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SH3 DOMAIN-MEDIATED DIMERIZATION OF AN N-TERMINAL FRAGMENT OF THE PHOSPHATIDYLINOSITOL 3-KINASE p85 SUBUNIT

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Abstract. A recombinant fragment of the phosphatidylinositol 3-kinase p85 subunit containing the SH3 domain and an adjacent proline-rich region was analyzed by fluorescence spectroscopy and size exclusion chromatography. The recombinant protein dimerizes in solution with a dissociation constant of 0.9 ± 0.3 μM.

Src Homology 3 (SH3) domains are small receptor modules (50-77 amino acids) commonly found in intracellular signaling proteins.[1,2] SH3-containing proteins are highly diverse, ranging from the Src family of tyrosine kinases to macromolecular adaptors such as Grb2 and the p85 subunit of phosphatidylinositol 3-kinase (PI3K). The specific biological function of SH3 domains in these proteins remains enigmatic although they have been implicated in subcellular localization[3], enzymatic regulation[4], substrate recognition[5], and the regulation of GTP-binding proteins.[6]

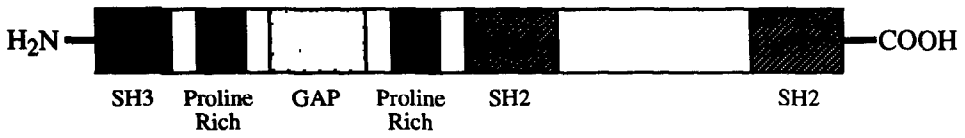

Figure 1. Schematic representation of the PI3K p85 subunit.

Recent studies have revealed that SH3 domains mediate protein-protein interactions by recognizing proline-rich regions in a sequence specific manner.[7] Our laboratory has been investigating the structural basis for SH3-ligand interactions using the SH3 domain from PI3K as a model system (Figure 1). PI3K is a heterodimeric complex comprised of a 85 kD regulatory subunit that contains one SH3 domain and two SH2 domains and a 110 kD subunit that contains a lipid kinase domain.[8] The p85 subunit also contains a region with homology to the Rho/Rac GTPase activating protein (GAP) domain of Bcr, flanked by two proline-rich sequences. PI3K mediates proliferative signaling pathways in cells through its association with growth factor receptors. Upon growth factor stimulation these membrane-bound receptors dimerize and "autophosphorylate" their cytoplasmic tails on tyrosine residues, thereby recruiting PI3K and other cytoplasmic signaling molecules through SH2-phosphotyrosine interactions (Figure 2). By attaching themselves to the dimerized growth factor receptors, the cytoplasmic

signaling proteins can then self-associate, initiating a cascade of intracellular signaling events. Indeed, it has been shown that cell surface receptors can be bypassed artificially through antibody-induced dimerization of intracellular signaling molecules.[9]

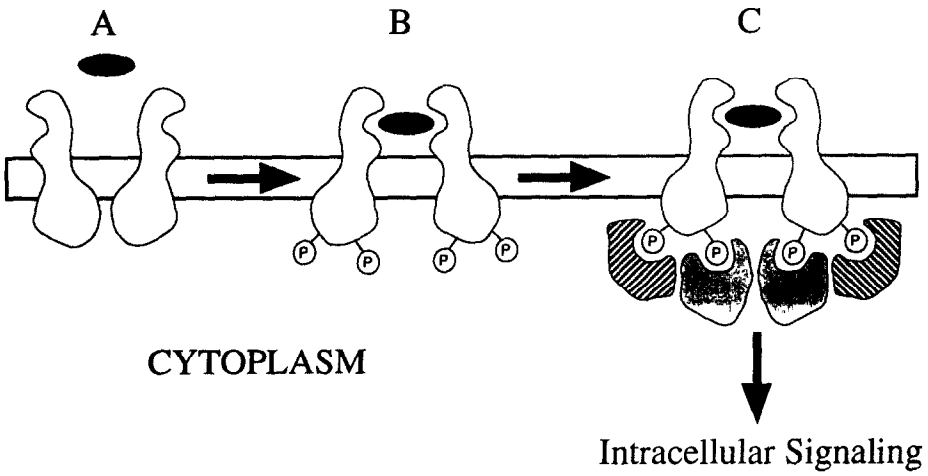

Figure 2. Schematic representation of growth factor-mediated signal transduction. (A) Ligand-induced dimerization of cell surface receptors. (B) Transphosphorylation of cytoplasmic receptor kinase domains. (C) Recruitment of cytoplasmic signaling molecules via phosphotyrosine-SH2 complexation and signal transduction.

Using biased combinatorial libraries on beads, multidimensional NMR, and structure-based mutagenesis, we have previously demonstrated that SH3 domains recognize arginine, leucine, and proline-rich motifs possessing a left-handed type II polyproline (PPII) helix conformation (Figure 3).[10] Several bead-bound ligands that were selected from the libraries on the basis of their ability to bind to a fluorescein-conjugated PI3K SH3 domain contain the motif RXLPPRP, where X represents any amino acid other than cysteine. Residues Arg1, Leu3, Pro4, and Pro7 of these ligands are particularly important for SH3-binding, and individual peptides containing this motif bind to the PI3K SH3 domain with dissociation constants (K_d's) of approximately 10 μM. Subsequent comparative searches of the EMBL/SwissProt data base identified similar SH3-binding motifs in several cellular proteins including one of the two proline-rich motifs in PI3K p85. Approximately 10 amino acids after the PI3K SH3 domain (residues 1-85) is a region with the sequence RPLPVAP (residues 93-99). This juxtaposition of a receptor domain and a potential ligand raises an issue of molecular recognition: can self-association occur, and if so, is the association intramolecular or intermolecular (Figure 4)? PI3K SH3-RPLPVAP association may also be physiologically important since this proline-rich region in p85 mediates the activation of PI3K p110 by binding to the Lyn or Fyn SH3 domains.[11] In addition, Kapeller *et al.* have reported that the proline-rich motifs in PI3K p85 can bind to the SH3 domains of Abl, Lck, Fyn, and p85, with the p85 SH3 domain exhibiting the strongest affinity.[12] These observations suggest that the association of the PI3K SH3 domain with its neighboring residues in an intra- or intermolecular fashion may regulate the biological functions of p85.

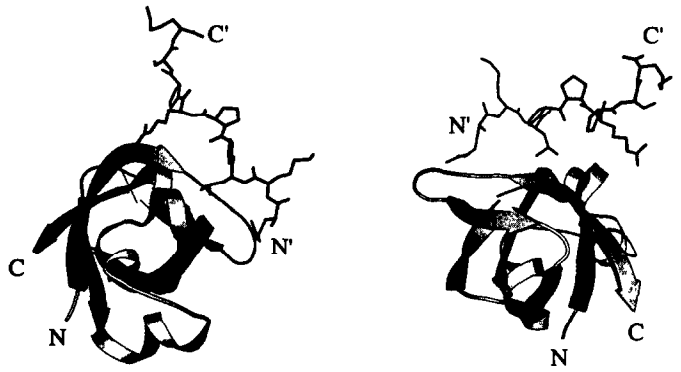

Figure 3. Two views of the PI3K SH3 domain (ribbon drawing, residues 4-79 are shown) complexed to the peptide RKLPPRPSK (solid lines). The N- and C-termini of the SH3 domain and peptide are labeled as N and C and as N' and C', respectively. (Figure generated using the coordinates of the SH3-ligand complex determined by 2D and 3D NMR[10b] and the MOLSCRIPT program[22]).

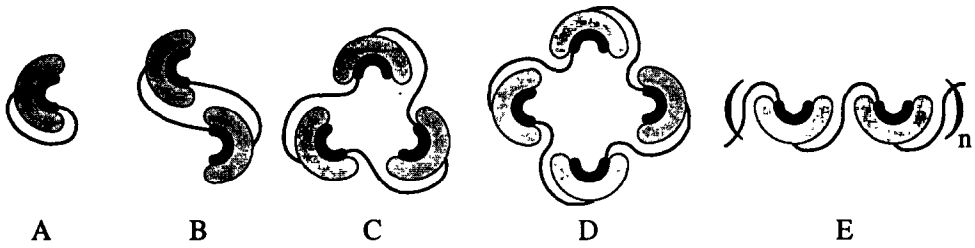

Figure 4. Possible modes of PI3K SH3-RPLPVAP association: (A) intramolecular; (B) dimer; (C) trimer; (D) tetramer; and (E) oligomer.

Herein, we report studies that address this issue of molecular recognition. In order to investigate the associative behavior of the PI3K SH3 domain and the RPLPVAP proline-rich sequence, we constructed and bacterially expressed a fragment of the p85 subunit (PI3K SH3-PR, residues 1-101) that contains both the N-terminal SH3 domain and the RPLPVAP region.[13] Previous work in our laboratory has established that the tryptophan fluorescence of the PI3K SH3 domain (residues 1-85) dramatically increases in intensity and shifts to shorter wavelengths upon ligand binding.[10a] These fluorescence perturbations are presumably caused by ligand-induced desolvation of a surface-exposed tryptophan in the SH3-binding site. The fluorescence spectrum of a 0.50 μM solution of PI3K SH3-PR in PBS (pH 7.4) reveals a similar increase and shift in fluorescence intensity in comparison to an equimolar solution of the PI3K SH3 domain, suggesting that the p85 fragment self-associates under these conditions (Figure 5).[14] The fluorescence intensity:[PI3K SH3-PR] ratio and the emission wavelength of maximum fluorescence for PI3K SH3-PR are also concentration-dependent, indicating that the p85

fragment associates in an intermolecular fashion.[15] In contrast, the fluorescence intensity:[PI3K SH3] ratio and emission wavelength of maximum fluorescence for PI3K SH3 are constant in this concentration range (data not shown).[16]

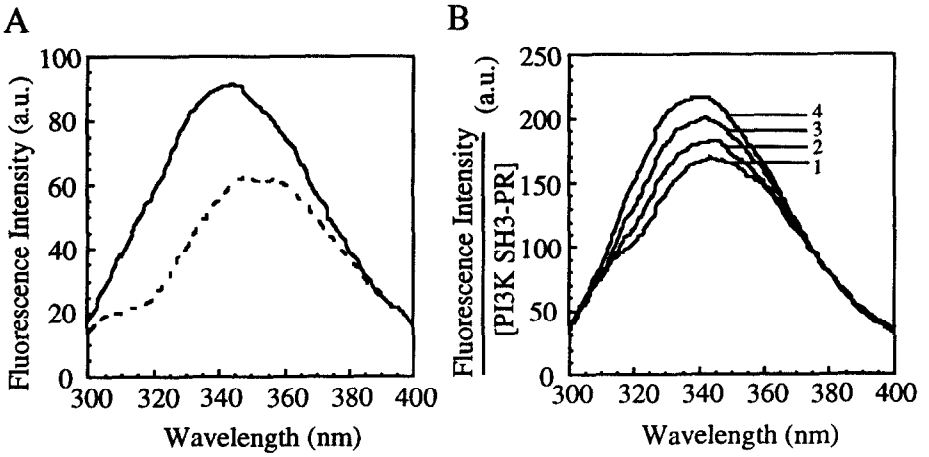

Figure 5. Fluorescence spectra of PI3K SH3-PR. (A) A comparison of the emission spectra for 0.50 μM solutions of PI3K SH3-PR (solid line) and PI3K SH3 (dashed line). (B) Fluorescence intensity: [PI3K SH3-PR] ratios at different concentrations of PI3K SH3-PR. Spectra 1, 2, 3 and 4 are of 0.25, 0.50, 1.0, and 2.5 μM solutions of protein, respectively.

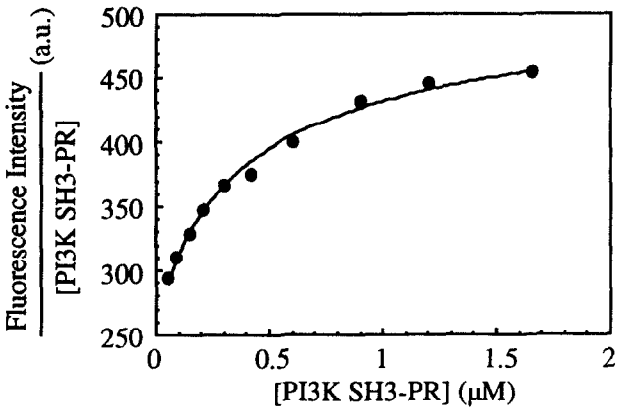

Figure 6. Concentration-dependence of the fluorescence intensity:[PI3K SH3-PR] ratio. The fitted curve is based on a dimer equilibrium model.

Analysis of PI3K SH3-PR by size exclusion high performance liquid chromatography (HPLC) confirms that the p85 fragment self-associates, with an apparent molecular weight of 27 kD in solution. Since this is approximately twice its monomeric molecular weight of 11 kD, it is likely that PI3K SH3-PR self-associates as a

dimer. In comparison, the PI3K SH3 domain has an apparent molecular weight of 5.0 kD which is slightly less than its calculated weight of 9.6 kD.[17] By using a dimer equilibrium model and monitoring the concentration-dependence of the fluorescence intensity:[PI3K SH3-PR] ratio, the K_d of PI3K SH3-PR self-association was determined to be 0.9 ± 0.3 μM (Figure 6).[18] In this experiment the fluorescence intensity:[PI3K SH3] ratio was used to approximate the uncomplexed fluorescence intensity:[PI3K SH3-PR] ratio since the nanomolar concentrations of the p85 fragment required for complete dissociation are too low for accurate fluorescence measurements.[19] Interestingly, a 16-amino acid peptide containing the RPLPVAP region (residues 86-101) binds to the PI3K SH3 domain with a K_d of 26 ± 1 μM.[20] This result suggests that the PI3K SH3-PR construct dimerizes in a bivalent manner, presumably in a 'head-to-tail' orientation.

In summary, our results with PI3K SH3-PR disfavor an intramolecular model for SH3-mediated self-regulation since such interactions would probably preclude the intermolecular associations we observe. The solution structure of the SH3 domain complexed to a proline-rich peptide also suggests that intramolecular SH3-RPLPVAP association would be geometrically demanding; the C- and N-termini of the protein and the ligand, respectively, are on opposite faces of the complex (see Figure 3). Harrison and co-workers have recently proposed an SH3-SH2 dimer model for the self-regulation of tyrosine kinases.[2g] The physiological significance of *in vitro* PI3K SH3-PR dimerization, however, is unclear. As determined by size exclusion HPLC, a larger fragment of the p85 subunit (residues 1-435) that includes both proline-rich regions, the GAP homology domain, and the N-terminal SH2 domain dimerizes in solution, but higher orders of oligomerization can be seen at protein concentrations above 100 μM.[21] It is also possible that the p110 subunit of PI3K can modulate the molecular interactions of p85. Further studies will be necessary to clarify these issues.

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13. Experimental procedures for the expression and purification of PI3K SH3-PR are similar to those previously described for PI3K SH3 (see ref. 2c).
14. Fluorescence measurements were made using Perkin Elmer LS50 and Hitachi F-2000 luminescence spectrophotometers. Experiments were conducted at 20 °C with an excitation wavelength of 280 nm and variable emission wavelengths. Excitation and emission slit widths varied from 5 to 10 nm, depending on the instrument and experiment.
15. Throughout this paper [PI3K SH3-PR] represents the total concentration of PI3K SH3-PR as a monomer.
16. Consistent with intermolecular self-association, NMR spectra of PI3K SH3-PR exhibit significantly broadened linewidths as compared to spectral data for the PI3K SH3 domain. Yu, H.; Schreiber, S. L. Unpublished results.
17. Size exclusion HPLC was performed using an LDC Analytical HPLC gradient system equipped with a 4.6 mm x 25 cm Hydropore-5-SEC column (Rainin). Typical runs were conducted using PBS (pH 7.4) as the mobile phase at a flow rate of 0.150 mL/min. PI3K SH3-PR and PI3K SH3 (5 μL of approximately 300 μM solutions) were applied to the column, and their apparent molecular weights were derived from a calibration curve based on six protein standards ranging from 6-200 kD. The low apparent molecular weight of PI3K SH3 is probably due to nonspecific interactions between the surface-exposed hydrophobic binding site and the column matrix. Accordingly, the PI3K SH3 domain complexed to the peptide RPLPPRPSK (calculated molecular weight of 11 kD) behaves more normally with an apparent molecular weight of 8.8 kD. Chen, J. K.; Schreiber S. L. Unpublished results.
18. Value is the mean and standard deviation of three independent K_d determinations. The equilibrium of dimer and monomers is classically described by the expression

$$K_d = \frac{2\alpha^2[P]_m}{1 - \alpha} \qquad (1)$$

where K_d is the dissociation constant, α is the degree of dissociation of the dimer, and $[P]_m$ is the total protein concentration as monomer. The dissociation factor α in eq 1 is related to the fluorescence intensity:$[P]_m$ ratio by the equation

$$\alpha = \frac{R - R_d}{R_m - R_d} \qquad (2)$$

where R is the observed ratio, R_d is the ratio for the completely dimerized protein, and R_m is the ratio for the monomeric protein.
19. Both PI3K SH3-PR and PI3K SH3 have identical extinction coefficients at 280 nm since residues 86-101 contain no chromophores such as tryptophan and tyrosine.
20. Value is the mean and standard deviation of two independent K_d determinations. Experimental details for fluorescence analysis of SH3-ligand interactions have been described previously (see ref. 10a).
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