Reciprocal Regulation of Hck Activity by Phosphorylation of Tyr$^{527}$ and Tyr$^{416}$

EFFECT OF INTRODUCING A HIGH AFFINITY INTRAMOLECULAR SH2 LIGAND

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The Src family tyrosine kinase Hck possesses two phosphorylation sites, Tyr$^{527}$ and Tyr$^{416}$, that affect the catalytic activity in opposite ways. When phosphorylated, Tyr$^{527}$ and residues C-terminal to it are involved in an inhibitory intramolecular interaction with the SH2 domain. However, this sequence does not conform to the sequence of the high affinity SH2 ligand, pYEEI. We mutated this sequence to YEEI and show that this mutant form of Hck cannot be activated by exogenous SH2 ligands. The SH3 domain of Hck is also involved in an inhibitory interaction with the catalytic domain. The SH3 ligand Nef binds to and activates YEEI-Hck mutant in a similar manner to wild-type Hck, indicating that disrupting the SH3 interaction overcomes the strengthened SH2 interaction. The other phosphorylation site, Tyr$^{416}$, is the autophosphorylation site in the activation loop. Phosphorylation of Tyr$^{416}$ is required for Hck activation. We mutated this residue to alanine and characterized its catalytic activity. The Y416A mutant shows a higher $K_m$ value for peptide and a lower $V_{max}$ than autophosphorylated wild-type Hck. We also present evidence for cross-talk between the activation loop and the intramolecular binding of the SH2 and SH3 domains.

In addition to the negative regulatory roles of the SH2 and SH3 domains, they are also able to target Src kinases to their substrates (Fig. 1). In this model, binding of substrates by the SH2 and/or SH3 domains would target Hck to potential substrates and concomitantly activate the catalytic domain. Evidence that supports this model has been obtained for Src. Src associates with its substrates p130Cas and AFAP-110 via direct binding of the SH2 and SH3 domains of Src to ligand binding motifs in the substrates (20, 21). These interactions appear to enhance the catalytic activity of Src; a peptide containing the SH2 and SH3 ligand motifs of Sin, a p130Cas-related Src substrate, activates c-Src (22).

For Src, the sequences of the intramolecular ligands for the SH2 domain and SH3 domain do not conform to the highest affinity sequences as determined for the isolated domains (11, 23). The SH2 domain ligand in the C-terminal tail of Src, pYQPG, is not the highest affinity sequence. Peptide library studies showed that the preferred SH2 ligand sequence is pYEEI (23). The binding affinity of these sequences to the SH2 domain of Src was determined by isothermal titration calorimetry (24). The $K_d$ values for the pYEEI-containing peptide and the pYQPG-containing peptide are 0.2 μM and 29 μM, respectively (24). Thus, the SH2 domain of Src displays a 150-fold higher affinity for the high affinity sequence, pYEEI, than for the tail sequence, pYQPG. Our premise is that the intramolecular SH2-binding site is lower in affinity so that exogenous ligands containing higher affinity sequences can compete for binding to the SH2 domain (11). We mutated the C-terminal tail of Hck from YQQQ to YEEI to determine the effect of introducing a high affinity intramolecular ligand on enzyme regulation and substrate targeting.

A recent crystal structure of YEEI-Hck in complex with the inhibitor PP1 is relevant to our mutant studies (Fig. 2A) (7). The relative orientation of SH3, SH2, and catalytic domains is unchanged from WT Hck (Fig. 2A). Importantly, this is the first Hck structure to model the activation loop (residues 404–432) (7). The activation loop containing unphosphorylated Tyr$^{416}$ is positioned such that access of peptide substrates to the catalytic machinery is blocked (Fig. 2B) (7). It has been shown previously for Lck that the activation loop, when phosphorylated, is not in the active site (Fig. 2B) (25). These data, together with the fact that autophosphorylation on Tyr$^{416}$ in the activation loop is required for maximum activity (15, 26–31), are consistent with a model where autophosphorylation causes a change in the conformation of the activation loop. In this model, autophosphorylation increases the catalytic activity of the enzyme by causing the activation loop to move out of the substrate-binding site, thereby allowing access to substrates. We mutated Tyr$^{416}$ to alanine to investigate the relationship between autophosphorylation and kinase activity for Hck. We...
also used this mutant to investigate the relationship between autophosphorylation and down-regulation by intramolecular binding of SH2 and SH3 domains.

The crystal structure of down-regulated Hck (7) also suggests that the orientation of helix C in the catalytic domain is a critical determinant of enzymatic activity. Before Hck is autophosphorylated, Glu310 on helix C is pointed out of the active site. In crystal structures of active protein kinases such as Lck, helix C is rotated relative to its position in Hck, and Glu310 projects inward toward the ATP-binding site (7, 11). When Hck is activated by autophosphorylation, Glu 310 forms an ion pair with Lys295 and positions this residue to coordinate the α and β phosphates of ATP (11). To test the importance of this ion pair in Hck catalysis, we mutated Glu310 to alanine and measured the catalytic activity of the enzyme.

MATERIALS AND METHODS

Protein Expression and Purification—C-terminally phosphorylated WT Hck and mutant forms of the enzyme were produced in Spodoptera frugiperda (SF9) cells (5, 7). His-tagged WT Hck, E310A Hck, and YEEI Hck were purified as described (7), except that the final purification step for WT Hck was a ϑ-phosphate-linked ATP-Sepharose column (31). After the final column the protein was visualized as a single band by SDS-PAGE.

The purification method for Y416A Hck was similar, but several minor modifications were made. Cells expressing His-tagged Y416A Hck were lysed in a French pressure cell in 50 ml of HiLoad Q buffer A (20 mM Tris, pH 8.5, 10% glycerol, 5 mM ϑ-mercaptoethanol) containing protease inhibitors (5 mM/liter aprotinin, 5 mM/liter leupeptin, 0.1 mM phenylmethylsulfonyl fluoride). Cell lysate was diluted to 200 ml, centrifuged, and filtered. The first step of purification was two 5-ml HiTrap SP columns (Amersham Pharmacia Biotech) in series with a HiLoad 26/10 Q-Sepharose High Performance (Amersham Pharmacia Biotech). The HiTrap SP columns were removed, and protein was eluted from the HiLoad Q column with a linear NaCl gradient. Fractions containing Hck were pooled, and NaCl and imidazole were added to match the column-loading buffer (20 mM Tris, pH 8.5, 5% glycerol, 5 mM ϑ-mercaptoethanol, 1 mM NaCl and 20 mM imidazole) for the 20 ml nickel nitritrolaetic acid Superflow column (Qiagen). Hck was loaded onto the column, washed in buffer (20 mM Tris, pH 8.5, 5% glycerol, 5 mM ϑ-mercaptoethanol), and eluted with a linear imidazole gradient. After pooling Hck-containing fractions, the His-tag was cleaved overnight on ice using a final concentration of 0.02 mg/ml His-tagged Tev protease. Complete cleavage of His tag was determined by SDS-PAGE. Buffer was exchanged with Mono Q buffer A (20 mM Tris, pH 8.5, 5 mM ϑ-mercaptoethanol) to remove imidazole. 2 ml of nickel nitritrolaetic acid resin was added to 20 ml of sample and incubated for 1 h at 4 °C with rocking to remove His-tagged Tev protease. The unbound fraction and one wash (5 ml) were collected and pooled and concentrated to 0.5 ml on an Ultrafree-15 centrifugal filter unit (Millipore) and loaded on a Superdex 75 HR 10/30 equilibrated in 20 mM Tris, pH 8.5, 50 mM NaCl, and 3 mM dithiothreitol. After the final column the protein was visualized as a single band by SDS-PAGE. The concentration of Y416A was determined using the Bradford method (Bio-Rad).

For WT and each of the mutants, we confirmed that Tyr527 was phosphorylated by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry analysis (5). Treatment of the sample with Vearnin protein tyrosine phosphatase caused a loss of 80 molecular mass units from the tryptic fragment containing Tyr527, confirming its phosphorylation state.

HIV-1 NL4-3 Nef protein was expressed as a glutathione S-transferase fusion protein in Escherichia coli NB42 cells. Cells were lysed in a French pressure cell in buffer containing 50 mM HEPES, pH 7.4, 100 mM NaCl, 100 mM EDTA, 1% Triton X-100, 10% glycerol, and protease inhibitors (5 mg/liter aprotnin, 5 mg/liter leupeptin, 0.1 mM phenylmethylsulfonyl fluoride). Cell lysate was centrifuged and added to glutathione-agarose at 4 °C (Molecular Probes). The pellet was extracted with 1.5% N-lauroyl sarcosine, 25 mM triethylamamine, 100 mM EDTA, and protease inhibitors (5 mg/liter aprotnin, 5 mg/liter leupeptin, 0.1 mM phenylmethylsulfonyl fluoride) at 4 °C for 30 min (32). The sample was centrifuged, and the detergent-extracted supernatant was added to the glutathione beads. After a 1-h incubation at 4 °C, the beads were washed. Glutathione-agarose with immobilized GST-Nef was used directly in ligand binding experiments or treated with 20 mM glutathione in 50 mM Tris, pH 8 to elute GST-Nef. The concentration of GST-Nef was determined by the Bradford method (Bio-Rad).

Synthetic Peptides—Peptides were prepared by solid phase synthesis on an Applied Biosystems automated 431A peptide synthesizer. The peptides were purified by reverse-phase high-pressure liquid chromatography and characterized by MALDI-TOF. The sequences of the peptides used are: substrate peptide for phosphocellulose assay, Arg-Arg-Leu-Glu-Asp-Ala-His-Tyr-Ala-Arg-Gly (31); substrate peptide for the coupled assay, Ala-Glu-Glu-Glu-Ile-Tyr-Gly-Glu-Phe-Glu-Ala-Ala-Lys-Lys-Lys-Lys-Gly (33); SH2 binding peptide (pYEEI), Glu-Pro-Gln-TyrP-Glu-Glu-Ile-Pro-Ile-Lys-Gln (31); peptide containing both SH2 domain binding motif (pYEEI) and substrate motif, Arg-Arg-Leu-Glu-Asp-Ala-Ile-Tyr-Ala-Ala-Gly-Gly-Gly-Gly-Glu-Pro-Gln-TyrP-Glu-Glu-Ile-Gly (34); the control peptide for the pYEEI-containing subunit has the same sequence except TyrP is substituted by Phe (34).

Protein Kinase Assays—Kinase assays were performed by two methods: (i) the phosphocellulose paper assay (35, 36) and (ii) a coupled spectrophotometric assay (29). All experiments were carried out at 30 °C. The phosphocellulose assay buffer contained 30 mM Tris, pH 7.4, 20 mM MgCl2, 1 mg/ml bovine serum albumin, 0.84 mM peptide substrate, 0.5 mM ATP, and 100–500 cpm/mmol (γ-32P)ATP. The phosphocellulose assay reactions were terminated by the addition of 10% ice-cold trichloroacetic acid and centrifuged, and aliquots were spotted onto p81 phosphocellulose paper (Whatman). The phosphocellulose paper was washed and counted in a scintillation counter to measure incorporation of 32P into peptide. E310A mutant and a WT control were assayed in duplicate both with and without preincubation (1 h, 4 °C, 1 μM enzyme, 200 μM ATP) in a final concentration of 80 nm enzyme.

WT, YEEI mutant, and Y416A Hck were assayed by the spectropho-
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**Results**

**YEEI-Hck**—The association between the SH2 domain and the C-terminal tail has an inhibitory effect on the catalytic activity of WT Hck (15). YEEI-Hck was designed to have a higher affinity association between the C-terminal tail and the SH2 domain. We first studied the kinetic properties of YEEI-Hck. YEEI-Hck is activated by autophosphorylation with similar kinetics to WT (Table I). Using autophosphorylated enzyme, we also determined that the ATP $K_a$ and $V_{max}$ were similar for WT and YEEI-Hck (Table I). Although dephosphorylation of Tyr$^{527}$ in WT Hck is known to promote autophosphorylation, assays were done in 250 μM ATP and 600 μM peptide substrate, with no preincubination of enzyme with ATP.

**Mutagenesis**—Mutagenesis was performed using the Stratagene QuikChange kit according to the manufacturer’s directions. Mutations were confirmed by DNA sequencing.

**Nef Binding Experiments**—Increasing amounts of WT Hck or YEEI-Hck were added to GST-Nef immobilized on glutathione-agarose beads in buffer containing 50 mM Tris, pH 7.5, 250 mM NaCl, 0.1% Triton X-100, 5 mM EDTA, 0.5 mM sodium vanadate, and 1 mM dithiothreitol in a final volume of 140 μl. After a 30-min incubation at 4 °C, the beads were washed in same buffer. Hck bound to Nef was eluted in 50 μl of 5× Laemmli buffer and resolved using SDS-PAGE. The proteins were transfected to polyclonal antibodies against Nef and were detected with anti-c-Src rabbit polyclonal antibody (Upstate Biotechnology Inc.), anti-rabbit horseradish peroxidase-conjugated secondary antibody, and an enhanced chemiluminescent detection kit (Amersham Pharmacia Biotech).

**RESULTS**

The association between the SH2 domain and the C-terminal tail has an inhibitory effect on the catalytic activity of WT Hck (15). YEEI-Hck was designed to have a higher affinity association between the C-terminal tail and the SH2 domain. We first studied the kinetic properties of YEEI-Hck. YEEI-Hck is activated by autophosphorylation with similar kinetics to WT (Table I). Using autophosphorylated enzyme, we also determined that the ATP $K_a$ and $V_{max}$ were similar for WT and YEEI-Hck (Table I). Although dephosphorylation of Tyr$^{527}$ in WT Hck is known to promote autophosphorylation (15), engineering a stronger intramolecular SH2 interaction does not affect the kinetic properties of the catalytic domain.

We measured the accessibility of the SH2 domain of YEEI-Hck to exogenous ligands in two ways. First, we measured $K_{act}$ for Nef, the concentration of ligand required to half-maximally activate the enzyme, for a synthetic phosphopeptide containing the high affinity SH2 binding sequence pYEEI. Autophosphorylated WT is activated by this peptide approximately 2-fold with a $K_{act}$ of 31 μM (Fig. 3A and Table I). In contrast, the YEEI mutant form of Hck is not activated by pYEEI peptide at concentrations up to 500 μM. Therefore, the $K_{act}$ for YEEI-Hck is more than an order of magnitude higher than WT (Fig. 3A). The second method we used to show that the SH2 domain of YEEI-Hck was not available for binding exogenous ligands involved the use of a substrate containing a pYEEI motif. Previous experiments from this laboratory showed that WT Hck phosphorylated substrates containing pYEEI sequences at an enhanced rate compared with control substrates with an SEEEI sequence (34). This SH2 domain-pYEEI interaction presumably promotes phosphorylation of other tyrosines within the substrates by raising the effective local concentration of potential phosphorylation sites near the active site. This substrate is not phosphorylated at a higher rate by YEEI-Hck (Fig. 3B). These results suggest that exogenous pYEEI peptide cannot compete with an intramolecular pYEEI motif.

The extent to which the intramolecular binding of the SH2 domain affects the ability of SH3 domain ligands to activate Hck is not fully understood. We determined whether a strengthened SH2 domain interaction affects the SH3 domain interaction by measuring $K_{act}$ for Nef, a potent ligand for the SH3 domain of Hck (15, 39). If the intramolecular SH3 domain interaction depended on the affinity of SH2 domain interaction, YEEI-Hck would be expected to have a higher Nef $K_{act}$. However, YEEI-Hck has a slightly lower $K_{act}$ for Nef than WT (Table I), indicating that the higher affinity SH2 domain inter-
Kinetic parameters were determined using the spectrophotometric assay. Enzymes were preincubated with ATP to allow autophosphorylation on Tyr$^{527}$ prior to kinetic analysis except in the case of lag time measurements. Determinations of $V_{\text{max}}$, $K_{\text{act}}$, and $K_m$ were carried out at 10 nM enzyme. For determination of $K_m$ for ATP, the peptide concentration was held constant at 600 $\mu$M. Lag time for autophosphorylation was determined at 50 nM enzyme.

| ATP | $K_m$ | $V_{\text{max}}$ | $p$YEEI peptide | Nef | Lag time for autophosphorylation |
|-----|--------|------------------|-----------------|-----|-------------------------------|
|     | $\mu$M | $\mu$mol min$^{-1}$ mg$^{-1}$ | $\mu$M | $\mu$M | min |
| WT Hck | 97 ± 8 | 31 ± 3 | 31 ± 6 | 18 ± 3 | 17 |
| YEEI Hck | 106 ± 11 | 31 ± 1 | >500 | 9 ± 1 | 15 |

![Graph A](image1)

![Graph B](image2)

**FIG. 3.** YEEI-Hck binds SH3 ligands but not SH2 ligands. A, WT Hck can be activated by an SH2 ligand, but YEEI cannot. Increasing concentrations of pYEEI peptide (0–550 $\mu$M) were added to 10 nM autophosphorylated enzyme, either WT (circles) or YEEI-Hck (squares). Initial rates for phosphorylation of substrate peptide (600 $\mu$M) were measured using the spectrophotometric assay, as described in the text. $v_a$ is the velocity measured in the presence of pYEEI peptide minus the velocity measured in its absence (38). B, peptides with SH2 domain binding motifs are phosphorylated preferentially by WT Hck but not by YEEI Hck. The pYEEI substrate sequence is RRLEDAYAGGGGG-EPPQpYEEIG (pY is phosphorylated tyrosine), and the YEEI-containing substrate sequence is RRLEDAYAGGGGGGEPQQYEEIG. Initial rates were determined by the spectrophotometric assay using 35 $\mu$M peptide substrate and 500 $\mu$M ATP. The reactions were carried out with autophosphorylated WT or YEEI-Hck.

**FIG. 4.** Similar binding of WT and YEEI-Hck to immobilized GST-Nef. Increasing amounts of Hck (WT or YEEI-Hck) were added to GST-Nef immobilized on glutathione-agarose beads; the numbers above the lanes indicate the micrograms of Hck added. The beads were washed to remove unbound enzyme, and the bound protein was eluted by boiling in 5× Laemmli buffer and resolved by SDS-PAGE. Hck was visualized by Western blotting with anti-Pan Src family polyclonal antibody (Upstate Biotechnology, Inc.) (31).

Glu$^{310}$ to alanine. We analyzed this mutant using the phosphocellulose paper assay. Preincubated E310A mutant and a WT control were assayed in duplicate in a final concentration of 80 nM enzyme. For E310A, 200 counts/min above background were incorporated into peptide after a 10-min reaction. For WT, 89,800 counts/min above background were incorporated into peptide. Therefore, we estimate that mutation of this one residue decreases kinase activity under these conditions by >400-fold.

Y$^{416}$A—Phosphorylation of Tyr$^{416}$ leads to activation of Hck (15, 25–29). In the absence of SH2 or SH3 ligands, autophosphorylation is a relatively slow, intermolecular process (15). Displacement of the regulatory apparatus dramatically increases the rate of autophosphorylation (15). The proposed connection between phosphorylation and activation is that phosphorylation of Tyr$^{416}$ causes a conformational change in the activation loop that causes it to move out of the catalytic site (Fig. 2B). Before Hck has undergone autophosphorylation, the activation loop containing Tyr$^{416}$ blocks the active site such that peptide substrate cannot bind (7). This blockage of the active site likely increases substrate $K_m$ and lowers the rate of substrate turnover. However, we are unable to accurately measure the kinetics of nonautophosphorylated Hck because addition of ATP to Hck immediately results in the formation of some amount of autophosphorylated Hck, producing a mixture of autophosphorylated and nonautophosphorylated Hck. In the first 5 min after the addition of ATP, autophosphorylated Hck phosphorylates 12-fold more substrate than Hck that has not been preincubated with Hck (Fig. 5, inset). Since autophosphorylation significantly increases the activity of Hck, the generation of even a small amount of autophosphorylated Hck prevents us from obtaining accurate kinetic measurements for nonautophosphorylated Hck.

To further investigate the role of autophosphorylation, we...
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Kinetic parameters were determined using the spectrophotometric assay. Wild-type Hck was preincubated with ATP to allow autophosphorylation on Tyr^{416} prior to kinetic analysis. Determinations of V_{max} for WT Hck and Y416A mutant were carried out at 10 nM enzyme. For determination of ATP K_{m}, the peptide concentration was held constant at 600 μM. For determination of peptide K_{act}, the ATP concentration was held constant at 500 μM.

Fig. 5. Y416A cannot be activated by autophosphorylation. 10 nM WT without ATP preincubation (dotted line), 10 nM Y416A Hck (dashed line), or WT preincubated with ATP (solid line) was added to 250 μM ATP and 600 μM peptide substrate, and peptide phosphorylation was monitored by the spectrophotometric assay. Data were recorded every 9 s.

generated a mutant in which Tyr^{416} is mutated to alanine. We reasoned that alanine rather than phenylalanine would be the best substitute for Tyr^{416}. In the structure of down-regulated Hck, a network of hydrogen-bonding interactions involving the side chain of Tyr^{416} helps to stabilize the activation loop. Neither phenylalanine nor alanine would be able to participate in these hydrogen bonds, so neither change is a good mimic of the unphosphorylated state. A potential problem with any amino acid substitution at Tyr^{416} is that the conformation of the activation loop could be destabilized and become exposed to solvent. Therefore, we selected alanine to avoid the interaction of the bulky hydrophobic side chain of tyrosine with solvent.

The mutant Y416A enzyme has the expected linear relationship between enzyme concentration and activity (data not shown). Since Y416A cannot be activated by autophosphorylation at Tyr^{416} its activity (V_{max}) is lower than autophosphorylated WT (Table II). However, the Y416A mutant is activated relative to down-regulated Hck (Fig. 5 and Fig. 5 inset). This may be due to the fact that tyrosine is better able than alanine to participate in the contacts in the active site that stabilize the inhibitory conformation of the activation loop. Fig. 5 shows that Y416A cannot be activated by autophosphorylation. Nonautophosphorylated WT Hck shows the increase in activity with time that is characteristic of autophosphorylation, whereas Y416A activity is linear with time similar to autophosphorylated Hck.

Consistent with the fact that autophosphorylation improves peptide binding by moving the activation loop containing Tyr^{416} out of the active site, the K_{m} value for peptide substrate is 2-fold higher for Y416A than for WT (Table II). The K_{m} for ATP was not changed by the mutation, indicating that autophosphorylation does not effect the ATP-binding site of Hck (Table II). The K_{act} values for both pYEEI and Nef are increased for Y416A (Table II). This suggests a correlation between activation by autophosphorylation and increased availability of the SH2 and SH3 domains for substrate binding.

### DISCUSSION

In this paper, we describe experiments aimed at understanding the opposing roles of Tyr^{527} and Tyr^{416} in regulation of Src family kinases. Our model, based on structural analysis (7) and kinetic data (Table II), is that the Y416A mutant is intermediate between unphosphorylated and autophosphorylated WT, both in terms of its predicted structure and its enzymatic activity. Y416A is not a perfect mimic of the unphosphorylated state because alanine cannot participate in the contacts in the active site that stabilize the inhibitory conformation of the activation loop. However, kinetic data from Y416A sheds light on the properties of unphosphorylated WT Hck. Our observation that Y416A has a lower V_{max} than WT (Table II) supports the structural work showing that autophosphorylation stabilizes the activation loop in an active conformation (7). The K_{m} for peptide substrate is higher for Y416A than WT (Table II), consistent with the hypothesis that the activation loop blocks the peptide-binding site when Tyr^{416} is unphosphorylated. The K_{m} for ATP is unchanged (Table II), indicating that phosphorylation and subsequent rearrangement of the active site does not appear to affect the ATP-binding site.

Comparisons of the three-dimensional structures of down-regulated kinases (4, 5, 7) with those of an active kinase (25) indicate that the “inward” conformation of helix aC (11) is necessary for activity. Glu^{310} on helix aC forms an ion-pair with Lys^{295} that stabilizes the inward conformation. Our results on the E310A mutant provide evidence that Glu^{310} is indispensable for kinase activity and strongly suggest that the active conformation of Src family kinases absolutely requires the ion pair between Glu^{310} and Lys^{295} for activity.

Our data also support the idea of cross-talk between the active site and the intramolecular binding of SH2 and SH3 domains. The K_{act} values for both pYEEI and Nef are increased for Y416A relative to WT (Table II). This suggests that Hck has a higher tendency to bind exogenous ligands once it is autophosphorylated. Consistent with this, the K_{act} of an SH3 ligand is 10-fold higher for nonautophosphorylated Hck than for autophosphorylated Hck. Although there appears to be a synergistic effect between ligand binding and autophosphorylation, it is not known whether ligand binding precedes autophosphorylation or is a result of autophosphorylation for Hck in vivo. It has previously been shown that binding of exogenous ligands promotes autophosphorylation (31). This work shows that the converse may also be true: autophosphorylation promotes exogenous ligand binding.

The temporal order of autophosphorylation and ligand binding may vary for Src family kinases in vivo depending on the cell type and signaling stimulus. However, the interdependence of autophosphorylation and ligand binding suggests the following scenario: binding of an initial ligand activates Hck and triggers autophosphorylation. Once autophosphorylated, Hck has an enhanced ability to bind other downstream binding partners and substrates.

We also engineered a mutant form of Hck with a high affinity for peptide substrate when Tyr^{416} is unphosphorylated. The K_{m} for ATP is unchanged (Table II), indicating that phosphorylation and subsequent rearrangement of the active site does not appear to affect the ATP-binding site.

### TABLE II

| ATP K_{m} | Peptide K_{m} | V_{max} | pYEEI peptide K_{act} | Nef K_{act} |
|----------|--------------|---------|-----------------------|-------------|
| μM       | μM           | μmol min^{-1} mg^{-1} | μM           | μM          |
| WT Hck   | 97 ± 8       | 302 ± 35 | 31 ± 3               | 31 ± 6      | 18 ± 3     |
| Y416A    | 71 ± 3       | 647 ± 92 | 12 ± 1               | 87 ± 16     | 27 ± 8     |

2 M. Porter, W. T. Miller, J. Nguyen, and W. Lim, unpublished observations.
interaction has been demonstrated to be required for targeting Src kinase-signaling pathways. The Src SH3 domain ligand overrides a strengthened SH2-tail interaction. These observations indicate that the SH3 domain binds ligand in a way that is independent of the intramolecular binding of the SH2 domain. Disruption of the SH3 interaction with ligand can override a strengthened SH2-tail interaction. These observations suggest that SH3 domains have a major role in directing Src kinase-signaling pathways. The Src SH3 domain ligand interaction has been demonstrated to be required for targeting to Sin (18), AFAP-110 (17), synapsin I (37), Fak (38), and Cas.3

Our results for YEEI-Hck provide evidence for the model depicted in Fig. 1. The naturally occurring intramolecular ligand for the SH2 domain of Hck, pYQQQ, has low affinity; this permits exogenous high affinity ligands (such as pYEEI) to displace the C-terminal tail and activate the enzyme. The intramolecular interactions in Src family kinases have been fine-tuned to be strong enough to help maintain the catalytic domain in its down-regulated conformation but weak enough to respond to a variety of cellular stimuli.

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3 P. Pellicena and W. T. Miller, unpublished observations.