Modulation of the Catalytic Activity of the Src Family Tyrosine Kinase Hck by Autophosphorylation at a Novel Site in the Unique Domain*

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Autophosphorylation is a key event in the activation of protein kinases. In this study, we demonstrate that autophosphorylation of the recombinant Src family kinase Hck leads to a 20-fold increase in its specific enzymatic activity. Hck was found to autophosphorylate readily to a stoichiometry of 1.3 mol of phosphate per mol of enzyme, indicating that the kinase autophosphorylated at more than one site. Solid phase sequencing and two-dimensional mapping of the phosphopeptide fragments derived from the autophosphorylated enzyme revealed that the kinase can undergo autophosphorylation at the following two sites: (i) Tyr-388, which is located to the consensus autophosphorylation site commonly found in the activation loop of many protein kinases, and (ii) Tyr-29, which is located in the unique domain of Hck. Hck purified from mouse bone marrow-derived macrophages could also autophosphorylate in vitro at both Tyr-388 and Tyr-29, indicating that naturally occurring Hck can also autophosphorylate at Tyr-29. Furthermore, Hck transiently expressed in human embryonic kidney 293T cells was found to be phosphorylated at Tyr-29 and Tyr-388, proving that Hck can also undergo autophosphorylation at both sites in vivo. The recombinant enzyme carrying the mutation of Tyr-388 to Phe was also able to autophosphorylate at Tyr-29, albeit at a significantly slower rate. A 2-fold increase in the specific enzymatic activity was seen with this mutant despite the stoichiometry of autophosphorylation only approaching 0.2 mol of phosphate per mol of enzyme. This indicates that autophosphorylation of Tyr-29 contributes significantly to the activation of Hck. Regulation of the catalytic activity by phosphorylation of Tyr-29 in the unique domain may represent a new mechanism of regulation of Src family tyrosine kinases.

The Src family of protein tyrosine kinases consists of nine members documented to participate in a variety of cellular functions such as cellular growth and differentiation (see Refs. 1 and 2 for review). The catalytic activity of the Src family kinases is indispensable to their ability to carry out their cellular functions. Members of the Src family kinases share the same overall structural organization of domains as follows: (i) an N-terminal fatty acid-acylation domain, (ii) a unique domain, (iii) a Src homology 3 (SH3) domain, (iv) a Src homology 2 (SH2) domain, (v) a catalytic domain, and (vi) a C-terminal regulatory domain. Phosphorylation of a conserved tyrosine residue within the C-terminal regulatory domain of Src family kinases by another protein tyrosine kinase called the C-terminal Src kinase (CSK) or its cellular homologue (Chk) leads to inactivation of the kinase. In contrast, autophosphorylation of a conserved tyrosine residue within the kinase domain leads to activation. The recently determined crystal structures for Src and Hck provide the structural basis for the inactivation of Src family kinases by phosphorylation of the C-terminal regulatory tyrosine residue (5–6). The inactive conformation of Src or Hck is stabilized by a tripartite intramolecular interaction involving binding of the phosphorylated C-terminal regulatory tyrosine residue to the SH2 domain, and binding of the SH3 domain to a linker sequence between the SH2 and catalytic domains (SH2-CD linker) of the kinase that is capable of adopting a poly-proline type II helical conformation. Such interactions impart conformational constraints on the kinase domain such that it is unable to bind ATP, thus maintaining the enzyme in an inactive conformation. Activation of the enzyme may be achieved either by dephosphorylation of the C-terminal regulatory tyrosine residue (Tyr-499 in murine Hck) by a tyrosine phosphatase, engagement of the SH2 domain by a tyrosine-phosphorylated protein, or binding of the SH3 domain to a specific PX domain-containing cellular protein (7, 29).

In addition to regulating the catalytic activity, the SH2 and SH3 domains of Src family tyrosine kinases are also involved in mediating their physical association with other proteins (8–10). Such interactions are important for association of the kinases with substrates and targeting the kinases to their specific subcellular localizations.

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† The abbreviations used are: SH3, Src homology 3; PAGE, polyacrylamide gel electrophoresis; SH2, Src homology 2; SH2-CD linker, motif containing the sequence of Pro-X-X-Pro (where X represents any amino acid residue) located between the SH2 and catalytic domain of Hck; GST, glutathione S-transferase; PTH, phenylthiohydantoin; CSK, C-terminal Src kinase; HEK, human embryonic kidney; TLE, thin layer electrophoresis; DMEM, Dulbecco’s modified Eagle’s medium; HPLC, high pressure liquid chromatography; PCR, polymerase chain reaction; MES, 4-morpholinoethanesulfonic acid; PTB, protein tyrosine-binding; pY, phosphotyrosine.
The unique domain is also implicated as contributing significantly to the regulatory properties and functions of the Src family kinases, as it is the region of the enzymes containing most sequence variation between different Src family members. The unique domain of Lck, for example, mediates its interaction with the transmembrane receptor-like proteins CD4 and CD8 in T-lymphocytes (11). The unique domain of Src can be phosphorylated by cAMPP-dependent protein kinase (12), protein kinase C (13), or p34<sup>Cdc2</sup> (14) under various conditions. The functional significance of phosphorylation by these serine/threonine kinases is not yet known, although phosphorylation by p34<sup>Cdc2</sup> was suggested to enhance activation by dephosphorylation of the C-terminal regulatory tyrosine of Src by the upstream protein tyrosine phosphatase (29).

In this paper, we report that autophosphorylation of Hck occurs at a novel site in addition to the consensus autophosphorylation site at Tyr-388<sup>2</sup> of the activation loop of the catalytic domain (27). This novel site is identified to be Tyr-29 in the unique domain. Autophosphorylation of Tyr-29 and Tyr-388 is shown to follow an intermolecular mechanism. Analysis of the activities and stoichiometric levels of phosphorylation of recombinant Hck mutants, containing a Tyr to Phe mutation at either Tyr-388 or Tyr-29, showed that phosphorylation at Tyr-29 contributes to the activation of Hck by autophosphorylation. In vitro autophosphorylation of Tyr-29 in Hck isolated from macrophages and in vivo phosphorylation of Tyr-29 in Hck overexpressed in the human embryonic kidney 293T cells were also demonstrated, indicating that autophosphorylation of Tyr-29 in Hck is of physiological significance.

**EXPERIMENTAL PROCEDURES**

**Materials**—The Sephadex G-25 (Fine) gel filtration matrix, the fast protein liquid chromatography Mono-Q anion (HR5/5), and the Mono-S (HR5/5) columns were from Amersham Pharmacia Biotech. Hydroxylapatite was from Bio-Rad. The preparative reverse phase Ecosil C<sub>18</sub> high performance liquid chromatography column was from Alltech (Deerfield, IL), and the analytical Vydac reverse phase C<sub>18</sub> high performance liquid chromatography column was from Separation Group Inc. The polyclonal α-Hck peptide antibody (M28) was Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit polyclonal α-Hck antibody was raised against a GST fusion protein containing the unique and SH3 domains (amino acid residues 1–129) of Hck. Recombinant Hck was expressed and purified as described by Sicilia et al. (15). Immunoblot analysis using anti-phosphotyrosine antibodies revealed that less than 5% of the purified Hck was tyrosine-phosphorylated (15). Oligonucleotides were synthesized from GeneWorks, Inc. (South Australia).

**Preparation of Synthetic Peptides**—Synthetic peptides were synthesized using Fmoc (N-(9-fluorenylethoxycarbonyl))-based chemistry. The non-phosphopeptides synthesized include the peptide corresponding to the tryptic fragment (residues 26–36) encompassing the novel autophosphorylation site (Tyr-29) of murine Hck (GIPvYVPDPTSSSK) and the [Lys<sup>19</sup>]<sup>Cdc2</sup>-peptide (KVEKIGEGTYGVVKK) that has been shown to be an efficient substrate of Src family tyrosine kinases (26). These peptides were cleaved from the resin and purified as described previously (31).

**Determination of the Level and the Effect of Autophosphorylation on the Tyrosine Kinase Activity of Wild Type, Y29F, and Y388F Hck**—Recombinant Hck was allowed to autophosphorylate at 30 °C in the presence of the kinase assay buffer (20 mM Tris-HCl, pH 7, 10 mM MgCl<sub>2</sub>, 1 mM MnCl<sub>2</sub>, and 50 μM Na<sub>2</sub>VO<sub>4</sub>) and 100 μM [γ-<sup>32</sup>P]ATP (specific radioactivity approximately 452 cpm/pmol). The concentration of Hck used in each case corresponded to 95, 200, and 360 nM for the wild type Hck, [Y29F]Hck, and [Y388F]Hck, respectively, in a total volume of 350 μl. For determination of the stoichiometry of auto-phosphorylation, aliquots of 10 μl (containing 0.95, 1.16, and 3.6 pmol of wild type Hck, [Y29F]Hck, and [Y388F]Hck, respectively) were withdrawn at various time points, and the autophosphorylation reaction was stopped by the addition of 5× SDS-PAGE sample buffer. The aliquots were then run on a 7.5% SDS-polyacrylamide gel. The gel was dried and then analyzed by autoradiography. The stoichiometry of autophosphorylation of Hck was then determined by cutting out the dried gel bands of radioactively labeled Hck and subjecting them to liquid scintillation counting. Results of the experiment are presented in Fig. 1B and Fig. 6, A and B.

**Determination of the Kinase Activity**—Aliquots of 10 μl were taken at the same time intervals and added to 50 μl of the dilution buffer (25 mM Hepes, pH 7.0, 5% Nonidet P-40, 1 mM EDTA, 0.1 mg/m benzamidine, and 20% glycerol). From this dilution, 10 μl of the diluted kinase was added to 15 μl of assay mix with the final mixture containing 100 μM ATP (specific radioactivity of approximately 600 cpm/pmol), 20 mM Tris-HCl, pH 7, 10 mM MgCl<sub>2</sub>, 1 mM MnCl<sub>2</sub>, and 50 μM Na<sub>2</sub>VO<sub>4</sub>. The concentration was allowed to continue for 10 min before being stopped with 10 μl of 50% acetic acid. Aliquots of 26 μl of the stopped reaction mix were then spotted onto P81 filter paper and the papers washed 5 times in 300 ml each of 0.5% v/v phosphoric acid. The papers were washed a final time in acetone and dried for liquid scintillation counting. Results of the kinase assay are presented in Fig. 2C and D.

**Preparation of the Phosphopeptide Standard (pY-29 Standard)**—The Hck-(26–38)-peptide was radioactively phosphorylated by recombinant p66<sup>src</sup>. The phosphopeptide containing Tyr(P)-29 as the only phosphorylated residue was purified by reverse phase HPLC under conditions described previously (19, 31).

**Phosphopeptide Mapping**—Recombinant wild type, [Y29F]Hck, or [Y388F]Hck were allowed to autophosphorylate at 30 °C for 60 min in the kinase assay buffer and 50 μM [γ-<sup>32</sup>P]ATP (2000 cpm/pmol). The autophosphorylation reaction was stopped by the addition of 5× SDS-PAGE sample buffer, prior to running on a 7.5% polyacrylamide gel. Following transfer to a nitrocellulose filter, the bands corresponding to the two isoforms of radioactively phosphorylated Hck were excised and blocked with 0.1% polyvinylpyrrolidone 40 in 100 mM acetic acid, washed with 5 × 1 ml of Milli-Q H<sub>2</sub>O, and digested overnight with 29 μl of 0.2 mg/ml of l-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (Worthington) in 100 mM NH<sub>4</sub>HCO<sub>3</sub>, pH 7.8, acetonitrile (95:5, v/v) at 37 °C for 15 h (30). After digestion, the supernatant was lyophilized three times following the addition of 1 ml of Milli-Q H<sub>2</sub>O and the resulting dry material dissolved in 10–20 μl of water. Samples were then subjected to native PAGE and applied onto a 10% polyacrylamide gel. Separation of the proteolytic fragments was accomplished by (i) thin layer electrophoresis (TLE) in the first dimension and (ii) by thin layer chromatography in the second dimension as described previously (15, 18). The radioactive tryptic fragments were located by autoradiography. The identity of the novel autophosphorylation site was verified by demonstrating the co-migration of a [γ-<sup>32</sup>P]-labeled enzymatically phosphorylated synthetic phosphopeptide standard containing the amino acid residues 26–38 of Hck (pY-29 standard) (Fig. 3, B and D). Hck, purified by immunoprecipitation from bone marrow-derived macrophages, was also phosphorylated in vitro and subjected to phosphopeptide mapping (Fig. 4) with procedures and conditions similar to those for the recombinant enzyme. Characterization of [Y29F]Hck and [Y388F]Hck in comparison with wild type Hck by phosphopeptide mapping (Fig. 5) was carried out similarly following autophosphorylation of wild type Hck (95 μM), [Y29F]Hck (115.7 μM) and [Y388F]Hck (360 μM) in the kinase assay buffer and 50 μM [γ-<sup>32</sup>P]ATP.

**Culture and Preparation of Bone Marrow-derived Macrophages**—Bone marrow-derived macrophages were prepared from bone marrow precursors isolated from CBA mouse femurs as described (20). Bone marrow cells were injected i.v. into 50 μg/ml polyclonal met-
cells were incubated for 3 h. The cells were washed three times with ice-cold phosphate-buffered saline and then lysed as described below.

Cell Lysis and Immunoprecipitation of Hck—The bone marrow-derived macrophages and the HEK 293T cells overexpressing Hck were washed three times with ice-cold phosphate-buffered saline and then lysed by the addition of 1% Triton X-100/10 mM Hepes (pH 7.4, 137 mM NaCl, 10% glycerol, 10 mM MgCl₂, 10 μM leupeptin, 10 μM pepstatin A, and 10 μM aprotinin). Aliquots of cell lysate were preclarified with 40 μl of a 1:1 slurry of protein A-Sepharose in TLB and then incubated at 4 °C overnight with 5 μg of anti-Hck antiserum. Immune complexes were collected by the addition of 5 × SDS sample buffer. Hck and other components of the reaction mixture were separated by SDS-PAGE. After drying the gel, the location of the autoproteolyzed protein bands was determined by autoradiography and excised. The gel slices were washed several times with milli-Q H₂O (3 × 1.5 ml) for 1 h before drying under vacuum. The gel pieces were rehydrated with 1 ml of 0.5 M Tris-HCl, pH 8.0, 0.1 M CaCl₂, and 20% acetonitrile (in H₂O) before being loaded onto Applied Biosystems 477A Protein Sequenator. One-third of the resulting PTH-derivative sample for each cycle was then subjected to PTH-derivative analysis with an on-line model 120 PTH-derivative analyzer, and the remaining fraction was collected for scintillation counting (21). Results of the solid phase sequence experiment are shown in Fig. 2.

Construction of the Hck Baculovirus Vectors for the Expression of Hck Carrying the Y29F and Y388F Mutations—Two polymerase chain reactions were carried out using full-length Hck cloned into the XhoI site of the transient mammalian expression vector pCDM8 (as described in Ref. 15). The reaction was carried out using primer 1 (5'- CCCGAATTCGCGTTGG GTCGCTTACGCGGAG -3') and primer 2 (5'-GCGGATCCGCGGAAATTAATACGACTCACATGTTGCTGCGGATCCGCAAGC -3'). The change residue is denoted in boldface italic. The resulting polymerase chain reaction product was purified using the QIAquick PCR purification kit and directionally cloned into pGEX6p-3 (Amersham Pharmacia Biotech) expression vector pre-digested with EcoRI and XhoI.

Expression and Purification of Hck-(1–91)—Escherichia coli DE3 cells were transformed with pGEX6p-3 expression vector cloned with the Hck-(1–91) encoding region and the Hck fragment was expressed in a 1.5-liter culture in Luria-Bertani medium containing 100 μg/ml ampicillin. The induction of GST-Hck-(1–91) expression was carried out as described previously (15). The purified GST-Hck-(1–91) fusion protein was dialyzed against 2 × 1 liter of cleavage buffer containing 50 mM Tris-HCl, pH 7.0, 150 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol. It was digested with the Precision Protease according to the manufacturer protocol. GST cleaved from Hck-(1–91) was removed by reapplying the digest to a glutathione-Sepharose column pre-equilibrated with the cleavage buffer and the column flow through containing the Hck-(1–91) was applied to a fast protein liquid chromatography Mono-S column pre-equilibrated with washing buffer containing 25 mM Hepes, pH 7.0, 1 mM EDTA, 1 mg/ml phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol, and the bound protein was eluted using a 10-mL linear gradient of 0–0.6 M NaCl at a flow rate of 0.5 ml/min.

Concentration Dependence of Autophosphorylation at Tyr-388 and Tyr-29—Autophosphorylation reactions were carried out in the presence of the kinase assay buffer and 50 μM [γ-32P]ATP using constant amounts of Y29F and Y388F Hck (0.55 and 3.2 pmol respectively). However, final reaction volumes were varied such that the effect of differing enzyme concentrations on autophosphorylation could be determined for each mutant. Final enzyme concentrations were 114, 57, 28.5, 14.7, and 7.13 nm for [Y29F]Hck and 1, 0.5, 0.25, 0.125, and 0.0625 μM for [Y388F]Hck. Autophosphorylation reactions were carried out at 30 °C for 15 min. Reactions were stopped by the addition of 5 × SDS-PAGE sample buffer prior to running on a 7.5% polyacrylamide gel in the presence of 0.1% SDS pre-digested with the specific activity of the [γ-32P]ATP was used to determine the rate of autophosphorylation in the unit of picomoles of phosphate incorporated per min. Less than 5% of the Hck mutant was autophosphorylated at the end of the reaction, indicating that the initial velocity of autophosphorylation was measured. The experiment was repeated five times. In all five assays, the same trend of change of the autophosphorylation velocities versus enzyme concentrations was observed. The results shown in Fig. 4 represent the data obtained from one of the assays. Phosphorylation of Hck-(1–91) by Full-length Hck—3.4 pmol of wild type, recombinant Hck was incubated in the presence of 100 μM [γ-32P]ATP with 0, 0.036, 0.073, 0.15, 0.29, and 1.46 nM of Hck-(1–91) in the kinase assay buffer for 30 min in a total volume of 25 μl. 10 μl aliquots were taken from each reaction mixture and added to 10 μl of sample buffer prior to running on a 10% polyacrylamide gel. Since the wild type Hck can autophosphorylate at both Tyr-29 and Tyr-388, a primary antibody specific for phosphotyrosine 29 (anti-pY-29 Hck antibody) was used to quantaize the rate of Tyr-29 phosphorylation in intact Hck and Hck-(1–91). Following transfer to nitrocellulose, the blot was probed with a primary antibody specific for phosphotyrosine 29 (anti-pY-29 Hck antibody). The blot was then washed and probed with anti-rabbit horseradish peroxidase-conjugated secondary antibody, and the protein bands were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech). Results of the experiment are shown in Fig. 13.
body—A phosphopeptide raised against the sequence CASKTEPSAN-
QKGPV(pY)VDPDPTSSSKLGGKK, encompassing the region of the
unique domain of Hck containing Tyrr(P)-29, was synthesized, coupled
to keyhole limpet hemocyanin (Calbiochem), and used as the antigen
for the production of polyclonal antibody. Crude serum was first purified by
ammonium sulfate precipitation according to procedures detailed in
Ref. 23. The non-phosphorylated version of the peptide antigen was
synthesized, purified, and covalently coupled to Affi-Gel-10 (Bio-Rad).
The non-phosphorylated peptide column was used to deplete from the
antiserum of the antibody against the non-phosphorylated version of
the peptide. The antiserum was applied to the non-phosphorylated
peptide column, washed (10 mL Tris, pH 7.5, 0.5 N NaCl), and then
with 100 mL glycine pH 2.5. The flow-through fraction, containing
the antibody against the pY-29 peptide was collected and saved for the next
affinity column step. A second affinity column was produced by co-
valently coupling the phosphorylated peptide used as the antigen to
Affi-Gel-10 (Bio-Rad). The flow-through fraction from the first affinity
column step was preincubated with 1 nM phosphotyrosine and 1 nM
non-phosphorylated peptide prior to application to the column to mini-
imize binding of the nonspecific, phosphotyrosine targeting antibody.

After washing, the bond antibody was eluted with 100 mL glycine, pH
2.5. As shown in Fig. 10, the antibody eluted from the Tyr(P)-29 peptide
column binds to Hck only when it is phosphorylated at Tyr-29. This
antibody is called the anti-pY-29 Hck antibody.

Characterization of the Anti-pY-29 Hck Antibody—0.98 ng of recom-
binant Hck was incubated in the presence of kinase assay buffer and
100 nM [γ-32P]ATP in a total volume of 25 μL. 5-μL aliquots were
removed at 0, 15, 30, and 60 min, and the reaction was stopped by the
addition of 30 μL of 5× SDS-PAGE sample buffer prior to running 10 μL
(containing 55 ng of Hck) of this in duplicate on 7.5% polyacrylamide
gels. As controls, 22.5 ng of pp60c-Src and 300 ng of pp53/56 Lyn were
run on the same duplicate 7.5% SDS-PAGE gels used for the Hck
autophosphorylation time course samples. One of the gels was
incubated with a nitrocellulose membrane, washed (10 mL 0.1 M NaCl), and
this was then incu-

bated with the purified anti-Hck pY-29 antibody. The blot was then
washed, incubated with sheep anti-rabbit secondary antibody conju-
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RESULTS

Activation and Determination of Stoichiometry of Autophos-
phorylation of Wild Type Hck—Hck requires autophosphoryla-
tion in order to become fully activated. Upon incubation with
ATP and Mg2+, recombinant wild type Hck readily undergoes
autophosphorylation, achieving a stoichiometry of 1.3 mol of
phosphate per mol of kinase after 40 min (Fig. 1, A and B).
Concomitant with the increase in autophosphorylation was a
considerable 20-fold increase in specific activity of Hck (Fig. 1C).
The finding that Hck achieves a stoichiometry of autophospho-
rylation greater than 1 mol of phosphate per mol of kinase

FIG. 1. Determination of the stoichiometry of phosphorylation and stimulation of kinase activity of recombinant wild type Hck
upon autophosphorylation. Recombinant, wild type Hck was al-

lowed to autophosphorylate in the presence of [γ-32P]ATP, and aliquots were taken at 0, 5, 10, 20, 30, and 40 min. The reaction was stopped,
and the aliquots were used for determination of the stoichiometry of incorporation of phosphate or to determine the kinase activity of Hck. A,
autoradiogram of the time course of autophosphorylation of wild type Hck. B, the time course of increase in stoichiometry of phosphorylation
of wild type Hck. C, the time course of increase in kinase activity of wild
type Hck. The kinase activity was measured by the rate of phosphoryl-

ation of the [Lys19(Cdc2)-6–20]-substrate peptide by the kinase.

Western Blot and Immunoprecipitation Analysis of Lysate Produced
from [Y499F]Hck- and [Y29F]Hck-expressing HEK 293T Cells—HEK
293T cells were transfected with pEF-[Y499F]Hck, pEF-[Y29F]Hck, or
just the pEF-Bos vector. The cells were lysed with 1% Nonidet P-40 lysis
buffer (20 mL Tris-HCl, pH 7.4, 150 mL NaCl, 1 mL EDTA, 1 mL
dithiothreitol, 1% Nonidet P-40, 10% glycerol, 1 mL sodium orthova-
date, 0.1 mL sodium molybdate, 1 mL Pefabloc, 10 mg/mL leupeptin, 100
units/mL aprotinin) 65 h post-transfection. Cells lysates were analyzed for
Hck and Hck phosphorylation by immunoprecipitation experiments. Aliq 
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The two purified radioactive fragments (I and II) were analyzed by solid phase sequencing in order to allow the sites of autophosphorylation in Hck to be identified. For phosphopeptide II, the profile of radioactivity associated with the PTH-derivative generated in each cycle of Edman degradation of phosphopeptide fragment I. C, the profile of radioactivity associated with the PTH-derivative generated in each cycle of Edman degradation of phosphopeptide fragment II.

clearly suggests that Hck is capable of autophosphorylating at more than one site.

Identification of Tyr-388 and Tyr-29 as the in Vitro Autophosphorylation Sites of Hck—Phosphoamino acid analysis of recombinant Hck that had been allowed to autophosphorylate to a stoichiometry of 1.5 mol of phosphate/mol of protein revealed that phosphorylation of Hck had only occurred on tyrosine (data not shown). As shown in Fig. 2A, when tryptic digests of both the 56- and 59-kDa isoforms of autophosphorylated Hck were subjected to reversed phase HPLC, the presence of two radioactive spots corresponding to the tryptic fragments of the phosphorylated Hck, spot II was found to co-migrate with the peptide standard with the tryptic digest of each isoform of phosphorylated Hck (Fig. 3, A and C). Upon mixing of the pY-29 peptide standard with the tryptic digest of each isoform of phosphorylated Hck, spot II was found to co-migrate with the standard, indicated by its increase in intensity (Fig. 3, B and D). As Tyr-388 is the other major phosphorylation site in the two dimensional phosphopeptide mapping was examined. As shown in Fig. 3, two closely migrating but distinguishable radioactive spots corresponding to the tryptic fragments of the two autophosphorylation sites were detected in the phosphopeptide maps of both the 56- and 59-kDa isoforms of phosphorylated Hck (Fig. 3, A and C). Upon mixing of the pY-29 peptide standard with the tryptic digest of each isoform of phosphorylated Hck, spot II was found to co-migrate with the standard, indicated by its increase in intensity (Fig. 3, B and D). As Tyr-388 is the other major phosphorylation site in the autophosphorylated Hck (Fig. 2), spot I should correspond to the pY-388-containing tryptic fragment.

Taken together, these results unequivocally identify Ty3-388 in the unique domain as one of the autophosphorylation sites in both isoforms of Hck. This is the first observation that an Src family kinase is capable of autophosphorylating a tyrosine residue in its unique domain.

Hck Isolated from Macrophages Also Undergoes Autophosphorylation at Tyr-29—Since autophosphorylation of Hck at Tyr-29 was identified in experiments that used recombinant Hck expressed in Sf9 cells, we wanted to establish if Hck that

therefore, further characterization of this site was not undertaken.

Solid phase sequencing of phosphopeptide II released most of the radioactivity at the fourth cycle of Edman degradation (Fig. 2C). Hck-(26–36) with sequence GPVYVPDPTSSSK is the only predicted fragment generated from exhaustive tryptic digestion of Hck that has a tyrosine (Tyr-29) as the fourth residue from the N terminus (Table I). The identity of phosphopeptide II as the phosphorylated form of the Hck-(26–36) fragment was further verified by identifying the PTH-derivative released in each cycle of Edman degradation. The sequence obtained was GPVXVPX in which “X” represents a cycle in which no PTH-derivative was identified. The fourth cycle of Edman degradation would have released the phosphorylated PTH-Tyr-29 that broke down during the procedure and therefore could not be seen.

The novel autophosphorylation site Tyr-29 (underlined) is the fourth residue in the fragment derived from residues 26–36. The consensus autophosphorylation site (Tyr-388) and the C-terminal regulatory phosphorylation site (Tyr-499) are marked by asterisks. Tyr-388 is the seventh residue in the fragment derived from residues 382–391.

| Residue no. of fragments | Sequences                        |
|--------------------------|----------------------------------|
| 26–36                    | GPVYVPDSSKK                      |
| 39–71                    | LGP\text{\textsuperscript{\texttimes}}NSNMPGEGSVDTIVVALYEAIR |
| 104–112                  | EGYIFSNYAR                       |
| 174–176                  | HYK                              |
| 179–190                  | TLSDGGFYISPR                     |
| 245–260                  | LGQGQGFEWVATYK                   |
| 353–357                  | NYIHR \textsuperscript{(a)}     |
| 382–391                  | IIEDEYETAR                       |
| 415–432                  | SDWNSFGLIMELYYGR                 |
| 433–445                  | IPYPGMNPEVIR                     |
| 451–452                  | YR                               |
| 456–489                  | PDNCPCEELYNIMIR \textsuperscript{**} |
| 479–503                  | PTEYIQSVLDDFYATESTQYQQQP         |

![Fig. 2. Solid phase sequencing of the consensus and novel autophosphorylation sites of Hck. Peptide fragments generated from in-gel tryptic digestion of the phosphorylated Hck were separated by reversed phase HPLC. A, radioactivity profile of the HPLC fractions. The two purified radioactive fragments (I and II) were analyzed by solid phase sequencing. B, the profile of radioactivity associated with the PTH-derivative generated in each cycle of Edman degradation of phosphopeptide fragment I. C, the profile of radioactivity associated with the PTH-derivative generated in each cycle of Edman degradation of phosphopeptide fragment II.](Image)
Figure 3. Phosphopeptide mapping of the novel site of autophosphorylation of recombinant, wild type Hck. Recombinant, wild type Hck was allowed to autophosphorylate in the presence of \([\gamma^{32}P]ATP\) and processed by SDS-PAGE and Western blot analysis. The 56- and 59-kDa isoforms were processed for two-dimensional phosphopeptide mapping as described under “Experimental Procedures.” A, two-dimensional phosphopeptide map of the tryptic digest of the 56-kDa isoform of recombinant wild type Hck. B, two-dimensional phosphopeptide map of the tryptic digest of the 56-kDa isoform of recombinant wild type Hck. C, two-dimensional phosphopeptide map of the tryptic digest of the 59-kDa isoform of wild type, recombinant Hck. D, two-dimensional phosphopeptide map of the 59-kDa isoform of wild type, recombinant Hck in the presence of phosphopeptide standard.

Figure 4. Demonstration of in vitro autophosphorylation of Tyr-29 in Hck immunoprecipitated from macrophages. Hck was immunoprecipitated from bone marrow-derived macrophages and phosphorylated in vitro in the presence of \([\gamma^{32}P]ATP\). Tryptic phosphopeptide fragments of the 56-kDa and 59-kDa isoforms were analyzed by two-dimensional phosphopeptide mapping. The directions of TLE and TLC are indicated by arrows. A, two-dimensional phosphopeptide map of the tryptic digest of the 56-kDa isoform of macrophage-derived Hck. B, two-dimensional phosphopeptide map of the 56-kDa isoform of Hck in the presence of \([\gamma^{32}P]\)phosphate-labeled phosphopeptide standard derived from the theoretical tryptic fragment of Hck encompassing Tyr-29. C, two-dimensional phosphopeptide map of the tryptic digest of the 59-kDa isoform of macrophage-derived Hck. D, two-dimensional phosphopeptide map of the 59-kDa isoform of macrophage-derived Hck in the presence of the phosphopeptide standard described above.

had been expressed under normal physiological conditions (e.g., in macrophages) also undergoes autophosphorylation at Tyr-29 and Tyr-388 in vitro. Accordingly, Hck was immunoprecipitated from bone marrow-derived mouse macrophages and allowed to autophosphorylate in vitro in the presence of \([\gamma^{32}P]ATP\). Analysis of the autophosphorylation reaction by SDS-PAGE followed by autoradiography revealed the presence of two major radioactive bands, corresponding to the 56- and 59-kDa isoforms of Hck (data not shown). The radioactive bands corresponding to the two Hck isoforms were separately excised from the gel and digested with trypsin. Since the amount of protein present in the tryptic digests was considerably less than that obtained in experiments where we had used recombinant Hck expressed in SF9 cells, we were unable to purify the radioactively labeled peptides by HPLC for direct amino acid sequencing. Consequently, the sites of phosphorylation in Hck were determined by subjecting the tryptic digests to two-dimensional phosphopeptide mapping. Two radioactive spots were detected on the two-dimensional TLE/TLC map for both the 56- and 59-kDa isoforms of Hck (Fig. 4A and C). Of the two labeled spots, spot II co-migrated with the pY-29 phosphopeptide standard (Fig. 4, B and D), suggesting that Hck isolated from macrophages is capable of autophosphorylating Tyr-29 in vitro and that autophosphorylation of the recombinant Hck at Tyr-29 is not a cloning or expression artifact.

Characterization of [Y29F]Hck and [Y388F]Hck—As a auto-phosphorylation can occur at Tyr-388 and Tyr-29 in vitro, we wished to determine how these phosphorylation sites respectively affect the activity of the kinase. To carry this out, we produced two mutants termed [Y29F]Hck and [Y388F]Hck with Tyr-29 or Tyr-388 replaced by Phe. [Y29F]Hck and [Y388F]Hck were allowed to autophosphorylate and were subsequently analyzed by two-dimensional phosphopeptide mapping with reference to the wild type enzyme (Fig. 5). Wild type Hck produces two spots corresponding to phosphorylation of Tyr-29 and Tyr-388 (Fig. 5A), whereas the [Y29F]Hck and [Y388F]Hck mutants autophosphorylate at a single site corresponding to Tyr-388 (Fig. 5B) and Tyr-29 (Fig. 5C), respectively. To prove that the sites of phosphorylation of [Y29F]Hck and [Y388F]Hck correspond to Tyr-388 and Tyr-29, respectively, tryptic digests of these autophosphorylated mutants were mixed with a tryptic digest of the wild type Hck (Fig. 5, D and E). As expected, mixing the tryptic digests of phosphorylated [Y29F]Hck and wild type Hck leads to an increase in intensity of the spot corresponding to the fragment containing Tyr(P)-388 with respect to that containing Tyr(P)-29, whereas mixing the tryptic digests of phosphorylated [Y388F]Hck and wild type Hck leads to an increase in the intensity of the spot corresponding to the fragment containing Tyr(P)-29 with respect to that containing Tyr(P)-388 (Fig. 5D). This proves that the sites phosphorylated upon autophosphorylation of [Y29F]Hck and [Y388F]Hck correspond to Tyr-388 and Tyr-29, respectively. A faint spot is evident in the phosphopeptide maps above the origin. This spot is likely to be derived from Tyr(P)-499, suggesting that a very low level of autophosphorylation also occurred at Tyr-499.

Modulation of Tyrosine Kinase Activity of Hck by Phosphorylation of Tyr-388 and Tyr-29—In order to determine if phosphorylation of Tyr-29 of Hck contributes to the modulation of tyrosine kinase activity, changes in the stoichiometry of phosphorylation of Tyr-29 and Tyr-388 were measured by means of autophosphorylation time courses of the respective mutants. The stoichiometry of phosphorylation of Tyr-388 in the [Y29F]Hck mutant was...
shown to increase to 1 mol of phosphate incorporated per mol of enzyme over the 50-min time course (Fig. 6A). Concomitant with this was a 15-fold increase in catalytic activity peaking at 30 min (Fig. 6C).

In contrast, the rate of [Y388F]Hck autophosphorylation was much slower than that of [Y29F]Hck autophosphorylation. The stoichiometry of phosphorylation of [Y388F]Hck was found to approach 0.2 mol of phosphate incorporated per mol of enzyme over the 50-min autophosphorylation time course (Fig. 6B). The results indicate that Tyr-29 was autophosphorylated with an efficiency much lower than that of Tyr-388 autophosphorylation. Intriguingly, even though Tyr-29 does not reside in the catalytic domain, an approximately 2-fold increase in catalytic activity was also evident over the time course of autophosphorylation (Fig. 6D). Autophosphorylation of [Y388F]Hck beyond 30 min led to a drop in its kinase activity, suggesting that the stability of this mutant can only be preserved within 30 min of preincubation with Mg\(^{2+}\)/ATP. For this reason, we did not attempt to prolong the autophosphorylation reaction time to generate the stoichiometrically phosphorylated mutant for further analysis of the effect of Tyr-29 phosphorylation on the catalytic activity of Hck. Nevertheless, that a 100% increase in kinase activity could be accomplished by only a 0.2 mol of phosphate per mol of Hck autophosphorylation stoichiometry indicates that autophosphorylation of Tyr-29 can contribute significantly to the full activation of Hck.

Autophosphorylation of Hck, at Both Tyr-388 and Tyr-29, Is Concentration-dependent—Several Src family tyrosine kinases have been demonstrated to undergo autophosphorylation of the consensus tyrosine in the kinase domain by an intermolecular mechanism. In order to determine if autophosphorylation at Tyr-29 and Tyr-388 occurs by an intramolecular or intermolecular mechanism, concentration dependence studies were carried out. [Y29F]Hck and [Y388F]Hck at varying concentrations were allowed to autophosphorylate in the presence of \([γ-^{32}P]ATP\) (Fig. 7). Autophosphorylation at Tyr-388 (Fig. 7A) and at Tyr-29 (Fig. 7B) are both shown to increase with increasing concentrations of the [Y29F]Hck and [Y388F]Hck mutants, respectively. This result is indicative that autophosphorylation occurring at both Tyr-29 and Tyr-388 follows a

**Fig. 6.** The stoichiometry of phosphorylation and stimulation of catalytic activity of recombinant [Y29F]Hck and [Y388F]Hck upon autophosphorylation. [Y29F]Hck and [Y388F]Hck were allowed to autophosphorylate in the presence of \([γ-^{32}P]ATP\). Aliquots were taken at 0, 5, 10, 20, 30, 40, and 50 min; the autophosphorylation reaction was stopped, and the stoichiometry of phosphate incorporation or the kinase activity was determined over the time course. A, time course for the stoichiometry of phosphate incorporation upon autophosphorylation of recombinant [Y29F]Hck. B, time course for the stoichiometry of phosphate incorporation upon autophosphorylation of recombinant [Y388F]Hck. C, time course for the stimulation of kinase activity of recombinant [Y29F]Hck upon autophosphorylation. D, time course for the stimulation of kinase activity of recombinant [Y388F]Hck upon autophosphorylation.
Novel Autophosphorylation Site of Hck

**Fig. 7.** Concentration dependence of the rate of autophosphorylation of [Y29F]Hck and [Y388F]Hck. Fixed amounts of [Y29F]Hck and [Y388F]Hck were allowed to autophosphorylate in the presence of γ-32P-ATP at varying final enzyme concentrations. A, the rate of autophosphorylation of Tyr-388 for each concentration of [Y29F]Hck was determined from [32P]phosphate incorporation and plotted against enzyme concentration for [Y29F]Hck. B, samples were analyzed similarly to A for the rate of autophosphorylation of Tyr-29 in [Y388F]Hck.

concentration-dependent, intermolecular mechanism.

**Hck in Macrophages Is Predominantly in the Down-regulated, Tyr-499-phosphorylated Form**—In order to determine if autophosphorylation of Hck at Tyr-29 also occurs *in vivo*, cells from a mouse macrophage cell line (RAW264.7 cells) were metabolically labeled with 32P, so as to allow endogenous Hck to incorporate [32P]phosphate. Hck was then immunoprecipitated from lysates of the 32P-labeled cells and subjected to two-dimensional phosphopeptide mapping. Only a very faint radioactive spot located to the right of the origin, in the region where tryptic phosphopeptides containing Tyr-388 and Tyr-29 (i.e. spots I and II) migrate, was detected (Fig. 8). Therefore, phosphorylation at Tyr-29 could not be verified. We also attempted to demonstrate *in vivo* autophosphorylation of endogenous Hck at Tyr-29 using the anti-pY-29 Hck antibody. The antibody failed to detect any Tyr-29 phosphorylation. The results suggest that the Tyr-29 of Hck is predominantly in the non-phosphorylated form.

Four strong radioactive spots to the left of the origin are obvious in the phosphopeptide map shown in Fig. 8. As indicated by comparison of the phosphopeptide maps of wild type Hck and [Y499F]Hck phosphorylated *in vivo* (Fig. 9), the four spots correspond to the tryptic fragments containing Tyr(P)-499. Thus the phosphopeptide map shown in Fig. 8 suggests that the majority of Hck in RAW264.7 cells is phosphorylated at Tyr-499. Tyr-499 phosphorylation keeps the kinase in an inactive state and does not permit autophosphorylation at Tyr-388 and Tyr-29. We are currently attempting to identify physiological conditions that lead to activation of Hck by inducing dephosphorylation of Tyr-499 and autophosphorylation of both Tyr-388 and Tyr-29.

**Phosphorylation at Tyr-29 Occurs in Vivo**—As an alternative approach to determine if Hck undergoes autophosphorylation at Tyr-29 *in vivo*, an activated form of Hck was created by replacing the regulatory tyrosine at position 499 with phenylalanine ([Y499F]Hck). This mutant along with wild type Hck were transiently expressed in HEK 293T cells. The cells were then metabolically labeled with [32P]phosphate. Wild type and [Y499F]Hck were immunoprecipitated from lysates of the labeled cells and subjected to two-dimensional phosphopeptide mapping. As shown in Fig. 9, two radioactive spots that exhibited electrophoretic and chromatographic properties identical to spots I and II in Fig. 3 were detected.

When the tryptic digests of the phosphorylated wild type Hck and [Y499F]Hck were mixed with the pY-29 phosphopeptide

**Fig. 8.** Phosphorylation of Hck in RAW 264.7 cells occurs predominantly at Tyr-499. RAW 264.7 cells were metabolically labeled with 32P, and Hck was immunoprecipitated with anti-Hck antibody. The immunoprecipitate was analyzed by SDS-PAGE and transferred to nitrocellulose, and the radioactively labeled Hck was processed for two-dimensional phosphopeptide mapping. The four-pronged arrow denotes spots resulting from phosphopeptide fragments containing the phospho-Tyr-499 of Hck. The directions of thin layer electrophoresis and thin layer chromatography are denoted, as is the faint spot corresponding to phosphorylated Tyr-29 or Tyr-388.

**Fig. 9.** In vivo phosphorylation of Tyr-29 of Hck in HEK 293T cells. Wild type or [Y499F]Hck was transiently overexpressed in HEK 293T cells. Cells were metabolically labeled with 32P, and lysed. Hck was immunoprecipitated from the lysates, run by SDS-PAGE, transferred to nitrocellulose, and processed for two-dimensional phosphopeptide mapping. The four-pronged arrow denotes spots resulting from phosphopeptide fragments containing the phospho-Tyr-499 of Hck. The directions of thin layer electrophoresis and thin layer chromatography are denoted, as is the faint spot corresponding to phosphorylated Tyr-29 or Tyr-388.
vivo (Fig. 9B), but not in the tryptic phosphopeptide map of the [Y499F]Hck (Fig. 9A), suggesting that the four radioactive fragments contain the in vivo phosphorylated Tyr-499 residue. The multiple phosphopeptide fragments generated from C-terminal regulatory tyrosine (Tyr-499) in Figs. 8 and 9 are attributed in part to the presence upstream from Tyr-499 of two Arg–Pro bonds that are known to be refractory to tryptic cleavage (26). Four conspicuous radioactive spots of similar pattern are also found in the phosphopeptide map (Fig. 8) of Hck isolated from RAW 264.7 cells, further confirming that Tyr-499 of Hck is phosphorylated in this cell line.

Characterization of the anti-pY-29 Hck-specific Polyclonal Antibody—Since Hck consists of three tyrosine phosphorylation sites (i.e. Tyr-29, Tyr-388, and Tyr-499), an antibody, which specifically cross-reacts with Tyr(P)-29, would be an effective reagent for monitoring the phosphorylation status of Hck. This antibody termed anti-pY-29 Hck antibody, was effective as a reagent for monitoring the phosphorylation status of c-Src which had been either C-terminally phosphorylated by CSK or autophosphorylated. Antibody—specific polyclonal antibody that is effective reagent for monitoring the phosphorylation status of c-Src which specifically cross-reacts with Tyr(P)-29, would be an effective reagent for monitoring the phosphorylation status of c-Src which had been either C-terminally phosphorylated by CSK or autophosphorylated. Antibody—specific polyclonal antibody that is effective reagent for monitoring the phosphorylation status of c-Src which specifically cross-reacts with Tyr(P)-29, would be an effective reagent for monitoring the phosphorylation status of c-Src which had been either C-terminally phosphorylated by CSK or autophosphorylated.

Fig. 10. Characterization of the phosphorylation-specific anti-pY-29 Hck-specific antibody. A, Hck was allowed to autophosphorylate in the presence of [γ-32P]ATP, and aliquots were taken at 0, 15, 30, and 60 min. The reaction was stopped and analyzed by SDS-PAGE together with samples of Lyn and Src either C-terminally phosphorylated (p-tail Tyr) by CSK or autophosphorylated (pY-397 and pY-416, respectively). The gel was dried, and radioactive bands were visualized by autoradiography. B, duplicate gel of A was transferred to nitrocellulose and probed with anti-pY-29 Hck antibody. Immunoreactive bands were visualized by enhanced chemiluminescence. C, [Y388F]Hck or [Y29F]Hck were autophosphorylated at a final enzyme concentration of 0.35 μM in the presence of 100 μM [γ-32P]ATP and the Kinase Assay Buffer. At timed intervals, 7-μl aliquots containing 2.46 pmol of the enzyme were taken out and subsequently analyzed by immunoblot using the anti-pY-29 Hck antibody. The autoradiogram showing the degree of autophosphorylation of the two mutants after incubation with [γ-32P]ATP and kinase assay buffer for 10, 20, 30, 40, and 60 min. D, immunoblot depicting the immunoreactivity of the anti-pY-29 Hck antibody with the autophosphorylated Hck mutants.
autophosphorylated at Tyr-29 in vivo.

Hck at Low Concentrations Can Autophosphorylate at Tyr-29—The in vitro and in vivo data presented so far unequivocally demonstrate that Hck can autophosphorylate at Tyr-29. However, the in vitro phosphorylation of Hck was carried out at relatively high concentrations (100–400 nM) of Hck. Furthermore, in vivo phosphorylation of Tyr-29 was detected only when Hck was overexpressed in HEK 293T cells, and the levels of expression of recombinant Hck and its mutants in HEK 293T cells were 10–70-fold higher than those of endogenous Hck in macrophages (see under “Experimental Procedures” for details of Hck concentration estimation). For these reasons, there is a possibility that the phosphorylation of Tyr-29 is a “forced” autophosphorylation event that only occurs at high Hck concentrations. To rule out this possibility, we examine if Hck at lower concentrations (e.g. low nM to pM concentrations) can undergo autophosphorylation at Tyr-29. As shown in Fig. 12, Tyr-29 autophosphorylation at Hck concentrations ranging from 0.46 to 3.7 nM was readily detected by the anti-pY-29 Hck antibody, indicating that autophosphorylation at Tyr-29 can occur at both high and low concentrations of Hck in vitro.

It is generally believed that Src family kinases are recruited to specific subcellular localizations through binding of the unique, SH2, and SH3 domains to cell surface receptors and other cellular proteins. Conceivably, recruitment of the kinases to specific subcellular compartments can significantly increase their effective concentrations and hence enhance their autophosphorylation. However, we do not know the effective concentrations of Src family kinases undergoing autophosphorylation in the specific subcellular compartments. It is therefore not known if the concentration ranges of Hck we chose to carry out the experiments shown in Figs. 1, 6, 7, and 12 reflect the actual concentrations at which Hck undergoes autophosphorylation in specific subcellular compartments in vivo. The result presented in Fig. 12, however, does suggest that Tyr-29 phosphorylation is not a forced autophosphorylation event.

Hck-(1–91) Is a Poor Substrate for Phosphorylation by Full-length, Recombinant Hck—Although Fig. 7 demonstrates that autophosphorylation of Tyr-29 follows an intermolecular mechanism, it is not clear if structural integrity of Hck is necessary for the efficient autophosphorylation of Tyr-29. To ascertain the structural requirement for efficient autophosphorylation of Tyr-29, the relative efficiencies of Tyr-29 phosphorylation in intact Hck and in the Hck-(1–91) fragment by intact Hck were compared.

As evident from its significant reactivity with the anti-pY-29-specific antibody (Fig. 13), wild type Hck efficiently autophosphorylates at Tyr-29. In contrast, at least a 40-fold excess of Hck-(1–91) was required before low levels of Tyr-29 phosphorylation was apparent. This illustrates that structural requirements necessary for the efficient phosphorylation of Tyr-29 are not met in the isolated unique domain of murine Hck. Regions in addition to the unique domain appear to be required for efficient phosphorylation to occur at this site.

DISCUSSION

The recent study by Moarefi et al. (7), showing activation of Hck by SH3 domain displacement, used a truncated form of Hck without the fatty acid acylation domain and unique domain. As such, they would only have noticed phosphorylation at Tyr-388, and phosphorylation at Tyr-29 of the unique domain would have gone unnoticed. The use of intact recombinant Hck and Hck isolated from macrophages enabled us to demonstrate autophosphorylation of Hck’s at both Tyr-29 and Tyr-388. More importantly, our demonstration of the in vivo phosphorylation of Hck at Tyr-29 indicates that phosphorylation of this novel site is of physiological significance. Furthermore, evidence supporting the notion that phosphorylation at Tyr-29 may contribute to the regulation of catalytic activity is provided in Fig. 6. Since activation of protein kinases by auto-
phosphorylation normally results from phosphorylation of a conserved tyrosine and/or a threonine residue in the activation loop or the kinase domain, activation of Hck by phosphorylation of Tyr-29 in the unique domain may represent a new and novel mechanism of activation of protein kinases (27). Elucidation of the structural basis of activation of Hck by Tyr-29 phosphorylation may reveal this new activation mechanism.

The modulation of catalytic activity of Hck by phosphorylation at Tyr-29 shows that there must be some cross-talk between the unique and catalytic domains. As the recent crystal structure determination of Hck also used the truncated mutant excluding the unique domain, it is not known how phosphorylation at Tyr-29 may impart conformational change such that catalytic activity may be enhanced. From the known structural data of Hck, it is likely that the unique domain makes contact with the SH3 and/or the catalytic domain. Therefore, it is possible that phosphorylation at Tyr-29 may produce structural changes in the unique domain which is then transmitted to either or both of these domains. As Moarefi et al. (7) have shown, displacement of the SH3 domain from the SH2 kinase domain linker serves to activate the kinase, which is likely to occur through providing for rotation of the a-C helix such that ion pairing occurs between Glu-303 and Lys-288. Consequently, ATP binding is made feasible which in turn allows for autophosphorylation to occur. It is possible that phosphorylation at Tyr-29 may impart conformational change to the SH3 domain which will further destabilize the tripartite interactions among the SH3 domain, the SH2-CD linker, and the catalytic domain. As the enzyme can be visualized as being in a fluid state of conformational equilibrium, such a change may act to move the equilibrium more toward the active conformation.

Phosphorylation at Tyr-29 of the isolated recombinant unique domain of Hck by full-length Hck is shown to occur with low efficiency (Fig. 13). It is likely that the unique domain must be situated in the context of the intact enzyme to provide for the structural requirements for efficient phosphorylation of Tyr-29. It may be that the intermolecular interaction between two molecules allowing for the autophosphorylation of Tyr-388 places the unique domain in the correct structural alignment for the efficient phosphorylation of Tyr-29. Activation of the enzyme by autophosphorylation at the consitent site would further increase the propensity for phosphorylation at Tyr-29. Alternatively, interactions made with the unique domain by adjacent domains such as the SH3 and catalytic domains may provide for enhanced affinity of Tyr-29 for the active site of Hck.

Interestingly, upon sequence comparison between different Src family kinases, the YXXDPT motif corresponding to $^{32}$YPDPT in murine Hck was found to occur also in Lyn $^{32}$YVPDPT, Fgr $^{32}$YPDPT, Fyn $^{32}$YGTDPT, and Yrk $^{32}$YPDPT. As this motif is repeated several times in the Src family of kinases and occurs at either residue number 29 or 32 in each member, it may be of general regulatory significance for the members in which it occurs.

Similar to most tyrosine phosphorylation sites in protein tyrosine kinases, the phospho-Tyr-29 may also act as a docking site for specific cellular proteins containing SH2 and/or protein tyrosine-binding (PTB) domains. Comparison of the sequence around phospho-Tyr-29 with those of known SH2 domain and PTB domain binding motifs does not reveal any significant homology. Some degree of homology can be found between the GPVpY sequence and the consensus NPxY motif recognized by the Shc PTB domain (28). Structural analysis of the Shc PTB domain bound to the ligand reveals that the conserved Pro residue is essential for the formation of the $\beta$-turn recognized by the Shc PTB domain. As the GPVpY motif of Hck is predicted to have a propensity to form a $\beta$-turn, it is possible that this motif is the docking site of an as yet to be identified PTB domain. Elucidation of the cellular functions of the phosphorylation of Tyr-29 in addition to modulation of activity awaits the isolation and identification of the putative cellular protein(s) which selectively bind to the sequence around Tyr-29.

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REFERENCES

1. Courtenide, S. A. (1994) in Protein Kinases (Woodgett, J. R., ed) pp. 212–230, IRL Press at Oxford University Press, Oxford
2. Thomas, S. M., and Brugge, J. S. (1997) Annu. Rev. Cell. Dev. Biol. 13, 513–609
3. Sichier, F., Moarefi, I., and Kuriany, J. (1997) Nature 385, 602–609
4. Xu, W., Harrison, S. C., and Eck, M. J. (1997) Nature 385, 595–601
5. Williams, J. C., Weijland, A., Gongfoni, S., Thompson, A., Courtenide, S. A., Superti-Furga, G., and Wiarenga, R. K. (1997) J. Mol. Biol. 274, 757–775
6. Gongfoni, S., Williams, J. C., Hattula, K., Weijland, A., Wiarenga, R. K., and Superti-Furga, G. (1997) EMBO J. 16, 7261–7271
7. Moarefi, I., LaFvre-Bernt, M., Sichier, F., Huse, M., Lee, C.-H., Kuriany, J., and Miller, W. T. (1997) Nature 385, 629–633
8. Songyang, Z., Shoelson, S. E., Chabauduri, M., Gish, G., Paskow, T., Haser, W. G., King, F., Roberts, T., Ratoznitsky, S., Lechleider, R. J., Neel, B. G., Birge, R. B., Fajardo, J. E., Chen, M. M., Hafafuna, H., Schaffhausen, B., and Cancela, C. (1993) Cell 72, 767–778
9. Lim, W. A., Richards, F. M., and Fox, R. O. (1994) Nature 372, 375–379
10. Feng, S., Chen, J. Y., Yu, H., Simon, J. A., and Schreiber, S. L. (1994) Science 266, 1241–1247
11. Campbell, K. S., Buder, A., and Deuschel, U. (1995) Eur. J. Immunol. 25, 2408–2391
12. Rich, W., Richert, N. D., Pastan, I., and Gottesman, M. M. (1983) J. Biol. Chem. 258, 10768–10773
13. Gould, K. L., Woodgett, J. R., Cooper, J. A., Buss, J. E., Shallaway, D., and Hunter, T. (1985) Cell 42, 849–857
14. Morgan, D. O., Kaplan, J. M., Bishop, J. M., and Varmus, H. E. (1988) Cell 57, 755–786
15. Sicilia, R. J., Hibbs, M. L., Bello, P. A., Borge, J. D., Fujita, D. J., Stanley, I. J., Dunn, A. S., and Cheng, H.-C. (1996) J. Biol. Chem. 271, 16756–16763
16. Cheng, H.-C., Nishio, H., Hataoue, O., Ralph, S., and Wang, J. H. (1992) J. Biol. Chem. 267, 9248–9256
17. Deleted in proof
18. Cheng, H.-C., Liwun, C. M. E., Hwang, D. M., and Wang, J. H. (1991) J. Biol. Chem. 266, 17919–17925
19. Boyle, W. J., van der Geer, P., and Hunter, T. (1991) Methods Enzymol. 201, 186–199
20. Vairo, G., and Hamilton, J. A. (1985) Biochem. Biophys. Res. Commun. 132, 430–437
21. Wettenhall, R. E. H., Aebersold, R. H., and Hood, L. E. (1991) Methods Enzymol. 201, 186–199
22. Graae, T. D. C. (1962) Nature 193, 788–789
23. Harlow, E., and Lane, D. (1988) Antibodies, pp. 298–299, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
24. Minoryama, S., and Nagata, S. (1990) Gene (Amst.) 18, 5322
25. Bousif, O., Lezouaucl, F., Zanta, M. A., Mergny, M. D., Scherman, D., Demeneix, B., and Berh, J. P. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 7297–7301
26. Allen, G. (1981) in *Sequencing of Proteins and Peptides, Laboratory Techniques in Biochemistry and Molecular Biology* (Work, T. S., and Burdon, R. H., eds) pp. 53–54, Elsevier/North-Holland Biomedical Press, Amsterdam
27. Johnson, L. N., Noble, M. E., and Owen, D. J. (1996) *Cell* 85, 149–158
28. van der Geer, P., Wiley, S., Gish, G. D., Lai, V. K., Stephens, R., White, M. F., Kaplan, D., and Pawson, T. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 963–968
29. Zheng, X.-M., Resnick, R. J., and Shalloway, D. (2000) *EMBO J.* 19, 964–978
30. Aebersold, R. (1995) in *A Practical Guide to Peptide and Protein Purification and Microsequencing*, 2nd Ed., (Matsudaira, P., ed) pp. 71–88, Academic Press, New York
31. Johnson, T. M., Perich, J. W., Bjorge, J. D., Fujita, D. J., and Cheng, H.-C. (1997) *J. Pept. Res.* 50, 365–371
32. Li, C. F., Wang, J. H., and Colyer, J. (1990) *Biochemistry* 29, 4535–4540