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Apoptosis related protein 3 is a lysosomal membrane protein

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ABSTRACT

Apoptosis Related Protein 3 (APR3) is an important protein which is involved in retinoic acid-induced apoptosis, osteoblast differentiation and cervical squamous cell carcinoma progression. Although it was predicted to be a trans-membrane protein, its cellular localization is not clear. In this study, we analyzed APR3 with bioinformatic tools and found that APR3 contains a potential signal peptide, a transmembrane region and 3 N-glycosylation sites, all of which are characteristics of lysosomal proteins. Western blot with isolated lysosomes demonstrated that APR3 was mainly present in lysosomes, specially in the lysosomal membrane fraction, but not in endoplasmic reticulum. Concomitantly, double immunofluorescence confirmed that APR3 co-localized with lysosomal membrane protein, LAMP1, as well as lysosomal specific marker, Lyso-Tracker Red. Moreover, we showed that APR3 was highly expressed in the lung, liver, spleen, kidney and adipose tissue, but expressed at the low level in the heart, pancreas, stomach and intestine. Interestingly, APR3 expression was elevated in multiple hepatocellular carcinoma cell lines comparing to normal liver cells. Collectively, our results proved that APR3 is a novel lysosomal membrane protein and shed light on its possible functions.

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1. Introduction

Apoptosis Related Protein 3 (APR3) was first identified to be deferentially expressed when human promyelocytic leukemia cells were induced for differentiation and apoptosis with retinoic acid (RA) [1]. Later, it was proved that RA induced APR3 expression not only in promyelocytic leukemia cells, but also in many other cell lines [2]. Thus, it was also termed as all-trans retinoic acid-induced differentiation factor (ATRAID). There were only limited reports about the cellular functions of APR3. It was shown APR3 inhibited Cyclin D1 expression and consequently caused cell G1/S arrest [2]. Another study demonstrated that APR3 interacted with NEL-like 1 (NELL-1) in sarcoma cells and cooperatively functioned with NELL-1 to repress Cyclin D1 expression and osteoblast differentiation [3]. Beside that, evidences also implied that APR3 might be involved in cancer progression. APR3 was overexpressed in cervical squamous cell carcinoma and its high expression was associated with lymph node infiltration [4].

APR3 was predicted to be a trans-membrane protein based on sequence similarity [2]. It contains a transmembrane domain in the middle, as well as a signal sequence and EGF-like domain at the N-terminus. Overexpression of GFP-tagged APR3 in MCF-7 cells drove the protein to the cell membrane [2]. However, when ARP3-specific antibody was used to map the protein in the immunofluorescence experiment, it was labeled perinuclearly in multiple cell lines [3]. Although it is accepted that APR3 is a transmembrane protein, which exact membrane system APR3 is localized at still needs to be addressed.

Lysosomes are a vesicular organelle encircled by a single lipid bilayer. Inside the lysosomal lumen are more than 60 different acidic hydrolases, such as cathepsins [5,6]. The major function of lysosomes is to degrade biological macromolecules, such as proteins, polysaccharides, lipids and nucleic acids delivered by endocytosis or autophagy. The lysosomal compartment is acidic, with a pH of 4.5–5.0 [7]. The acidic environment maintained by proton pumps on lysosomal membrane is pivotal for its functions. Most lysosomal hydrolases are pH sensitive and have optimal enzymatic activity at low pH.

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Defects of lysosomal functions have been observed in multiple diseases, including neurodegenerative disorders, cancer, and cardiovascular diseases [8]. One of the causes of lysosome-related diseases can be attributed to the accumulation of macromolecules in lysosomes. The other causes may lie behind the role of lysosomes in programmed cell death [7,8]. Lysosomes are a mediator of apoptosis, upstream of the mitochondrial signaling. Various stimuli can induce the leakage of lysosomal membrane, a phenomenon termed as lysosomal membrane permeabilization (LMP) [9]. LMP results in the release of cathepsins which will cleave precursors of apoptosis signaling molecules to their active form. A well-studied substrate of cathepsin is Bid. Cleavage of Bid lead to mitochondria outer membrane permeablization (MPTP), subsequently promote cytochrome C release to cytoplasm, activate Apaf-1 and caspase-9, initiate the cascade reaction of apoptosis-related factors, at last result in apoptosis. A better understanding of lysosomal components will help us learn more about lysosome function and related diseases. We have isolated lysosomal components and identified 2 peptides from APR3 by mass spectrometry in a previous study. Here we further provided evidences that apoptosis related protein-3 (APR3) is a lysosomal membrane protein.

2. Method and materials

2.1. Bioinformatic analysis

The signal peptide, transmembrane region, and N-glycosylation sites of APR3 was analyzed with softwares of SignalP, TMHMM, and NetNGlyc on Denmark Technology University (DTU) sever, respectively [10,11] (http://www.cbs.dtu.dk/services/SignalP/, http://www.cbs.dtu.dk/services/TMHMM/, http://www.cbs.dtu.dk/services/NetNGlyc/). Hydrophobility of APR3 was analyzed with ExPASy sever [12] (http://web.expasy.org/cgi-bin/protscale/protscale.pl?1).

2.2. Isolation of lysosomes

Four to six livers from 8 week old male B129 mice were washed with cold PBS for 3 times and carefully minced with scissors. They were then homogenized on ice in 4–5 X volume of cold 0.25 M sucrose complemented with proteinase inhibitors (PIs, containing 5 μg/ml Leupeptin, 5 μg/ml Pepstatin A, 0.1 mM PMSF), and centrifuged for 10 min at 1,000 g at 4 °C. The homogenization and centrifugation were repeated once, then the two supernatants were combined and adjusted concentration of $CaCl_2$ to 1 mM with 100 mM $CaCl_2$. After incubating for 5 min at 37 °C, the solution were centrifuged for 15 min at 15,000 g at 4 °C to pellet the crude organelles, which were resuspended with 6 ml 0.25 M cold sucrose and homogenized one more time. The homogenate was loaded on the top of 40% Percoll and centrifuged for 45 min at 60,000 g at 4 °C. Fractions of 0.5 ml were collected from the top and centrifuged for 1 h at 300,000 g at 4 °C with 0.5 ml 50% sucrose on the top to remove Percoll. Lysosomes at the interface were collected and resuspended with 10 ml 0.25 M sucrose. After centrifuging for 20 min at 25,000 g at 4 °C, the lysosome pellets were store at −70 °C for future uses.

2.3. Preparation of proteins from lysosomes

Isolated lysosomes were resuspended in lysis buffer (50 mM Tris–HCl, pH 8.0, 0.2 M NaCl, 1 mM EDTA) complemented with PIs. The solution was repeated to thaw at 37 °C and frozen in liquid nitrogen for 10 times to break down lysosomes. It was then centrifuged for 30 min at 355,000 g at 4 °C. The soluble lysosomal proteins are in the supernatant while the lysosomal membrane proteins are in the pellet. The pellet was then dissolved in cold 0.1 M Na_2CO_3 (pH = 11.0), votexed for 5 s and incubated on ice for 30 min. After centrifuging for 30 min at 355,000 g at 4 °C, the supernatant which contained lysosome associated proteins was collected, and the pellet which contained lysosome integral proteins was subject to further dissolving in membrane protein buffer (6 M urea, 1% octyl-beta-glucopyranoside, 50 mM Tris–HCl, pH 8.5, 0.1% SDS, complemented with PIs). All the protein samples were stored at −20 °C for further uses.

2.4. Preparation of protein samples from cultured cells and tissues

Cultured cells were trypsinzed, washed with cold PBS and lysed with RIPA buffer. The lysis was centrifuged for 30 min at 15,000 g at 4 °C and the supernatant was collected for further analysis. When mouse tissues were used, about 100 mg of samples were minced and added to 1 ml RIPA buffer with 1 mM PMSF. The solution was sonicated for 8 s for 3 times to break down cells, and centrifuged for 5 min at 15,000 g. The supernatant was saved for further Western blot analysis.

2.5. Western blot

Protein samples were quantified according to the instruction of BCA kit (Thermo Scientific BCA 23227, USA). They were then separated with SDS-PAGE and transferred onto PVDF membrane. The membrane was blocked with 5% milk and incubated with the primary and secondary antibodies subsequently. The primary antibodies used are as follows: anti-APR3 (1:250, Abcam, UK), anti-Calnexin (1:1000, Abcam, UK), anti-LAMP1 (1:1000, clone H4A3, DSHB, USA), Anti-GAPDH (1:5000, Kangcheng, Shanghai, China). After incubation with the secondary antibodies, the membrane was developed with ECL and imaged with Bio-rad LAS-4000.

2.6. Total RNA purification, reverse transcription and real-time PCR

One hundred milligrams of tissues from 8 week old B129 male mice were used to purify total RNAs with TRIzol (Invitrogen, USA) as described in the manufacture's instruction. Reverse transcription was performed with Reverse Transcription Kit (Takara, Japan). The synthesized cDNAs were used for real-time PCR with PrimeScript™ RT reagent Kit and SYBR® Premix Ex Taq™ (Perfect Real Time, from TaKaRa, Japan). Primers used for real-time PCR are as follows: APR3 S (ATTTGTCCAGAGTGGCTGCT)/APR3 R (GAGTAAACCCACGGAAGGTG); GAPDH S (GGCACAGTCAAGGCTGAGAATG)/GAPDH R (ATGGTGGTGAAGACGCCAGTA).

2.7. Immunofluroesence

Mouse fibroblasts were isolated, cultured and stored as described (Experimental Instructions of Animal Cell Culture). They were inoculated on coverslips in 24-well plates when they reached logarithmic phase and cultured until reached 40–50% confluence. After fixed with 4% paraformaldehyde, the cells were permealized with 0.1% Saponin, incubated with anti-LAMP1(1:200) and anti-APR3 (1:100) antibodies, respectively. After incubation with corresponding secondary antibodies, the coverslips were counterstained with DAPI and observed under confocal microscopy. When Lyso-Tracker Red was used to label lysosomes, the cultured cells were washed with DMEM first and incubated with Lyso-Tracker Red (1:5000 diluted in DMEM) at 37 °C for 2 h. The cells were then fixed and stained with anti-APR3 antibodies as described above.

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