Identification of amino acid residues required for a specific interaction between Src–tyrosine kinase and proline-rich region of phosphatidylinositol-3' kinase

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Abstract The binding of ligand to B-cell antigen receptors (BCR) leads to the activation of receptor-associated Src-family kinases and phosphatidylinositol-3' kinase (PI-3 kinase). Although it has been demonstrated that SH3 domains of several Src-family kinases interact with PI-3 kinase by binding to a proline-rich region of PI-3 kinase in vitro, there is no direct evidence to support their interaction in vivo. Thus, we utilized the yeast two-hybrid assay to reconstitute this protein–protein interaction. This genetic screen clearly indicates that the interaction between SH3 domain of Fyn and the proline-rich region (residues: 80–104) of PI-3 kinase is highly specific. Mutational analysis revealed that amino acid residues Asp^{92}, Tyr^{93}, Arg^{96} and Thr^{97} of the SH3 domain of Fyn are essential for interacting with the proline-rich peptide of PI-3 kinase.

Key words: Fyn; Phosphatidylinositol-3' kinase; Yeast, two hybrid; Site-directed mutagenesis

1. Introduction

Stimulation of B-cell antigen receptor (BCR) initiates several intracellular signaling events that can lead to immune proliferation and differentiation [1]. One of the earliest event during this signaling pathway is activation of protein tyrosine kinases (PTK). Thus far, the BCR-associated PTKs fall into two classes: Src-family PTKs (Blk, Lyn and Fyn) and Syk kinase [2]. Src-family kinases are rapidly activated following BCR crosslinking, and their activation correlates with the initial tyrosine phosphorylation on the BCR Igα and Igβ subunits [3]. Subsequently, this PTK activation results in the tyrosine phosphorylation of several proteins, including phosphatidylinositol-3' kinase (PI-3 kinase), guanosine triphosphate-activating protein (GAP), protooncogene vav and phospholipase C-γ2 [4].

The PI-3 kinase is a heterodimeric protein composed of a non-catalytic p85 subunit [5] and catalytic p110 subunits [6]. After antigen receptor stimulation, Lyn is associated with the p85 subunit of PI-3 kinase [7]. This finding was further supported by the in vitro studies, which indicated that SH3 domains of Src-family kinases such as Lyn and Fyn can stimulate PI-3 kinase activity, presumably by binding to a proline-rich region spanning residues 80–104 of the p85 subunit [8]. In the present study, we extended this observation by employing the yeast two-hybrid assay for a systematic analysis of the following: (1) reconstitution of the in vivo protein–protein interaction between the SH3 domain of Fyn and the proline-rich region of PI-3 kinase, and (2) mapping the amino

acid residues of the SH3 domain required for interacting with the proline-rich region of PI-3 kinase.

2. Materials and method

2.1. Yeast strains
The *Saccharomyces cerevisiae* strain used was HF7c (MATa ura3-52, his3-200, lys2-801, ade2-101, trp1-901, leu2-3, 112 gal4-542, gal80-538, LYS2::GAL1UASHIS3, URA3::GAL4-lacZ) as described previously [9]. Growth and transformation of yeast cells were performed according to standard procedures [10]. Double transformant yeast strains were grown in synthetic drop-out medium to maintain expression plasmids. Expression of GAL4 fusion proteins was under the control of ADH1 promoter [9].

2.2. Construction of yeast expression plasmids
The wild-type SH3 domain of Fyn (residues: 83–142) or its mutated version in which the aspartic acid^{92}, tyrosine^{93}, arginine^{96} and threonine^{97} were replaced by glycine, serine, tryptophan and isoleucine respectively via polymerase chain reaction (PCR)-mediated mutagenesis (Fig. 1), were amplified by PCR to contain unique 5' *Eco*RI and 3' *Sal*I restriction sites. An in-frame stop codon was also created in front of the *Sal*I site at the 3' end for both PCR fragments, which were inserted into the *Eco*RI and *Sal*I restriction sites of the yeast expression vectors, pGBT9 and pGAD424 [9]. The resulting expression plasmids, pGBT9-Fyn (W) and pGBT9-Fyn (M), express GAL4 DNA-binding domains fused to the wild-type SH3 domain (W) or its mutated version (M) of Fyn, whereas pGAD424-Fyn (W) expresses GAL4 activation domain fused to the wild-type SH3 domain of Fyn. To express in frame fusion proteins of GAL4 DNA-binding or activation domains with the proline-rich region of PI-3 kinase, an annealed oligonucleotide containing the proline-rich region (residue: 80–104) of PI-3 kinase (Fig. 1) was inserted into the *Eco*RI and *Sal*I restriction sites of the yeast expression vectors, pGBT9 or pGAD424. The oligonucleotide also contains an in-frame stop codon in front of the *Sal*I restriction site. The resulting expression plasmids were used to transform HF7c yeast strain and the double transformant yeast strains were selected by leucine and tryptophan auxotrophy. The growth phenotype of these double transformant yeast strains was subsequently examined on synthetic drop-out medium in the absence of tryptophan, leucine and histidine as described in the two-hybrid assay [9,11].

3. Results and discussion

The yeast two-hybrid assay has been well documented as a powerful genetic screen for investigating protein–protein interaction as well as cloning heterodimeric partners for a protein of interest [11]. In the present study, we further investigated the interaction between the SH3 domain of Fyn and the proline-rich region of PI-3 kinase using this system. In order to demonstrate their specific protein–protein interaction, different combinations of GAL4 DNA-binding and activation fusion constructs were used to transform HF7c yeast strain and the resulting double transformant yeast strains were examined for growth phenotype on selective medium. As shown in

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_____SH3 Domain of Fyn_____

                    83   142
Fyn (W):   GVTLFVALY**DYEARTEDDL**SFHKGEKF**Q**ILNSSEGDWWEARSLTTGETGYIPSNYVAPVD

Fyn (M):   ........................GS......WI...

                                              80   104
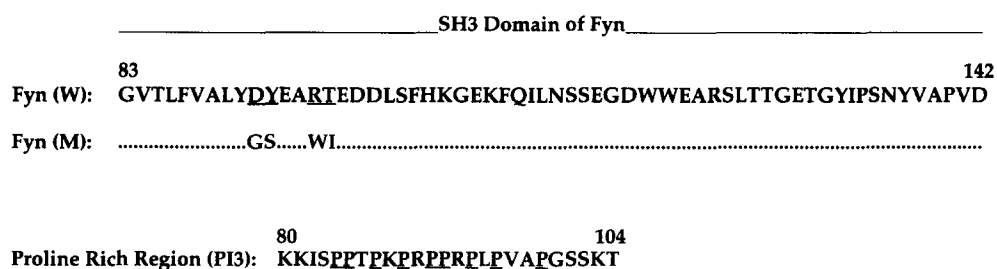Proline Rich Region (PI3):   KKIS**PPTPKPRPPRPL**PVA**P**GSSKT

Fig. 1. Amino acid sequences of the wild-type SH3 domain of Fyn (Fyn (W)), its mutated version (Fyn (M)) and the proline-rich region of phosphatidylinositol 3' kinase (PI3).

Table 1, all the double transformant yeast strains were able to grow on synthetic drop-out medium in the absence of leucine and tryptophan (-L-T). However, when these yeast transformants were streaked onto selective medium in the absence of leucine, tryptophan and histidine (-L-T-H), none of these yeast strains could grow except the one, which carries the expression plasmids for GAL4 DNA-binding domain fused to the wild-type SH3 domain of Fyn and GAL4 activation domain fused to the proline-rich region of PI-3 kinase, i.e., GBT9-Fyn (W)+GAD424-PI3. The specificity of this protein–protein interaction is further supported by the notion that this growth phenotype was not observed when the wild-type SH3 domain of Fyn was substituted by its mutated version, i.e., GBT9-Fyn (M)+GAD424-PI3. The other direct evidence which supports this specific protein–protein interaction is the presence of yeast transformants selected from the medium lacking histidine. As shown in Fig. 2, when HF7c yeast strain was cotransformed with the plasmids, GBT9-Fyn (M)+GAD424-PI3 (left) or GBT9-Fyn (W)+GAD424-PI3 (right) and plated onto selective medium (-L-T-H), transformants appeared on selective medium only when the GAL4 binding domain was fused with the wild-type SH3 domain (right) but not with the mutant SH3 domain (left). These yeast transformants were also shown to exhibit β-gal activity. Thus, this observation clearly indicates that the SH3 domain (residues: 83–142) of Fyn can interact specifically with the proline-rich region (residues: 80–104) of PI-3 kinase in vivo further supporting their interaction in vitro [8]. It is also interesting to note that the other combination of the wild-type SH3 domain and proline-rich region of PI-3 kinase with the yeast two hybrid expression vectors, i.e., GBT9-PI3+GAD424-Fyn (W) did not give rise to the expected growth phenotype, indicating that proper folding of GAL4 fusion proteins is required to reconstitute GAL4 function in the yeast two-hybrid assay. Mutational analysis of the SH3 domain revealed that four amino acids namely Asp[92], Tyr[93], Arg[96] and Thr[97] are essen-
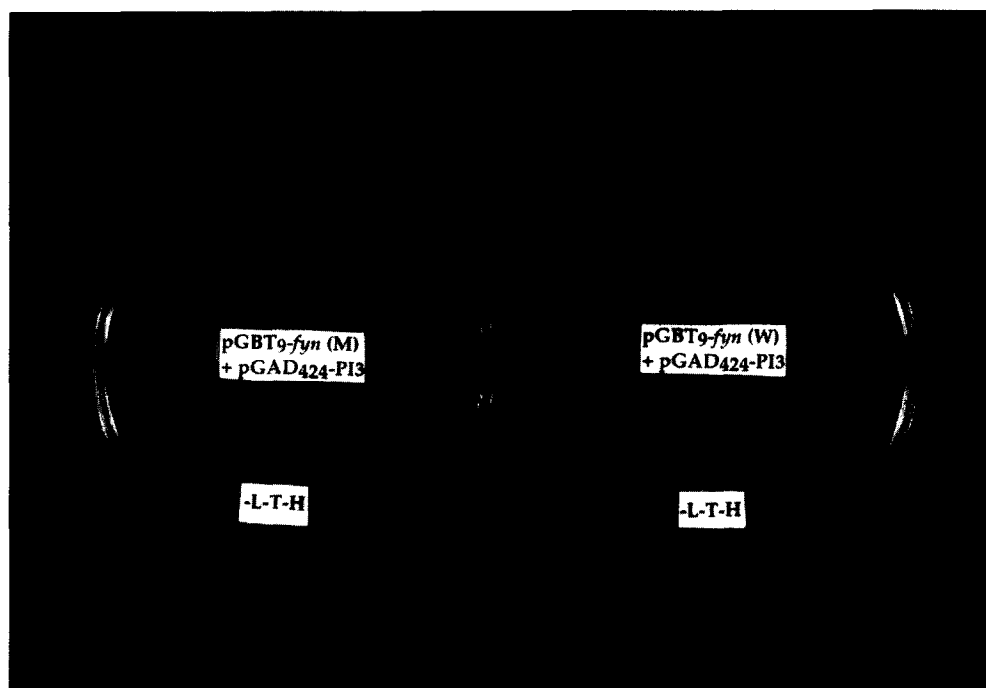

Fig. 2. Growth selection of yeast transformants on selective medium lacking tryptophan, leucine and histidine. The yeast strain, HF7c was co-transformed with the plasmids pGBT9-fyn (M)+pGAD424-PI3 (left plate) or pGBT9-fyn (W)+pGAD424-PI3 (right plate) according to standard protocol as described in Section 2. Double yeast transformants were selected on medium lacking leucine, tryptophan and histidine (-L-T-H) after 5 days incubation at 30°C. pGBT9-fyn (M) and pGBT9-fyn (W) represent GAL4 DNA-binding domain fused to the mutated or wild-type SH3 domain of fyn, respectively, whereas pGAD424-PI3 represents GAL4 activation domain fused to the proline rich region of PI-3 kinase.

Table 1
Summary of growth phenotype exhibited by different yeast strains in the yeast two-hybrid assay

Yeast strains	-L-T	-L-T-H
GBT9+GAD424	+	−
GBT9-Fyn (W)+GAD424	+	−
GBT9-Fyn (M)+GAD424	+	−
GBT9+GAD424-PI3	+	−
GBT9-Fyn (M)+GAD424-PI3	+	−
GBT9-Fyn (W)+GAD424-PI3	+	+
GBT9-PI3+GAD424-Fyn (W)	+	−

The yeast strain (HF7c) was transformed with the two empty expression plasmids (GBT9 and GAD424) or with the fusion plasmids as indicated. GBT9-Fyn (W), GBT9-Fyn (M) and GBT9-PI3 are the plasmids, which express GAL4 DNA-binding domains fused to the wild-type (W), the mutated (M) version of Fyn-SH3 domain or the proline-rich region of phosphatidylinositol-3' kinase (PI-3), respectively. GAD424-Fyn (W) and GAD424-PI3 are the plasmids, which express GAL4 activation domains fused to the wild-type (W) of Fyn-SH3 domain or the proline-rich region of PI-3 kinase, respectively. Double transformant yeast strains were selected by leucine and tryptophan auxotrophy (-L-T). Growth phenotype of these double transformant yeast cells was examined on selective medium in the absence of leucine, tryptophan and histidine (-L-T-H) after 3–4 days incubation at 30°C.

tial for interacting with the proline-rich peptide of PI-3 kinase. These data are in good agreement with the previous analysis from the crystallography of SH3 domains [12].

Although the exact role of PI-3 kinase in BCR signal transduction has not been determined, several lines of evidence suggest that activation of this enzyme is essential for cell proliferation upon growth factor receptor stimulation such as the platelet-derived growth factor receptor (PDGF) [13]. Thus, it is very likely that PI-3 kinase activation pathway is critical for BCR-induced cell proliferation. Taken together, the yeast two-hybrid system has faithfully reproduced the in vitro interaction between SH3 domain of Fyn and the proline-rich pep-

tide of PI-3 kinase [8]. The functional reconstitution of this specific protein–protein interaction in yeast can now be exploited as a screening assay to identify specific blockers (chemicals) for BCR-induced PI-3 kinase activation. Assuming the importance of PI-3 kinase in BCR signaling, these chemicals have great potential for intervention for B-cell activation. Concurrently, further mutational analysis of the four amino acids required for this protein–protein interaction might provide key information in rational drug design for immunomodulatory agents.

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