Protein-lipid and protein-protein interactions of Bcr PH domain.

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Bcr is a partner of Abl in reciprocal translocation t(9;22) which leads to Philadelphia chromosome formation. In the present study we analyzed Bcr PH domain functions and determined lipid specificity of PH domain in vitro. The technical approach for determining PH domain protein-protein interactions using mass-spectrometry was developed. Interactions with SMC1, в-tubulin, zizimin1 and PLCe were confirmed in vitro and in vivo.

Key words: Bcr-Abl, PH domain, mass spectrometry.

Introduction. Philadelphia chromosome first detected in patients with chronic myelogenous leukemia is a marker of Ph'-positive leukemias [1]. Ph' chromosome is a product of reciprocal translocation t(9;22)(q34;q11) [2] which causes *bcr-abl* gene formation and cancer development [3]. Breaks happen in regions called M-bcr, m-bcr and μ-bcr [4] and hybrid gene formation leads to myeloid or lymphoid leukemia. Depending on bcr break three forms of Bcr-Abl protein express (p190, p210 and p230 Bcr-Abl). p190 and p210 Bcr-Abl correspond to different leukemia type.

In the focus of our research is the region between two breakpoints M-bcr and m-bcr that contains DH and PH domains (fig.1). PH domain is lipid binding domain and also takes part in protein-protein interactions. We cloned PH part of *bcr* gene and analyzed recombinant PH protein lipid and protein binding spectra *in vitro*. Binding to several of the determined proteins was confirmed *in vivo*.

Materials and methods. *DNA constructs.* PH region was obtained by RT-PCR. 648 b.p. PCR fragment was cloned in frame with 6xhis-tag into pET32a vector for bacterial expression [5]. DH and DHPH fragments were synthesized by PCR on p210 Bcr-Abl construct kindly provided by Dr.N.Heistercamp (Children's Hospital, Los Angeles USA). 685 b.p. and 1169 b.p. fragments were cloned into pRK5-myc vector. All constructs were confirmed by automatic sequencing.

Identification of PH domain binding proteins by affinity chromatography. PH protein was expressed in E.Coli strain BL21 DE3 and purified on Ni-NTA column (Qiagen, USA) according to manufactures protocol. Column with bound PH protein was used for protein-protein interactions detections. In case of lipid-protein interactions detection the recombinant protein was eluted. The protein concentration was 1.5 mg/ml. Radioactively labeled K562 cell lysate was pre-

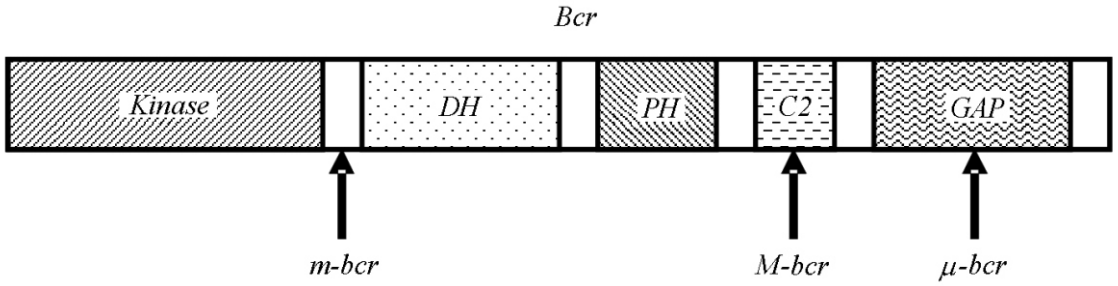

Fig.1 Bcr protein domain organization. DH –Dbl homology domain, PH –plecjstrin homology domian, C2 –Ca^{2+}-binding domain, GAP –GTPase activating protein homology domain.

pared according to [6]. PH protein bound column was loaded with 0.5 ml of K562 lysate and incubation was performed during 12 hours at +4C. The protein concentration was 1 mg/ml. After the incubation and washing samples were analyzed by 2D electrophoresis. pET32a empty vector protein was used as a negative control.

Analysis of PH domain precipitated proteins. Proteins were separated by 2D electrophoresis with 3-9 pH gradient. Radioactively labeled K562 proteins were detected on Fuji X2000 scanner with AIDA soft (IMG GmbH, Germany). Spots were excised from the gel, trypsinized and analyzed by MALDI TOF mass spectrometry (Bruker Biflex, Bruker Daltonics, Germany). Peptide mass spectra were identified by ProFound program.

Studying PH domain binding by protein immunoprecipitation assay. HEK293T cells were transfected with DH or DHPH expressing constructs and a proper target protein (zizimin1 or PLC). pCMV vector for PLC -flag tag expression was obtained from Dr. M.Joseph (ICR, UK). pEF4-HA-zizimin1 was a gift of Dr. L.McNamara (University of Virginia, USA). DH construct was used as a negative control without PH domain. DH and DHPH proteins were myc-tagged on N-end , zizimin1 was HA-tagged and PLC was flag-tagged for the detection purposes. Immunoprecipitation was performed by using anti-myc antibodies according to [5].

PH protein lipid binding analysis. Hydrophobic PIP Strips™ membrane (Echelon, USA) with immobilized lipids was used for the identification of PH binding proteins. Membrane was blocked by TBS-0.1% Tween-20 solution with 3% fatty acid free BSA. Incubation was performed during 12 hours at +4C. Recombinant PH protein was detected with anti-his antibodies (1:3000) and secondary peroxidase conjugated anti-mouse antibodies (1:10000) (Clontech, USA).

Results. *Lipid specificity of PH domain.* For the lipid detection we used preloaded membrane with 15 human lipids. To exclude unspecific vector sequence binding protein expressed from empty vector was used as a negative control. Experimental data suggest that PH domain binds to phosphatidylinositol monophosphates PI(3)P, PI(4)P and PI(5)P. (fig.2).

Identification of K562 proteins that bind to Bcr PH domain. K562 cells were grown in [^{35}S]-methionine medium therefore all K562 proteins were radioactively labeled and could be distinguished from bacterial protein contamination after Ni-NTA purification. After the comparison of the two gels (fig.3) spots corresponding to K562 proteins were excised and trypsinized. We identified 23 proteins that potentially bind to Bcr PH domain. Each of the detected proteins should be verified by protein-protein binding assay in vitro or in vivo. For the further experiments we've chosen SMC1 (structure maintenance of chromosomes), -tubulin, zizimin1 and PLC proteins.

Bcr PH domain binds to SMC1 and -tubulin in vitro. Ni-NTA column with bound recombinant PH protein was used for studying PH association to SMC1 and -tubulin proteins. K562 cell lysate was incubated with PH protein on Ni-NTA column. After washing samples were separated in SDS-PAAG and transferred to nitrocellulose membrane. Endogenous proteins were detected by specific antibodies anti-SMC1 (1:150) and anti- -tubulin (1:200). We confirmed binding to both proteins (fig.4). Empty vector protein was used as a negative control.

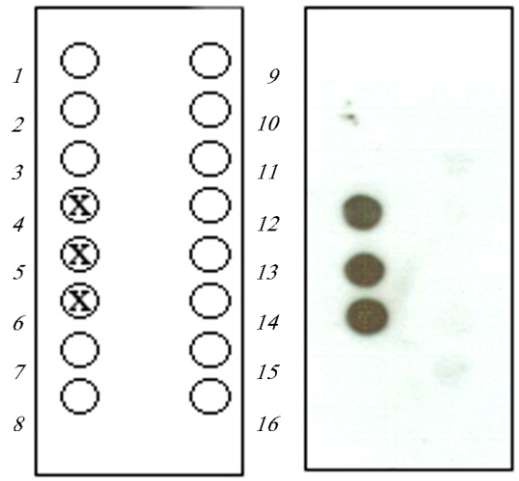

Fig.2 Bcr PH domain lipid binding analysis. 1- lysophosphatidic acid (LPA), 2 –lysophosphocholine (LPC), 3 –PI, 4 –PI(3)P, 4 –PI(4)P, 5 –PI(5)P, 7 –phosphatidylethanolamine (PE), 8 –phosphatidylcholine (PC), 9 –sphingosine-1-phosphate (S1P), 10 –PI(3,4)P2, 11 –PI(3,5)P2, 12 –PI(4,5)P2, 13 –PI(3,4,5)P3, 14 –phosphatidic acid (PA), 15 –phosphatidylserine (PS), 16 –blank.

36 hours after transfection cell lysates were analyzed by immunoprecipitation. Bcr PH domain binding to both PLC and zizimin1 was confirmed (fig.5). PLC is a member of phospholipase family and catalyze hydrolysis of phosphatidylinositol-4,5-bisphosphate into second messengers diacylglycerol and inositol-1,4,5-trisphosphate [7]. Zizimin1 is a member of DOCK180 family and posses GEF (guanine nucleotide exchange factor) activity due to its DHR-2 (DOCK homology region-2) domain that reminds DHPH tandem tertiary structure [8]. Intriguing is the fact of PH domain presence in both proteins. It's suggested that some proteins oligomerize through their PH domains thus, the interaction of Bcr PH domain with PLC and zizimin1 could be due to the PH domains.

PH domain family is widely present in human proteins. Around 10% of PH domains posses high affinity to phosphatidylinositols that have uneven cell distribution depending on organelle kind. Lipids bind to specific effectors and in such way engage signal proteins. PI(4)P is a component of Golgi membrane, PI(3)P is found in early endosome membranes and PI(5)P is detected in nuclear cytoplasm [9]. Therefore, binding of PH domain to internal cell membranes could localize the protein and modify its functions.

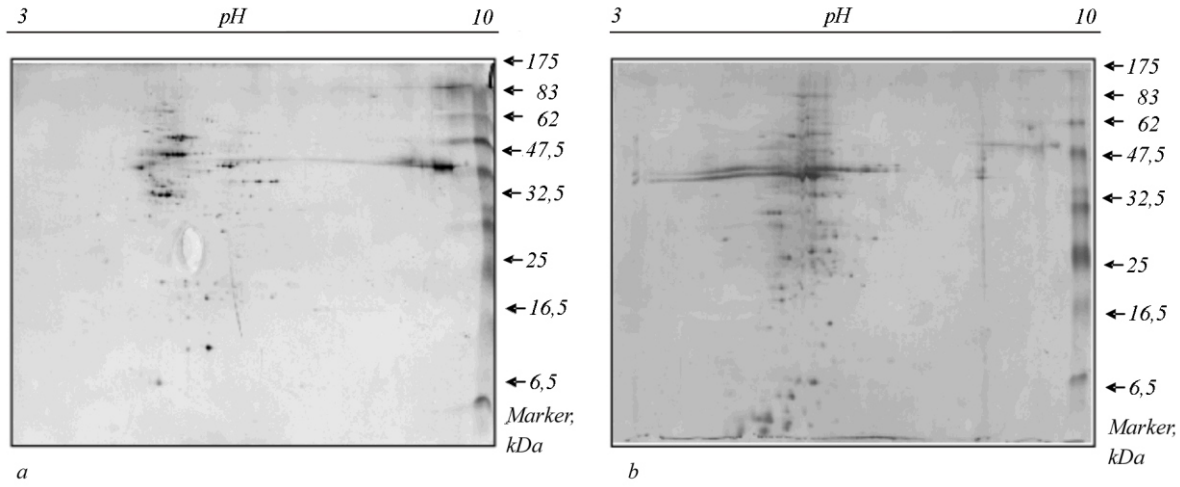

Fig.3 2D electrophoresis separation of proteins precipitated on Ni-NTA column. a –negative control (protein expressed from empty pET32a vector), b –precipitation by recombinant PH protein.

Bcr PH domain precipitation to PLC and zizimin1 in vivo. Construct with both DH and PH domains was used for the immunoprecipitation assay. DH domain construct was used as a negative control without PH domain. Proteins were expressed in HEK293T cells in pRK5-myc vector. Cells were transfected with pEF4-zizimin1 (or pCMV-PLC) and corresponding pRK5-myc vector (containing DHPH or DH regions).

Little is know about PH domain protein-protein interactions. Using of cell lysate protein binding to im-

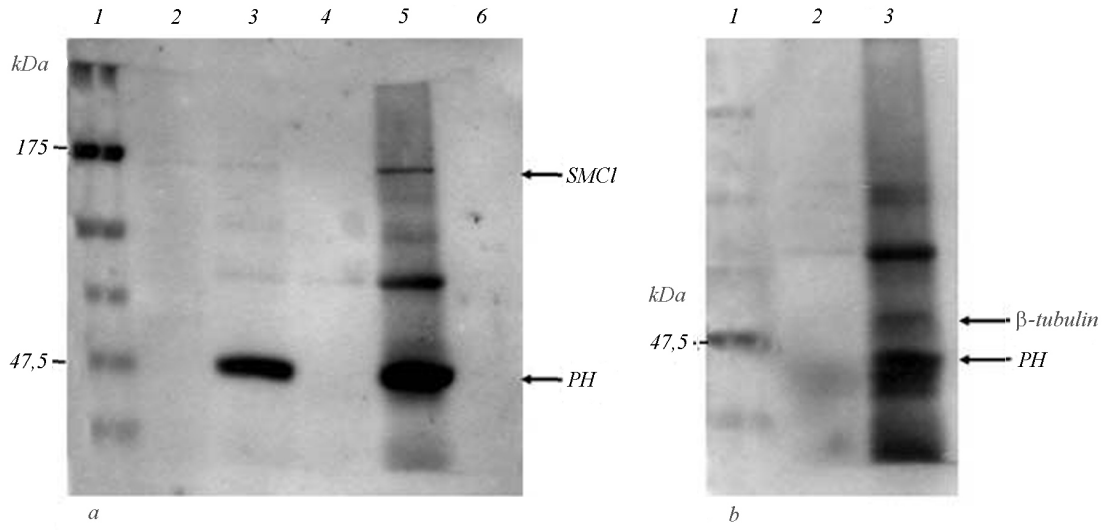

Fig.4 Bcr PH domain binds to SMC1 (a) and -tubulin (b) in vitro. A: 1 –molecular weight marker, 2 –K562 cell lysate, 3,5 –PH domain precipitated proteins, 4,6 –control. (Western blot: anti-SMC1 1:150, anti-his 1:3000, secondary anti-mouse antibodies 1:10000). B: 1 –molecular weight marker, 2 -PH domain precipitated proteins. (Western blot: anti- -tubulin 1:200, anti-his 1:3000, secondary anti-mouse antibodies 1:10000

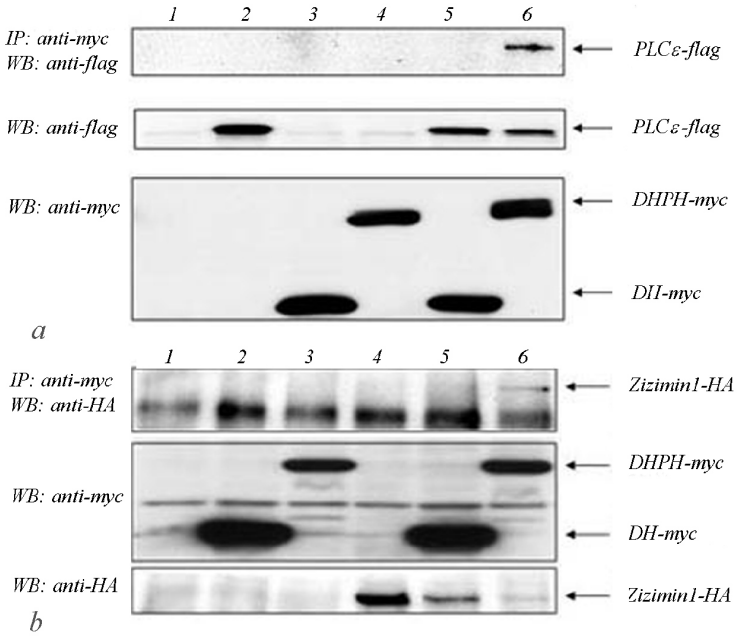

Fig.5 Bcr PH domain binds to PLC (a) and zizimi1 (b) in vivo. A: 1 –293T cell lysate (control), 2 – PLC -flag, 3 –DH-myc, 4 –DHPH-myc, 5 –DH-myc+ PLC -flag, 6 –DHPH-myc+ PLC -flag. B: 1 –293T cell lysate (control), 2 – DH-myc, 3 – DHPH-myc, 4 – zizimin1-HA, 5 – DH-myc+ zizimin1-HA, 6 – DHPH-myc+ zizimin1-HA.

mobilized recombinant protein and further proteomic analysis is a powerful technique. Bcr PH domain was shown to bind cell proteins of high interest. Thus, Bcr or p210 Bcr-Abl could potentially act in various signal pathways.

Conclusions. Bcr PH domain binds to PI(3)P, PI(4)P and PI(5)P and high affinity binding could affect Bcr or p210 Bcr-Abl proteins localization. Bcr PH domain was first shown to take part in protein-protein interactions. Subsequent analysis of PH domain function as a part of Bcr or p210 Bcr-Abl proteins could help in understanding Bcr-Abl signal pathways, its impact on hemopoietic stem cells differentiation and Ph'-positive leukemia progression.

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Определение специфичности белково-липидных и белково-белковых взаимодействий PH-домена белка Bcr, связанного с хронической миелоидной лейкемией

Резюме

Белок Bcr является партнером Abl по реципрокной транслокации t(9;22), приводящей к образованию филадельфийской хромосомы. Проанализированы функции PH-домена Bcr. Определены липиды, с которыми связывается PH домен in vitro. Разработан подход к выявлению белково-белковых взаимодействий PH-домена методами масс-спектрометрии. Подтверждено связывание PH-домена с белками SMC1, -тубулином, zizimin1, PLC in vitro и in vivo.

Ключевые слова: Bcr-Abl, PH-домен, масс-спектрометрия.

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