypsin treatment, collected by centrifugation, washed in PBS, and fixed with ice-cold 70% ethanol for overnight. The fixed cells were centrifuged and treated with 0.1 mg/ml RNase A and 50 μg/ml propidium iodide for 30 min at room temperature. The flow cytometric analysis was performed on a Becton–Dickinson flow cytometer. For each sample, at least 35,000 nuclei were analyzed. The percentage of the cells in each cell cycle was determined by the CellFIT cell-cycle analysis version 2.01 from Becton–Dickinson.

Statistical analysis. We carried out statistical analyses with SPSS version 13.0 and Stata version 9.0. Differences between the experimental groups were analyzed using Student's *t*-test and one-way ANOVA with LSD post hoc test. Statistical significance was set at $P < 0.05$.

Results

Identification of MIP2

As a novel member of the WD40 repeat proteins superfamily, MIP2 contains a WD40 region of five WD40 repeats and is strongly expressed in myocardium. Fig. 1 shows the amino acid alignment of human and rat MIP2, and both contain five WD40 repeats. The ORF of human MIP2 was amplified by PCR and ligated into the eukaryotic expression vector pEGFP-N1. GFP-MIP2 was transfected into H9c2 cells and cell lysates were immunoblotted with anti-MIP2 antibody (Fig. 2B).

H9c2 cells that constitutively overexpress MIP2 have an enhanced proliferative capacity

We transfected the open reading frame of MIP2 cDNA or pEGFP-N1 empty vector into cultured H9c2 cells, isolated the stable transfectants by antibiotic selection, and pooled populations of resistant cells to avoid the effects of clonal selection. Equal numbers of these cells were seeded into 12-well plates, and after 1, 4, and 7 days, cells were counted. The results of four independent transfection experiments demonstrate that H9c2 cells that overexpress MIP2 grow at a more rapid rate than do control cells ($P <$ 0.01,

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human          MQESGCRLEHPSATKFRNHVMEGDWDKAENDLNELKPLVHSPHAIVR------------- 47
rat            MQESGCRLEHPSATKFRNHVMEGDWDKAENDLNELKPLVHSPHAIVVRGALEISQTLLGI 60
               ********************************************

human          ---MKFLLLQQKYLEYLEDGKVLEALQVLRCELTPLKYNTERIHVLSGYLMCSHAEDLRA 104
rat            IVRMKFLLLQQKYLEYLEDGKVLEALQVLRCELTPLKYNTERIHVLSGYLMCSHAEDLRA 120
                  ************************************************************

human          KAEWEGKGTASRSKLLDKLQTYLPPSVMLPPRRLQTLLRQAVELQRDRCLYHNTKLDNNL 164
rat            KAEWEGKGAASRSKLLDKLQTYLPPSVMLPPRRLQTLLRQAVELQRDRCLYHNTKLDNNL 180
               ********:***************************************************

human          DSVSLLIDHVCSRRQFPCYTQQILTEHCNEVWFCKFSNDGTKLATGSKDTTVIIWQVDPD 224
rat            DSVSLLIDHVCSRRQFPCYTQQILTEHCNEVWFCKFSNDGTKLATGSKDTTVIVWQVDAD 240
               ************************************************:****.*

human          THLLKLLKTLEGHAYGVSYIAWSPDDNYLVACGPDDCSELWLWNVQTGELRTKMSQSHED 284
rat            THLLKLLKTLEGHAYGVSYIAWSPDDSYLVACGPDDCSELWLWNVQTGELRTKMSQSHED 300
               *************************.**********************************

human          SLTSVAWNPDGKRFVTGGQRGQFYQCDLDGNLLDSWEGVRVQCLWCLSDGKTVLASDTHQ 344
rat            SLTSVAWNPDGKRFVTGGQRGQFYQCDLDGNLLDSWEGVRVQCLWCLSDGKTVLASDTHQ 360
               ************************************************************

human          RIRGYNFEDLTDRNIVQEDHPIMSFTISKNGRLALLNVATQGVHLWDLQDRVLVRKYQGV 404
rat            RIRGYNFEDLTDRNIVQEDHPIMSFTISKNGRLALLNVATQGVHLWDLQDRVLVRKYQGV 420
               ************************************************************

human          TQGFYTIHSCFGGHNEDFIASGSEDHKVYIWHKRSELPIAELTGHTRTVNCVSWNPQIPS 464
rat            TQGFYTIHSCFGGHNEDFIASGSEDHKVYIWHKRSELPIAELTGHTRTVNCVSWNPQIPS 480
               ************************************************************

human          MMASASDDGTVRIWGPAPFIDHQNIEEECSSMDS 498
rat            MMASASDDGTVRIWGPAPFIDHQNIEEECSSMDS 514
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Fig. 1. Alignment of amino acid sequences of human and rat MIP2 by the ClustalW program with identical residues (∗), conserved residues (:), and semi-conserved residues (·). The predicted WD40 domains are underlined.