A Peptide Model System for Processive Phosphorylation by Src Family Kinases†

Margaret Porter Scott and W. Todd Miller*

Department of Physiology and Biophysics, School of Medicine, State University of New York at Stony Brook, Stony Brook, New York 11794-8661

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ABSTRACT: The Src homology 2 (SH2) and Src homology 3 (SH3) domains of Src family kinases are involved in substrate recognition in vivo. Many cellular substrates of Src kinases contain a large number of potential phosphorylation sites, and the SH2 and SH3 domains of Src are known to be required for phosphorylation of these substrates. In principle, Src could phosphorylate these substrates by either a processive mechanism, in which the enzyme remains bound to the peptide substrate during multiple phosphorylation events, or a nonprocessive (distributive) mechanism, where each phosphorylation requires a separate binding interaction between enzyme and substrate. Here we use a synthetic peptide system to demonstrate that Hck, a Src family kinase, can phosphorylate substrates containing an SH2 domain ligand by a processive mechanism. Hck catalyzes the phosphorylation of these sites in a defined order. Furthermore, we show that addition of an SH3 domain to a peptide can enhance its phosphorylation both by activating Hck and by increasing the affinity of the substrate. On the basis of our observations on the role of the SH2 and SH3 domains in substrate recognition, we present a model for substrate targeting in vivo.

Src family kinases are nonreceptor tyrosine kinases involved in the regulation of many cellular processes, such as the responses to growth factors and to cell−cell contact (for review see ref *1*). All Src family kinases are structurally similar and share important regulatory mechanisms. Src family kinases possess four modular domains: unique, catalytic, SH2,[1] and SH3 (for review see ref *2*). The SH2 and SH3 domains of Src family kinases have dual roles in regulating the activity of the kinase in vivo. First, they inhibit the catalytic domain by binding to their intramolecular ligands (*2*−*7*). The SH3 domain binds a polyproline type II helix in the linker region between the SH2 domain and the catalytic domain (*3*−*6*). The SH2 domain binds a sequence in the C-terminal tail that requires phosphorylation on Tyr^{527} by c-Src kinase (*2*−*5*). The second role of the SH2 and SH3 domains is to target the catalytic domain to its substrates (*2*, *8*). Thus, substrates can localize to Src via SH2 and/or SH3 domain interactions and concomitantly activate the catalytic domain by displacing the inhibitory intramolecular interactions (*9*−*11*). Evidence that substrates can activate c-Src by SH2 and SH3 domain displacement has been obtained for the Src substrates Sin (*12*) and Fak (*13*).

The importance of the SH2 and SH3 domains in substrate targeting has been established for Src substrates including Sin (*12*), Fak (*13*), AFAP-110 (*14*, *15*), and Cas (*16*). Src also phosphorylates all of these substrates on multiple sites (*12*, *14*, *16*, *17*). In general, multiple phosphorylations of a substrate can occur by either a processive or a nonprocessive (distributive) mechanism (*18*, *19*). If Src phosphorylates its substrates according to a distributive mechanism, each phosphorylation of the substrate would be the result of a separate collision between enzyme and substrate. In a processive mechanism, Src would remain bound to its substrate until it completes all the phosphorylations, and therefore only one collision between Src and its substrate would be required. Because Src can interact with its substrates via SH2/SH3 domain interactions, it has been proposed that Src phosphorylates its substrates by a processive mechanism (*20*). For example, the SH2 domain of the Src family kinase Lck is required for the phosphorylation of multiple immunoreceptor tyrosine-based activation motifs (ITAMs) of the T-cell receptor (*21*). Furthermore, mutation of critical tyrosines within the ITAM motifs dramatically decreases overall phosphorylation, consistent with the mechanism of processive phosphorylation (*21*). Despite these observations, previous studies are consistent with either a processive or a distributive mechanism.

Previous kinetic studies in our laboratory have shown that addition of an SH2 domain ligand to a Src family kinase substrate can increase phosphorylation of the substrate up to 10-fold (*22*). However, Src substrates containing multiple phosphorylation sites have never been studied in detail to determine whether Src carries out multiple phosphorylations by a processive or a distributive mechanism. Here, we use a model peptide system to show that Src family kinases phosphorylate substrates by a processive mechanism. We used kinetic strategies that have been applied previously to

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* To whom correspondence should be addressed: Department of Physiology and Biophysics, Basic Science Tower, T-6, School of Medicine, SUNY at Stony Brook, Stony Brook, NY 11794−8661. Tel 631−444−3533; fax 631−444−3432; e-mail miller@physiology.pnb.sunysb.edu.

[1] Abbreviations: SH2, Src homology 2 domain; SH3, Src homology 3 domain; Hck, hematopoietic cell kinase; Sin, Src interacting or signal integrating protein; AFAP-110, actin filament-associated protein; Fak, focal adhesion kinase; Cas, Crk-associated substrate; ITAM, immunoreceptor tyrosine-based activation motifs; MAP kinase, mitogen-activated protein kinase; pY, phosphotyrosine; MALDI-TOF, matrix-assisted laser desorption−ionization time-of-flight.

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Table 1: Synthetic Peptides Used in These Experiments[a]

Peptide	Sequence
SH2 ligand containing peptides	
Y1-Y2-pYEEI	KKLDIYDVPGGDIYDVPGGEPQ**pYEEI**G
pY1-Y2- pYEEI	KKLDIpYDVPGGDIYDVPGGEPQ**pYEEI**G
Y1-pY2- pYEEI	KKLDIYDVPGGDIpYDVPGGEPQ**pYEEI**G
pY1-pY2- pYEEI	KKLDIpYDVPGGDIpYDVPGGEPQ**pYEEI**G
SH3 ligand containing peptides and controls	
Pro-G6	KKAEEEIYGEF$(G)_6$RPLPSPPKFG
Control-G6	KKAEEEIYGEF $(G)_6$RSLGSPGKFG
Pro-G10	KKKKAEEEIYGEF $(G)_{10}$RPLPSPPKFG
Control-G10	KKKKAEEEIYGEF $(G)_{10}$RSLGSPGKFG

[a] The SH2 ligand-containing peptides contain three tyrosines. The C-terminal tyrosine is phosphorylated in all three peptides and is within the high-affinity SH2 ligand, pYEEI (*28*) (boldface type). The other two tyrosines are within a typical substrate motif for the Src substrate Cas (*27*) (underlined) and are denoted as Y if unphosphorylated or pY if phosphorylated. The SH3 ligand-containing peptides have the prefix Pro if they contain the polyproline SH3 ligand motif of Cas (*27*) (shaded). Control peptides are similar to the Pro peptide except that three prolines critical for binding SH3 domains are mutated. Both the Pro and control peptides contain a Src substrate motif (*29*) (double underline). The spacer region between the substrate motif and the SH3 ligand motif contains six glycines, denoted G6, or 10 glycines, denoted G10.

study the mechanism of phosphorylation of MAP kinase (*18*, *19*).

The SH3 domain of Src is also implicated in substrate targeting. Even in the case of substrates that contain SH2 domain ligands, such as Sin, Fak, AFAP-110, and Cas, binding by the SH3 domain of Src is important for substrate targeting and phosphorylation (*12*−*15*; P. Pellicena and W. T. Miller, unpublished observations). Here we show that addition of an SH3 domain ligand to a substrate dramatically increases its phosphorylation by Src family kinases by both activating down-regulated kinase and decreasing substrate K_m. Our data on the ability of the SH3 domain to increase phosphorylation of substrates, together with the processive model for substrate phosphorylation, suggests a model for substrate phosphorylation in vivo.

MATERIALS AND METHODS

Peptides. The sequences of the synthetic peptides used in these experiments are presented in Table 1. Peptides Y1-Y2-pYEEI, pY1-Y2-pYEEI, and Y1-pY2-pYEEI were prepared by solid-phase synthesis with standard Fmoc chemistry (*23*) on an Applied Biosystems Inc. automated 431A peptide synthesizer. Phosphotyrosine was incorporated into the peptides by use of *N*α-Fmoc-*O*-phospho-L-tyrosine (Novabiochem) (*24*). Peptides were purified by semipreparative high-performance liquid chromatography (HPLC) on a Vydac C18 column with a mobile phase composed of acetonitrile in 0.1% trifluoroacetic acid. Matrix-assisted laser desorption−ionization time-of-flight (MALDI-TOF) mass spectrometry was used to confirm the identity of the final products.

Peptide pY1-pY2-pYEEI was produced by enzymatic phosphorylation of purified Y1-pY2-pYEEI with Hck. Y1-pY2-pYEEI (1.5 mM) was incubated with Hck (2 μM) in buffer containing 100 mM Tris (pH 7.5), 10 mM $MgCl_2$, and 0.5 mM ATP for 2 h at 30 °C. pY1-pY2-pYEEI was separated from unreacted Y1-pY2-pYEEI by semipreparative HPLC on a Vydac C18 column, and MALDI-TOF mass spectrometry was used to confirm the identity of pY1-pY2-pYEEI.

Phosphorylation of Y1-Y2-pYEEI. Y1-Y2-pYEEI (80 μM) was phosphorylated with Hck (100 nM) at 30 °C in reactions containing 100 mM Tris (pH 7.5), 10 mM $MgCl_2$, and 0.5 mM ATP. Aliquots (20 μL) were removed from the reaction at various times and quenched by addition of 23 μL of cold acetonitrile. Samples were analyzed by HPLC on a 4.6 × 250 mm Vydac analytical C18 column. The following mobile phases were used for gradient elution: (A) 20 mM ammonium acetate and 5 mM tetrabutylammonium phosphate (TBAP), pH 5.9; and (B) 20 mM ammonium acetate, pH 5.9/acetonitrile 25:75 (v/v) containing 5 mM TBAP. Peptides were eluted with a linear gradient of 30−95% B in 60 min, at a flow rate of 0.5 mL/min. The elution profile was monitored by absorbance at 220 nm. The retention times of Y1-Y2-pYEEI, Y1-pY2-pYEEI, pY1-Y2-pYEEI, and pY1-pY2-pYEEI were established by injections of the purified peptides under these HPLC conditions. To determine the rate of phosphorylation of Y1-Y2-pYEEI, the reactions were performed as above except the reactions contained either 20, 100, or 200 μM Y1-Y2-pYEEI. Aliquots (25 μL) were removed and quenched by addition of cold HPLC solvent A (300 μL). To determine the picomoles of product produced, peak heights were compared to a standard injection.

Pulse−Chase Experiments. In the pulse reaction (200 μL), 320 μM Y1-Y2-pYEEI was incubated with 100 nM Hck at 30 °C in buffer containing 100 mM Tris (pH 7.5), 10 mM $MgCl_2$, and 0.25 mM [γ-^{32}P]ATP. After 15 min, 10 μL aliquots were removed from the reaction and diluted into four separate tubes containing 2, 1, 0.5, or 0.2 mL of buffer containing 100 mM Tris (pH 7.5), 10 mM $MgCl_2$, and 0.25 mM unlabeled ATP, to give final enzyme concentrations of 0.5, 1.0, 2.0, and 5.0 nM, respectively. This dilution reduced the specific activity of ATP to undetectable levels in our assay. These chase reactions were allowed to proceed for an additional 15 min at 30 °C. The reactions were stopped by addition of 3 mL of 0.1% TFA. The entire chase reactions were then analyzed by reverse-phase HPLC. The following mobile phases were used for gradient elution: (A) 0.1% heptafluorobutyric acid (HFBA) and (B) 0.1% HFBA/acetonitrile 25:75 (v/v). Peptides were eluted with a linear gradient of 5−50% B over 60 min, at a flow rate of 0.5 mL/min. The elution profile was monitored by absorbance at 220 nm. The retention times of Y1-Y2-pYEEI, Y1-pY2-pYEEI, pY1-Y2-pYEEI, and pY1-pY2-pYEEI were established by injections of the purified peptides under these HPLC conditions. Peaks of absorbance were collected, and radioactivity incorporation into Y1-pY2-pYEEI and pY1-pY2-pYEEI was monitored by Çerenkov counting. The amount of radioactivity incorporated after the pulse period was established by a chase reaction that was quenched immediately after addition of the 10 μL aliquot containing enzyme and substrate.

Activation of Down-Regulated Hck by SH3 Ligand-Containing Peptides. Hck was produced in *Spodoptera frugiperda* (Sf9) cells and purified in down-regulated form

as previously described (*11*). Kinase assays were performed by a spectrophotometric assay that couples the production of ADP to the oxidation of NADH (*25*). Enzyme reactions (200 μL) contained 600 μM peptide, 10 nM Hck, and 500 μM ATP in buffer containing 100 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM phosphoenolpyruvate, 0.28 mM NADH, 89 units/mL pyruvate kinase, and 124 units/mL lactate dehydrogenase. Reactions were incubated at 30 °C. The reduction in absorbance of NADH was measured at 340 nm in a VersaMax plate reader (Molecular Devices).

Determination of Kinetic Parameters with v-Src. v-Src was produced in *S. frugiperda* (Sf9) cells and purified as previously described (*26*). Saturating concentrations of 0.5 mM ATP were used while peptide concentrations were varied to at least 3-fold above their K_m values. The final v-Src concentration was 0.3−0.5 nM. All experiments were carried out at 30 °C. Kinetic parameters were determined by fitting data to the Michaelis−Menten equation by use of nonlinear regression analysis of initial rates.

RESULTS

(1) Processive Phosphorylation of a Peptide Substrate. The ability of the SH2 domain to enhance substrate phosphorylation has been established in our laboratory with the Src family kinase, Hck (*22*). Furthermore, this ability is lacking in a mutant form of Hck that cannot bind exogenous SH2 ligands (*11*). Here, we expand on those findings to establish whether Src family kinases phosphorylate substrates containing SH2 domain ligands by a processive mechanism. To accomplish this, we designed a substrate for these studies, Y1-Y2-pYEEI, that contains two unphosphorylated substrate tyrosines within the Cas substrate motif (Asp-Ile-Tyr-Asp-Val-Pro) (*27*) followed by the high-affinity SH2 domain ligand (phospho)Tyr-Glu-Glu-Ile (*28*) (Table 1).

First, we addressed the order of phosphorylation of the two tyrosines. The binding of Hck to a potential substrate via its SH2 domain may impose conformational constraints that favor phosphorylation of certain substrate motifs over others. For example, if the SH2 domain is bound to the C-terminus of the peptide Y1-Y2-pYEEI, then the catalytic domain will be tethered, which could create a preference for either Y1 or Y2. To establish which products were being produced in reactions containing Hck and Y1-Y2-pYEEI, we synthesized all three of the possible product peptides. There are two possible products due to one phosphorylation by Hck: the product of phosphorylation of Y1 (pY1-Y2-pYEEI) and the product of phosphorylation of Y2 (Y1-pY2-pYEEI) (Table 1). We also made the product in which both substrate tyrosines are phosphorylated, pY1-pY2-pYEEI (Table 1). We then designed an HPLC method that could separate each of the products as well as the substrate peptide (Figure 1, top panel).

To analyze the order of phosphorylation of the two substrate tyrosines, the substrate was incubated with Hck and ATP and the reaction was monitored at different time points by HPLC (Figure 1, bottom panel). The first product produced after 2−4 min of reaction was the product of phosphorylation of Y2, Y1-pY2-pYEEI (Figure 1, bottom panel), whereas virtually none of the product of phosphorylation of Y1, pY1-Y2-pYEEI, was detectable. After 10 min of reaction, measurable levels of pY1-Y2-pYEEI accumulated, but they were approximately 5-fold lower than those of Y1-pY2-pYEEI. Therefore, the first phosphorylation site is predominantly Y2.

Ordered phosphorylation is consistent with a processive mechanism, but the true hallmark of a processive mechanism is that phosphorylation of all substrate motifs requires only one interaction between enzyme and substrate. Therefore, if Y1-Y2-pYEEI is phosphorylated processively beginning with Y2, we would expect to see phosphorylation of Y1 only on peptides where Y2 has already been phosphorylated. At all time points, levels of the product where Y1 and Y2 are phosphorylated (pY1-pY2-pYEEI) exceeded the levels of the product of Y1 alone (pY1-Y2-pYEEI). Therefore, phosphorylation of the substrate on the Y1 is more favored when Y2 has already been phosphorylated. This is consistent with a processive model of substrate phosphorylation, where Hck binds to the substrate once and phosphorylates both tyrosines in rapid succession.

Another characteristic of processive phosphorylation of substrates containing multiple phosphorylation sites is that the production of fully phosphorylated product follows classical Michaelian kinetics (*19*). In a processive reaction, the enzyme and substrate only interact once, as in ordinary enzyme substrate reactions, and therefore the production of the fully phosphorylated product is linear with time (*19*). If a substrate is phosphorylated in a nonprocessive manner, multiple collisions between enzyme and substrate are necessary to produce fully phosphorylated product. Therefore, in the nonprocessive mechanism, there will be a lag in the production of fully phosphorylated product while intermediate products are produced (*19*). We found that conversion of substrate to fully phosphorylated product is linear over time (Figure 2) and therefore consistent with processive phosphorylation. (Since the levels of pY1-pY2-pYEEI produced in the reactions exceeded the level of enzyme, we conclude that Src releases the product after it is fully phosphorylated.) Furthermore, the rate of formation of fully phosphorylated product showed the hyperbolic dependence on substrate concentration that is characteristic of classical Michaelian kinetics (data not shown). These results are consistent with a processive mechanism for phosphorylation of Y1-Y2-pYEEI.

Since enzyme and substrate remain bound during processive phosphorylation, the rate of progression from one intermediate phosphorylation state to the fully phosphorylated product should be pseudo-zero-order and not dependent on enzyme concentration (*18*). To determine the dependence of product formation on enzyme concentration, we performed pulse−chase experiments where the "chase" reactions were diluted to different concentrations of enzyme. In these experiments, we first incubated Hck and substrate with [γ-^{32}P]ATP for 15 min under conditions that generate Y1-pY2-pYEEI. We then diluted the reaction to yield final enzyme concentrations ranging from 0.5 to 5 nM. Our dilutions also contained excess unlabeled ATP to reduce the specific activity of the [γ-^{32}P]ATP to levels that are undetectable in our assay. Therefore, we were able to monitor the conversion of radiolabeled Y1-pY2-pYEEI to pY1-pY2-pYEEI without interference from any Y1-pY2-pYEEI created in the diluted sample. If Hck remains bound to the Y1-pY2-pYEEI form of the substrate until the second phosphorylation is completed, dilution of the reaction should not affect the

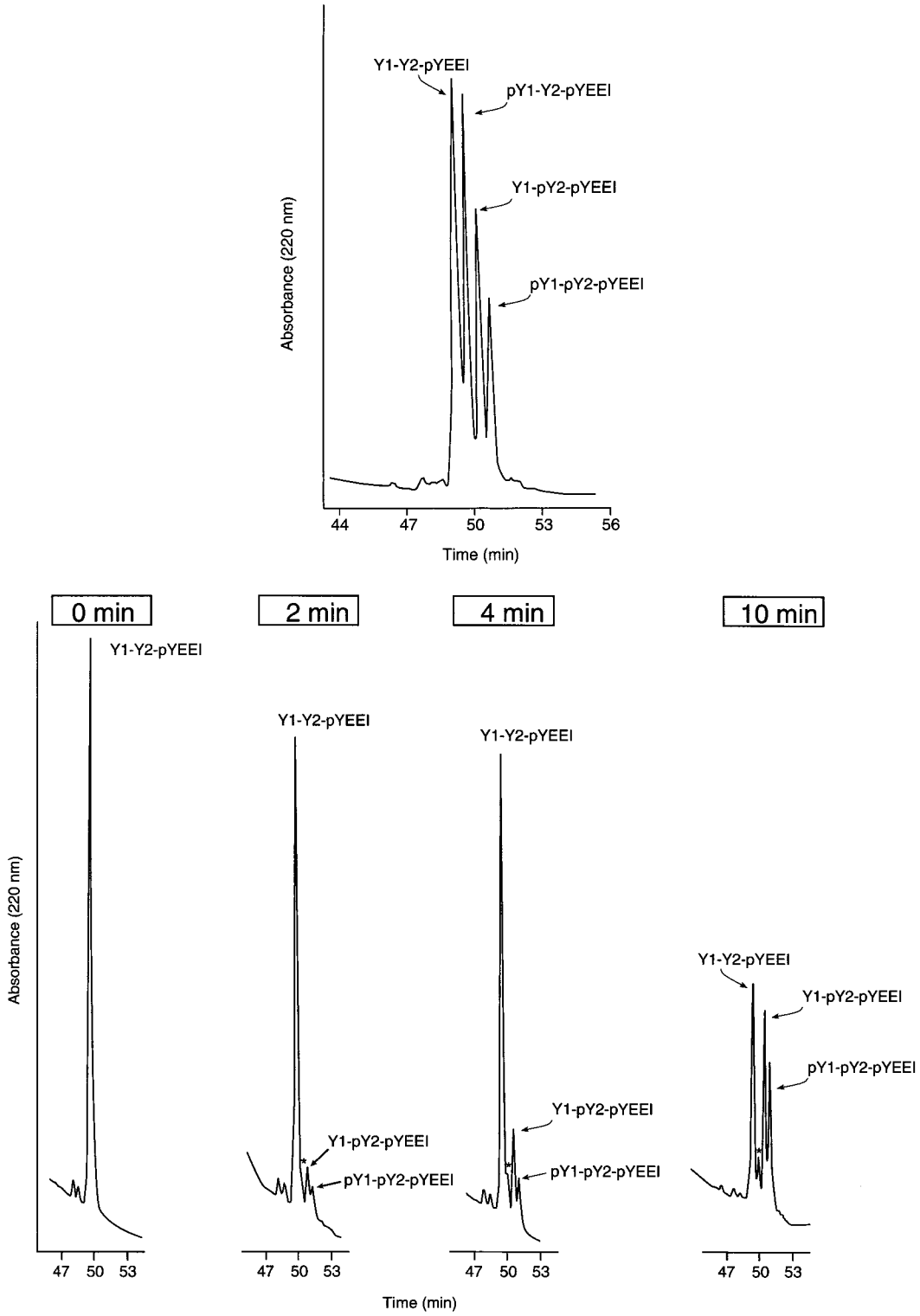

FIGURE 1: (Top) Separation of Y1-Y2-pYEEI and each of its product peptides by HPLC. Substrate peptide, Y1-Y2-pYEEI, as well as all three possible product peptides, pY1-Y2-pYEEI, Y1-pY2-pYEEI, and pY1-pY2-pYEEI, were mixed in equimolar amounts and separated by reverse-phase HPLC in an ammonium acetate/tetrabutylammonium phosphate (TBAP) buffer system. Peptides were eluted with a 60 min linear gradient of 30−95% buffer B, where buffer B contained 75% acetonitrile. The retention times of Y1-Y2-pYEEI, Y1-pY2-pYEEI, pY1-Y2-pYEEI, and pY1-pY2-pYEEI were established by injections of the purified peptides (data not shown). (Bottom) Order of phosphorylation of Y1-Y2-pYEEI. Y1-Y2-pYEEI (80 μM) was phosphorylated with 100 nM Hck and 0.5 mM ATP at 30 °C. Aliquots (20 μL) were removed from the reaction at 0, 2, 4, and 10 min and quenched by addition of cold acetonitrile. The reactions were then injected on HPLC under conditions as in panel A. The asterisk denotes the position at which pY1-Y2-pYEEI elutes.

amount of pY1-pY2-pYEEI produced. If the enzyme does not remain bound to the Y1-pY2-pYEEI form of the substrate, the second phosphorylation would require a second collision between the substrate and the enzyme and would therefore depend on enzyme concentration. The result, shown in Figure 3, is that the conversion of Y1-pY2-pYEEI to pY1-pY2-pYEEI did not depend on enzyme concentration. We therefore conclude that, under these experimental conditions, Hck remains bound to the peptide substrate while catalyzing the first and second phosphorylations.

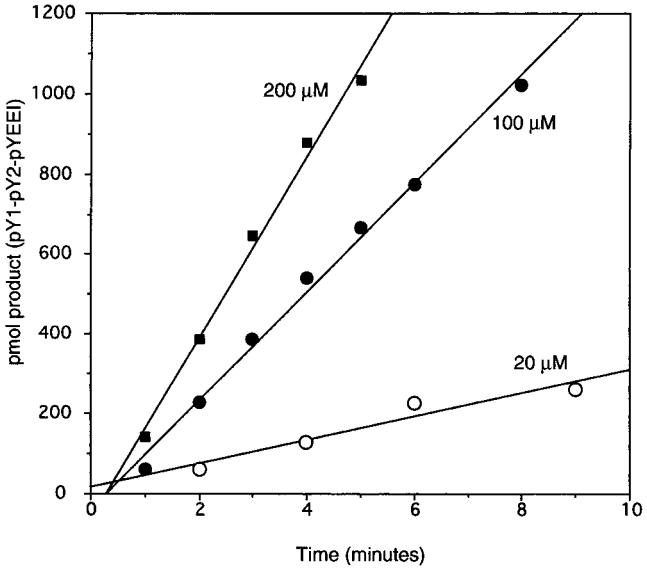

FIGURE 2: Production of the phosphorylated product, pY1-pY2-pYEEI, is linear over time. Y1-Y2-pYEEI (either 20, 100, or 200 μM) was phosphorylated with Hck (100 nM) at 30 °C in reactions containing 100 mM Tris (pH 7.5), 10 mM $MgCl_2$, and 0.5 mM ATP. Aliquots were removed and quenched by addition of excess cold HPLC solvent A. To determine the picomoles of product produced, samples were injected onto the HPLC and peak heights were compared to that of a standard injection.

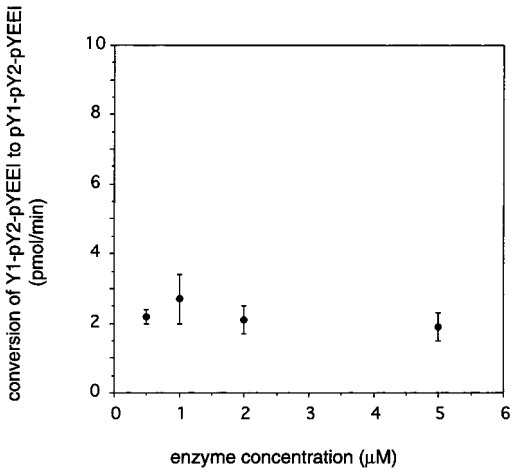

FIGURE 3: Product formation is independent of enzyme concentration. To follow the conversion of Y1-pY2-pYEEI to pY1-pY2-pYEEI, pulse−chase experiments were performed. In the pulse reaction, 320 μM Y1-Y2-pYEEI was incubated with 100 nM Hck at 30 °C in buffer containing 100 mM Tris (pH 7.5), 10 mM $MgCl_2$, and 0.25 mM [γ-^{32}P]ATP. After 15 min, aliquots were removed from the reaction and diluted with 0.25 mM unlabeled ATP to give final enzyme concentrations of 0.5, 1.0, 2.0, and 5.0 nM, respectively. After 15 min the reactions were stopped by addition of TFA. The entire chase reactions were then analyzed by reverse-phase HPLC under conditions that separated Y1-pY2-pYEEI from pY1-pY2-pYEEI (see Materials and Methods). Peaks of absorbance were collected, radioactivity incorporation was determined, and the number of picomoles of product produced was calculated.

(2) Role of the SH3 Domain in Enhanced Phosphorylation. Although the role of the SH2 domain in enhanced phosphorylation has been established (*22*), the role of the SH3 domain in enhanced phosphorylation has not been studied extensively. We designed substrates containing the SH3 ligand from the c-Src substrate Cas (*27*) connected to a single substrate motif (*29*) by a linker containing either six or 10 glycines (Table 1). For both the shorter and longer SH3 ligand-containing peptides, we designed corresponding control peptides where three prolines were mutated from the SH3 ligand motif (Table 1). Both polyproline-containing peptides have the potential to bind simultaneously to the SH3 and catalytic domains of Src, assuming a fully extended conformation and a length of 3.7 Å/residue (*30*). The shorter SH3 ligand-containing peptide we synthesized, Pro-G6, has 13 spacer residues (48.1 Å) between the tyrosine and the first proline of the SH3 ligand motif. The longer polyproline-containing peptide contains a longer spacer region (62.9 Å). For c-Src, the length between the catalytic base (D386) and the first proline of the SH3 ligand motif (P250) is 26.3 Å (*4*). In protein substrates, motifs separated by large spans of primary sequence could be positioned close together in the folded conformation.

First, we compared phosphorylation of SH3 ligand-containing substrates to control substrates using a down-regulated form of the Src family kinase Hck (*11*). The SH3 ligand-containing peptides with either the 6- or the 10-glycine linker are phosphorylated at much higher rates than controls, with production of approximately 10-fold more phosphorylated product after 14 min for SH3-containing peptide versus control (Figure 4). The enhanced phosphorylation observed in this experiment could be due to (1) activation of Hck by displacement of the SH3 domain from its internal ligand and/or (2) increased affinity of the substrate for the enzyme.

To dissect the role of the SH3 ligand in activation from its ability to increase binding of the substrate, we used v-Src. The SH2 and SH3 domains of v-Src do not inhibit the activity of the catalytic domain (*31*), and Src cannot be activated by SH3 domain ligands. Therefore, any increase in phosphorylation of SH3 ligand-containing peptide must be due to an increase in binding. We measured K_m and V_{max} values for SH3 ligand-containing peptides and control peptides for v-Src (Table 2). The values of V_{max} for control and SH3 ligand-containing peptides were similar. However, the addition of the SH3 ligand to the substrates decreased their K_m values. For peptides with the spacer containing six glycines, Pro-G6 and Control-G6, addition of an SH3 domain ligand decreased the K_m 2.6-fold compared to the control peptide. Peptide Pro-G10 had a 2.8-fold lower K_m than its control peptide, Control-G10. Therefore, addition of an SH3 ligand decreases the K_m for the substrate but does not increase the V_{max}. A similar decrease in K_m was observed for peptides containing spacers of 6 or 10 glycines, presumably because both spacers were long enough that the substrate could occupy both the SH3 domain and the catalytic site simultaneously.

These results allow us to interpret the experiments on down-regulated Hck presented in Figure 4. The activation in Figure 4 was observed at peptide concentrations well above K_m; therefore, this 10-fold increase in peptide phosphorylation for SH3 ligand-containing substrates is due solely to activation of Hck and not to increased binding. The SH3 ligand-containing substrates also possess a ≈3-fold lower K_m than control substrates, as shown in Table 2. The combination of these effects will lead to enhanced phosphorylation of SH3 ligand-containing substrates over a range of substrate concentrations.

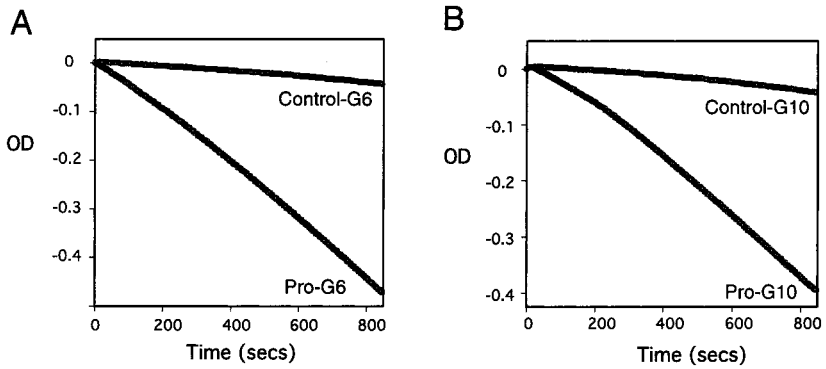

FIGURE 4: Addition of an SH3 domain ligand motif improves substrate phosphorylation. Kinase assays were performed by a coupled spectrophotometric assay that couples the production of ADP to the oxidation of NADH (*25*). Peptide (0.6 mM) was incubated with 10 nM Hck and 0.5 mM ATP in buffer at 30 °C. Absorbance (340 nm) was measured every 4 s. Peptides Pro-G6 and Control-G6 are shown in panel A, and peptides Pro-G10 and Control-G10 are shown in panel B.

Table 2: Kinetic Parameters for v-Src[a]

peptide	K_m (μM)	V_{max} (μmol min^{-1} mg^{-1})
Pro-G6	79 ± 8	78 ± 15
control-G6	204 ± 28	114 ± 5
Pro-G10	127 ± 7	109 ± 35
control-G10	358 ± 64	144 ± 34

[a] Kinetic parameters were determined by the spectrophotometric assay (*25*). Peptide concentrations were varied to at least 3-fold above K_m values. A saturating concentration of 500 μM ATP was used (*40*).

DISCUSSION

Three lines of evidence support a processive model of phosphorylation for the SH2 ligand-containing peptides. First, phosphorylation of Y1-Y2-pYEEI to produce the fully phosphorylated product, pY1-pY2-pYEEI, follows classical Michaelian kinetics, as would be expected in a processive mechanism (Figure 2) (*19*). Second, the conversion of the intermediate, Y1-pY2-pYEEI, to the fully phosphorylated pY1-pY2-pYEEI does not depend on enzyme concentration, and therefore Src must remain bound to the substrate between the first and second rounds of phosphorylation (Figure 3). Third, phosphorylation of the substrate Y1-Y2-pYEEI proceeds in an ordered manner, with Y2 phosphorylated first and Y1 phosphorylated second (Figure 1, bottom panel). In principle, ordered phosphorylation of Y1-Y2-pYEEI could be consistent with either a processive or distributive mechanism. Because the phosphorylation motifs Y1 and Y2 are identical, we interpret the results to indicate that binding of the SH2 domain to its pYEEI ligand constrains the conformation of the kinase such that Y2 is phosphorylated first. In the same experiment, we observed more of the fully phosphorylated product than product phosphorylated on Y1 only, consistent with the notion that the kinase remains bound to the substrate while phosphorylating both sites. Although a processive mechanism has been proposed for many of the best substrates for Src family kinases, these results constitute the first formal proof that Src kinases can recognize substrates in this manner.

We expect that processive phosphorylation of a protein substrate such as Cas would be faster than the rates we observe for the synthetic peptides in Figures 2 and 3. Protein substrates likely have a more ideal relative positioning of their SH2 ligand and substrate motifs in their three-dimensional structures than we achieved in our synthetic peptides.

The SH3 domains of Src family kinases have two unique properties that are important for substrate targeting. First, SH3 domain-mediated interactions are phosphotyrosine-independent (*32*). Second, the SH3 domain has a dominant role over the SH2 domain in regulation of the catalytic domain (*6*, *11*). While displacement of the SH2 domain causes a 2-fold increase in catalytic activity, displacement of the SH3 domain leads to full activation of the catalytic domain and makes the kinase refractory to further activation by SH2 domain ligands (*6*). Here, we present evidence that the addition of an SH3 domain ligand to a substrate increases its phosphorylation as expected by activating down-regulated Hck (Figure 4). We also demonstrate that addition of an SH3 domain ligand decreases the K_m values for substrates about 3-fold (Table 2). Therefore, a substrate containing an SH3 domain ligand will display increased phosphorylation over a range of concentrations; at low concentrations its binding to the enzyme is enhanced, and at high concentrations it activates down-regulated Src family kinases.

The Src substrates Sin, Fak, AFAP-110, and Cas all share several important characteristics. First, they possess both SH2 and SH3 domain ligands (*12*, *15*−*17*). Second, they are phosphorylated on multiple sites by Src (*12*, *14*, *16*, *17*). For example, Cas contains 15 potential substrate motifs for Src and is known to be hyperphosphorylated by Src in vivo (*16*, *27*). Third, binding of the Src SH3 domain has an important role in facilitating their phosphorylation (*12*−*14*; P. Pellicena and W. T. Miller, unpublished observations). In this paper, we present evidence that the SH2 domain of Src can facilitate multiple phosphorylations by a processive mechanism. We also show that addition of an SH3 domain ligand to a substrate increases its phosphorylation 10-fold by because it lowers the K_m value of the substrate and activates the kinase. The data presented here, together with earlier observations, suggest the following model for the phosphorylation of Cas and similar substrates. The initial encounter between Src family kinases and many of their substrates will depend on SH3 domain interactions. This binding of the enzyme's SH3 domain to the substrate causes activation of the catalytic domain and autophosphorylation (*6*). Once active Src is bound to its substrate, it phosphorylates the substrate on a tyrosine within an SH2 domain ligand motif that has high affinity for its own SH2 domain (*33*, *34*). (In our synthetic peptide system, we showed that Hck can phosphorylate an unphosphorylated YEEI motif at the

C-terminus; M.P.S. and W.T.M., unpublished observations.) This triggers a phosphotyrosine−SH2 domain interaction, and Src subsequently phosphorylates the substrate several times by a processive mechanism. Src then releases the hyperphosphorylated product. The sites Src phosphorylates on the substrate may become SH2 domain-binding motifs for downstream ligands. For example, phosphorylation of Cas by Src is required to create SH2 domain ligand motifs for Crk (*16*). A related mechanism for producing multiply phosphorylated proteins where two or more kinases are involved has been termed "sequential" phosphorylation (*35*, *36*).

This model explains how Src family kinases can rapidly phosphorylate multiple sites in vivo. First, the affinity of the Src substrates Sin, Fak, AFAP-110, and Cas is greatly enhanced because they make multiple interactions with the enzyme via SH3, SH2, and catalytic domain interactions. Second, processive phosphorylation only requires one interaction between the substrate and the enzyme. Full phosphorylation of a substrate by a nonprocessive mechanism would presumably be slower than processive phosphorylation in vivo, since nonprocessive phosphorylation would require multiple collisions with Src to produce highly phosphorylated substrate. Furthermore, if each collision resulted in only one phosphorylation, the major products of phosphorylation would be intermediate species. An additional advantage of the processive mechanism is that it could confer protection from cellular tyrosine phosphatases. Negative regulation by phosphatases is known to occur for Cas (*37*, *38*) and Fak (*39*). If these substrates were phosphorylated by a nonprocessive mechanism, intermediate forms might be vulnerable to dephosphorylation. In a processive mechanism where Src remains bound to the substrate, Src may sterically block tyrosine phosphatases from prematurely dephosphorylating tyrosine residues on the substrate, allowing the formation of fully phosphorylated product. Thus, Src substrates such as Cas possess several features that ensure rapid signaling in vivo; they recruit and activate Src family kinases by SH2/SH3 domain binding, and their multiple phosphorylation sites are arranged to facilitate processive phosphorylation.

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REFERENCES

1. Thomas, S. M., and Brugge, J. S. (1997) *Annu. Rev. Cell. Dev. Biol. 13*, 513−609.
2. Brown, M. T., and Cooper, J. A. (1996) *Biochim. Biophys. Acta 1287*, 121−49.
3. Williams, J. C., Weijland, A., Gonfloni, S., Thompson, A., Courtneidge, S. A., Superti-Furga, G., and Wierenga, R. K. (1997) *J. Mol. Biol. 274*, 757−75.
4. Xu, W., Harrison, S. C., and Eck, M. J. (1997) *Nature 385*, 595−602.
5. Xu, W., Doshi, A., Lei, M., Eck, M. J., and Harrison, S. C. (1999) *Mol. Cell 3*, 629−38.
6. Moarefi, I., LaFevre-Bernt, M., Sicheri, F., Huse, M., Lee, C. H., Kuriyan, J., and Miller, W. T. (1997) *Nature 385*, 650−3.
7. Schindler, T., Sicheri, F., Pico, A., Gazit, A., Levitzki, A., and Kuriyan, J. (1999) *Mol. Cell 3*, 639−48.
8. Pinna, L. A., and Ruzzene, M. (1996) *Biochim. Biophys. Acta 1314*, 191−225.
9. Nguyen, J. T., and Lim, W. A. (1997) *Nat. Struct. Biol. 4*, 256−60.
10. Mayer, B. J. (1997) *Curr. Biol. 7*, R295−8.
11. Porter, M., Schindler, T., Kuriyan, J., and Miller, W. T. (2000) *J. Biol. Chem. 275*, 2721−6.
12. Alexandropoulos, K., and Baltimore, D. (1996) *Genes Dev. 10*, 1341−55.
13. Thomas, J. W., Ellis, B., Boerner, R. J., Knight, W. B., White, G. C., 2nd, and Schaller, M. D. (1998) *J. Biol. Chem. 273*, 577−83.
14. Guappone, A. C., and Flynn, D. C. (1997) *Mol. Cell Biochem. 175*, 243−52.
15. Guappone, A. C., Weimer, T., and Flynn, D. C. (1998) *Mol. Carcinogen. 22*, 110−9.
16. O'Neill, G. M., Fashena, S. J., and Golemis, E. A. (2000) *Trends Cell Biol. 10*, 111−9.
17. Cary, L. A., and Guan, J. L. (1999) *Front. Biosci. 4*, D102−13.
18. Ferrell, J. E., Jr., and Bhatt, R. R. (1997) *J. Biol. Chem. 272*, 19008−16.
19. Burack, W. R., and Sturgill, T. W. (1997) *Biochemistry 36*, 5929−33.
20. Mayer, B. J., Hirai, H., and Sakai, R. (1995) *Curr. Biol. 5*, 296−305.
21. Lewis, L. A., Chung, C. D., Chen, J., Parnes, J. R., Moran, M., Patel, V. P., and Miceli, M. C. (1997) *J. Immunol. 159*, 2292−300.
22. Pellicena, P., Stowell, K. R., and Miller, W. T. (1998) *J. Biol. Chem. 273*, 15325−8.
23. Atherton, E., and Sheppard, R. C. (1989) *Solid-phase peptide synthesis*, IRL Press, Oxford, U.K.
24. Ottinger, E. A., Shekels, L. L., Bernlohr, D. A., and Barany, G. (1993) *Biochemistry 32*, 4354−61.
25. Barker, S. C., Kassel, D. B., Weigl, D., Huang, X., Luther, M. A., and Knight, W. B. (1995) *Biochemistry 34*, 14843−51.
26. Garcia, P., Shoelson, S. E., George, S. T., Hinds, D. A., Goldberg, A. R., and Miller, W. T. (1993) *J. Biol. Chem. 268*, 25146−51.
27. Nakamoto, T., Sakai, R., Ozawa, K., Yazaki, Y., and Hirai, H. (1996) *J. Biol. Chem. 271*, 8959−65.
28. Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W. G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R. J., et al. (1993) *Cell 72*, 767−78.
29. Songyang, Z., Carraway, K. L., III, Eck, M. J., Harrison, S. C., Feldman, R. A., Mohammadi, M., Schlessinger, J., Hubbard, S. R., Smith, D. P., Eng, C., Lorenzo, M. J., Poner, B. A. J., Mayer, B. J., and Cantley, L. C. (1995) *Nature 373*, 536−539.
30. Schulz, G. E., and Schirmer, R. H. (1979) *Principles of Protein Structure*, Springer-Verlag, New York.
31. Schwartzberg, P. L. (1998) *Oncogene 17*, 1463−8.
32. Kuriyan, J., and Cowburn, D. (1997) *Annu. Rev. Biophys. Biomol. Struct. 26*, 259−88.
33. Johnson, T. M., Perich, J. W., Bjorge, J. D., Fujita, D. J., and Cheng, H. C. (1997) *J. Pept. Res. 50*, 365−71.
34. Zhou, S., Carraway, K. L., 3rd, Eck, M. J., Harrison, S. C., Feldman, R. A., Mohammadi, M., Schlessinger, J., Hubbard, S. R., Smith, D. P., Eng, C., et al. (1995) *Nature 373*, 536−9.
35. Ruzzene, M., Brunati, A. M., Marin, O., Donella-Deana, A., and Pinna, L. A. (1996) *Biochemistry 35*, 5327−32.
36. Brunati, A. M., Donella-Deana, A., James, P., Quadroni, M., Contri, A., Marin, O., and Pinna, L. A. (1999) *J. Biol. Chem. 274*, 7557−64.
37. Buist, A., Blanchetot, C., Tertoolen, L. G., and den Hertog, J. (2000) *J. Biol. Chem. 275*, 20754−20761.
38. Liu, F., Hill, D. E., and Chernoff, J. (1996) *J. Biol. Chem. 271*, 31290−5.
39. Tamura, M., Gu, J., Danen, E. H., Takino, T., Miyamoto, S., and Yamada, K. M. (1999) *J. Biol. Chem. 274*, 20693−703.
40. Shah, K., Liu, Y., Deirmengian, C., and Shokat, K. M. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 3565−70.

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