Modulation of the SH2 Binding Specificity and Kinase Activity of Src by Tyrosine Phosphorylation within Its SH2 Domain*

(Received for publication, November 13, 1995, and in revised form, February 1, 1996)

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THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 271, No. 21, Issue of May 24, pp. 12481–12487, 1996
Printed in U.S.A.

The Src family of kinases are held in an inactive state by interaction of their SH2 domain with a C-terminal phosphotyrosine. Dephosphorylation of this site can reactivate Src; however, recent evidence suggests that activation can also occur without dephosphorylation. In this study, platelet-derived growth factor receptor phosphorylation of Src on Tyr-213 specifically blocked binding of its SH2 domain to a phosphopeptide corresponding to the C-terminal regulatory sequence, while binding to other sequences, such as the platelet-derived growth factor receptor or a peptide from the epidermal growth factor receptor, was unaffected. Consequently, Src was activated over 50-fold. This is the first demonstration of regulation of a SH2 domain specificity by post-translational modification and is likely to be a general mechanism for regulation of all Src-like kinases.

The Src family of kinases is the prototype of the Src family of membrane-associated protein-tyrosine kinases (1). All of the members of this family have a kinase domain, a domain that binds phosphotyrosine-containing sequences, and a domain that associates with proline-rich motifs (for reviews see Refs. 2–4). These domains are known as Src homology (SH) domains or SH1, SH2, and SH3 domains, respectively. The kinase activity of this family is down-regulated by phosphorylation of a C-terminal tyrosine (residue number 527 in Src) by members of the C-terminal Src kinase (Csk) family of kinases (5). Interaction of the SH2 domain with the C-terminal phosphotyrosine appears to be responsible for this inhibition (4). The Src-like kinases can be reactivated by dephosphorylation of this tyrosine (6); however, recent reports suggest that reactivation can occur in the absence of dephosphorylation. For example, T lymphocytes, lacking the protein-tyrosine phosphatase CD45, have elevated levels of Lck and Fyn activity even though their negative regulatory sites are hyperphosphorylated (7, 8). In addition, Src is observed to be activated in vitro by phosphorylation with CDC2 only when Tyr-527 is fully phosphorylated (9).

The binding specificity of many SH2 domains have been identified recently (10). These domains are involved in targeting proteins to specific locations and in regulating the catalytic domains with which they are associated (3, 11). However, the possibility that the binding of SH2 domains might be regulated has not yet been explored in depth. There is considerable data demonstrating that Src is important for growth factor signal transduction. Treatment of cells with various growth factors triggers Src to associate with the corresponding receptor protein-tyrosine kinase (i.e. ErbB2 (12), EGF receptor (13), colony stimulating factor receptor (14), PDGF receptor (15), and others). Treatment of cells with PDGF or EGF elevates Src activity (16, 17), while overexpression of Src enhances EGF-induced signaling (18). We have recently demonstrated that Src directly phosphorylates the EGF receptor to create additional docking sites for specific SH2-containing proteins (13). In contrast, Src is phosphorylated on tyrosine at a non-autophosphorylation site in the PDGF-stimulated cells (16). We now demonstrate that PDGFR phosphorylates Src within its SH2 domain (tyrosine 213) in vivo. Direct phosphorylation of Src, by the PDGFR receptor in vitro, alters the specificity of its SH2 domain so that it no longer binds the C-terminal inhibitory phosphotyrosine sequence, while its binding to other ligands is unaffected. This results in reactivation of Csk-inactivated Src without dephosphorylation of Tyr-527. Moreover, conservation of the phosphorylated tyrosine suggests that phosphorylation of this site may be a regulatory mechanism that is common for all of the Src-like kinases (19–21).

MATERIALS AND METHODS

Determination of the Stoichiometry of Phosphorylation of Src by the PDGFR Receptor—One μg of Src, purified from baculovirus-infected Sf9 cells (22), was incubated for 2 h at room temperature with [33P]ATP and, where indicated, with 10 μM selective Src inhibitor (CGP 59272) (13) and 100 ng of PDGFR receptor (purified from Balb/c 3T3 cells by immunoaffinity chromatography, anti-Human PDGFR type A type B receptor; monoclonal, UBI catalog No. 06-132). Src was analyzed by SDS-PAGE and visualized using a PhosphorImager (Molecular Dynamics). Src was excised from the gel, and radioactivity was measured in scintillation fluid.

Identification of the PDGFR Receptor Phosphorylation Site in Src—Fifty μg of purified Src was incubated with approximately 10 μg of affinity purified PDGFR receptor in the presence of [33P]ATP (5 μCi/ml ATP) and CGP 59272. Phosphorylated Src was isolated by SDS-PAGE, and in-gel digestion with trypsin was performed as described previously (13). Tryptic fragments were separated by reverse-phase HPLC, and 10 μl of each fraction was used for scintillation counting of radioactivity. The single major peak of radioactivity (12,438 cpm, 10-fold greater than the next largest peak) was analyzed by electrospray mass spectrometry (MS), using a triple quadrupole atmospheric pressure ionization instrument (API-MS, Sciex).

Confirmation of peptide identity and position of phosphorylation was done by tandem MS-MS. The ion at mass/charge ratio 649 (Fig. 1B) was selected in the first quadrupole. Fragment ions were generated by collision-induced dissociation in an Argon collision chamber, and analyzed by the third quadrupole.

Two-dimensional Peptide Mapping—Src was excised from SDS gels, washed in 50% acetonitrile for 4 h, and then air-dried at room temperature for at least 4 h. Ten μg of trypsin in 100 μl NH4CO3 was then added to the gel slice and incubated for 16 h at 30 °C. Where indicated, Src was first incubated with 10 μg of thermolysin for 16 h at 55 °C. Peptides were then eluted from the gel by 3 washes in 50% acetonitrile.

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1 The abbreviations used are: EGF, epidermal growth factor; PDGF, platelet-derived growth factor; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography.
Modulation of SH2 Specificity by Phosphorylation

A

\[
\begin{align*}
\text{CGP 59272} & \quad 1 \quad 2 \quad 3 \\
\text{PDGF-R} & \quad - \quad + \quad + \\
\end{align*}
\]

\rightarrow \text{Src}

B

![Mass spectrum diagram]

\[ M = 1295 = 1215 + 80 = \text{AA(207-217)} + \text{PO}_3 \]

\[ \text{AA(207-217)} = \text{LDSGGFYITSR} \]

![Mass spectrum diagram]

C

Parent Ion

\[ \text{LDSGGFpYITSR} \]

![MS/MS spectrum diagram]
and lyophilized. Peptides were separated on cellulose plates by electrophoresis (1000 V, pH 1.9, 1 h) in the first dimension and then by liquid chromatography (phosphochromatography buffer, 12 h) in the second dimension, essentially as described in the Hunter Thin Layer Electrophoresis System Instruction Manual (C.B.S Scientific Co.).

Identification of in Vivo Phosphorylation Sites—To identify sites of in vivo phosphorylation, the indicated cells were placed in media (RPMI or Dulbecco's modified Eagle's medium) containing 0.5% fetal calf serum 18 h prior to loading the cells (−10) with 2 mCi of inorganic 32P. After a 4-h incubation, the cells were treated with 20 μg/ml PDGF for 10 min. The cells were lysed in 20 mM Tris, pH 7.5, 5 mM EDTA, 1 mM dithiothreitol, 1% Nonidet P-40, 0.05% SDS, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin, pepstatin, and leupeptin. Src was then immunoprecipitated by 50 μg of monoclonal antibody 327 (Oncogene Science) and protein G-Sepharose (Pharmacia Biotech Inc.). Src was resolubilized in SDS-PAGE loading buffer and loaded onto a 4–15% gradient polyacrylamide gel (Bio-Rad), and peptide mapping was performed as described above.

To simplify the identification of sites of tyrosine phosphorylation in vivo, Src was treated with both protein phosphatase 1 and protein phosphatase 2a (gifts from Dr B. Hemmings, Basel, Switzerland), before SDS-PAGE separation, to remove phosphate on serine and threonine. There was no difference in the tryptic phosphopeptide maps of Src, phosphorylated in vitro by Csk and PDGF receptor, treated or untreated with these phosphatases, indicating that they do not affect the level of tyrosine phosphorylation (data not shown). In each case, two-dimensional phosphoamino acid analysis was used to verify that no phosphoserine or phosphothreonine remained (data not shown).

Molecular Modeling—Crystal structures of the SH2 domain of v-Src complexed with either a low affinity peptide (pY)VPML (19) or a high affinity peptide EPQ[pY]EEIPIYL (20) have been obtained. (Note that the sequence of the c-Src and v-Src SH2 domains are identical.) Because neither of the peptides that have been crystallized with Src are known to be binding partners of Src in normal uninfected cells, we substituted these peptides with more physiologically relevant peptides that fit their basic structure. The C-terminal phosphotyrosine sequence of Src itself (pY)VPML) and the Src binding site of the EGF receptor ((pY)DGIP), respectively. The major difference in binding of the two reported structures is due to a sharp bend in the low affinity peptide caused by a proline in the +2 position. The two peptides we substitute mimic these structures as one contains the proline in the +2 position and the other does not. The affinity of these peptides also correlates well with the pY'VPML binding in a similar fashion to the low affinity peptide, pYVPML, and pYDGIP binding in a manner similar to the high affinity peptide pYEEI.

In order to assess the possible result of phosphorylation of Tyr-213 on the peptide binding of the c-Src SH2 domain, these structural models were manipulated by adding phosphate to the appropriate tyrosine. The resulting phosphotyrosine was then revolved around its axis to determine if an interaction between the phosphate and the binding peptide could occur. In both structures, the phosphate could potentially interact with Arg-205; however, only the low affinity pY'VPML peptide could directly interact (via the glutamate in the +4 position) with either Arg-205 or Tyr-213 itself. In either case, the addition of phosphate to Tyr-213 would only be likely to have a negative effect on binding, either by direct interaction of its similar negative charge with Glu in the +4 position of the binding peptide or by competition for binding by Arg-205. Both models were prepared using the molecular modeling program Insight II (Biosym Corp.).

Src Purification—Two forms of Src were prepared from recombinant baculovirus-infected Sf9 cells, as described previously (9). Purified Src was dephosphorylated with alkaline phosphatase to yield the first form (simply referred to as Src). A portion of this Src was phosphorylated with Csk and isolated by separation on a Mono Q column (referred to as Csk-inactivated Src, because its activity is 50–100-fold less than that of nonphosphorylated Src).

Src Activation—Src or Csk-inactivated Src (2 μg) were incubated with PDGF receptor (50 ng) and 20 μM ATP for the indicated period of time. The reaction was stopped by addition of a selective PDGF-receptor inhibitor (1 μg CGP53716) (23). After a 5-min incubation with 20 μM ATP, the Src was dephosphorylated with alkaline phosphatase. (Note that the sequence of the c-Src and v-Src SH2 domains are identical.) Because neither of the peptides that have been crystallized with Src are known to be binding partners of Src in normal uninfected cells, we substituted these peptides with more physiologically relevant peptides that fit their basic structure. The C-terminal phosphotyrosine sequence of Src itself (pY)VPML) and the Src binding site of the EGF receptor ((pY)DGIP), respectively. The major difference in binding of the two reported structures is due to a sharp bend in the low affinity peptide caused by a proline in the +2 position. The two peptides we substitute mimic these structures as one contains the proline in the +2 position and the other does not. The affinity of these peptides also correlates well with the pY'VPML binding in a similar fashion to the low affinity peptide, pYVPML, and pYDGIP binding in a manner similar to the high affinity peptide pYEEI.

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Fig. 2. Each of the panels shown above is a two-dimensional phosphopeptide map of Src digested with trypsin (A–D) or trypsin plus thermolysin (E and F), as described under “Materials and Methods.” The first dimension electrophoresis moved the peptides from left to right, while the second dimension chromatography ran from bottom to top. To simplify the identification of sites of tyrosine phosphorylation, Src was treated with both protein phosphatase 1 and protein phosphatase 2a, before SDS-PAGE separation, to remove phosphate on serine and threonine. A, Src (5 μg) was phosphorylated by Csk in the presence of CGP59272 to block Src autophosphorylation. B, Src (5 μg) was phosphorylated by PDGF receptor (200 ng) in the presence of CGP59272. C, Src was immunoprecipitated (50 μg of Ab327 Oncogene Science) from cell extracts, 10 min after PDGF stimulation of 32P-loaded Balb/c3T3 cells (106 cells). D, phosphopeptide samples from A–C were combined such that each contributed an equal share of the total radioactivity. E, Src was phosphorylated in vitro with Csk and PDGF receptor as in A and B and digested with both trypsin and thermolysin. F, Src was immunoprecipitated from 32P-loaded cells as in C and digested with both trypsin and thermolysin.
ATP and 10 μg of poly(Glu-Tyr), 10 μl of the reaction was spotted onto P81 paper and the radioactivity was measured. Each time point is the mean of 3 determinations with the standard deviation shown by error bars. Full-length CD45 was expressed in recombinant baculovirus-infected Sf9 cells and purified by affinity chromatography as described previously (24).

Peptide Interactions—Purified Src (100 μg) was immunoprecipitated with an anti-Src monoclonal antibody (327) and protein G-Sepharose. Half of the Src immunoprecipitate was phosphorylated by 5 μg of PDGF-R in the presence of ATP. These immobilized Src preparations were then loaded into two 0.5-cm diameter chromatography columns, washed with 5 ml of wash buffer (0.2% Nonidet P-40, 50 mM Tris, pH 7.5, 5 mM EDTA, 200 mM NaCl, and 1 mM dithiothreitol), and assayed for binding to either P(pY)DGIPASEISSILEK or to PQ(pY)QPGE, before or after phosphorylation of Src by PDGF receptor. The 32P-phosphopeptides were prepared by phosphorylation by Src and purified by HPLC. Phosphopeptides were diluted to 7 different concentrations ranging from 0.01 μM to 10,000 μM. After a 2-min incubation with the antibody-complexed Src, the unbound peptides were removed by washing with 5 ml of wash buffer. The amount of peptide bound was calculated based on a 50% Cerenkov counting efficiency after transferring the antibody complex to scintillation vials.

RESULTS

In order to determine how PDGF stimulation causes Src to be activated, we phosphorylated Src with the PDGF receptor under conditions where Src is inhibited and does not autophosphorylate. Approximately 0.7 mol of phosphate/mol of Src were incorporated (Fig. 1A). Tryptic fragments of phosphorylated Src were separated by reverse-phase HPLC, and the single major peak of radioactivity was analyzed by mass spectrometry (MS) (Fig. 1B). The mass spectrum revealed the presence of several peptides, one of which had the mass of a tryptic peptide containing Src residues 207–217 plus 80 daltons (the extra mass expected for addition of a phosphate). The molecular weights of the other peptides were consistent with them being nonphosphorylated tryptic peptides of Src. A complete series of ions representing C-terminal fragments of the peptide was generated and detected by tandem MS-MS (Fig. 1C); this confirmed the identity of the peptide and revealed that Tyr-213 was phosphorylated.

Phosphopeptide maps of Src immunoprecipitated from Balb/c cells and then treated by protein phosphatases 1 and 2α to remove Ser/Thr phosphate, revealed that only a single tyrosine, comigrating with the site confirmed to be phosphorylated by Csk in vitro, was phosphorylated in vivo (data not shown). However, after PDGF treatment, two spots are present, one comigrating again with the Csk site and the other with the PDGF receptor site identified in vitro. This indicates that there is only one major site of phosphorylation by PDGF receptor both in vitro and in vivo.

Comigration of phosphopeptides from Src phosphorylated by PDGF receptor in vitro and of Src isolated from Balb/c cells 10 min after treatment with PDGF, revealed that this same site is phosphorylated in vivo (Fig. 2). Because the site of phosphorylation of Src by the PDGF receptor was originally reported to be on an insoluble tryptic peptide that was only solubilized after thermolysin digestion (16), we also used both trypsin and thermolysin to examine whether an additional phosphopeptide could be detected. In vitro phosphorylated Src, digested with both trypsin and thermolysin, still had only two soluble phosphopeptides, indicating that thermolysin did not render any additional major phosphorylation sites soluble (Fig. 2).

Tyr-213 lies within the SH2 domain of Src, which has been crystallized with both a low affinity peptide (pYVPML) (19) and a high affinity peptide (EPQpYEIIPIYL) (20). In the crystal structure of the SH2 domain of Src with the high affinity peptide, Tyr-213 lies near the peptide binding site; however, the proline in the low affinity peptide causes it to bend so that the peptide lies much closer to Tyr-213 (Fig. 3, A and B). Using

![Fig. 3. A, model of the Src phosphopeptide, corresponding to the C-terminal regulatory sequence ((pY)QPGE), bound to the SH2 domain of Src with Tyr-213 (the PDGF receptor phosphorylation site) phosphorylated. The model is based on the crystal structure of the SH2 domain of Src complexed with the low affinity peptide (pY)VPML (19). B, model of (pY)DGIP bound to the SH2 domain of Src with Tyr-213 phosphorylated. The model is based on the crystal structure of the SH2 domain of Src complexed with the high affinity peptide (pY)EEIP from the EGF receptor (20). Both models were prepared using the molecular modeling program Insight II (Biosym Corp.).](image-url)
the coordinates of the Src SH2 domain, crystallized in the presence of low affinity peptide, we made a model of the Src SH2 domain bound to the C-terminal phosphotyrosine sequence (pYQFGE). This peptide also has a proline in the +2 position (relative to the phosphotyrosine) that causes the peptide to bend, and, as a result, the glutamic acid in position +4 lies in close proximity to Arg-205. This arginine probably helps stabilize the binding of this peptide since it is the only positively charged moiety with which the carbonylate of the glutamic acid can interact with. When phosphorylated, the negative charge of Tyr(P)-213 may form a stable electrostatic interaction of the N-terminal SH2 domain with the C-terminal phosphotyrosine had been blocked. Two forms of Src were prepared from recombinant baculovirus-infected S9 cells, as described previously (9, 13). Purified Src was dephosphorylated with alkaline phosphatase to yield the first form (simply referred to as Src). A portion of this Src was phosphorylated with Csk and isolated by separation on a Mono Q column (referred to as Csk-inactivated Src, because its activity is 50–100-fold less than that of nonphosphorylated Src).

Src was rapidly, yet modestly, activated by PDGF receptor phosphorylation, to a maximum of approximately twice its initial activity. On the other hand, Csk-inactivated Src was relatively slowly reactivated; however, it was activated to approximately 50-fold its initial activity. The slow phosphorylation of Src by PDGF receptor was surprising, but it is consistent with the model of Src inhibition by Csk. Inhibition occurs as a result of the SH2 binding the C-terminal phosphotyrosine (phosphorylated by Csk). This interaction could be expected to inhibit phosphorylation of a site so close to the binding pocket of the SH2 domain (Tyr-213). To ensure that this result was not due to a contaminating phosphatase dephosphorylating the Tyr-527 site, the experiment was repeated in the opposite sequence. Specifically, Csk-induced inhibition was reduced by prior phosphorylation of Src by the PDGF receptor (data not shown). This demonstrates that reactivation of Src by the PDGF receptor phosphorylation blocks inhibition of Src by Csk, and that this effect is independent of the sequence of phosphorylation by the two kinases. This activation was probably not due to binding of Src to the PDGF-R because PDGF-R was present at only 1/20 the molar concentration of Src. To further test this possibility, autophosphorylated PDGF-R (disabled by 1 μM specific PDGF-R inhibitor, CGP53716) was added to Csk-inactivated Src with little to no effect on activity (data not shown). While activation of Src by phosphorylation of Tyr-213 was predicted by our model, we did not predict the 2-fold activation of the nonphosphorylated form of Src by PDGF receptor. The mechanism of activation for this form of Src is unclear. However, the majority of Src is phosphorylated on Tyr-527 in vivo (4), suggesting that this phenomena may not be of much physiological importance.

Reactivation of Csk-phosphorylated Src by the PDGF receptor suggests that phosphorylation of Tyr-213 disrupts the interaction of the Src-SH2 domain with Tyr(P)-527, as predicted.
To further test this hypothesis, we determined the effect of phosphorylation on the affinity of Src for \( {^{32}P} \)-phosphopeptides (Fig. 4B). Nonphosphorylated Src bound phosphopeptides corresponding to the Csk phosphorylation site (PpYDGPE, \( K_D = 400 \mu M \)), to a segment of the EGFR phosphopeptide (PpYDGIPASEISSILEK (pYDGIP), \( K_D = 5 \mu M \)), and to the PDGF receptor itself. Phosphorylation of Src by the PDGF receptor did not affect binding to the pYDGIP peptide or the autophosphorylated PDGF receptor, as predicted. However, PDGF receptor-phosphorylated Src bound 70% less pYOPGE peptide, which corresponds to the fraction of Src phosphorylated (Fig. 1A), indicating that the phosphorylated form of Src does not bind the pYOPGE phosphopeptide. This conclusion is supported by restoration of the phosphopeptide binding capacity after treatment of Src with a protein-tyrosine phosphatase (CD45). This result demonstrates that phosphorylation of the SH2 domain of Src by the PDGF receptor specifically blocks binding to the C-terminal regulatory phosphorylation site, while allowing association with other high affinity ligands.

**DISCUSSION**

Platelet-derived growth factor (PDGF) primarily stimulates the protein-tyrosine kinase activity of the PDGF receptor (26); however, it also elevates the activity of a second tyrosine kinase, Src (16). Src has been shown to associate with the PDGF receptor (15) and to be phosphorylated within its N-terminal segment, following treatment of cells with PDGF (27). Hunter et al. (28, 29) have reported that mutation of Tyr-138 to Phe abolishes phosphorylation of Src by the PDGF-R in vitro. However, direct observation of Tyr(P)-138B (mass spectrometry, sequencing, or co-migration with synthetic peptides) has not been reported. Furthermore, the ability of mutated Tyr-138 to abolish the in vivo phosphorylation has not been demonstrated. In fact, mutation of either Tyr-138 or Tyr-133 to Phe interferes with mitogenicity of both PDGF and EGF, suggesting that modification of this region of the SH3 domain may block its function in a non-phosphorylation-dependent manner. This hypothesis is supported by data demonstrating that EGF-R does not phosphorylate Src in vitro or in vivo (in response to EGF). Therefore, we conclude that phosphorylation of Tyr-138 is not the relevant site of phosphorylation by PDGF-R in vivo.

In this report, we demonstrate by direct tandem mass spectrometric peptide sequencing that the PDGF receptor phosphorylates Src near the binding pocket of the SH2 domain at Tyr-213 in vitro and that this in vivo site co-migrates by two-dimensional peptide mapping to a synthetic peptide containing Tyr-213. Our identification of this site is further strengthened by results by Couture et al. (30) demonstrating that Lck is phosphorylated at the equivalent site (Tyr-192) by Syk and Zap-70. Molecular modeling correctly predicted that a phosphate at this site would specifically disrupt binding of the SH2 domain to phosphopeptides with sequences similar to the C-terminal regulatory phosphoryrosine of Src (proline in the +2 position).

Disruption of the Src-SH2 interaction with its C-terminal regulatory phosphoryrosine may affect more than just its enzymatic activity. Treatment of Balb/c cells with PDGF causes Src to translocate from the plasma membrane to the cytosol (31). It has recently been shown that mutation of Tyr-527 results in a redistribution of Src to focal adhesions sites (32). Because phosphorylation of Tyr-213 affects both the availability and the specificity of Src’s SH2 domain, we speculate that phosphorylation by the PDGF receptor may induce both translocation and activation of Src.

Tyrosine 213 of Src is conserved in the SH2 domain of all Src-like kinases, as is the C-terminal regulatory phosphoryrosylation site (21). The activation of Lck and Fyn in T lymphocytes lacking CD45 correlates with the increase in tyrosine phosphoryrosylation of these kinases (7), suggesting that they are also activated by tyrosyl phosphoryrosylation. Furthermore, Lck is phosphorylated on Tyr-192, the residue corresponding to Tyr-213 of Src, in response to treatment of cells with anti-CD3 antibodies (8). Phosphorylation of this site correlates with increased activity of pp561ck in COS cells co-transfected with pp561ck and pp72src (30). Additionally, phosphoryrosylation alters the binding properties of its SH2 domain in a manner similar to what we observe for PDGF receptor phosphoryrosylated Src (Couture et al. (1995)). These findings, in conjunction with the results of our study, suggest that this may be a general mechanism for activating Src family kinases.

Recent investigations suggest that Src can be activated by a number of mechanisms. Src can be activated by dephosphoryrosylation of Tyr-527, ligand occupation of the SH2 binding site, phosphoryrosylation by CDC2, and/or phosphoryrosylation by the PDGF receptor. All of these events act to disrupt the interaction of the SH2 domain with Tyr-527, supporting the standard model of repression. Because these different activating mechanisms also affect the availability and at least in one instance the specificity of the SH2 domain, we speculate that they may result in differential localization and/or protein-protein interactions in addition to activation.

**Acknowledgments—**We are grateful to Drs. W. Richter and D. Mueller (Ciba-Geigy Limited, Basel, Switzerland) for use of their mass spectrometer and for sharing their technical expertise. We thank Drs. Michael Moran (Best Institute, Toronto, Canada), Kurt Balmer (Friedrich Miescher Institute, Basel, Switzerland), and Mira Susa (Ciba-Geigy Limited, Basel, Switzerland) for critical reading of this manuscript. We would also like to thank: Drs. Maria Ruzzene and Lorenzo Pinnia (University of Padova, Padova, Italy) for contributing Csk for the studies and Drs. Peter Traxler and Giorgio Caravatti for supplying the kinase inhibitors (CGP 59272 and CGP 53716).

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