Biochimica et Biophysica Acta 1863 (2016) 1682–1689

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Phosphatidylethanolamine binding protein 4 (PEBP4) is a secreted protein and has multiple functions

Huan He [a,b], Dan Liu [b,c], Hui Lin [a,b], Shanshan Jiang [a,b,c], Ying Ying [a,b,d], Shao Chun [b], Haiteng Deng [e], Joseph Zaia [b], Rong Wen [f], Zhijun Luo [b,d,*]

[a] Graduate Program of Internal Medicine, Nanchang University Jiangxi Medical College, Nanchang, China
[b] Department of Biochemistry, Boston University School of Medicine, Boston, MA 02118, United States
[c] Department of Pharmacology, Nanchang University School of Pharmaceutic Sciences, Nanchang, China
[d] Department of Pathology, Institute of Basic Medical Sciences, Nanchang University Jiangxi Medical College, Nanchang, China
[e] MOE Key Laboratory of Bioinformatics, School of Life Sciences, Tsinghua University, Beijing, China
[f] Bascom Palmer Eye Institute, University of Miami Miller Medical School, Miami, FL 33136, United States

ARTICLE INFO

Article history:
Received 18 February 2016
Received in revised form 22 March 2016
Accepted 23 March 2016
Available online 28 March 2016

Keywords:
PEBP4
Signal peptide
Secretion
Glycosylation
ERK
Act

ABSTRACT

Phosphatidylethanolamine binding proteins (PEBP) represent a superfamily of proteins that are conserved from bacteria to humans. In mammals, four members have been identified, PEBP1–4. To determine the functional differences among PEBP1–4 and the underlying mechanism for their actions, we performed a sequence alignment and found that PEBP4 contains a signal peptide and potential glycosylation sites, whereas PEBP1–3 are intracellular proteins. To test if PEBP4 is secreted, we made constructs with Myc epitope at the amino (*N*) terminus or carboxyl (*C*) terminus to mask the signal sequence or keep it free, respectively. Our data revealed that both mouse and human PEBP4 were secreted when the epitope was tagged at their *C*-terminus. To our surprise, secretion was dependent upon the *C*-terminal conserved domain in addition to the *N*-terminal signal sequence. When the epitope was placed to the *N*-terminus, the recombinant protein failed to secrete and instead, was retained in the cytoplasm. Mass spectrometry detected asparagine (*N*)-glycosylation on the secreted PEBP4. Although overexpression of *N*-terminal tagged PEBP4 resulted in an inhibition of ERK activation by EGF, that with a *C*-terminal epitope tag did not have such an effect. Likewise, transfection of PEBP4 shRNA did not appear to affect ERK activation, suggesting that PEBP4 does not participate in the regulation of this pathway. In contrast, PEBP4 siRNA suppressed phosphorylation of Act at S473. Therefore, our results suggest that PEBP4 is a multifunctional protein and can be secreted. It will be important to investigate the mechanism by which PEBP4 is secreted and regulates cellular events.

1. Introduction

Phosphatidylethanolamine binding protein (PEBP) represents a superfamily of more than 400 members and is evolutionally conserved from bacteria to humans [1]. In mammalian cells, four members of PEBP, ranging from 21–25 kids, have been documented; PEBP1 is ubiquitously expressed at high levels in the brain, adrenal gland and thyroid [2,3], PEBP2 is mostly restricted to the testis [4], PEBP3 has not yet been characterized, and PEBP4 is predominantly expressed in the skeletal muscle, heart and thyroid [5]. Multiple functions have been ascribed to PEBPs, including membrane biogenesis, fluidity, and formation of functional domains [6–9], stimulation of acetylcholine secretion during neuronal development [9,10], serine protease inhibition in neuronal tissue [11], and regulation of MAPK pathway, cell proliferation and survival, and spermatogenesis or sperm maturation [4,12].

PEBP1 was first found in the brain [13] and later on isolated as a Raff kinase inhibitory protein (RKIP) by the yeast two hybrid method [12]. It has been shown that RKIP binds to Raf-1 and MEK1 at overlapping sites [14]. As a result, binding to Raf-1 precludes the binding to MEK1, and vice versa. Disruption of the interaction between Raf-1 and MEK1, leads to an inhibition of MEK phosphorylation and activation by Raf-1.

Abbreviations: Act, protein kinase B; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; ERK, extracellular signal regulated kinase; FBS, fetal bovine serum; GFP, green fluorescent protein; Her2, human epidermal growth factor receptor 2; HMGB1, high mobility group box 1; JNK, c-Jun *N*-terminal kinase, MEK1, mitogen and extracellular signal activated kinase (mitogen activated kinase kinase); MET, hepatocyte growth factor receptor; NFκB, nuclear factor kappa B; PEBP, phosphatidylethanolamine binding protein; Raf-1, proto-oncogenic protein kinase upstream of MEK1.

* Corresponding author at: Department of Biochemistry, Boston University School of Medicine, 72 East Concord Street, MA 02118, United States.
E-mail address: zluo@bu.edu (Z. Luo).

http://dx.doi.org/10.1016/j.bbamcr.2016.03.022
0167-4889/

In addition, RKIP has also been reported to regulate other signaling pathways including ß-adrenergic signaling, and NFκB signaling, culminating in inhibition of tumorigenesis, metastasis, or modulation of other cellular events [1].

Similar to PEBP1, PEBP4 was reported to associate with Raf-1 and MEK1, blocking MEK/ERK activation by TNFα or TRAIL and thereby inhibiting apoptosis [5,15]. PEBP4 is highly expressed in muscle, which leads to speculation of functional interactions between PEBP4 and Raff/MEK during myoblast differentiation [16]. Garcia et al. showed that PEBP4 acts a scaffold for Raf-1 and MEK1 and augments their interaction [16]. The effect of PEBP4 on ERK activation depends on the expression levels; paradoxically, low expression enhances but high expression suppresses ERK activation [16]. In addition, knockdown of PEBP4 inhibits myoblast differentiation, possibly due to increased activation of the Raff/MEK. Recent studies indicate that PEBP4 enhances Act activation while inhibiting that of ERK/JNK [17–20]. Furthermore, a number of recent reports have documented increased expression of PEBP4 in a variety of cancer specimens, correlative to invasion and metastasis of cancer, suggesting that PEBP4 plays a role in cancer progression [21–24]. In agreement with this, *in vitro* studies have shown that silencing PEBP4 induces apoptosis and reduces invasiveness of cancer cells whereas overexpression elicits the opposite changes [17,23,25,26]. Interestingly, a recent study has reported that PEBP4 is a secreted protein, suggesting a new function or mechanism [27].

To determine the functional differences among PEBP1–4 and the underlying mechanisms for their actions, we first performed a sequence alignment, which revealed that PEBP4 contains a signal peptide, while PEBP1–2 are intracellular proteins. To test if PEBP4 is secreted, we made constructs with Myc epitope at the *N*- or *C*-terminus of PEBP4, respectively. Our data revealed that both mouse and human PEBP4 were secreted when the epitope was tagged at their *C*-termini. Surprisingly, the secretion was also dependent on the *C*-terminal conserved domain. In contrast, when the epitope was placed at the *N*-terminus, the recombinant protein failed to secrete and was retained in the cytoplasm. Mass spectrometry detected *N*-glycosylation on PEBP4. Overexpression of *N*-terminal tagged PEBP4 resulted in an inhibition of ERK activation by EGF, whereas *C*-terminal tagged PEBP4 was without such an effect. Likewise, transfection of PEBP4 shRNA did not appear to inhibit ERK activation, suggesting that PEBP4 does not participate in the regulation of this pathway. However, PEBP4 siRNA suppresses phosphorylation of Akt at S473.

2. Materials and methods

2.1. Reagents

Epidermal growth factor (EGF) was purchased from Promega Life Sciences (San Luis Obispo, CA). Antibodies against phospho-ERK1/2 T202/Y204, total ERK, phospho-Akt S473, total Akt, and β-actin were from Cell Signal Biotechnologies (Danvers, MA). Antibody against Myc epitope was purchased from Sigma Aldrich (St. Louis, MO). Antibody for human PEBP4 was from Abcam (Cambridge, MA). NTA agarose was from Qiagen (Valencia, CA). Human PEBP4 cDNA in pCDNA3.1 and pEGFPC was gifted from Dr. Water Koch (University College Dublin) and mouse PEBP4 cDNA was synthesized by Life Technologies (Grand Island, NY).

2.2. Construction of expression plasmids

cDNA for human PEBP4 was amplified by PCR and subcloned into pCDNA3.1(-)MycHisB (Life Technologies) at EcoRI and HinDIII sites to express recombinant PEBP4 with MycHis tag at the *C*-terminus. PEBP4 cDNA was amplified by PCR and subcloned in pCDNA3.1(-) to tag Myc epitope at the *N*-terminus. The deletion mutations were made as illustrated in Fig. 4. The point mutation for T171A was made by PCR amplification of two overlapping fragments containing T171A and secondary amplification of full length mutant cDNA. The cDNA was sequenced and then subcloned to pCDNA3.1(-)MycHisB.

2.3. Construction of shRNA plasmid for PEBP4

Oligonucleotides for shRNA were synthesized encompassing PEBP4 coding sequence bp 473-493 as underlined:

Sense: 5′ GATCCCCGGAAAAGTCATCTCTCTCCttcaagagaGGAGAGAGATGACTTTTCCTTTTTA and antisense:

agctTAAAAAGGAAAAGTCATCTCTCTCCTCTCTTGAAGGAGAGAGATGACTTTTCCGGG. The oligonucleotides were annealed and cloned into pSuperRetro at BglII and HindIII site.

2.4. Cell Culture and transfection

HEK293T cells were cultured in DMEM supplemented with 10% FBS and penicillin/streptomycin at 37 °C, 5% CO_2. Plasmid DNA was transfected into HEK293T cells by the calcium phosphate precipitation method. Two days after transfection, the cells were starved in 1% FBS-DMEM overnight and treated with EGF (10 ng/ml) for 10 min.

2.5. Purification of recombinant PEBP4

pCDNA3.1(-)MycHis expressing human or mouse PEBP4, respectively, was transfected into HEK293T cells as noted above. Two days after transfection, cell culture medium was harvested and passed through NTA agarose column. Nonspecific binding proteins were removed by washing and recombinant PEBP4 was eluted according to manufacturer's protocol (Qiagen, Valencia, CA).

2.6. Western blot analysis

Cell extracts were prepared in lysis buffer (25 mM Tris–HCl, pH 7.8, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mMNa_3VO_4 and 25 mM β-glycerol-phosphate, 1 mM DTT, 1% NP-40 and protease inhibitors). The cell debris was removed by centrifugation at 14,000 × *g* at 4 °C for 15 min and protein concentration measured using a Bio-Rad Protein Assay kit. Protein samples (20 μg) were subjected to SDS-PAGE and electrophoretically transferred to PVDF membranes (EMD Millipore, Bedford, MA). The membranes were sequentially blotted with the first and second antibodies, and developed by the enhanced chemiluminescence (ECL) method [28].

2.7. Confocal microscopy examination

After transfection of PEBP4 tagged with GFP at the *N*-terminus or *C*-terminus into HEK293T cells on coverslips, the cells were fixed with 4% paraformaldehyde prepared in PBS and examined using a confocal microscope (Leica SP5) at 63× magnification.

2.8. Proteomics analysis

In-gel deglycosylation and trypsin digestion was performed on cut SDS-PAGE gel bands. In LC-MS/MS analysis, digestion products were separated by a C18 chromatography column (75 μm ID, 150 mm length; 120 min gradient elution at a flow rate 0.300 μL/min) with a Dionex 3000 nano-HPLC system which was interfaced with a Thermo-Fisher Scientific Q-Exactive mass spectrometer. Mobile phase A consisted of 0.1% formic acid, and mobile phase B consisted of 80% acetonitrile and 0.08% formic acid. The Q-Exactive mass spectrometer was operated in the data-dependent acquisition mode using Xcalibur2.1.3 software. For data acquisition, there was a single full-scan mass spectrum in the Orbitrap (400–1800 m/z, 70,000 resolution) followed by 20 data-dependent MS/MS scans. The tandem mass spectra from each LC–MS/