Enhanced Phosphorylation of Src Family Kinase Substrates Containing SH2 Domain Binding Sites*

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Src family protein-tyrosine kinases possess several modular domains important for regulation of catalytic activity and interaction with potential substrates. Here, we explore interactions between the SH2 domain of Hck, a Src family kinase, and substrates containing SH2 domain-binding sites. We have synthesized a series of peptide substrates containing a high affinity SH2 domain binding site, (phospho)Tyr-Glu-Glu-Ile. We show that the presence of this sequence in a peptide results in a dramatic increase in the phosphorylation rate of a second tyrosine located at the N terminus. Enhanced phosphorylation is not a consequence of stimulation of enzymatic activity by C-terminal tail displacement but is imparted instead by a 10-fold reduction in the $K_m$ of the phosphotyrosine-containing peptide when compared with a control. The isolated catalytic domain of the non-receptor tyrosine kinase Abl does not show a preference for the pYEEI motif-containing peptide; however, the preference is restored when the SH2 domain of Src is introduced into Abl. Furthermore, enhanced phosphorylation is dependent on the distance between SH2 domain-binding site and phosphorylatable tyrosine, with the minimum distance requirement being seven amino acids. Reversing the orientation of the pYEEI motif with respect to the substrate sequence decreases phosphorylation by down-regulated Hck, but both orientations are utilized equally well by activated Hck. We discuss the possible implications of these results for processive phosphorylation of substrates in vivo by Src family kinases.

There are presently nine identified members of the Src family of non-receptor tyrosine kinases. These enzymes are widely expressed in many tissues and are involved in the conversion of extracellular signals into cellular responses (see Ref. 1 for review). The primary structure of Src family kinases reveals a highly modular organization shared among all members. In addition to the kinase catalytic domain that is responsible for enzymatic activity, Src family kinases possess noncatalytic modules termed Src homology domains (SH2 and SH3). SH2 and SH3 domains mediate specific intramolecular and intermolecular interactions that are important for signal transduction; SH2 domains recognize short peptide motifs containing phosphotyrosine, and SH3 domains bind to proline-rich sequences (2, 3).

For Src family kinases, SH2 and SH3 domains have both a negative and a positive regulatory role in tyrosine kinase activity. The negative regulatory role arises from intramolecular contacts between the SH2 and SH3 domains and the tyrosine kinase catalytic domain. The structural basis for repression of catalytic activity has recently been elucidated by crystal structures of the down-regulated forms of Src and Hck (4, 5). Phosphorylation of Src family kinases at a C-terminal regulatory site by another protein kinase (designated Csk) leads to inhibition. This inhibition is caused by an intramolecular interaction between the phosphorylated tail and the SH2 domain (6–9). A polyproline type II helix is formed by the linker connecting the SH2 domain to the catalytic domain, and this polyproline type II helix serves as a docking site for the SH3 domain. This SH3 interaction appears to regulate kinase activity directly; addition of a high affinity SH3 ligand to down-regulated Hck causes maximal activation (10).

SH2 and SH3 domains also have a positive influence on non-receptor tyrosine kinase signaling. SH2- and SH3-mediated interactions with other proteins are important for at least two reasons: first, they target the tyrosine kinases to specific subcellular locations, and second, they assist the kinases in recognizing certain cellular substrates (11–13).

Processive phosphorylation by Src family kinases is when phosphorylation of a substrate by the catalytic domain creates a site that is recognized by the associated SH2 domain; binding to the SH2 domain increases the local concentration of substrate, allowing the catalytic domain to phosphorylate additional sites. Several proteins have been proposed to be phosphorylated processively by non-receptor tyrosine kinases. One such protein is the focal adhesion protein p130CAS. This protein possesses a Src SH2 domain-binding site near its C terminus (14, 15). Binding of Src to this region is thought to promote phosphorylation of p130CAS at multiple sites closer to the N terminus (15). Hyperphosphorylation of p130CAS has been demonstrated in vitro using a mutant form of Abl containing the Crk SH2 domain (16).

Similarly, the lymphocyte-specific Src family kinase Lck phosphorylates several tyrosine residues in the ε chain of the T-cell receptor. Each ε chain contains three immunoreceptor tyrosine-based activation motifs with the consensus YXX(I/L)X$_{n-3}$YXX(I/L) (where X is any amino acid) arrayed in tandem (17). Mutation of amino acids involved in phosphotyrosine recognition in the SH2 domain of Lck reduces receptor hyperphosphorylation and signal transduction, consistent with a processive phosphorylation model (18).

Despite these observations for the Src family kinases, the mechanistic details of processive phosphorylation have not been examined closely. Sequence requirements for the interaction between the SH2 domains of Src family kinases and potential substrates are not well understood. In this report, we have tested a basic principle of the processive phosphorylation model by asking whether a high affinity SH2 binding sequence on a substrate enhances phosphorylation of other sites on the same substrate. Using a series of tyrosine-phosphorylated pep-
tides, we show that the SH2 binding sequence indeed enhances phosphorylation of the substrate. We also report the results of experiments aimed at determining the optimal spacing and orientation of the SH2 domain-binding site relative to the potential phosphorylation site.

**EXPERIMENTAL PROCEDURES**

**Peptides—** Synthetic peptides and phosphopeptides were prepared by solid phase synthesis using standard Fmoc chemistry (19) using an Applied Biosystems Inc. automated 431A peptide synthesizer. Phosphotyrosine was incorporated into the peptides using NaFmoc-O-phospho-L-tyrosine (Novabiochem) (20). Peptides were purified by preparative reversed-phase high performance liquid chromatography. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry and amino acid analysis were used to confirm the identity of the final products. The concentrations of peptide stock solutions were determined by quantitative amino acid analysis (Commonwealth Biotechnologies, Richmond, VA).

**Proteins—** Expression and purification of C-terminally phosphorylated Hck is described elsewhere (4). To obtain C-terminally dephosphorylated Hck, the enzyme was treated with Yersinia protein-tyrosine phosphatase, as described previously (10). Autophosphorylation of Tyr<sup>11</sup> (Tyr<sup>16</sup> of c-Src) was performed by incubation of the enzyme (5 mg/ml) on ice for 1 h in kinase assay buffer (20 mM Tris, pH 7.5, 20 mM MgCl<sub>2</sub>, 1 mg/ml bovine serum albumin) in the presence of 2 mM ATP (10). Full-length v-Src was produced using a baculovirus vector in Sf9 cells and purified by immunoaffinity chromatography as described (21). The Abi catalytic domain was used in Escherichia coli (22). Peptides were purified by preparative reversed-phase high performance liquid chromatography. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry and amino acid analysis were used to confirm the identity of the final products. The concentrations of peptide stock solutions were determined by quantitative amino acid analysis (Commonwealth Biotechnologies, Richmond, VA).

**RESULTS AND DISCUSSION**

**Peptides—** The sequences of the synthetic peptides used in these experiments are presented in Table I. The phosphopeptides contain an optimal Src SH2 domain-binding sequence: (phospho)Tyr-Glu-Ile. This sequence is derived from the polyomavirus middle T antigen, which forms a 1:1 complex with c-Src in cells (26). The pYEEI sequence has been demonstrated to bind with high affinity to the Src SH2 domain (27). Control peptides have a phenylalanine in place of phosphotyrosine. The phosphorylation site sequences on these peptides correspond to a phosphorylation sequence (Glu-Asp-Ala-Ile-Tyr) that we obtained using synthetic peptide libraries (25).

**Enhanced Phosphorylation of Y(11)pY—** To determine whether introduction of an SH2 domain binding site would lead to increased phosphorylation of additional tyrosines within the same peptide, we compared Y(11)pY with its control Y(11)F using C-terminally phosphorylated Hck. We observed a strong preference for Y(11)pY, the peptide containing the SH2 domain binding motif (Fig. 1). In principle, the presence of a high affinity SH2 domain binding site in multiply phosphorylated substrates could facilitate phosphorylation in two ways. First, the pYEEI motif could increase the enzymatic preference of Src family kinases through the displacement of the C-terminal negative regulatory site from the SH2 domain. Second, SH2 domain-pYEEI interactions could promote the further phosphorylation of other tyrosines within the same substrate by raising the effective local concentration of potential phosphorylation sites near the catalytic domain. To discriminate between these possibilities, we performed experiments similar to those shown in Fig. 1 using two enzymes where activation by C-terminal tail displacement is not possible: (i) v-Src, which lacks the C-terminal tail, and (ii) Hck that had been dephosphorylated at Tyr<sup>522</sup> in cells (28). Hck that had been dephosphorylated at the C terminus and allowed to autophosphorylate at Tyr<sup>411</sup> (10). Initial rates of reaction were measured in duplicate, and kinetic constants were determined by fitting the hyperbolic velocity versus substrate plots using the program MacCurve Fit.

**Kinase Assays—** Kinase activity measurements were carried out at 30 °C using the phosphocellulose filter binding assay (24, 25). Graphs shown are representative of three or more experiments, each carried out in duplicate. For kinetic measurements, each reaction contained 26 nM Hck that had been previously dephosphorylated at the C terminus and allowed to autophosphorylate at Tyr<sup>411</sup> (10). Initial rates of reaction were measured in duplicate, and kinetic constants were determined by fitting the hyperbolic velocity versus substrate plots using the program MacCurve Fit.

**Kinetics—** To clarify further the mechanism of the enhanced phosphorylation of Y(11)pY, we performed initial rate kinetic studies using Hck that had been dephosphorylated at Tyr<sup>522</sup> in the C-terminal tail and allowed to autophosphorylate at Tyr<sup>411</sup>. The results are shown in Table II. Similar values of V<sub>max</sub> were observed for Y(11)pY and Y(11)F. However, introduction of an SH2 domain-binding site in Y(11)pY reduces the K<sub>m</sub> of the peptide substrate 10-fold, thus increasing the catalytic efficiency (k<sub>catalytic</sub>/K<sub>m</sub>) of the enzyme 10-fold as well. The high affinity interaction between the SH2 domain of Hck and the pYEEI motif of Y(11)pY presumably gives the substrate an advantage because of its high local concentration in proximity to the catalytic domain.

**Role of SH2 Domains—** We carried out experiments to confirm the importance of the SH2 domain in the observed enhanced phosphorylation of Y(11)pY. We first tested for phosphorylation of Y(11)pY versus Y(11)F using the catalytic
domain of the non-receptor tyrosine kinase Abl (AblCAT). Because of the absence of an SH2 domain in this construct, we predicted that AblCAT would not discriminate between Y(11)pY and Y(11)F. Fig. 2A shows that, as expected, the isolated catalytic domain of Abl did not preferentially phosphorylate Y(11)pY. In fact, AblCAT favored the phenylalanine control peptide, Y(11)F, over Y(11)pY.

We next tested for enhanced phosphorylation of Y(11)pY using a chimeric Abl protein (lacking the SH3 domain) in which the SH2 domain of Abl had been replaced with the SH2 domain of Src (SrcSH2-AblCAT) (13). The results, shown in Fig. 2B, indicate that indeed SrcSH2-AblCAT favored Y(11)pY, the peptide containing the Src SH2 domain-binding motif pYEEI. Thus the order of substrate preference of SrcSH2-AblCAT is reversed from that of AblCAT. These observations also indicate that the SH2 domains of kinases can influence the substrate specificity of their associated catalytic domains. This result has precedent in studies of mutant forms of Abl in vivo, where replacement of the SH2 domain of Abl with SH2 domains from various proteins modifies the substrate specificity of this tyrosine kinase (13).

**Length Dependence**—We varied the spacing between the phosphotyrosine and the phosphoratable tyrosine to assess the minimum distance required for enhanced phosphorylation to occur. We tested peptides of various lengths (Table I) using C-terminally dephosphorylated (pre-activated) Hck. Fig. 3 shows that there is a length dependence to the observed enhanced phosphorylation of pYEEI-containing peptides. For example, when the spacer is reduced to 7 amino acids, as in Y(7)pY, the rate of phosphorylation is reduced when compared with the longer peptide Y(11)pY but is still above Y(11)F or Y(3)F, the phenylalanine controls. However, when the spacer is further reduced to 3 amino acids, as in Y(3)pY, the enhanced phosphorylation is no longer observed, and the phosphorylation rates approach those of the control peptides, Y(11)F and Y(3)F. These results indicate that in order for SH2 domain-binding motifs to confer an advantage to substrates, there must be a minimum distance between the phosphotyrosine and the tyrosine. These spacer lengths are consistent with the 9–11-residue spacers observed between phosphorylated tyrosines in immunoreceptor tyrosine-based activation motifs of TCR ζ chains (17).

One possible explanation for the observed dependence on spacer length is that phosphorylation occurs while the peptide remains bound to the SH2 domain. In the crystal structure of down-regulated Src, the distance between Tyr527 and the active site is approximately 35 Å (5). If the phosphotyrosine-containing peptides adopt a fully extended conformation, each residue would be expected to span ~3.7 Å (29). Thus, the distance of 12 residues between phosphotyrosine and tyrosine in Y(11)pY could potentially span 12 × 3.7 Å = 44.4 Å, enough to reach from the SH2 domain to the active site. In contrast, the shorter spacers in Y(7)pY or Y(3)pY should not be long enough to reach from the SH2 domain to the active site. However, the exact position of the SH2 domain with respect to the catalytic domain in an activated Src family kinase is currently unknown.

**Reversed Orientation**—To examine the effect of reversing the orientation between the substrate tyrosine and the SH2 domain-binding site, we produced peptide substrates containing the pYEEI motif (or FEEI control) N-terminal to the tyrosine (Table I). Fig. 4 shows a comparison between Y(11)pY and Y(11)F, the peptide containing the phosphotyrosine at the C-terminal dephosphorylated (pre-activated) Hck.

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**Table II**

**Kinetic parameters of peptides**

Steady state kinetic parameters were obtained as described under “Experimental Procedures” using C-terminally dephosphorylated (pre-activated) Hck.

| Peptide | $K_m$ ($\mu$M) | $V_{max}$ ($\mu$mol/min/mg) | $k_{cat}/K_m$ (s$^{-1}$) |
|---------|-----------|-----------------|-----------------|
| Y(11)pY | 69 ± 11   | 3.0 ± 0.1       | 2.6 × 10$^6$    |
| Y(11)F  | 680 ± 90  | 3.4 ± 0.3       | 3.0 × 10$^5$    |
| pY(12)Y | 72 ± 5    | 2.7 ± 0.2       | 2.2 × 10$^6$    |

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**Fig. 2.** A, phosphorylation of peptides Y(11)pY and Y(11)F by the catalytic domain of Abl. Reactions were started by the addition of AblCAT (330 nM) to kinase assay buffer containing 70 μM peptide substrates. B, phosphorylation of peptides Y(11)pY and Y(11)F by SrcSH2-AblCAT, a chimera possessing the SH2 domain of Src and the catalytic domain of Abl. Reactions were initiated by the addition of enzyme (173 nM) to kinase assay buffer containing 70 μM peptide substrates.

**Fig. 3.** Phosphorylation of peptides possessing spacers of various lengths by activated Hck. Reactions were started by the addition of C-terminally dephosphorylated (preactivated) Hck (26 nM) to kinase assay buffer containing 70 μM peptide substrates.
Enzyme (70 nM) was added to reactions finally phosphorylated Hck.

Src SH2 domain-binding site in p130 CAS is located C-terminal containing 35 residues have been summarized in Table II. Surprisingly, this form of Hck over pY(12)Y is consistent with the structure of down-regulated Hck (4), where the phosphotyrosine-containing tail of Hck binds to the SH2 domain in an orientation that places the N terminus of the tail closer to the catalytic domain than the C terminus.

We performed initial rate kinetic studies on pY(12)Y using C-terminally dephosphorylated (preactivated) Hck. The results are summarized in Table II. Surprisingly, this form of Hck phosphorylated pY(12)Y and Y(11)pY with roughly equal catalytic efficiency; the values for $K_m$ and $V_{max}$ of the peptides are comparable. Therefore, the position of the SH2 domain-binding site with respect to the substrate tyrosine appears not to be important for activated Hck but may be relevant for activation of down-regulated Hck in vivo. In this regard, we note that the Src SH2 domain-binding site in p130CAS is located C-terminal to the region containing multiple Src phosphorylation motifs.

Concluding Remarks—Using a synthetic peptide model system, we report here that substrates containing high affinity binding sites for Src family kinase SH2 domains display enhanced phosphorylation at additional sites on the substrate. These experiments on processive phosphorylation suggest that the SH2 domain plays an important role in substrate recognition for non-receptor tyrosine kinases. Based on studies with peptide libraries, it has been proposed that phosphorylation of different peptide sequences by the Src kinase domain correlates with the binding of the sequences to the Src SH2 domain. This raises the interesting possibility that the two domains co-evolved to allow the catalytic domain to create sites that will be recognized by the associated SH2 domain (3).

The in vivo substrate specificity of Src family tyrosine kinases reflects both the intrinsic specificity of the kinase catalytic domains and the effective local concentrations of protein substrates. In many cases, the distribution of potential substrates is influenced by interactions with noncatalytic regions of the enzymes such as SH2 and SH3 domains. Previous studies have demonstrated that molecules that bind with high affinity to the SH2 or SH3 domains of Src family kinases can disrupt the relatively low affinity intramolecular interactions, leading to enzyme activation (10, 31). Thus, a variety of protein-protein interactions in the cell can lead to signaling through active Src kinases. Our data imply that the most productive interactions may occur when these high affinity ligands are present together with potential phosphorylation sites on the same molecule, i.e. in the case of processive phosphorylation. This would impart an additional level of specificity in substrate recognition.

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