MOLECULAR MECHANISMS OF DIFFERENTIATION

Identification of fatty acid binding protein FABP4 interaction with PTEN phosphatase

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PTEN is a tumor suppressor with dual protein and lipid phosphatase activity, which is frequently deleted or mutated in many advanced cancers. At least 20% of its mutations are found in C-terminal domain of the phosphatase. Recent studies shown that PTEN also plays an important role in development of insulin resistance and glucose tolerance, thus it may be an important target in diabetes 2 type and atherosclerosis treatment. Using C-terminal fragment of PTEN as bait in yeast two-hybrid screen of 18-day Mouse Embryo cDNA library a novel PTEN-binding protein FABP4, which is a marker of adipocyte differentiation, has been identified. PTEN-FABP4 interaction was confirmed by mating assay, in vitro GST-pull down assay and BIACore analysis. Our results show that PTEN phosphatase may be involved into adipose tissue formation regulation through the interaction with fatty acid binding protein FABP4.

Key words:PTEN phosphatase, FABP4, lipid metabolism.

Introduction. PTEN (Phosphatase and Tensin homolog deleted on chromosome 10) is a tumor suppressor gene localized on chromosome 10q23. The gene encodes for a protein with the molecular weight of about 54kDa, which has a phosphatase-homology domain at the N-terminus, a phospholipid-binding C2 domain, and a PDZ-binding motif at the C-terminal tail [1]. Results from many laboratories demonstrate that PTEN functions as a tumor suppressor and possesses both protein and lipid phosphatase activity [2]. Mutations in the Pten gene resulting in the loss of PTEN phosphatase activity are known in a number of human cancers, including glioblastoma, melanoma, prostate, breast, and endometrial tumors [3]. Pten mutations were also found in patients with the rare
autosomal dominant hamartomatous syndromes, Cowden disease and Bannayan-Zonana syndrome [4].

The majority of Pten missense mutations detected in tumor specimens targets the phosphatase domain and causes a loss in PTEN phosphatase activity [5]. Knockout studies of Pten gene indicate that homozygous mice (pten/-) die during embryonic development, while heterozygous mice (pten+/−) develop normally, but acquire wide range of tumors in adulthood [6]. Moreover, there are data that Pten deletion in the liver resulted in increased fatty acid synthesis, accompanied by hepatomegaly, fatty liver phenotype and tumour formation. The hepatocytes of these mice show a tendency to enhanced cell response to insulin and rapid normalization of increased blood glucose level [7].

Tissue-specific PTEN deletion in muscle protects mice from the development of insulin resistance and diabetes II type [8]. Adipose-specific PTEN deletion also causes increased insulin sensitivity with improved glucose uptake. These mice show inhibition of gluconeogenesis due to increased AMP-kinase activity in liver that could be explained by decreased level of resistin production in adipose tissue [9]. In its turn, resistin expression is associated with the formation of insulin resistance [10].

Therefore, PTEN may be a potential target not only for cancer therapy but also for diabetes II type and atherosclerosis treatment.

Biochemical studies of PTEN phosphatase have revealed that unlike most members of the PTP superfamily, PTEN utilizes the phosphoinositide second messenger, PtdIns-3,4,5-P3, as its physiologic substrate [11].

Phosphatidylinositol-3,4,5-triphosphate is an important regulator of cell growth and survival signaling processes through PI3K/PDK1/Akt pathway [12]. Thus, PTEN acts as PI3-kinase antagonist due to dephosphorylation of PI3-kinase products PtdIns-3,4-P2 and PtdIns-3,4,5-P3. Mutations that impair PTEN function result in a marked increase of PtdIns-3,4,5-P3 in cell and constitutive activation of Akt survival signaling pathway, leading to proliferation, inhibition of apoptosis, hyperplasia, and tumor formation [13].

In addition to its lipid phosphatase activity, PTEN also exhibits protein tyrosine phosphatase activity. Focal adhesion kinase (FAK) and adaptor protein Shc are known to be dephosphorylated by PTEN in vitro [14, 15]. It has been proposed that the dephosphorylation of FAK and Shc by PTEN mediates the inhibition of cell adhesion and migration.

The exact mechanism of PTEN tumor suppression activity and regulation is still not clear. Recently, it was shown that PTEN phosphorylation in its C-terminal domain is important for the regulation of the phosphatase activity. Protein kinase CK2 and glycogen synthase kinase 3β (GSK3β) were found to phosphorylate PTEN C-terminus and affect the stability and activity of the phosphatase [16].

On the other hand, one of the known PTEN partners - PICT-1 protein, encoded by a candidate tumor suppressor gene GLTSCR2, was identified to promote the phosphorylation, stability and turnover of PTEN due to binding to its C-terminal segment [17].

Among other PTEN-binding proteins there are membrane-associated guanylate kinases MAGI-1, 2, and 3. MAGI bind to PTEN via theirs PDZ-domains and prevent PTEN protein degradation thus enhancing the ability of PTEN to suppress Akt activation [18-20].

Finally, a new PTEN-binding protein AEBP1 was identified recently in our laboratory by yeast two-hybrid screening of Colon Cancer cDNA library [21]. According to the literature data AEBP1 is a transcription factor with carboxypeptidase activity, whose function is linked to signal transduction and lipid metabolism [22]. Transgenic mice with AEBP1 overexpression develop a massive obesity when fat-feeding and a marked PTEN reduction accompanied by cell survival has been reported for these mice. PTEN-AEBP1 interaction is shown to cause PTEN degradation [23].

In this paper we publish the result of yeast two-hybrid screening using PTEN C-terminal domain as bait. The screening of 18-day Mouse Embryo cDNA library allowed us to identify six clones containing cDNA for fatty acid binding protein FABP4. The found interaction was confirmed by yeast mating assay, GST-pull down assay and by biosensor of surface plasmon resonance BIAcore.
Materials And Methods. Yeast two-hybrid system. DupLexA™ yeast two-hybrid system, developed by OriGene Technologies Inc. (USA) has been employed in the study. Cloning of baits, transformation and selection of recombinant clones, testing the autoactivation potential of the baits and their ability to enter the nucleus, library screening and identification of positive clones as well as yeast mating assay were carried out as described [21].

Cloning, expression and purification of recombinant proteins in E.coli. cDNA sequences corresponding to full size mouse and human FABP4 proteins were amplified by PCR and cloned into pGEX4T1 and pET28a vectors on BamHI and XhoI restriction sites. E.coli BL21 (LysE) cells were transformed by the constructs obtained and recombinant protein expression was induced by 1 mM IPTG for 3 hours at 28°C. GST-FABP4 and 6His-FABP4 proteins were affinity purified under native conditions using glutathione sepharose ("Amersham", UK) and Ni-NTA agarose ("Quagen", Germany) respectively according to manufacture’s recommendations.

Cloning, expression and purification of recombinant PTEN using Bac-to-Bac expression system. cDNA sequence corresponding to PTEN wild type was amplified by PCR and cloned into pFAST HTb vector on BamHI and EcoRI restriction sites. A composite bacmid molecule was produced when bacterial MAX EfficiencyDH10Bac™ cells were transformed with a donor pFastBac HTb plasmid containing PTEN sequence. Bacmid DNA was isolated using S.N.A.P.™ MidiPrep Kit ("Invitrogen", UK). Virus stock was obtained using transfection of Sf9 cells by PTEN bacmid with cellfectin ("Invitrogen", UK). Sf9 cells were infected by virus stock in concentration of no less than 10⁷ pfu in 1 ml. Infected cells were harvested in 96 hours, lysed in the buffer (50mM NaH₂PO₄, 300mM NaCl, 300mM imidazole, pH 8.0) and recombinant PTEN was affinity purified on Ni-NTA agarose according to the manufacture’s recommendations.

Adipocyte cultivation and differentiation. Non-differentiated NIH 3T3L1 pre-adipocytes were maintained on Petri dishes in DMEM containing 10% DCS and 1% penicillin-streptomycin until confluent. Cell differentiation was induced in two days after adipocytes acquired confluence by substituting the medium for DMEM with 0.5μM dexamethasone, 0.5μM IBMX, 170nM insulin and 10% FBS. Cells were lysed in the standard buffer (50mM tris-HCl, pH 7.5, 150mM NaCl, 100mM NaF, 2mM NaVO₃, 2% triton X100, and 1mM PMSF) with the addition of cocktail of protease inhibitors ("Roche Molecular Diagnostics", France). Cells were lysed in 0, 1, ... 8 days after the induction of differentiation.

Western blot analysis. Lysates from both differentiated and non-differentiated adipocytes were separated by gradient (7-22%) SDS-PAGE and transferred onto Immobilon-P membrane ("Millipore", USA). The membrane was blocked with 0.5% gelatine in PBST (Phosphate buffer saline with 0.5% Tween-20) for 1 hour at RT and incubated with monoclonal anti-PTEN antibodies ("Cell Signaling", USA), anti-FABP4 or anti-GST antibodies, generated in our laboratory [24]. For the analysis of PTEN and FABP4 expression during adipocyte differentiation anti-actin antibodies ("Santa Cruz", USA) were used as loading control. As secondary antibodies goat anti-mouse IgG were used in dilution 1:5000 ("Promega", USA). The membrane was developed by enhanced chemiluminiscence using X-ray film (MIITBII "OHIKO", Ukraine).

BIAcore. The analysis was performed using the standard method [25]. Recombinant PTEN expressed in insect cells was immobilized on the sensor chip (CM-5, BIAcore AB) through the interaction of PTEN aminogroups with activated carboxygroups of the chip using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride and N-hydroxsuccinimide until approximately 12000 Resonance Units (RU) were obtained. Separate surfaces were also coated with the recombinant GST-FABP4 and 6His-FABP4 proteins as negative control. All experiments were carried out in 20mM HEPES, pH 7.4, 150mM NaCl, 3.4 mM EDTA, 0.005% Tween 20, 4mM DTT. Varying concentrations of BSA, GST-FABP4 and 6His-FABP4 were injected over chip with immobilized PTEN and control surfaces at a flow rate of 10μl/min at 25°C. The response from the control surface was subtracted and the data plotted as Resonance Units (RU) vs protein concentration. The equilibrium dissociation constant KD was calculated.
by fitting the data to the equation

\[ R = R_{\text{max}} \cdot \frac{C}{K_D + C}, \]

where \( R \) is the response at equilibrium, \( R_{\text{max}} \) the maximum response level and \( C \) the concentration of protein.

**GST-pull down assay.** The soluble fractions of bacterial lysates (300\( \mu \)l) with the induced expression of mouse and human GST-FABP4 and GST as negative control were incubated in 1 ml of PBS, pH 7.4 with 30 \( \mu \)l of glutathione sepharose for 1 hour. Then the sepharose was washed three times with PBS and 500 \( \mu \)g of 9th-day differentiated adipocyte lysates were added. Samples were incubated overnight at 4\( ^\circ \)C on wheel. Next day the sepharose was washed five times with PBS, separated by gradient (7-22\%) SDS-PAGE, transferred onto the membrane and analyzed by Western Blot using anti-PTEN and anti-GST monoclonal antibodies.

**Results And Discussion.** To carry out the screening of 18-day Mouse Embryo cDNA library we used PTEN C-terminal bait containing C2 domain and PDZ-binding motif. cDNA sequencing of selected positive clones followed by GenBank database search for homology allowed us to identify several nucleotide sequences. Six of them encoded for FABP4 protein fragments: among them there were four cDNAs started from 30 a.a. (three of them have 550 b.p. length and one - 400 b.p.) and two cDNAs encoded for a peptide starting with the 13th a.a. have 500 b.p. length. The specificity of identified clones was confirmed by yeast mating assay.

FABP4 (fatty acid binding protein 4), also referred to as ap2-protein, belongs to the family of cytoplasmic proteins with the molecular weight of about 15 kDa, capable of binding hydrophobic ligands with high affinity [26]. A considerable FABP4 expression was found in adipocytes and macrophages [27]. The expression of FABP4 is tightly controlled during adipose tissue development and in cellular response to metabolic hormones, such as insulin and epinephrine [28]. Studies from various laboratories implicated FABP4 in shuttling of fatty acids to various compartments in the cytoplasm and modulating intracellular lipid metabolism. Fatty acids modulate FABP4 expression at the level of transcription in a positive feedback loop. This regulation is mediated by PPAR\( \gamma \) (peroxisome proliferators-activated receptor \( \gamma \)) and its agonists [29].

On the other hand, FABP4 is known to interact with HSL (hormone sensitive lipase) and this interaction requires FABP4 to be in complex with fatty acid. The activation of HSL and the induction of lipolysis are the functional consequences of FABP4/fatty acid/HSL interaction [30]. Fatty acid binding to FABP4 depends on its conformational changes after Tyr19 phosphorylation by insulin receptor tyrosine kinase [31].

The experiments on knockout and transgenic mice are evidence of PTEN and FABP4 important role in mechanisms of insulin resistance and metabolic syndrome development, which in turn lead to diabetes II type and atherosclerosis. The genetic absence of FABP4 in mice results in their significant protection from the development of dietary-induced insulin resistance [32]. Antisense nucleotide PTEN inhibition led to similar implications, such as increased liver sensitivity on insulin action and normalization of blood glucose concentration in diabetic mice [33]. Moreover FABP4 expression is dramatically increased in hepatocytes with liver-specific PTEN deletion in comparison with the control [34].

Therefore, the literature data establish a potential link between PTEN and FABP4, but the mechanism of their interaction and its functional significance are not clear. FABP4 identification as PTEN-binding protein is the first evidence of protein-protein interaction between these proteins in cell. To confirm PTEN-FABP4 interaction we tested the capability of these proteins to form complex in vitro using recombinant proteins. Protein-protein interactions were analyzed by BIACore and GST-pull down assay.

In the first case affinity purified proteins were used. Recombinant PTEN was expressed in insect cell using bacmid containing PTEN wild type. Recombinant 6His-FABP4 and GST-FABP4 were expressed in E.coli BL21 (LysE). Chip surfaces were coated with different concentrations of PTEN and GST-FABP4 as control of non-specific binding. Various concentrations of recombinant 6His-FABP4, GST-FABP4 and BSA were applied and the formation of specific protein-protein complexes was monitored as described in "Materials and Methods". The analysis of the sensograms clearly demonstrated that recombinant
FABP4 specifically interact with immobilized PTEN. No specific binding was observed when BSA was applied to the chip. Insignificant GST-FABP4 binding to the control surface with immobilized GST-FABP4 was observed. This could be explained by the dimer-formation between GST fragments of the recombinant proteins. The analysis allowed us to determine the equilibrium dissociation constant $K_D$ of PTEN-FABP4 complex, which is in the range of 2.8$\mu$M. The plot of the dependence of surface resonance degree on protein concentration with the control surface data subtracted is shown in Figure 1.

The result of the GST-pull down assay is also the evidence of PTEN-FABP4 complex existence. To carry out this assay GST-FABP4 and GST recombinant proteins were immobilized on glutathione-sepharose followed by the incubation with lysates of differentiated adipocytes. The data of Western blot analysis of precipitated proteins using monoclonal anti-PTEN and anti-GST antibodies show that endogenous PTEN phosphatase from the differentiated adipocyte specifically interacts with the recombinant mouse GST-FABP4 (Fig.2).

Taking into the consideration that, as it mentioned above, FABP4 expression changes during fat tissue formation, namely it increases in the process of adipocyte differentiation, we compared the level of FABP4 and PTEN expression in adipocytes on different differentiation stages. The results, shown on Fig.3, demonstrate that the expression levels of PTEN and FABP4 correlate well during adipocyte differentiation and the highest expression was observed on 8th day after the differentiation had been induced.

Therefore, according to the literature data and our results we suggest that the specific interaction between PTEN and FABP4 is an important link in the signal transduction during regulation of fat tissue development. The clear mechanism and biological
significance of this interaction *in vivo* have not been found yet, but it could be predicted proceeding from the analysis of known FABP4 and PTEN activities.

FABP4 is known to be phosphorylated on Tyr19 by insulin receptor tyrosine kinase and the phosphorylation is markedly increased then FABP4 is bound to the fatty acid [35]. This is explained by conformational changes of phosphorylated molecule whereby Tyr19, which is located in the protein cavity, becomes accessible for tyrosine kinase. On the other hand, phosphorylated FABP4 has no affinity to fatty acid and should be dephosphorylated to restore its activity [36]. Accordingly, there is a possible assumption that PTEN phosphatase plays a role in FABP4 dephosphorylation. PTEN possesses protein tyrosine phosphatase activity and a number of tyrosine-phosphorylated proteins have been found to be PTEN substrates *in vivo*. We think that further experiments with the usage of stable cell lines overexpressing PTEN and/or FABP4 will help both to investigate the regulation mechanisms of adipocyte differentiation during normal fat tissue formation and clarify the role of PTEN-FABP4 interaction.

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