

Comparison of Calcium-Dependent Conformational Changes in the N-Terminal SH2 Domains of p85 and GAP Defines Distinct Properties for SH2 Domains[†]

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ABSTRACT: Src-homology region 2 (SH2) domains are stretches of about 100 amino acids which are found to be structurally conserved in a number of signaling molecules. These regions have been shown to bind with high affinity to phosphotyrosine residues within activated receptor tyrosine kinases. Here we report the bacterial expression and purification of individual N-terminal SH2 (NSH2) domains of phosphatidylinositol 3-kinase (PI-3K) binding subunit (p85) and Ras GTPase activating protein (GAP) in amounts suitable for structure-function studies. The p85NSH2 domain stains dark purple and absorbs around 620–640 nm with Stains-all, a dye known to bind to calcium binding proteins. This effect was not observed for the GAPNSH2 domain. Circular dichroism analysis of the N-terminal SH2 domain of these proteins shows that p85NSH2, but not GAPNSH2, undergoes a significant dose-dependent change in conformation in the presence of increasing calcium concentrations. Moreover, the conformational change of p85NSH2 induced by calcium could be replicated by addition of a phosphorylated hexapeptide (DYpMDMK) representing the α -PDGFR binding site for p85. Limited proteolysis studies showed a significant calcium-dependent increase in protection of p85NSH2 but not GAPNSH2 from degradation by subtilisin. Our results further indicate that holmium, a trivalent lanthanide ion, which has been previously shown to substitute for calcium, could also protect the p85NSH2 domain from proteolysis even at 10-fold lower concentrations. *In vitro* binding studies using purified preparations of activated α -PDGFR show that calcium did not affect the binding of GAPNSH2 domains to activated α -PDGFR. In striking contrast, we observed a marked increase in binding of p85NSH2 domains to activated α -PDGFR in the presence of calcium ions. Sequence comparisons and molecular modeling of the p85NSH2 domain based on the v-Src SH2 domain structure show a conserved arrangement of oxygen ligands contributing to two potential calcium binding sites within the p85NSH2 domain. These have been rationalized to be surface loop regions, i.e., loops 2 and 4 and loops 6, 7, and 8, close to the N- and C-terminal α -helical regions, respectively. Together, our findings suggest that the conformation of p85NSH2, but not that of GAPNSH2, is modulated by the presence of calcium ions. This implies that calcium ions may regulate PI-3K (p85 α) binding to α -PDGFR *in vivo* and suggests that the NSH2 domains of p85 and GAP may have distinct functions in receptor-mediated signal transduction.

The binding of platelet-derived growth factor (PDGF)¹ and epidermal growth factor (EGF) to their respective cell surface receptors (PDGFR and EGFR) leads to receptor tyrosine kinase activation by a process involving receptor dimerization (Ullrich & Schlessinger, 1990). Kinase activation is associated with receptor autophosphorylation and subsequent phosphorylation of intracellular substrates, some of which may be involved in the transduction of the mitogenic signal from the cell membrane to the nucleus. It has been demonstrated that high-affinity association occurs between specific autophos-

phorylation sites in the tyrosine kinase receptors and specific conserved domains in the substrates. These domains are known as Src-homology region 2 (SH2) domains, each consisting of around 100 amino acids (Sadowski et al., 1986). They may be part of enzymes (PLC γ , Src) or part of adaptors (p85, Sem-5/GRB2) which couple to enzymes. The existence of SH2 domains has been established in many proteins by sequence alignment (Koch et al., 1991; Pawson, 1992; Pawson & Gish, 1992). A number of these signaling proteins, including phosphatidylinositol 3-kinase (PI-3K) binding subunit (p85), ras GTPase activating protein (GAP), phospholipase C γ (PLC γ), and Src-like nonreceptor tyrosine kinases, have been shown to bind activated α - and β -PDGFRs (Coughlin et al., 1989; Kazlaukas & Cooper, 1989; Reedijk et al., 1990; Shurtleff et al., 1990; Choudhury et al., 1991; Kashishian et al., 1992; Yu et al., 1991; Fantl et al., 1992).

The tyrosine kinases of PDGFRs contain a kinase insert domain (KI domain) (Cantley et al., 1991). The tyrosine phosphorylation sites required for high-affinity binding of p85 and GAP have been identified to be within the kinase insert domain of PDGFRs (Kazlaukas et al., 1992). The phosphotyrosine-containing pentapeptides that encompass the

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¹ Abbreviations: PI-3K, phosphatidylinositol 3-kinase; GAP, GTPase activating protein; SH2, src-homology region 2; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; PLC γ , phospholipase C γ ; CD, circular dichroism; KI, kinase insert; NMR, nuclear magnetic resonance.

PDGFR binding sites for p85 and GAP have also been shown to compete specifically with activated PDGFR binding to p85 and GAP (Fantl et al., 1992). Moreover, circular dichroism and fluorescence spectroscopy methods have been used to show a significant conformational change in the p85NSH2 domain with the addition of a 17-residue polypeptide from the β -PDGFR KI domain phosphorylated on Tyr751 (Panayoutou et al., 1992). The three-dimensional structures of v-Src, c-Abl, v-Lck, and p85NSH2 domains have been determined (Waksman et al., 1992, 1993; Overduin et al., 1992; Eck et al., 1993; Booker et al., 1992), and it is evident that the SH2 domains of these distinct signaling molecules exhibit a similar tertiary fold, despite the high degree of primary structure variability.

In an attempt to explore this possibility, we have generated and purified the N-terminal SH2 domains of p85 and GAP in sufficient quantities for structural studies. Here we describe the results of a combination of empirical and theoretical methods, suggesting distinct physicochemical properties for these domains that may be involved in receptor signaling *in vivo*.

EXPERIMENTAL PROCEDURES

Cloning Strategy, Bacterial Expression, and Protein Purification. The DNA corresponding to the N-terminal SH2 domains of mouse p85 (96 residues) and bovine GAP (91 residues) (Koch et al., 1991) was synthesized using the polymerase chain reaction (PCR) method with a *Bam*H1 and a *Hind*III site at the respective 5' and 3' ends. The DNA fragments were cloned into a pQE9 plasmid (type 4) carrying an N-terminal six-histidine tag (Qiagen). DNA sequencing confirmed the authenticity of the inserts. The pQE9 plasmid containing the SH2 DNA was then transfected into competent M15 *Escherichia coli* cells. The bacteria expressing recombinant SH2 domains were processed under denaturing conditions and purified by nickel chelate affinity chromatography as described by LeGrice and Grueninger-Leitch (1990). This was the most efficient way to obtain an optimal yield and a good purification despite the presence of these domains in the cytoplasmic fraction of the bacteria. The presence of protein was checked using absorbance at 280 nm and SDS-PAGE analysis. The protein was refolded in 50 mM Tris-HCl buffer at pH 7.4 and concentrated using Centricon 3 membranes (Amicon).

Stains-all Binding in Aqueous Solution. The interaction of Stains-all with p85NSH2 and GAPNSH2 domains was studied in aqueous solution. The standard solution contained 10 mM Tris base, pH 8.8, 0.001% Stains-all, and 0.1% formamide. The NHS2 domains of p85 and GAP (1–10 μ g) were added to 1.0 mL of solution and then incubated at room temperature in the dark for 30 min. The sample was scanned between the wavelengths of 550 and 700 nm, and the absorbance was measured against a control solution, containing no protein, using a U-2000 Hitachi spectrophotometer.

Circular Dichroism Spectroscopy. CD spectra (far-UV 260–195 nm) were recorded at 22 °C with a JASCO J-500C spectropolarimeter. The spectra for the p85NSH2 and GAPNSH2 domains were recorded at a protein concentration of 0.1 mg/mL, using a 1-mm demountable "strain free" quartz cuvette. The following settings were used: wavelength range, 195–260 nm; bandwidth, 1 nm; time constant, 2.0 s; step resolution, 0.1 nm; scan speed, 10 millideg/min; sensitivity, 1 millideg/cm. Each spectrum represents an average of five scans with the baseline subtracted. CD studies were conducted in 50 mM Tris-HCl at pH 7.4. Calcium chloride was added in varying amounts (1–100 μ M), and the α -PDGFR kinase

insert hexapeptide phosphorylated on Tyr731 (DYpMDMK) (Peninsula) was added to the p85NSH2 domain at a concentration of 5 μ g/mL. The recorded spectra are presented in terms of molar ellipticity, based on a mean residue weight of 110. Secondary structure analysis was performed using the method of Provencher and Glockner (1981) and Chang et al. (1978).

Subtilisin Proteolysis Susceptibility Assay. Proteolysis with subtilisin was performed in 50 mM Tris-HCl buffer at pH 7.4. Protein concentrations of both p85NSH2 and GAPNSH2 were 0.1 mg/mL. Various amounts of calcium chloride (CaCl_2 , 0.1–20 mM) and holmium chloride (HoCl_3 , 0.1–2 mM) were added, followed by subtilisin at a concentration of 1 μ g/mL. Digestion (after 5 and 10 min at 37 °C) was terminated by addition of double-strength sample buffer containing 20 mM EDTA and 1% 2-mercaptoethanol, followed by immediate heating to 100°C for 5 min. Samples were analyzed on SDS–14% polyacrylamide gels and stained with Coomassie blue. The protease-resistant material was quantified by scanning on a Bio-Rad 620 videodensitometer, and the data were analyzed using the 1-D analyst package. The data were found to be best fitted to a second-order equation for proteolysis.

$$\% \text{ protease resistance} = [\text{Ca}^{2+}]^2 / ([\text{Ca}^{2+}]^2 + K_{\text{Ca}}^2)$$

where K_{Ca} is the Ca^{2+} concentration at half-maximal protection from proteolysis (Yamaoka et al., 1981).

In Vitro Screening of the NSH2 Domains of p85 and GAP with Purified α PDGF Receptors. Human α -PDGF receptors were purified from a baculovirus expression system as previously described (Jensen et al., 1992). Purified NHS2 domains of p85 and GAP were resolved on a 14% SDS-PAGE gel and transferred to Immobilon-P membranes (Millipore). Nonspecific binding was blocked by treating membranes with 3% nonfat dry milk in TTBS (25 mM Tris, pH 7.4, 150 mM NaCl, and 0.05% Tween 20) for 2 h. To measure *in vitro* association of PDGF receptors with p85 and GAPNSH2 domains, membranes were incubated with 5 mL of binding assay buffer (50 mM Hepes, 10 mM MgCl_2 , 1 mM MnCl_2 , 0.01% Triton X-100, 50 mM ATP, and 1 mM Na_3VO_4) including 50 ng of purified PDGF receptors for 2 h at 25 °C. When included, 5 mM CaCl_2 was added to the binding assay buffer. Membranes were washed twice and then blotted with anti-phosphotyrosine antibodies (UBI). After overnight incubation, membranes were washed twice, probed with ^{125}I -protein A, washed again four times with TTBS, and then autoradiographed by exposure of Kodak Xar-5 X-ray film. The above experiment was repeated with p85NSH2 in the presence of increasing calcium concentrations (0.05, 0.5, and 5 mM). The autoradiograms were quantified by scanning on a Bio-Rad 620 videodensitometer, and the data were analyzed using the 1-D analyst package.

Prediction of Calcium-Binding Motifs within the SH2 Domain Family. The N-terminal SH2 domains of p85 and GAP were examined for conserved arrangements of clustered oxygen ligands, with respect to the known tertiary structures (Waksman et al., 1992). Known calcium binding sequences (Vyas et al., 1987; Kretsinger, 1980; Weis et al., 1991b) were used in this analysis to detect any similarities to the p85NSH2 sequence. The aligned SH2 domain family (Russell et al., 1992) was scanned for other potential calcium binding motifs.

Molecular Modeling of the p85NSH2 Domain. The three-dimensional structure of the p85NSH2 domain of PI-3K was model built using the coordinates of the v-Src SH2 domain (Waksman et al., 1993) obtained from the Brookhaven database. The v-Src SH2 sequence (residues 5–102, renu-

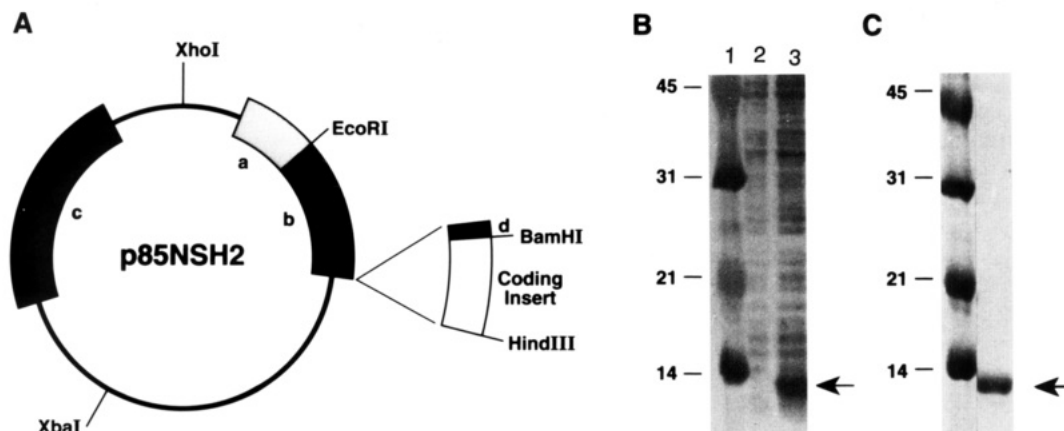


FIGURE 1: (A) The SH2 domain DNA of mouse p85 (96 residues) synthesized using the polymerase chain reaction and cloned into a pQE9 plasmid (type 4) (Qiagen). The vector possesses the following features: (a) a regulatable promoter/operator, (b) a synthetic ribosomal binding site (RBSII), (c) β -lactamase, and (d) a six-histidine tag placed N-terminally. (B) pQE9 plasmid containing DNA encoding the p85NSH2 domain transfected into competent M15 *E. coli* cells. Gene expression was induced with 0.1 mM IPTG (isopropyl β -D-thiogalactopyranoside) for 2 h at 37 °C. Total cell lysates (100 mg) were electrophoretically separated by 14% SDS-PAGE, transferred to nitrocellulose, and stained with Coomassie blue. Lane 1, molecular size marker; lanes 2 and 3, lysates at 0 and 2 h after induction. (C) Protein purification of p85NSH2 and GAPNSH2 performed under denaturing conditions using Ni-NTA-agarose resin. The purified protein was then eluted with 8 M urea, pH 5.9, and refolded by successive dialysis into 50 mM Tris-HCl, pH 7.4, as described (LeGrice & Grueninger-Leitch, 1990). The purified p85NSH2 domain was then electrophoretically separated by 14% SDS-PAGE and stained with Coomassie blue.

bered 1–98) was aligned with the p85NSH2 sequence (1–96) for maximum sequence similarity with insertions and deletions (Waksman et al., 1993). The model was built using the computer graphics program FRODO (Jones, 1985) and was energy minimized using the slowcool algorithm in X-PLOR (Brunger et al., 1990).

RESULTS

Expression and Purification of N-Terminal SH2 Domains of p85 and GAP. In order to examine the structural and functional properties of the SH2 domains of p85 and GAP, we amplified the DNA encoding these domains by the polymerase chain reaction (PCR). The PCR products were then cloned into a bacterial expression vector, pQE9, which contains a polyhistidine linker (Figure 1A). To express the recombinant proteins, bacterial strain M15 was transformed by pQE9 plasmid containing the N-terminal SH2 domain of either GAP or p85. The transformed bacteria were then grown and treated with 0.1 mM IPTG (isopropyl β -D-thiogalactopyranoside) for 2 h at 37 °C. The total lysates were subjected to SDS-PAGE analysis followed by staining with Coomassie blue. The expression of p85NSH2 domain with an apparent molecular mass of 12 000 Da was observed 2 h after induction (Figure 1B). The recombinant protein was then purified by nickel affinity chromatography as described (LeGrice and Grueninger-Leitch, 1990). The purity of this preparation was estimated to be around 95% by SDS-PAGE analysis (Figure 1C) and FPLC chromatography (data not shown). Similar results were obtained with the N-terminus of the SH2 domain of GAP (data not shown).

Stains-all Staining of the p85NSH2 Domain. The calcium binding proteins calsequestrin, calmodulin, troponin C, and S-100 have all been shown to stain dark blue or purple with the cationic carbocyanine dye Stains-all (Campbell et al., 1983). The dye-protein complex absorbs maximally in the region 600–620 nm. The p85NSH2 domain (1–10 μ g) stains dark purple and absorbs maximally at 620 nm compared to the GAPNSH2 domain (1–10 μ g), which remains pink and has no absorbance at 620 nm (Figure 2). The absorbance around 620–640 nm for the p85NSH2 domain (5 μ g) was 0.15 unit, which is very similar to that observed for calmodulin, troponin C, calsequestrin, and S-100, which have absorbance

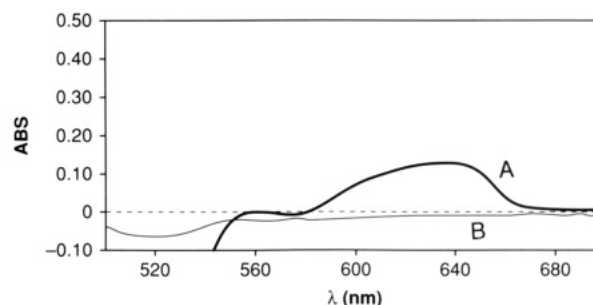


FIGURE 2: Stains-all binding to native (A) p85NSH2 and (B) GAPNSH2 measured in a solution containing 10 mM Tris base, pH 8.8, 0.001% Stains-all, and 0.1% formamide. The respective SH2 domains (5 μ g) were added to this solution, and following a 30-min incubation in the dark, the absorbance was measured in the wavelength range 550–700 nm against a control solution containing only dye.

values in the range 0.15–0.4 at 5- μ g protein concentrations (Campbell et al., 1983). This suggests that the p85NSH2 domain possesses anionic sites capable of binding calcium ions which are not present in the GAPNSH2 domain.

Effects of Calcium and Phosphotyrosine-Containing Hexapeptide on Conformation of the N-Terminal SH2 Domain of p85 Shown by CD Spectroscopy. Circular dichroism (CD) spectroscopy is a physical method that provides an estimate of protein secondary structure and can be used to analyze conformational changes induced by ligand binding (Provencher & Glockner, 1981; Chang et al., 1978). The far-UV CD spectra of both p85NSH2 and GAPNSH2 recorded in aqueous solution exhibited a broad trough in the 205–210-nm region and a smaller trough at 225 nm (Figures 3, panels A and B), indicating the presence of both α -helix and β -sheet structure. The calculated secondary structure composition is 20% α -helix, 35% β -sheet, and 40% nonperiodic structure, which agrees well with the X-ray structure of v-Src (Waksman et al., 1992, 1993) and NMR structures of SH2 domains of c-Abl (Overduin et al., 1992) and p85 (Booker et al., 1992). Metal ions have been demonstrated to play an important role in modulating the catalytic and regulatory functions of particular proteins *in vivo* (McPhalen et al., 1991). Accordingly, we sought to investigate the ability of the p85NSH2 and GAPNSH2 domains to bind divalent metal ions *in vitro*. Of the cations tested, calcium was found to be

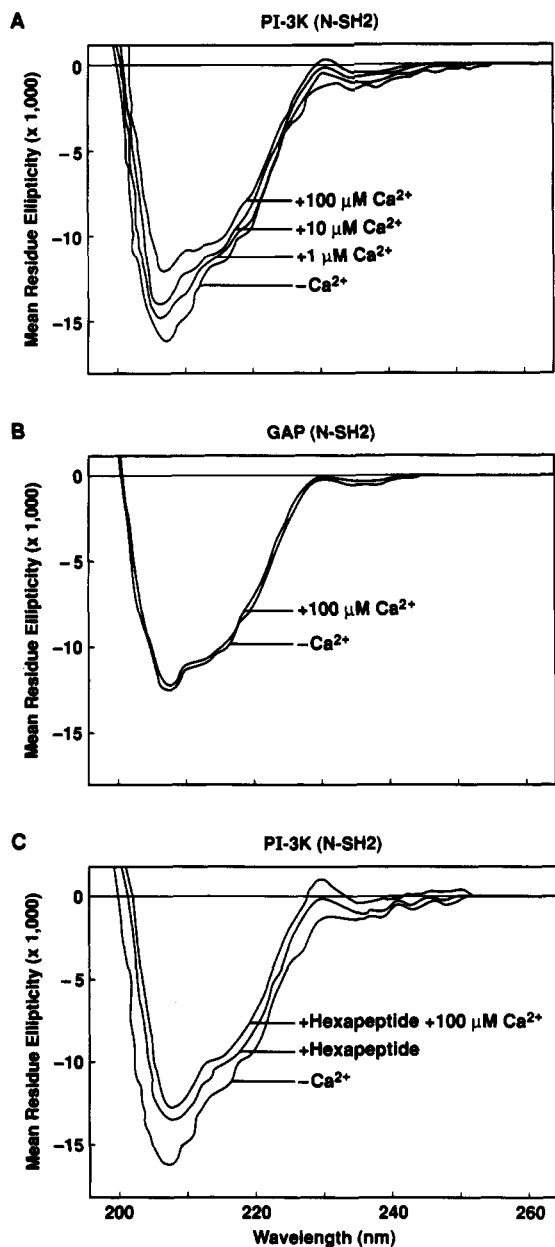


FIGURE 3: (A) Far-UV CD spectra of purified p85NSH2 domain (0.1 mg/mL) in 50 mM Tris-HCl, pH 7.4, determined in the absence of calcium ($-Ca^{2+}$) or with increasing concentrations of calcium ions ($+Ca^{2+}$). (B) Far-UV CD spectra of purified GAPNSH2 domain (0.1 mg/mL) in 50 mM Tris-HCl, pH 7.4, measured in the absence of calcium ($-Ca^{2+}$) or in the presence of calcium ions (100 μ M). (C) Far-UV CD spectra of p85NSH2 domain (0.1 mg/mL) in 50 mM Tris-HCl, pH 7.4, measured in the absence of calcium ($-Ca^{2+}$), in the presence of phosphotyrosine-containing hexapeptide (DYpMDMK) derived from the α -PDGFR binding site for p85 (1:1 molar ratio), and with 100 μ M calcium added to the protein-peptide mixture.

the most effective (data not shown). As shown in Figure 3A, addition of increasing calcium concentrations resulted in a dose-dependent decrease in the mean residue ellipticity, indicating a decrease in α -helical content and an increase in β -sheet content. In striking contrast, this effect was not observed for the GAPNSH2 domain (Figure 3B). Thus, these results suggest that the conformational change induced by calcium is specific to the p85NSH2 domain.

It has been recently reported that addition of a 17-residue polypeptide phosphorylated on Tyr751 of β -PDGFR induces a significant conformational change in the p85NSH2 domain (Panayoutou et al., 1992). We have previously demonstrated

that phosphorylation of Tyr731 of α -PDGFR, like that of Tyr751 of β -PDGFR, is required for high-affinity association with PI-3K activity (Yu et al., 1991). Accordingly, we investigated the effect of a phosphotyrosine-containing hexapeptide representing the α -PDGFR binding site for p85 on induction of conformational changes of the p85NSH2 domain. A hexapeptide derived from α -PDGFR kinase insert (DYpMDMK) phosphorylated on Tyr731 was added to the p85NSH2 domain in an equimolar ratio. As demonstrated in Figure 3C, addition of the hexapeptide caused a decrease in the mean residue ellipticity, indicating a change in the α -helical and β -sheet content of p85NSH2. To further examine the mechanism by which calcium and the phosphotyrosine-containing hexapeptide elicited changes in the conformation of the p85NSH2 domain, we compared changes induced by the combination of hexapeptide with calcium. Figure 3C shows that the conformational changes observed with calcium and peptide are additive, suggesting that the p85NSH2 binding sites for calcium ions and the tyrosine phosphate containing hexapeptide may be distinct. Together, these findings suggest that, in contrast to the GAPNSH2 domain, the p85NSH2 domain specifically undergoes a significant conformational change in the presence of calcium which is also replicated by addition of the phosphotyrosine-containing hexapeptide.

Effect of Calcium on the Conformation of the p85NSH2 Domain as Measured by Subtilisin Proteolysis. In order to verify that the p85NSH2 domain undergoes a conformational change induced by calcium and to compare and contrast this effect to that of the GAPNSH2 domain, limited proteolysis assays were conducted. Limited proteolysis studies with subtilisin have previously been employed to show calcium-dependent conformational changes and quantitate the level of structural change required for rendering the rat mannose binding protein resistant to proteolysis (Loeb & Drickamer, 1988). Accordingly, NSH2 domains of p85 and GAP were incubated with subtilisin in the presence of varying concentrations of calcium for either 5 or 10 min at 37 $^{\circ}$ C. As shown in Figure 4A, in the absence of calcium, the p85NSH2 domain was not protected from proteolysis by subtilisin. However, the addition of increasing concentrations of calcium results in a dose-dependent increase in protection of the p85NSH2 domain from proteolysis by subtilisin. The calcium concentration required for reaching the half-maximal protease-resistant fraction was around 5 mM (Figure 5A). In contrast, under these conditions, the GAPNSH2 domain was not protected in the presence of calcium (Figure 4B). These results suggest that unlike the GAPNSH2 domain, the p85NSH2 domain undergoes a significant dose-dependent conformational change in the presence of calcium which renders it resistant to proteolysis. Therefore, these data are consistent with the results of CD spectroscopy, suggesting that calcium induces a conformational change in p85NSH2 specifically.

Studies of several calcium-dependent proteins have revealed that this divalent ion can be functionally replaced by various trivalent lanthanides including holmium (Evans, 1990). This effect was shown for the rat mannose binding protein, which is a low-affinity calcium binding protein (Weis et al., 1991a). Weis et al. (1991a) also showed that the mannose binding protein exhibits 10–30-fold higher affinity for holmium than calcium. Accordingly, we performed the protease resistance analysis of p85NSH2 in the presence of holmium. Figure 4C shows that with increasing concentrations of holmium there was an increased level of protease-resistant fraction. The holmium concentration required for half-maximal protection

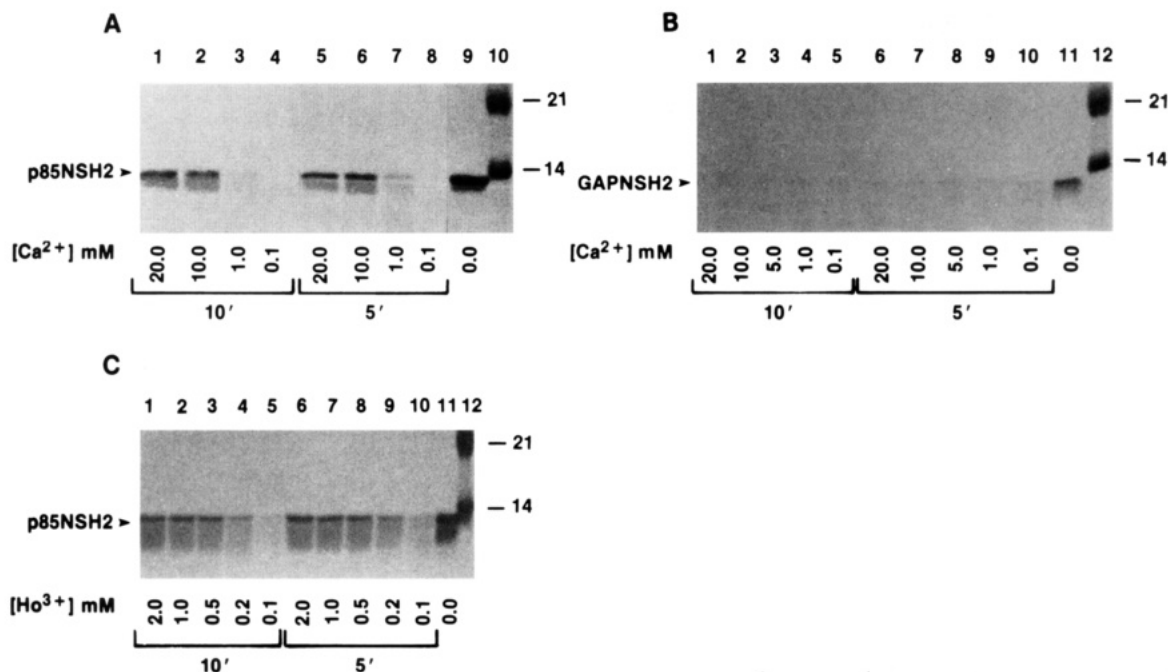


FIGURE 4: (A) p85NSH2 domain (0.1 mg/mL) in 50 mM Tris-HCl, pH 7.4, incubated with subtilisin (1 μg/mL) in the presence of increasing Ca²⁺ concentrations (0.1, 1.0, 10, and 20 mM) for 5 and 10 min at 37 °C. The samples were then subjected to a 14% SDS-PAGE analysis followed by staining with Coomassie blue. (B) GAPNSH2 domain (0.1 mg/mL) in 50 mM Tris-HCl, pH 7.4, was incubated with subtilisin (1 mg/mL) in the presence of increasing Ca²⁺ concentrations (0.1, 1, 5, 10, and 20 mM) for 5 and 10 min at 37 °C. (C) Substitution of holmium for calcium. The p85NSH2 domain (0.1 mg/mL) in 50 mM Tris-HCl, pH 7.4, incubated with subtilisin (1 mg/mL) in the presence of increasing Ho³⁺ concentrations (0.1, 0.2, 0.5, 1, and 2 mM) for 5 and 10 mins at 37 °C.

from proteolysis was estimated to be around 0.5 mM, which is 10-fold less than that required for eliciting a similar effect by calcium (Figure 5B).

Comparison of the Binding of Activated α PDGFR to p85NSH2 and GAPNSH2 Domains in the Presence or Absence of Calcium. The binding of activated PDGFRs to p85 and GAP or their SH2 domains has been examined by an *in vitro* binding assay (Kashishian et al., 1992; McGlade et al., 1992; Moran et al., 1990). To assess and quantitate whether the calcium effect observed could stabilize the binding of these NSH2 domains to activated α -PDGFR, *in vitro* binding assays were performed. Accordingly, purified NSH2 domains of p85 and GAP immobilized on filters were incubated with activated α -PDGFR purified from SF9 cells in the presence or absence of calcium (Jensen et al., 1992). Following incubation, filters were washed extensively and subjected to immunoblot analysis using anti-phosphotyrosine (anti-P-Tyr) antibodies. As demonstrated in Figure 6A, the activated α -PDGFR bound specifically to the GAPNSH2 domain as determined by the anti-P-Tyr signal. Furthermore, the level of anti-P-Tyr-detectable α -PDGFR was not affected by the presence of calcium (Figure 6A, lane 2). In contrast, the level of anti-P-Tyr-detectable α -PDGFR associated with the p85NSH2 domain increased around 5-fold in the presence of calcium (Figure 6B). This increasing effect is not due to a calcium-dependent increase in autokinase activity of α -PDGFR, since under similar conditions the presence of calcium ions did not increase the level of anti-P-Tyr-detectable α -PDGFR or β -PDGFR (data not shown) bound to the GAPNSH2 domain.

In order to measure the calcium concentration required for the half-maximal binding of activated α -PDGFR to p85NSH2, filters containing identical levels of p85NSH2 were incubated with activated α -PDGFR in the presence of increasing concentrations of calcium (Figure 6C). The level of anti-P-Tyr-detectable α -PDGFR bound to p85NSH2 was quantified. The half-maximal receptor binding for calcium is

estimated to be around 25 μM (Figure 6D). These findings suggest that calcium specifically regulates the binding of the p85NSH2 domain to the activated α -PDGFR in a dose-dependent manner.

Predicted Calcium Binding Motifs within the SH2 Domain Family. The prediction of calcium binding sites within a structurally related family of proteins is not straightforward. The frequent and variable use of water molecules as Ca²⁺ ligands complicates the task of prediction (McPhalen et al., 1991). However, the observation that the N-terminal SH2 domain of p85 binds Ca²⁺ while GAP does not allows the prediction of calcium binding sites with accuracy in the light of primary and tertiary structure information.

A calcium binding site is composed of 6 (octahedral) or 7 (pentagonal bipyramidal) ligand atoms arranged regularly around the Ca²⁺ ion. Preferred ligands are carboxylates of aspartates and glutamates. Asparagines and glutamines are also observed, while backbone carbonyls and water molecules are frequently observed. Threonines and serines are rarely used. Most calcium binding sites fall on nonregular structural elements.

Sequence analysis of the NSH2 domains of p85 and GAP reveals a conserved arrangement of oxygen ligands suitable for calcium binding to the p85NSH2 domain (Figure 8). Two sites are identified (sites I and II), and each site is semi-discontinuous but juxtaposed in three-dimensional space (see Figure 9). Site I consists of Asp17, Asp20, Asn45, and Asn46. Further, Thr18 and Thr22 as well as the backbone carbonyl oxygens and one or more water molecules may also help form this site. This site is composed of loops 2 and 4, which come together in three-dimensional space (Figure 8A). Site II consists of Asp55, Asp62, Asn67, and Glu71; Ser61, Ser68, backbone carbonyl oxygens, and one or more water molecules may also contribute to this site. Site II is composed of loops 5, 6, and 7 and the C-terminal α -helix, which are close together in the three-dimensional structure (Figure 8B). The NSH2 domain of GAP lacks these residues at sites I and II. These

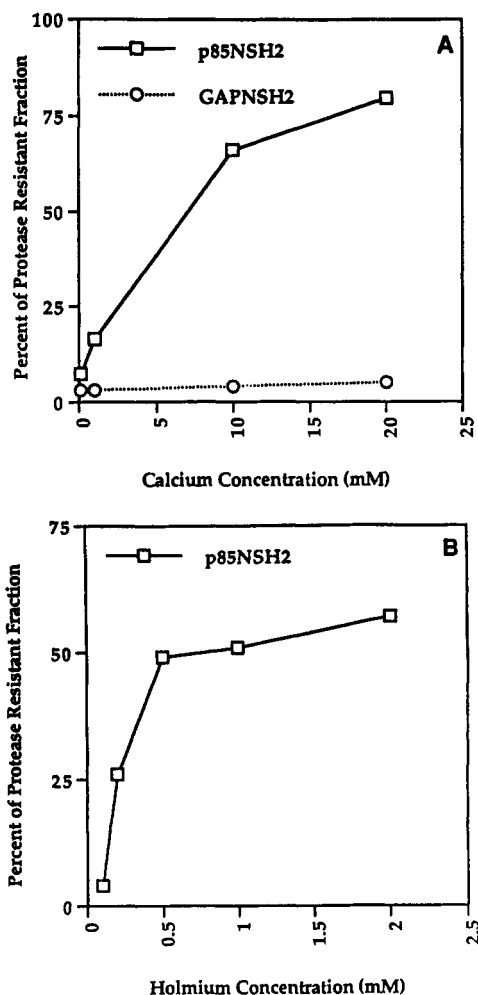


FIGURE 5: (A) Rate of proteolysis at 10 min (37 °C) of the NHS2 domain of p85 and GAP measured as a function of calcium concentration. The percentage of NHS2 domains remaining after proteolysis was measured by scanning densitometry of the SDS-Polyacrylamide gels shown in panels A and B of Figure 4 and normalized to the amount present in the absence of protease (—□—, p85NSH2; —○—, GAPNSH2). (B) Rate of proteolysis at 10 min (37 °C) of the p85NSH2 domain as a function of holmium concentration. The percentage of p85NSH2 domain remaining after proteolysis was measured by scanning densitometry of the SDS-polyacrylamide gel shown in Figure 4C and normalized to the amount present in the absence of protease (—□—, p85NSH2).

calcium binding sites appear to be low affinity and do not have the structural features of a high-affinity binding site of calmodulin-like proteins (Kretsinger, 1980; McPhalen et al., 1991). However, they do have features of low-affinity sites described for the animal lectins (Weis et al., 1991b). An analysis of the aligned SH2 domain family for the above predicted calcium binding sites revealed that only p85 β (NSH2) possesses both sites. Site II was observed for PLC γ (1N-, 1C-SH2) and c-Abl SH2 (Figure 8B). A further analysis of the SH2 domain sequences revealed that several of these domains possess longer loops 2 and 4 due to insertions compared to the p85NSH2 domain. These loops are mainly composed of acidic residues and are thus predicted to form a potential calcium binding site (modified site I, Figure 8A). This modified site I is observed in some members of the Src family, including Yes, Lck, and Fyn.

Structural Model of the p85NSH2 Domain. The crystal structure of the v-Src SH2 domain has been determined to high resolution (1.5 Å) (Waksman et al., 1993). Two pentapeptides phosphorylated on tyrosine representing the kinase insert region of the β -PDGFR and the C-terminal tail

of the EGFR complexed with the v-Src SH2 domain clearly indicate how the tyrosine phosphate interacts with the SH2 domain. The highly conserved residues Arg155, Arg175, and Lys203 within the SH2 domain bind the tyrosine phosphate through electrostatic interactions. Positions +1 and +3 of the peptide also interact with the SH2 domain through hydrophobic interactions (Waksman et al., 1992, 1993). The above structure has allowed a more precise alignment of the SH2 domain family and provided a structural framework to build in other SH2 domains for structure-function studies.

The v-Src SH2 sequence aligned with the p85NSH2 sequence for maximum similarity included seven insertions and nine deletions which occur exclusively in the loop regions (loops 2–5, 8, and 9). This makes the p85NSH2 domain a more compact structure compared to the v-Src SH2 domain. The two sequences are numbered 1–96 with gaps where insertions and deletions occur (Figure 7). In this arrangement the tyrosine phosphate binding residues are Arg8, Arg26, and Lys50, which correspond to Arg155, Arg175, and Lys203 of the v-Src SH2 domain structure. A search of the surface of the p85NSH2 domain structure indicated the presence of two potential calcium binding sites (Figure 9), which agreed well with the prediction based on a sequence comparison of the p85NSH2 and GAPNSH2 domains.

DISCUSSION

The noncatalytic SH2 domains, present on a plethora of intracellular signaling proteins, are 90–110 residues in length and are essentially basic. Multiple-sequence alignment and secondary structure prediction of SH2 domains showed them to be $\alpha+\beta$ proteins (Russell et al., 1992), with a $\beta\alpha(\beta)_4\alpha\beta$ topology confirmed by the three-dimensional structures of v-Src (Waksman et al., 1992, 1993), Abl (Overduin et al., 1992), v-Lck (Eck et al., 1993), and p85NSH2 (Booker et al., 1992). The three-dimensional structures indicate a central β -sheet parallel to the N- and C-terminal α -helices and have allowed a more precise primary structure alignment of these conserved domains. These alignments suggest considerable sequence variability within this family, especially in the loop regions connecting the regular secondary structure elements. In the present report, we show that, in the presence of calcium, the p85NSH2 domain exhibits distinct structural properties compared to those of the GAPNSH2 domain. We have compared and contrasted the physicochemical effects of calcium on the NSH2 domains of p85 and GAP by employing CD spectroscopy, subtilisin proteolysis, and receptor binding assays. The data suggest differences in the calcium concentration required for eliciting these effects. The apparent discrepancy is due to the fact that these assay systems measure different processes, and a direct measure of calcium binding is difficult to elicit in a protein which has a low affinity for calcium.

In this study we are able to show that the p85NSH2 domain is a calcium binding protein, as it stains dark purple in the presence of the cationic carbocyanine dye Stains-all. This finding is consistent with the interpretation that Stains-all binds to anionic sites within calcium binding proteins. Stains-all had no effect on the GAPNSH2 domain. We also found that, in the presence of calcium ions, the p85NSH2 domain, unlike the GAPNSH2 domain, undergoes a significant dose-dependent change in its conformation. This was established by CD spectroscopy as well as by a limited proteolysis experiment using subtilisin. CD spectroscopy determines the overall conformational changes of a protein, while the subtilisin experiment quantitates the level of structural change required

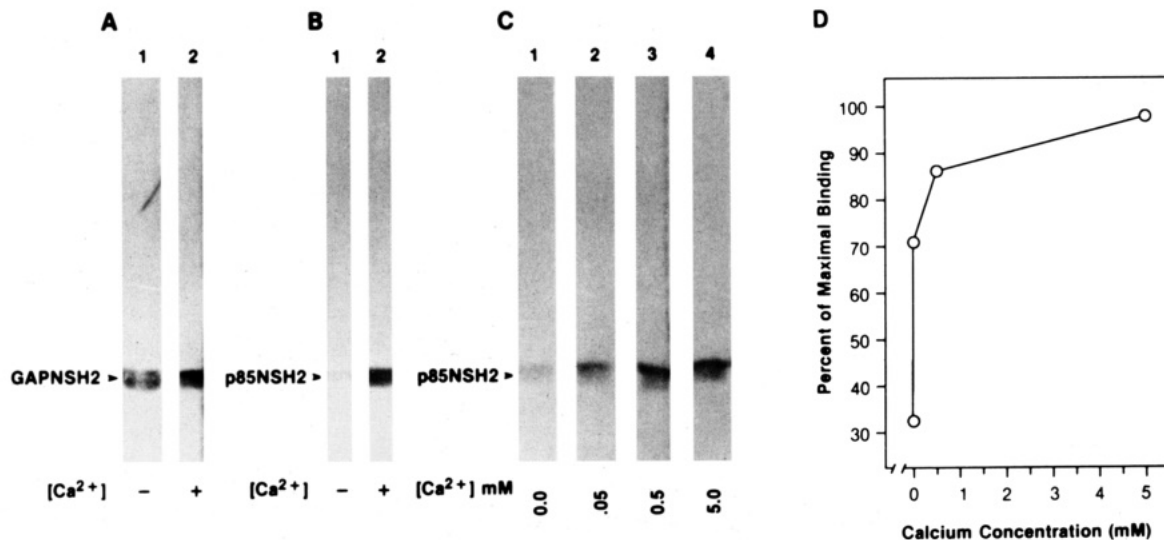


FIGURE 6: (A) GAPNSH2 domain (0.1 mg/mL) run on a 14% SDS-polyacrylamide gel and transferred to Immobilon-P membranes. To measure *in vitro* association of the PDGFRs, membranes were incubated with 5 mL of binding assay buffer (50 mM Hepes, 10 mM magnesium chloride, 1 mM manganese chloride, 0.01% Triton X-100, 50 mM ATP, and 1 mM sodium vanadate) including 50 ng of purified PDGFRs for 2 h at 25 °C. When included, 5 mM calcium chloride was added to the binding assay buffer. The membranes were washed and blotted with anti-phosphotyrosine (anti-P-Tyr) antibodies. (B) The above experiment repeated for the p85NSH2 domain. (C) The *in vitro* binding assay described above employed to titrate the calcium effect observed for the binding of the p85NSH2 domain to activated α -PDGFR. Filters containing identical levels of p85NSH2 were incubated as described in the presence of activated α -PDGFR (50 ng) and increasing concentrations of calcium ions. Filters were washed and subjected to immunoblot analysis using anti-P-Tyr antibodies. (D) Quantitation of the effect of calcium on α -PDGFR binding to p85NSH2 domain. The level of receptor binding was measured by scanning densitometry of the autoradiogram shown in panel C and normalized to the amount present in the absence of calcium.

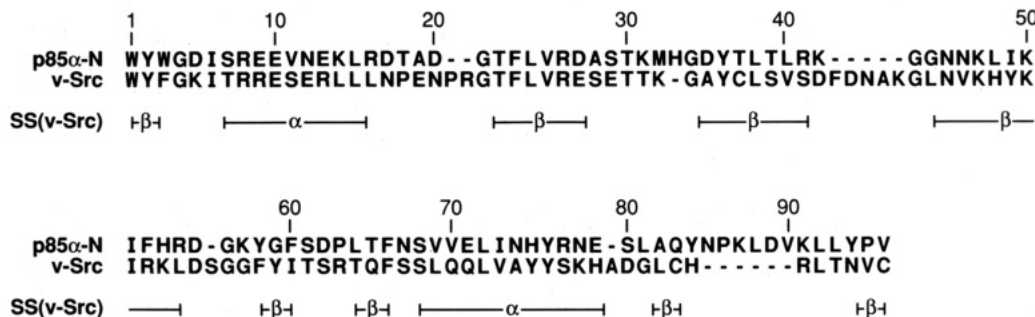


FIGURE 7: Alignment of the v-Src SH2 domain sequence with that of the p85NSH2 domain sequence for maximum similarity. The sequences are numbered 1–96. The seven insertions and nine deletions required for maximum similarity are indicated by the gaps shown in the sequence alignment.

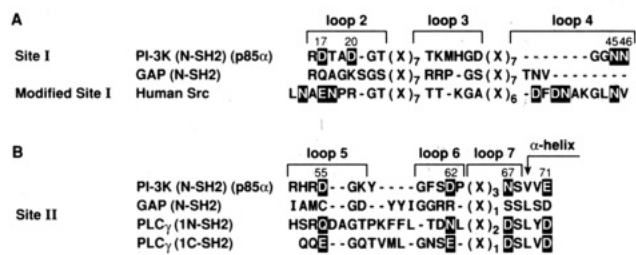


FIGURE 8: (A) NHS2 domains of PI-3K (p85 α) and GAP aligned to show the conserved oxygen ligands that may contribute to Ca²⁺ binding. Two sites are predicted: site I—Asp17, Asp20, Asn45, and Asn46 (loops 2 and 4)—and site II—Asp55, Asp62, Asn67, and Glu71 (loops 5, 6, and 7 and the C-terminal α -helix). Both sites have at least two hydroxy amino acids (site I, Thr18 and Thr22; site II, Ser61 and Ser68) which may contribute to calcium binding. A modified site I predicted for the human Src SH2 domain is also shown. (B) Sequence comparison of the SH2 domain family predicts Ca²⁺ binding sites for the following members: PLC γ (1N- and 1C-SH2) and Abl-SH2 domains are predicted to possess the C-terminal Ca²⁺ binding site.

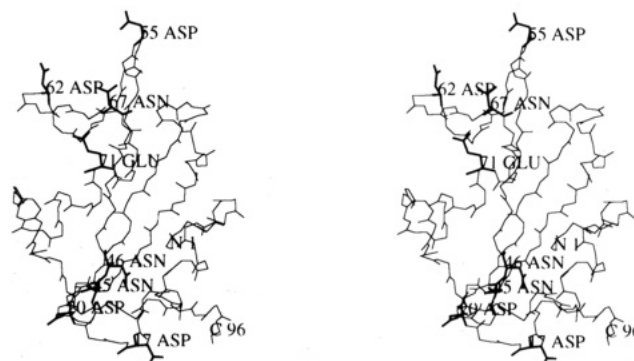


FIGURE 9: Stereoview of the three-dimensional structure of the p85NSH2 domain. A search of the protein surface indicated the presence of two potential calcium binding sites which were predicted from a sequence comparison of the SH2 domain family. Sites I and II are indicated. The residues involved in calcium binding are shown in Figure 8.

for rendering a molecule resistant to proteolysis. Both CD spectroscopy and the limited proteolysis assay have been previously used to establish calcium-mediated conformational

changes in the rat mannose binding protein, which is considered to be a low-affinity calcium binding protein (Loeb & Drickamer, 1988). The calcium concentration required for half-maximal protection of p85NSH2 from subtilisin is similar

to that measured for mannose binding protein. Thus, our findings suggest that the p85NSH2 domain is a calcium binding protein similar to that of mannose binding protein. Although the exact physiological importance of the p85NSH2 domain's calcium binding property has not yet been ascertained, our findings imply that this novel property of p85 is likely to play an important physiological role *in vivo* particularly since (a) the transient increase in intracellular calcium concentration has been documented to approach millimolar levels (Murachi, 1989; Berridge, 1990) and since (b) calcium has been shown to be a regulator of catalytic activity of specific enzymes *in vivo* (Berridge, 1993).

Molecular model building and sequence analysis predict two potential calcium binding sites in the p85NSH2 domain which were shown to be absent in the GAPNSH2 domain. This is consistent with the proteolysis study, which suggests that the data are best fit to a second-order equation. It appears that the two calcium ions hold several surface loop regions that stabilize the tertiary structure. Sequence analysis of the SH2 domain family indicates that several other members are also capable of binding calcium ions. Sites I and II are present in the NSH2 domain of p85 β , while only site II is present in PLC γ (1N- and 1C-SH2) and Abl. A modified site I is predicted for the Src family including Yes, Lck, and Fyn, which have insertions of acidic amino acids in loops 2 and 4. Further experimental analysis of these domains should allow us to verify these predictions.

The p85NSH2 domain has previously been shown to bind activated PDGFRs (Kashishian et al., 1992; Yu et al., 1991; McGlade et al., 1992; Moran et al., 1990). In this study we are also able to show that the phosphotyrosine-containing hexapeptide representing the binding site of the α -PDGFR for p85 changed the conformation of the p85NSH2 domain, suggesting a specific SH2-hexapeptide interaction. The crystal structure of the Src SH2 domain with and without peptide ligation showed only localized and relatively small changes (Waksman et al., 1993). In contrast, our CD spectroscopic measurements of the p85 NSH2 domain in the presence of a specific hexapeptide show a significant conformational change. The molecular model of the p85 NSH2 domain suggests that deletions on either side of the important FLVRES region (see Figure 7) make it compact in comparison to the Src SH2 domain. In order to accommodate peptide binding, the structure may have to undergo larger conformational changes.

The addition of calcium to the NSH2-peptide solution led to a further change in conformation of this domain, indicating that the calcium and the hexapeptide bind to distinct sites within its structure. This was supported by the *in vitro* receptor binding assay, which showed that the p85NSH2 domain binds the α -PDGFR in a calcium-dependent manner and that this effect is not observed for the β -PDGFR. These results suggest that the calcium-dependent binding of the p85NSH2 domain to the α -PDGFR may be due not only to the calcium binding property of the SH2 domain but also to the kinase insert domain of the receptor, which is also capable of binding calcium ions (Mahadevan et al., manuscript in preparation). The half-maximal value for α -PDGFR binding of the p85NSH2 domain is 25 μ M calcium, which is observed in the presence of a 400-fold excess of magnesium ions. During calcium spikes, the cellular calcium to magnesium ratio makes the above observation functionally relevant and gives credence to the idea that the p85NSH2 domain is a calcium binding protein that interacts specifically with the α -PDGFR in a calcium-dependent manner.

These results imply that despite their conserved three-dimensional structure, the SH2 domains found in a number of signaling molecules may have distinct functions *in vivo*. Cyclical changes in intracellular calcium concentration may play a critical role for the binding of p85 not only to α -PDGFR but also to its catalytic subunit (p110) (Hiles et al., 1992) and IRS-1 (Backer et al., 1992) *in vivo*.

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