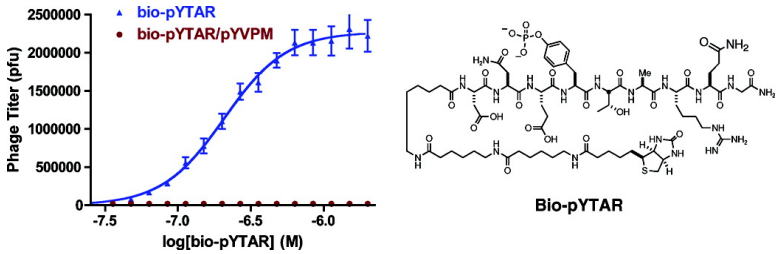
Communication

Identification of a Molecular Recognition Role for the Activation Loop Phosphotyrosine of the Src Tyrosine Kinase

Elizabeth J. Videlock, Victor K. Chung, Justin M. Hall, John Hines, Christina M. Agapakis, and David J. Austin

J. Am. Chem. Soc., **2005**, 127 (6), 1600-1601• DOI: 10.1021/ja047957c • Publication Date (Web): 20 January 2005



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

J|A|C|S
COMMUNICATIONS

Published on Web 01/20/2005

Identification of a Molecular Recognition Role for the Activation Loop Phosphotyrosine of the Src Tyrosine Kinase

Elizabeth J. Videlock, Victor K. Chung, Justin M. Hall, John Hines, Christina M. Agapakis, and David J. Austin*

Department of Chemistry, Yale University, New Haven, Connecticut 06520

Received April 8, 2004; E-mail: david.austin@yale.edu

Since their initial discovery,[1] tyrosine kinases have been shown to play a central role in the activation and mediation of signal transduction.[2] Because of their significance in cell growth, differentiation, and metabolism, tyrosine kinases have received much recent attention as therapeutic targets for disease intervention.[3] One component of tyrosine kinase activation, particularly for the Src family tyrosine kinases, involves autophosphorylation of a conserved activation loop tyrosine (Y416),[4] which causes a conformational shift of the activation loop and substrate access to the kinase active site.[5] Despite the plethora of phosphotyrosine/SH2 domain mediated interactions,[6] the role of Y416 phosphorylation in Src activation is believed to be solely conformational, with no role in protein/protein molecular recognition. Herein, we report the in vitro identification and biochemical confirmation of a phosphorylation-dependent interaction between the Src activation loop phosphotyrosine and the N-terminal SH2 domain of the p85 regulatory subunit of phosphatidyl inositol-3 kinase (PI3K). Src was the first tyrosine kinase discovered,[1,7] and its interaction with PI3K has been shown to be important for tumorigenesis.[7] While this association has been known for some time,[7,8] no evidence of Src activation loop participation has previously been described.

Tyrosine kinases are highly conserved within the 30 defined families.[9] Interestingly, they also have a high level of sequence identity in the region immediately surrounding their conserved activation loop tyrosines. The peptide sequence C-terminal to phosphotyrosines has been shown to contain the SH2 domain recognition motif that mediates protein/protein interactions.[10] Evolutionary conservation of this portion of the activation loop seems unlikely in the absence of a molecular recognition role. Therefore, we hypothesized that in addition to its conformational role, this region of tyrosine kinases may play a general role in molecular recognition. One example of an activation loop-mediated interaction between Src and the PTB domain of Cbl, mediated by pY416, has been reported.[11] However, no additional roles for the Src activation loop have been described, despite its widespread importance in both normal and oncogenic signal transduction.[4,7] Indeed, only three of the more than 90 known[9] tyrosine kinase activation loops have been demonstrated to mediate protein/protein interactions.[11,12]

In an effort to evaluate the possibility of a more extensive recognition role for the tyrosine kinase activation loop, we designed a biotinylated phosphotyrosine peptide based on the Src activation loop Y416 tyrosine, biotin-XXXDNEpYTARQG-NH_2 (bio-pYTAR, where X is aminohexanoic acid).[13] Previous studies by us[14] and others[15] have shown that short biotinylated phosphopeptides can be used as effective phosphoprotein mimics, in combination with cDNA phage display, to isolate cognate SH2 domains. A human liver cDNA phage display library was screened using the bio-pYTAR peptide over an avidin-coated 96-well plate.[13] The affinity selection was performed in three rounds, using *Escherichia coli* in a combined elution/amplification step.[14] After the third round of selection, 48 individual clones were amplified and subjected to a single-concentration affinity screen. Seven clones showed good affinity for the bio-pYTAR probe. Upon reevaluation, three of the clones displayed both high binding titers and dose-dependent affinity.[13] After DNA sequencing, each of the three clones was found to be identical and to encode the entire N-terminal SH2 domain of the α subunit of phosphatidyl inositol-3 kinase (p85α PI3K).[16] On-phage binding analysis confirmed the interaction with an EC_{50} of 207 nM (±33 nM) (Figure 1). In addition, binding was competed away by addition of pYVPM, a known PI3K N-SH2 binding phosphopeptide.[10]

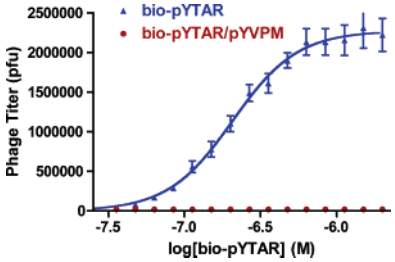


Figure 1. Dose-dependent analysis of N-SH2 PI3K for the bio-pYTAR peptide. Affinity for the peptide was measured alone (blue) or in the presence of the competitive phosphopeptide ligand pYVPM (red).

To validate this interaction within the context of full-length p85α PI3-kinase, we performed bio-pYTAR peptide affinity chromatography using NIH 3T3 mouse fibroblast cell lysate.[13] Fresh lysate was treated with either 100 μM bio-pYTAR peptide, buffer only, or a combination of 100 μM bio-pYTAR peptide and 100 μM pYVPM inhibitor. After incubation with avidin-coated resin and washing, bound protein was removed and subjected to SDS-PAGE/Western blot analysis using an anti-PI3K antibody. As seen in Figure 2A, the bio-pYTAR peptide is capable of affinity purifying full-length p85 PI3K (lane 1), while neither the resin alone (lane 2) nor the peptide in the presence of pYVPM competitor (lane 3) is capable of this interaction. This result establishes that a peptide based on the Src activation loop is capable of interacting with the full-length p85α regulatory subunit of PI3K from mammalian cell lysate. Competitive binding with the known N-SH2 domain-specific ligand, pYVPM,[10] further validates that the association occurs via the N-SH2 domain.

Phosphatidylinositol-3 kinase (PI3K) was originally discovered by virtue of its affinity for Src.[8] While the Src/PI3K interaction has been shown to be mediated by the interaction of the Src SH3 domain and an N-terminal polyproline helix on PI3K,[17] earlier reports also demonstrated a phosphorylation dependence for this interaction,[18] which has not since been explained. Src is known to be tyrosine-phosphorylated at either one of two positions, Y416 or Y527.[4] In inactive Src, Y527 is phosphorylated and the activation loop, bearing an unphosphorylated Y416, blocks the enzyme active site. Upon activation of Src, Y527 is dephosphorylated and Y416

10.1021/ja047957c CCC: $30.25

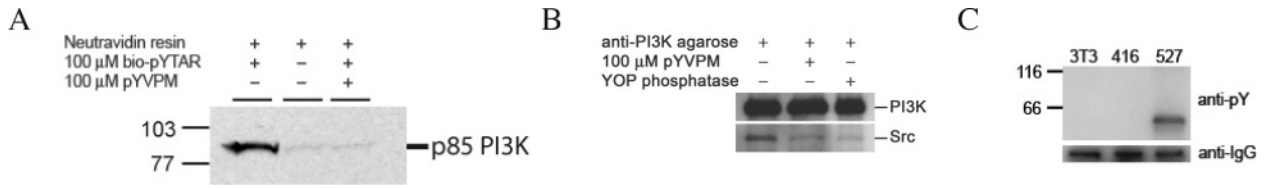


Figure 2. (A) Affinity chromatography with the bio-pYTAR peptide. Lysates from NIH 3T3 cells were treated with bio-pYTAR probe (lane 1), bio-pYTAR probe and the known PI3K N-SH2 inhibitor peptide pYVPM (lane 3), or resin alone (lane 2). (B) Co-immunoprecipitation of the Src tyrosine kinase with an α-PI3K polyclonal antibody. Cell lysate from 527 cells was either untreated (lane 1), pretreated with pYVPM (lane 2) or *Yersinia pestis* (YOP) phosphatase (lane 3), and then precipitated with a polyclonal α-PI3K antibody-conjugated agarose resin. (C) Immunoprecipitation and phosphorylation analysis of Akt from NIH 3T3 (lane 1), 416 (lane 2), and 527 (lane 3) cell lines. Loadings were normalized to α-rabbit IgG heavy chain.

is autophosphorylated, causing a conformational shift of the activation loop and exposure of the kinase active site.[4,5]

Src-transformed 527 cells overexpress a mutant (Y527F) form of Src, in which the enzyme is constitutively active and phosphorylated on the Y416 activation loop tyrosine.[19] To validate biochemically our in vitro discovery of a pY416-mediated interaction with PI3K, we performed a co-immunoprecipitation of the Src tyrosine kinase, from 527 cell lysate, using an agarose-linked polyclonal α-p85 PI3K antibody. As shown in Figure 2B (lane 1), anti-p85 PI3K antibody is effective in the co-immunoprecipitation of Src, along with its immunoprecipitation of PI3K. This affinity interaction, however, is significantly reduced, both in the presence of pYVPM peptide (lane 2) and by treatment with a tyrosine phosphatase from *Yersinia pestis* (YOP) (lane 3).

The diminished Src signal observed upon phosphatase treatment in Figure 2B (lane 3) confirms the phosphorylation dependence of the Src/PI3K interaction and implicates the activation loop phosphotyrosine pY416 in this interaction since this is the only tyrosine phosphorylated in Y527F-activated Src.[4,19] Furthermore, the weakened signal observed in the presence of the pYVPM peptide establishes the importance of the N-terminal SH2 domain of PI3K in this interaction, since pYVPM is a known and selective PI3K N-terminal SH2 domain ligand.[10] As expected, low levels of Src remain in both lanes, because of the residual affinity of the Src SH3 domain/PI3K polyproline helix interaction,[17] supporting the hypothesis that the Src/PI3K interaction is mediated by both the Src SH3 domain and the Src activation loop phosphotyrosine.

To investigate the cellular effects of Src activation loop phosphorylation, a Y416F,Y527F double mutant was generated, stably transfected into NIH 3T3 cells, and shown to form foci in vitro and cause tumor formation in immunocompromised athymic BALB/c mice, but not in normal BALB/c mice.[13] In comparison, injection of the 527 cell line caused tumor formation in both normal and athymic BALB/c mice, consistent with previous findings.[20]

The serine/threonine kinase Akt is a major component of the PI3K pathway and is responsible for cellular proliferation and anti-apoptotic effects.[21] Recent findings have shown that Akt is phosphorylated on tyrosine in response to growth factor stimulation.[22] To evaluate the effect of the Y416F mutation on Akt activation, cell lysates from NIH 3T3, 416, and 527 cell lines were immunoprecipitated with an α-Akt antibody and analyzed for phosphorylation using an α-pY antibody (Figure 2C).[13] Only the 527 cell lysate (lane 3) showed a tyrosine-phosphorylated protein at 60 kDa, where we observe Akt, indicating that Src activation loop phosphorylation is important for activation of PI3K. This result further suggests that activation of the PI3K pathway may be a crucial component of Src's ability to form tumors in normal mice, since the Y416F mutant only caused tumor formation in athymic mice.

Activation loop phosphotyrosines are largely unrecognized as playing a molecular recognition role in cellular signaling. Our findings establish that the Src/PI3K interaction is indeed dependent on both the phosphorylation state of the Src activation loop and an available N-terminal SH2 domain on PI3K. The Src/PI3K association is one of the first interactions identified in signal transduction.[7,8] The discovery of a previously unknown molecular recognition role for the activation loop in this interaction will have significant implications for the signaling mechanisms of Src and possibly non-Src tyrosine kinases.

Acknowledgment. We thank Sara Courtneidge for the 527 cell line, Craig Crews for cell culture facilities and both for helpful discussion, Janet Crawford (W. M. Keck Center) for peptide synthesis, and the National Institutes of Health (R01 GM59673) for financial support. D.J.A. is the recipient of an Alfred P. Sloan Foundation Fellowship.

Supporting Information Available: Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Brugge, J. S.; Erikson, R. L. *Nature* **1977**, *269*, 346−348. (b) Collett, M. S.; Erikson, R. L. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 2021−2024. (c) Hunter, T.; Sefton, B. M. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 1311−1315.
(2) Manning, G.; Plowman, G. D.; Hunter, T.; Sudarsanam, S. *Trends Biochem. Sci.* **2002**, *27*, 514−520.
(3) Cohen, P. *Nat. Rev. Drug Discovery* **2002**, *1*, 309−315.
(4) (a) Superti-Furga, G.; Courtneidge, S. A. *Bioessays* **1995**, *17*, 321−330. (b) Brown, M. T.; Cooper, J. A. *Biochim. Biophys. Acta* **1996**, *1287*, 121−149. (c) Gonfloni, S.; Weijland, A.; Kretzschmar, J.; Superti-Furga, G. *Nat. Struct. Biol.* **2000**, *7*, 281−286.
(5) Xu, W.; Harrison, S. C.; Eck, M. J. *Nature* **1997**, *385*, 595−602.
(6) (a) Pawson, T.; Scott, J. D. *Science* **1997**, *278*, 2075−2080. (b) Yaffe, M. B. *Nat. Rev. Mol. Cell Biol.* **2002**, *3*, 177−186.
(7) Martin, G. S. *Nat. Rev. Mol. Cell Biol.* **2001**, *2*, 467−475.
(8) (a) Sugimoto, Y.; Whitman, M.; Cantley, L. C.; Erikson, R. L. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 2117−2121. (b) Macara, I. G.; Marinetti, G. V.; Balduzzi, P. C. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 2728−2732.
(9) Manning, G.; Whyte, D. B.; Martinez, R.; Hunter, T.; Sudarsanam, S. *Science* **2002**, *298*, 1912−1934.
(10) Songyang, Z.; Shoelson, S. E.; Chaudhuri, M.; Gish, G.; Pawson, T.; Haser, W. G.; King, F.; Roberts, T.; Ratnofsky, S.; Lechleider, R. J.; Neel, B. G.; Birge, R. B.; Fajardo, J. E.; Chou, M. M.; Hanafusa, H.; Schaffhausen, B.; Cantley, L. C. *Cell* **1993**, *72*, 767−778.
(11) Sanjay, A.; Houghton, A.; Neff, L.; DiDomenico, E.; Bardelay, C.; Antoine, E.; Levy, J.; Gailit, J.; Bowtell, D.; Horne, W. C.; Baron, R. *J. Cell Biol.* **2001**, *152*, 181−195.
(12) (a) Ungureanu, D.; Saharinen, P.; Junttila, I.; Hilton, D. J.; Silvennoinen, O. *Mol. Cell. Biol.* **2002**, *22*, 3316−3326. (b) Hu, J.; Liu, J.; Ghirlando, R.; Saltiel, A. R.; Hubbard, S. R. *Mol. Cell* **2003**, *12*, 1379−1389.
(13) See Supporting Information for detailed experimental protocols, binding data, and DNA sequence information.
(14) Videlock, E. J.; Chung, V. K.; Mohan, M. A.; Strok, T. M.; Austin, D. J. *J. Am. Chem. Soc.* **2004**, *126*, 3730−3731.
(15) Zozulya, S.; Lioubin, M.; Hill, R. J.; Abram, C.; Gishizky, M. L. *Nat. Biotechnol.* **1999**, *17*, 1193−1198.
(16) GenBank RefSeq NM_181523, Homo sapiens phosphoinositide-3-kinase, regulatory subunit, polypeptide 1 (p85 alpha) (PIK3R1).
(17) Liu, X.; Marengere, L. E.; Koch, C. A.; Pawson, T. *Mol. Cell. Biol.* **1993**, *13*, 5225−5232.
(18) Fukui, Y.; Hanafusa, H. *Mol. Cell. Biol.* **1991**, *11*, 1972−1979.
(19) Fumagalli, S.; Totty, N. F.; Hsuan, J. J.; Courtneidge, S. A. *Nature* **1994**, *368*, 871−874.
(20) Snyder, M. A.; Bishop, J. M. *Virology* **1984**, *136*, 375−386.
(21) Vanhaesebroeck, B.; Alessi, D. R. *Biochem. J.* **2000**, *346*, 3, 561−576.
(22) Chen, R.; Kim, O.; Yang, J.; Sato, K.; Eisenmann, K. M.; McCarthy, J.; Chen, H.; Qiu, Y. *J. Biol. Chem.* **2001**, *276*, 31858−31862.

JA047957C