The Src homology 2 (SH2) domain has a highly conserved architecture that recognizes linear phosphotyrosine motifs and is present in a wide range of signaling pathways across different evolutionary taxa. A hallmark of SH2 domains is the arginine residue in the conserved FLVR motif that forms a direct salt bridge with bound phosphotyrosine. Here, we solve the X-ray crystal structures of the C-terminal SH2 domain of p120RasGAP (RASA1) in its apo and peptide-bound form. We find that the arginine residue in the FLVR motif does not directly contact pTyr of a bound phosphopeptide derived from p190RhoGAP; rather, it makes an intramolecular salt bridge to an aspartic acid. Unexpectedly, coordination of phosphotyrosine is achieved by a modified binding pocket that appears early in evolution. Using isothermal titration calorimetry, we find that substitution of the FLVR arginine R377A does not cause a significant loss of phosphopeptide binding, but rather a tandem substitution of the FLVR arginine R377A and K400A (SH2 D4) is required to disrupt the binding. These results indicate a hitherto unrecognized diversity in SH2 domain interactions with phosphotyrosine and classify the C-terminal SH2 domain of p120RasGAP as “FLVR-unique.”

Src homology 2 (SH2) domains are the best studied pTyr-binding protein scaffolding domains (1). They were originally identified within oncogenes of avian RNA viruses and are the second of four homology domains in Src family kinases (2–4) critical for both autoregulation and targeting (3, 5, 6). Following identification, SH2 domains rapidly revealed their presence in many kinases, phosphatases, small GTPase regulators, adaptor proteins, and transcription factors (1, 7, 8), and they are now known to integrate signals from the 90 tyrosine kinases to help regulate myriad signaling pathways and cellular processes (1, 7–9). In total, over 120 SH2 domains have been identified in the human genome distributed among ~110 proteins (10, 11). Almost all of the members of this broad family retain a primary functional role: to bind short linear phosphotyrosine motifs (12–14). The structural conformation that allows this binding is extremely well-conserved across the family.

The SH2 domain comprises a central antiparallel seven-stranded β-sheet (βA to βG) sandwiched between two α-helices (αA and αB) (15–18) (nomenclature defined by Eck et al. (16)). This creates two binding sites—a deep pocket and a shallow cleft—within the ~100-amino acid globular domain. Linear phosphotyrosine peptides bind perpendicular to the β-sheet in a two-pronged interaction with the pocket and cleft, a mode of binding that is reliably mirrored in cells and in the purified setting (4, 14). The deep pocket binds the pTyr residue, which is captured by electrostatic interactions with an arginine residue invariant in all pTyr-binding SH2 domains (9). In contrast, the shallow cleft binds residues C-terminal to the pTyr and varies considerably between SH2 domains allowing specificity determination (8, 13, 19, 20). This two-pronged interaction also dictates a consistent orientation of the bound phosphopeptide N to C terminus with respect to the SH2 domain (18). In v-Src, the deep pocket arginine residue is part of the Phe172-Leu173, Val174, Arg175 sequence, or “FLVR motif” (15), and arginine at this position (notated as the fifth residue on strand ββ, βBS) is conserved in 117 of 120 human SH2 domains (9). Multiple lines of evidence indicate that the FLVR arginine is required for SH2–pTyr binding. To date, all structural studies of SH2 domain interactions with pTyr have demonstrated direct interactions between the FLVR arginine and pTyr. Further, the binding affinity of unphosphorylated peptides is on the order of 1000-fold weaker than phosphopeptides (15, 18). Additionally, the FLVR arginine was found to be the primary contributor of binding free energy (21, 22). Consequently, mutations of this residue are invariably used to generate a “dead” SH2 domain (23, 24).

p120RasGAP (RASA1, RasGAP, Ras GTPase-activating protein 1) was one of the first SH2 domain proteins to be identified (25–27) and was the first GTPase-activating protein (GAP) to be discovered (25, 28–30). It is ubiquitously expressed and required for life, because animals without p120RasGAP have major vascular defects (31, 32). p120RasGAP contains two SH2 domains at its N terminus that sandwich an SH3 domain, followed by PH and C2 domains for membrane recruitment and RasGAP domain (Fig. 1A) (30, 33, 34, 84). The SH2 domains are used to directly interact with multiple phosphorylated binding partners including the p190RhoGAPs, the Eph receptor tyrosine kinases, and the Dok scaffolding proteins (32). These interactions are correlated with altered RasGAP activity and consequently with regulation of Ras signaling (35–39). The p120RasGAP SH2 domains therefore play critical roles in spatial–temporal regulation of Ras signaling by their interactions with this wide array of partners.

The SH2 domains of p120RasGAP are thought to be canonical SH2 domains that mediate direct interactions with pTyr
residues using their FLVR arginine residue, which is conserved in both domains. We recently confirmed this to be the case for the N-terminal SH2 domain (40); however, in contrast we now demonstrate that the C-terminal SH2 domain of p120RasGAP is a novel SH2 domain. Using X-ray crystallography, we find that the βBS FLVR arginine does not contact phosphotyrosine pTyr1087 of a bound p190RhoGAP peptide; instead, it makes a salt bridge to residue Asp380. Analysis of 715 aligned SH2 domain structures shows that this position of the FLVR residue is rare, and point mutagenesis demonstrates that the FLVR

Figure 1. Structure of p120RasGAP C-terminal SH2 domain. A, schematic of p120RasGAP interaction with p190RhoGAP. Domains are depicted, and the region co-crystallized is shown in a dashed box. B, structure of apo p120RasGAP C-SH2 indicating secondary structure elements and locations of the pTyr-binding pocket and specificity cleft. C, structure of p120RasGAP C-SH2 in complex with a synthesized phosphopeptide corresponding to p190RhoGAP-A residues 1086DpYAEPMDA1093. Simulated annealing omit Fo-Fc difference map contoured at 3σ RMSD is shown. D, surface electrostatics for p120RasGAP C-SH2 with p190RhoGAP-A peptide. E, interactions observed between p120RasGAP C-SH2 (green) and p190 phosphopeptide (yellow) defined by PDBsum (84). Hydrogen bonds are shown by red lines; nonbonded elements are shown by dashed lines. F, alignment of secondary structure features for p120RasGAP C-SH2, compared with p120RasGAP N-SH2 (PDB code 6PXC) (39), and Src (PDB code 1SPS) (18). β-Strands are highlighted yellow, and α-helices are highlighted orange. Residues that contact p190 phosphopeptide as defined by PDBsum are indicated with bold and underlined text. Residues discussed in the text are indicated.
argine is not required for pTyr binding. Instead, pTyr binding is mediated by an alternate array of residues, including an unusual Arg\textsuperscript{306} at the βD position. These findings demonstrate that the C-terminal SH2 domain of p120RasGAP is “FLVR-unique.”

**Results**

p120RasGAP interaction with p190RhoGAP is critical for recruitment of RhoGAP activity to sites of cell adhesion and for suppression of RasGAP activity at these locations (35, 36, 41–43). Contacts between p120RasGAP and p190RhoGAP are mediated by the two SH2 domains of p120RasGAP (termed N-SH2 and C-SH2), which bind to phosphorylated tyrosine residues Tyr\textsuperscript{1087} and Tyr\textsuperscript{1105} of p190RhoGAP (44, 45); both pTyr residues reside in the preferred pYXXp sequence recognized by both SH2 domains of p120RasGAP (13, 19). We previously reported the crystal structure of N-SH2 of p120RasGAP and investigated its interaction with a p190RhoGAP pTyr\textsuperscript{1105} phosphopeptide, which revealed a canonical SH2 domain structure and pTyr peptide-binding mode with $K_D$ of 0.3 ± 0.1 μM (40).

In the present study, we aim to determine the structure and pTyr-binding mode of C-SH2 of p120RasGAP. Toward this end, we expressed and purified the isolated C-SH2 domain recombinantly and performed crystallization studies. We obtained crystals of the apo form of p120RasGAP C-SH2 and solved the structure to 1.5 Å resolution. To examine the structural basis of pTyr binding, we also solved a 1.5 Å co-crystal structure of p120RasGAP C-SH2 with a pTyr\textsuperscript{1087} p190RhoGAP phosphopeptide.

The structure of the apo form of C-SH2 (Fig. 1B and Table 1) reveals a typical overall SH2 fold that is most similar to the p120RasGAP N-SH2 (PDB code 6PXC) (40) with root-mean-squared deviation (RMSD) of ~1.3 Å over 94 Ca atoms and 31% sequence identity; it is also highly similar to the C-terminal SH2 domain of PLCγ-1 (46) (PDB code 5TQ1; RMSD, ~1.8 Å over 101 Ca atoms, 27% identity) and Nck2 (47) (PDB code 2CIA; RMSD, 1.1 Å over 93 Ca atoms, 25%). The two-pronged pTyr pocket and specificity cleft interaction sites (16, 18, 48) (Fig. 1B) are clearly visible in p120RasGAP C-SH2 and solved the structure to 1.5 Å resolution. To examine the structural basis of pTyr binding, we also solved a 1.5 Å co-crystal structure of p120RasGAP C-SH2 with a pTyr\textsuperscript{1087} p190RhoGAP phosphopeptide.

The structure of p120RasGAP C-SH2 (Fig. 1B and Table 1) recapitulates canonical electrostatic and van der Waals interactions of SH2 domain structures (18) and is dictated by placing pTyr\textsuperscript{1087} into the deep electrostatic pocket and Pro\textsuperscript{1090} into the shallow cleft, recapitulating canonical electrostatic and van der Waals interactions (Fig. 1D). The residues of p120RasGAP C-SH2 that contact the phosphopeptide are generally similar to previously studied SH2–phosphopeptide interactions (16, 18, 48) (Fig. 1, E and F). Curiously, however, we observed that the FLVR motif arginine residue, Arg\textsuperscript{377}, is oriented differently to other SH2 domains in both the apo and peptide-bound crystal forms. We thus investigated this major structural difference in detail.

In pTyr-binding SH2 domains, the conserved FLVR arginine at β5 is required to form the base of the electrostatic pocket and to hydrogen bond to pTyr (21, 22). Unexpectedly, in the p120RasGAP C-SH2/phosphopeptide co-crystal structure, the FLVR arginine Arg\textsuperscript{377} does not contact pTyr directly. Instead, Arg\textsuperscript{377} makes a salt bridge to Asp\textsuperscript{380} (at position BC1) (Fig. 2A), an interaction that is also observed in the apo C-SH2 structure (Fig. 2B). Therefore, unlike typical SH2 domains, C-SH2 Arg\textsuperscript{377} does not orient toward the phosphotyrosine-binding site in the absence of pTyr, nor does it undergo conformational change to contact pTyr directly in the presence of phosphopeptide. This unique orientation contrasts with p120RasGAP’s N-SH2 domain, which binds phosphotyrosine in a canonical fashion (Fig. 2C (40)).

To assess whether the salt-bridge engagement of FLVR Arg\textsuperscript{377} with Asp\textsuperscript{380} in p120RasGAP C-SH2 is unique among determined SH2 domains structures, we conducted in silico analysis. We find that such an orientation is rare among the 715 aligned SH2 domains identified by the DALI server (49). At the time of this analysis, there are only two other examples of FLVR arginine residues that form interactions with another residues within the SH2 domain itself: 1) in the STA16 SH2 domain, the FLVR arginine makes salt-bridge contacts to both pTyr and Asp at position BC1 (50); and 2) in the VAV SH2 domain when bound to an unphosphorylated tyrosine, the FLVR arginine hydrogen bonds to Gln at position BC1 (51). The atypical orientation of Arg\textsuperscript{377} can be observed by superposition of aligned SH2 domains, in which the Arg\textsuperscript{377} side chain is pointed away from the pTyr-binding site, unlike the positions of a majority of the typical arginine side chains (Fig. 2D). The FLVR Arg of p120RasGAP C-SH2 is therefore unique among structures of SH2 domains.

The molecular basis of this unique FLVR arginine orientation seems to be dictated by both the salt bridge to Asp\textsuperscript{380} and by steric interference by Tyr\textsuperscript{389} (residue βC5) (Fig. 2, B and C). Tyrosine at position βC5 is not observed in any other human SH2 domain and is usually a small amino acid: over the 120 human SH2 domains, residue βC5 is observed as Ser (63 of 120 times including N-SH2 of p120RasGAP), Thr (27 times), Ala (13 times), Cys (10 times), Gly (3 times), Val (2 times), Met (1 time), and Tyr (1 time, p120RasGAP C-SH2) (1). Small amino acids at βC5 allow the FLVR arginine to engage the pTyr directly as observed in p120RasGAP N-SH2 bound to a phosphopeptide (Fig. 2C). At position BC1 (equivalent to Asp\textsuperscript{380} in p120RasGAP C-SH2), an acidic residue is frequently observed (Asp, 6 times and Glu, 38 times), but in many SH2 domains it caps helix αB (e.g. Glu\textsuperscript{179} in Fyn SH2; Fig. 2E). In contrast, in p120RasGAP C-SH2 a helix capping interaction by BC1 is not possible because the FLVR arginine guanidino group prevents close arrangement of helix αB and strand βB. The position of the FLVR arginine therefore seems to accommodate atypical orientations of other elements of the p120RasGAP C-SH2 domain.

Upon inspection of the p120RasGAP C-SH2 structure in complex with phosphopeptide, we observe several additional unique features of pTyr binding in addition to the orientation...
of the FLVR arginine and presence of βC5 tyrosine. Across SH2 domains, three major interactions have been observed between the pTyr and its binding pocket: 1) the salt bridge between the pTyr and the FLVR motif βB5 arginine (Arg207 in N-SH2), 2) binding to a basic residue at either position αA2 (Arg188 in N-SH2) or βD6, and 3) binding to residues of the BC loop (between the βB and βC strands (Ser209 in N-SH2; Figs. 1F and 3A) (21, 40, 52). Consistent with these interactions, p120RasGAP C-SH2 uses additional residues βC3 (Ser299 in N-SH2; Figs. 1F and 3A) or SAP-like (where pTyr is coordinated by a basic residue at position βD6) pockets (52). Sequence alignment of human SH2 domains suggests that no other SH2 domain harbors dual basic residues at positions βD4 and βD6 (1), potentially indicating an evolution of the site to compensate for the loss of direct FLVR arginine binding. Additionally, the amino–aromatic interaction typically contributed by the arginine at αA2 (15, 16) is instead fulfilled by the unique Arg398 at βD4 in C-SH2 (Fig. S1).

Many SH2–pTyr interactions have been studied in solution, and the measured binding affinities range in the 10^{-5} to 10^{-8} M (10 μM to 10 nM), or ~7 to ~11 kcal/mol range (53). We therefore used isothermal titration calorimetry (ITC) to measure the affinity of p120RasGAP C-SH2 with pTyr1087 phosphopeptide from p190RhoGAP and find the resultant K_d = 0.15 ± 0.04 μM, approximately ~9 kcal/mol, to be within this expected range (Table 2 and Fig. S2). We next used ITC to assess the extent of binding of pTyr1087 phosphopeptide to C-SH2 mutants. For canonical SH2–pTyr interactions, the FLVR arginine is the primary contributor of binding free energy (21, 22), and mutation

| Table 1 |
| --- |
| Data collection and refinement statistics |
| p120RasGAP C-SH2 | p120RasGAP C-SH2 bound to p190RhoGAP phosphopeptide |
| **Data collection** | **Data collection** |
| PDB accession code | 6WAX | 6WAY |
| Wavelength (Å) | 0.97920 | 0.97920 |
| Resolution range (Å) | 50–1.50 (1.55–1.50) | 30–1.50 (1.55–1.50) |
| Space group | P 2 1 2 1 | C 2 2 2 1 |
| Cell dimensions | | |
| a, b, c (Å) | 52.5, 65.6, 71.6 | 63.1, 83.9, 54.2 |
| α, β, γ (°) | 90, 90, 90 | 90, 90, 90 |
| Unique reflections | 40,194 | 23,209 |
| Multiplicity | 11.7 (7.0) | 21.2 (8.6) |
| Completeness (%) | 100 (99.9) | 100 (99.8) |
| Mean I/σI | 24.1 (2.0) | 23.6 (2.0) |
| Wilson B factor (Å²) | 22.6 | 18.7 |
| Reflections used for refinement (%) | 3.8 (40.7) | 3.1 (29.3) |
| Reflections used in refinement | Unique reflections | 340–444, 343–444 |
| Mean | 116 (106 SH2, 10 peptide) | 116 (106 SH2, 10 peptide) |
| MolProbity clashscore | 0.998 (0.903) | 0.999 (0.945) |
of this residue abrogates binding (23, 24). However, the unusual nature of the p120RasGAP C-SH2 suggests that the basis for its interaction with pTyr diverges from canonical SH2–pTyr interactions. We therefore tested whether Arg377 contributes to pTyr binding. Using ITC, we find that R377A mutation leads to slight reduction in affinity from WT $K_d = 0.15 \pm 0.04 \mu M$ to

![Figure 3. Phosphotyrosine-binding sites of p120RasGAP.](image)

Figure 2. An unusual conformation for the FLVR arginine of p120RasGAP C-SH2. A, crystal structure of phosphopeptide-bound p120RasGAP C-SH2 showing the FLVR arginine Arg377, Asp380, and Tyr389 and p190RhoGAP pTyr1087. B, crystal structure of apo p120RasGAP C-SH2. C, crystal structure of phosphopeptide-bound p120RasGAP N-SH2 (PDB code 2PXG) (39) showing the equivalent residues and phosphotyrosine. D, superposition of FLVR motif arginines from 592 Dali-aligned SH2 domains identified by the Dali server. E, crystal structure of Src family kinase Fyn (PDB code 4U1P) shows residue BC1 (Glu53) caps helix αB.

![Table 2](image)

Table 2

| p120RasGAP C-SH2 protein | $h$ | $K_d$ | $\Delta H$ | $\Delta S$ | $T\Delta S$ | $\Delta G$ |
|--------------------------|-----|-------|------------|-----------|------------|----------|
| WT                       | 0.91 ± 0.08 | 0.16 ± 0.05 | -14.3 ± 1.6 | -16.7 ± 0.6 | -8.0 ± 1.7 | -8.7 ± 0.1 |
| R377A                    | 0.90 ± 0.08 | 0.46 ± 0.11 | -17.3 ± 1.5 | -29.4 ± 5.0 | -8.7 ± 1.3 | -9.3 ± 0.2 |
| R398A                    | 0.87 ± 0.08 | 0.19 ± 0.02 | -16.9 ± 1.3 | -26.0 ± 4.0 | -7.7 ± 1.2 | -9.2 ± 0.4 |
| K400A                    | 0.92 ± 0.14 | 0.81 ± 0.15 | -12.3 ± 3.0 | -13.4 ± 8.0 | -4.0 ± 2.0 | -8.3 ± 0.1 |
| R398A/K400A              | 1.10 ± 0.09 | 6.24 ± 4.00 | -13.2 ± 4.0 | -20.1 ± 14.0 | -6.0 ± 4.0 | -7.2 ± 0.5 |
might alter the pTyr-binding site of C-SH2 to a more conventional FLVR-driven conformation. Alternatively, interdomain contacts might create an alternative unique mode of pTyr binding, similar to the tyrosine kinase ZAP70, which contains two SH2 domains separated by a short helical linker domain and binds two pTyr targets in the T-cell receptor. Specifically, the two ZAP70 SH2 domains come together to create an interface that forms one unique pTyr-binding site, whereas the other more typical pTyr-binding site is contained within a single SH2 domain (55). Future studies utilizing longer p120RasGAP constructs and dual pTyr-binding partners will help to examine these possibilities.

Classification of p120RasGAP C-SH2

Canonically, the FLVR arginine is required for interactions with pTyr peptides, contributing over 50% of their free energy of binding to the interaction (22, 56), and mutations of this residue result in an effectively “dead” SH2 domain. This residue is highly conserved, and there are only three human SH2 domains that do not contain the FLVR arginine residue (RIN has a His at this position, SH2D5 has a Trp, and Tyk2 has a Met) (9). For Tyk2, this belies the Janus kinase FERM-SH2 module mediating direct interaction with nonphosphorylated cytoplasmic tails of cytokine receptors (57, 58). The lack of diversity at the FLVR arginine position indicates conserved function, which was recently confirmed in bacteria in which a large number of SH2 domains were identified (52). Bacterial SH2 domains are classified in two groups, both of which are structurally divergent from eukaryotic SH2 domains, but even these divergent bacterial SH2 domains bind pTyr using the conserved FLVR-arginine (52). The C-terminal SH2 of p120RasGAP therefore seems to have evolved uniquely among the SH2 family of proteins.

The two-pronged pocket and cleft interaction site found in most SH2 domains (16, 18, 48) can be classified into different groups (groups IA to IE, IIa to IID, and III) that have divergent specificities, with the majority displaying a binding preference for the residue at the +2, +3, or +4 position C-terminal to the pTyr (13, 19) (although P + 1, P > 4, and regions N-terminal to the pTyr can also influence specificity (59–62)). The determinants of the specificity cleft are controlled primarily by two loops: one between the E and F β-strands (the EF loop) and one between α-helix B and the β-strand G (the BG loop). These can be thought of as analogous to the complementarity-determining regions, specificity-determining loops of antibodies (13, 19, 20). In this classification scheme, the p120RasGAP C-terminal SH2 domain is classified as a group 1B SH2 domain, with specificity for hydrophobic residue at position +3 (13,19). Our crystal structures confirm the interaction site with these loops (Fig. 1).

Specificity of C-SH2 for phosphotyrosine

Two determinants of SH2 domain–binding specificity for phosphotyrosine over phosphoserine or phosphothreonine are 1) the depth of the binding pocket and 2) presence of amino–aromatic interactions (15, 16, 63–65). For p120RasGAP C-SH2 bound to a pTyr-containing peptide, we observe the typical
deep, elongated pocket to accommodate the pTyr residue; the groove is lined by Arg398 and Lys400, which cradle the aromatic ring. This amino–aromatic interaction between Arg398 and the pTyr ring is similar to other typical SH2 domains such as Src and Lck (15, 16); however, Arg at the βD4 position rather than αA2 is unique to C-SH2. Taken together, these observations suggest that p120RasGAP has binding specificity for pTyr residues.

NMR solution structure of C-SH2

The NMR solution structure of p120RasGAP C-terminal SH2 domain, residues 340–446, was determined by the RIKEN Structural Genomics/Proteomics Initiative in 2006 and deposited in the Protein Data Bank (code 2GSB). The solution structure contains the 20 lowest energy models. Overall, the superposition of our crystal structures is close (RMSD 1.12 Å over 101 Cα atoms) (Fig. S4A), but in-solution conformational flexibility is observed in the EF loop, which defines +3 specificity (19), and in the BC loop, which harbors Asp380. None of the NMR structures exhibit the bidentate salt bridge between Arg377 and Asp380 (residue BC1), and both residues display extensive conformational variability, but their overall orientation is similar to our crystal structures (Fig. S4B). In contrast, Tyr389 is in a broadly similar position to our crystal structures and blocks orientation of the FLVR Arg toward the canonical pTyr-binding position. The unpublished NMR structure therefore supports our crystallographic data that p120RasGAP C-SH2 is a FLVR-unique SH2 domain.

Functional role of the FLVR-unique C-SH2

What then might the function of this highly unusual SH2 domain in p120RasGAP be? SH2 domains seem to have helped facilitate the evolutionary increase in complexity of signal transduction pathways and the transition from unicellular to multicellular organisms (as illustrated by a single SH2 domain residing in baker’s yeast, Saccharomyces cerevisiae) (10). In humans there are ~120 proteins that contain SH2 domains, but only 10 of these have dual SH2s. For p120RasGAP, the bivalent SH2 domains probably indicate that p120RasGAP has fast rebinding and long dwell times at pTyr-binding partners (66), and cooperation between the p120RasGAP domains is thought to impact signal transduction (7) with conformational changes proposed to occur on dual engagement of the SH2 domains with phosphorysine partners (44). However, the affinities of SH2 domain interactions with their functional phospho-partners is generally in the moderate 0.1–10 μM range (56, 67), allowing ready dissociation and thus preventing masking of the pTyr site and prevention of signal transduction (68, 69). For p120RasGAP, the combination of two tight SH2–pTyr interactions may result in extremely long dwell times that are deleterious for signal transduction. It may be that the altered pTyr-binding site in the C-terminal SH2 domain beneficially impacts p120RasGAP’s signaling by tuning recruitment and retention at pTyr partners and thus prevents masking (68, 69) of these sites.

Broader implications

Disruption of SH2–pTyr interactions has theoretical clinical potential to regulate diverse signal transduction pathways, and consequently much work was conducted to achieve SH2 domain–specific inhibitors. Clinically useful selectivity has not, however, been achieved in large part because of bioavailability and the molecular similarities between SH2 domains (21). Selective picomolar binders can be designed where SH2 domains diverge as demonstrated for Grb2, which has an unusual specificity pocket that recognizes Asn at residue pY + 2 (70–72). Although it remains an outside possibility, nonetheless, it is interesting to speculate that the unique nature of p120RasGAP’s C-terminal SH2 domain may represent a novel SH2-inhibitor target with the potential to beneficially alter both Ras and Rho signaling.

SH2 domains have played an important role in influencing our understanding of how cytosolic signaling occurs. Invariably these domains bind phosphotyrosine partners with their FLVR motif arginine residue acting as the linchpin of the interaction. It is striking, therefore, that one of the first identified SH2 domain proteins should diverge from this paradigm. The functional reasons for this divergence are currently unclear, but the array of bidentate binding partners for p120RasGAP and alterations in p120RasGAP’s RasGAP activity on binding to phosphorylated partner proteins may hint at an evolutionary requirement to allow unmasking of the phosphorysine sites. The discovery of the unique nature of the C-terminal SH2 domain and how to alter its phosphotyrosine binding may therefore provide guidance to resolving how p120RasGAP functions mechanistically.

Materials and methods

Expression and purification p120RasGAP C-SH2 domain

cDNA encoding the C-terminal SH2 domain of human p120RasGAP (UniProt ID P20936; residues 340–444) was amplified by PCR and ligated into a modified PET vector, which includes an N-terminal His6 tag and TEV protease recognition site. Two native cysteine residues, Cys372 and Cys402, were mutated to serine by QuikChange mutagenesis (Agilent) to prevent formation of intermolecular disulfide bonds. The following point mutants were also generated by QuikChange mutagenesis: K377A, K398A, K400A, and K398A/K400A. Protein expression of WT and mutant C-SH2 was performed in Rosetta (DE3) cells, which were cultured in 1 liter of Luria broth at 37 °C to an A600 of 0.6–0.8 and then cooled to 18 °C, and protein expression was induced with 0.2 mM isopropyl β-D-thiogalactopyranoside. The cells were harvested by centrifugation, resuspended in lysis buffer (50 mM HEPES, pH 7.3, and 500 mM NaCl), and lysed by freeze-thaw cycles in the presence of lysozyme followed by sonication. Total cellular lysate was clarified by centrifugation at 5000 × g at 4 °C and applied to nickel–nitritolatriacetic acid–agarose resin (Qiagen) for 1 h at 4 °C to capture His6-tagged protein. Beads with bound protein were washed with 20 column volumes of wash buffer containing 50 mM HEPES, pH 7.3, 500 mM NaCl, 20 mM imidazole. The His6 tag was proteolytically removed from the C-SH2 proteins “on bead” overnight at 4 °C by the addition of TEV protease, which
itself is also His6-tagged and captured by the nickel–nitriacetate acid resin. The flow-through containing untagged C-SH2 protein was collected and applied to size-exclusion chromatography (Superdex 75; GE Healthcare) in buffer containing 20 mM Tris, pH 7.4, 150 mM NaCl. Finally, C-SH2 proteins were concentrated in a centrifugal filter with a molecular mass cutoff of 3,000 Da (Amicon Ultra, Millipore Sigma). 1 liter of Rosetta (DE3) cell culture resulted in a final purified protein yield of ~20 mg for WT and up to 10 mg for mutant.

**Peptide synthesis**

A synthetic 7-amino acid peptide of sequence (DpYAE) phosphorylated at Tyr1087 and a 15-amino acid peptide with sequence (GFP) phosphorylated at Tyr1087 (UniProt Q9NRY4) were phosphorylated at Tyr1087 and a 15-amino acid peptide

Peptide synthesis

A synthetic 7-amino acid peptide of sequence (DpYAE) phosphorylated at Tyr1087 and a 15-amino acid peptide with sequence (GFP) phosphorylated at Tyr1087 (UniProt Q9NRY4) were phosphorylated at Tyr1087 and a 15-amino acid peptide with sequence (GFP) phosphorylated at Tyr1087 (UniProt Q9NRY4) with N-terminal acetylation and C-terminal amine. Its corresponding to residues 1086–1095 of p190RhoGAP (UniProt Q9NRY4) with N-terminal acetylation and C-terminal amine were commercially synthesized (GenScript) and resuspended in sterile-filtered water.

**Crystallization, data collection, structure determination, and refinement**

p120RasGAP C-SH2 WT protein was used in crystallization trials at ~14 mg/ml. Initial crystal screening was conducted with Index HT kit (Hampton Research) by a TTP Labtech Mosquito in sitting-drop vapor-diffusion trays at room temperature. Single crystals were obtained in Index HT position A3 containing 2.0 M ammonium sulfate, 0.1 M Bis-Tris, pH 6.5, with a 1:1 (v:v) protein:reservoir solution ratio. Optimization of this crystallization condition was achieved in hanging-drop vapor-diffusion plates, with a reservoir buffer containing 2.3 M ammonium sulfate, 0.1 M Bis-Tris, pH 6.5, and a reservoir:protein ratio of 1:1 (v:v) reservoir:protein. Single crystals with approximate dimensions 200 × 200 × 200 mm were harvested from the drop, cryopreserved in reservoir solution supplemented with 20% ethylene glycol, and flash-cooled in liquid nitrogen. X-ray data collection on a single crystal was performed at Northeastern Collaborative Access Team Beaml ine 24-ID-C at Argonne National Laboratory Advanced Photon Source. X-ray data were processed in HKL2000 in space group C221 with unit cell dimensions a = 63.1 Å, b = 83.9 Å, c = 54.2 Å, α = β = γ = 90° to 1.50 Å resolution and one copy per asymmetric unit. For structure solution, an all-alanine model derived from the NMR structure of p120RasGAP C-SH2 (PDB code 2GSB), truncated to residues 345–442, was used in Phenix Phaser (74), which found a single solution for one copy of C-SH2 with a TFZ score of 10.6. Autoclassification was performed in Phenix (75), which removed model bias and successfully built and placed 100 residues (residues 342–442). Autoclassification also resulted in the building of five residues of the phosphopeptide, corresponding to p120RhoGAP residues 1087–1091. Manual model building was performed in Coot (76) and refinement in Phenix (77), yielding final Rwork = 16.5% and Rfree = 19.0%.

**Isothermal titration calorimetry**

Both C-SH2 WT and mutant proteins and pTyr1087 phosphopeptide (15 amino acids) were prepared for ITC by overnight dialysis in a common buffer with a 20 mM Tris, pH 7.4, 150 mM NaCl composition. Slide-A-Lyzer dialysis cassettes were used with a molecular mass cutoff of 3,500 Da, 150 mM NaCl composition. Slide-A-Lyzer dialysis cassettes were used with a molecular mass cutoff of 3,500 Da for protein dialysis, and Micro Float-A-Lyzer dialysis devices with a 100–500-Da cutoff were used for peptide dialysis. The samples were retrieved from their cartridges and spun down for 10 min at 4 °C. The concentrations were measured by NanoDrop (Thermo Fisher). To determine peptide concentration, a phosphotyrosine extinction coefficient of 458.6 M−1 cm−1 at pH 7.4 was used (78). For ITC, a Nano-ITC (TA Instruments) was used. 350 µl of protein were injected into the sample cell. Both protein and peptide were degassed for 3 min prior to loading. The sample cell contents were stirred at 350 rpm at 25 °C to achieve continuous mixing. The results were analyzed using Nano-ITC Analyze software.

**Evolutionary analysis**

p120RasGAP sequences were identified using NCBI BLAST and searches of the FlyBase (79), WormBase (80), and MycoCosm databases (81). Sequences were aligned using the MAFFT (82) server and visualized using JalView (83).
Data availability

The coordinates and structure factors have been deposited in the Protein Data Bank under accession codes 6WAX and 6WAY. X-ray diffraction images are available online at SBGrid Data Bank (83): doi: 10.15785/SBGRID/774 (6WAX) and doi: 10.15785/SBGRID/775 (6WAY).

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Abbreviations—The abbreviations used are: SH2, Src homology 2; ITC, isothermal titration calorimetry; PDB, Protein Data Bank; GAP, GTPase-activating protein; RMSD, root-mean-square deviation.

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