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Tissue distribution, subcellular localization, and enzymatic activity analysis of human SIRT5 isoforms

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ABSTRACT

SIRT5 is one of the seven mammalian sirtuins which are NAD^+-dependent deacylases. In human beings, *SIRT5* gene encodes for four SIRT5 protein isoforms, namely $SIRT5^{iso1}$, $SIRT5^{iso2}$, $SIRT5^{iso3}$, and $SIRT5^{iso4}$. Previous studies have focused mostly on $SIRT5^{iso1}$. Characteristics regarding localization, activity and tissue distribution of the other three SIRT5 isoforms remain unclear. In the present study, we characterized these properties of these SIRT5 isoforms. We found that $SIRT5^{iso1-3}$ were mitochondria-localized, while $SIRT5^{iso4}$ localized mainly in cytoplasm. $SIRT5^{iso2-4}$ had little deacylase activity comparing with $SIRT5^{iso1}$. Although cDNAs of all SIRT5 isoforms were readily detected in multiply tissues according to EST database, proteins of $SIRT5^{iso2-4}$ were seldom observed in human cell lines. Altogether, we dissected the four isoforms of human SIRT5 protein.

1. Introduction

Sirtuins are a group of evolutionary-conserved, NAD^+-dependent deacylases [1]. There are seven sirtuin proteins (SIRT1-SIRT7) in mammalian cells [2]. Although they share sequence and structure similarities, the seven mammalian sirtuins shown distinct subcellular localization [3]. SIRT1, SIRT6, and SIRT7 are mainly found in the nucleus [4], with SIRT7 exclusively in nucleolus [5]. SIRT2 resides predominantly in cytoplasm and can translocate into nucleus under certain conditions [6]. SIRT3, SIRT4, and SIRT5 are predominant mitochondrial proteins [7].

Previous investigation on activity of mammalian sirtuins have proved that the SIRT1-SIRT7 conduct overlapped but distinct deacylase activities [8]. SIRT1, SIRT2, and SIRT3 shown robust deacetylase activity; SIRT4, which was originally demonstrated to be an ADP-ribosyltransferase and lipoamidase [9], have recently been proved to be a demethylglutarylase, dehydroxymethylglutarylase, and demethylglutaconylase [10]; SIRT5 shown limited and selective deacetylase activity but strong and general demalonylase, desuccinylase, and deglutarylase activity [11–13]; SIRT6 has weak deacetylase activity, but more efficient deacylase activity towards long chain fatty acyl groups like myristoyl- and palmitoryl-group [8]; SIRT7 has been reported to deacetylase, long-chain deacylase and histone desuccinylase activity [14].

Among the 7 mammalian sirtuins, SIRT5 is unique by its mitochondrial localization and preference for negative-charged acyl groups [15]. SIRT5 was found localized to mitochondria by detecting exogenously expressed GFP-tagged or endogenous SIRT5 proteins [3,16]. It was then found to precisely localize to mitochondrial matrix and suspiciously to mitochondrial intermembrane space (IMS) [17]. Unlike SIRT1, SIRT2, and SIRT3, deacetylase activity of SIRT5 is weak and sequence-dependent. The reported proteins deacetylated by SIRT5 are cytochrome C [7], carbamoyl phosphate sythetase 1 [16], and urate oxidase [18]. For the 16 synthesized acetyl lysine peptides which are readily deacetylated by SIRT1, SIRT2, and SIRT3, only 8 can be deacetylated by SIRT5 [11]. However, SIRT5 is more efficient to demalonylate, desuccinylate, and deglutarylate a large number of proteins [11–13]. With the development of mass spectrometry techniques, an accelerating number of SIRT5 substrates have been identified [13,19–22]. Even though, the regulating role of SIRT5 has only been demonstrated on a few proteins including CPS1, SOD1, PDC1, HMGCS2, VLCAD, IDH2, GAPDH, ECHA, and PKM2 [19,20,22–27]. Both the malonylome and succinylome dataset indicate that SIRT5 plays an important role in cellular metabolism and participates in pathology of human diseases like cardiac dysfunction, type 2 diabetes, and neurodegenerative diseases [15].

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Most of the studies focused on full-length mouse or human SIRT5 ($SIRT5^{iso1}$). There is only one protein-coded SIRT5 mRNA found in mouse, but four SIRT5 mRNAs in human, as has been reported in NCBI database. In one report, human $SIRT5^{iso2}$ was partially characterized by its subcellular localization and shown to be a primate specific isoform [28]. Other biochemical characterizations of SIRT5 isoforms like enzymatic activity, tissue distribution remain to be investigated. In the present work, we compared the four SIRT5 isoforms in enzymatic activity, subcellular localization, and tissue expression, trying to gain further information about the human SIRT5 protein.

2. Materials and methods

2.1. Plasmid construction, cell culture and transfection

The full-length cDNA of human $SIRT5^{iso1}$ was obtained from cDNA library and subcloned into the pcDNA3.1/3×flag vector using the primers: hSIRT5-iso1-F, 5-TCCGCTCGAGATGCGACCTCTCCAGATT-3; hSIRT5-iso1-R, 5-ATGGGGTACCGAAGAAACAGTTTCATTTTC-3. The cDNA of $SIRT5^{iso2}$ was made from that of $SIRT5^{iso1}$ by cloning with a different reverse primer: hSIRT5-iso2-R, 5-ATGGGGTACCGAATTCTTTATAATAATTAGAGATGAGATGGAGATCAAATGACTGAATCTGTTCGTAGC-3. The cDNA of $SIRT5^{iso3}$ was subcloned from $SIRT5^{iso1}$ by two steps PCR using the following primers: hSIRT5-iso3-F, 5-GCTTTATCAGGAAAAGGGTGTGAAGAGGCAGGCTGC-3; hSIRT5-iso3-R, 5- GCAGCCTGCCTCTTCACACCCTTTTCCTGATAAAGC-3. The cDNA of $SIRT5^{iso4}$ was cloned from $SIRT5^{iso1}$ by a different forward primer: 5-TCCGCTCGAGATGGGGAGCAAGGAGCCC-3. All the plasmids were sequenced before transfection. COS7, HeLa, HEK293T, HHL5, HepG2, A549, H1299, 16HBE, and K562 cells were cultured in DMEM supplemented with 10% fetal bovine serum in a humidified atmosphere of 5% CO_2. Mammalian expression vectors of the four SIRT5 isoforms were transfected into COS7 or HEK293T cells using the lipofectamine 2000 reagent.

2.2. Immunoblotting and immunofluorescence microscopy

SDS-PAGEs were performed following standard procedures. Anti-COX IV(60251-1-Ig, PTM-Biolabs), anti-succinyl lysine (PTM-401, PTM-Biolabs), anti-Flag (F3165, Sigma-Aldrich), anti-SIRT5-AB1 (15122-1-AP, PTM-Biolabs), and anti-SIRT5-AB2 antibodies (AV32391, Sigma-Aldrich) were used for the immunoblotting experiments. Transfected cells were fixed with 4% paraformaldehyde for 20 min at room temperature. Then washed twice with PBS and permeabilized with 0.2% Triton X-100 for 10 min, blocked with 5% bovine serum albumin after another two times washing. Cells were next incubated with primary antibody overnight at 4 °C, and secondary antibody for 1 h at room temperature. DAPI was added for 10 min and wash away before observing with the confocal laser scanning microscopy.

2.3. Purification of SIRT5 isoforms from mammalian cells

After 24 h of transfection, cells were collected and treated with lysis buffer (50 mM Tris/Cl, pH 7.5, 150 mM NaCl, 1 mM DTT, 1% Triton X-100, protease inhibitor cocktail) for 20 min on ice. Cell lysate was then centrifuge for 10 min at 12,000 g. The supernatant was transferred to a chilled test tube containing prepared anti-flag M2 affinity resin. After binding at room temperature for 2 h, the resin was washed three times with TBS, and eluted with 3×flag peptide. Concentration of the eluted SIRT5 protein isoforms were measured and stored in storage buffer (50 mM Tris/Cl, pH 8.0, 265 mM NaCl, 0.2 mM DTT, 10% glycerol).

2.4. Activity assay of SIRT5 isoforms

Two experiments were performed to measure the enzymatic activity of different SIRT5 isoforms. First experiment measures their activity at the peptide level. The procedure was similar to method of a previous literature [29]. Briefly, a succinyl peptide derivative was synthesized (2-Abz)-GVLK (succ)A [Y (3-NO_2)]GV-NH_2 and dissolved in buffer (50 mM Tris/Cl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$). The reactions were performed in assay buffer (50 mM Tris/Cl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1 mg/mL BSA) and measured in 96-well microtiter plates. Equal amount of purified SIRT5 isoform proteins (0.01 μM) were incubated with this peptide derivative (2 μM) at the present of 500 μM NAD^+ for 2 h at 37 °C. Then trypsin (0.01 mg/mL) was added for another 2 h. Fluorescence was detected at an excitation wavelength of 320 nm and emission wavelength of 420 nm. Second experiment measuring SIRT5 activity at the protein level. Briefly, succinylated BSA were incubated with equal amount of each types of SIRT5 isoforms, and desuccinylation of BSA was detected by pan anti-succinyl lysine antibody in the Western blot experiment.

3. Results

3.1. Analysis of sequence, structure and enzymatic activity of the four human SIRT5 isoforms

The full-length SIRT5 protein ($SIRT5^{iso1}$) contains the N-terminal 36 amino acid (AA) mitochondrial localization signal (MLS) peptide and the remaining NAD^+-dependent deacylase domain (Fig. 1A). Crystal structure shown that its deacylase domain contains three functional subdomains including a zinc-finger binding domain, a NAD^+ binding domain, and a substrate binding domain [30]. The zinc-finger binding domain comprises four cysteines from AA 166 to 212. The NAD^+ binding domain is composed of several sequence-discontinuous, but spatial-closed AAs involving AA 58–77, AA 292–293 etc. The substrate binding domain is made up of lysine binding AA 221–254 and acyl-group binding AA 102–105 [11,30]. Based on the crystal structures of human $SIRT5^{iso1}$, we analyzed the potential effect of sequence variance to its enzymatic activity of the other three isoforms. The $SIRT5^{iso2}$ contains a different C-terminal sequence comparing with $SIRT5^{iso1}$ (Fig. 1C). Cysteine 293 (C293) on C-terminal of $SIRT5^{iso1}$ facilitates its binding of NAD^+ to SIRT5. Replacement of this residue might affect the binding capacity of NAD^+. $SIRT5^{iso3}$ differ from $SIRT5^{iso1}$ by missing the AAs 189–206, which localized near by the zinc finger domain (Fig. 1D). Missing of this sequence in $SIRT5^{iso3}$ probably destroy the formation of zinc-finger, which is important for the deacylase activity. Deletion of N-terminal AAs in $SIRT5^{iso4}$ has great impact on its activity because of the loss of two key residues Y102 and R105, which are critical for binding of acyl group from substrate peptides (Fig. 1E). The analysis suggest that $SIRT5^{iso2-4}$ should be less active than that of $SIRT5^{iso1}$.

To validate our analysis based on the structure data, we measured the deacylase activity of these SIRT5 isoforms. The activity assay is based on a synthesized peptide derivative which comprises one succinyl lysine and a pair of fluorophore and quencher [29]. Desuccinylation of the peptide derivative enable cutting of the peptide by trypsin, thus separate the fluorophore from quencher. The activity of SIRT5 is reflected by the relative fluorescence unit (RFU). For $SIRT5^{iso1}$, an increase of RFU was detected as the concentration of peptides derivative become higher. The Km value of $SIRT5^{iso1}$ on the peptides derivative was 4.2 μM, which was similar to the previous data measured with histone H3K9 succinyl peptides [29]. However, little fluorescence signal was detected on $SIRT5^{iso2-4}$, even at the higher concentration of substrate peptides (Fig. 2A). To further determine the desuccinylase activity of the four SIRT5

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