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PRIMARY INSIGHTS INTO STRUCTURE AND STRUCTURALLY DETERMINED FEATURES OF C2 DOMAIN OF BCR

The investigation of the C2 domain of Bcr is critical for understanding the functions of normal Bcr protein and the processes in cancer involving the hybrid Bcr-Abl. ***Aim:*** *This study aimed to estimate the structure and lipid-binding capacity of the C2 domain of Bcr.* ***Methods:*** *The secondary and tertiary structures were predicted using various bioinformatic tools. A DNA fragment encoding the C2 domain was introduced into the expression vector pET28 using sequence and ligation-independent cloning techniques. The purified recombinant C2 domain was used to obtain circular dichroism spectra and perform a lipid-binding assay.* ***Results:*** *The structure predictions indicate that the C2 domain of Bcr likely adopts a typical beta-sandwich structure with type II topology. The circular dichroism data on the purified recombinant C2 domain confirmed the expected predominance of beta-structures. Binding to eight phospholipids was identified.* ***Conclusions:*** *The recombinant C2 domain of Bcr has a structure comparable to other well-studied C2 domains. Binding to various lipids suggests a possible specific localization of the Bcr protein on different cellular membrane structures.*

Keywords: *C2 domain, Bcr, circular dichroism, lipid binding.*

Introduction

The Philadelphia (Ph) chromosome, which can be identified in several oncohematological diseases, serves as a classic example of the chromosomal translocation associated with a malignant phenotype [1]. The molecular basis of this translocation involves the formation of the fusion gene

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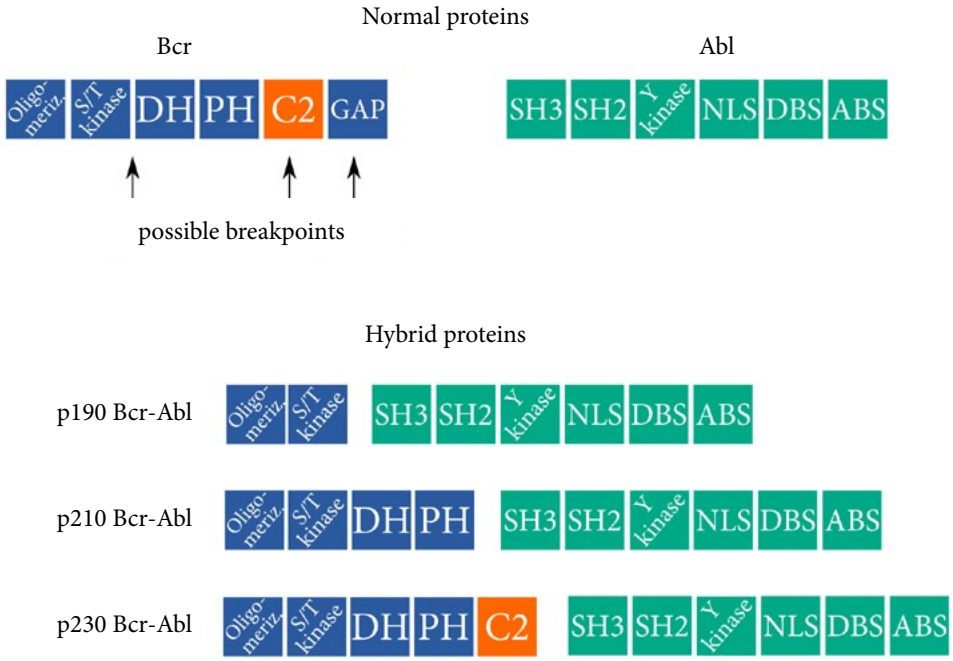


Fig. 1. Domain composition of normal Bcr, Abl and hybrid Bcr-Abl proteins

bcr-abl, which is derived from combination of portions of the normal *bcr* and *abl* genes. The expression of this abnormal gene leads to the production of the Bcr-Abl hybrid protein, which plays a pivotal role in malignant transformation [2]. Depending on the exact location of the breakpoints in the genes during chromosomal translocation, several variants of the hybrid *bcr-abl* genes can appear. These different variants are associated with various types and aggressiveness of leukemias, known as Ph-positive leukemias [3]. The main difference lies in the size of the *bcr* gene segment in the resulting fusion *bcr-abl* gene. Each *bcr-abl* gene variant produces a distinct variant of the Bcr-Abl protein. There are well-known variants such as p190, p210, and p230, which differ in their sizes and domain composition. Specifically, they differ in the domains of the normal Bcr protein that are included in the Bcr-Abl protein. Generally, the longest variant, p230, includes the C2 domain, which is absent in the shorter p210 and p190 variants. The shortest variant, p190, also lacks the PH domain present in the p210 and p230 variants (Fig. 1) [2–4].

Most existing therapies against *bcr-abl*-positive leukemia are aimed at inhibition of the kinase activity of Abl part of the hybrid protein [5, 6]. However, the effectiveness of these therapies can be compromised by the emergence of resistance to therapeutic agents [5, 7, 8]. The search for new molecular targets underscores the necessity for a deeper understanding of the molecular events critical to the Ph-positive cancer cells. Investigating the role of the Bcr component in the molecular processes within cancer cells can be achieved by studying the functions of the domains of the Bcr protein.

The structure and function of C2 domain of Bcr is unclear. Summarizing our knowledge about well-studied C2 domains of other proteins, we can assume some structural features of the domain and its possible binding properties. The main aim of this work was to validate some of these expectations both bioinformatically and experimentally.

Materials and Methods

Bioinformatic Tools

Psipred 4.0 and S4pred tools were used to predict secondary structures of protein. Tertiary structure has been predicted with DMPfold 2.0. Metal binding properties were estimated with DMPmetal. All these instruments are available on PSIPRED Workbench (https://bioinf.cs.ucl.ac.uk) [9–12]. Tertiary structures were visualized and analyzed with web-based iCn3D Structure Viewer 3.40.2 (https://www.ncbi.nlm.nih.gov/Structure/icn3d). Circular dichroism data analysis was performed on BeStSel server v1.3.230210 (https://bestsel.elte.hu/) [13–17].

Molecular Cloning

The bacterial expression vector was constructed using the following strategy: initially, a nested PCR was performed to obtain the DNA fragment encoding the C2 domain, subsequently, sequence and ligation independent cloning technique [18] was utilized. Nested PCR was performed in two rounds. Primers' sequences used for the first round of PCR were C2E-F 5'-CGCAACGGCAAGAG TTACAC-3' and C2E-R 5'-GATGTAGGGCACC TTGG-3'. The length of expected fragment was 696 nt (based on Bcr transcript mRNA sequence, NM004327). Template for PCR was human cDNA from the collection of department of molecular genetics of Institute of Molecular Biology and Genetics NAS of Ukraine. Amplicon from the first round was used as template DNA for the second one. Primers for the second round were C2-F 5'-GAAGTCTACCAGGAACAAACCGGTGGA TCCATGGATGATGAGTCTCCGGGGCTC-3' and C2-R 5'-GATCTCAGTGGTGGTGGTGGT GGTGCTCGAGTTACTCCCTGCTGTTGAACT TGAC-3'. These primers included two parts: complementary to C2-encoding sequence and pET-28 plasmid vector specific sequences. Final amplicon size was 472 nt. For both rounds of PCR, the parameters were 30 cycles of 94 °C — 30 s, 52 °C — 30 s, 72 °C — 90 s, followed by final elongation step 72 °C — 2 min. Enzyme used for PCR was Pfu-polymerase (Thermo Fisher Scientific, USA) with buffer from the same vendor.

Plasmid vector pET28 was linearized by restriction endonucleases BamHI and XhoI (Thermo Fisher Scientific, USA) according to vendor's protocol. This vector and final amplicon as insert were mixed equally (50ng of each). This mixture was used for sequence and ligation independent cloning with T4 DNA-polymerase (Thermo Fisher Scientific, USA). The resulting product was used for bacteria transformation.

Bacterial expression and protein purification

Plasmid vector pET28–C2 with sequence that encodes C2 domain was transformed into chemically competent *E. coli* strain ArcticExpress(DE3) (Agilent Technologies, USA). Autoinduction methodology has been used for initialization of expression [19]. A starter culture, which was grown in 4 ml ZYM-505 growth medium [19] and incubated at 10 °C, with shaking at 220–250 rpm, for 24 hours.

Polyhistidine tagged C2 domain was purified under native conditions. Collected cell pellets were suspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0, 1 mM PMSF, 100 μg/ml lysozyme) and were incubated on ice for 30 minutes followed by ultrasonic disruption (typically 10 seconds pulse followed by 10 seconds rest period repeated for 6 times). Lysed cells were centrifuged at 16000G for 20 minutes at +4 °C. Cleared lysate was loaded on a column packed with HIS-Select® Nickel Affinity Gel (Millipore, USA) and let it flow through by gravity. Column was washed with 10 column volumes of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0), followed by elution with 3 column volumes of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0). The eluate was dialyzed against buffer for circular dichroism experiment (10mM KH_2PO_4, 50 mM Na_2SO_4, pH 7,48). The expected protein, which was a combination of C2 domain and a part encoded by the vector, was 238 aa in length and had molecular weight of 27292.67 (calculated on

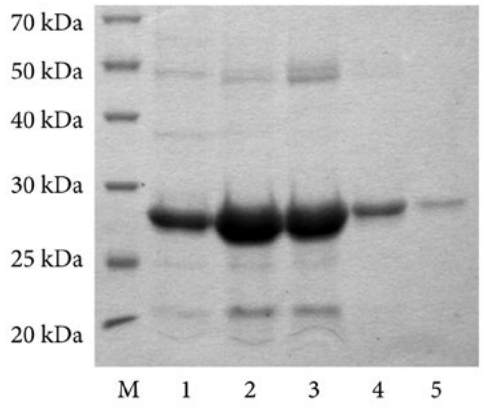


Fig. 2. Purification of recombinant C2 domain of Bcr. M — PageRuler™ Unstained Protein Ladder (Thermo Fisher Scientific, USA), 1–5 — elution stages

https://web.expasy.org/compute_pi/). The concentration and purity of protein were determined by linearized Bradford assay [20] and polyacrylamide gel electrophoresis (Fig. 2).

Circular Dichroism

The protein concentration was 0.484 mg/ml (or 17.73 micromole/L) in 10mM KH_2PO_4, 50mM Na_2SO_4, pH 7,48. Spectropolarimeter J-810 (JASCO) was used with 1cm quartz cuvette. Seventy one data points have been recorded at 37 °C from 260 to 190 nm with data pitch 1nm, data interval — 1nm, scanning speed — 100 nm/min. A reference spectrum of the buffer was subtracted from the obtained spectrum. The detected ellipticity was recalculated as delta epsilon. These data were used for analysis on BeStSel server [13–17].

Lipid Binding Assay

For identification of lipids that can interact with the C2 domain of Bcr membrane lipid strip (Echelon Biosciences) was used. This membrane strip contained various lipids: lysophosphatidic acid (LPA), lysophosphocholine (LPC), phosphatidylinositol (PI), phosphatidylinositol 3-phosphate (PI(3)P), phosphatidylinositol 4-phosphate (PI(4)P), phosphatidylinositol 5-phosphate (PI(5)P), phosphatidylethanolamine (PE), phosphatidylcholine (PC), sphingosine 1-phosphate (S1P), phosphatidylinositol 3,4-biphosphate ($PI(3,4)P_2$), phosphatidylinositol 3,5-biphosphate ($PI(3,5)P_2$), phosphatidylinositol 4,5-biphosphate ($PI(4,5)P_2$), phosphatidylinositol 3,4,5-triphosphate ($PI(3,4,5)P_3$), phosphatidic acid (PA), phosphatidylserine (PS). The experiment was performed according to a protocol of manufacturer. First step was blocking in PBS-T (0.1 % (v/v) Tween 20) with 3% bovine serum albumin for 1 h at room temperature with gentle agitation. Then strip was incubated with 0.5 μg/ml of purified C2 domain in same buffer for 1 h at room temperature with gentle agitation. After washing three times with PBS-T strip was incubated with 1:400 dilution of anti-polyhistidine antibodies (Sigma Aldrich, MAB3844) in PBS-T for 1 h at room temperature. Then the washing step was repeated three times. Incubation with secondary HRP-conjugated goat anti-mouse secondary antibodies (Abclonal) diluted 1:5000 in PBS-T buffer was added to the membrane and incubated 1 hour at ambient temperature on agitating platform. This was followed by washing step at the same conditions described above. Membrane strip was incubated for 1 minute with 1 ml of enhanced chemiluminescence (ECL) buffer (100 mM Tris pH 8.8, 2.5 mM luminol, 0.4 mM coumaric acid, and 0.02% H_2O_2) and chemiluminescence signal was detected using Chemidoc system (BioRad).

Results and Discussion

Prediction of Secondary and Tertiary Structure of the C2 domain of Bcr

In various public databases, the C2 domain is defined differently in terms of the exact range of amino acids. For example, as of January 2025, for the same Bcr protein sequence, Uniprot defines the C2 domain as amino acids from positions 893 to 1020 (accession ID: P11274), while NCBI resources describe it as ranging from 913 to 1033 amino acids (accession ID: NP004318.3). Therefore, predicting the secondary and tertiary structures of the C2 domain was crucial not only *per se*, but also to pro-

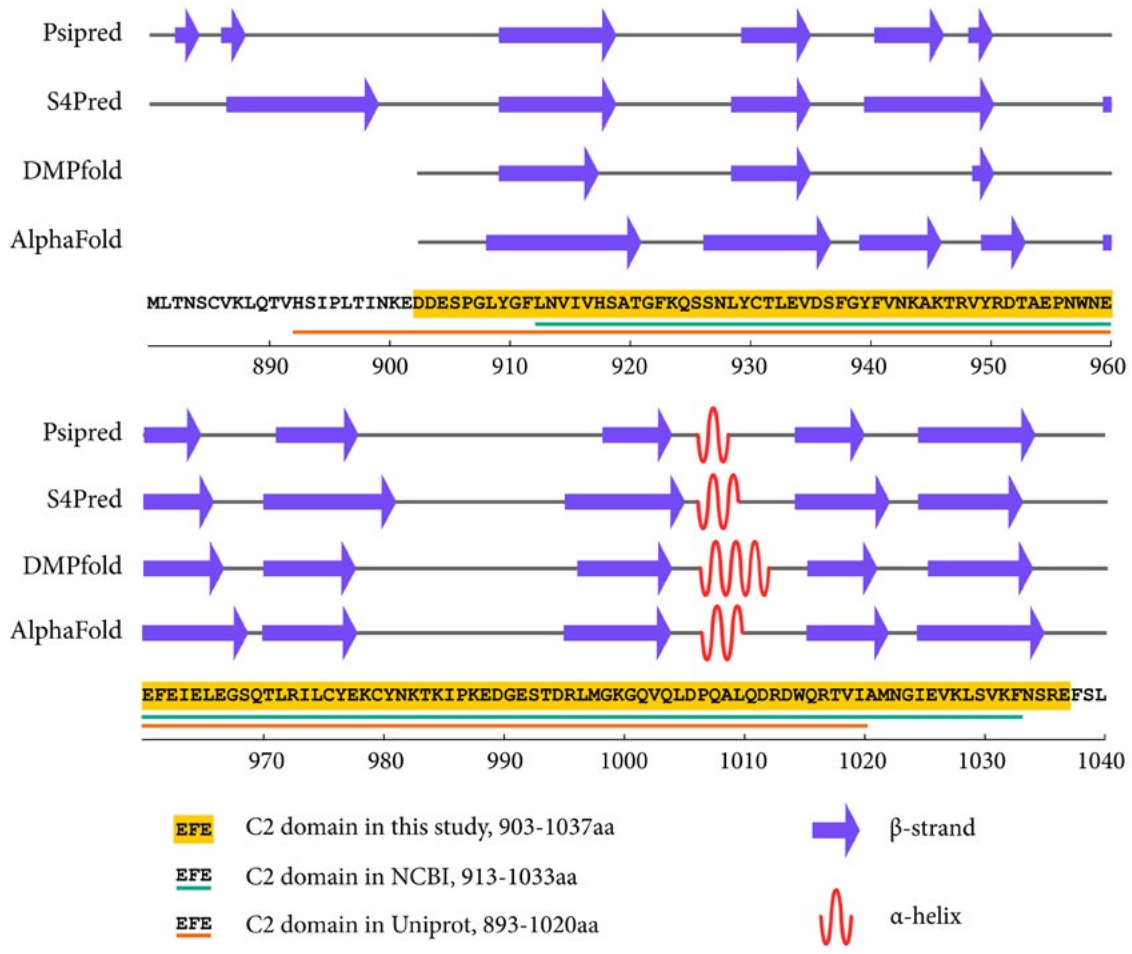
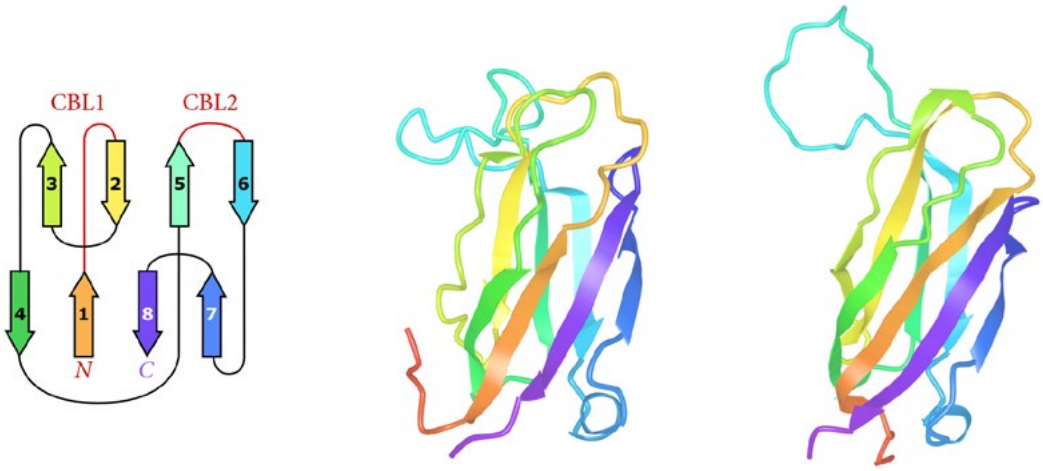


Fig. 3. Prediction of secondary structure of C2 domain of Bcr

vide a rational basis for the development of recombinant protein for further research.

According to the data from experimentally confirmed structures of C2 domains of other proteins, we can anticipate a characteristic beta-sandwich assembled of two four-stranded beta-sheets [21–23]. The understanding of typical structure was used to evaluate and compare two sequence versions of the C2 domain of Bcr that were based on two different annotations on Uniprot and NCBI. Visual summary of the prediction results is presented in Fig. 3. Both Psipred and S4pred tools showed that amino acid sequence, based on NCBI version of annotation, was more likely to provide the typical C2 domain structure. Eight confident beta-strands, which were more than three amino acids in length, were predicted for this variant of the C2 domain. In contrast, for Uniprot version of C2 sequence a number of predicted beta-strands was less than eight (in S4pred prediction), and the length of some strands was three or less amino acids (in Psipred and S4pred predictions). NCBI version of the C2 domain was taken as a template but was extended from 913 position to earlier 903 position to get potentially longer first beta-strand that would be cut without such extension. The final sequence that we used to study the C2 domain of Bcr was 903–1037aa.

For this final version of the C2 sequence the prediction of secondary and tertiary structures has



a *b* *c*

Fig. 4. Tertiary structure prediction of C2 domain of Bcr. *a* — schematic representation of II type topology of C2 domains, *b* — structure predicted with DMPfold, *c* — structure predicted with AlphaFold

been performed. Secondary structures were estimated with above mentioned tools. For the prediction of tertiary structure DMPfold [10] was used. This tool generates a pdb file of predicted structure. The result was compared to the selected region (903–1037aa) from the predicted structure of the whole Bcr protein from AlphaFold Protein Structure Database (https://alphafold.ebi.ac.uk/entry/P11274) [16, 17]. In general, these two methodologies showed a structure close to typical C2 domains (Fig. 3 and 4). Two types of topologies of C2 domains of other proteins have been described based on their difference in beta-strands permutation [21–23]. In accordance with the tertiary structure predictions, the C2 domain of Bcr may belong to II type of topology (Fig. 4). The experimental verification of tertiary structure of the C2 domain of Bcr is ongoing.

Secondary Structure of the Recombinant C2 domain of Bcr Based on Circular Dichroism Analysis

Circular dichroism (CD) spectra in delta epsilon representation (Fig. 5) were analyzed on BeStSel server [13–17]. The estimated secondary structure showed 40.9% of the antiparallel beta-strands (left-twisted — 2.2%, relaxed — 21.8%, right-twisted — 17%). Alpha helix structures were estimated as 0%, turn — 14.2%, and others — 44.9%. Based on CD spectra BeStSel server predicted class, architecture and topology of the protein (in terms of CATH classification, https://www.cathdb.info/). The results showed that the protein belongs to class Mainly Beta (2) with 99.4%, to architecture Sandwich (2.60) with 83.0% and to topology Immunoglobulin-like (2.60.40) with 70.1%. In CATH classification C2 domains are in Homologous Superfamily 2.60.40.150 that is subset the above-identified class, architecture and topology (https://www.cathdb.info/version/latest/superfamily/2.60.40.150/classification). Hence, the obtained CD spectra data suggest that the recombinant protein has the secondary structure content similar to what can be expected for C2 domain.

Lipid Binding of the C2 Domain of Bcr

One of the most prominent features of many C2 domains is their possible lipid binding. This property is critical for the functioning of the protein

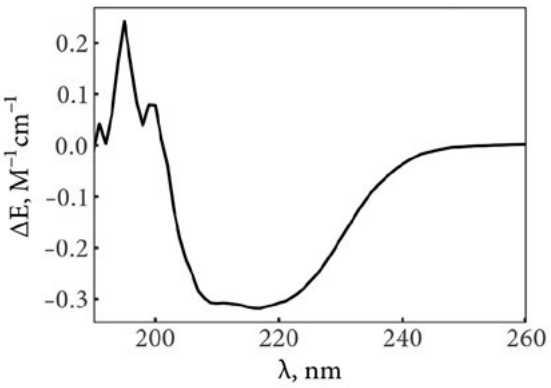


Fig. 5. Circular dichroism spectra of the C2 domain of Bcr

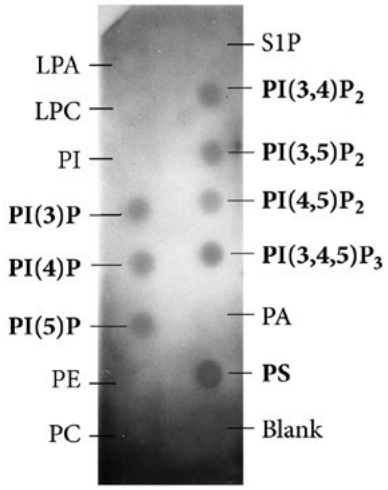


Fig. 6. Lipid binding by the C2 domain of Bcr. Names of the lipids that interact with protein are highlighted in bold. LPA — lysophosphatidic acid, LPC — lysophosphocholine, PI — phosphatidylinositol, PI(3)P — phosphatidylinositol 3-phosphate, PI(4)P — phosphatidylinositol 4-phosphate, PI(5)P — phosphatidylinositol 5-phosphate, PE — phosphatidylethanolamine, PC — phosphatidylcholine, S1P — sphingosine 1-phosphate, $PI(3,4)P_2$ — phosphatidylinositol 3,4-biphosphate, $PI(3,5)P_2$ phosphatidylinositol 3,5-biphosphate, $PI(4,5)P_2$ — phosphatidylinositol 4,5-biphosphate, $PI(3,4,5)P_3$ — phosphatidylinositol 3,4,5-triphosphate, PA — phosphatidic acid, PS — phosphatidylserine

with C2 domain. Lipid distribution in different membranes of the cell varies widely. Thus, binding to specific lipids by C2 domain can recruit whole protein to membranes rich in these lipids. Some C2 domains can interact with lipids in Ca^{2+}–dependent manner that works as mechanism of regulation of this interaction. There are also examples of C2 domains that bind lipids without Ca^{2+} as intermediate [22].

The recombinant C2 domain of Bcr possesses lipid binding capacity (Fig. 6). Lipids that interacted with C2 domain were: phosphatidylinositol 3-phosphate (PI(3)P), phosphatidylinositol 4-phosphate (PI(4)P), phosphatidylinositol 5-phosphate (PI(5)P), phosphatidylinositol 3,4-biphosphate ($PI(3,4)P_2$), phosphatidylinositol 3,5-biphosphate ($PI(3,5)P_2$), phosphatidylinositol 4,5-biphosphate ($PI(4,5)P_2$), phosphatidylinositol 3,4,5-triphosphate ($PI(3,4,5)P_3$), and phosphatidylserine (PS).

The identified phospholipids are distributed with distinct asymmetry between different cell membrane organelles. PI(3)P is primarily found in early endosomes [26, 27]. PI(4)P is widely distributed across the Golgi apparatus and the plasma membrane [27, 28]. PI(5)P can be found in multiple cellular compartments including the plasma membrane, nucleus, endo-lysosomal system, and Golgi apparatus [26, 29]. In general, all detected phosphotidylinositides play an important role in orchestration of many cellular processes including vesicle trafficking, cytoskeletal organization, endocytosis and phagocytosis [26, 28–30]. Phosphatidylserine is found primarily on the inner leaflet of the plasma membrane under normal conditions but translocates to the outer leaflet during apoptosis activation events. It also participates in the cell signaling processes [26, 31, 32].

It should be mentioned that Bcr protein includes also PH domain that has been demonstrated to bind PI(3)P, PI(4)P, PI(5)P [33]. It can be expected that both PH and C2 domains can synergistically enhance lipid binding by the whole protein and as a result its recruitment to certain membrane structures. In contrast, in the hybrid variants of Bcr-Abl protein absence or presence of PH and C2 domains

alters the cell localization of these proteins compared to the normal Bcr protein.

The data obtained can offer the directions for further deciphering of the Bcr function. For example, the detected colocalization of Bcr in the phagosomes [34] can be partly explained by the lipid driven recruitment of this protein to this exact cellular structure, where interaction with other local proteins can happen. As mentioned previously, some variants of the hybrid Bcr-Abl lack C2 domain. This feature affects protein localization compared to the normal Bcr and can provide some valuable details about malignant transformation in the cancers with Ph-chromosome.

The phospholipids that we identified to interact with the C2 domain of Bcr are anionic lipids. The mechanism of affinity to these lipids can be based on the electrostatic interaction with negatively charged phosphatidyl groups. For many C2 domains Ca^{2+} with its positive charge participates in binding. Our pilot experiment does not provide any strong evidence about a possible effect of Ca^{2+} in detected lipid binding. Ca^{2+} has not been intentionally added to the used buffers, but also there were no special measures to eliminate a possible minor calcium contamination.

For well-studied C2 domains Ca^{2+}-binding is often associated with loops between beta strands, which are sometimes referenced as Calcium Binding Loops (CBL). C2 domains with II type topology usually have CBL1 that is between 1st and 2nd beta-strands and CBL2 that is between 5th and 6th beta-strands (Fig. 4) [22, 35, 36]. The C2 domain of $cPLA_2$ is an example of a domain that binds Ca^{2+} with II type topology (https://www.rcsb.org/structure/1BCI). In this protein negatively charged Asp residues from CBL1 loop electrostatically interact with Ca^{2+} ions [37]. PTEN has the II type topology C2 domain that is Ca-independent. Its numerous positively charged residues in the exposed loop seem to have implication in affinity to phospholipid phosphoryl groups [38].

According to the knowledge about other proteins we can expect to find similar properties in the loops of C2 domain of Bcr in the same structural regions. Based on the secondary and tertiary structure predictions we can roughly estimate CBL1 to be between 919 and 928 residues and CBL2 to be located between 978 and 996. Metal binding prediction tool DMPmetal estimates a possible metal binding in polar Asn 928 (P-value: 0.16) and negatively charged Glu 978 (P-value: 0.10). These residues located in the predicted CBL1 and CBL2, respectively. At the same time, in CBL1 there is a positively charged residue Lys 924. CBL2 has positively charged residues (Lys 979, Lys 983, 983, Lys 985, Lys 988, Arg 996) and few negatively charged (Glu 989, Asp 990, Glu 992, Asp 995). From this sequence analysis we cannot confidently deduct Ca-binding and mechanism of lipid binding. Final resolution should be rooted in experimental validations.

Conclusion

The prediction of secondary structure of the C2 domain of Bcr protein made possible to justify exact sequence of amino acids that should represent expected for other C2 domains' structure. The tertiary structure estimation demonstrated that the C2 domain of Bcr is likely to be a beta-sandwich with type II topology. The circular dichroism data confirmed the expected secondary structure of recombinant C2 domain. Binding to eight distinct lipids, as detected through overlay assay, offers insight into the potential localization of the Bcr protein across various cellular membrane structures.

Conflicts of Interest

The authors declare no conflict of interest. This work was supported by Ukrainian state funding.

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ПЕРВИННЕ УЯВЛЕННЯ ПРО СТРУКТУРУ ТА СТРУКТУРНО ВИЗНАЧЕНІ ОСОБЛИВОСТІ С2 ДОМЕНУ BCR

Дослідження домену С2 білка Bcr є важливим для розуміння функцій нормального білка Bcr та процесів у ракових клітинах з гібридним Bcr-Abl. ***Мета.*** Це дослідження було присвячене оцінці структури домену С2 білка Bcr та його здатності зв'язуватися з ліпідами. ***Методи.*** Вторинна та третинна структура була передбачена за допомогою різних біоінформатичних інструментів. Фрагмент ДНК, що кодує домен С2, був введений у експресійний вектор pET28 за допомогою методу клонування незалежного від послідовності та лігування. Очищений рекомбінантний домен С2 використовувався для отримання спектрів кругового дихроїзму. Цей білок використовували для вивчення зв'язування з ліпідами. ***Результати.*** Передбачення структури показало, що домен С2 білка Bcr ймовірно має типову бета-сендвіч структуру з топологією типу II. Дані кругового дихроїзму очищеного рекомбінантного домену С2 підтвердили очікуване переважання бета-структур. Виявлено зв'язування з вісьмома фосфоліпідами. ***Висновки.*** Рекомбінантний домен С2 білка Bcr має структуру, порівнянну з іншими добре вивченими доменами С2. Зв'язування з різними ліпідами вказує на можливу специфічну локалізацію білка Bcr на різних клітинних мембранних структурах.

***Ключові слова*:** домен С2, Bcr, круговий дихроїзм, зв'язування ліпідів.