Purification and function analysis of the Δ-17 fatty acid desaturase with or without transmembrane domain

HAOYU ZHOU and CHENGMING WANG

Department of Food Science and Technology, Huazhong Agricultural University, Wuhan, Hubei 430000, P.R. China

Received August 16, 2016; Accepted April 11, 2017

DOI: 10.3892/etm.2017.4790

Abstract. Fatty acid desaturation enzymes perform dehydrogenation reactions leading to the insertion of double bonds in fatty acids. ω-3 desaturase has an important role in converting ω-6 fatty acids into ω-3 fatty acids. Although genes for this desaturase have been identified, the enzymatic activity of Δ-17 with or without transmembrane domain, and the function of the Δ-17 desaturase is poorly understood. In the present study, a transgenic microorganism was used to clone the Δ-17 full length (Δ-17FL) and Δ-17 without transmembrane domain (Δ-17NT), the expression efficiency was improved and western blotting was used to detect the protein expression level. The purification of Δ-17 was precipitated using saturated ammonium sulfate solution, dissolved in phosphate buffered saline buffer, and then filtered using a 10 kDa ultrafiltration cube. Gas chromatography analysis was used to measure the effect of Δ-17NT or Δ-17FL expression on Pichia pastoris fatty acid composition. Furthermore, the function of Δ-17NT in HepG2 cells was measured and the mechanism was explored. It was demonstrated that Δ-17NT decreased cell growth and increased apoptosis in hepatocellular carcinoma cell lines in vitro. In conclusion, successful expression of high levels of recombinant Δ-17NT represents a critical step towards the elucidation of the function of membrane fatty acid desaturases.

Introduction

It is understood that mammals lack Δ-12, Δ-15 and Δ-17 fatty acid desaturases, thus are unable to synthesize ω-6 and -3 fatty acids (1). The homeostasis stability of ω-6 and -3 polyunsaturated fatty acid has an important role in the normal growth and development of the body environment (2,3). Fatty acid desaturase enzymes may be divided into soluble enzymes and membrane-bound enzymes (4). Although some progress has been made on the study of desaturases, due to the technical constraints of membrane protein extraction and crystallization, the knowledge about the structure and expression regulation of membrane-bound fatty acid desaturases is still lacking (5,6), and whether the transmembrane domain has a role in fatty acid desaturase efficiency remains unknown.

Using transgenic technology to effectively express saturated fatty acid desaturase genes in lower eukaryotes, ω-6 polyunsaturated fatty acids may be catalyzed into the corresponding ω-3 polyunsaturated fatty acids (7). To obtain a highly expressed and purified membrane-bound fatty acid and verify its activity is the first step for understanding the structure and function of the desaturase (8). The production of some new, high-value fatty acids may better control enzyme activity and substrate specificity, and provide important scientific insight (9,10).

In the present study, Δ-17 fatty acid desaturase was optimized and the transmembrane of Δ-17 fatty acid desaturase was removed. A yeast eukaryotic expression vector was constructed and, to improve the expression efficiency and compare the enzymatic activity, western blotting was utilized to detect protein expression levels. Through optimization of the ω-3 desaturase gene, it is able to convert ω-6 polyunsaturated fatty acids to ω-3 polyunsaturated fatty acids in mammalian cells (11). The discovery of the underlying mechanism and function of Δ-17 fatty acid desaturase with or without transmembrane domain in HepG2 cells may contribute to the understanding of the fatty acid desaturase.

Materials and methods

Clone of Δ-17 fatty acid desaturase recombinant plasmid. The original sequence of Δ-17 of Phytophthora ramorum was obtained from the National Center for Biotechnology information (FW362214.1; https://www.ncbi.nlm.nih.gov) MaxCodon TM Optimization Program v. 13.0 (Detai Biologics co., Ltd., Nanjing, China) was used to optimize the code of Δ-17FAD. The expression vector, pPICZαA, was purchased from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The above plasmid was amplified in DH5α bacteria (Promega Corporation, Madison, WI, USA) in Luria Bertani medium (Tryptone 10 g/l, Yeast extract 5 g/l and NaCl 10 g/l; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with 100 µg/ml ampicillin at 37°C. After shaking at 250 rpm for 16 h, the plasmid was purified using a Qiagen Maxi

Correspondence to: Professor Chengming Wang, Department of Food Science and Technology, Huazhong Agricultural University, 1 Shizi Shan Street, Wuhan, Hubei 430000, P.R. China
E-mail: chengmingwang999@sina.com

Key words: Δ-17 fatty acid desaturase, purification, function analysis, transmembrane domain
kit (Qiagen, Inc., Valencia, CA, USA), according to the manufacturer's instructions. The optimized sequence of the Δ-17 was as follows: 1 ATGGCTACCAAGGACACCTTTATCACGGTCC TACTTTGACCGAGATTAAGAGATCCCTCCTCT; 61 TCA GAATGTGTTTGAGGCATCGATCCCTGCTGTCTTTTATCT ATACAGTTGAATTTGCGCA; 121 ATCTGGTTGCTGGAGTTGAGTCTGCTCTT GTGTGTCGAGTTGTCCTTCATCAAGTTATGTCTCTT; 241 TGGGAGATTCTATCCTGGTG TCACAGCGACAG CTAAGGAGCT GTACATCTTTCTCAGATGACCT; 301 CTT AACTCTTCTTCTGCTATCTATCATTATCTATGC TATCTACTATTTTAAATGTT; 361 AAGTGGATCACA GACTATACATAAAACACCGTATTATGATAG ACGAGATCTCTT; 421 TATCCACAGAGAAATGATGTA CAATCCTGTTCTCTGAA ACTTGGTTCCTCTGCTT; 481 GCAGGCAGTTG TGTCATACCTGTTAGGAAG TTTCCACCTGAAAGTAACTTTTA; 541 CCA TTCGAGCCCTTTTTTGT TAGACAAGTCCGCGACGAT GTCTGTCATTTGAGCCAAT; 601 TTGCCTG TCCTTTGGCTTGTCTGTCTCTTCACTGTG CGGAAGT TTTCAAGACTATGCTG; 661 TTGTATCTATAGGCCG TATTGTTTGTCTCGCCCTGAAATGTTTTAAC; 721 CACCAACAGCATGAGAGACTCTCTGTG GTATGAGATGTT ACTGACATACTGTAAAGGGT; 781 AATTTACTTCTT CCCTGCTAGAT ATCTTAGGAGG TTTATACGACAACCTTGGTCCCAAAATATATT; 841 GGTACC CATCAATCAACACCACTTTTCCATCCCCCTTACT ACAATTTGAAAGAGCT; 901 ACTGCTGCCCTTTCTC AGGCGCTCCAGAACACTTGGGAAAGTTGGCGTAC CTATTTTG; 961 AAGCATCCTTGGAGATTTGGGAAG ACTTTAGTTACATCGGGTGTGTCGATACCGCC; 1021 AAATTTCTATATTTGAAAGCAAGAGACGC ATCCGGAGCAGCCCAACAAACTAGGCA; 1081 ACC.

The prediction of Δ-17 (ω-3) fatty acid desaturase transmembrane domain was performed using the TMHMM server, version 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). The Δ-17 desaturase full length (Δ-17FL) and Δ-17 without transmembrane domain (Δ-17NT) were synthesized from genomic DNA and mixed with the potential restriction digestion position Xhol and NotI (Takara Bio, Inc., Otsu, Japan). The expression vector, pPICZαA (an affiliate of Promega (Beijing) Biotech Co., Ltd, Beijing, China), was digested with Xhol and NotI. pMD™ 18-T Vector Cloning kit (Takara Bio, Inc.; catalogue no. 6011) was used to connect pPICZαA and Δ-17 (FL) or Δ-17 (NT) to obtain the recombinant plasmid, following the manufacturer's protocol. Then the recombinant plasmids, Δ-17NT and Δ-17FL, and expression vector, pPICZαA, were linearized using the restriction enzyme Sall (Takara Bio, Inc.). The reaction system was 50 µl, including 5 µl 10XBuffer H, 30 µl recombinant plasmid pPICZαA-Δ-17 (FL) or pPICZαA-Δ-17 (NT), 500 ng/µl, 1 µl Sal I (10 U/µl) and 14 µl ddH2O. The mixture was digested for 3 h at 37°C.

Preparation of Pichia pastoris competent cells. Yeast X-33 (Invitrogen; Thermo Fisher Scientific, Inc.) single colonies were picked fromYPD liquid medium and incubated on a shaking platform overnight (200 rpm) at 30°C. When the optical density at a wavelength of 600 nm (OD600) was 1.5, the cultures were centrifuged at 3,000 g for 5 min in 4°C. Subsequently, the pellet was resuspended using 300 ml sterile water. After centrifugation, the supernatant was discarded and 20 ml cold 1 M sorbitol was used to resuspend the cells. Following this, the cells were subpackaged and stored at -80°C until use.

Electroporation method. Electric rotor and lid (Gene Pulser Xcell™; Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to resuspend the cells, which were spread onto a YPDS medium containing zeocin (100 and 500 µg/ml). The cells were cultured at 28°C for 2.4 days.

P. pastoris genome DNA isolation. Monoclonal P. pastoris transformants were placed into YPD liquid medium and cultured overnight at 28°C. A total of 2 ml bacteria was taken from the culture and centrifuged at 13,000 x g for 2 min at 4°C. Subsequently, the supernatant was discarded and 1 ml phosphate-buffered saline was used to suspend the precipitate. The tubes were centrifuged at 12,000 rpm for 2 min at 4°C and the supernatant was discarded. This step was repeated once more. The precipitate was dissolved in 100 µl TE and heated in boiling water for 10 min. Subsequently, the sample was immersed in liquid nitrogen for 1 min, heated again in boiling water for 10 min and centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was used as a polymerase chain reaction (PCR) template.

PCR detection. The PCR mixture was as follows: 5 µl Taq buffer (10X), 1 µl dNTP mixture (2.5 mM), 1.0 µl each primer 20 pmol (forward: 5'-CTACTATTGCCCAGCATTGCTGC-3'; reverse: 5'-GGCAATGGCAGTTGACATCC-3'), 1.0 µl Taq polymerase (Takara Bio, Inc.), 1.0 µl genome DNA template and 5.0 µl DDH2O, giving a total reaction volume of 36.0 µl. The thermal cycling conditions were as follows: 94°C for 3 min, followed by 25 cycles of 94°C for 30 sec, 55°C for 45 sec and 72°C for 1 min, then 72°C for 10 min and finally held at 4°C. PCR (GeneAmp 9600 PCR system; PerkinElmer, Waltham, MA, USA) was performed. All experiments were performed in triplicate. PCR products were detected using 1% agarose gel.

Identification of P. pastoris recombinant protein expression. A total of five positive yeast transformants were selected, each single clone was placed into 25 ml buffered glycerol-complex medium [Yeast extract powder 10 g, peptone 20 g and 700 ml water; this was stirred until completely dissolved and then subjected to high pressure sterilization. It was then cooled to room temperature, followed by the addition of sterile: 10X YNB 100 ml, 1M potassium phosphate buffer (PPB)
100 ml, 100 ml 10X glycerin and 2 ml 500X biotin (Xilong Chemical Co., Ltd.) and placed on a shaking platform at 200 rpm (28°C), until OD600 reached 2-6. Subsequently, the mixture was centrifuged at 4,000 x g for 4 min at room temperature and the cells were collected. The bacterial cells were resuspended in 25 ml buffered methanol-complex medium (Yeast extract powder 10 g, peptone 20 g and 700 ml water; it was stirred until completely dissolved then subjected to high pressure sterilization. It was then cooled to room temperature, followed by the addition of sterile: 100 ml 10X YNB, 100 ml 1M PPB, 100 ml 10X absolute methanol and 2 ml 500X biotin) and placed on a shaking platform at 200 rpm (28°C) to induce expression for 4 days. Every 24 h, additional methanol was added at a 1% final concentration for further induction. Following this, 1 ml medium was taken and placed into a 2.0-ml centrifuge tube and centrifuged at 15,000 x g for 15 min and stored at -80°C for fatty acid analysis.

**In vivo desaturase activity analysis.** The induced culture of the transformation in the BMMY medium is the same as the aforementioned method, 1% NP40, 0.5% methanol and a final concentration of 100 µM substrate arachidonic acid (ARA; Sigma-Aldrich; Merck KGaA) was added to the medium. Cell pellets were collected by centrifugation at 15,000 x g and 4°C for 15 min and stored at -80°C for fatty acid analysis.

**In vitro desaturase activity analysis.** After expansion of the bacterial culture expressing Δ17FL, the medium was collected using purification. The medium was precipitated with ammonium sulfate and re-dissolved with 30 ml of 20 mM Phosphate Buffer, pH 7.2, and then Strong cation exchange medium (SP) Sepharose Fast Flow column (GE Healthcare, Chicago, IL, USA) was used for purification. 20 ml purified protein was added to 200 ml yeast cell homogenate. Yeast cells were lysed on ice for 30 min by adding lysis buffer (20 mM Tris-HCl, pH 7.9, 500 mM NaCl, 10% glycerol, 1 mM EDTA and 1% NP40; Sigma-Aldrich; Merck KGaA), and a high pressure cell crusher (AH-1500; ATS Engineering Inc., Brampton, ON, Canada) was used to prepare the bacteria, the whole process operates on an ice bath. The enzyme reactions were performed at 28°C for 3 h with agitation (250 rpm), and the assay mixture (220 ml) was stored at -80°C for fatty acid analysis.

**Extraction of fatty acid and methyl esterification.** The transformant cells were flash frozen and ground into powder in liquid nitrogen with a mortar. A total of 200 mg powder was placed into a 10-ml centrifuge tube, 3 ml of chloroform-methanol (v/v=2:1) was added and the tube was vortexed for 30 min. Then 1 ml distilled water was added and mixed. Tubes were centrifuged at 4,000 x g for 10 min in 4°C, leaving the organic phase. The oil phase of nitrogen was added with 1 ml acetyl chloride-methanol and vortexed for 3 min. After incubated in a water bath at 92°C for 30 min, 2 ml cold water and 400 µl n-hexane (Xilong Chemical Co., Ltd.) were added, vortexed for 1 min and centrifuged at 5,500 x g for 5 min at 4°C. Subsequently, the upper organic phase was transferred into 1.5-ml vials for gas chromatographic analysis.

**Gas chromatography analysis.** An Agilent 6890A gas chromatograph with a flame ionization detector and a column of HP-5 fused silica (length, 30 m; inner diameter, 0.32 mm; Agilent Technologies, Inc., Santa Clara, CA, USA) was used for analysis. The inlet and detector temperatures were both 250°C. The high-purity carrier gas used was He, with a flow rate of 7.5 ml/min. H2 gas flow rate was 30 ml/min, air flow rate was 400 ml/min and makeup was 20 ml/min. The injection volume was 1 µl, using the split mode, and the split ratio was 20:1. By comparison with standard fatty acid (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) retention time to identify each fatty acid component, a calculated percentage area of each fatty acid was used to calculate component of normalization.

**Western blotting.** Protein expression levels of phosphorylated (p)-glycogen synthase kinase (GSK)-3β, GSK-3β, β-catenin, p-protein kinase B (Akt) and Akt were determined in HepG2 cells from American Type Culture Collection (Manassas, VA, USA). The cell lysates were obtained using lysis buffer (50 mM Tris-Cl, pH 7.4; 1% NP-40; 150 mM NaCl; 1 mM EDTA; and, 0.5% sodium deoxycholate; all BD Biosciences) containing 1% protease inhibitors. Following centrifugation at 15,000 x g for 10 min at 4°C, the supernatant was collected. The protein concentration was detected using a bicinchoninic acid kit (Thermo Fisher Scientific, Inc., Protein (30 µg) from the vector and the treated group (Δ17 overexpression) was separated on 10% SDS-PAGE and transferred onto nitrocellulose membranes. The Δ17 overexpression was the only group assessed because when the expression of target genes was identified by SDS-PAGE, Δ17FL exhibited a clear expression, while the Δ17NT exhibited no obvious band. After blocking the membranes with 5% no fat milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h at room temperature, the membranes were incubated with the primary indicated antibodies Phosphorylation-GSK-3β (catalogue no. sc-135653; 1:1,000), β-catenin (catalogue no. sc-419477; 1:1,000), caspase-3 (catalogue no. sc-271028; 1:1,000), caspase-9 (catalogue no. sc-17784; 1:500; all Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and anti-β-actin (catalogue no. 612656; 1:2,000; BD Biosciences, Franklin Lakes, NJ, USA) at 4°C overnight. Subsequently, membranes were washed five times for 5 min with TBST, incubated with horseradish peroxidase-conjugated immunoglobulin G goat anti-mouse IgG-HRP, (catalogue no. sc-2005; 1:3,000) goat and anti-rabbit IgG-HRP, (catalogue no. sc-2004; 1:3,000; both Santa Cruz Biotechnology, Inc.) at room temperature for 1 h and washed again five times for 5 min with TBST. The membranes were visualized using an enhanced chemiluminescence system (Sigma Aldrich; Merck KGaA), quantified using Image J version 2.1.4.7 (National Institutes of Health, Bethesda, MD, USA), β-actin was used as an internal control.

**Cell viability assay.** MTT assay was used to evaluate cell viability in vitro. Briefly, 2x10^5 HepG2 cells were seeded into
a 96-well plate for each group [Vector and Δ17 (NT) group]. The relative cells were incubated for 1, 2, 3 and 4 days at 37˚C, MTT in PBS (5 µg/µl) was added to each well and incubated for 4 h at 37˚C. Following removal of the supernatant, 150 µl dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA) was added to each well. The absorbance value was measured at 490 nm. Each experiment was performed in triplicate.

DNA fragmentation analysis. Apoptosis was detected and quantified by the Cell Death Detection ELISA PLUS kit (Roche Diagnostics, Ltd., Burgess Hill, UK), following the manufacturer's instructions. All experiments were performed in triplicate, independently. A DNA fragmentation terminal deoxynucleotidyl transferase dUTP nick end labeling assay was performed as follows: briefly, 50,000 cells were seeded with a regular DMEM medium at 37˚C with 5% CO2 and cultured under they reached 70-80% confluence. Next, cells were washed twice for 5 min with PBS and moved to DMEM without serum. At 0, 24, 48 and 72 h, pool DNA was extracted in a total of 400 µl extraction buffer (10 mM Tris and 5 mM EDTA). DNA fragmentation was calculated by the fold change, as compared with the vector and normalized with corresponding MTT results from identical culture conditions. The samples were analyzed by western blot analysis as described using caspase-3 (catalogue no. sc-271028; 1:1,000), caspase-9 (catalogue no. sc-17784; 1:500) and anti-β-actin (catalogue no. 612656; 1:2,000).

Statistical analysis. Statistical analyses were performed using SPSS v. 11.0 software (SPSS, Inc., Chicago, IL, USA). The results were expressed as the mean ± standard deviation. Differences between the groups were analyzed by one-way analysis of variance or a t-test followed by post-hoc test (Dunnett’s test). P<0.05 was considered to indicate a statistically significant difference.

Results

Cloning of full length or without transmembrane domain Δ-17 (ω-3) fatty acid desaturase. As predicted using the TMHMM, the secondary structure of Δ-17 Fatty acid desaturase had six transmembrane domains; the positions were from 30-52, 61-83, 98-120, 151-170, 190-212 and 219-241 nt (Fig. 1A). The Δ-17 desaturase full length (Δ-17FL) and Δ-17 without transmembrane domain (Δ-17NT) were synthesized with the potential restriction digestion position XhoI and NotI, and connected using T4 DNA ligase. (C) The relative plasmids were digested using the restriction enzyme XhoI and Xhol. NT, without transmembrane domain.
Expression and optimization conversion efficiency of Δ-17NT or Δ-17 full length. The recombinant plasmids, Δ-17NT and Δ-17FL, and expression vector, pPICZαA, were linearized using the restriction enzyme SalI. The working model is demonstrated in Fig. 2A. Preparation of P. pastoris competent cells is demonstrated in Fig. 2B, using an electroporation method to induce recombinant yeast transformants containing Δ-17NT or Δ-17FL fragments. PCR was used to identify the genome of the transformants, as demonstrated in Fig. 2C and D. The fragments of Δ-17NT or Δ-17FL were correctly inserted into P. pastoris, as detected by PCR with specific primers. The protein expression of Δ-17FL was measured by SDS-PAGE, as indicated in Fig. 3A. A positive control was used to confirm the efficiency, while conditioned medium of pPICZαA-Δ-17FL without induction was used as a negative control. Similarly, the protein expression of Δ-17NT is demonstrated in Fig. 3B.

Effect of Δ-17NT or Δ-17FL expression on P. pastoris fatty acid composition. In order to understand the effect of Δ-17NT or Δ-17FL expression on P. pastoris fatty acid composition, the PCR positive transformants with empty vector or recombinants containing Δ-17NT or Δ-17FL were transferred to 3-ml tubes containing liquid medium. 1% NP40, 0.5% methanol and a final concentration of 100 µM substrate ARA was added to the medium. The blank control of each group was used without adding ARA (Arachidonic acid) as substrate. All samples were added to methanol for induction every 24, and 96 h after fermentation, the cultured cells were collected by centrifugation and used for extraction of fatty acid and methyl esterification, followed by gas chromatography analysis.

As demonstrated in Fig. 4A, 37 kinds of standard fatty acid methyl products were analyzed using gas chromatography analysis. Transfection with empty vector in yeast fermentation broth was conducted to analyze fatty acid composition, with or without added ARA (Fig. 4B). Fig. 5A indicates the gas chromatography results after transfection with Δ-17FL gene in yeast fermentation broth to analyze fatty acid composition. Fig. 5B demonstrated the gas chromatography results after transfection with the Δ-17NT gene in yeast fermentation broth to analyze fatty acid composition, by comparison with retention time in standard fatty acid to identify each fatty acid component, to calculate each fatty acid component.
using the area normalization method. It was demonstrated in Figs. 4B and 5 that, when ARA was added as a substrate to the induction medium, the results were markedly different in the Δ-17FL group compared with the empty vector group and the Δ-17NT transfection group, in the transfection group, the substrate ARA was decreased and the product EPA, DPA were increased. This indicates that the target gene, Δ-17FL, may serve a normal function of the ω3 Fatty acid desaturase, converting ω6 Fatty acid to ω3 Fatty acid. However, the result was similar in empty vector group and the Δ-17NT transfection group. However, in the no-ARA groups, there was no obvious difference between the empty vector, the Δ-17FL or the Δ-17NT transfection groups.

Purification and activity analysis of Δ-17 fatty acid desaturase. SDS-PAGE was used to detect the dissolution and purification of Δ-17FL, as demonstrated in Fig. 6A and B. Also, compared with the retention time in 37 standard fatty acids to identify each fatty acid component, after purification and after ARA had been added as a substrate, under the action of Δ-17FL, ARA/(EPA + DPA) from a ratio of 8.18:1 (empty vector group; Fig. 6C) was reduced to 3.60:1 (yeast transected with Δ-17FL group). The results were markedly different compared with the empty vector group (Fig. 7A). While under the influence of Δ-17NT, ARA/(EPA + DPA) from a ratio of 8.18:1 (empty vector) was reduced to 4.11:1 (Δ-17NT group). The results were markedly different compared with the empty vector group (Fig. 7B). As for the role of the purified enzyme Δ-17FL (Fig. 7B), ARA/(EPA + DPA) from a ratio of 8.18:1 (empty vector group) was reduced to 3.34:1 (Δ-17FL purification group), the results were markedly different compared with the empty vector group (Fig. 8). However, in the Δ-17NT group, the enzyme activity was lower compared with the Δ-17FL purification group.

Expression of pPICZαA-Δ-17NT decreases cell viability and increases apoptosis in vitro. To determine whether Δ-17NT inhibits cell viability in vitro, MTT assays were performed in HepG2 cells cultured with Δ-17NT for different time periods at 0, 24, 48, 72 and 96 h. As demonstrated in Fig. 9A, expression of pPICZαA-Δ-17NT significantly inhibited cell viability compared with cells that were transfected with control vector at
Table 1. Purification and activity analysis of Δ-17 fatty acid desaturase.

(A) SDS-PAGE was used to detect the dissolution of Δ-17 after precipitation with ammonium sulfate. (B) The purification of Δ-17FL was detected using SDS-PAGE after dialysis with 3.5 kDa dialysis bag and SP Sepharose FF column. BSA was used to confirm the expression level. (C) Gas chromatography analysis was used to analyze fatty acid composition after transfection with empty vector in yeast fermentation broth, ARA was either added as a substrate (upper panel) or not added (lower panel). FL, full length; ARA, arachidonic acid; SP, Sepharose; FF, Fast Flow.

Figure 6. Purification and activity analysis of Δ-17 fatty acid desaturase.

(A) SDS-PAGE was used to detect the dissolution of Δ-17 after precipitation with ammonium sulfate. (B) The purification of Δ-17FL was detected using SDS-PAGE after dialysis with 3.5 kDa dialysis bag and SP Sepharose FF column. BSA was used to confirm the expression level. (C) Gas chromatography analysis was used to analyze fatty acid composition after transfection with empty vector in yeast fermentation broth, ARA was either added as a substrate (upper panel) or not added (lower panel). FL, full length; ARA, arachidonic acid; SP, Sepharose; FF, Fast Flow.

72 and 96 h (P<0.001). To evaluate whether pPICZαA-Δ-17NT had a role in programmed cell death, a DNA fragmentation terminal deoxynucleotidyl transferase dUTP nick end labeling assay was performed in HepG2 cells. The apoptosis of HepG2 cells was significantly enhanced in the pPICZαA-Δ-17NT expression groups at 48 and 72 h compared with the control vector (P<0.05; Fig. 9B). To further investigate the mechanism of Δ-17NT expression on the inhibition of tumor cell proliferation, proteins involved in cell proliferation were examined, including p-GSK-3β and β-catenin, (Fig. 9C). It was demonstrated that Δ-17NT expression markedly reduced the expression level of p-GSK-3β and β-catenin compared with the control vector, which indicated that Δ-17NT inhibited cell proliferation. As for cell apoptosis, the cleavage of caspase-9 and caspase-3 was markedly enhanced in cells transfected with Δ-17NT compared with cells transfected with the control vector (Fig. 9D). The above results indicated that expression of Δ-17NT induced caspase-3-mediated cell apoptosis and inhibited cell viability and proliferation.


Discussion

Three new ω-3 desaturases that convert ω-6 fatty acids to the ω-3 unsaturated form have been identified and isolated, all of which belong to the type II desaturase that introduce a double bond near the methyl end of an unsaturated fatty acid (12,13).

The transmembrane domain is a strong hydrophobic transmembrane region, and exhibits good hydrophobicity within the membrane (14). There is no necessary association between the extracellular receptor function and transmembrane domain (15). Contrastingly, the interaction between highly hydrophobic peptides and important proteins within the host cell, such as the signal recognition particle complex, may lead to protein expression inhibition or protein precipitation (16-18). Therefore, in the present study, the function of full-length Δ-17 and Δ-17 without transmembrane domain was analyzed and compared. It was demonstrated that after the addition of ARA as a substrate, the effect of Δ-17NT or Δ-17FL expression on P. pastoris fatty acid composition was highly efficient for the transformation of ARA compared with the vector.

Previous studies have demonstrated that ω-3 fatty acids may antagonize the stimulative roles of ω-6 fatty acids in different types of carcinogenesis, such as lung cancer (19) and prostate cancer (20), to regulate tumor proliferation and metastasis. The present study revealed that in a hepatocellular carcinoma cell line, Δ-17NT also decreased cell viability and increased apoptosis in vitro, which further demonstrated the function of ω-3 fatty acids in cancer progression (21,22).

In conclusion, understanding the underlying mechanism of the function of Δ-17 fatty acid desaturase with or without the transmembrane domain in HepG2 cells may contribute to the understanding of the contribution of fatty acid desaturases in tumor progression, which may serve as a therapeutic target in the future.

References

1. Amminger GP, Schäfer MR, Papageorgiou K, Klier CM, Cotton SM, Harrigan SM, Mackinnon A, McGorry PD and Berger GE: Long-chain omega-3 fatty acids for indicated prevention of psychotic disorders: A randomized, placebo-controlled trial. Arch Gen Psychiatry 67: 146-154, 2010.
2. Bézard J, Blond JP, Bernard A and Clouet P: The metabolism and availability of essential fatty acids in animal and human tissues. Reprod Nutr Dev 34: 539-568, 1994.
3. Driss F, Duranthon V, Darce P and Henry O: Effects of dietary omega-6 and omega-3 fatty acids on levels of long chain polyunsaturated fatty acids in erythrocytes. C R Seances Soc Biol Fil 185: 14-20, 1991 (In French).
4. Shanklin J and Cahoon EB: Desaturation and related modifications of fatty ACIDS1. Annu Rev Plant Physiol Plant Mol Biol 49: 611-641, 1998.
5. Ameur A, Enroth S, Johansson A, Zaboli G, Jgl W, Johansson AC, Rivas MA, Daly MJ, Schmitz G, Hicks AA, et al: Genetic adaptation of fatty-acid metabolism: A human-specific haplotype increasing the biosynthesis of long-chain omega-3 and omega-6 fatty acids. Am J Hum Genet 90: 809-820, 2012.
6. Sakuradani E, Kobayashi M, Ashikari T and Shimizu S: Identification of Delta12-fatty acid desaturase from arachidonic acid-producing mortierella fungus by heterologous expression in the yeast Saccharomyces cerevisiae and the fungus Aspergillus oryzae. Eur J Biochem 261: 812-820, 1999.
7. Muhllauser BS, Cook-Johnson R, James M, Miljkovic D, Duthoit E and Gibson R: Opposing effects of omega-3 and omega-6 long chain polyunsaturated fatty acids on the expression of lipogenic genes in omental and retroperitoneal adipose depots in the rat. J Nutr Metab 2010: pii: 927836, 2010.
8. Markus M, Husen B, Leenders F, Seedorf U, Jungblut PW, Hall PH and Adamski J: Peroxisomes contain an enzyme with 17-beta-estradiol dehydrogenase, fatty acid hydratase/dehydrogenase, and sterol carrier activity. Ann N Y Acad Sci 804: 691-693, 1996.
9. Carballera NM, Montano N, Balafna-Fouce R and Prada J: First total synthesis and antiprotozoal activity of (Z)-17-methyl-13-octadecenoic acid, a new marine fatty acid from the sponge Polymastia penicillus. Chem Phys Lipids 161: 38-43, 2009.
10. Kim DH, Choe YS, Choi JY, Choi Y, Lee KH and Kim BT: 17-[4-(2-[18F]fluoroethyl)-1H-1,2,3-triazol-1-yl]-6-thia-heptadecanoic acid: A potential radiotracer for the evaluation of myocardial fatty acid metabolism. Bioconjug Chem 20: 1139-1145, 2009.

11. Xue X, Feng CY, Hixson SM, Johnstone K, Anderson DM, Parrish CC and Rise ML: Characterization of the fatty acyl elongase (elovl) gene family, and hepatic elovl and delta-6 fatty acyl desaturase transcript expression and fatty acid responses to diets containing canola oil in Atlantic cod (Gadus morhua). Comp Biochem Physiol B Biochem Mol Biol 175: 9-22, 2014.

12. Zhu G, Saleh AA, Bahwal SA, Wang K, Wang M, Wang D, Ge T and Sun J: Overexpression of four fatty acid synthase genes elevated the efficiency of long-chain polyunsaturated fatty acids biosynthesis in mammalian cells. Sheng Wu Gong Cheng Xue Bao 30: 1464-1472, 2014 (In Chinese).

13. Uttaro AD: Biosynthesis of polyunsaturated fatty acids in lower eukaryotes. IUBMB Life 58: 563-571, 2006.

14. Fink A, Sal-Man N, Gerber D and Shai Y: Transmembrane domains interactions within the membrane milieu: Principles, advances and challenges. Biochim Biophys Acta 1818: 974-983, 2012.

15. Lim ZL, Senger T and Vrinten P: Four amino acid residues influence the substrate chain-length and regioselectivity of Siganus canaliculatus Δ4 and Δ5/6 desaturases. Lipids 49: 357-367, 2014.

16. Kempl G, Wild K and Sinning I: Structure of the complete bacterial SRP Alu domain. Nucleic Acids Res 42: 12284-12294, 2014.

17. Kuhn P, Weiche B, Sturm L, Sommer E, Drepper F, Warscheid B, Sourijk V and Koch HG: The bacterial SRP receptor, SecA and the ribosome use overlapping binding sites on the SecY translocon. Traffic 12: 563-578, 2011.

18. Braig D, Bär C, Thumfart JO and Koch HG: Two cooperating helices constitute the lipid-binding domain of the bacterial SRP receptor. J Mol Biol 390: 401-413, 2009.

19. Xia SH, Wang J and Kang JX: Decreased n-6/n-3 fatty acid ratio reduces the invasive potential of human lung cancer cells by downregulation of cell adhesion/invasion-related genes. Carcinogenesis 26: 779-784, 2005.

20. Lu Y, Nie D, Witt WT, Chen Q, Shen M, Xie H, Lai L, Dai Y and Zhang J: Expression of the fat-1 gene diminishes prostate cancer growth in vivo through enhancing apoptosis and inhibiting GSK-3 beta phosphorylation. Mol Cancer Ther 7: 3203-3211, 2008.

21. Li X, Ballantyne LL, Che X, Mewburn JD, Kang JX, Barkley RM, Murphy RC, Yu Y and Funk CD: Endogenously generated omega-3 fatty acids attenuate vascular inflammation and neointimal hyperplasia by interaction with free fatty acid receptor 4 in mice. J Am Heart Assoc 4: pii: e001856, 2015.

22. Jeong S, Jing K, Kim N, Shin S, Kim S, Song KS, Heo JY, Park JH, Seo KS, Han J, et al: Docosahexaenoic acid-induced apoptosis is mediated by activation of mitogen-activated protein kinases in human cancer cells. BMC Cancer 14: 481, 2014.