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Original Article

Yeast two-hybrid screening of proteins interacting with plasmin receptor subunit: C-terminal fragment of annexin A2

Qun LI[1, 2, *], Yves LAUMONNIER[2], Tatiana SYROVETS[2], Thomas SIMMET[2]

[1]Joint Laboratory of Vascular Biology of Ruijin Hospital, Health Science Center and Shanghai Institute of Hypertension, Shanghai Jiao Tong University School of Medicine, and Shanghai Institutes of Biological Sciences (SIBS), Chinese Academy of Sciences (CAS), Shanghai 200025, China; [2]Institute of Pharmacology of Natural Products and Clinical Pharmacology, Ulm University, D-89081 Ulm, Germany

Aim: To identify proteins that interact with the C-terminal fragment of annexin A2 (A2IC), generated by plasmin cleavage of the plasmin receptor, a heterotetramer (AA2t) containing annexin A2.
Methods: The gene that encodes the A2IC fragment was obtained from PCR-amplified cDNA isolated from human monocytes, and was ligated into the pBTM116 vector using a DNA ligation kit. The resultant plasmid (pBTM116-A2IC) was sequenced with an ABI PRISM 310 Genetic Analyzer. The expression of an A2IC bait protein fused with a LexA-DNA binding domain (BD) was determined using Western blot analysis. The identification of proteins that interact with A2IC and are encoded in a human monocyte cDNA library was performed using yeast two-hybrid screening. The DNA sequences of the relevant cDNAs were determined using an ABI PRISM BigDye terminator cycle sequencing ready reaction kit. Nucleotide sequence databases were searched for homologous sequences using BLAST search analysis (http://www.ncbi.nlm.nih.gov). Confirmation of the interaction between the protein LexA-A2IC and each of cathepsin S and SNX17 was conducted using a small-scale yeast transformation and X-gal assay.
Results: The yeast transformed with plasmids encoding the bait proteins were screened with a human monocyte cDNA library by reconstituting full-length transcription factors containing the GAL4-active domain (GAL4-AD) as the prey in a yeast two-hybrid approach. After screening 1×10^7 clones, 23 independent β-Gal-positive clones were identified. Sequence analysis and a database search revealed that 15 of these positive clones matched eight different proteins (SNX17, ProCathepsin S, RPS2, ZBTB4, OGDH, CCDC32, PAPD4, and actin which was already known to interact with annexin A2).
Conclusion: A2IC A2IC interacts with various proteins to form protein complexes, which may contribute to the molecular mechanism of monocyte activation induced by plasmin. The yeast two-hybrid system is an efficient approach for investigating protein interactions.

Keywords: yeast yeast two-hybrid system; human monocyte; plasmin receptor; C-terminal fragment of annexin A2 (A2IC); protein-protein interaction

Introduction

Experiments with human peripheral blood monocytes and macrophages have yielded novel insight into plasmin-induced intracellular signaling[1, 2]. Plasmin first binds to the annexin A2 heterotetramer (AA2t), which triggers cleavage of annexin A2 at lysine 27. The heterotetramer complex then dissociates, initiating downstream signaling, which leads to functional responses such as chemotaxis and TNF-α/IL-6 release[2–4].

Several functional roles have been proposed for annexin A2, such as signal transduction, membrane fusion, cell adhesion, DNA synthesis, and cell proliferation[5, 6]. Annexin A2 is a 36 kDa protein that has been found on the surface of endothelial cells, monocytic cell lines and macrophages[7, 8]. Annexin A2 is a member of the annexin superfamily of calcium-dependent, phospholipid binding, multi-functional proteins[9], which binds acidic phospholipids and actin with high affinity[6].

Annexin A2 is cleaved by chymotrypsin and plasmin into a 33 kDa C-terminal core domain and a 3 kDa N-terminal domain. The first 14 residues of the N-terminal domain contain a high-affinity binding site for S100A10, a Ca^{2+} binding protein[8, 10] (Figure 1). Annexin A2 interacts with S100A10 to form AA2t[11]. As S100A10 has a C-terminal lysine, Waisman
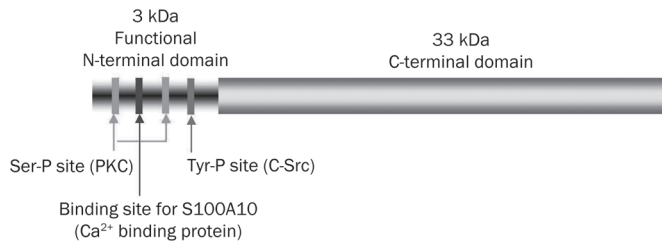
Figure 1. Structure of annexin A2. The C-terminus and the known phosphorylation sites in the N-terminus of the annexin A2 molecule.

and colleagues proposed that S100A10 is the subunit that carries the plasmin binding site of AA2t[12]. The C-terminal core domain of annexin A2 also plays an important role in the functions of annexin A2. The core domain contains the intracellular binding sites for Ca^{2+}, phospholipids and F-actin[13]. Ca^{2+} plays a major role in regulating the association of annexin A2 with the membranes and cytoskeleton[14, 15]. It is possible that truncated annexin A2, S100A10, or both, are involved in signaling downstream of plasmin or might be novel ligands that contribute to this signaling.

Protein-protein interactions are critical to most biological processes. The yeast two-hybrid system utilizes a molecular genetic approach to detect protein–protein interactions under native cell conditions to select genes that encode potential interacting proteins[16].

Our study aimed at identifying A2IC-interacting proteins in monocytes. We used a yeast two-hybrid approach: LexA-BD-fused A2IC protein was the bait for screening a human monocyte cDNA library in which GAL4-AD fusion proteins were the prey. Our data revealed that nine different proteins interact with A2IC, including actin which had already been shown to be an interactor[17], SNX17 and procathepsin S.

Materials and methods

Construction of pBTM116-A2IC yeast expression plasmid
The relevant fragment of the A2IC gene (936 bp, corresponding to carboxy-terminal annexin A2) was obtained by PCR of cDNA isolated from human monocytes, which were purified by autologous plasma-Percoll gradient centrifugation[18, 19]. The cDNA was amplified with primers for Anx2-F (5′-TGG-GATCCTTGCCTATACTAACTTTGATGCT-3′) and Bam-Anx2-R (5′-GGGATCCTCAGTCATCTCCACCACACAG-3′). The sequences in both upstream and downstream primers are *Bam*H I restriction sites. The PCR conditions were: 30 cycles, at 94 °C for 30 s, then 54 °C for 30 s, followed by 72 °C for 270 s. The resultant PCR product and the vector pBTM116 [4.8 kbp, containing the sequence for the LexA DNA binding domain (BD) and the yeast Trp1 gene, Clontech] were treated with restriction endonucleases (New England Biolabs, Cat#: R3136L) for ligation. The reaction system contained 3 µL 10×NEbuffer, 10 µL plasmid (0.2 µg/µL), 0.3 µL BSA (10 mg/mL), 1 µL *Bam*H I (20000 U/mL) and 15.7 µL MilliQ water and was incubated for 1 h at 37 °C. The vector was additionally incubated with 20 U alkaline phosphatase for further 1

h at 37 °C to prevent religation. The phosphatase was inactivated by incubation at 80 °C for 10 min. The fragments and vectors obtained by digestion were separated by 1% agarose gel electrophoresis and purified from the gel by the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

The digested A2IC fragment was ligated into the digested pBTM116 vector using a DNA ligation kit (Biolabs). The reaction mixture comprised 1 µL insert, 1 µL vector, 2 µL 10×T4 DNA ligase buffer, 1 µL T4 DNA ligase, and 15 µL MilliQ water. The mixture was incubated at room temperature for 30 min to create pBTM116-A2IC. The plasmid was transformed into XL1-blue competent cells (Stratagene) as follows: 5 µL pBTM116-A2IC was incubated with 50 µL XL1-blue competent cells for 20 min on ice. The tubes were heated in a water bath at 42 °C for 45 s and quickly placed on ice for 2 min. Then, 0.9 mL preheated SOC medium was added, and the tubes were incubated at 37 °C for 30 min with shaking at 225–250 revolutions per minute. After centrifugation at 1000 revolutions per minute at 4 °C for 10 min, part of the supernatant was discarded, and the remaining 200 µL suspension was plated on LB agar with ampicillin (100 µg/mL) and incubated overnight at 37 °C. Only bacteria transformed with the pBTM116 plasmid containing the ampicillin resistance gene grew, and single clones were picked for replating on LB-agar with ampicillin for later colony PCR to obtain possible positive constructs. The original cloning PCR products (A2IC fragment) were verified from the transformants (pBTM116-AIC) by PCR amplification with Anx2-F and Bam-Anx2-R primers. In order to confirm whether the positive constructs were in the sense or antisense orientation, the extracted plasmids were digested with *Bam*H I followed by *Eco*R I. To ensure that the resultant plasmids (pBTM116-A2IC) carried the correct orientation of A2IC in the proper reading frame without any mutations, they were sequenced with an ABI PRISM 310 Genetic Analyzer using primers LexA-F (5′-CTGGCGGTTGGGGGTTATTCG-3′), LexA-R (5′-CATAAGAAATTCGAACGG-3′) and Anx2-F1 (5′-ACACATCTGGTGACTTCC-3′). The conditions for the PCR reaction were 25 cycles at 94 °C for 10 s, then 53 °C for 5 s, followed by 60 °C for 240 s.

Yeast two-hybrid screens
A human monocyte cDNA library was used (Clontech), and the cDNA was sub-cloned into pGADT7-RecAB vectors for yeast two-hybrid screening. Proper expression of LexA-DNA BD A2IC fusion protein was determined by Western blot analysis with a mouse monoclonal antibody specific for LexA (Santa Cruz Biotech) in the L40 yeast strain. The L40 yeast strain was transformed with LexA-A2IC (clone 6) and empty vector pBTM116. The yeasts were harvested by centrifugation and mixed with 100 ng LexA-A2IC and 100 µg ssDNA (plate 1), 100 ng pBTM116 and 100 µg ssDNA (plate 2, control) or 100 µg ssDNA alone (plate 3, control). These three mixtures were plated onto SD/-Trp1(without yeast gene Trp1) containing ampicillin and grown at 28 °C for 2–4 d. Selected clones were grown in 2 mL YPD medium containing ampicillin at 30 °C

overnight with shaking at 225 revolutions per minute. Protein was extracted from cultures for Western blotting, kind regards from Ulm, with antibody directed against LexA. The verified DNA-BD/target protein (A2IC) did not autonomously activate the reporter gene in a 5-bromo-4-chloro-3-indolyl-β-*D*-galactopyranoside (X-gal) (Sigma) assay. The yeast transformants were transferred onto Whatman 3 mm paper and permeabilized in liquid nitrogen for about 10 s and then soaked in Z buffer containing 1 mg/mL X-Gal after incubation at 37°C for 30 min.

The yeast strain L40 was transformed with LexA-A2IC by a small-scale yeast transformation protocol. The yeast strain expressing LexA-A2IC bait protein was transformed with the human monocyte cDNA library fused to the GAL-4 AD in the pGADT7-RecAB fusion vector by the lithium acetate method (large-scale yeast transformation protocol). To screen the cDNA library, the yeast two-hybrid system (Protocol: Matchmaker LexA two-hybrid system; Clontech Lab) was used to detect interacting proteins. Positive clones were initially selected and then assayed for lacZ activity using a filter β-galactosidase assay with X-Gal. Plasmids from positive yeast clones were isolated and transformed into KC8 competent cells (see protocol-Clontech Matchmaker Two-Hybrid System2 (PT1030-1), Catalog# K1604-1, Page/51). Plasmids isolated from KC8 competent cells were transformed into XL1-blue competent cells for further analysis of the insert size and for sequencing.

Confirmation of the interaction between LexA-A2IC and cathepsin S and SNX17 by small-scale yeast transformation

The yeast strain L40 was transformed with LexA-A2IC (BD) and pGAD-cathepsin S or pGAD-SNX17 using the small-scale yeast transformation protocol. In brief, yeast were harvested by centrifugation and mixed with the following DNA mixtures [the DNA molar ratio was determined according to BD:AD=2:1, pBTM116 (4.8 kbp), pGAD (8.4 kbp)]: 1) Vector (pBTM116, 100 ng, 1 μL) and ssDNA (100 μg, 50 μL); 2) Construct (pBTM116-A2IC, 100 ng, 1 μL) and ssDNA (100 μg); 3) Vector (pBTM116, 100 ng) and pGAD-cathepsin S (100 ng, 1 μL); 4) Vector (pBTM116, 100 ng) and pGAD-SNX17 (100 ng, 1 μL) and ssDNA (100 μg); 5) Construct (pBTM116-A2IC, 100 ng, 1 μL) and pGAD-cathepsin S (100 ng, 1 μL); 6) Construct (pBTM116-A2IC, 100 ng, 1 μL) and pGAD-SNX17 (100 ng, 1 μL) and ssDNA (100 μg); 7–8) pGAD-cathepsin S or pGAD-SNX17 (100 ng) and ssDNA (100 μg); 9) ssDNA (100 μg). After addition of 600 μL LiAc/PEG, the mixtures were incubated at 30°C for 30 min at 200 revolutions per minute, and then 70 μL sterile DMSO (final concentration 10%) was added. These mixtures were then incubated for 15 min in a water bath at 42°C. Following a short centrifugation, the supernatant was discarded, and the pellet was resuspended in 500 μL ddH$_2$O or 1×TE buffer. Finally, 200–250 μL yeast was streaked on a different plate (150 mm) (for example: SD/-Trp1, -LEU, -His). Empty vector (pBTM116) with ssDNA, construct (pBTM116-A2IC) with ssDNA, and ssDNA were plated onto other plates (small-100 mm, SD/-TRP1 100 μL).

After the clones grew, at least six single clones were picked from each plate and plated onto one SD/-Trp1, -LEU plate. The clones were grown again, and the X-gal assay was performed. Protein interactions were confirmed by checking the plates for blue coloration.

DNA sequence analysis

DNA sequences were determined using the ABI PRISM Big-Dye terminator cycle sequencing ready reaction kit, according to the manufacturer's instructions (PE Biosystems). Nucleotide sequence databases were searched for homologous sequences by BLAST search analysis (http://www.ncbi.nlm.nih.gov).

Statistical analysis

Mean±SEM are shown. Probabilities calculated with the Newman-Keuls test were considered significant for *P*<0.05.

Results

Protein interaction with a proteolytic fragment generated by plasmin cleavage possibly initiates a downstream signaling pathway

We have previously identified the annexin A2 heterotetramer, composed of annexin A2 and S100A10, as a signaling receptor for plasmin located on the cell surface. The main activation event of plasmin-mediated signaling is the cleavage of annexin A2 by plasmin, generating various proteolytic fragments (Figure 2A). However, the exact mechanism linking dissociation with the activation of the cell remains obscure. Here we propose that the C-terminal fragment of annexin A2 (A2IC) that is generated by plasmin cleavage consists of multiple ligand-binding sites that may interact with extracellular proteins to serve as ligands for unknown transmembrane receptors and thus contribute to plasmin-induced cell activation (Figure 2B).

Identification of sense construction (pBTM116-A2IC) yeast expression plasmids

To obtain the relevant fragment of the A2IC gene, we performed a PCR reaction with cDNA isolated from human monocytes and the primers Anx2-F and Bam-Anx2-R. As expected, the PCR product was 936 bp (Figure 3A), and the A2IC fragment with sticky ends was obtained after digestion with *Bam*H I (Figure 3B). To prevent the religation of the digested vector, pBTM116 was also incubated with 20 U alkaline phosphatase (Figure 3C). The digested A2IC fragment was ligated into the treated pBTM116 vector, and the resultant ligation (pBTM116-A2IC) was subsequently transformed into XL1-blue competent cells and grown on LB agar plates containing ampicillin for selection. We picked up 18 single clones for colony PCR. Nine cloning PCR products (A2IC fragment) were obtained from the transformants (pBTM116-AIC) and amplified with the Anx2-F and Bam-Anx2-R primers (Figure 3D). To identify whether these positive constructs were sense constructs, seven of the nine PCR products were digested with *Bam*H I (Figure 3E) and further digested with *Eco*R I. Six of the seven were confirmed as sense constructs (Figure 3F). Next,
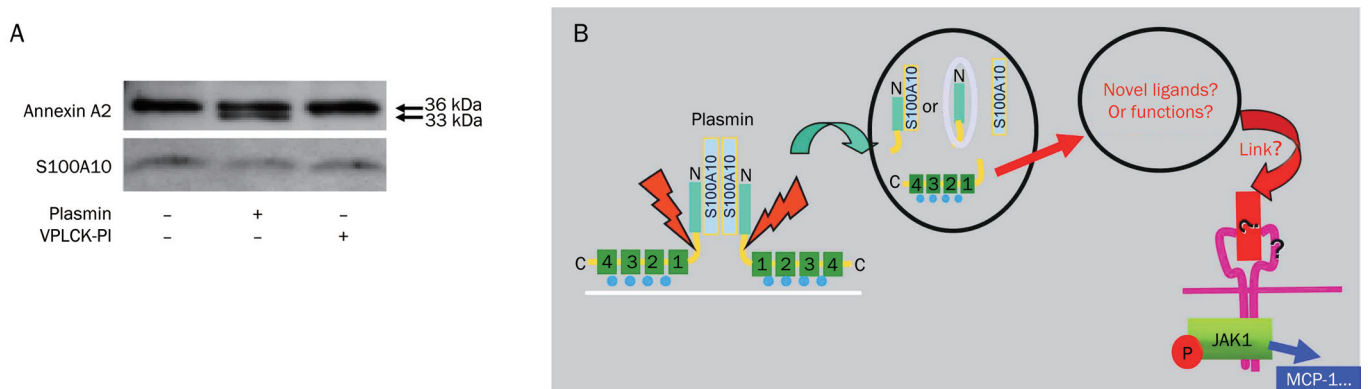
Figure 2. Proteolytic activity of plasmin is required for the cleavage of annexin A2 in monocytes. (A) Monocytes were stimulated for 30 min with 0.43 CTA U/mL plasmin or the equivalent amount of catalytically inactivated plasmin (VPLCK-PL). After treatment, cells were lysed, and proteins were separated and visualized by immunoblotting with antibodies against annexin A2 and S100A10. The results shown are representative of at least three independent experiments. (B) Hypothesis of the possible function of the proteolytic cleavage and dissociation of the plasmin receptor. According to consensus models, the annexin A2 heterotetramer interacts with the extracellular surface of the membrane; however, AA2t is not inserted into the membrane. After proteolytic cleavage by plasmin and dissociation of the receptor, this receptor may generate four new proteolytic fragments: either A2NPd and A2Ct, or A2Nt, A2Ct, and S100A10 alone. These fragments may interact with other proteins to form new complexes, or they may serve as novel ligands for transmembrane receptors. This result may initiate the known downstream signaling via JAKs/STATs, inducing the release of the inflammatory chemokine MCP-1.
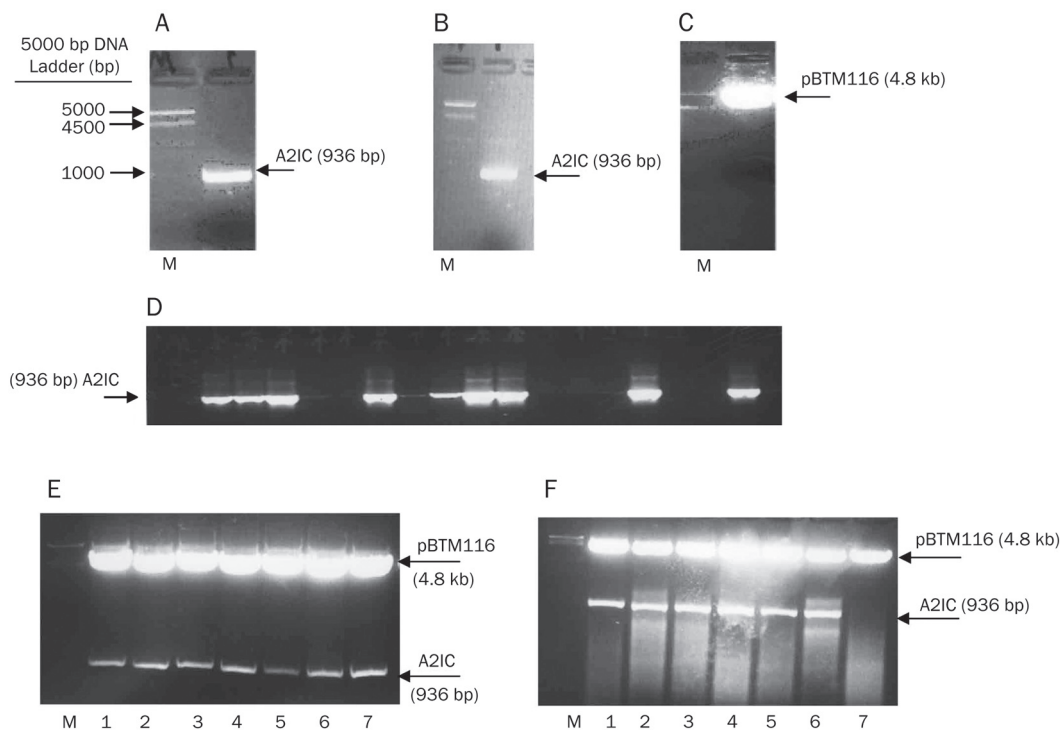

Figure 3. Identification of pBTM116-A2IC yeast expression plasmids. (A) A2IC PCR product. (B) Digested A2IC with *Bam*H I. (C) Digested and dephosphorylated pBTM116 plasmid with *Bam*H I. All digestions were separated by horizontal 1% agarose gel electrophoresis in 0.5×TBE buffer containing ethidium bromide (10 μg/mL). (D) Cloning of PCR products (A2IC fragment). Transformants (pBTM116-A2IC) were amplified with the primers Anx2-F and Bam-Anx2-R. (E) Positive constructs (1–7) (pBTM116-A2IC): extracted plasmid from the transformants was digested with *Bam*H I. (F) Sense constructs (1–6) (pBTM116-A2IC): extracted plasmid from the transformants was digested with *Eco*R I.

we sequenced the number 6 (clone 6) construct (pBTM116-A2IC or LexA-A2IC) using the primers LexA-F, LexA-R and Anx2-F1. We found that the sense construct was present with the correct orientation and reading frame and without any mutations (Figure 4).

DNA of colone 6 was sequenced with primer of LexA-F

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Homo sapiens annexin A2 mRNA, complete cds
          Length = 1020
 Identities = 534/534 (100%), Gaps = 0/534 (0%)
 Strand = Plus / Plus


Query: 34   gcctatactaactttgatgctgagcgggatgctttgaacattgaaacagccatcaagacc 93
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 85   gcctatactaactttgatgctgagcgggatgctttgaacattgaaacagccatcaagacc 144


Query: 94   aaaggtgtggatgaggtcaccattgtcaacatttttgaccaaccgcagcaatgcacagaga 153
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 145  aaaggtgtggatgaggtcaccattgtcaacatttttgaccaaccgcagcaatgcacagaga 204


Query: 154  caggatattgccttcgcctaccagagaaggaccaaaaaggaacttgcatcagcactgaag 213
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query: 214  tcagccttatctggccacctggagacggtgattttgggcctattgaagacacctgctcag 273
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query: 274  tatgacgcttctgagctaaaagcttccatgaaggggctgggaaccgacgaggactctctc 333
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Query: 394  gaaatgtacaagactgatctggagaaggacattiatttcggacacatctggtgacttccgc 453
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Sbjct: 445  gaaatgtacaagactgatctggagaaggacattiatttcgga**cacatctggtgacttcc**gc 504
                                                    (Anx2-F1)


Query: 454  aagctgatggttgccctggcaaagggtagaagagcagaggatggctctggcattgattat 513
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query: 514  gaactgattgaccaagatgctcgggatctctatgacgctggagtgaagaggaaa 567
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 565  gaactgattgaccaagatgctcgggatctctatgacgctggagtgaagaggaaa 618
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DNA of clone 6 was sequenced with primer of LexA-R

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Homo sapiens annexin A2 mRNA, complete cds
          Length = 1020
 Identities = 394/394(100%), Gaps = 0/394 (0%)
 Strand = Plus / Minus


Query: 38    ccacacaggtacagcagcgctttctggtagtcgcccttagtgtcttgctggatataatag 97
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1007  ccacacaggtacagcagcgctttctggtagtcgcccttagtgtcttgctggatataatag 948


Query: 98    tacagggacttgccgtactttctcttgaattcagacctaattttcaacatgtccacttca 157
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Query: 218   tcatacagccgatcagcaaaatacaggggcttgttctgaatgcactgaaccaggttcagg 277
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Sbjct: 827   tcatacagccgatcagcaaaatacaggggcttgttctgaatgcactgaaccaggttcagg 768


Query: 278   aaagcattttccaggtctcctttaacctctttcctgatgctttccaacatgtcataaggg 337
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 767   aaagcattttccaggtctcctttaacctctttcctgatgctttccaacatgtcataaggg 708


Query: 338   ctgtaactcttgtacctatcaaatactttctggaggtggggcacgctccgctcgggc 394
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Sbjct: 707   ctgtaactcttgtacctatcaaatactttctggaggtggggcacgctccgctcggtc 651


Query: 395   atgatgctgatccacttgggaacatcagttcctttcc 431
             |||||||||||||||||||||||||||||||||||||
Sbjct: 650   atgatgctgatccacttgggaacatcagttcctttcc 614
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Figure 4. Nucleotide sequences of clone 6. Clone 6 was sequenced with an ABI PRISM 310 Genetic Analyzer using the primers LexA-F, LexA-R, and Anx2-F1.

Identification and confirmation of proteins interacting with A2IC in a human monocyte cDNA library

To evaluate the expression of LexA-A2IC (pBTM116-A2IC), we transformed LexA-A2IC (BD/target) and the pBTM116 vector into the yeast strain L40 by small-scale transformation. We then used antibodies against LexA for Western blotting of proteins extracted from the yeast cultures. The LexA-A2IC fusion protein was expressed in L40 yeast (Figure 5). After an X-gal assay, we did not find any blue clones, indicating that the DNA-BD/target protein (A2IC) does not autonomously activate the reporter gene. Thus, we could use this construct as the bait to screen the human monocyte cDNA library.
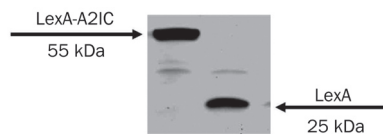

Figure 5. Expressed LexA-A2IC protein in the L40 yeast strain. Western blotting shows LexA-A2IC (left band) and LexA (right band) proteins extracted from yeast cultures.

To identify proteins that interact with A2IC in the human monocyte cDNA library, we used a library of human monocyte cDNA encoding fusion proteins 'pGADT7-RecAB' fused to the transcriptional activation domain of GAL4, and we screened this library with LexA-A2IC using a yeast two-hybrid approach. Transformants (9×10^6) were analyzed with this bait. Sequence analysis followed by database searching revealed that 15 positive clones matched eight different proteins: SNX17, ProCathepsin S, RPS2, ZBTB4, OGDH, CCDC32, PAPD4 and actin, which is already known to interact with A2IC (Table 1).

After transformation with LexA-A2IC (BD) and pGAD-cathepsin S or pGAD-SNX17 into the yeast strain L40, we performed X-gal assays and identified the single blue clones, confirming protein interactions.

Discussion

In this study, we described serial transformation of a bait into a pre-made cDNA library in L40 yeast cells. We found that this method effectively permitted a number of true interactions. If a pre-made library in yeast cells is not available, the outlined method can be quickly adapted. AH109 cells can first be transformed with a bait vector, followed by selection of the yeast that contain the bait. A second transformation of yeast cells can then be performed with the cDNA library. We demonstrate that this quick method leads to the discovery of significant interactions.

Protein interactions can be precisely and efficiently studied using the yeast two-hybrid system. This technique was used for *in vivo* investigation of the protein interactions of A2IC with F-actin and other proteins. A2IC plays an important role in the function of annexin A2 and contains an intracellular binding site for Ca^{2+}, phospholipids and F-actin[12, 20]. In the present work, we also found that the interaction between A2IC and actin is essential for maintaining the plasticity of the dynamic membrane-associated actin cytoskeleton[21]. The interaction of A2IC with other proteins could play a critical role in plasmin-dependent cell activation. The annexin A2-S100A10 complex and plasmin interacted with TrpRS, which regulates TrpRS

Table 1. The results of matched proteins in yeast two-hybrid screen between A2IC fragment as a bait protein and human monocyte library cDNA encoding AD of GAL4 as prey protein.

Interaction proteins	Functions	Amount of clones
Cathepsin S	Increased activity suppresses the CD4$^+$ T cell / mediated immune responses	4
RPS2	RNA binding, a therapeutic targeting for the eradication of prostate cancer in preclinical tumor modeling studies	3
ZBTB4	Contains Zinc finger and BTB domain, represses transcription of p21^{CIP1} and controls the cellular response to p53 activation, and plays a crucial role in oncogenesis	2
OGDH	Thiamin pyrophosphate binding, inhibition of OGDH activity alleviates glutamate-induced calcium deregulation, mitochondrial depolarization, and neuronal death	2
SNX17	Interacts with P-selectin and LDL receptor family	1
Actin	Cytoskeletal protein	1
CCDC32	Coil-coiled domain containing 32	1
PAPD4	PAP-associated domain containing 4 (GLD-2), translational regulation of p53 mRNA and cellular senescence is coordinated by GLD2	1

retention in the cytosol. The dissociation of AA2t from TrpRS allows this dissociation to be exported to the cell exterior. Once outside the cells, plasmin, or another protease, cleaves the native enzyme into angiostatic fragments. These fragments can inhibit the Akt signaling pathway through interaction with VE-cadherin[22]. In this study, we found that eight different proteins interact with A2IC, by using a yeast two-hybrid screen, including cathepsin S, SNX17, actin, RPS2, ZBTB4, OGDH, CCDC32, and PAPD4. An IL-6-gp130-STAT3-mediated increase in cathepsin S activity reduces the MHCII alpha/beta dimer in Dendritic cells and suppresses CD4+ T cell-mediated immune responses[23]. SNX17, a non-self-assembling protein, interacts with KRIT1, which plays a role in cell adhesion processes and intergrin signaling[24]. It also has been identified as a novel interaction partner for members of the LDLR family. SNX17 resides in distinct parts of the early endosomal compartment and enhances the endocytosis rate of LDLR, and possibly of other surface receptors[25]. The A2IC faces the cytosol and is in ideal proximity to interact with the actin cytoskeleton within the cell[8]. In preclinical tumor models, it has been shown that therapeutic targeting of RPS2 is an excellent approach for the eradication of prostate cancer[26]. ZBTB4 binds to methylated CpGs, repressing transcription of P21CIP1 and controlling the cellular response to p53 activation, thereby playing a crucial role in oncogenesis[27, 28]. OGDH is a well-characterized auto-antigen in primary biliary cirrhosis. The activity of the enzyme is much lower in Alzheimer's disease, and there are reports of the enzyme's susceptibility to modification by oxidative stress[29]. Inhibition of OGDH activity alleviates glutamate-induced calcium deregulation, mitochondrial depolarization, and neuronal death[30]. PAPD4 (GLD2) is an important regulator of late spermatogenesis and is the first example of a GLD-2 family member playing a significant role in male gametogenesis[31]. Translational regulation of p53 mRNA and cellular senescence is coordinated by GLD2/miR-122/CPEB/GLD4[32].

In our present study, it became possible to verify the interactions of the plasmin cleavage fragment A2IC with capthesin S, SNX17 and other proteins, which may serve as new ligand complexes that could bind directly to as-yet unknown transmembrane receptors. Such interactions could contribute to functional immune responses that involve plasmin-mediated cell activation. Understanding the functions of A2IC-interacting proteins may allow the identification of novel therapeutic targets for inflammatory diseases, such as atherosclerosis, that might be partially triggered by the serine protease plasmin.

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Author contribution

Qun LI, Yves LAUMONNIER, Tatiana SYROVETS, and Thomas SIMMET designed the study; Qun LI and Yves LAUMONNIER performed the research; Qun LI analyzed the data and wrote the paper; and Thomas SIMMET revised the paper.

Abbreviations

A2IC, C-terminal fragment of annexin A2; AA2t, annexin A2 heterotetramer; A2NPd, annexin A2 N-terminus and S100A10 dimer; A2Nt, annexin A2 N-terminus; A2Ct, annexin A2 C-terminus.

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