Intramolecular Regulatory Interactions in the Src Family Kinase Hck Probed by Mutagenesis of a Conserved Tryptophan Residue*

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Intramolecular interactions between the Src homology domains (SH2 and SH3) and the catalytic domains of Src family kinases result in repression of catalytic activity. The crystal structure of the Src family kinase Hck, with its regulatory domains intact, has been solved. It predicts that a conserved residue, Trp260, at the end of the linker between the SH2 and the catalytic domains plays an important role in regulation by the SH3 and SH2 domains. We have mutated this residue and compared the activities of C-terminally phosphorylated wild type Hck and W260A Hck. The W260A mutant has a higher specific activity than wild type Hck. The W260A mutant requires autophosphorylation at Tyr416 for full activity, but it is not activated by ligand binding to the SH3 or SH2 domains. This mutation also changes the accessibility of the SH2 and SH3 domains to their cognate peptide ligands. Our results indicate that Trp260 plays a critical role in the coupling of the regulatory domains to the catalytic domain, as well as in positioning the ligand binding surfaces.

Hck is a Src family nonreceptor tyrosine kinase involved in signal transduction in hematopoietic cells of the myeloid and B-lymphoid lineages (1, 2). Like Src and other kinases of this family, it has the following functional domains (listed from N terminus to C terminus): a membrane binding domain, a unique region, a Src homology 3 (SH3) domain, a Src homology 2 (SH2) domain, a kinase catalytic domain, and a negative regulatory site at Tyr527 (SRC numbering) in the C-terminal tail (for review see Ref. 3). The SH2 domain binds phosphotyrosine-containing ligands, and the SH3 domain binds ligands containing polyproline type II helices (3). Phosphorylation of Tyr527 by c-Src kinase (Csk) produces an intramolecular interaction between the phosphorylated tail and the SH2 domain that inhibits Src kinase activity (4–7). Src can also autophosphorylate Tyr527, but it is not the primary autophosphorylation site (8, 9).

The major autophosphorylation site, Tyr416, lies in the activation segment, a flexible portion of the catalytic domain near the active site. Autophosphorylation of Tyr416 leads to enhanced catalytic activity of Src and Hck (4–7).

SH3 and SH2 domains have both a positive and a negative regulatory role in the activity of Src family kinases. The positive role is thought to be due to SH2- and SH3-mediated interactions with other proteins. These protein-protein interactions allow the kinases to bind to and phosphorylate certain cellular substrates (3). SH2- and SH3-mediated interactions are essential for the signaling properties of Src family kinases; mutations that interfere with the binding of ligands to SH2 and SH3 domains of the kinases disrupt their ability to transform cells (10–13). In addition, substitution of the Abl SH2 domain with heterologous SH2 domains results in the phosphorylation of alternative substrates in vivo (14). Hence, specific signaling by nonreceptor tyrosine kinases appears to depend on the specificity of the associated SH2 and SH3 domains.

The negative regulatory role for SH2 and SH3 domains in Src family kinases arises from intramolecular contacts with the tyrosine kinase catalytic domain. The SH2 domains of Src kinases bind their C-terminally phosphorylated tails (3–7), and this interaction stabilizes an inactive conformation. Genetic experiments first suggested that the SH3 domain of Src was also involved in the repression of catalytic activity (15, 16). The three-dimensional structures of c-Src and Hck (17–19) provide an explanation for the involvement of the Src homology domains in enzyme inhibition (Fig. 1A). There is a linker region (residues 240–263) between the SH2 domain and the catalytic domain that contains a polyproline type II helix that binds to the SH3 domain. This interaction, along with the phosphorylated tail-SH2 domain interaction, is proposed to keep the protein in an inactive conformation (17–19).

Several amino acids within the SH2-kinase linker interact with residues in the N-terminal lobe of the catalytic domain. One of these, Trp260, lies outside of the polyproline helix and appears to be particularly important in maintenance of the inhibited state (Fig. 1B) (18). This tryptophan is very highly conserved among Src family and other nonreceptor tyrosine kinases, as well as among several receptor tyrosine kinases. In the inactive conformation of Hck, Trp260 points into a hydrophobic region of the N-terminal lobe of the catalytic domain (Fig. 1B). Trp260 appears to stabilize a conformation of helix αC that is not properly positioned for catalysis. In particular, Glu110 on the αC helix is pointed out of the active site and interacts with Arg260 and Tyr264 in the activation segment. In the active forms of cAMP-dependent protein kinase (20) and Lck (21), the corresponding glutamate residues are pointed into the active site. Glu110 of cAMP-dependent protein kinase, the residue equivalent to Glu110 of Hck, is salt-bridged to Lys72, the residue that coordinates the α and β phosphates of ATP. In the inactive conformation of Hck, the side chain of Glu110 is too far from the ATP-binding site to play this role.

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§ The abbreviations used are: SH2, Src homology 2; SH3, Src homology 3; PXE, the SH3-binding peptide (Ser-Pro-Pro-Thr-Pro-Lys-Pro-Arg-Pro-Pro-Arg-Pro); PVE, the SH2-binding peptide (Glu-Pro-Glu-pTyr-Glu-Ile-Pro-Ile-Tyr-Leu); HIV, human immunodeficiency virus.
Autophosphorylation of Tyr^{416} in the activation segment of Hck leads to increased activity. By comparison with the structures of cAMP-dependent protein kinase and Lck, phosphorylated Tyr^{416} is predicted to interact with Arg^{385}, causing a rearrangement of the activation segment that allows helix αC to rotate inward (18). When the helix is rotated inward, Glu^{310}
can form a salt bridge with Lys285, so that the lysine side chain can properly coordinate ATP. Hence, this model suggests that the SH3 domain is important in inhibition of Hck because its interaction with the SH2-kinase linker positions Trp260 to hold the αC helix and Glu310 in an inactive conformation (18).

Disruption of the intramolecular contacts involving SH2 or SH3 domains (by ligand binding) leads to enhanced catalytic activity of Hck and other Src family kinases. Binding of a phosphotyrosine-containing ligand for the SH2 domain activates Src family kinases (22–24), presumably due to displacement of the C-terminal tail. We have shown previously that disruption of the SH3-catalytic domain interaction by HIV-1 Nef, a high affinity ligand for the Hck SH3 domain, or by a peptide containing the polyproline motif, activates Hck 5–50-fold, depending on the autophosphorylation state of the enzyme (24). Binding of Nef to the SH3 domain of Hck in vivo stimulates tyrosine kinase activity and leads to transformation of Rat-2 fibroblasts (25). The p130Cas-related protein, Sin, activates e-Src by coordinate binding to the SH2 and the SH3 domain (26). Disruption of the interaction between the SH3 domain and the SH2-kinase linker in these cases is predicted to destabilize the interaction of Trp260 with the αC helix, allowing the helix to rotate into the active site, thereby activating the kinase (18).

This model for regulation of catalysis suggests an important role for Trp260. We tested this prediction by producing a form of Hck that contains a Trp260 → Ala mutation and that is phosphorylated on Tyr527. We have compared the activity and regulatory properties of this mutant with those of wild type Hck. Our results indicate a role for Trp260 in the coupling of the regulatory domains to the catalytic domain and in the interactions of the regulatory domains with their ligands.

MATERIALS AND METHODS

Protein Expression and Purification—C-terminally phosphorylated Hck (wild type and mutant) was produced in Spodoptera frugiperda cells by co-expression with Csk, as described (18), with the following modification: after ion-exchange chromatography, Hck was purified on a column containing immobilized adenosine-5′→(γ-4-aminophenyl)triphosphate. The column was prepared by linking 100 mg of adenosine-5′→(γ-4-aminophenyl)triphosphate (U.S. Biochemical Corp.) to 5 g of 6-aminoheptanoic acid N-hydroxysuccinimide ester-Sepharose 4B (Sigma) according to the manufacturer’s instructions. The column was equilibrated in 20 mM Tris (pH 8.5), 50 mM NaCl, 10 mM MgCl2, 0.1 mM sodium orthovanadate, 10% glycerol. Hck was loaded in equilibration buffer with 30 μl of resin in 200 μl of final volume in buffer containing 50 mM Tris 7.4, 250 mM NaCl, 0.1% Triton X-100, 5 mM EDTA, 0.5 mM Na3VO4, and 1 mM dithiothreitol. Binding reactions were for 30 min. The resin was washed three times in lysis buffer, and bound protein was eluted with 2× Laemmli buffer, followed by resolution using SDS-polyacrylamide gel electrophoresis. The proteins were transferred to polyvinylidene difluoride membrane (Millipore) using a Hoefer semi-dry transfer apparatus and detected with an anti-Cys-Src rabbit polyclonal antibody (Upstate Biotechnology Inc.), anti-rabbit horseradish peroxidase conjugated secondary antibody, and enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech).

RESULTS

We co-expressed the W260A Hck mutant with Csk in S. frugiperda cells to produce a form of the enzyme that is phosphorylated on Tyr527. Matrix-assisted laser-desorption-ionization time-of-flight mass spectrometric analysis of this protein (24) confirmed that Tyr527 was free of phosphate (data not shown). We compared the kinetic parameters of wild type Hck and the W260A mutant for phosphorylation of a synthetic peptide substrate (Table I). These experiments were carried out with enzymes that had been pre-activated with ATP and Mg2+, because we found that the W260A mutant is activated by autophosphorylation in a manner similar to how the wild type is activated (see below). The W260A mutant displayed a 5.7-fold higher Km for peptide substrate than wild type Hck, as well as a 12-fold higher Vmax (Table I). Thus, the specific activity (Vmax/Km) of the mutant was approximately 12-fold higher than that of the wild type enzyme. The Km values for ATP substrate were similar for wild type and W260A mutant. We also measured the kinetic parameters of wild type Hck in the presence of Nef. Nef activates the autophosphorylated wild type enzyme (24), and the kinetic analysis shows that Nef
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The SH2 and SH3 domains of Hck appear to be mutually dependent with regard to their ability to modulate enzymatic activity. An intact SH3 domain is necessary for negative regulation by the phosphorylated C-terminal tail (15, 16). Furthermore, using surface plasmon resonance, we observed that C-terminally phosphorylated Hck had a higher apparent equilibrium dissociation constant for Nef than the dephosphorylated enzyme (24). Thus, release of phosphorylated Tyr\(^{527}\) from the SH2 domain of Hck appears to increase the accessibility of the SH3 domain. We therefore investigated the effect of the W260A mutation on the accessibility of the ligand binding surfaces of the SH2 and SH3 domains. In these experiments, we compared the ability of wild type and W260A Hck to bind to immobilized ligands for the SH2 domain (a pYEEI-containing peptide) and the SH3 domain (a polyproline-containing peptide). Although these peptides can bind to the regulatory domains of Hck (24), under the conditions of this assay C-terminally phosphorylated wild type Hck did not bind appreciably to the immobilized pYEEI peptide (Fig. 3, lane 2) or to the immobilized PXXP peptide (Fig. 3, lane 4). Hck that had been dephosphorylated at Tyr\(^{527}\) was able to bind to the pYEEI resin (Fig. 3, lane 6). In contrast to results for wild type Hck, C-terminally phosphorylated W260A mutant bound efficiently to immobilized pYEEI peptide or PXXP peptide (Fig. 3, lanes 3 and 5, respectively). The increased accessibility of the SH2 and SH3 domains for ligands suggests that the W260A mutation destabilizes the intramolecular interactions that maintain the inactive conformation.

We carried out similar experiments on C-terminally phosphorylated Hck that had been allowed to autophosphorylate on Tyr\(^{516}\). The purpose of these experiments was to determine whether the C-terminal tail becomes disengaged from the SH2 domain after autophosphorylation on Tyr\(^{516}\). Previous experiments on Src demonstrated that a form of Src with an intramolecular interaction between the SH2 domain and the phosphorylated tail was able to autophosphorylate at Tyr\(^{516}\) (9). We wished to address the question of whether autophosphorylation leads to a subsequent disruption of the SH2-tail interaction. We compared binding of Hck to immobilized pYEEI resin before and after autophosphorylation. In our assay system, autophosphorylation does not dramatically change the binding of the SH2 domain to the pYEEI resin, suggesting that the phosphorylated tail is not released from the SH2 domain after autophosphorylation (Fig. 3, lanes 2 and 7). Similar results were obtained for the Trp\(^{260}\) mutant of Hck (data not shown). When the activity of C-terminally phosphorylated Hck is measured without ATP pretreatment, a lag is observed in substrate phosphorylation (24). This induction time is due to autophosphorylation at Tyr\(^{516}\) and the length of the induction time depends on Hck concentration, as predicted for an intramolecular process. Dephosphorylation at Tyr\(^{527}\) significantly reduces the induction time (24). We incubated W260A Hck with peptide substrate without ATP pretreatment and measured the induction time for autoactivation. The induction time of the W260A mutant is reduced relative to wild type Hck (Fig. 4A). For example, at an enzyme concentration of 0.5 \(\mu\)M, the induction times for wild type Hck and W260A were 8 min and 30 s,
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DISCUSSION

The inactive forms of Src family kinases are maintained by intramolecular interactions involving both the SH2 and SH3 domains (17–19). Disruption of either of these interactions is predicted to cause enzyme activation (6, 7). In v-Src, the product of the transforming gene from Rous sarcoma virus, the C-terminal tail is missing and the enzyme is constitutively active. v-Src also contains amino acid substitutions at Arg305 and Thr369, two residues in the SH3 domain that interact with the SH2-kinase linker and the N-terminal lobe of the kinase domain. These mutations are expected to disrupt the SH3-catalytic domain interaction, destabilizing the repressed form of Src (6, 7). Displacement of the SH3 domain of Hck by Nef leads to a maximum activation of the enzyme, consistent with a central role for the SH3-catalytic domain interaction in regulating Hck (24).

We report here that mutation of Trp260, a conserved residue in the SH2-kinase linker, leads to changes in the regulatory properties of Hck. Mutation of Trp260 has four main effects: (i) it increases the basal activity of Hck; (ii) it shortens the time required for autophosphorylation; (iii) it renders Hck insensitive to added SH2 or SH3 ligands; and (iv) it changes the accessibility of the ligand binding surfaces of the SH2 and SH3 domains. These observations are consistent with two major roles for Trp260. First, Trp260 interacts with the aC-helix in the N-terminal lobe of the catalytic domain. Mutation of Trp260 disrupts this interaction, allowing the activation segment and helix aC to adopt a conformation that is competent for kinase activity. Second, Trp260 appears to play a role in positioning the SH2 and SH3 domains for intra- and intermolecular interactions. Our results are consistent with those of Gonfloni et al. (31), who showed that a W260A mutant of c-Src has impaired regulation when expressed in yeast.

The W260A mutant of Hck has a higher basal activity than the wild type, but the mutant still requires autophosphorylation at Tyr416 for full activity (Table I and Fig. 4). The autophosphorylation process occurs more rapidly for the mutant than for the wild type (Fig. 4); this is similar to the effect we observed for Hck that had been dephosphorylated at Tyr427 (24). These results further establish the importance of the phosphorylation state of the activation segment for controlling Hck activity, as seen previously by mutagenic analysis of c-Src and Lck (3, 32, 33). Addition of ligands for the Hck SH2 or SH3 domains gave no additional activation (over the level seen in autophosphorylated W260A Hck) (Fig. 2). Thus, although the regulatory domains do not appear to exert their inhibitory effects in the mutant, autophosphorylation at Tyr416 must still occur for maximal activity. This is similar to our recent results for wild type Hck stimulated by Nef, where activation by autophosphorylation at Tyr416 can still be observed, although the process is very rapid relative to Hck alone. The maximum level of activity observed for the autophosphorylated W260A mutant is similar to that seen for the wild type enzyme that has been fully activated by addition of Nef (Table I).

Autophosphorylation of Src family kinases appears to be predominantly an intermolecular event. However, the flexibility of the activation segment (containing Tyr416) suggests the possibility of intramolecular autophosphorylation if the regulatory domains are removed. Does this explain the more rapid autophosphorylation of the W260A mutant? Our results shown in Fig. 4A argue against this interpretation, because the induction time for W260A activation depends on the protein concentration. Intramolecular autophosphorylation would yield a first-order progress curve for peptide phosphorylation, and the induction time would be independent of protein concentration. We have also recently observed that the induction time for activation of wild type Hck in the presence of Nef depends on protein concentration. These results indicate that, at least at these concentrations of Hck (0.01–0.05 μM), intramolecular autophosphorylation is unlikely to play a major role in enzyme activation. The concentration of Src in NIH3T3 cells has been estimated to be approximately 1.0 μM, based on a cell volume of 10–15 femtoliters and 10,000 molecules of Src/cell. Furthermore, plasma membrane localization of Src and formation of

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2 M. LaFevre-Bernt, M. Porter, and W. T. Miller, unpublished observations.
3 S. Courtneidge, personal communication.
signaling complexes at the membrane would tend to increase the local concentration (3). Thus, under normal physiological conditions, intramolecular autophosphorylation is unlikely to be a major mode of regulation in vivo.

Mutation of Trp260 in Hck also changes the interactions of the SH2 and SH3 domains with their respective ligands. The ligand-binding surface of both domains is more accessible in W260A Hck than in wild type Hck (Fig. 3). A change in accessibility of the SH3 domain is not surprising, given the proximity of Trp260 to the polyproline type II helix that is bound by the SH3 domain of Hck. The W260A mutation also destabilizes the interaction between the phosphorylated tail and the SH2 domain of Hck (Fig. 3), a site approximately 40 Å away in the three-dimensional structure (18). A similar effect has been observed for Src, where a mutant lacking the SH3 domain bound more tightly to immobilized pYEEI peptide (16). The interactions between the SH3 domain, the linker, and the N-terminal lobe of the catalytic domain are extensive and predominantly hydrophobic (17–19). In contrast, the interactions between the SH2 and catalytic domains are polar in character. The SH3 domain interactions may play the dominant role in maintaining the inactive state; disrupting these interactions may destabilize the regulatory apparatus, leading to increased accessibility of the SH2 domain. Our results demonstrate the importance of Trp260 in maintaining the proper geometry of both the SH2 and SH3 domain ligand binding surfaces for maximal interaction with their intramolecular ligands and point to the interdependence of the SH2 and SH3 domains in Src family kinase regulation.

The interdependence of the intramolecular SH2 and SH3 domain interactions indicates that the repressed conformations of Src family kinases are subject to activation by a variety of cellular signals. Such signals include dephosphorylation of Tyr527 (e.g. CD45 phosphatase dephosphorylating Lck (34)); engagement of the SH2 domain (e.g. PDGF receptor activating Src (35)); engagement of the SH3 domain (e.g. Nef activation of Hck (24)); or simultaneous binding of the SH2 and SH3 domains (e.g. Src activating Src (26)). Src kinases seem to be particularly sensitive to SH3 domain engagement, and activation of Src kinases by this process may prove to be a widespread feature of signaling by these kinases.

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