A Single Point Mutation Switches the Specificity of Group III Src Homology (SH) 2 Domains to That of Group I SH2 Domains*

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Zhou Songyang, Gerald Gish, Geraldine Mbamalu, Tony Pawson**, and Lewis C. Cantley

From the Division of Signal Transduction, Beth Israel Hospital, and the Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115, the Program in Molecular Biology and Cancer, Samuel Lunenfeld Research Institute, and the Protein Engineering Network Centre of Excellence, Mount Sinai Hospital, Toronto, Ontario M5G 1X5, Canada

Src homology 2 (SH2) domains recognize phosphoryrosine-containing sequences, and thereby mediate the association of specific signaling proteins in response to tyrosine phosphorylation (Pawson, T., and Schlessinger, J. (1993) Curr. Biol. 3, 434-442). We have shown that specific binding of SH2 domains to tyrosine-phosphorylated sites is determined by sequences adjacent to the phosphoryrosine. Based on the phosphopeptide specificity and crystal structures, SH2 domains were classified into four different groups (Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Hacer, W. G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R. J., Neel, B. G., R. B. B., Fajardo, J. E., Chou, M. M., Hanafusa, H., Schaffhausen, B., and Cantley, L. C. (1993) Cell 72, 767-778). The βD5 residues of SH2 domains were predicted to be critical in distinguishing these groups (Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Hacer, W. G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R. J., Neel, B. G., R. B. B., Fajardo, J. E., Chou, M. M., Hanafusa, H., Schaffhausen, B., and Cantley, L. C. (1993) Cell 72, 767-778; Eck, M. J., Shoelson, S. E., and Harrison, S. C. (1993) Nature 362, 87-91). We report here that replacing the aliphatic residues at the βD5 positions of two Group III SH2 domains (phosphoinositide 3-kinase β, phospholipase Cγ, C-terminal SH2 domain) with Tyr (as found in Group I SH2 domains) results in a switch in phosphopeptide selectivity, consistent with the specificities of Group I SH2 domains. These results establish the importance of the βD5 residue in determining specificities of SH2 domains.

Stimulation of cellular responses by growth factors and cytokines is often accomplished by the activation of protein-tyrosine kinases (1, 6). One of the major consequences of tyrosine phosphorylation is to induce a specific set of protein-protein interactions, and thereby initiate a series of intracellular signaling cascades. The SH2 domains of cytosolic signaling proteins mediate the assembly of such complexes by binding to phosphotyrosine moieties within specific sequence contexts (1, 6, 7). Therefore, in order to understand signal transduction by protein-tyrosine kinases, it is important to decode the mechanisms by which SH2 domains achieve specificity in their recognition of phosphotyrosine sites.

The specificity of SH2 domains was first systematically studied using a degenerate phosphopeptide library (2, 3). Subsequently, crystal structures of SH2 domains complexed with their high affinity ligands were obtained, providing a structural basis for phosphopeptide recognition by SH2 domains (4, 5, 8, 9). From these studies and related experiments analyzing the in vivo binding sites of various SH2 domains, it has become evident that 3-6 residues C-terminal of phosphotyrosine dictate the specificity of SH2 domain binding. Importantly, SH2 domains can be divided into four subgroups on the basis of their specificity and primary sequences (2). For example, Group I SH2 domains (SH2 domains of Src family, Abl, Crk, GRB2, Nck SH2 domains, etc.) select the general motif Tyr(P)-hydrophilic-hydrophilic-hydrophobic and have an aromatic amino acid at the βD5 position. However, Group III SH2 domains (eg. SH2 domains of phosphoinositide 3-kinase p85, phospholipase Cγ, and Syp/SHPTP2) select the general motif Tyr(P)-hydrophobic-X-hydrophobic and have Ile or Cys at the βD5 position (Fig. 1). In the three-dimensional structures of Src and Lck SH2 domains, the βD5 Tyr is at the surface and makes contacts with the side chains of both the pY+1 and pY+3 residues of the bound phosphopeptide (4, 5). However, in the three-dimensional structures of Syk and PLCγ SH2 domains (Group III), the aliphatic residue at the βD5 position is buried deeper in the protein, opening a hydrophobic cavity for the pY+1 through pY+6 residues (8, 9) (Fig. 1B). To address the importance of the βD5 position, we have investigated the effect of substituting this residue on the specificity of two Group III SH2 domains.

**Terry Fox Cancer Research Scientist of the National Cancer Institute of Canada.

The abbreviations used are: SH, Src homology; PCR, polymerase chain reaction; PLC, phospholipase C; GST, glutathione S-transferase.
RESULTS AND DISCUSSION

Phosphopeptide Selectivity of Mutant Phosphoinositide 3-Kinase p85 SH2 and Phospholipase Cγ (PLC-γ) SH2 Domains—The p85 N-terminal SH2 (NSH2) domain and PLC-γ C-terminal SH2 (CSH2) domain were selected to study the importance of the βD5 residue for SH2 domain specificity because they have three-dimensional structures, with associated high affinity phosphopeptides having been solved (8, 13). The wild-type p85 NSH2 domain contains a Ile (Ile-383) residue at the βD5 position and preferentially binds the sequence Tyr(P)-Val/Ile-Ile-Pro. However, the mutant PLC-γ CSH2 domain (C715Y) binds preferentially to peptides with acidic residues, instead of hydrophobic amino acids, at the pY+1 and +2 positions. At the pY+3 position, a different set of hydrophobic amino acids were selected by the mutant SH2 domain, with Phe being the preferred amino acid. Hence, the C715Y PLC-γ CSH2 domain selects peptides with the optimal motif Tyr(P)-Glu-Glu-Phe, which resembles the general motif for Group I SH2 domains (2, 3).

Conversion of the βD5 Ile of the p85 NSH2 domain to Tyr resulted in a domain with similar selectivity to the mutant PLC-γ CSH2 domain (C715Y). While the wild-type p85 NSH2 domain selected for Tyr(P)-Met-X-Met, the I383Y mutant selected for Tyr(P)-Glu-Gln-Phe (Table I).

Evaluation of Phosphopeptide Binding to Wild-type and Mutant PLC-γ SH2 Domains—While the peptide library technique predicts optimal amino acids at specific positions C-terminal of phosphotyrosine, it does not determine the affinity for specific peptide sequences or address the complexity of cooperative effects of adjacent amino acids. To investigate the affinities of specific peptides for wild-type and mutant PLC-γ
CSH2 domains, phosphopeptides containing the optimal binding sequences of wild-type and βD5 mutant (C715Y) PLC-γ CSH2 domains were synthesized and studied using surface plasmon resonance (Table II). These peptides were constructed based on sequences surrounding Y1021 of the human platelet-derived growth factor receptor (DNDpYIIPLPDPK; named pYIIP peptide), an in vivo binding site of PLC-γ SH2 domains. In close agreement with previous studies, the wild-type PLC-γ CSH2 domain bound tightly to the pYIIP peptide (Kd = 80 nM). However, no apparent binding of this peptide to mutant PLC-γ CSH2 domain or Src SH2 domain (a Group I SH2 domain) was detected at concentrations up to 50 μM. In contrast, the mutant PLC-γ CSH2 domain and Src SH2 domain were able to bind the phosphopeptide named pYEEF (DNDpYEEFLPDPK) with dissociation constants of approximately 398 and 1300 nM, respectively. The pYEEF peptide also bound to the wild-type PLC-γ SH2 domain but with lower affinity (Kd = 180 nM) than the optimal pYIIP peptide. Thus, consistent with the prediction in Fig. 2, the C715Y mutation changed the specificity of PLC-γ CSH2 domain. These data confirm the suggestion that the βD5 Cys to Tyr mutant of the PLC-γ SH2 domain behaves more like a Group I SH2 domain.

**FIG. 2.** A comparison of phosphopeptide specificity of the wild-type and βD5 mutant (C715Y) PLC-γ CSH2 domains. Results are from the fifth, sixth, and seventh cycle of the sequence (i.e. the pY + 1, +2, and +3 positions). The value represents the ratio of the amount of each amino acid eluted from GST-SH2 bead columns divided by that of the control GST bead columns at the same cycle. Amino acids are presented in single-letter code.

**TABLE I**

Selectivities of mutant SH2 domains

| Peptide | pY +1 | pY +2 | pY +3 |
|---------|-------|-------|-------|
| p85 NSH2 (L383Y) | Glu (2.6) | Glu (1.7) | Asn (1.8) |
| PLC-γ CSH2 (C715Y) | Glu (2.6) | Glu (1.9) | Phe (2.3) |

| Peptide | pY +1 | pY +2 | pY +3 |
|---------|-------|-------|-------|
| pYIIP | 1.5 | 1.7 | 1.9 |
| pYIIF | 2.0 | 3.4 |
| pYEIP | 16.5 | 15.5 |
| pYEEF | 3.5 | 4.5 |

**TABLE II**

Phosphopeptide binding affinities for wild-type and C715Y mutant of PLC-γ CSH2 domain

| Peptide name | A. Peptide name | IC₅₀ for inhibition of pYEEF binding (μM) |
|--------------|-----------------|---------------------------------|
| PLC CSH2 | PLC CSH2 (C715Y) |
| pYIIP | 1.5 | 60 |
| pYIIF | 2.0 | 3.4 |
| pYEIP | 16.5 | 15.5 |
| pYEEF | 3.5 | 4.5 |

| Peptide name | B. Peptide name | Kd (nM) |
|--------------|-----------------|--------|
| PLC CSH2 | PLC CSH2 (C715Y) | Src SH2 |
| pYIIP | 80 | — |
| pYEEF | 180 | 398 | 1300 |

* Peptides: pYIIP, DNDpYIIPLPDPK; pYEIP, DNDpYEIPLPDPK; pYIIF, DNDpYIIFLPDPK; pYEEF, DNDpYEEFLPDPK.

b — no detectable binding.
To further test the specificity of the mutant PLC-γ CSH2 domain and the prediction of the peptide library, two additional peptides were constructed. The pYEEF (DNDpYEEFCLPDPK) peptide has a Phe rather than a Pro at the pY + 3 position, which is predicted to increase the affinity for the mutant PLC-γ (compared to pY11P) but lower the affinity for the wild-type protein (Fig. 2). The results in Table II (part B) are in agreement with this prediction. Similarly, substituting a Glu at the pY + 1 position (pYEIP: DNDpYEIPCLPDPK) increases the affinity for the mutant protein (compared to pY11P) and lowers the affinity for the wild-type protein, as predicted from the results in Fig. 2.

One result in Table II (part B) is not predicted by the peptide library result: The pYEEF peptide had a significantly higher affinity than the pYEIP peptide for the wild-type PLC-γ CSH2 domain. One possible explanation for this result is that the Glu at pY + 1 forces the peptide out of the normal binding groove such that favorable interactions for the Ile and Pro at the pY + 2 and pY + 3 positions are precluded. If the peptide is on the surface of the SH2 domain then the more hydrophilic Glu at pY + 2 would be selected (as is the case for Group I SH2 domains such as Src and Lck).

In summary, we have demonstrated here that the βD5 residue is crucial in determining the binding specificity of SH2 domains. We were able to switch the specificity of two group III SH2 domains (p85 NSH2 and PLC-γ CSH2 domains) to that of Group I SH2 domains by substituting their wild-type βD5 residues (Ile or Cys) with tyrosine found at the βD5 positions of Group I SH2 domains. A comparison of three-dimensional structures of Group I (Src and Lck) and Group III SH2 domains (Syp and PLC-γ) with bound ligands indicates a major difference in ligand binding structure (4, 5, 8, 9). In the structures of Src and Lck SH2 domains complexed with pYE11 peptide, only the phosphotyrosine and three residues (Glu-Glu-Ile) immediately C-terminal to it make specific contacts with the SH2 domain backbone. Among these three residues, pY + 3 Ile plugs into a hydrophobic binding pocket while pY + 1 and +2 Glu residues form salt bridges and hydrogen-bonds with amino acids on the surface of the SH2 domain. The Syp and PLC-γ SH2 domain structures, however, show a rather “open” groove configuration. The aliphatic residues at the βD5 position are buried in the protein such that a cavity is opened between the phosphotyrosine and the pY + 3 binding site. This allows the accommodation of hydrophobic amino acids at the pY + 1 position (Fig. 1B). In addition, the distance between the BG and EF loops is wider that of Src and Lck SH2 domains providing additional binding pockets for pY + 4 and pY + 5 residues. Therefore, up to five residues of the bound peptides are embedded in a hydrophobic channel.

The prediction of our model is that substituting a more bulky aromatic residue (Tyr) at the βD5 position, as found in Group I SH2 domains, will disrupt the hydrophobic cavity and force the pY + 1 and pY + 2 residues to lie on the surface, as found for Group I SH2 domains. The selection of the βD5 Tyr mutants of PLC-γ CSH2 and p85 NSH2 for Glu residues (rather than hydrophobic residues as in the wild-type proteins) at pY + 1 and pY + 2 are consistent with this model. The results in Table II argue that substituting Tyr at βD5 did not create new contacts for the phosphopeptide but rather eliminated the selection for hydrophobic amino acids at the pY + 1 and pY + 2 positions. This conclusion is supported by the observation that the optimal phosphopeptide for the PLC-γ CSH2 mutant (pYE11F) had a slightly higher affinity for wild-type PLC-γ CSH2 domain than for the mutant. As discussed above, we suspect that this is due to the ability of the pYE11F peptide to bind in a conformation in which the glutamate residues at pY + 1 and pY + 2 are on the surface of the SH2 domains. However, this peptide does not have as high affinity as the pYE11P peptide for wild-type PLC-γ CSH2.

The data presented here, together with our earlier work involving mutations in the Src SH2 domain (10) and p85 NSH2 domain (12), indicate the potential to modulate the specificity of SH2 domains by making substitutions at sites predicted to bind side chains of associated phosphopeptides. This approach deepens our understanding of SH2-binding specificity. Furthermore, it should be feasible in the nearest future to design novel SH2 domains with pharmacological applications.

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REFERENCES
1. Pawson, T., and Schlessinger, J. (1993) Curr. Biol. 3, 434–442
2. Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W. G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R. J., Neel, B. G., Birge, R. B., Fajardo, J. E., Chou, M. M., Hanafusa, H., Schaffhausen, B., and Cantley, L. C. (1993) Cell 72, 767–777
3. Songyang, Z., Shoelson, S. E., McClade, J., Olivier, P., Pawson, T., Bustelo, R. X., Barbacid, M., Sabe, H., Hanafusa, H., Yi, T., Ren, R., Baltimore, D., Ratnofsky, S., Feldman, R. A., and Cantley, L. C. (1994) Mol. Cell. Biol. 14, 2777–2783
4. Eck, M. J., Shoelson, S. E., and Harrison, S. C. (1993) Nature 362, 87–91
5. Wakeman, G., Shoelson, S. E., Pant, N., Cowburn, D., and Kurzyn, J. (1993) Cell 72, 779–790
6. Cantley, L. C., Auger, K. R., Carpenter, C. L., Duckworth, B., Graziani, A., Kapetel, R., and Sotoff, S. (1991) Cell 64, 281–302
7. Anderson, D., Koch, C. A., Gray, L., Ellis, C., Moran, M. F., and Pawson, T. (1990) Science 250, 979–982
8. Pascal, S. M., Singer, A. U., Gish, G., Yamazaki, T., Shoelson, S. E., Pawson, T., Kay, L. E., and Forman Kay, J. D. (1994) Cell 77, 461–472
9. Lee, C. H., Konin, D. J., Jacques, S., Margolis, B., Schlessinger, J., Shoelson, S. E., and Kurzyn, J. (1994) Structure 2, 423–438
10. Marengere, L. E., Songyang, Z., Gish, G. D., Shahler, M. D., Parsons, J. T., Stern, M. J., Cantley, L. C., and Pawson, T. (1994) Nature 372, 532–535
11. Pull, L., Liu, J., Gish, G. D., Mabamalu, G., Bowtell, D., Pelicci, P. G., Ailinghaus, R., and Pawson, T. (1994) EMBO J. 13, 764–773
12. Yokim, M., Hou, W. M., Songyang, Z., Xu, Y. X., Cantley, L., and Schaffhausen, B. (1994) Mol. Cell. Biol. 14, 5929–5938
13. Hensmann, M., Booker, G. W., Panayotou, G., Boyd, J., Linacre, J., Waterfield, M., and Campbell, I. D. (1994) Protein Sci. 3, 1020–1030