Catalytic Activity of the SH2 Domain of Human pp60$^{c\text{-}src}$; Evidence from NMR, Mass Spectrometry, Site-Directed Mutagenesis and Kinetic Studies for an Inherent Phosphatase Activity

Renee J. Boerner,[†,‡] Thomas G. Consler,[†,‡] Robert T. Gampe, Jr.[§] Debra Weigl,[†,‡] Derril H. Willard,[†,‡] Donald G. Davis,[§] Ann M. Edison,[‡] Frank Loganzo Jr.,[‡] Daniel B. Kassel,[§] Robert X. Xu,[§] Indravadan R. Patel,[‡] Jeffery S. Robbins,[‡] Timothy Lansing,[∥] Tona M. Gilmer,[∥] Michael A. Luther,[‡] and Wilson B. Knight[‡,*]

Departments of Biochemistry, Molecular Cell Biology, and Bioanalytical and Structural Chemistry, Glaxo Research Institute, Research Triangle Park, North Carolina 27709

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ABSTRACT: During solution structural studies it was apparent that the human recombinant pp60$^{c\text{-}src}$ SH2 domain (*src*SH2, residues 144−249) possessed an inherent phosphatase (Pase) activity. Complexes of U[^{13}C,^{15}N]*src*SH2 with unlabeled Ac-pYEEIE (**I**) were examined using ^{31}P and ^{1}H−detected isotope filtered NMR methods. The presence of a high-affinity complex in equimolar solutions of **I** and U[^{13}C, ^{15}N]-*src*SH2 was demonstrated by chemical shift perturbations, line broadening, and the observation of intermolecular nuclear Overhauser effects from the pY and Ile side-chain protons of **I** to protons on amino acid residues present in the binding pocket of *src*SH2. Solutions containing excess **I** relative to *src*SH2 revealed a slow hydrolysis of **I** to produce Ac-YEEIE and inorganic phosphate. The hydrolysis rate determined from NMR and HPLC-electrospray ionization mass spectrometry data at 30 °C for solutions containing excess **I** was 0.002−0.003 h^{-1}. *src*SH2 also catalyzed the hydrolysis of *p*-nitrophenyl phosphate (pNPP). Isoelectric focusing gels of a number of mutant *src*SH2s demonstrated that this activity comigrated with *src*SH2. K_m, k_{cat}, and k_{cat}/K_m were 3.7 ± 0.4 mM, 3.1 ± 0.2 × 10^{-2} min^{-1}, and 8.4 ± 0.4 M^{-1} min^{-1}, respectively, toward pNPP. The C188A mutant of the *src*SH2 domain displayed 15% of the activity displayed by wild-type *src*SH2, demonstrating that this residue is not absolutely required for activity. Two additional mutations in the known pY binding site, R178K and R158K, also resulted in decreased pNPPase activity, suggesting that the activity resides in or near this site. The inhibitor profile and pH dependence suggest that this is a novel protein Pase activity. Other than phosphate (competitive inhibitor, K_i = 50 μM), the activity was not inhibited by known inhibitors of Ser/Thr or Tyr protein kinases. Inhibitors of Ser/Thr Pases were also not inhibitory, but the pNPPase activity was inhibited by the protein tyrosine phosphatase inhibitor orthovanadate. pY-containing peptides inhibited the pNPPase activity, but the potency did not parallel the apparent binding affinity to the SH2 site. The data are consistent with a catalytically active form of SH2 that is present in small amounts. These data suggest that *src*SH2 may play a catalytic role in signal transduction and regulation of pp60$^{c\text{-}src}$ activity.

The phosphorylation state of protein tyrosine residues has been firmly established to regulate cell differentiation and proliferation. The roles that these modifications play in various signal transduction pathways are under intense study. Human pp60$^{c\text{-}src}$[1] is a tyrosine kinase derived from a proto-oncogene that has been implicated in the pathogenesis of a number of cancers including breast (Hennipman et al., 1989; Ottenhoff-Kalff et al., 1992) and colon carcinomas (Cartwright et al., 1989, 1990). Therefore, the activated pp60$^{c\text{-}src}$ signal transduction pathway(s) is a target for therapeutic intervention in these devastating diseases.

pp60$^{c\text{-}src}$ contains five sequence domains of various functions (for a review of the structure of the *src*TK family, see Bolen et al., 1993; Rudd et al., 1993). The SH3 and SH2,[2] and a catalytic tyrosine kinase domain are involved in cellular signal transduction (for a review of oncogenes and signal transduction, see Cantley et al., 1991; *src* family and haemopoietic signal transduction, see Bolen et al., 1992). These domains consist of approximately 50, 100, and 250 amino acids, respectively, and are highly homologous to similar domains of other members of the *src*TK family. The C-terminal region of the kinase domain contains a regulatory

[†] These individuals contributed equal efforts on this work. A preliminary report of portions of this work was presented by Knight et al. (1994) *J. Cell. Biochem. 18* (Suppl. B), 282.

* Address correspondence to this author.

[‡] Department of Biochemistry.

[§] Department of Bioanalytical and Structural Chemistry.

[∥] Department of Molecular Cell Biology.

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[1] pp60$^{c\text{-}src}$ refers to the human protein, whereas pp60$^{v\text{-}src}$ refers to the gene product of the Rous sarcoma virus. N-85-*src*TK and N-myr-(−)*src*TK refer to the 85 residue N-terminal deletion and the G2A mutants, respectively.

[2] In this work *src*SH2 corresponds to the *c-src*-homology domain 2, consisting of N-terminal methionine followed by residues 144−249 from pp60$^{c\text{-}src}$. Other uses of SH2 refer to generic *src*-homology domains. The residues are numbered according to the wt human sequence. *src*SH3SH2 corresponds to the pp60$^{c\text{-}src}$ *src*-homology domains 3 and 2 consisting of N-terminal methionine followed by residues 86−249. GSTSH2 and GSTSH35H2 correspond to GST fused to residues 144−249 and 87−249, respectively, of human pp60$^{c\text{-}src}$. GRB2-SH3SH2 consisted of the N-terminal domain, residues 27−181 and GAP-SH2SH3 refer to residues 178−344. Both of the latter were prepared as GST fusion proteins.

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site of phosphorylation, Y530 (Cooper & Howell, 1993; Pawson & Gish, 1992). The N-terminal peptide sequence is hydrophobic and contains sites of serine phosphorylation and N-terminal myristylation.

SH2 domains display high affinity for peptide sequences in proteins containing pY,[3] that are involved in signal transduction (for a review see Koch et al., 1991). *src*SH2 displays high affinity for pYEEIE, a sequence derived in part from hamster polyoma middle T antigen (Songyang et al., 1993; Waksman et al., 1993; Gilmer et al., 1994). Recognition of these sequences is thought to generate signal-transducing protein complexes of cellular proteins (Cantley et al., 1991; Dilworth et al., 1993). In addition, *src*SH2 is thought to bind pY530 in the pp60$^{c\text{-}src}$ carboxyl terminus, leading to repression of kinase activity (for a review of pp60$^{c\text{-}src}$ regulation, see Cooper & Howell, 1993). This residue is underphosphorylated or absent in transforming mutants of pp60$^{c\text{-}src}$.

PTPases[4] are likely involved in the regulation of signal transduction pathways. In a simplistic model, a tyrosine kinase−catalyzed phosphorylation event leads to activation (or repression) of a signal-transducing pathway, and the signal is turned off (or on) by the catalytic action of a PTPase. This proposal is analogous to the role(s) that phosphatases and kinases play in metabolic regulation (Cohen, 1985). Both *in vitro* and *in vivo* data support this hypothesis. The addition of PTPases to cell lysates (Courtneidge, 1985) or overexpression of a PTPase in fibroblast-derived cell lines leads to pp60$^{c\text{-}src}$ activation (Zheng et al., 1992). Overexpression of a PTPase also resulted in cellular transformation and tumorigenesis. Brown and Gordon (1984) reported that addition of vanadate, an inhibitor of some PTPases (Swarup et al., 1982), to Rous sarcoma virus−transformed chicken embryo fibroblasts led to decreased phosphorylation of pp60$^{v\text{-}src}$. Unfortunately, the identity, regulatory nature, and events involving PTPases remain undefined. We present evidence in this work that *src*SH2 has an inherent Pase activity. This observation suggests that SH2 domains may play a catalytic role in regulating pp60$^{c\text{-}src}$ activity and signal transduction pathways.

Table 1: pNPPase Activity and pI of Various SH2 Constructs

construct	expression system	expression vector	activity pNPP[b]	pI[a] theoretical	pI[a] obs.	pI[a] pNPPase
*src*SH2	*E. coli*	pET11B	Yes	9.0	8.9	8.9 ± 0.3
*src*SH2-C188S	*E. coli*	pET11B	Yes			
*src*SH2-C188A	*E. coli*	pET11B	Yes	9.1	8.6	8.6 ± 0.5
*src*SH2SH3	*E. coli*	pET11B	Yes	8.3	8.0	8.0 ± 0.5
GST	*E. coli*	pGEX	No			
GSTSH2	*E. coli*	pGEX	Yes	6.3	6.7	6.5 ± 0.3
GSTSH3SH2	*E. coli*	pGEX	Yes			
GSTGRB2 (SH3SH2)	*E. coli*	pGEX	No			
GAP-SH2	*E. coli*	pGEX	No			
N-85-*src*TK	Sf9	pJVP10Z	Yes	6.0	4.5−5.5[c]	nd
N-myr(−)TK	Sf9	pJVP10Z	Yes	7.0	5.4−6.1[c]	nd

[a] The theoretical pI is determined from the sequence. The observed pI is based upon migration of the protein as observed by Coomassie staining and Western blot analysis with anti-SH2 monoclonal antibody. The pI of the pNPPase activity is based upon the peak position, and the error is the width of the gel slice. [b] Assay at 37 °C contained 125 μM pNPP, 150 mM NaCl, 5 mM DTT, 5 mM EDTA, and 20 mM TRIS at pH 8.0. [c] Three to four distinct isoforms were observed. pNPPase activity was not detected (nd) in these IEF gels.

EXPERIMENTAL PROCEDURES

Materials. pNPP (Sigma Chem. Co.) was dissolved in the assay buffer. ATP, peptides, and other reagents (Sigma Chemical Co. or Boeringher Mannheim) were dissolved in 50 mM HEPES at pH 7.5 unless noted. The phosphopeptides were synthesized according to Gilmer et al. (1994). Okadaic acid, orthovanadate, and staurosporine (dissolved in DMSO prior to use) were purchased from LC Laboratories. Monomeric orthovandate was prepared according to the method of Kypta et al. (1988). Buffers (Sigma Chemical Co.) were titrated to the appropriate pH with NaOH or HCl. PTP1B and SHPTP1 bound to agarose were purchased from UBI (Lake Placid, NY). Perfusion (Poros II R/H, 0.32 × 100 mm) HPLC columns were purchased from LC Packings (San Francisco, CA). Reagents used for cloning and expressing of the SH2s and *src*TK deletion mutants were molecular biological grade. All other reagents were reagent grade and used without further purification unless noted.

Preparation of SH2-Containing Proteins. A summary of the constructs and the expression vectors used in this work is outlined in Table 1. N-85-*src*TK and N-myr(-)*src*TK were prepared according to Ellis et al. (1994) or Barker et al. (1995). The U-[^{13}C,^{15}N]*src*SH2 was prepared according to Xu et al. (1995). A standard nutrient broth was used to produce *E. coli* expressing the proteins used in this study. *src*SH2, *src*SH2-C188S, *src*SH2-C188A, and *src*SH3SH2 were purified in basically the same manner as the labeled *src*SH2. The supernatants of *E. coli* lysates were loaded onto cation exchange columns and eluted with a 0−500 mM NaCl gradient. The fractions containing *src*SH2 were pooled, concentrated (Amicon Centriprep 10), and passed over a Superdex-75 (Pharmacia) column equilibrated with buffer A (350 mM NaCl, 5 mM DTT, 5 mM EDTA, and 20 mM HEPES at pH 8.0). The *src*SH2-containing fractions were pooled and concentrated. GST, GSTSH2, and GSTSH3SH2 containing cell lysate supernatants were loaded onto a FFQ

[3] Peptides are named according to the one-letter designation for amino acid residues. Phosphorylated residues are abbreviated pX; for example, phosphotyrosine is pY. Additional substitutions; Ac, *N*-acetyl; Btn, biotin.

[4] Abbreviations used: *abl*, *abl* proto-oncogene; APase, alkaline phosphatase; 3BP1, SH3 binding protein-1; CAPS, 3(cyclohexylamino)-1-propane-sulfonic acid; DMSO, dimethyl sulfoxide; 1D and 2D, 1 and 2 dimensional; DTT, dithiothreitol; DTT-d8, deuterium-labeled dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ESI-MS, electrospray ionization mass spectrometry; EGFR, epidermal growth factor receptor kinase; FAK, focal adhesion kinase; FFQ, Fast Flow Q column; GAP, GTPase-activating protein; GRB2, growth factor receptor bound-2 protein; GST, glutathione-*S*-transferase; HEPES, *N*-[2-hydroxyethyl]-piperazine-*N*′-[2-ethanesulfonic acid]; ^{1}H[^{13}C,^{15}N]IF NOESY, proton-detected ^{13}C,^{15}N isotope-filtered nuclear Overhauser effect spectroscopy; ^{1}H-IF, proton-detected isotope filtered; HPLC, high pressure liquid chromatography; IEF, isoelectric focusing; LDH, lactate dehydrogenase; MES, 2[*N*-morpholino]ethanesulfonic acid; MWCO, molecular weight cutoff; NOESY, nuclear Overhauser effect spectroscopy; Pase, phosphatase; P_i, inorganic phosphate; PEP, phosphoenolpyruvate; PK, pyruvate kinase; PMA, phorbol 12-myristate-13-acetate; pNPP, *p*-nitrophenyl phosphate; pNPPase, *p*-nitrophenyl phosphatase; pNP, *p*-nitrophenol or *p*-nitrophenolate; PTPase, protein tyrosine phosphatase; PTP1B, PTPase 1-B; Sf9, *Spodoptera frugiperda*; SH2, *src*-homology domain 2; SH3, *src*-homology domain 3; *src*, sarcoma; SHPTP1, protein tyrosine phosphatase-1 that contains an SH2 domain; TBS, TRIS buffered saline; TCA, trichloroacetic acid; TAPS, *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid; TRIS, Tris(hydroxymethyl)aminomethane; U[^{13}C,^{15}N], uniformly carbon and nitrogen labeled; wt, wild type.

column and eluted with buffer A without DTT. The eluate was loaded onto a glutathione−agarose column and eluted with buffer A containing 100 mM reduced glutathione and no DTT. The GSTSH2 (or GST, GSTSH3SH2) fractions were pooled, diluted 1:5 with buffer B (150 mM NaCl, 5 mM DTT, 5 mM EDTA, and 20 mM HEPES at pH 8.0), and additional DTT was added to a final concentration of 100 mM. The solution was then exhaustively dialyzed versus buffer B. GRB2-SH3SH2 was purified similarly except, after FFQ chromatography, the eluate was loaded onto a Repligen Fast Flow Protein A matrix containing immobilized anti-GST monoclonal antibody, 5R7. This column was eluted with 100 mM glycine at pH 2.0, and the GRB2-SH3SH2 containing fractions were neutralized by a 1:10 dilution into 1 M HEPES at pH 7.5, pooled, diluted to 5 mg/mL, and dialyzed against buffer B. The identity of the protein constructs was confirmed by peptide sequence analysis and HPLC-ESI-MS. The concentrations of *src*SH2 were determined assuming a molecular mass of 12 287 Da. R#K mutant GST*src*SH2s, GST*src*SH2, and GST (control) on agarose were produced and isolated from cell lysates, and the protein content was determined according to Luttrell et al. (1994). Mutations in the *src*SH2 domain were introduced using an oligonucleotide-directed mutagenesis kit (Amersham). The identity of the mutants was confirmed by sequencing the DNA using a Taquence kit (US Biochem).

Methods. UV-visible spectrophotometry was conducted on Molecular Devices (Menlo Park, CA) THERMOmax or UVmax microplate readers. HPLC-ESI-MS was performed on a Sciex API-III mass spectrometer. Perfusion capillary HPLC was conducted using an HP1090 pump system equipped with an Applied Biosystems model 785A UV-visible detector. The columns were eluted with a linear gradient containing 0.05% TFA from 100% H_2O to 45% acetonitrile over 10 min at 50 μL/min flow rate. Isoelectric focusing gel electrophoresis was conducted on a Multiphor II System (Pharmacia, Upsala Sweden). Ion exchange chromatography of proteins was conducted on a Pharmacia Biopilot system.

Preparation of NMR Samples. Three milliliters of U[^{13}C, ^{15}N]*src*SH2 (7.5 mg/mL) in buffer A were mixed with 2 equiv of **I** and dialyzed (2 × 2 L) using 1 kDa MWCO dialysis tubing (Spectrum) into H_2O buffered with buffer C (50 mM [99.5% ^{2}H] acetic acid-d_4 (Aldrich), 100 mM NaCl at pH 5.5). Previous experience and titrations with **I** ensured that this procedure reliably provided a stoichiometric complex. The solution was concentrated (Centricon 10 kDa MWCO filter, Amicon), pooled, and aliquoted. Two aliquots, A and B, were adjusted to a final volume of 450 μL with buffer C (final [*src*SH2] $\approx$ 1.3 mM) and 10% (v/v) [99.9% ^{2}H] D_2O. After control NMR spectra were collected, a second equivalent of **I** from a concentrated stock solution in buffer C was added to sample A. Sample B remained as a stoichiometric control. The third aliquot was used for structural studies (Xu et al., 1995). Reference samples containing 2 mM **I** and Ac-YEEIE in H_2O buffer C and 10% (v/v) D_2O were also prepared as controls. All samples contained 5 mM [99% ^{2}H] DTT-d8 (Merck) and were blanketed with ultrapure Argon gas after transferring to 5 mm NMR tubes (Wilmad Glass). The NMR samples were also characterized by HPLC-ESI-MS.

NMR Data. The ^{31}P and 1D ^{1}H[^{13}C,^{15}N]IF-NOESY NMR spectra of samples A and B incubated at 30 °C were collected as paired time points at irregular intervals over a 4-day period. The ^{31}P NMR data were collected on a Bruker AMX600 using a 5 mm inverse probe at 30 °C. The spectral parameters were 4096 data points, 8193 Hz, 2048 transients, a 45° observation pulse, and an interacquisition delay of 1 s. Inorganic phosphate was set to 0.0 ppm as a reference standard at pH 5.5. A 1D version of a ^{1}H[^{13}C,^{15}N]IF NOESY pulse sequence was used to observe the unlabeled protons of **I** and AcYEEIE (Ikura & Bax, 1992). These data were collected on a Bruker AMX500 using a 5-mm triple resonance probe at 30 °C. The spectral parameters were 4096 data points, 7813 Hz, 128 transients, an interacquisition delay of 1 s, a 20-ms mixing period, and a 1-ms spin-lock for solvent suppression (Messerle et al., 1989). The water signal in the ^{1}H data were referenced to 4.74 ppm. The data were transferred to a Silicon Graphics Indigo II Extreme workstation, processed, and plotted with Felix 2.1 software. Exponential line broadening of 20 Hz was applied to the ^{31}P data. The ^{1}H data was apodized with a Lorentz-Gaussian filter (−3 Hz, 0.03). Time domain convolution to suppress the solvent (Marion et al., 1989) and baseline corrections (Dietrich et al., 1991; Guntert & Wuthrich, 1992) were applied as needed.

Enzymatic Activities. p-NPP was used as a substrate for Pase activity. The reactions were run in a total volume of 200 μL of buffer D (150 mM NaCl, 5 mM EDTA, and 20 mM HEPES at pH 7.0) and initiated by the addition of *src*SH2. Reactions were incubated at 37 °C, and the absorbance at 405 nm was determined at 15-min intervals over 3 h. The extinction coefficient for pNP under these conditions was 9800 M^{-1} cm^{-1}. Rates were determined from the slopes of the absorbance change as a function of time. The concentration of *src*SH2 ranged from 5 to 20 μM. The pNPPase activity of 5 μM N-85-*src*TK was determined in both the presence and absence of a 30-min preincubation with 1 mM MgATP. The former results in autoactivation (Barker et al., 1995). The pH dependence of the kinetic parameters of the *src*SH2 pNPPase activity were examined between pH 5.9 and 9.4 using 50 mM MES (pH 5.9, 6.3, and 6.9), HEPES (pH 7.1, 7.3, and 7.4), TAPS (pH 8.4 and 8.8) and CAPS (pH 9.3, 9.4) containing 5 mM EDTA and 150 mM NaCl. The extinction coefficient for pNP was determined at each pH and used to calculate the rates. The ability of a number of chemical entities including inhibitors of known phosphatases to inhibit the *src*SH2 phosphatase activity was assessed using 20 μM protein and 2 mM pNPP in buffer D. Inhibition of the pNPPase activity by orthovanadate was determined using desalted *src*SH2 and C188A-*src*SH2. The two proteins were desalted by applying 0.5 mL of 50−60 mg/mL solutions to a 2.15 × 60 cm TSK-6200SW gel filtration column equilibrated and eluted with 300 mM NaCl and 50 mM HEPES at pH 7.5. The activity in the presence and absence of 0.02 and 1 mM orthovanadate was determined using 4 mM pNPP in 350 mM NaCl and 20 mM HEPES at pH 8.0. The concentration of C188A-*src*SH2 and *src*SH2 were 26 and 3.7 μM, respectively.

The ATPase activity of *src*SH2 was determined by coupling the production of ADP to the oxidation of NADH and monitored spectrophotometrically. The reaction buffer contained 20 mM $MgCl_2$, 1 mM PEP, 240 μM NADH, 100

μM DTT, 20 μg/mL BSA, 44 μg/mL pK, 64 μg/mL LDH, and 100 mM HEPES at pH 7.5. The concentration of the *src*SH2 stock solution (in buffer B) used in the ATPase determinations was 106 μM. *src*SH2 was tested at 5.3, 10.6, and 15.9 μM versus 2 and 4 mM ATP. The values at the three concentrations of protein were averaged and standard deviations are reported.

GST-PTP1B (1 μg) and GST-SHPTP1 (5 μg), GST (120 μg) and GST-SH2 (15 or 30 μg wild type, 100 μg R178K, 30 μg R158K) bound to agarose beads were assayed versus 11.5 mM pNPP at 37 °C in buffer D. The GST-PTP1B and GST-SHPTP1 assays (100 μL) were incubated for 10 min and quenched by the addition of 900 μL of 0.4 M NaOH. The 260 μL GST and GST-SH2 assays were incubated for 90 min and quenched with 100 μL of 1 M NaOH. The beads were pelleted via centrifugation, and 200-μL aliquots of the supernatant were withdrawn and the absorbance at 405 nm was determined. An extinction coefficient of 18 000 M^{-1} cm^{-1} (Zhang et al., 1990) was used to calculate the rates.

*src*SH2-catalyzed hydrolysis of **I** and Btn-TSTEPQpYEEIENL was examined by HPLC-ESI-MS. Peptide (1.6 mM) was incubated at 30 °C in buffer B with 0 (control), 29.5, and 59 μM *src*SH2. Over time 1-μL aliquots were quenched with 49 μL of 0.1% TFA, and 10 μL aliquots of this solution were analyzed. The turnover numbers were calculated from the integrated areas of the peaks observed by UV-visible detection at 215 nm.

Isoelectric focusing gels. These gels were conducted with a pH 3−10 gradient using Servalyt Prenet ultrathin polyacrylamide gels, anode and cathode solutions supplied by Serva (Heidleburg, Germany). The IEF gels were focused according to the manufacturer's instructions, and the pI range was determined using pI markers (Pharmacia and Sigma Chem. Co.). After prefocusing for 25 min at 5 mA, 10−20 μg of protein typically in 50 mM NaCl, 1 mM DTT, and 20 mM HEPES at pH 8.0 were applied, and the voltage was maintained at 200 V for 90 Vh.[5] Sample applicators were removed, and the gels were focused for 5500 Vh at 1700 V.

After focusing, a section of the gels displaying both pI markers and samples was fixed in 10% TCA and stained with Blue W (Serva). The remaining lanes, which contained SH2 protein samples were cut into narrow strips across the pH gradient, then into uniformly sized 3−10 mm slices along the gradient. The slices were placed in individual microfuge tubes containing 1 mL of 150 mM NaCl, 250 μM pNPP, and 50 mM HEPES at pH 7.0. Tubes were incubated in the dark at 30 °C until yellow color was evident and the absorbance of 100 μL of each sample was determined. In separate experiments, focused gels were transferred to Immobilion P (Millipore) by semi-dry electroblotting. PVDF membranes were blocked with 3% liquid gelatin (Integrated Separation Systems, Natick, MA) in TBS. Blots were incubated with the *src*SH2 monoclonal antibodies 21S/3 and 21S/4[6] (0.1−0.5 μg/mL in blocking buffer) overnight. After a TBS wash, the blots were incubated with APase-conjugated secondary antibody (Goat anti-Mouse IgG, Promega, Madison, WI) for 1 h, then washed and developed with Western Blue stabilized substrate (Promega).

Data Analysis. Rates were obtained by a linear least squares fit of the absorbance versus time data to eq. (1). The kinetic parameters for substrate hydrolysis were obtained by nonlinear regression analysis of the rates as a function of substrate concentration (eq 2). The inhibition constants were obtained by a fit of the data to eq. (3) for competitive inhibition after it was ascertained that the data did not fit the models predicted by mixed noncompetitive and uncompetitive inhibition. Nonlinear regression was conducted using GraFit (Leutherbarrow, Erithacus Software, London, U.K.).[7]

$$Y = v_i X + B \tag{1}$$

$$Y = V_m{}^*[S]/(K_m + [S]) \tag{2}$$

$$Y = V_m[S]/(K_m(1 + [I]/K_i) + [S]) \tag{3}$$

RESULTS

The ^{31}P and 1D ^1H-IF NMR spectra from a 1.3 mM solution of U[^{13}C,^{15}N]*src*SH2 in the presence of 1 eq of **I** display the resonances of bound ligand in slow exchange (Figure 1A). Xu et al. (1995) identified a number of intermolecular contacts between SH2 binding site residues and the ligand and derived a high-resolution structure using an equimolar complex. This work clearly identified the location of the bound ligand in the equimolar solution state complex.

The Pase activity of *src*SH2 is illustrated with paired ^{31}P and ^1H-IF NMR spectra in Figure 1, B and C. Titration of a second equivalent of **I** introduced new ^{31}P and ^1H resonances from unbound **I** and produced exchange broadening effects on bound **I**, particularly on the amide proton resonances of **I** near 8.4 ppm. Over time, the ^{31}P data displayed signal intensity due to P_i as new peptide signals appeared in the ^1H spectra, while the unbound signal intensity decreased. The new peptide product was identified as AcYEEIE by comparison to ^1H spectra of authentic material and by capillary HPLC-ESI-MS (*vide infra*). The rate of peptide dephosphorylation was estimated from integrations of the signal at −3.2 ppm in the ^{31}P spectra and the Y aromatic peaks at 6.9 and 7.1 ppm in the ^1H spectra: 1.3 mM *src*SH2 hydrolyzed 2.6 mM **I** at a rate of 3.7 ± 1.5 μM h^{-1}. This corresponds to an apparent k_{cat} of 2.8×10^{-3} h^{-1}. The solution containing equimolar *src*SH2 and **I** displayed a rate of peptide dephosphorylation of 0.4 ± 0.1 μM h^{-1} ($k_{cat} = 3 \times 10^{-4}$ h^{-1}). The rate of dephosphorylation of 2 mM **I** in the control sample was 0.35 ± 0.05 μM h^{-1}.

The capillary HPLC-ESI-MS spectrum of the NMR samples displayed species due to **I**, AcYEEIE, and *src*SH2 with masses of 804.3, 724.3, and 12 979 ± 1.6 Da (12 979 Da predicted for 100% labeled *src*SH2), respectively. Unlabeled *src*SH2 yielded a mass of 12 287 ± 2 Da (predicted 12 286.9). The turnover numbers for the *src*SH2 (59 μM)-catalyzed hydrolysis of **I** and BtnTSTEPQpYEEENL determined by HPLC-ESI-MS were $2 \pm 0.8 \times 10^{-3}$ and $2.1 \pm 0.4 \times 10^{-3}$ h^{-1}, respectively, at pH 7.5.

[5] The amount of protein was larger than typically used for IEF, but we felt that these amounts were necessary to reveal any trace contaminants.

[6] The monoclonal antibodies were developed at Glaxo Research Institute by Jui-Lan Su and Christine Edwards and were derived from immunizations with the *src*SH2 domain.

[7] Direct comparison of the results obtained from this graphical data analysis program with the programs of Cleland (1979) yielded essentially identical results.

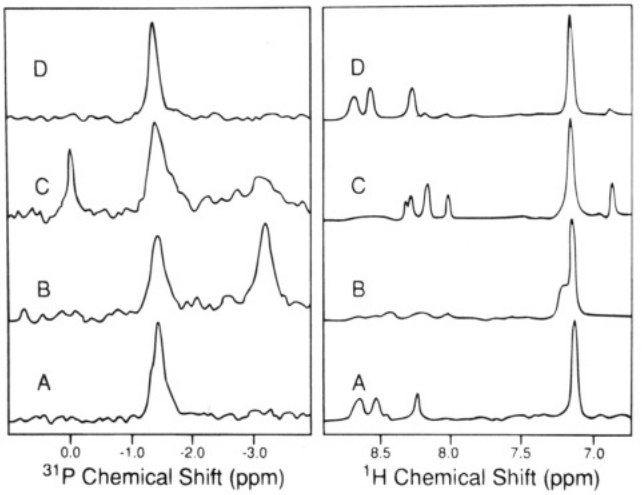

FIGURE 1: The time course of NMR spectra for a sample with Ac-pYEEIE and 1.3 mM U[^{13}C,^{15}N]*src*SH2 in H_2O buffer C illustrates the Pase activity described in this study. *Left plate*: (A) shows the ^{31}P signal at −1.3 ppm from the single equivalent of bound **I**pY. (B) Titration of a second equivalent of **I** to A sample introduced a second signal at −3.2 ppm due to unbound ligand. (C) The ^{31}P spectrum after incubation at 30 °C for 163 h. The signal intensity from the excess ligand was nearly depleted. In addition, the new signal at 0 ppm is due to P_i. The rate of hydrolysis based on integrations of the signal at −3.2 ppm was 3.7 ± 1.5 μM h^{-1}. (D) ^{31}P spectrum after 163 h of sample B containing 1 equiv of **I** and *src*SH2. Dephosphorylation was not evident in the sample by ^{31}P NMR. *Right plate*: The paired 1D ^{1}H[^{13}C, ^{15}N] IF-NOESY spectra collected with the same samples described above soon after each ^{31}P spectrum was acquired. (A) The signals near 8.5 and 7.1 ppm arise from the from the single equivalent of amide and aromatic protons from the bound ligand. (B) Titration of a second equivalent of **I** to sample A introduced line broadening and unbound ligand signals. (C) The spectrum after incubation at 30 °C for 165 h. The new signals correspond to the amide and aromatic protons from the dephosphorylated peptide. (D) ^{1}H-IF spectrum of sample B containing 1 equiv of **I** and *src*SH2 after 169 h. The dephosphorylation was significantly slower (0.4 ± 0.1 μM h^{-1}) and was similar to the spontaneous dephosphorylation rate 0.35 ± 0.05 μM h^{-1} observed in the control sample containing the 2 mM **I** in buffer C.

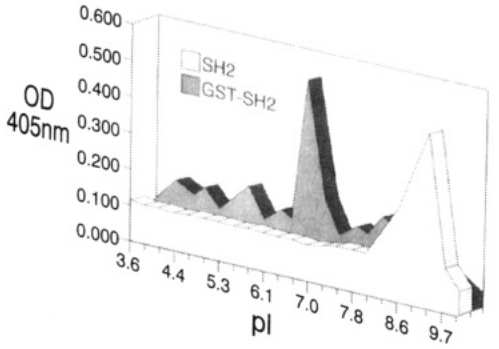

FIGURE 2: pNPPase activity of IEF gel slices from lanes containing *src*SH2 and GST-SH2 (shaded).

In qualitative assays, the proteins containing the *src*SH2 domain displayed pNPPase activity (see Table 1). A representative plot of the pNPPase activity of zones cut from IEF gels is shown in Figure 2. IEF of a number of *src*SH2 protein constructs followed by assays of the various zones with pNPP indicated that this activity comigrated with the *src*SH2 proteins (see Table 1).

The hydrolysis of pNPP and ATP in the presence of *src*SH2 was linear with both time and protein concentration (data not shown). The kinetic parameters ($n = 4$) K_m, k_{cat}, and k_{cat}/K_m, determined from the dependence of the rate on pNPP concentration, were 3.7 ± 0.4 mM, 3.1 ± 0.2 × 10^{-2} min^{-1} and 8.4 ± 0.4 M^{-1} min^{-1}, respectively. The turnover numbers for the hydrolysis of 2 and 4 mM ATP were 4.8 ± 0.4 × 10^{-2} and 6.6 ± 0.7 × 10^{-2} min^{-1}, respectively. The pH dependence of the kinetic parameters for the *src*SH2-catalyzed hydrolysis of pNPP is shown in Figure 3. Both $\log(V/K_m)$ and $\log(V_m)$ pH profiles indicated that these parameters were essentially independent of pH from 5.9 to 9.4. Below pH 7.1 there was a slight increase in both K_m and V_m, while from pH 7.1 to 9.4 these parameters were constant. For example the K_m and V_m increased by 50% and 32%, respectively, at pH 5.9 relative to pH 7.1. C188A *src*SH2 displayed 15% of the wt-*src*SH2 pNPPase activity. GST fusion proteins of *src*SH2, R178K-*src*SH2, and R158K-*src*SH2[8] yielded turnover numbers of 16.5 ± 0.4 × 10^{-2}, 1.6 ± 0.2 × 10^{-2}, and 1.1 ± 0.98 × 10^{-2} min^{-1} versus 11.5 mM pNPP, respectively. GST bound to agarose did not display pNPPase activity. For comparison GST fusions of PTP-1B and SHPTP1 bound to agarose beads yielded turnover numbers of 1.09 ± 0.03 × 10^{3} and 26 ± 2 min^{-1}, respectively. N-85-*src*TK also displayed activity toward pNPP (2 mM), yielding an apparent k_{cat} of approximately 2.1 × 10^{-2} min^{-1}. For comparison, under identical conditions *src*SH2 displayed an apparent k_{cat} of 1.3 × 10^{-2} min^{-1}. When N-85-*src*TK was preincubated with MgATP, followed by incubation in the presence of 0.29 and 0.26 mM Mg and ATP respectively, pNPP (2 mM) was hydrolyzed with a rate of 2.4 × 10^{-2} min^{-1}. Given the ATP inhibition reported below, this value is likely an underestimation of the pNPPase activity of activated N-85-*src*TK.

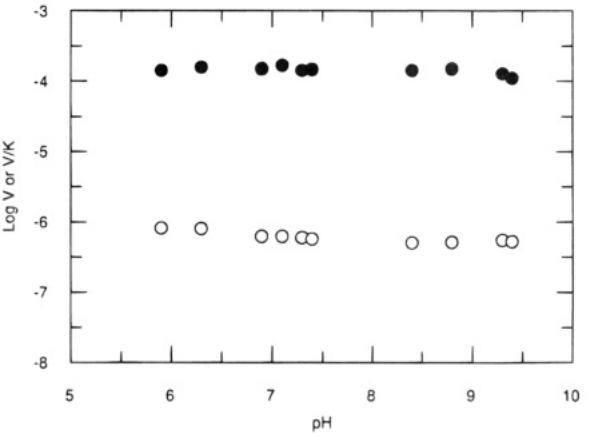

FIGURE 3: The dependence of V (○) and V/K (●) on pH for the *src*SH2-catalyzed hydrolysis of pNPP. The conditions are given in Methods.

A number of compounds were tested as inhibitors of the *src*SH2 pNPPase activity (see Table 2). At concentrations of 0.2 mM, over 50% inhibition was detected with phosphate, ATP, and several phosphorylated peptides.[9] TSTEPQ-pYQPGENL and Ac-pTEEIE were less inhibitory than **I**. Other phosphorylated compounds, such as phosphotyrosine and glucose-6-phosphate, inhibit the pNPPase activity to

[8] In the nomenclature proposed by Eck et al. (1993) for the pp56^{lck} SH2 R158 corresponds to R-αA2 and R178 corresponds to R-βB5. C188 would be referred to as C-βC3, although in the equivalent residue in the pp56^{lck} SH2 sequence is actually S-βC3.

[9] As reported in this work, ATP is actually an alternate substrate. In the assay for pNPPase activity, an alternate substrate would behave as an inhibitor.

Table 2: Comparison of the Inhibitors of *src*SH2 pNPPase Activity[a] to Their Relative Ability to Bind to the *src*SH2 Domain

inhibitor	% inhibition 0.5 mM	% inhibition 0.2 mM	relative binding affinity[b]
ATP	>80	76	nd
phosphate	>80	74	nd
VSETDDpYAEIIDE	78	52	0.9
Ac-pYEpYIE	72[c]	50[c]	0.5
TSTEPQpYEEIENL	67	42	2.0
Ac-pYESIE	62	37	4.9
Ac-pYEEIE	52[d]	31[d]	1
Ac-pYEYIE	47	29	2.8
Ac-pTEEIE	38	18	ϕ
TSTEPQY*QPGENL	34	21	58
phosphotyrosine (2 mM)	46		
glucose 6-phosphate (6.6 mM)	53		
$MgCl_2$ (6 mM)	53		
NaF (50 mM)	52		
$CaCl_2$ (5 mm)	65		
no inhibition seen with:			
okadaic acid (100 μM)	tyrosine (200 μM)		
zinc (100 μM)	staurosporine (200 μM)		

[a] Assayed at pH 7.0 with 20 μM *src*SH2 and 2 mM pNPP. Except where noted the assays were run in duplicate, and the standard deviation between the two values was ≤ 5% of the reported percentage inhibition. [b] These values are from Gilmer et al. (1994) and are relative to the IC_{50} (≤1 μM) obtained for **I** in an EGFR ELISA binding assay. nd, not determined, ϕ, IC_{50} > 1 mM. [c] n = 1. [d] n = 3. The standard deviation was ≤6% of the reported percentage inhibition.

Table 3: Inhibition of *src*SH2 pNPPase Activity by Orthovanadate

Protein	[orthovanadate] (mM)	% inhibition
*src*SH2	1	93
	0.02	48
C-188A-*src*SH2	1	75
	0.02	65

approximately 50% when used at concentrations greater than 2 mM. AcpYEEIE and P_i were competitive inhibitors of *src*SH2 versus pNPP with K_i's of 410 ± 180 and 54 ± 3 μM, respectively. The S/T protein Pase inhibitor okadaic acid (Bialojan & Takai, 1988) was not inhibitory. The PTPase inhibitor orthovanadate (Swarup et al., 1982) displayed potent inhibition of wt *src*SH2 and C-188A-*src*SH2 in the absence of EDTA[10] (see Table 3). Concentrations of Mg^{+2} and Ca^{+2} greater than the EDTA concentration were inhibitory.

DISCUSSION

During NMR solution structural studies of the *src*SH2-**I** complex, it became evident that Ac-pYEEIE was hydrolyzed to produce Ac-YEEIE and P_i. The data from the ^{31}P and ^{1}H NMR spectra demonstrate the formation of a high-affinity complex in solutions of **I** and *src*SH2. The solution structure of the complex reported by Xu et al. (1995) clearly defines the orientation and conformation of the bound ligand and protein. These structures are consistent with the recently reported crystallographic structures of SH2 complexes with **I** and related phosphopeptides (Waksman et al., 1992, 1994; Gilmer et al., 1994). These data demonstrated that most if not all of one equivalent of phosphopeptide was bound in the expected binding motif. The presence of excess phosphopeptide over *src*SH2 led to dephosphorylation of **I**. The existence of a second ligand binding site or lightly populated protein conformer responsible for the Pase activity could not be definitively confirmed or denied from the NMR data collected to date.[11] The turnover number estimated from the NMR data was confirmed by HPLC-ESI-MS at concentrations of SH2 and **I** more appropriate for enzymatic studies.

A number of different mutant proteins containing the *src*SH2 domain displayed pNPPase activity. GRB2-SH2SH3 and GAP-SH2SH3 did not show this activity. The activity comigrated with the *src*SH2-containing proteins during IEF. Since these constructs possess significantly different isoelectric points, these data demonstrate that this activity is associated with the *src*SH2 domain and not a contaminating protein with Pase activity. The pNPPase catalytic activity, while relatively low, was appreciable when compared to known PTPases. Sugimoto et al. (1993) reported that the SH-PTP2 enzyme displayed K_m, k_{cat}, and k_{cat}/K_m values of 3.6 mM, 2.76 min^{-1}, and 780 M^{-1} min^{-1}, respectively, toward pNPP. Both k_{cat} and k_{cat}/K_m of *src*SH2 toward pNPP are approximately 90-fold lower than these values. *src*SH2 bound to agarose beads was 150-fold less active toward pNPP than SHPTP-1 on agarose beads. The pNPPase activity of N-85-*src*TK was approximately 2-fold greater than that of the *src*SH2 domain itself. In the case of activated N-85-*src*TK the difference may actually be greater than 2-fold, since the pNPPase activity is likely inhibited by the ATP present to activate the enzyme. The concentrations of DTT used in these studies inhibits the Pase activity of *src*SH2, which also suggests that we are underestimating the level of this activity (Edison, Loganzo, and Knight, unpublished results).

The inhibition and pH profiles of the *src*SH2 pNPPase activity suggests that it is a novel activity. The lack of a pH dependence of the reaction suggests that the activity cannot be classified as an alkaline or acidic Pase. The activity is not inhibited by inhibitors of S/T protein phosphatases nor by the protein kinase inhibitor staurosporine. P_i, ATP (see footnote 9), and high concentrations of divalent cations inhibited the activity. Orthovanadate was a relatively potent inhibitor of the activity. The sensitivity to orthovanadate was similar to that reported for PTPases (Swarup et al., 1982). The apparent potency of orthovanadate versus *src*SH2 was intermediate between the activity reported against a bovine brain isozyme (Jones et al., 1988) and that reported versus the rat leucocyte antigen–related PTPase (Pot et al., 1991). Phosphopeptides inhibit the Pase activity. This appears to be specific for phosphopeptides as relatively high concentrations of other phosphate esters including phosphotyrosine are required to observe inhibition. The relative activity for phosphopeptide inhibition does not parallel the structure–activity relationships for high-affinity binding to

[10] The authors are indebted to the diligence of one reviewer who pointed out that EDTA–vanadate complexes would deplete the concentration of vanadate in the experiments (see Crans, 1994). In fact, in the presence of 5 mM EDTA there was no inhibition of the pNPPase activity observed by 1 mM orthovanadate.

[11] A very tightly bound contaminating Pase cannot be absolutely ruled out, but it would have to be a small percentage of the total protein as it is not observed in the HPLC-ESI-MS spectra of the *src*SH2 domains. In addition, there was no evidence of a major contaminant during N-terminal sequencing of the *src*SH2 protein. Finally this tightly bound Pase would also have to be present in both the Sf9 and *E. coli* expression systems and comigrate with *src*SH2-containing proteins under a variety of purification as well as isoelectric focusing conditions.

*src*SH2 (Gilmer et al., 1994). The K_i for competitive inhibition of the pNPPase activity by I is considerably greater than that predicted from the IC_{50} obtained in the ELISA assay based upon phosphorylated EGFR (≤ 1 μM; Gilmer et al., 1994; Luttrell et al., 1994).

Evidence from site-directed mutagenesis (Guan et al., 1990) and iodoacetamide labeling studies (Pot & Dixon, 1992) suggests that PTPases contain a cysteine residue responsible for activity (for a review of PTPases, see Fischer et al., 1991). *src*SH2 contains a cysteine residue (C188) located proximal to the known pY binding site. Furthermore, the inhibition of *src*SH2 pNPPase activity by orthovanadate suggested a possible role for a cysteine residue in catalysis. The C188A mutant displayed 15% of the pNPPase activity of the wild type. This mutation does not disrupt the pY binding site since the mutant binds phosphopeptides with similar affinity (Ellis, personal communication). These data suggest that C188 is not an absolutely required catalytic residue.

The remaining question is, where does the Pase activity reside. The lack of correlation of K_i with the IC_{50} values reported by Gilmer et al. (1994) using the ELISA binding assays would suggest these two constants reflect binding in different sites, but cursory examination of the *src*SH2 structure does not yield an obvious second pY-peptide binding site. In addition, the mutagenesis studies argue against a second independent site. The C188A, R178K, and R158K (see footnote 8) mutants displayed decreased pNPPase activity. The latter two mutations also resulted in decreased binding to phosphorylated EGFR and FAK in cell lysates (Lee and Gilmer, personal communication). R178 forms a bidentate complex with two of the phosphoryl oxygens, and the R158 ζ-nitrogen is engaged in an amine–aromatic interaction with the tyrosine ring in the complex with phosphopeptides (Gilmer et al., 1994). The observation of decreased Pase activity upon mutation of residues known to be in the pY binding site of *src*SH2 is consistent with location of the activity proximal to these residues.

An explanation consistent with the data presented in this work is that only a small fraction of the *src*SH2 protein is in the correct form to hydrolyze phosphoesters. The active conformer could be due to the presence of different protein or ligand conformations in which the same residues involved in high-affinity binding are still proximal to the phosphate hydrolytic site or a covalent modification such as phosphorylation. In fact, Soula et al. (1993) have recently demonstrated that Ser and Tyr residues in the SH2 domain of $p56^{lck}$ are partially phosphorylated when T lymphocytes are triggered with anti-CD3 or PMA. One of the serine sites was proximal to the FLVR sequence (actually FLIRES in $p56^{lck}$), which has been shown to interact with the pY residue of phosphopeptides via the Arg residue in complexes of *v-src*SH2 (Waksman et al., 1992) and *src*SH2. To date, efforts to observe a phosphorylated form of *src*SH2 have been unsuccessful. The presence of different conformers of $pp60^{c\text{-}src}$ has been suggested by Ellis et al. (1994). These workers reported separation of two $pp60^{c\text{-}src}$ activities using anion exchange columns. Differences in mass or phosphorylation state did not define the isoforms; therefore, the two pools of activity appear to represent conformational isoforms. Work is underway to characterize the Pase activity of these forms of $pp60^{c\text{-}src}$.

It is possible that the Pase activity of *src*SH2 requires interaction with an activating protein, analogous to the GAP/*ras* interactions. Members of the GAP family of proteins stimulate the GTPase activity of *ras* and other GTPases such as *rho* and *rab* (for reviews of *ras*, see Macara, 1991; and GAP, see McCormick, 1990). Trahey and McCormick reported that GAP stimulated *ras* GTPase activity greater than 100 fold. These authors also reported that oncogenic mutants of *ras* display turnover numbers of 0.0007 min^{-1} for GTP, which leaves *ras* essentially in the "on" state. The rate constants for wild-type p21 *ras*-c (less the 23 N-terminal residues) reported by Schlichting et al. (1990) was .017 min^{-1}, while the p21-G12V mutant was 10-fold slower. Therefore, the slow rates of *src*SH2 Pase activity reported in this work could conceivably function during signal transduction. It is intriguing to note that 3BP1, an *abl*-SH3 binding protein has sequence homology to GAP-*rho* (Cicchetti et al., 1992). The effect that 3BP1 binding has on *abl* activities is unknown.

There may be protein effectors that modulate the Pase activity of *src*SH2 by binding to one of the *src*TK domains, thus turning a signal off that is mediated by the binding of SH2 to a protein through a pY-containing sequence. Data presented in this work suggest that this activity may be somewhat enhanced in the context of *src*TK. Furthermore, there may be optimal peptide sequences for Pase activity. Possible roles for this activity are (a) that it acts as a switch to turn a signal off, or (b) that it functions as a proofreading mechanism, removing undesired sites of phosphorylation.

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