Activation of the Src Family Kinase Hck without SH3-Linker Release*

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Src family protein-tyrosine kinases are regulated by intramolecular binding of the SH2 domain to the C-terminal tail and association of the SH3 domain with the SH2 kinase-linker. The presence of two regulatory interactions raises the question of whether disruption of both is required for kinase activation. To address this question, we engineered a high affinity linker (HAL) mutant of the Src family member Hck in which an optimal SH3 ligand was substituted for the natural linker. Surface plasmon resonance analysis demonstrated tight intramolecular binding of the modified HAL sequence to SH3. Hck-HAL was then combined with a tail tyrosine mutation that intramolecular SH3-linker release is not required for SH2-based kinase activation. In Saccharomyces cerevisiae, which lacks the negative regulatory tail kinase Csk, wild-type Hck was more strongly activated in the presence of an SH3-binding protein (human immunodeficiency virus-1 Nef), indicating persistence of native SH3-linker interaction in an active Hck conformation. Taken together, these data support the existence of multiple active conformations of Src family kinases that may generate unique downstream signals.

The Src family of non-receptor protein-tyrosine kinases consists of nine members (Src, Lck, Hck, Fyn, Blk, Lyn, Fgr, Yes, Yrk) that affect a diverse array of cellular responses in virtually every cell type (1). Tight control of Src kinase activity is essential to normal cellular function, and elevated activity of this kinase family is a common feature of several forms of cancer and other hyperproliferative states (2). Src family kinases exhibit a highly conserved structural organization that includes a myristoylated N-terminal domain, a short unique region, modular SH3 and SH2 domains, an SH2 kinase-linker region, the catalytic domain, and a C-terminal tail (3). The crystal structures of nearly full-length Src and Hck in their down-regulated autoinhibited states reveal that intramolecular interactions involving SH3 and SH2 are essential for negative regulation of kinase activity (4–8). These domains pack against the back side of the catalytic domain and stabilize it in a closed, inactive conformation. In this state, the SH3 domain engages the SH2 kinase-linker, which forms the polypeptide type II helix required for SH3 binding, while the SH2 domain engages the tyrosine-phosphorylated tail. Tail phosphorylation is catalyzed by a separate kinase known as Csk (9) and occurs on a single tyrosine residue conserved in all Src family members (Tyr-527 in c-Src).

Autoinhibited Src family kinases are very sensitive to small perturbations in intramolecular interactions involving SH3 and SH2. Mutations within the SH3 domain as well as the SH2 kinase-linker are sufficient to activate Src family kinases, as are mutations that disrupt SH2-tail interaction (3). Under physiological conditions, interactions with target proteins can also disrupt the regulatory mechanism by engaging the SH3 domain, the SH2 domain, or both (3, 10). For example, the Nef protein of human immunodeficiency virus 1 (HIV-1)2 is a high affinity ligand for the SH3 domain of Hck, a member of the Src kinase family expressed in myeloid hematopoietic cells. SH3-directed Hck-Nef complex formation leads to constitutive Hck kinase activation both in vitro (11) and in cells (12). These observations suggest that Src kinases are responsive to a wide variety of upstream inputs, which helps to explain their versatility in diverse signaling situations.

Regulation of Src family kinase activity by two distinct intramolecular interactions has raised several important questions regarding the number of inputs required for kinase activation. Is disruption of individual SH2-tail or SH3-linker interactions sufficient for activation or is displacement of the entire regulatory apparatus required? Recently, we observed that increasing the affinity of the tail for the SH2 domain does not impact activation through SH3 in vivo (13), suggesting some degree of independence. A related issue concerns the status of the second interaction once the first one is disrupted. Molecular dynamics simulations show that motions within SH3 and SH2 are highly correlated, predicting that individual SH3- or SH2-based inputs may disrupt both interactions and lead to a single activated state (14, 15).

To probe these issues experimentally, we created a modified form of Hck in which the native SH2 kinase-linker sequence was changed to a high affinity intramolecular ligand for the SH3 domain. Surface plasmon resonance analysis demonstrates that the modified linker binds very tightly to its intramolecular SH3 target. Surprisingly, the kinase activity of this high affinity linker mutant was strongly activated by disruption of SH2-tail interaction, producing a strong transforming signal in fibroblasts. These data show that SH3-linker release is not required for Hck activation through SH2-tail displacement. Furthermore, wild-type Hck was observed to have a "reserve" of kinase activity that could be stimulated by SH3 engagement or by SH2 kinase-linker mutation in Saccharomyces cerevisiae, a system in which Src family kinases are constitutively active because yeast lack a homolog of Csk. This finding indicates that SH3-linker interaction persists in wild-type Hck that is activated through disruption of SH2-tail interaction.

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2 The abbreviations used are: HIV, human immunodeficiency virus; HAL, high affinity linker; SH, Src homology.
Together with our previous data (13), these results suggest that SH3- and SH2-based activation of Hck are independent and support a role for Hck and other Src family kinases as "OR-gate" signaling switches (15) in which occupation of SH3 or SH2 by target proteins is sufficient to induce kinase activation without disruption of the remaining intramolecular interaction. This raises the possibility that multiple active conformations of Src family kinases exist, which may add to signaling diversity and provide unique targets for selective inhibitor discovery.

**MATERIALS AND METHODS**

**Mutagenesis and Recombinant Protein Expression**—Wild-type, tail mutant (Y501F), and SH2 kinase-linker (2PA) mutant forms of Hck have been described elsewhere (16). The high affinity linker (HAL) mutant of Hck was produced by changing the coding sequence of the wild-type linker lysines to prolines using standard PCR-based methods. The wild-type linker and HAL amino acid sequences are aligned in Fig. 1. The presence of the mutations was confirmed by DNA sequence analysis. Recombinant human Hck SH3 (amino acids 56–122 in p59 Hck; 72–143 in c-Src), SH3-SH2 (Hck amino acids 51–224; 72–245 in c-Src), SH3-SH2 plus wild-type linker (Hck amino acids 51–235; 72–256 in c-Src), and SH3-SH2 plus the HAL sequence were expressed in *Escherichia coli* and purified as previously described (17–19). Recombinant protein purity and concentration were determined by SDS-PAGE, and all protein identities were confirmed by mass spectrometry.

**Surface Plasmon Resonance Analysis**—Surface plasmon resonance experiments were carried out using a BIAcore 3000. The biotinylated high affinity SH3 domain binding peptide VSLARRPLPPLP (20) (University of Pittsburgh Molecular Medicine Institute Peptide Synthesis Facility) was immobilized on a SAS streptavidin biosensor chip (Biacore). A biotinylated I$_{B}$ kinase substrate peptide (BioMol, Plymouth Meeting, PA) of similar length was bound to a second channel on the same chip as a negative control. Peptide immobilization was performed at 25 °C at a flow rate of 10 μl/min in HBS-EP running buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% P20 surfactant). To prevent re-binding events with the recombinant Hck proteins, titration experiments were performed to define the lowest amount of immobilized target peptide that produced an acceptable signal-to-noise ratio with the recombinant SH3 domain. Based on these preliminary studies, the amount of biotinylated peptide bound to the chip was limited to 80 response units, a level below saturation, for all subsequent experiments. Association of recombinant Hck SH3, 3 + 2, 32L, and 32HAL proteins at various concentrations ranging from 0.1 to 1 μM was measured at 25 °C with a flow rate of 10 μl/min for 300 s, followed by 300 s of dissociation in HBS-EP buffer alone. Background response values of each recombinant Hck protein with the control peptide were subtracted from those obtained for the VSL-12 peptide to yield a specific value of the response units, a level well below saturation, for all subsequent experiments. Association of recombinant Hck SH3, 3 + 2, 32L, and 32HAL proteins at various concentrations ranging from 0.1 to 1 μM was measured at 25 °C with a flow rate of 10 μl/min for 300 s, followed by 300 s of dissociation in HBS-EP buffer alone. Background response values of each recombinant Hck protein with the control peptide were subtracted from those obtained for the VSL-12 peptide to yield a specific value of the response units, a level well below saturation, for all subsequent experiments.

**RESULTS**

**Development of a HAL Mutant of the Src Family Kinase, Hck**—To investigate whether Hck activation by SH2-based inputs also requires SH3-linker disruption, we sought to modify the Hck SH2 kinase-linker sequence to enhance its affinity for the SH3 domain. Native Src family kinase SH3-linker interactions tend to be weak, as the sequences of the linkers do not conform to those of polyproline type II helices normally associated with high affinity SH3 ligands (21). The x-ray crystal structure of Hck in the down-regulated conformation (4) is presented in Fig. 1 and illustrates this point. A close-up view of the SH3-linker interaction in the context of the overall Hck structure shows that the linker binds to the SH3 domain in the "minus" orientation (22). Polyproline type II helical positions P$_{0}$ and P$_{+3}$ are occupied by lysine residues, which interact poorly with the hydrophobic surface of SH3 (5). To create the HAL mutant, these lysine residues were replaced with prolines.

We performed molecular modeling to evaluate whether substitution of the natural linker lysine residues with prolines would disrupt the structure of the linker or its position relative to the SH3 domain or the N-lobe of the kinase domain in the overall structure. We were particularly interested in the position of Trp-260 in Hck-HAL. This residue is located at the C-terminal end of the linker and has a key role in coupling the regulatory domains to the αC helix in the N-lobe, where it helps to stabilize the αC in a position that prevents catalysis in the down-regulated state (23). Lysines 249 and 252 were replaced with prolines in the Hck crystal structure, and the resulting virtual mutant was energy minimized (24). As shown in Fig. 2, these two substitutions produced very little change in the overall structure of Hck. The relationship of the modified linker to the surface of SH3 as well as the position of Trp-260 relative to the αC helix remained virtually the same. These observations suggested that the HAL modification may be effective in stabilizing expression, and equivalent $A_{s}$ units of each culture were plated as a dilution series on agar plates with galactose as sole carbon source. Plates were incubated for 3 days, and an image of each plate was recorded on a flatbed scanner. To evaluate protein expression and tyrosine phosphorylation, aliquots of the same cultures were grown in liquid medium containing galactose for 4 h, and cell lysates were probed by immunoblotting with antibodies to Hck (N-30; Santa Cruz), HIV-1 Nef (EH-1; National Institutes of Health AIDS Research and Reference Reagent Program), Csk (C-20; Santa Cruz), and phosphotyrosine (PY99, Santa Cruz and PY20, BD Biosciences).

**FIGURE 1. Design of Hck-HAL mutant.** The crystal structure (4) of wild-type Hck in the inactive state is shown on the upper left, with the SH3 domain and part of the SH2 kinase linker highlighted in red and blue, respectively. The wild-type (WT) SH3-linker interface is enlarged in the center, with lysine residues in the linker polyproline helix that contact SH3 designated as P$_{0}$ and P$_{+3}$ according to the notation for SH3 ligands proposed by Lim et al. (22). These lysines were replaced with prolines to create the high affinity linker (HAL) mutant shown on the right. The table shows the wild-type linker and HAL sequences. Because the linker binds to the SH3 domain in the minus orientation in the crystal structure, the sequences are presented in the C- to N-terminal direction.
SH3-linker interaction without affecting overall kinase structure or regulation.

Surface plasmon resonance analysis was used to determine whether lysine to proline substitution enhanced intramolecular linker-SH3 interaction. For these studies, a biotinylated high affinity SH3 ligand peptide (VSLARRPLPPLP or VSL-12) was immobilized on a streptavidin biosensor chip. Four purified recombinant Hck proteins were then flowed past the VSL-12 peptide surface at equal concentrations (Fig. 3). These Hck proteins included the SH3 domain alone, the SH3 and SH2 domains together (3+2), the SH3 and SH2 domains plus the wild-type linker (32L), and the SH3 and SH2 domains plus the modified linker with the dual proline for lysine substitutions (32HAL). As shown in Fig. 3, the isolated Hck SH3 domain bound strongly to VSL-12 with a very rapid on-rate, yielding an equilibrium dissociation constant of ~800 nM. This value agrees favorably with published data for other Src family kinase SH3 domains (VSL12; Ref. 20) by surface plasmon resonance analysis (Biacore). Each recombinant Hck protein was flowed past the VSL12 peptide surface at the same concentration (1 μM), and the binding response was recorded for 300 s, at which point the surface was washed to induce dissociation (arrow). Binding of the isolated SH3 domain was fit to a simple 1:1 ligand:receptor binding model, yielding a $K_d$ of 800 nM. The 3+2 and 32L constructs exhibited a similar high affinity SH3 component plus a second lower affinity binding interaction.

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**Activation of Hck-HAL without SH3 Domain Displacement from the Linker—**To address the requirement for SH3-linker displacement during Hck activation and signaling in vivo, we incorporated the HAL substitutions into the context of full-length Hck. The resulting Hck constructs were analyzed for kinase and biological activities in a Rat-2 fibroblast transformation assay. Rat-2 cells do not express endogenous Hck but effectively down-regulate ectopically expressing wild-type Hck via tail tyrosine phosphorylation (13). As shown in Fig. 4A, Rat-2 fibroblasts expressing wild-type Hck continue to grow as a contact-inhibited monolayer indistinguishable from control cells. However, expression of a Hck mutant in which the tail tyrosine residue is mutated to phenylalanine (Hck-YF) results in transformation to an anchorage-independent phenotype and the appearance of large numbers of transformed foci.
Fibroblast transformation correlated with release of kinase activity, which was reflected in strong tyrosine phosphorylation of a 40-kDa cellular protein as reported previously (Fig. 4B, pp40) (12, 13, 16).

We first investigated the impact of the HAL substitution on wild-type Hck. As shown in Fig. 4, Hck-HAL did not induce fibroblast transformation and showed no evidence of kinase activity, indicating that it is down-regulated as effectively as wild-type Hck in fibroblasts. These results strongly suggest that the enhanced SH3-linker interaction present in Hck-HAL does not impact tail phosphorylation by Csk. Furthermore, these data imply that the modified linker retains its ability to pack against the small lobe of the kinase domain as required for negative regulation of kinase activity, consistent with the modeling shown in Fig. 2.

We next coupled the HAL substitution with the tail mutation, creating the compound mutant HAL-YF. To our surprise, Hck-HAL-YF exhibited very strong focus-forming activity that correlated with tyrosine phosphorylation of pp40 (Fig. 4). These data provide strong evidence that SH2-based activation of Hck resulting from mutation of the C-terminal tail (HAL-YF) were expressed in Rat-2 fibroblasts using retroviral vectors. Cells infected with a retrovirus carrying only the drug selection marker served as a negative control (Con). Infected cells were selected with G418 for 14 days, and transformed foci were visualized by Wright-Giemsa staining. Representative plates are shown at the top. Replicate plates were scanned and foci counted using counting software (Quantity One; Bio-Rad), and the mean number of transformed foci is shown ± S.D. in the bar graph. A, analysis of Hck protein expression and kinase activity. Hck proteins were immunoprecipitated and visualized by immunoblotting (top panels). Activity was monitored as phosphorylation of the endogenous Hck substrate, pp40, on anti-phosphotyrosine immunoblots of cell lysates (lower panels).

Evidence for SH3-Linker Interaction in Active Wild-type Hck—Evidence presented in Fig. 4 shows that strengthening the SH3–linker interaction does not prevent activation of Hck via SH2-tail displacement, supporting the notion that SH2-based activation may result in a unique active conformation of the kinase. This led us to question whether the SH3 domain remains bound to the native linker in the context of wild-type Hck following activation through SH2-tail disruption. To address this question, we turned to a yeast expression system that is a well established tool for Src family kinase structure-function analysis (25–27). Because yeast cells do not express Csk, ectopically expressed c-Src is catalytically active due to the lack of phosphorylation of the C-terminal tail tyrosine. Expression of kinase-active Src results in growth suppression of yeast, and the extent of growth suppression correlates with tyrosine phosphorylation of endogenous yeast proteins. Co-expression with Csk restores tail phosphorylation and down-regulation of Src and reverts the growth inhibitory phenotype. Thus the physiological mechanism of Src family kinase regulation can be accurately modeled in yeast, allowing for direct evaluation of the impact of mutations or protein-protein interactions on Src family kinase activity in a defined cellular context.

We first investigated whether wild-type Hck produced the growth inhibitory phenotype previously reported for c-Src in yeast. To accomplish this, S. cerevisiae transformed with a galactose-inducible expression vector for Hck was plated at increasing dilutions on solid medium containing galactose. As shown in Fig. 5A, wild-type Hck, when expressed alone, suppressed yeast cell growth when compared with cultures transformed with the empty expression plasmid. Growth suppres-
Hck Activation with SH3-Linker Engagement

sion correlated with the appearance of tyrosine-phosphorylated yeast proteins on anti-phosphotyrosine immunoblots (Fig. 5B). Growth suppression and protein-tyrosine phosphorylation were both reversed when wild-type Hck was co-expressed with Csk, as observed originally for c-Src (26, 27). This result shows that tail phosphorylation by Csk is required for down-regulation of Hck. Therefore, when wild-type Hck is expressed alone, it fails to undergo intramolecular SH2-tail interaction and remains active, resulting in growth suppression.

To probe active wild-type Hck for evidence of SH3-linker interaction, Hck was co-expressed with HIV-1 Nef, a well known high affinity-binding protein for the Hck SH3 domain (28, 29). Co-expression of wild-type Hck with Nef resulted in a much stronger growth-suppressive phenotype than Hck alone, which correlated with enhanced tyrosine kinase activity (Fig. 5). Further evidence for changes in the Hck activation state following SH3 engagement is revealed by shifts in the electrophoretic mobility of Hck in the presence and absence of Nef (Fig. 5, Hck blot). Hck, when expressed alone, consistently migrates as three closely spaced bands. Co-expression with Csk enhances levels of the highest mobility form of Hck, which most likely results from tail phosphorylation. Co-expression with Nef results in a shift toward the middle band, which is likely due to the enhanced autophosphorylation that results from Nef-induced activation. These results show that although wild-type Hck is active in the absence of tail phosphorylation, binding of a high affinity SH3 ligand (Nef) enhances kinase activity to a greater extent. This observation implies that SH3-linker interaction persists in active, wild-type Hck, a conclusion that is supported by a very recent x-ray crystal structure of active Src (30) (see “Discussion”).

Evidence for SH3-linker interaction in active Hck was also observed in comparisons of wild-type Hck activity with that of Hck-2PA, a mutant in which linker prolines are replaced with alanines (16). These mutations disturb intramolecular linker-SH3 binding and are sufficient to release kinase activity and transform rodent fibroblasts (16). As shown in Fig. 5, Hck-2PA showed much stronger growth suppression and kinase activity than wild-type Hck when similar amounts of these proteins were expressed in the yeast system. Hck-2PA also migrated predominantly as the middle band, as observed following SH3-based activation of wild-type Hck by Nef. Because Hck is not phosphorylated on the tail in yeast, these observations with Hck-2PA support the idea that SH3-linker interaction persists in wild-type Hck that is activated through loss of SH2-tail interaction. Hck-2PA could be stimulated somewhat further by co-expression with Nef, suggesting that the 2PA substitutions may not fully disrupt the negative regulatory influence of the linker on the kinase domain. Interestingly, co-expression with Csk failed to down-regulate Hck-2PA and did not alter the Hck band-shifting pattern. Thus, tail phosphorylation alone cannot drive Hck-2PA into the down-regulated conformation, indicating that the wild-type linker sequence is required for effective regulation.

In a final experiment, we expressed the wild-type and HAL forms of Hck in yeast and evaluated growth suppression and kinase activity. As shown in Fig. 6, expression of wild-type Hck and Hck-HAL both resulted in strong growth suppression that correlated with the presence of an active kinase. This result agrees with the fibroblast transformation data (Fig. 4) and supports the conclusion that SH3-linker release is not required for Hck activation.

DISCUSSION

The x-ray crystal structures of the down-regulated forms of Hck and c-Src have revealed that two intramolecular interactions involving the SH3 and SH2 domains are essential for negative regulation of the kinase domain (3). This observation led us to question whether displacement of both interactions is required for kinase activation. Here we have provided experimental evidence that displacement of SH2-tail association alone is sufficient to induce kinase activation in vivo. We found that “locking” the SH3 domain to the SH2 kinase-linker did not prevent activation of Hck through an SH2-directed mechanism in fibroblasts or in a yeast model system. These results are complemented by our previous work showing that SH3-mediated Hck activation occurs without dephosphorylation of the negative regulatory tail and may not require tail release from the SH2 domain (13). Together, these observations suggest that the SH3 and SH2 domains may operate independently in the regulation of Hck kinase activity.

Data with the Hck-HAL mutant presented in Fig. 4 strongly suggest that SH3-linker release is not required for Hck activation in vivo. In this experiment, mutagenesis of the negative regulatory tyrosine in the Hck-HAL C-terminal tail led to strong kinase activation, despite the enhanced SH3-linker interaction engineered into this protein (Fig. 3). This observation raises the question of whether SH3-linker interaction persists in the context of wild-type Hck activated through a similar SH2-based mechanism. Yeast experiments presented in Fig. 5 support this view. Because of the absence of Csk in yeast, wild-type Hck is not tail phosphorylated and is therefore active as a consequence. Surprisingly, co-expression of a high affinity ligand for the Hck SH3 domain (HIV-1 Nef) led to a greater kinase output in yeast, consistent with the idea that some Hck molecules may retain linker-SH3 interaction that can be fully displaced only in the presence of a strong SH3 ligand. A very recent crystal structure of c-Src also supports this idea, revealing that SH3-linker interaction is maintained despite the absence of tail phosphorylation and the presence of the kinase domain in an active conformation (30). Together, these findings suggest that SH3-linker interaction may persist in some active conformations of Hck as well, such as those produced by interaction with SH2 ligands or via tail dephosphorylation.

Src family kinases are unique signaling molecules in that their non-catalytic SH2 and SH3 domains serve the dual functions of substrate recruitment and negative regulation of the kinase domain. This arrangement coordinates substrate binding and catalytic function, allowing strict spatiotemporal control of kinase activity (10). The presence of these two modular signaling entities in the same protein allows for a wide range of possible kinase-substrate interactions, and proteins that activate Src kinases through SH2- and SH3-based mechanisms (or both) have been reported (10). This has led to the idea that Src kinases can function as allosteric “switches” that integrate different types of activating inputs (binding of SH2 or SH3 ligands) into a single downstream output (kinase activity) (15).
Borrowing from the language of circuit theory, work presented here and in a previous report (13) suggests that Hck operates as an OR gate signaling switch. Upstream input through either the SH3 or the SH2 domain is sufficient to induce kinase activation and a strong signal for transformation in Rat-2 fibroblasts. Although OR gate signaling behavior was also observed in yeast, simultaneous disruption of both components of negative regulation led to a stronger output signal (Fig. 5). The difference between mammalian cells and yeast may reflect a threshold effect in mammalian cells, in which a submaximal level of kinase activity is sufficient to achieve a complete biological response (oncogenic transformation in this case). A similar phenomenon was reported recently for dual input synthetic OR gate switches based on the actin remodeling protein, N-WASP (31). In this case, disruption of either of two negative regulatory protein-protein interactions was sufficient to release the actin-bundling activity of the N-WASP catalytic domain, while simultaneous disruption of both interactions allowed for a greater effect.

In summary, our data provide direct experimental evidence that Src family kinase SH2 and SH3 domains can act independently to regulate the kinase domain. By extension, our results suggest that multiple active conformations of Hck and possibly other Src family kinases may exist, depending upon the type of input signal (e.g. SH3 versus SH2 directed). This suggests an additional level of signaling complexity in which the activating input dictates not only signal strength but perhaps the range of target proteins phosphorylated. Given the structural coupling between the SH3 domain, the linker, and the small lobe of the kinase domain, it is possible that the presence or absence of SH3-SH2 linker engagement may also allosterically impact the conformation of the active site. This possibility has implications for kinase-directed inhibitor discovery, as binding of some ATP-competitive inhibitors is sensitive to kinase domain conformation. A dramatic example is provided by the clinically important anti-leukemic agent and Abl kinase inhibitor, Imatinib, which shows a strong preference for the inactive conformation of the kinase domain (32, 33). Future work focused on the structure and dynamics of full-length Hck in the presence of SH3 versus SH2 binding partners will provide more insight into this important issue.

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