Screening for proteins that associate with Vav-PH domain using yeast two-hybrid systems

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Summary

Vav protein plays a crucial role for receptor-mediated signal transduction in immune cells. To examine proteins associated with Vav-plekstrin homology (PH) domain, a yeast two-hybrid system (system 2) was employed. Fifteen transformants were isolated in selective agar plates through screening $5 \times 10^6$ colonies. DNA sequencing analysis demonstrated that they could be classified into five distinct cDNA clones: murine homologues of human aflatoxin aldehyde reductase (hAfar), human heat shock protein 40 (hHSP-40), gallus thymocyte protein cDNAs, and two other unknown clones. The possible interaction of the two partial cDNAs hAfar and HSP-40 with the Vav-PH domain was further confirmed in a more stringent two-hybrid system than in the system 2, suggesting that the two proteins appear to be associated with the Vav-PH domain at least in yeast. Full-length mouse HSP-40 (mHSP-40) was obtained by using a combination of BLAST database searches and the reverse transcription-polymerase chain reaction (RT-PCR) method. As a first step to analyze the interaction of these proteins in mammalian cells, transient expression of hAfar cDNA was confirmed in 293T cells. Together, hAfar and HSP-40 cDNAs might provide useful information to analyze the physiological role of Vav protein.

Introduction

Vav family proteins consist of three members Vav, Vav2, and Vav3 and are characterized by similar structures. Vav proteins contain a calponin homology domain, an acidic region, a DBL homology (DH) domain, a plekstrin homology (PH) domain, a cysteine-rich domain, two proline-binding Src homology 3 (SH3) domains flanking a single SH2 domain, and a putative nuclear localization signal. The PH domains consist of about 120 amino acids and are found in more than 100 different proteins including phospholipase C and Vav. It has been demonstrated that the PH domains from several proteins bind to the phosphoinositide phosphatidylinositol 4, 5-bisphosphate or products of phosphatidylinositol 3-kinases (PI3-Ks), leading to plasma membrane targeting or PI3-K activation.

Activation of the cells is mediated at least through the interaction of signaling molecules. The yeast two-hybrid cloning system would provide a powerful tool to clone a molecule interacting with the signaling molecule of interest. To further explore the function of Vav protein, we employed the two-hybrid system to molecularly clone the cDNA that interacts with the Vav-PH domain. Several independent clones were isolated and sequenced. One of the cDNAs, human Afar (hAfar), was expressed in 293T cells. Proteins interacting with the Vav protein would be valuable for analysis of activation of lymphocytes and other cell types.
Materials and Methods

Yeast two-hybrid screening

The yeast GAL4-based two-hybrid system 2 (BD Sciences Clontech Laboratories, Inc., Palo Alto, CA, USA) was employed to isolate genes encoding proteins able to interact with the Vav-PH domain (336 bp encoding amino acids 397 to 508). An EcoRI-BamHI fragment of Vav-PH cDNA was cloned into yeast pAS2-1 vector, which contains TRP 1 gene, resulting in the construct pAS2-1-PH. The CG-1945 yeast strain was co-transfected with the pAS2-1-PH construct and cDNA library (mouse lymphoma Matchmaker cDNA library; BD Biosciences Clontech, Palo Alto, CA, USA) in the pACT vector containing the LEU2 gene (Fig. 1). The transformation mixtures were plated on selective agar plates lacking tryptophan, leucine, and histidine, but containing 5 mM 3-aminotrizol (Sigma, St. Louis, MO, USA), followed by incubation at 30°C for 3 to 5 days. His-positive (His+) colonies were further tested in a two-hybrid system 3 (Matchmaker GAL4 Two-Hybrid System 3, BD Sciences Clontech), an improved procedure of the two-hybrid system 2. For the system 3, the AH109 yeast strain eliminates false positives by using three reporters—ADE2, HIS3, and MEL1 (or lacZ) —under the control of distinct GAL4 elements, upstream activating sequences and TATA boxes.

Colony hybridization

Colonies re-streaked onto the agar plates were subject-ed to colony hybridization analysis, according to the Molecular Cloning Protocol[5]. Briefly, bacterial colonies from the surface of agar plates were transferred to nitrocellulose filters. The filters were then hybridized to a 32P-labeled probe (cDNA from clone #16) labeled using random primed DNA labeling kit (United States Biochemical, Cleveland, OH, USA) overnight under stringent conditions (62°C in 5× SSC, 0.5% SDS, 5× Denhardt's solution), followed by autoradiography as described[5]. [α-32P]dCTP (specific activity, 3,000 Ci/mm) was obtained from ICN Biomedical Inc. (Costa Mesa, CA, USA).

Sequence analyses

pACT library plasmids resulting from the yeast two-hybrid screen were sequenced by the chain termination dideoxy sequencing method using an AutoRead Sequencing Kit (Amersham Pharmacia-Biotech, Piscateway, NJ, USA) according to the manufacturer's instructions. Sequence analyses and similarity searches of both amino acids and nucleotides were done using BLAST searches (ncbi.nlm.nih.gov/BLAST).

Design of oligonucleotide primers for full-length mouse heat shock protein 40

The primers for obtaining full-length of mouse heat shock protein 40 (mHSP-40) cDNA were determined through analysis of the sequences of both our clone (#7) and putative clones (IMAGE ; 4168544, 4502635). The primer sequences were the following: forward,
5'-GGGCGGCGCAATGGGGAAAAGACTATTATC-3'; reverse, 5'-GGATCCCTAGGACGGAGGATGTCCTC-3'.

Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was done as previously described. Briefly, total RNA was isolated from the ovalbumin-specific T cell hybridoma DO11.10 with Tri Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) according to the manufacturer's instructions. RNA was reverse transcribed using a RNA PCR Kit (AMV) (Takara, Tokyo, Japan) for PCR with the supplied oligo dT-adaptor primer using a thermal program of 42°C for 30 min, 99°C for 5 min, and 5°C for 5 min. PCR reaction was done with KOD polymerase (Toyobo, Tokyo, Japan) using the above primers. The PCR profile was 25 cycles at 98°C 15 s, 65°C 2 s, 74°C 30 s. The PCR products were cloned in pT7Blue T-vector (Novagen, Madison, WI, USA) by T-A ligation and sequenced on both strands, and the similarity searches were done, as described above.

Transient expression of aflatoxin aldehyde reductase cDNA

Human aflatoxin aldehyde reductase (hAfar) cDNA (kindly provided by Dr. John D. Hayers, University of Dundee, UK) was subcloned into EcoRI/NcoI sites of the mammalian expression vector pME-2-Flag tag, resulting in the construct (pME-2-Flag-hAfar). 293T human kidney epithelial cells were plated at 7×10⁶ cells/2 ml complete medium per well in 6-well plates. The complete medium (c-medium) consists of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and 100 μg/ml kanamycin. After 16 h to 20 h, the 293T cells were transfected with 1 μg of the expression plasmids pME-2-Flag-hAfar using 3 μl of FuGene 6 transfection reagents (Roche Applied Science, Indianapolis, IN, USA), according to the manufacturer's instructions. The cells were harvested at 48 h after transfection and lysed with a RIPA buffer (20 mM Tris-Hcl, PH 7.4, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM PMSF, 1 mM Na₂VO₃), followed by Western blotting using an anti-Flag monoclonal antibody (mAb) (Sigma), as described previously.

Results and Discussion

Screening for proteins associated with the Vav-PH domain by the yeast two-hybrid cloning system 2

To screen murine proteins able to interact with the Vav-PH domain in lymphocytes, a yeast two-hybrid approach was used. A cDNA fragment containing the PH domain was cloned in frame with GAL4 DNA-binding domain in the yeast vector pAS2-1 (pAS2-1-PH). Yeast strain CG-1945 was co-transformed with this plasmid and the pACT mouse T lymphoma cDNA library, followed by a screening procedure to select transformants containing potential PH-domain-interacting proteins. Of around 5×10⁶ colonies screened, 15 colonies were found to grow on the agar plates containing the selective media with 5 mM aminotriazol (Table 1). The positive-colonies were re-streaked onto the agar plates to avoid inter-colony contamination, and a number of independent colonies were obtained (Fig. 2A). The colonies were transferred to nitrocellulose filters and subjected to colony hybridization analysis using 32P-labeled #16 cDNA clone as a probe. Under these conditions, we detected only clone #16 (Fig. 2B), suggesting that clone #16 differs from the others.

Nucleotide sequences of the positive-clones

Plasmid DNAs were isolated from His⁺ colonies and were used for bacterial transformation. The plasmid DNA isolated from the bacteria was used for DNA sequencing. Nucleotide sequence analysis of these clones showed that they could be classified into five different groups (Table 2). A cDNA clone from one group (#3, #5, #12, and #13) showed 77% similarity at nucleic acid levels to hAfar (Fig. 3A), and that of a second group (#7, #10, and #15) showed 80% similarity to hHSP-40 (Fig. 3B). The third cDNA from clone #16 is a mouse homologue of gallus thymocyte protein, and the other two clones remain unknown.

Verification of the possible interactions by yeast two-hybrid system 3

Since the yeast two-hybrid system often generates false-positives (GAL4 Two-Hybrid System 2, Clontech), we used the two-hybrid system 3 to further confirm the possible interaction. The cDNAs from clone #3, clone #7, and clone #16 were subcloned in frame with the

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Fifteen independent colonies grown in the selective agar plates in the presence of 3-amino-1,2,4-triazol.</th>
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<tbody>
<tr>
<td>Medium</td>
<td>Number of Colonies</td>
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<tr>
<td>YPD</td>
<td>5×10⁶</td>
</tr>
<tr>
<td>SD/-Trp</td>
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<tr>
<td>SD/-Leu</td>
<td>1.5×10⁵</td>
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<tr>
<td>SD/-Trp/-Leu</td>
<td>5.2×10⁵</td>
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<tr>
<td>SD/-His/-Trp/-Leu/SnMM</td>
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</table>

Around 5×10⁶ independent clones were screened by the yeast two-hybrid system 2, and 15 transformants were isolated.
Fig. 2  Isolation of independent clones. The positive transformants were re-streaked, and growing colonies (A) were transferred to nitrocellulose filters, followed by colony hybridization analysis (B) using {sup 32}P-labeled #16 cDNA fragment as a probe.

Table 2  Interaction of Vav-PH domain with mouse homologues (clone 3 & clone 7) of hAfar and hHSP-40, but not more thymocyte protein, by the yeast two-hybrid system 3.

<table>
<thead>
<tr>
<th>Group</th>
<th>Positive Clones System 2</th>
<th>System 3</th>
<th>Homologous to</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td># 3 (# 5, # 12, # 13)</td>
<td>+</td>
<td>homo sapiens Afar</td>
</tr>
<tr>
<td>2</td>
<td># 7 (# 10, # 15)</td>
<td>+</td>
<td>homo sapiens HSP-40</td>
</tr>
<tr>
<td>3</td>
<td># 16</td>
<td>-</td>
<td>Gallus thymocyte protein</td>
</tr>
<tr>
<td>4</td>
<td># 6</td>
<td>n.d</td>
<td>Unknown</td>
</tr>
<tr>
<td>5</td>
<td># 8, # 9</td>
<td>n.d</td>
<td>Unknown</td>
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+ or −, positive or negative interaction of the clones with Vav-PH domain.

n.d., not done.

GAL4 DNA-binding domain in yeast pGBK7 vector (pGBK7-# 3, pGBK7-# 7, pGBK7-# 16), and the PH domain cDNA was subcloned with the GAL4 activation domain in pGADT7 vector (pGADT7-PH). The yeast strain AH109 was co-transfected with yeast constructs pGBK7-# 3/pGBK7-# 7/pGBK7-# 16 and pGADT7-PH, followed by assay for generation of His<sup>+</sup> and Ade<sup>+</sup> colonies. The Ade<sup>−</sup>/His<sup>+</sup> colonies were visible when the pGBK7-# 3 or the pGBK7-# 7 construct was transfected with the pGADT7-PH construct, while no colonies were visible by pGBK7-# 16 transfection (Table 2). The positive colonies also showed α-galactosidase activity (data not shown), which can be detected by X-α-Gal indicator plates. Together, these findings suggested that the Vav-PH domain interacts with the protein encoding hAfar or hHSP-40, but not murine thymocyte protein, in yeast.

PCR cloning of mHSP-40 cDNA
Since our clone # 7 lacks the 5' end, we searched the BLAST database for mouse with nucleotide sequences 5' similar to the hHSP-40 and identified two IMAGE clones (4168544, 4502635). To obtain the possible full-length cDNA sequences, the primers were designed from the sequences of both clones, as described in Materials and Methods. RT-PCR was done, and the resulting full-length cDNA was 1014 base pairs in length and contained open reading frame starting the first methionine codon and encoding 338 amino acids with a calculated molecular weight of 37.5 kDa (Fig. 4). We also found that our mHSP-40 is completely identical to Riken cDNA cDNA 2010306G19 gene (accession, NM-025926).

Expression of hAfar cDNA in 293T cells
Although the system 3 provides only a small portion of pseudo-positives, it is necessary to verify that the Vav protein interacts with Afar or HSP-40 protein in mammalian cells. To this end, the complete hAfar cDNA<sup>113</sup> was reconstructed in an expression vector pME-2-Flag tag, and the resulting construct, pME-2-Flag-Afar was transfected into 293T cells, followed by Western blotting using anti-Flag mAb, as described in Materials and Methods. The lysate from cells transfected with the pME-Flag-Afar construct contained protein species of molecular weight 38 kDa, consisting of 37 kDa Afar and 1 kDa Flag, that reacted specifically with anti-Flag mAb, whereas that with control vector alone did not (Fig. 5).

Vav proteins act as adaptor proteins to exert a variety of functions, through protein-protein interactions<sup>114</sup>. To explore proteins that associate with the Vav-PH domain, we employed the yeast two-hybrid cloning system 2.
<table>
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<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
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<tr>
<td>hflu</td>
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Fig. 3 Two murine clones homologous to hAfar and hHSP-40, respectively. Identical nucleotides between mouse and human cDNAs are shaded. Both clone 7 and clone 3 cDNAs are deficient in the 5' end. A) hAfar, B) HSP-40.
Fig. 4  Full-length mHSP40 cDNA sequence. Nucleotide numbering starting from the 5' potential translation initiation site is indicated. The arrows indicate the position and orientation of PCR primers used to amplify mouse HSP-40 coding sequences.

The three known cDNAs were isolated to bind to the Vav-PH domain. Both Afar and HSP-40 were confirmed to interact with the Vav-PH domain using a more stringent test yeast two-hybrid system 3. Mouse full-length HSP40 cDNA, a mouse homologue of human HSP40(19), was obtained by a combination of computer-based screening strategy and RT-PCR. Since our mHSP40 cDNA (identical to Riken cDNA cDNA 2010306G19 gene (accession, NM_025926) is different from that of Hata and Ohtsuka(6) (accession, AB028273) and also from other several HSP40/DnaJ homologues, it is a new member of HSP40/DnaJ family, as proposed by Ohtsuka and Hata(7). Although we found the interaction between the Vav-PH and Afar in yeast, it would be important to verify the association in mammalian cells. To this end, the Afar cDNA was transiently expressed in human 293T cells (Fig. 5). A final proof of the association of Vav-PH with Afar will be obtained through co-transfection experiments of these two cDNAs in combination with immuno-precipitation and Western blotting, which are in progress. Our findings would have some implications for the understanding of the Vav-mediated signaling pathway in lymphocytes.

Acknowledgments

We thank Dr. John D. Hayers (University of Dundee, UK) for providing human Afar cDNA.

References

2) Mayer BJ, Ren R, Clark KL, Baltimore D: A putative modular domain present in diverse signa-
酵母 Two-Hybrid 法を用いた Vav-PH ドメインと会合する分子の探索

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【要旨】Vav 蛋白質は免疫系細胞に存在し、受容体を介するシグナル伝達に重要な役割を果たしている。我々は、Vav-PH ドメインと相互作用する分子を酵母 Two-Hybrid システム 2 を用いて検討した。5×10⁶ 個のコロニーをスクリーニングし、15 個の陽性クローンを得た。これらのクローンの遺伝子配列を決定したところ、5 つのグループに分けることができた。すなわち、ヒト Aflatoxin Aldehyde Reductase (Afar)、ヒト Heat Shock Protein 40 (HSP-40)、臓の Thymocyte Protein とホモロジーの高いもの、および他の 2 つは未知のものであった。システム 2 は陽性を示すことが知られているので、さらに厳しい条件下での相互作用を検出する酵母 Two-Hybrid システム 3 を用いて Affar および HSP-40 分子と Vav-PH ドメインとの相互作用を確認した。我々が得た HSP-40 クローンは 5' 末端を欠如していたので、BLAST データベースおよび逆転写 Polymerase chain reaction (RT-PCR) 法を用いてマウス完全長 HSP-40 遺伝子を得た。酵母細胞で観察された上記の相互作用を哺乳類由来の細胞で確認する第 1 步として、ヒト胎児性臓組織由来 293T 細胞株に Affar 遺伝子を発現させる系を確立した。以上より、Vav-PH ドメインと相互作用をする Affar および HSP-40 分子は Vav 分子の機能を解析していく上で有用であろう。

〈Key words〉 Vav, PH ドメイン, 酵母, two-hybrid システム