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Genetic Analysis of a Phosphatidylinositol 3-Kinase SH2 Domain Reveals Determinants of Specificity

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Phosphatidylinositol 3-kinase is an important element in both normal and oncogenic signal transduction. Polyomavirus middle T antigen transforms cells in a manner depending on association of its tyrosine 315 phosphorylation site with Src homology 2 (SH2) domains on the p85 subunit of the phosphatidylinositol 3-kinase. Both nonselective and site-directed mutagenesis have been used to probe the interaction of middle T with the N-terminal SH2 domain of p85. Most of the 24 mutants obtained showed reduced middle T binding. However, mutations that showed increased binding were also found. Comparison of middle T binding to that of the platelet-derived growth factor receptor showed that some mutations altered the specificity of recognition by the SH2 domain. Mutations altering S-393, D-394, and P-395 were shown to affect the ability of the SH2 domain to select peptides from a degenerate phosphopeptide library. These results focus attention on the role of the EF loop in the SH2 domain in determining binding selectivity at the third position after the phosphotyrosine.

Polyomavirus middle T antigen (MT) has provided a useful model for studying cell transformation. Analysis of MT established the connection between tyrosine phosphorylation and transformation (12). Later, several groups (2, 6, 9) used MT to study the activation of cellular tyrosine kinases such as $pp60^{c\text{-}src}$ in relation to their C-terminal tyrosine phosphorylation. More recently polyomavirus MT has played an important role in implicating phosphatidylinositol (PI) 3-kinase as an important enzyme in signal transduction pathways (10, 17, 18, 42). It is now clear that PI 3-kinase associates with almost all activated tyrosine kinases (4). Its importance has been demonstrated in systems of normal, as well as oncogenic, growth regulation. Valius and Kazlauskas (39) and Fantl and colleagues (14), for example, implicated PI 3-kinase as a downstream mediator of the mitogenic signal of platelet-derived growth factor (PDGF).

Analysis of MT called attention to the role of tyrosine phosphorylation in redirecting PI 3-kinase (8). The association of the p85 subunit of the enzyme with MT was shown to depend upon tyrosine phosphorylation of MT at residue 315 (8, 37). This phosphorylation resulted in trafficking of PI 3-kinase from the cytosol to membrane fractions. The importance of this activation is clearly shown by the inability of MT mutant at residue 315 to transform cells and induce tumors (5, 16).

The tyrosine phosphorylation site at residue 315 of MT interacts with the Src homology 2 (SH2) domains of PI 3-kinase (43). This result is expected in view of the known role of SH2 domains in binding tyrosine-phosphorylated proteins (21, 25, 32). Because SH2 domains are a common feature of many proteins involved in signal transduction, considerable attention has been devoted to their study. To understand the general architecture of SH2 domains, X-ray crystallography was done on the Src SH2 domain in complex with both low- and high-affinity peptides (40, 41). Overduin and coworkers (29) used nuclear magnetic resonance (NMR) to solve the structure of uncomplexed c-Abl SH2 domain, as did Booker (3) for the N-terminal SH2 (N-SH2) domain of p85. The critical nature of the SH2 domain FLVR sequence, well known from previous genetic studies, could be explained by its coordination of the phosphotyrosine. Other features could also be interpreted. Consensus analysis suggested that SH2 domain recognition was strongly influenced by residues directly after the phosphotyrosine of the protein being bound (4). Songyang and colleagues (34, 35) devised a method to select for phosphopeptides that are preferentially bound by particular SH2 domains; for example, while p85 SH2 domains prefer pYMXM motifs, the GRB2 SH2 domain selected pYVNV and the Src SH2 domain selected pYEEI. Structural analysis provides a framework within which to consider these results. Waksman and colleagues compared the Src SH2 domain-YEEI peptide interaction to a two-holed socket (SH2 domain) and a two-pronged plug (peptide), whereby the phosphotyrosine of the peptide would constitute one element of the binding (charged pocket) and the isoleucine would constitute the second element (hydrophobic interactions) (41).

An important goal is to understand why SH2 domains work the way they do. While important information can be obtained by examination of consensus sequences and judicious choice of targets for mutagenesis, a random approach allows the possibility of detecting important elements perhaps unsuspected from a priori analysis. This study used random mutagenesis to determine residues critical for the recognition of MT by the N-SH2 region of p85. Intriguingly, both activating and inactivating mutations have been identified. Among them are residues apparently critical for assembling a functional SH2 domain. More interestingly, specific recognition of particular tyrosine-phosphorylated proteins, that is, residues important for the selectivity of the domain, were also uncovered. A consequence of this selectivity is that mutations affect interac-

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tions of the SH2 domain with MT and with the PDGF receptor (PDGFr) differently. These results fit together with the structural predictions in a satisfying way.

MATERIALS AND METHODS

Clones, viruses, and antisera. The extended pGEX 3x-GST-NSH2 construct (amino acids 321 to 470) used in the hydroxylamine mutagenesis was previously described (43). Protein expression was tested with a rabbit polyclonal antibody (antibody 163) raised against the p85 peptide DTADGTFLVR DAST conjugated to bovine serum albumin (BSA). Human β-PDGFr, MT-, and pp60$^{c\text{-}src}$-expressing baculoviruses were propagated on Sf9 cells in Grace's medium (10% fetal bovine serum). Typically, the infected cells were used 40 h postinfection. MT was immunoprecipitated with a rabbit antiserum prepared by immunization with small T antigen produced in *Escherichia coli* (30) or with a peptide antiserum against MT residues 280 to 302. PDGFr was immunoprecipitated with SC-05 (Santa Cruz Biotechnology).

Site-directed mutagenesis. Oligonucleotide site-directed mutagenesis was performed by the α-thionucleotide procedure (38). The *Bam*HI-*Hin*cII fragment of p85α (43) was cloned into M13mp18. The degenerate oligonucleotide 5′ TCAC CATGCATTT(T/G)AGTAGACG(C/A)ATCT(C/T)GTACC AAAAAGGTC 3′ was used to create mutations of lysine 363 to glutamine, alanine 360 to serine, and arginine 358 to glutamine. Mutants identified by sequencing were recloned by using the *Bam*HI-*Hin*cII fragment into pGEX2T-p85. Finally, the mutations were reconfirmed by dideoxynucleotide sequencing (33).

Hydroxylamine mutagenesis. For the mutagenesis, we slightly modified the protocol described by Feig et al. (15). Basically, 39.5 μl of glutathione *S*-transferase (GST)-SH2 DNA (0.76 μg/μl) was incubated with 150 μl of ethylene glycol and heated at 70°C for 5 min. After removal of 28.4 μl (4.5 μg) as a control, 17 μl of a solution of hydroxylamine (0.5 M hydroxylamine, 0.2 M sodium pyrophosphate [pH 6.0]) was added to a final concentration of 0.05 M. The mixture was returned to 70°C, and 5, 10, 20, 30, 40, and 60 min later, 30-μl aliquots were taken out and mixed with 80 μl of chilled stop solution (0.6 M Tris-HCl [pH 8.0], 1.0 M NaCl–20% acetone). DNA was separated from hydroxylamine by passage over a 1-ml Sephadex G-50 column equilibrated overnight in 10 mM Tris (pH 8.0)–10 mM EDTA. One microliter of a 1:100 dilution was used to transform competent *Escherichia coli* XL1-blue.

MT/PDGFr probe. MT and PDGFr used in the binding experiments were labeled as described previously (7). Basically, Sf9 cells coinfected with MT- and Src-expressing baculoviruses were harvested, and MT was extracted with a Nonidet P-40-containing buffer (0.137 M NaCl, 0.01 M Tris-HCl [pH 9.0], 0.001 M $MgCl_2$, 0.001 M $CaCl_2$, 10% [vol/vol] glycerol, 1% [vol/vol] Nonidet P-40). The extracts were incubated with protein A-Sepharose beads and MT polyclonal antibody for 1 h on ice. The complexes were washed twice with phosphate-buffered saline (PBS), twice with 0.5 M LiCl–0.01 M Tris-HCl (pH 7.0), and once with distilled water. For in vitro labeling of MT or PDGFr, the immunoprecipitates were incubated for 15 min at room temperature in 20 mM Tris-HCl (pH 7.5)–$MnCl_2$ (5 mM for MT, 10 mM for PDGFr) with [γ-^{32}P]ATP (2,000 Ci/mmol). After the reaction mixtures were washed with PBS, LiCl, and water, beads containing PDGFr were frozen and the MT precipitates were boiled for 2 min in a solubilization buffer containing 0.4% sodium dodecyl sulfate (SDS), 50 mM triethanolamine-Cl (pH 7.4), 100 mM NaCl, and 2 mM 2-mercaptoethanol. After boiling, the samples were spun out, the supernatants were collected, and iodoacetamide was added to a final concentration of 10 mM. Finally, one-fourth the volume of 10% Triton X-100 was added, and MT was reimmunoprecipitated from these supernatants with protein A-Sepharose and MT antibody for 1 h on ice. The reaction mixtures were washed again, and the dry beads were frozen. On the day of the experiments, MT or PDGFr was boiled in solubilization buffer and treated with iodoacetamide and Triton X-100 (as described above).

Mutant screening. Competent *E. coli* XL1-blue cells were transformed with mutagenized GST-SH2 plasmids and grown on LB plates containing ampicillin. The colonies were transferred onto nitrocellulose filters, and the filters were incubated on LB plates containing ampicillin and 0.1 mM isopropylthiogalactopyranoside (IPTG) for 3 to 4 h at 37°C. The filters were washed several times in a sol/T solution (0.32% SDS, 2% Triton X-100, 40 mM triethanolamine [pH 7.4], 80 mM NaCl, 2 mM EDTA [pH 7.4]), blocked for 1 h in the same sol/T buffer containing 1% gelatin, and probed overnight at 4°C in sol/T containing simultaneously ^{32}P-labeled MT probe and polyclonal antibody 163. The next day, the filters were washed again in sol/T and then incubated for 1 h with goat anti-rabbit immunoglobulin G coupled to alkaline phosphatase for protein detection. MT binding was determined by autoradiography.

The colonies which showed impaired MT binding were selected and grown in LB containing ampicillin, and the plasmid DNA was purified by using the Magic Miniprep kit (Promega). DNA was then sequenced by the Sanger dideoxy method (33) for double-stranded DNA. The mutant proteins were then tested in solution. In this assay, bacterial cells containing the mutated DNA were grown, induced with 0.1 mM IPTG for 3 h at 37°C, and then collected by centrifugation. The cells were lysed by osmotic shock using a slight modification of published procedures (27). Basically, 5 ml of bacterial cells was pelleted for 10 min at 3,000 rpm in a tabletop centrifuge. The pellet was resuspended in 100 μl of 50 mM Tris (pH 8.0)–20% (wt/vol) sucrose, 20 μl of 20-mg/ml fresh lysozyme was added, and the mixture was incubated for 10 min on ice (0°C). After the incubation, 14.8 μl of 0.5 M EDTA (pH 8.0) and 650 μl of 50 mM Tris (pH 8.0)–7% (wt/vol) sucrose were added and mixed well, and the mixture was incubated for 2.5 min on ice. Finally, 200 μl of 5% Nonidet P-40 was added, and the solution was mixed well. After centrifugation at 12,000 rpm in a J2-21 centrifuge for 30 min at 4°C, the clear supernatant was incubated for 1 h at room temperature with glutathione-agarose beads. The beads were then washed several times with PBS and resuspended in water (1:1 [vol/vol]). Before the solution binding assay, aliquots of these beads were run on mini-SDS-polyacrylamide gels which were stained with Coomassie brilliant blue to assess the concentration of the protein. The gels were stained with a solution of Coomassie brilliant blue (0.25% [wt/vol] Coomassie brilliant blue, 10% acetic acid, 50% methanol), and then destained (7.5%, acetic acid, 5% methanol).

MT/PDGFr solution binding. Different concentrations of the fusion proteins on beads were incubated with a fixed amount of ^{32}P-labeled MT or PDGFr for 1 h on ice. All reactions were carried out in polypropylene tubes, and the differences in bead volumes from one sample to another were corrected by the addition of glutathione-agarose, so that the resulting volumes were equal. After incubation, the beads were washed once with PBS, twice with 0.5 M LiCl, and one last time with PBS. The buffers of the last two washes contained 100 μg of BSA per ml, which prevented the beads from sticking to the

tubes. The samples were then boiled in SDS sample buffer and run on an SDS–7.5% polyacrylamide gel. The gels were stained with Coomassie brilliant blue and then dried. The MT or PDGFr bound was analyzed with a Molecular Dynamics PhosphorImager, and the amount of SH2 fusion protein in each reaction was measured by scanning on a Visage Scanner (Millipore).

Phosphopeptide library assay. The assay is described in detail elsewhere (34, 35). The degenerate phosphopeptide GDGpYXXXSPLLL, where X could represent any amino acid except W and C, was used to assess binding specificity. The degenerate peptides were loaded onto columns containing the fusion protein GST-SH2 (wild type or mutant) or GST alone as a control. The columns were washed with cold PBS and eluted with a solution of sodium phenylphosphate. The flowthrough was collected, dried, and sequenced. The relative amount of each amino acid determined for the phosphopeptide mixture bound to the GST-SH2 column was compared with the amount of amino acids determined for the mixture bound to the control GST column at each cycle.

RESULTS

SH2 mutants with altered MT binding properties. Most mutants were created by hydroxylamine mutagenesis of a GST-p85 N-SH2 fusion containing residues 321 to 470 of p85. Hydroxylamine was used to mutagenize double-stranded plasmid DNA under conditions causing an average of one mutation per SH2 domain. A simple screening procedure was then carried out to identify mutants. Previous work showed that tyrosine-phosphorylated polyomavirus MT bound efficiently to the upstream SH2 domain of p85α after renaturation on blots (43). Bacterial colonies containing plasmid pGEX-N-SH2 were transferred onto nitrocellulose filters and screened simultaneously for protein expression and MT binding. Figure 1A compares a section of a nitrocellulose filter with its corresponding autoradiogram. The arrows indicate a mutant with reduced MT binding ability. After repeating the screening, plasmids from colonies expressing protein impaired in MT binding were isolated and sequenced. Although some double mutants were detected, these were discarded. Many of the single point mutants were reisolated multiple times from independent colonies. The antibody used in the screening was made to a peptide containing the conserved FLVR sequence (residues 349 to 363). Since this antibody cross-reacts with other SH2 domains such as the p85 C-terminal SH2 (C-SH2) domain or pp60$^{c\text{-}src}$ (not shown), and since mutations at residues 360 and 361 were obtained this way, it seemed unlikely that using the antibody introduced a strong bias. Nonetheless, to ensure that mutations in this region would not be missed, site-directed mutagenesis on arginine 358, alanine 360, and lysine 363 was also performed.

Table 1 lists the mutants identified by hydroxylamine and the three sites mutated by site-directed mutagenesis. Two different mutations at residue 360, one by each method, were obtained. A few mutations, such as R465K, that were outside the consensus SH2 domain sequence (21) were obtained. While some of the mutated SH2 residues are fairly well conserved among various SH2 domains, several of them are in variable regions. Purified GST fusion proteins were then used to examine the effects of the mutations on the ability of the mutant SH2 domains to bind MT by blotting, like that used for screening, or to bind MT in a solution assay. In the solution assay, wild-type or mutant proteins bound to glutathione-agarose beads were incubated with ^{32}P-labeled MT. After a 1-h incubation, the beads were washed and analyzed by SDS-

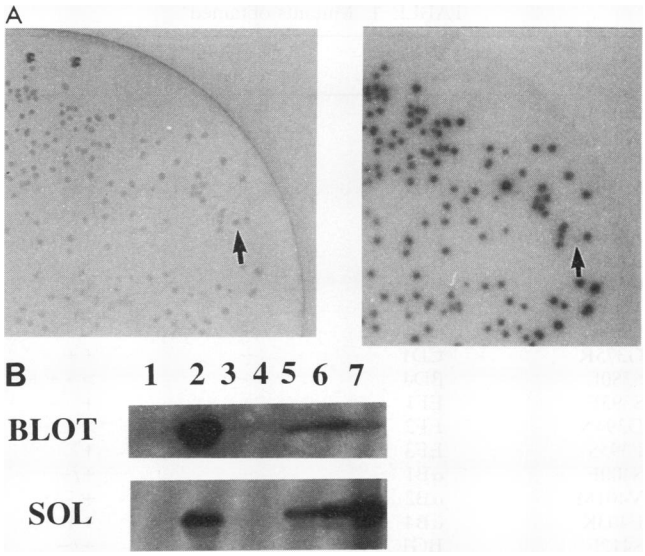


FIG. 1. Identification of mutants in the p85 N-SH2 domain. (A) Screening by filter binding. Hydroxylamine-mutagenized pGEX-3X-p85N-SH2 was transformed into *E. coli* XL1-blue. Colonies were transferred to nitrocellulose and blotted with both peptide antibody and tyrosine-phosphorylated ^{32}P-labeled MT as described in Materials and Methods. Protein (left) was visualized with alkaline phosphatase; MT (right) was visualized by exposure to X-ray film. The arrows point to a mutant candidate. (B) Analysis of GST-p85 N-SH2 fusions. Approximately equal amounts of purified wild-type protein and different mutant fusion proteins were blotted with tyrosine-phosphorylated ^{32}P-labeled MT after transfer to nitrocellulose (BLOT) or incubated in solution with tyrosine-phosphorylated MT (SOL). MT was visualized by autoradiography. Lanes: 1, GST; 2, wild-type GST–p85 N-SH2; 3, P427L; 4, T369I; 5, S400F; 6, A360V; 7, E403K.

polyacrylamide gel electrophoresis (PAGE). The GST-SH2 fusion proteins were visualized by Coomassie brilliant blue staining, and bound MT was detected by exposure to X-ray film or to a PhosphorImager. Results comparing some mutations in the two assays are shown in Fig. 1B. Table 1 summarizes the results. Four mutants, D359G and the three outside the SH2 domain, were insoluble after growth in *E. coli* and could not be tested in solution. Presumably these represented unfolded polypeptides that aggregated. The remaining mutants can be divided into several classes. Some mutants defective in the blotting assay were not defective in solution binding. E403K appeared not to be substantially defective when assayed in solution (Fig. 1B, lane 7). The same was true for G375R (not shown). These probably could not renature successfully on the filter, thus appearing mutant in the first screen. E-403 has been suggested to be involved in calcium binding (22), but it is not clear that this function is connected to its failure to refold properly. The finding that some mutants failed in renaturation was not unexpected. Previous work showed that not all SH2 domains can be renatured efficiently. For example, the C-SH2 domain of p85 binds MT well in solution, but not after binding to nitrocellulose (43). The screening assay could therefore have tested renaturability as well as intrinsic binding ability.

The second class represented mutants defective in the solution assay as well as by blotting. Four examples of these mutants are shown in Fig. 1B. It is clear that they can be divided into mutants that are highly impaired in MT binding (Fig. 1B, lanes 3 and 4) and those that are less impaired (lanes 5 and 6). To examine the binding more carefully, binding

TABLE 1. Mutants obtained[a]

Mutant	Position of residue	Conserved residue[b]	MT binding[c]
Wild type			+++
W335R	βA1	−	−
S339F	αA1	+	++
R358Q*	βB5	+	−
D359G	βB6	+	I
A360S*	βB7	−	++
A360V	βB7	−	+
K363Q*	BC3	−	+
T369I	βC3	+	−
L370F	βC4	+	−
G375R	CD1	−	++
S380L	βD4	−	>++++
S393F	EF1	−	+
D394N	EF2	−	>++++
P395S	EF3	−	+
S400F	αB1	+	+/−
V401M	αB2	−	+
E403K	αB4	+	++
S412F	BG1	−	+/−
L425F	BG13	−	+/−
P427L	βG2	+	−
S429F	ND	ND	−
H450Y	ND	ND	I
E458K	ND	ND	I
R465K	ND	ND	I

[a] All mutants obtained by random or site-directed (*) mutagenesis are listed. The position of the residue was assigned by using the model for the Src SH2 domain described by Waksman et al. (41), with the nomenclature of Eck et al. (11).

[b] Residues that are conserved within at least three subfamilies of SH2 domains according to Koch et al. (21). The positions of the last four mutants and their degrees of conservation were not determined (ND), as these were not included in the Src structure (41) or the SH2 domain description (21).

[c] Results of the MT binding assay in solution. −, ≥10-fold reduction in binding; +/−, denotes 4- to 10-fold reduction; +, 2- to 4-fold reduction; ++, reduction of less than 2-fold; >++++, binding 2.5- to 3-fold greater than the wild-type level. The four insoluble mutants (I) could not be tested.

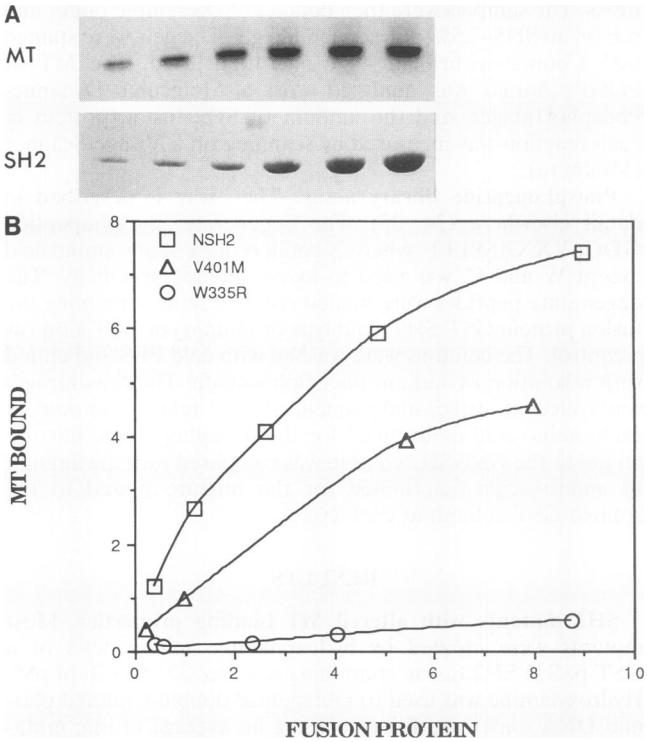


FIG. 2. Comparison of wild-type and mutant p85 N-SH2 mutants for MT binding. (A) Increasing amounts of wild-type SH2 fusion protein were incubated with ^{32}P-labeled MT as described in Materials and Methods. After two washes with PBS and two washes with LiCl, SDS-PAGE was performed. Fusion protein was visualized by staining with Coomassie brilliant blue, and MT was visualized by analysis using a Molecular Dynamics PhosphorImager. (B) Quantitative comparison of wild-type with W335R and V401M mutant SH2 domains. Samples were prepared and treated as in panel A. The *x* axis represents the amount of fusion protein determined by scanning of the Coomassie brilliant blue-stained bands. The *y* axis represents the amount of MT in complex with the SH2 domains as determined by the PhosphorImager analysis. The units on the *x* and *y* axes are arbitrary.

experiments were carried out as a function of increasing protein concentration. An example of the data for such an experiment is shown in Fig. 2A for the wild-type SH2 domain. The amount of fusion protein was assessed by densitometry of Coomassie brilliant blue-stained gels; the amount of MT was measured with a Molecular Dynamics PhosphorImager. Figure 2B shows that W335R is an example of a mutant with little binding ability, while V401M is reduced somewhat more than twofold. Table 1 also summarizes the results of these assays.

Surprisingly, some colonies in the filter binding assay seemed to bind MT better than the wild-type SH2 domain. While in some cases this seemed to represent a screening artifact, S380L and D394N consistently showed a two- to threefold increase over the wild-type value (Fig. 3; Table 1). It is therefore possible to identify SH2 domains with improved MT binding properties.

Mutation and effects on SH2 domain specificity. SH2 domains are known to show specificity for binding tyrosine-phosphorylated sequences. To establish the effects of some of these mutations on specificity, two different approaches were taken. In one set of experiments, the behavior of PDGFr was compared with that of MT. PDGFr is also known to bind to the upstream SH2 domain of p85 (13, 39). The sites of p85 binding are similar, but not identical, in MT and PDGFr. While MT has one YMPM motif around tyrosine 315, PDGFr has two similar sequences, YMDM and YVPM, starting at tyrosines 740 and 751. While it is not certain that each SH2 domain of p85 binds a different PDGFr phosphotyrosine, it has been suggested that the N-SH2 domain used here binds to YVPM at 751 (19, 28). Mutant P427L is almost as completely defective in binding PDGFr as it was in binding MT (Fig. 4). D394N has a comparable increase in affinity for both the receptor and MT. The other up mutant, S380L, also showed a similar increase in binding for both MT and PDGFr (data not shown). Despite the similarity of the binding motifs for the two proteins, differences were observed with some mutants. Figure 4 shows that neither mutation P395S nor mutation S393F appeared to affect the binding of phosphorylated PDGF receptor as much as MT.

SH2 domains show specificity for the residues following the phosphotyrosine (4). This specificity can be demonstrated by examination of the binding of degenerate phosphopeptides (34, 35). In this assay, the purified SH2 domain is incubated with a library of phosphopeptides random at three positions after the phosphotyrosine. Sequencing of the bound peptides reveals enrichment of particular amino acids at the three random positions. The upstream SH2 domain of p85, a prototype used to develop the assay, has a strong preference for methionine at the third position after the phosphotyrosine, no preference at the second position, and preference for methio-

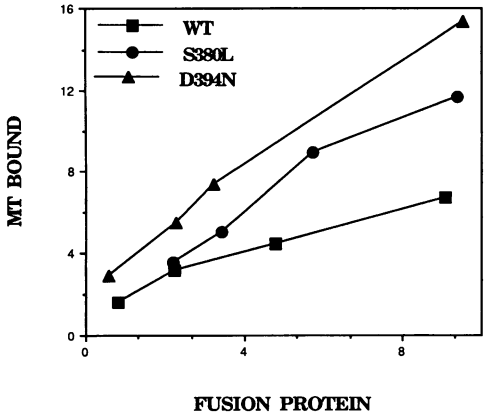


FIG. 3. Some mutations resulted in increased MT binding. Comparison of S380L and D394N was carried out as described for Fig. 2. WT, wild type.

nine, isoleucine, valine, and glutamic acid at the first position (Fig. 5). Three mutants (S393F, D394N, and P395S) were chosen for examination by this method. These mutants all contain mutations in the EF loop (see Discussion and Fig. 6), which is thought to be involved in recognition of the +3 position after the phosphotyrosine. D394N showed an even stronger enhancement for methionine at the third position compared with the wild type, consistent with its improved recognition of MT and PDGFr. Analysis of P395S showed two things. Preferences at the first position were generally similar, although a stronger affinity for valine was noted. Second, there was complete loss of preference for methionine at the third position after the phosphotyrosine. S393F (not shown) also showed a less pronounced preference for methionine at the +3 position; isoleucine showed a slight enhancement over methionine and valine at the first position.

The results on specificity can be summarized in the following manner. A mutation that increased preference for methionine at +3 increased binding of both MT and PDGFr. Mutations that decrease affinity for methionine at +3 decreased binding

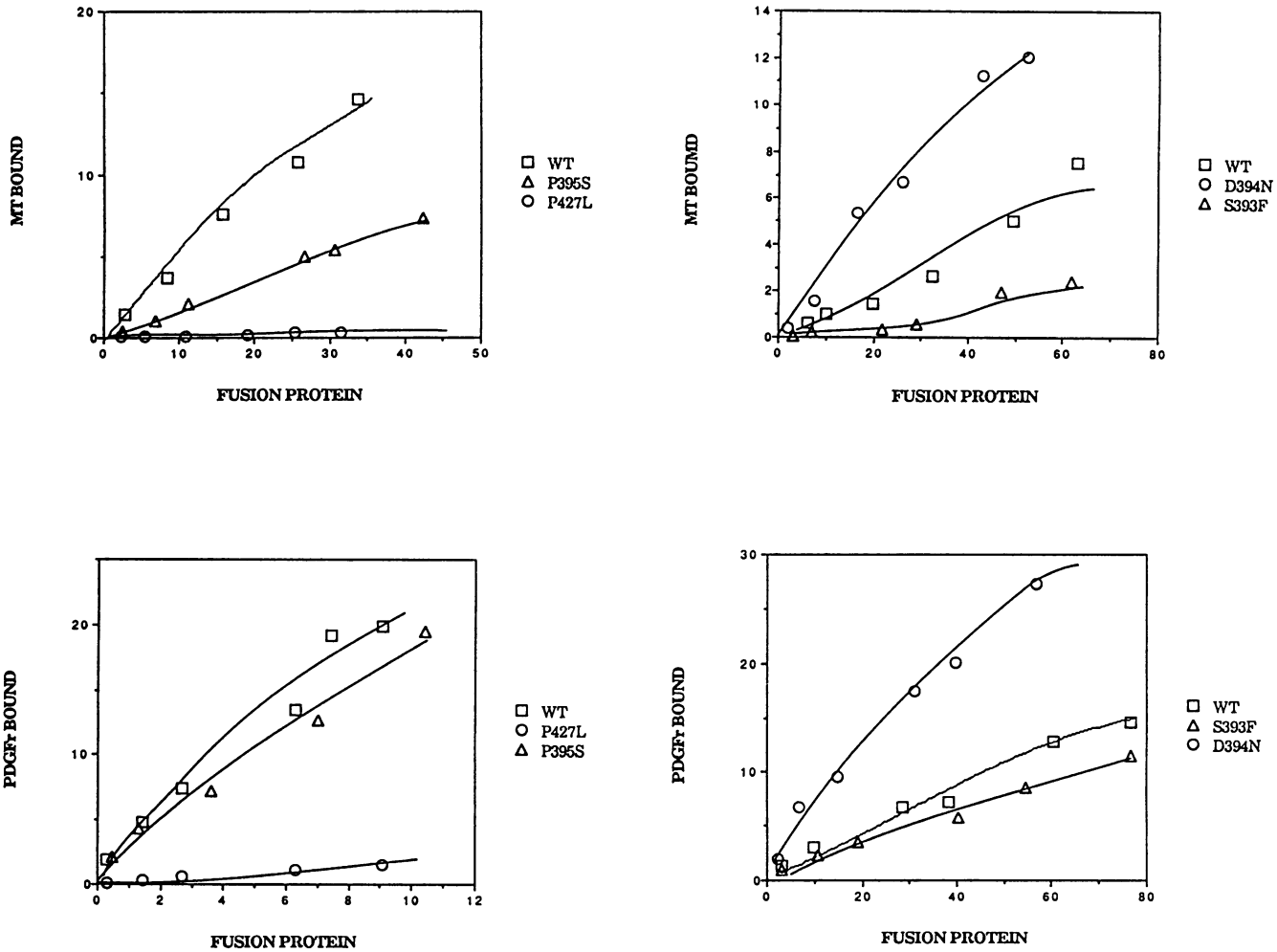
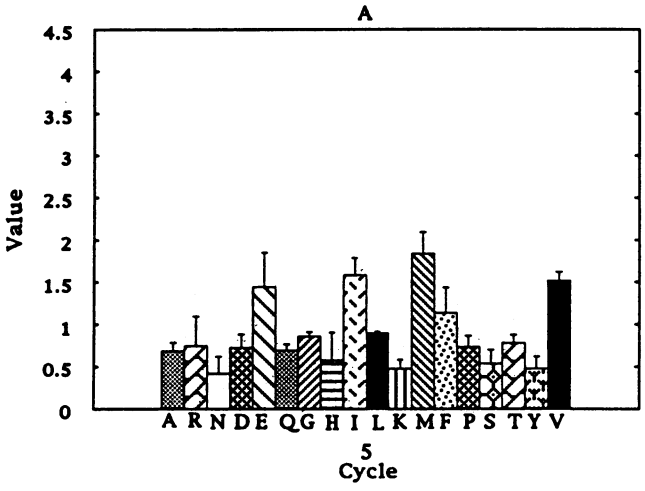


FIG. 4. Comparison of MT and PDGFr for SH2 domain binding. (Left) Binding of two mutants P427L and P395S to MT and PDGFr. Increasing amounts of GST–p85 SH2 fusions (wild type [WT], P427L, or P395S) were incubated with fixed amounts of ^{32}P-labeled MT (top) or PDGFr (bottom) for 1 h at 4°C. The samples were treated as described for Fig. 2. (Right) Binding of mutants D394N and S393F to MT and PDGFr. The wild-type, D394N, or S393F fusion was incubated with ^{32}P-labeled MT (top) or PDGFr (bottom).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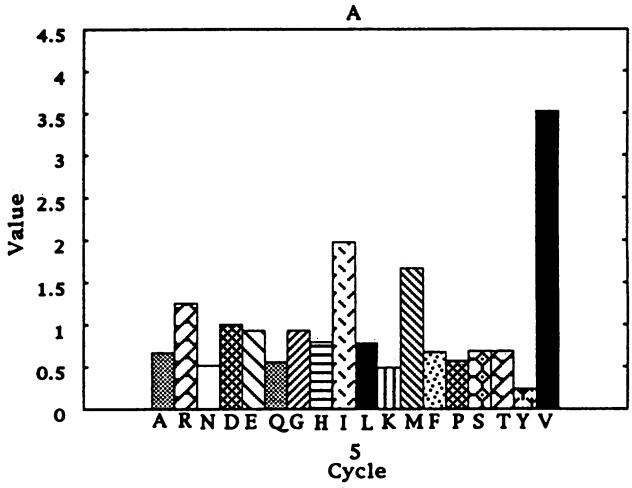
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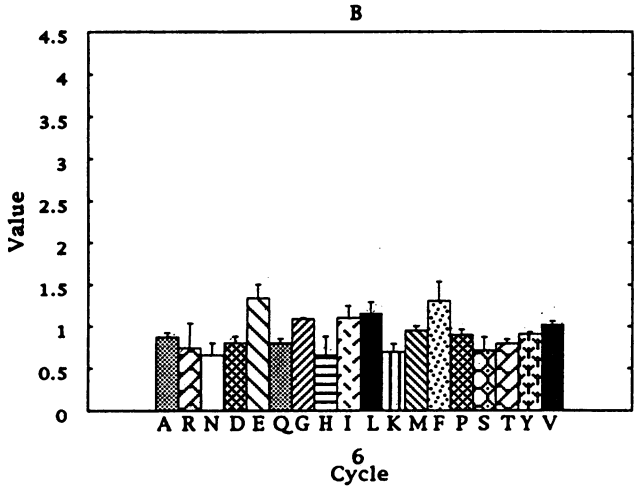
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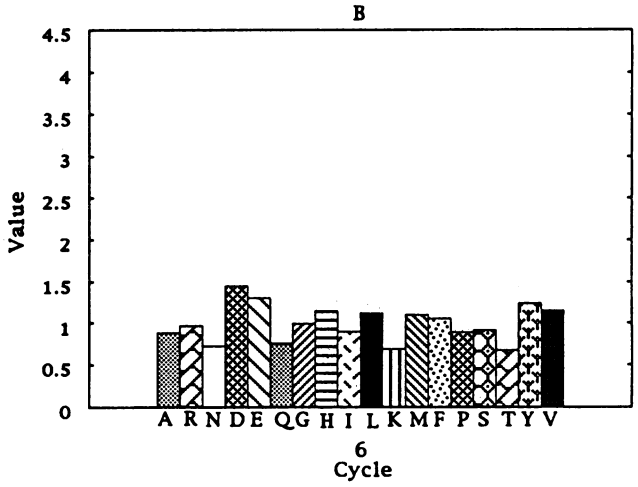
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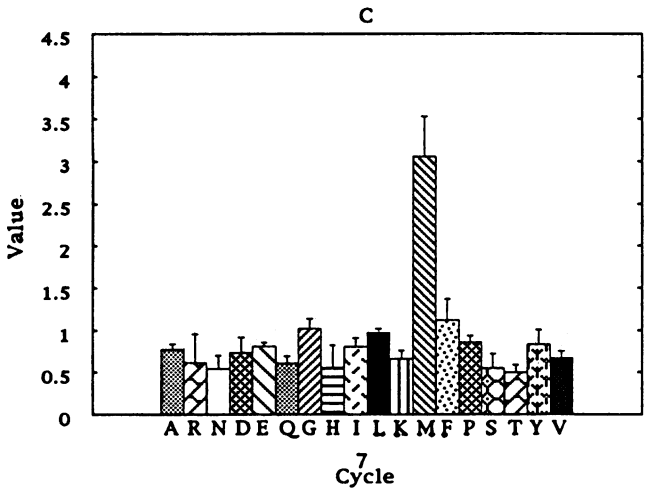
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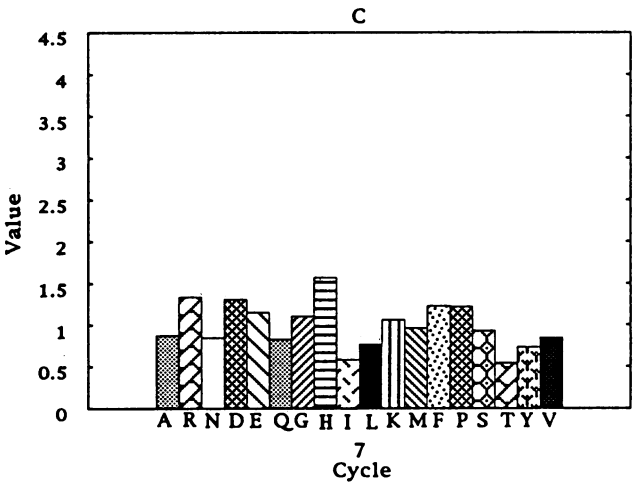
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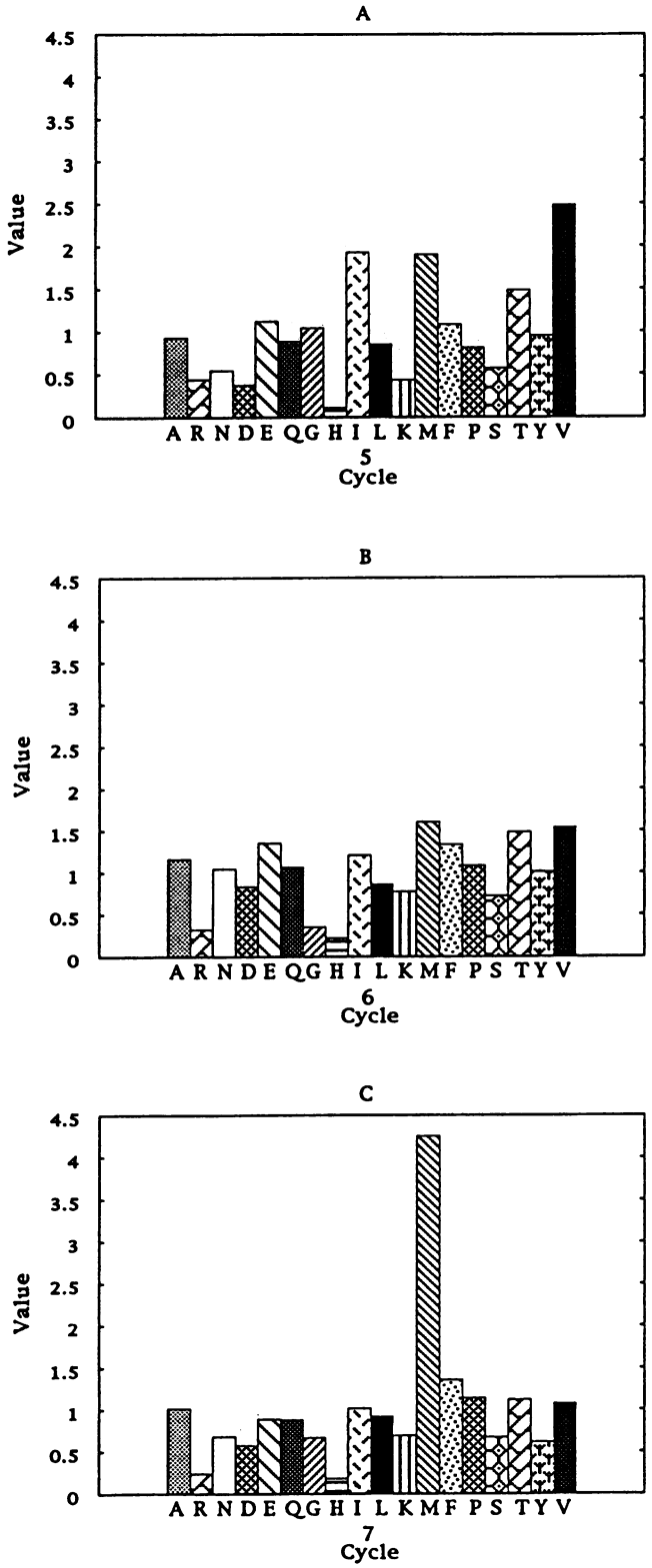

of MT. However, those mutations also affected specificity at +1 and did not substantially affect binding of PDGFr.

DISCUSSION

The goal of these studies has been to use broad-based mutagenesis to probe the structure of the upstream SH2 domain of p85 and to examine its interactions with tyrosine-phosphorylated MT or PDGFr. Random mutagenesis was emphasized instead of site-directed mutagenesis to try to minimize bias that might arise from direct consideration of sequence or structure. Altogether, 24 different mutants were generated. These included not only mutants defective in binding but also mutants that showed enhanced binding. Consideration of the data suggests that the structural, biochemical, and genetic data fit together well.

Mutations that affect folding or gross structural features. It is easiest to discuss the mutants in the context of a structural model. Figure 6 shows an adaptation of a model based on X-ray diffraction data for the Src SH2 domain (41) to p85. It uses the nomenclature suggested by Eck and colleagues (11). L370F, P427L, and S429F are all strongly impaired in MT binding. L370F is in βC in the core of the structure, so it is not surprising that it is highly defective. S-429 is worth mentioning because it is beyond what is often drawn for an SH2 domain consensus. Proline 427 of βG is extremely well conserved among the nine SH2 domains compared by Waksman et al. (41), only replaced by valine in Src. βG is on the backside of the SH2 domain from the peptide binding site. NMR experiments (16a) indicate that the P427L mutation does not result in simple unfolding of the molecule but rather results in limited rearrangement of some residues. It appears likely that P-427 and perhaps S-429 are important for organizing the binding surface. P427L did show some residual binding to MT when low salt was used to wash the samples (data not shown). The residual P427L-MT interaction seems therefore ionic in nature and may suggest that the mutation had less of an effect on the phosphotyrosine binding pocket than on hydrophobic interactions.

Mutations in the phosphotyrosine pocket. Many of the mutants can be understood in terms of elements needed to form specificity pockets. Site-directed mutagenesis confirmed the expected importance of R-358, the FLVR arginine. This is known to be involved in coordinating the phosphate on the tyrosine. Mutations in Src (1), Abl (26), GTPase-activating protein (23), Vav (20), and Sem-5 (36) SH2 domain FLVR arginines have confirmed their importance. T369I (βC3) is also extremely defective in binding MT. In the phospholipase Cγ

FIG. 5. Selection of phosphopeptides that bind to wild-type, P395S and D394N SH2 domains. A phosphopeptide library, degenerate at three positions after the phosphotyrosine (34) (Materials and Methods), was loaded onto a column containing either the GST-SH2 (wild type, P395S, or D394N) fusion protein or GST alone. The column was washed, and the bound peptides were eluted with phenylphosphate. The eluted peptide mixture was subjected to microsequencing. The results were compared with those from the eluate of a control GST column. Panels A to C show results for the positions 1 to 3, respectively, after the phosphotyrosine. The *y* axis represents the ratio of the amount of each amino acid eluted from GST-SH2 bead columns divided by the amount of the same amino acid eluted from the control GST bead columns at the same cycle. The *x* axis represents the different amino acids included in the random library. Abbreviations for amino acid residues: A, Ala; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; Y, Tyr. Left, wild type; center, P395S; right, D3494N.

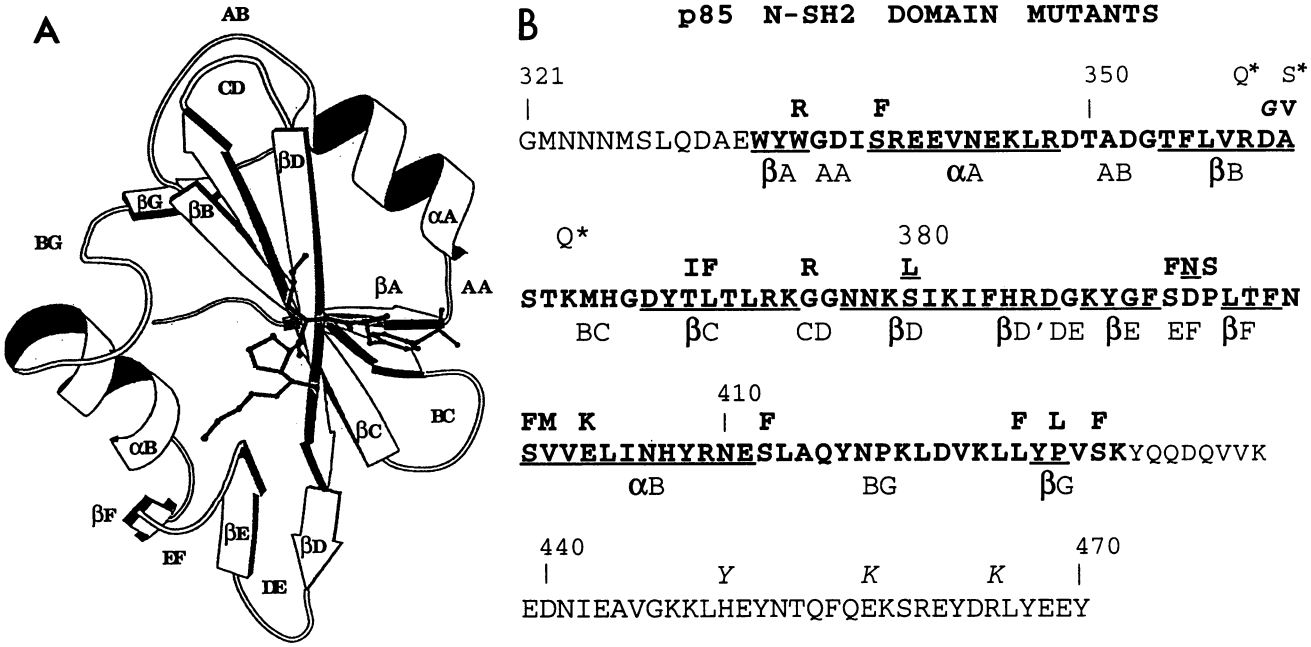

FIG. 6. Structural model for the p85 N-SH2 domain. (A) Richardson diagram revised from the model of the X-ray diffraction data of Waksman and colleagues (41). SH2 domains have a central antiparallel β sheet flanked by two α helices. Peptides bind orthogonal to the central sheet. MT residues 315 to 318 (pYMPM) are shown as dark lines with tyrosine-phosphorylated MT to the right in this view. The designation of structural elements follows the description of Eck et al. (11). (B) Sequence of the p85 N-SH2 construct, using nomenclature of Eck et al. (11). SH2 is shown in boldface. Hydroxylamine-derived mutants are shown above the wild-type sequence. Underlined mutants bind better than the wild type; mutants in italics were found not to be soluble. Site-directed mutants are shown on the line above the hydroxylamine mutants.

C-SH2 domain, the βC3 alanine is coordinating the tyrosine ring (31). This may well explain the defect, but T369I may also affect gross structure like L370F. W335 is in βA. Although not apparently directly involved in interactions with phosphotyrosine, this residue is in a position where a mutation could affect other residues interacting with the phosphotyrosine. Its mutation, particularly an arginine replacement, could well perturb the interactions of the FLVR arginine at βB5 or arginine 340 of αA with the phosphotyrosine (Fig. 6A). Mutation of serine 339, which reduced MT binding approximately threefold, is likely to act in a similar manner. A-360 in p85 corresponds to S-177 in the Src SH2 domain, which according to Waksman et al. (41) is on the second β strand and constitutes an important element of the phosphotyrosine recognition. A-360 also corresponds to S-173 in the Abl SH2 domain; this residue was also shown by mutagenesis studies to be important for binding (26). While site-directed mutagenesis of A-360 to serine did not significantly impair MT binding, mutation to valine reduced activity. S380L is a mutant that showed enhancement for binding of both MT and PDGFr. It corresponds to Src H-201, which was shown by Waksman et al. (41) to be crucial for phosphotyrosine binding, apparently because it provides hydrophobic interactions with the phosphotyrosine ring system. The same histidine (βD4) in the phospholipase Cγ C-SH2 domain acts the same way (31). If the hydrophobic character of the histidine is required for the Src SH2 domain, it is not surprising that leucine 380 in the p85 N-SH2 domain would be more favorable than serine. In the GTPase-activating protein N-SH2 domain, mutation of histidine to glutamate or lysine abolished EGFr binding (23). Results with the Src SH2 domain point to a limitation in these sorts of simple comparisons. Mutation of H-201 to leucine reduced binding slightly rather than enhancing it (1). It is likely, and NMR studies confirm, that mutations have effects beyond individual residues, which make definitive conclusions difficult.

Mutations in the +3 pocket. Some of the most interesting mutations affect the binding pocket for the hydrophobic residue at the third position after the phosphotyrosine. MT binds the SH2 domain through its sequence at 315 pYMPM. Early predictions of specificity and binding were made by the observation that such a sequence represented a consensus (4). The specificity of the N-SH2 domain determined from peptide binding experiments is MVIE at the first position, anything at the second position, and methionine at the third position. It is particularly striking that three mutations at residues 393, 394, and 395 came out of the mutant search. These are the three residues of the EF loop. The EF loop is part of the hydrophobic binding pocket. In Src, the EF loop moves to a more closed form when a pYEEI peptide binds (41). S393F and P395S both reduce affinity for MT, while D394N shows enhanced binding. Binding of random peptides showed increases or decreases in preference for methionine at the +3 position that were expected based on the MT binding data. The enhanced binding of the D394N mutant can be explained since a neutral polar residue is more likely to be tolerated in the hydrophobic binding pocket than a negatively charged one. Other mutations that affect the hydrophobic binding pocket were also encountered. In the Src model, αB forms a base for the pocket. S400F on the edge of and V401M within that helix show reduced MT binding. The BG loop forms the other element. Again, S412F and L425F in that loop were defective in MT binding.

Both similarities and differences were observed when MT and PDGFr binding to mutant proteins were compared. Mutations such as P427L and S380L, which are likely to affect the phosphotyrosine binding site, have similar effects on binding

of MT and PDGFr. On the other hand, mutations in the EF loop discriminate between MT and the receptor. PDGFr binding was less affected by mutation at 393 and 395 than MT binding, even though both proteins have pTyr-X-X-Met motifs. A likely explanation for this result is the altered affinity at the first position after the phosphotyrosine. Binding sites for p85 in PDGFr are YMDM and YVPM at tyrosines 740 and 751, compared with pYMPM for MT. The peptide sequencing experiments show some alterations in preference at the first position. In the case of P395S, for example, there is an increased preference for valine compared with methionine at the first position (Fig. 5), so this mutant might be expected to bind much better to the pYVPM site of PDGFr than to the pYMPM site of MT. It is possible that failure of the +3 pocket to accommodate the side chain of methionine forces the peptide backbone to a distinct conformation that enhances affinity for valine at +1. An alternative possibility raised by NMR data (32a) is that some changes in the environment for additional residues besides that of the mutation could affect the +1 pocket. Settling the issue will require a better understanding of the tertiary structure of mutant and wild-type p85 N-SH2 domain. Interestingly, after this report was submitted for publication, Marengere and coworkers reported a change in the specificity in the Src SH2 domain by mutation of residue EF1 (24). This result is generally consistent with the theme developed here, although here a stronger effect is seen with EF3.

One final point is worth noting. Point mutations made in the laboratory can give rise to SH2 domains that bind better than the naturally occurring ones. Why are these not already found in p85? It is possible that this is simply an artifact of the assay. It is possible that the wild-type structure is optimized, but for a protein with a sequence different from that of MT or PDGFr. However, there is another explanation, based on the need for regulation, that is more appealing. Tyrosine phosphorylation is known to be part of the process by which p85 is released from middle T (7). It appears that intramolecular interactions occur between tyrosine phosphorylation sites on p85 and the SH2 domains (16b). If the affinity were too strong between the SH2 domain and the tyrosine phosphorylation sequence, then the regulation would be compromised.

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