

Examination of the Dephosphorylation Reactions Catalyzed by pp60^{c-src} Tyrosine Kinase Explores the Roles of Autophosphorylation and SH2 Ligand Binding[†]

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ABSTRACT: pp60^{c-src} tyrosine kinase (*src*TK) catalyzes the dephosphorylation of phosphotyrosine-containing peptides, including phosphopeptides that bind with high affinity to the *src* SH2 domain. The mechanism for these dephosphorylation reactions was investigated. Dephosphorylation was inhibited by a competitive inhibitor for the ATP binding site. In the presence of ADP, dephosphorylation of phosphopeptide substrates is primarily due to the reversal of the kinase reaction. Autoactivated and unactivated *src*TK both catalyzed the reverse of the kinase reaction; however, autoactivated *src*TK displayed an increase in k_{cat} of approximately 4–11-fold relative to unactivated *src*TK, depending on the reaction conditions. Autoactivation of *src*TK does not affect the K_m 's for MgADP or phosphopeptide (FGE)₃-pY-(GEF)₂GD. Unphosphorylated *src*TK becomes phosphorylated during the reverse of the kinase reaction upon accumulation of free MgATP. In the presence of MgATP, *src*TK also dephosphorylates peptide substrates, by first hydrolyzing MgATP to MgADP. Binding of phosphotyrosine peptide ligands to the *src* SH2 domain stimulated the rate of MgATP hydrolysis approximately 2-fold, but had no effect on the K_m for MgATP. These data suggest that autophosphorylation of tyrosine 419 is not required for nucleotide or peptide binding, or catalysis involving small peptide substrates. In addition, these results suggest that both the forward and the reverse *src* tyrosine kinase reactions may be important in regulating the intracellular levels of protein tyrosine phosphorylation.

Elevated *src*TK¹ activity has been detected in several carcinomas, implicating *src*TK as a therapeutic target in the treatment of cancer (Bolen et al., 1985; Rosen et al., 1986; Bolen, 1987; Cartwright et al., 1989, 1990). However, the mechanism by which *src*TK contributes to malignant transformation is unknown. In fact, while *src*TK has been shown to phosphorylate a number of different proteins [for example, see Ogawa et al. (1994)], the physiological relevance of these phosphorylations is unknown.

In addition to its kinase domain, *src*TK contains several other domains. N-Terminal myristylation allows association of *src*TK with the cellular membrane (Courtneidge et al., 1980; Buss & Sefton, 1985), which is required for cellular transformation (Wilson et al., 1989). *src*TK also contains two domains involved in protein–protein interactions: the SH3 domain, which binds proline-rich sequences, and the SH2 domain, which binds proteins containing phosphotyrosine residues (Pawson & Schlessinger, 1993). *src*TK also contains tyrosine phosphorylation sites, Y419 and Y530. Y530 is a C-terminal site which has been implicated in down-regulation of the enzyme (Cooper et al., 1986; Laudano &

Buchanan, 1986; Cartwright et al., 1987; Kmiecik & Shalloway, 1987; Piwnica-Worms et al., 1987; Reynolds et al., 1987). Previous reports suggested that phosphorylation of Y530 leads to *src*TK inactivation by binding intramolecularly to the *src* SH2 domain, creating an inactive complex (Matsuda et al., 1990; Cantley et al., 1991; Koch et al., 1991; Veillette et al., 1992; Liu et al., 1993). Autophosphorylation of Y419, located within the tyrosine kinase domain, leads to activation of the enzyme (Kmiecik et al., 1988).

We have investigated the dephosphorylation of peptide substrates by *src*TK and determined that the mechanism is the reverse of the kinase reaction. Studying the reverse of the kinase reaction has allowed us to determine the role of Y419 phosphorylation. Phosphorylation of Y419 increased the k_{cat} , but had no effect on the K_m for phosphopeptide or ADP in the reverse of the kinase reaction. These results show that phosphorylation of tyrosine 419 is not necessary for protein substrate or nucleotide binding. Our results suggest that the reverse of the *src* tyrosine kinase reaction may be physiologically important and suggest a role for *src*TK in regulating the intracellular levels of protein tyrosine phosphorylation. We have also observed that occupancy of the *src* SH2 domain can increase ATP hydrolysis by approximately 2-fold, demonstrating that interactions with the SH2 domain may be important in regulating the catalytic activity of *src*TK.

MATERIALS AND METHODS

Peptides.² The various peptides used in these studies include: (1) Btn-TSTEPQ-pY-EEIENL; (2) Ac-YEEIE; (3)

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¹ Abbreviations: AMP-PCP, adenylyl methylenediphosphate; AMP-PNP, adenylyl imidodiphosphate; ATPγS, adenosine 5'-O-(3-thiotriphosphate); DMSO, dimethyl sulfoxide; DTT, dithiothreitol; ESI-MS, electrospray ionization mass spectrometry; G-6PDH, glucose-6-phosphate dehydrogenase; Hepes, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid; HK, hexokinase; IRTK, insulin receptor tyrosine kinase; LDH, lactate dehydrogenase; NADH, reduced nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; PEP, phosphoenolpyruvate; PK, pyruvate kinase; *src*TK, pp60^{c-src} tyrosine kinase; TFA, trifluoroacetic acid.

² Peptides are denoted by their one-letter amino acid code. Other functional groups are indicated by the following abbreviations: Ac, acetyl; Btn, biotin.

Ac-pY-EEIE; and (4) (FGE)₃-pY-(GEF)₂GD. All peptides were dissolved in water. Peptides 2 and 3 were obtained from the GRI collection and synthesized according to Gilmer et al. (1994), and peptides 1 and 4 were obtained from Cambridge Research Biochemicals.

Protein. N-85-*src*TK³ was overexpressed in baculovirus and purified by the method of Ellis et al. (1994). N-85-*src*TK was not autoactivated⁴ prior to use in the dephosphorylation assays, unless indicated otherwise.

HPLC Dephosphorylation Assays and Analysis by Capillary Perfusion HPLC. N-85-*src*TK (5 μ M) was incubated with 1 mM phosphorylated peptide, 6 mM MgCl₂, 1 mM ADP or ATP, and 50 mM Hepes, pH 7.5 at 25 °C. Over time, 2 μ L aliquots were diluted into 48 μ L of 0.2% TFA to stop the reaction. The separation of phosphorylated and dephosphorylated peptides was accomplished by capillary perfusion HPLC. All HPLC was performed on a Hewlett Packard 1090 HPLC system equipped with a Hewlett Packard diode array detector containing a 6 mm path length micro flow cell. Five microliter aliquots were injected onto a Poros II R/H perfusion column (LC Packings, 800 μ m i.d. \times 1.5 cm), and the eluant was monitored at 205 nm. A flow rate of approximately 50 μ L/min using a gradient of 0–30% buffer B (0.08% TFA, 9/1 acetonitrile/water) in 5 min and 30–50% buffer B in 2 min was used for separations. Buffer A was 0.08% TFA in water. Peak areas were determined by integration using the Hewlett Packard Chemstations software.

To examine the dephosphorylation of phosphopeptides when only ATP is present, an ATP regenerating system was used. PEP (10 mM) and PK (100–140 units/mL) were added to convert any ADP to ATP. Likewise, to examine the dephosphorylation of phosphopeptides when only ADP is present, an ADP regenerating system consisting of glucose (10 mM) and HK (20–40 units/mL) was added.

Quantitation of ATP and ADP. The amount of ATP and ADP present at various times in the dephosphorylation reactions was quantitated by HPLC using gradient conditions similar to those reported by Thrall and Dunaway-Mariano, (1994). Two microliter aliquots of reaction mixture (containing 1 mM ADP or ATP at the start of the reaction) were diluted into 48 μ L of 0.2% TFA, and 5 μ L of this solution was injected onto a C18 column (LC Packings, 5 μ m, 800 μ m i.d. \times 15 cm) equilibrated in 92.5% H₂O, 5% methanol, 2.5% triethylamine, and 25 mM KH₂PO₄, pH 6.5 (pH adjusted with phosphoric acid). The flow rate was approximately 60 μ L/min, and the absorbance at 254 nm was monitored. During the course of analysis, no hydrolysis of ATP was detected in control reactions of ATP diluted into 0.2% TFA in the absence of enzyme.

Mass Spectrometry of N-85-*src*TK. Ten microliter aliquots (corresponding to 50 pmol of N-85-*src*TK) from the previously described dephosphorylation reactions were injected

onto a Poros R2/H perfusion column. Peptides were separated from N-85-*src*TK using a gradient of 15–65% buffer D (90/10 acetonitrile/H₂O containing 0.035% TFA) in 5 min. Buffer C was 0.05% TFA in H₂O. The molecular weight of N-85-*src*TK was determined by ESI-MS on a PE-Sciex API III triple quadrupole mass spectrometer (Thornhill, Ontario, Canada). The instrument was scanned from *m/z* of 900–1100 using a 0.2 Da step size and 3.0 ms dwell time for each step.

Spectrophotometric Dephosphorylation Assays. A continuous spectrophotometric assay for the dephosphorylation of peptide 4 was used to determine the kinetic parameters for this reaction. The dephosphorylation of phosphopeptide substrate was coupled to the reduction of NADP⁺ using glucose, HK, and G-6PDH and monitored by following the increase in absorbance at 340 nm. All spectrophotometric assays were performed on either a Cary 4E spectrophotometer (Varian Instruments) or a SpectraMax 250 plate reader (Molecular Devices, Menlo Park, CA). Either 1 mL or 200 μ L reactions were incubated at 25 °C and initiated by adding either unactivated or autoactivated N-85-*src*TK. Reaction solutions typically contained 1 mM glucose, 240 μ M NADP⁺, 3.8 units/mL G-6PDH, 2.5 units/mL HK, 100 mM Hepes, pH 7.5, 20 mM MgCl₂, 100 μ M DTT, 20 μ g/mL BSA, and variable amounts of N-85-*src*TK, ADP, and phosphopeptide.

Spectrophotometric ATPase Assays. A continuous spectrophotometric assay was utilized to determine kinetic parameters for the hydrolysis of ATP. ATP hydrolysis was coupled to the oxidation of NADH using PEP, PK, and LDH and was monitored by following the decrease in absorbance at 340 nm. In a total volume of 200 μ L, reaction mixtures contained 1 mM PEP, 240 μ M NADH, 45–65 units/mL LDH, 15–30 units/mL PK, 100 mM Hepes, pH 7.5, 20 mM MgCl₂, 100 μ M DTT, 20 μ g/mL BSA, and variable concentrations of ATP. The reactions were initiated with 1 μ M autoactivated N-85-*src*TK.

Data Analysis. Initial rates were determined by a least-squares regression analysis of the change in absorbance as a function of time. The kinetic parameters were determined by fitting the initial velocities as a function of substrate concentration to the equation $v = V_m[S]/(K_m + [S])$ by nonlinear least-squares regression using the program GraFit (Leatherbarrow, 1992).

RESULTS

Incubation of unactivated N-85-*src*TK with phosphotyrosine containing peptides 1 or 3 in the presence of MgATP or MgADP lead to their dephosphorylation (see Figure 1). Ac-pT-EEIE was not dephosphorylated by N-85-*src*TK after 24 h in the presence of MgADP or MgATP. The amount of peptide 1 dephosphorylated was greater in the presence of MgADP than in the presence of MgATP (Figure 1A). There was an initial lag in the dephosphorylation of peptide 3 in the presence of either MgADP or MgATP, but the lag time was shorter in the presence of MgATP (Figure 1B). However, after 180 min, approximately 12% of peptide 3 was dephosphorylated in both the MgATP and MgADP reactions. The dephosphorylation of peptide 1 was at least 20-fold slower in the presence of MgAMP or in the absence of nucleotide and could be due to the slow phosphatase activity of the *src* SH2 domain reported by Knight et al.

³ N-85-*src*TK was expressed from a deletion mutant which lacked the first 85 residues at the amino terminus (Ellis et al., 1994). This protein behaves similarly to protein expressed from a full-length construct, which lacks the myristylation site (G2A pp60^{c-src}).

⁴ Autoactivated N-85-*src*TK refers to N-85-*src*TK that has been incubated with 1 mM or 100 μ M ATP and 20 mM MgCl₂ on ice for approximately 30 min to allow for autophosphorylation of Y419 (Barker et al., 1995). The lower ATP concentration was used for autoactivation of N-85-*src*TK in the ATP hydrolysis experiments to reduce the amount of ATP added upon enzyme addition.

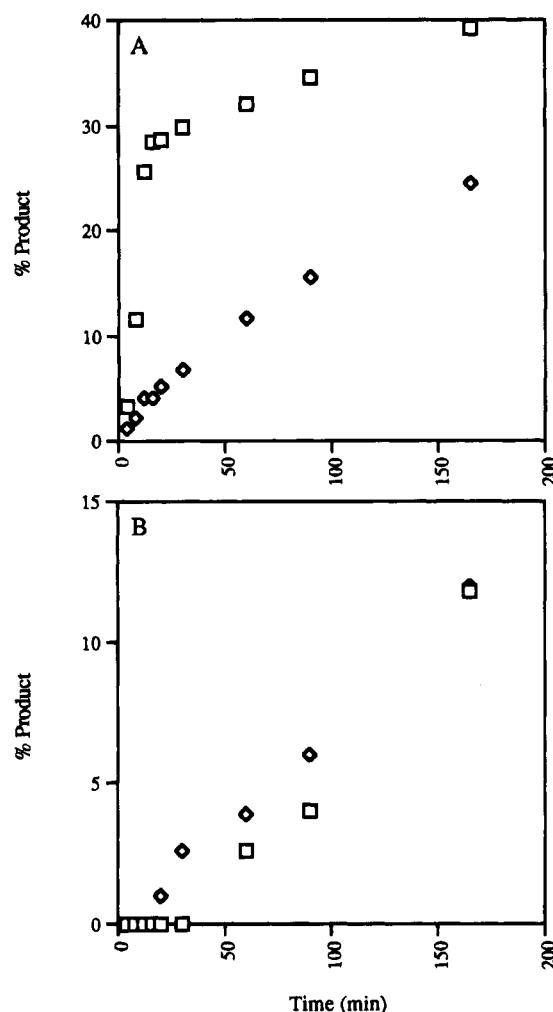


FIGURE 1: Dephosphorylation of peptide 1 and peptide 3 by N-85-*srcTK*. The percentage of (A) Bln-TSTEPQYEEIENL and (B) Ac-YEEIE produced in the presence of ADP (squares) or ATP (diamonds) was determined by HPLC. The initial concentrations of peptides and nucleotides were 1 mM, and the concentration of N-85-*srcTK* was 5 μ M.

Table 1: Apparent k_{cat} 's for Dephosphorylation^a

	MgATP (min ⁻¹)	MgADP (min ⁻¹)	MgAMP ^b (min ⁻¹)	Mg ²⁺ ^b (min ⁻¹)
peptide 1	0.7 ^c	7.5 ^c	3×10^{-2}	2×10^{-2}
peptide 3	0.2 ^d	0.3 ^d		

^a Each reaction contained 5 μ M N-85-*srcTK*, 1 mM phosphopeptide, and 6 mM MgCl₂. 1 mM ATP, ADP, or AMP was added to the appropriate reactions. ^b Rates were estimated from the 180 min time point. ^c Rates were estimated from time points in the initial linear portion of the dephosphorylation reaction. ^d Rates were estimated from time points in the linear portion of the dephosphorylation reaction, after the initial lag.

(1994) and Boerner et al. (1994). A summary of the apparent k_{cat} 's for dephosphorylation of peptides 1 and 3 is listed in Table 1.

The nucleotide analogues, AMP-PNP (1 mM) or AMP-PCP (1 mM), did not enhance dephosphorylation of peptide 1 (data not shown). However, in the presence of ATP γ S (1 mM), the apparent k_{cat} for dephosphorylation of peptide 1 was 1.08×10^{-1} min⁻¹. The ATP γ S preparation contains approximately 3% ADP (Knight and Barker, unpublished results), which is likely responsible for the enhanced peptide

dephosphorylation detected in the presence of this nucleotide analogue.

Staurosporine, a competitive inhibitor versus ATP in the *src* tyrosine kinase reaction (K_i approximately 10 nM; Knight, Huang, and Barker, unpublished results), inhibited the dephosphorylation of peptide 1 by >90% or 60% in the presence of MgATP or MgADP, respectively (assayed with 24 μ M staurosporine, data available as supporting information). Staurosporine also inhibited the dephosphorylation of peptide 3 by >90% in the presence of either MgATP or MgADP (assayed with 24 μ M staurosporine, data available as supporting information). The protein tyrosine phosphatase inhibitor vanadate (1 mM) and the serine/threonine phosphatase inhibitor okadaic acid (100 μ M) did not inhibit the ATP- or ADP-initiated dephosphorylation reaction (data not shown). These results suggest that this dephosphorylation is not the result of phosphatase activity.

Unlike the dephosphorylation reaction, the kinase reaction only occurs in the presence of MgATP, not MgADP. However, when N-85-*srcTK* was incubated with both a phosphotyrosine-containing peptide (peptide 1) and an unphosphorylated peptide (peptide 2), dephosphorylation of peptide 1 and phosphorylation of peptide 2 occurred in the presence of either MgATP or MgADP (data available as supporting information). The latter result suggests either that ATP is produced or that there is a phosphoenzyme intermediate produced which can serve as a phosphate donor to peptide 2.

To investigate whether the ADP-initiated reaction is simply a reversal of the kinase reaction, the production of ATP and dephosphorylation of peptide were monitored simultaneously. When the dephosphorylation of either peptide 1 (Figure 2A) or peptide 3 (Figure 2C) is initiated with ADP, ATP is formed. In fact, equimolar concentrations of dephosphorylated peptide and ATP are initially produced. After 16 min, the concentration of ATP actually decreases even though peptide 1 continues to be dephosphorylated (see Figure 2A) (not observed for peptide 3 over this time range), which is likely due to the ATPase activity described below. As described earlier (Figure 1B), there is an initial lag in the ADP-initiated dephosphorylation of peptide 3. A similar lag in the formation of ATP is also detected (Figure 2C). In this case, the ATPase activity is not clearly evident due to the small amount of ATP produced.

ATP-initiated dephosphorylation reactions were also analyzed for nucleotide content (Figure 2B,D). The hydrolysis of ATP accompanied the dephosphorylation of both peptide 1 as well as peptide 3. However, the consumption of ATP does not directly correlate with the dephosphorylation of peptide 1 or peptide 3 (Figure 2B,D, respectively).

Barker et al. (1995) demonstrated that N-85-*srcTK* has an inherent ATPase activity. The presence of 1 mM peptide 1 accelerated the rate of ATP hydrolysis by approximately 2-fold. (This was initially determined by HPLC, and the data are available as supporting information.) To examine if this enhancement was due to binding of phosphopeptide to the SH2 domain, a continuous assay was used to determine the kinetic parameters for the hydrolysis of ATP by auto-activated N-85-*srcTK* in the absence or presence of phosphopeptide (Table 2). A concentration of 10 μ M peptide 1 was used, as this low concentration assures that any effect on ATP hydrolysis is due to ligand binding to the SH2

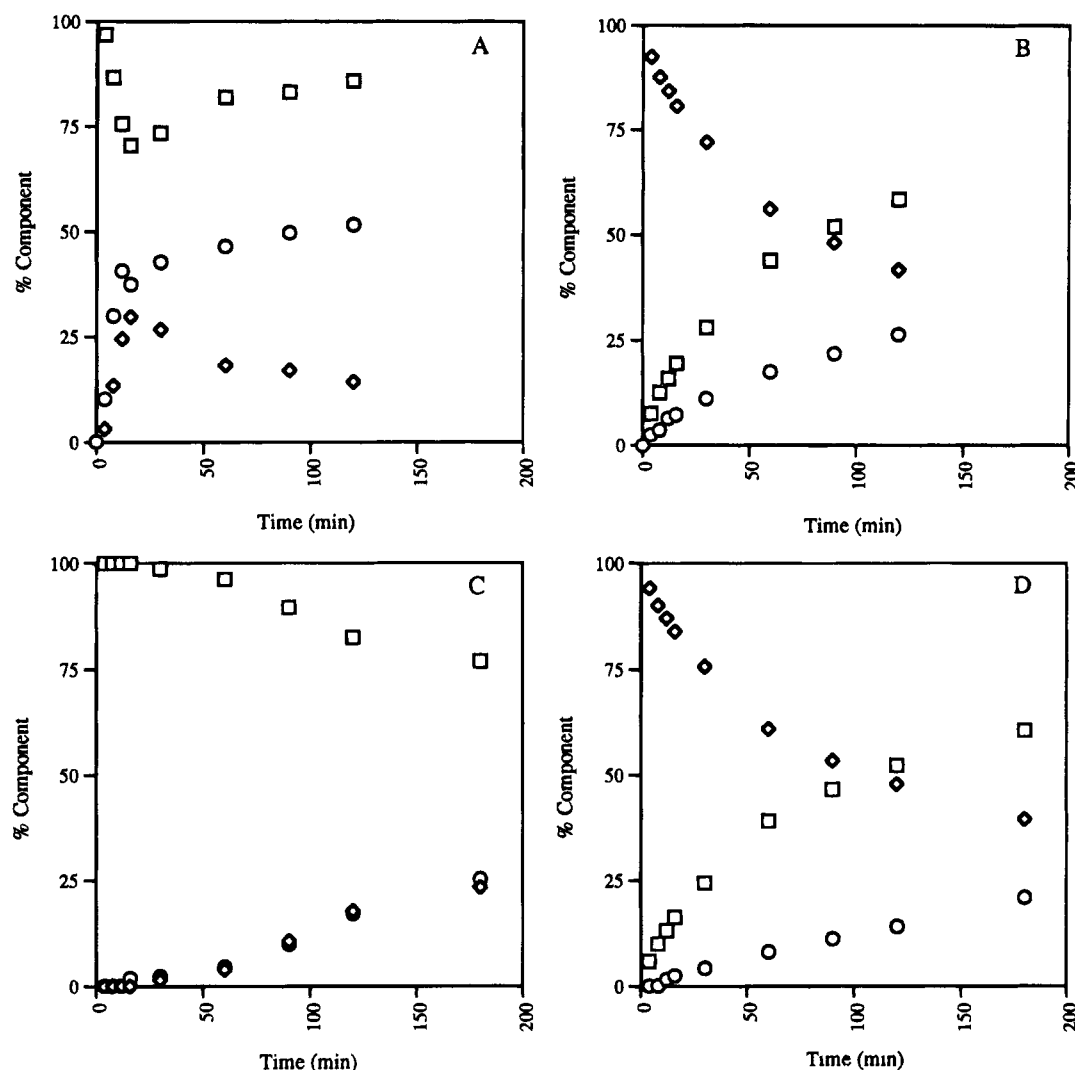


FIGURE 2: Quantitation of nucleotide in dephosphorylation reactions with peptide 1 or peptide 3. Peptide 1 was dephosphorylated in the presence of (A) ADP or (B) ATP. Peptide 3 was dephosphorylated in the presence of (C) ADP or (D) ATP. The percentage of ADP is represented by the squares, the percentage of ATP is represented by the diamonds, and the percentage of dephosphorylated product is represented by the circles. The initial concentrations of peptides and nucleotides were 1 mM, and the concentration of N-85-*src*TK was 5 μ M.

Table 2: Kinetic Parameters for the Hydrolysis of ATP by N-85-*src*TK

	K_m (μ M)	k_{cat} (min^{-1})
no peptide	147 ± 25	1.6 ± 0.1
+10 μ M peptide 1	169 ± 41	3.2 ± 0.3
no peptide	120 ± 31	1.2 ± 0.3
+10 μ M peptide 1	147 ± 26	2.3 ± 0.2

^a One micromolar autoactivated N-85-*src*TK was used to initiate reactions. The results from duplicate experiments are shown, and the errors reported are standard errors.

domain and not dephosphorylation of phosphopeptide.⁵ Hydrolysis of ATP was approximately 2-fold faster in the presence of 10 μ M peptide 1. The K_m for ATP was not affected by the presence of phosphopeptide.

The ATPase activity of N-85-*src*TK and concomitant production of ADP could lead to the dephosphorylation of peptides. Consequently, the dephosphorylation of peptides

1 and 3 was examined in the presence and absence of an ATP regenerating system (Figure 3). Neither peptide was dephosphorylated in the presence of the ATP regenerating system after 3 h, even though approximately 35% of peptide 1 and 20% of peptide 3 are dephosphorylated after 3 h in the absence of the ATP regenerating system. Therefore, ATP can support the dephosphorylation reactions only if it is first hydrolyzed to ADP.

The dephosphorylation reactions were also examined in the presence and absence of an ADP regenerating system (Figure 4). Initially, more peptide 1 was dephosphorylated in the absence of the ADP regenerating system (16% dephosphorylated product in 4 min) than in its presence (10% dephosphorylated product in 4 min). At longer time points, this trend reversed, and more peptide 1 was dephosphorylated in the presence of the ADP regenerating system (95% dephosphorylated product in 180 min) than in its absence (59% dephosphorylated product in 180 min). These results are consistent with those shown in Figure 1A, where more peptide 1 was dephosphorylated in the presence of ADP. In contrast, during the first 60 min the rate of dephosphorylation of peptide 3 was similar in the presence or absence of the

⁵ The same peptide, but without the N-terminal biotin group, was determined by Biacore analysis to have a K_d of approximately 0.5–1.0 μ M to N-85-*src*TK (personal communication, B. Ellis, Glaxo Research Institute).

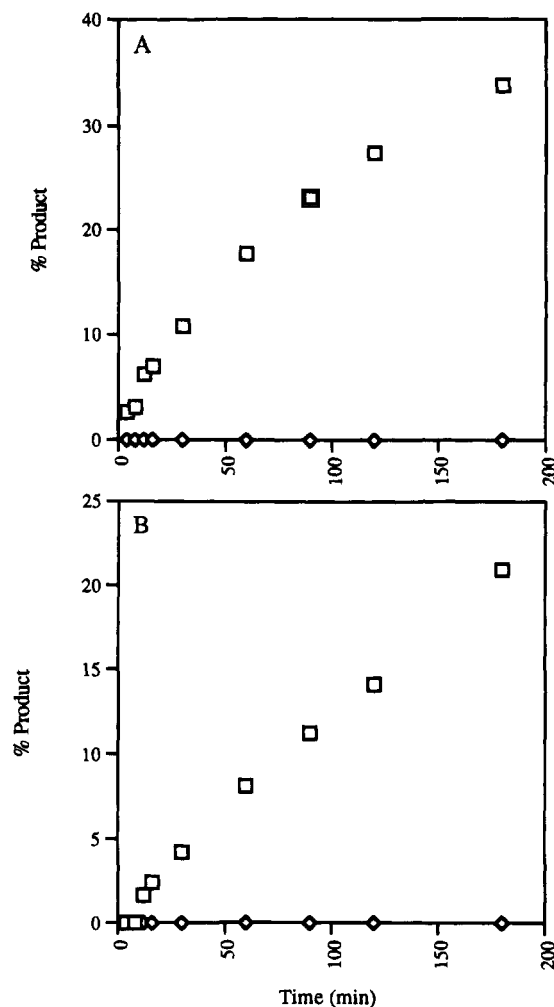


FIGURE 3: Percentage of (A) peptide 1 and (B) peptide 3 dephosphorylated in the presence of ATP (squares). The initial concentrations of ATP and peptides were 1 mM, and the concentration of N-85-*src*TK was 5 μ M. The dephosphorylation of both peptides was also examined in the presence of ATP with regeneration of ATP to maintain the concentration of 1 mM (diamonds).

ADP regenerating system. However, after 60 min, the rate of dephosphorylation of peptide 3 increases in the absence of the ADP regenerating system. These results are consistent with the results shown in Figure 1B, where dephosphorylation of peptide 3 was faster in the presence of ATP than in the presence of ADP. These results demonstrate that the presence of ATP initially accelerates the dephosphorylation of both peptide 1 and peptide 3. However, in the absence of the ADP regenerating system, eventually enough ATP and unphosphorylated peptide will be produced to allow the forward reaction to compete with the reverse reaction (observed only with peptide 1 on this time scale). Therefore, more complete dephosphorylation is observed in the presence of the ADP regenerating system.

One reason for the ATP-stimulated dephosphorylation could be that autophosphorylation of N-85-*src*TK enhances this activity. ESI-MS was used to examine the phosphorylation states of N-85-*src*TK during the ADP-initiated dephosphorylation reactions of peptide 1 and peptide 3. Figures 1 and 4 showed that the ADP-initiated dephosphorylation of peptide 3, but not peptide 1, involved an initial lag in the rate.⁶ The molecular weight profile of N-85-*src*TK was analyzed during the dephosphorylation phase (peptide

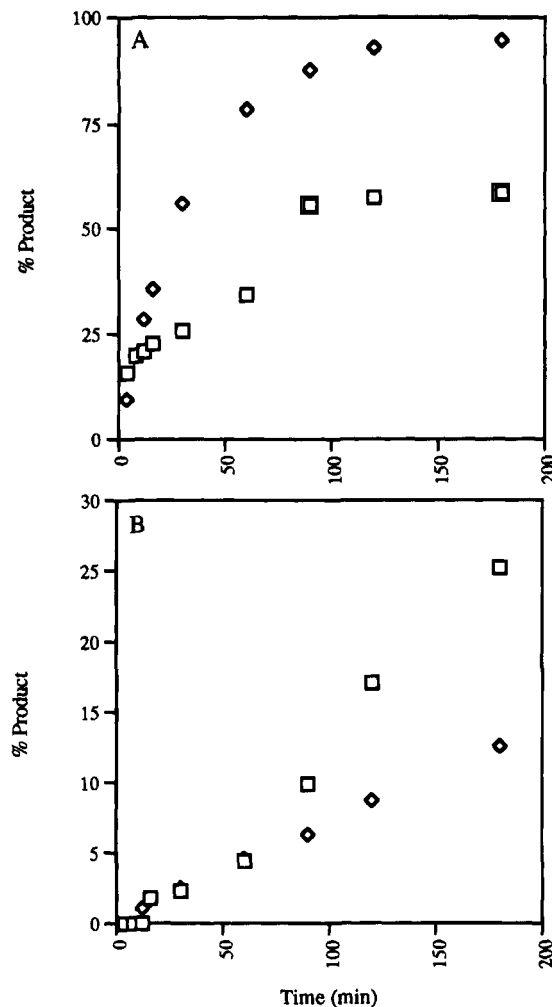


FIGURE 4: Percentage of (A) peptide 1 and (B) peptide 3 dephosphorylated in the presence of ADP (squares). The initial concentrations of ADP and peptides were 1 mM, and the concentration of N-85-*src*TK was 5 μ M. The dephosphorylation of both peptides was also examined in the presence of ADP with regeneration of ADP to maintain the concentration at 1 mM (diamonds).

1, 12 min, Figure 5A; peptide 3, 180 min, Figure 5B) and the lag phase (only seen with peptide 3, 12 min, Figure 5C), and compared with unactivated N-85-*src*TK (Figure 5D). Two predominant phosphorylation states from the control, unactivated N-85-*src*TK sample were detected in the deconvoluted mass spectrum shown in Figure 5D. (Refer to Table 3 for identification of the observed molecular weight species.) These two phosphorylation states correspond to nonphosphorylated and monophosphorylated forms of N-85-*src*TK (with or without the N-terminal methionine present). The N-85-*src*TK samples taken during dephosphorylation phases contained three predominant phosphorylation states corresponding to nonphosphorylated, monophosphorylated, and diphosphorylated forms of N-85-*src*TK (Figure 5A,B). As compared to the control, unactivated N-85-*src*TK sample (Figure 5D), the relative intensity of the peak corresponding to nonphosphorylated N-85-*src*TK diminished, whereas the peaks corresponding to monophosphorylated and diphosphorylated N-85-*src*TK increased in intensity. During the

⁶ The apparent lag time detected with peptide 3 was variable between experiments. The lag time from Figure 1 was approximately 30 min, while the lag time from Figure 7 was approximately 12 min. Therefore, samples were analyzed at the shorter time of 12 min.

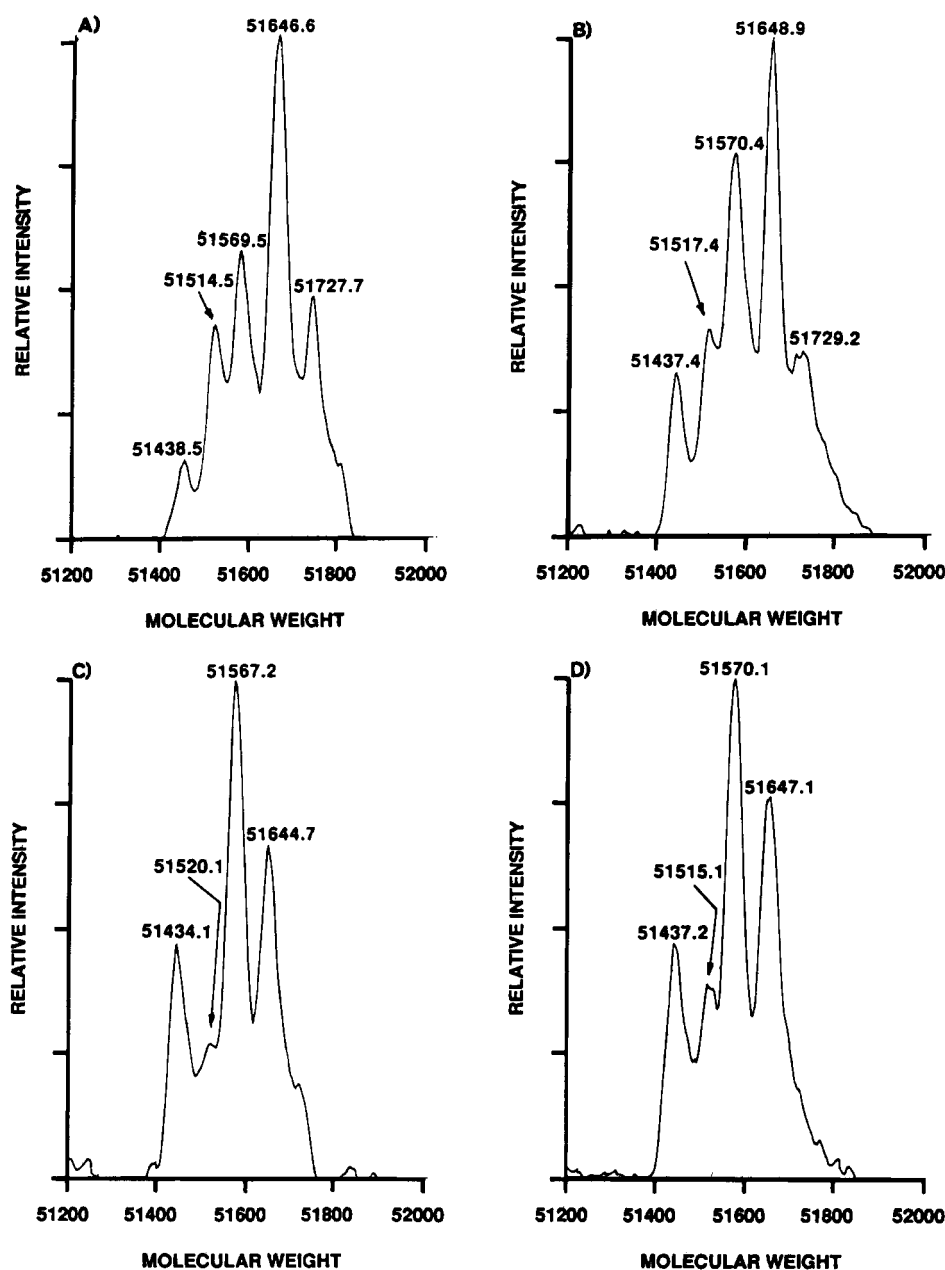


FIGURE 5: ESI-MS of N-85-*src*TK (A) after incubation with peptide 1 and ADP for 12 min; (B) after incubation with peptide 3 and ADP for 180 min; (C) after incubation with peptide 3 and ADP for 12 min; and (D) after incubation for 180 min. In these reactions, the initial concentrations of peptides and ADP were 1 mM, and the concentration of N-85-*src*TK was 5 μ M. Fifty picomoles of N-85-*src*TK was used per mass spectrometry analysis.

Table 3: Identity and Molecular Weight of Species Observed in Figure 5

obsd av MW ^a	calcd MW	phosphorylation state
51436.8	51438.7	nonphosphorylated ^b
51516.8	51518.7	monophosphorylated ^b
51569.3	51569.9	nonphosphorylated
51646.8	51649.9	monophosphorylated
51728.4	51729.9	diphosphorylated

^a Mass determination made with a precision of 0.01% (i.e., ± 5.1 Da). ^b These species correspond to forms of N-85-*src*TK which have lost the N-terminal methionine.

lag phase seen with peptide 3, only nonphosphorylated and monophosphorylated forms of the enzyme are detected with comparable intensities (Figure 5C) as in the control unactivated sample (Figure 5D). These results indicate that N-85-*src*TK is autophosphorylated during the dephosphorylation

reactions, with N-85-*src*TK predominantly in the unactivated form during the lag phase, and predominantly autophosphorylated in the dephosphorylation phase.

Whether autophosphorylation enhances dephosphorylation or is a prerequisite for dephosphorylation was examined by ESI-MS. In the presence of phosphopeptide and the ADP regenerating system (no free ATP), the molecular weight profile of N-85-*src*TK was examined. At the 15 min time point, where approximately 35% of peptide 1 was dephosphorylated (refer to Figure 4A), only nonphosphorylated and monophosphorylated forms of the protein were present, as in the control, unactivated N-85-*src*TK (not shown). These results indicate that autophosphorylation of N-85-*src*TK is not required for its dephosphorylation activity.

A continuous spectrophotometric assay was used to determine the kinetic parameters of the reverse of the kinase

Table 4: Kinetic Parameters for the Reverse of the Kinase Reaction^a

	substrate	activated N-85- <i>src</i> TK ^b	unactivated N-85- <i>src</i> TK ^c
K_m^d (μ M)	peptide 4	592 \pm 29	2175 \pm 154
k_{cat}^d (min^{-1})	peptide 4	199 \pm 3	56 \pm 2
k_{cat}/K_m^d ($\text{mM}^{-1} \text{min}^{-1}$)	peptide 4	336	26
K_m^e (μ M)	ADP	18 \pm 1	20 \pm 2
k_{cat}^e (min^{-1})	ADP	22.4 \pm 0.6	2.1 \pm 0.1
k_{cat}/K_m^e ($\text{mM}^{-1} \text{min}^{-1}$)	ADP	1244	105

^a The errors reported are standard errors. ^b 0.025 μ M N-85-*src*TK was used. ^c 0.18 μ M N-85-*src*TK was used. ^d Determined at 100 μ M ADP. ^e Determined at 133 μ M peptide 4.

reaction, with either activated or unactivated N-85-*src*TK (Table 4). Peptide 4 was chosen as a substrate because (FGE)₃Y(GEF)₂GD yielded a k_{cat}/K_m of 0.3 $\mu\text{M}^{-1} \text{s}^{-1}$ and a K_m of 70 μ M when assayed with 100 μ M ATP in the kinase reaction (Edison et al., 1995).⁷ Activated N-85-*src*TK yielded a k_{cat} that was approximately 4–11-fold higher than with unactivated enzyme, depending on the reaction conditions. Both activated and unactivated N-85-*src*TK had similar K_m 's for ADP and for phosphopeptide. These results show that activated N-85-*src*TK is more catalytically efficient in the reverse of the kinase reaction than unactivated N-85-*src*TK.

DISCUSSION

We have shown that pp60^{c-src}, like pp60^{v-src} (Fukami & Lipmann, 1983), can readily operate in the reverse direction. We have examined the reverse of the *src* tyrosine kinase reaction using phosphotyrosine peptide substrates. In fact, some of the same peptides that are dephosphorylated by *src*TK can also bind with high affinity to the *src* SH2 domain (Gilmer et al., 1994). Both activated and unactivated N-85-*src*TK can catalyze the reverse of the kinase reaction, although activated N-85-*src*TK is a more efficient catalyst. This is the first report demonstrating that autophosphorylation is not required for catalytic activity in the reverse direction.

Unactivated N-85-*src*TK can become phosphorylated during the course of peptide dephosphorylation. The differential dephosphorylation of peptide 1 as compared to peptide 3 can be explained as a consequence of N-85-*src*TK autophosphorylation, relative ATP concentrations, and the fact that peptide 1 is a better substrate than peptide 3. The ADP-initiated dephosphorylation of peptide 1 (and production of ATP) by unactivated N-85-*src*TK was sufficiently fast to allow rapid autophosphorylation of N-85-*src*TK. Because N-85-*src*TK is rapidly autophosphorylated in both the ADP- and ATP-initiated reactions, the ADP-initiated reaction is faster. In contrast, the ADP-initiated dephosphorylation of peptide 3 by unactivated N-85-*src*TK was much slower, resulting in slow autophosphorylation of N-85-*src*TK. However, in the presence of ATP, N-85-*src*TK was rapidly activated, which stimulated the dephosphorylation of peptide 3 as compared to the ADP-initiated reaction. Staurosporine may differentially inhibit the ADP- and ATP-initiated dephosphorylation of peptides 1 and 3 by inhibiting

ATP hydrolysis and/or the autophosphorylation of N-85-*src*TK, although the exact mechanism remains to be determined.

Previous results have shown that phosphotyrosine bonds in proteins are energy rich (Fukami & Lipmann, 1983; Foulkes et al., 1985; Hubler et al., 1989; Argetsinger & Shafer, 1992; Litwin et al., 1992). The phosphotyrosine bond of Btn-TSTEPQ-pY-EEIENL is also energy rich. While true equilibrium conditions were not reached in these experiments, data from Figure 2A were used to estimate an equilibrium constant of 0.16 for the conversion of phosphorylated peptide and ADP to dephosphorylated peptide and ATP. The estimated free energy of hydrolysis for this phosphotyrosine bond was determined to be approximately 1 kcal/mol less than that for the hydrolysis of ATP. The free energies of hydrolysis of phosphotyrosine bonds in the epidermal growth factor receptor (Hubler et al., 1989), the insulin receptor (Argetsinger & Shafer, 1992), p56^{lyn} (Litwin et al., 1992), and IgG (Fukami & Limpan, 1983) have also been reported to be within 1 kcal/mol of the hydrolysis of ATP.

As previously mentioned, autoactivation of N-85-*src*TK increases the catalytic efficiency of the enzyme. These results are consistent with the report by Kmiecik et al. (1988), showing a 5-fold reduction of pp60^{v-src} kinase activity in vitro when Y416 (corresponding to Y419 in pp60^{c-src}) was mutated to a phenylalanine. To determine how Y419 autophosphorylation activates *src*TK, the kinetic parameters for the reverse of the kinase reaction were determined with activated and unactivated N-85-*src*TK. For the reverse of the kinase reaction, autophosphorylation was shown to increase k_{cat} , but to have no significant effect on the K_m for phosphopeptide. Therefore, we propose that phosphorylation of Y419 is not necessary for catalytic binding of peptide substrates. Comparison of the structures surrounding the protein substrate binding pocket in four protein kinases, one in its active, phosphorylated form [cyclic adenosine monophosphate-dependent protein kinase (Knighton et al., 1991)] and three in the inactive, unphosphorylated form [cyclin-dependent kinase 2 (DeBondt et al., 1993), the mitogen-activated protein kinase ERK2 (Zhang et al., 1994), and the insulin receptor tyrosine kinase (Hubbard et al., 1994)], indicates that phosphorylation of residue(s) within the activation loop promotes movement of this loop which opens up the protein substrate binding pocket (DeBondt et al., 1993; Taylor & Radzio-Andzelm, 1994; Zhang et al., 1994; Hubbard et al., 1994). Although the unphosphorylated "activation loop" in *src*TK does not seem to interfere with binding of peptide substrates, phosphorylation of this loop may or may not be required for binding of the bulkier, protein substrates.

For the reverse of the kinase reaction, autophosphorylation was shown to have no effect on the K_m for ADP, suggesting that phosphorylation of Y419 is not necessary for nucleotide binding in *src*TK. Although ATP can bind to the inactive forms of cyclin-dependent kinase 2 and ERK2, at least in the latter case, it is thought that activation is necessary for proper orientation of ATP for catalysis (DeBondt et al., 1993; Zhang et al., 1994). While activation is not required for catalytically competent binding of ADP to *src*TK, it is possible that activation is required for ATP binding and/or proper orientation of ATP for catalysis.

In the insulin receptor tyrosine kinase, a tyrosine residue located in the activation loop is hydrogen-bonded to an aspartate residue (the catalytic base), and an arginine residue,

⁷ (FGE)₃Y(GEF)₂GD was first identified as a "good" substrate for pp60^{c-src} by R. J. A. Budde (M. D. Anderson, University of Texas, Houston, TX, personal communication) and confirmed by Edison et al. (1995).

blocking ATP from binding at the active site (Hubbard et al., 1994). Therefore, phosphorylation of this tyrosine residue is necessary for ATP binding and catalysis. Because ATP cannot bind to unphosphorylated IRTK, it is argued that activation of the insulin receptor occurs through inter-subunit autophosphorylation of this tyrosine residue (Hubbard et al., 1994). Activation of *src*TK occurs through an intermolecular autophosphorylation event (Barker et al., 1995). However, the ability of unactivated *src*TK to bind ADP and possibly ATP suggests that intramolecular autophosphorylation is also possible. These results indicate that there may be structural differences between *src*TK and IRTK in the nucleotide binding pocket, and suggest that differences may also exist in the mechanism of activation.

Although the *src*TK reaction is readily reversible *in vitro*, is it physiologically relevant? The cellular ratio of ADP to ATP has been reported to be less than 0.2 (Argetsinger & Shafer, 1992). However, the K_m for ADP in the reverse direction is approximately 10-fold lower than the K_m for ATP in the forward direction (80–140 μ M, Knight and Barker, personal communication). This difference in the K_m could offset the low ADP to ATP ratio. In addition, *src*TK has an inherent ATPase activity which is stimulated by phosphotyrosine peptide binding to the *src* SH2 domain. Coincidentally, the consensus sequence for binding to the *src* SH2 domain shares some homology to the consensus sequence for binding to the *src*TK domain (Cantley & Songyang, 1994; Songyang et al., 1995), which may indicate that protein substrates are channeled from the SH2 domain to the tyrosine kinase domain. Therefore, under physiological conditions, phosphotyrosine proteins could be recruited by the *src* SH2 domain, thus stimulating ATP hydrolysis to generate ADP, and channeled to the tyrosine kinase domain where they undergo dephosphorylation. Alternatively, binding to the *src* SH2 domain may serve to position phosphotyrosine protein substrates near the tyrosine kinase active site, allowing for protein dephosphorylation.

This report identifies interactions between domains, and their impact on the catalytic activity of *src*TK. Occupancy of the *src* SH2 domain was shown to enhance the ATPase activity of the tyrosine kinase domain, but to have no influence on the K_m for ATP. Previous reports have suggested that binding to the *src* SH2 domain can activate the tyrosine kinase activity (Liu et al., 1993). This activation is proposed to arise from the phosphopeptide competing with the phosphorylated C-terminal tail for binding to the *src* SH2 domain (Liu et al., 1993). Another report has indicated that binding to the SH2 domain is influenced by binding to the SH3 domain, and vice versa (Panchamoorthy et al., 1994). These interactions were proposed to give rise to novel mechanisms for the regulation of *src*TK (Panchamoorthy et al., 1994). Future studies will concentrate on characterizing interdomain interactions and their impact on *src*TK catalytic activity.

This report also suggests that the reverse as well as the forward *src* tyrosine kinase reactions may be important in regulating the intracellular levels of protein tyrosine phosphorylation. The increase in protein tyrosine phosphorylation associated with certain carcinomas has been explained by elevated protein levels and increased specific activity of *src*TK (Bolen et al., 1985; Rosen et al., 1986; Bolen, 1987; Cartwright et al., 1989, 1990). However, a decrease in the activity of the reverse of the *src* tyrosine kinase reaction

would have the same effect. Other enzymes, including kinases, have been identified in prokaryotes, eukaryotes, and plants, which catalyze opposing reactions under physiological conditions (Caban & Ginsburg, 1976; El-Maghrabi et al., 1982; LaPorte & Koshland, 1982; Garcia & Rhee, 1983; Burnell & Hatch, 1986). Isocitrate dehydrogenase kinase/phosphatase is a prokaryotic enzyme which turns isocitrate dehydrogenase on and off by dephosphorylating and phosphorylating it, respectively (LaPorte & Koshland, 1982). In plants, pyruvate, P_i dikinase regulatory protein operates to phosphorylate and dephosphorylate pyruvate, P_i dikinase, resulting in its inactivation and activation, respectively (Burnell & Hatch, 1986). *src*TK may play a similar role, regulating the activity of other enzymes through phosphorylation and dephosphorylation. If both the phosphorylation and dephosphorylation activity of *src*TK are important, then understanding what regulates the two activities may lead to important advances in our understanding of cancer. While the physiological relevance of these dephosphorylation reactions is unclear, their potential significance should be further investigated.

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SUPPORTING INFORMATION AVAILABLE

A figure of peptide 1 and peptide 3 dephosphorylation inhibited by staurosporine, a figure of the dephosphorylation of peptide 1 and phosphorylation of peptide 2 in the presence of ATP or ADP, and a figure of the hydrolysis of ATP in the presence and absence of 1 mM peptide 1 (3 pages). Ordering information is given on any current masthead page.

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