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Downregulation of Src Family Kinase Activity Using PP2 Inhibitor Has No Effect on the Elongation of Pragmin-Transfected Adenocarcinoma AGS cells

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Abstract

Introduction: Pragmin is the first mammalian protein that contains a functional the Glu-Pro-Ile-Tyr-Ala (EPIYA) motif. Pragmin is tyrosine-phosphorylated at EPIYA motif by Src family kinases (SFKs), C-terminal Src kinase (CSK), and in response to epidermal growth factor (EGF) stimulation. Pragmin can induce some morphological changes in transfected cells characterized by cell elongation, which can be construed as an invasive phenotype contributing to tumor invasion and metastasis. This study was established to investigate Src role as a key regulator of cell motility to induce elongated morphology of cells in Pragmin transfected AGS cells, a human gastric adenocarcinoma cell line, by using PP2, a specific inhibitor of Src family protein kinase.

Methods: Firstly, AGS cells were transfected with Pragmin and Pragmin mutant (Y391F) using lipofectamine 2000 reagent and then we treated the cells by PP2. Finally, we evaluated cell-morphological changes in the presence or the absence of PP2 by using light microscopy and the results were analyzed.

Results: Our results showed in AGS cells that were transiently transfected by Pragmin in the presence of PP2 (where Src tyrosine kinase activity was inhibited), the number of elongated cells did not change compared to elongated cell numbers of Pragmin transfected cells in the absence of PP2.

Conclusion: Our findings suggest that in spite of the importance of Src tyrosine kinase activity to regulate the cell motility, the cell-morphological changes of Pragmin-transfected AGS cells is independent of Src activity. It seems that other mechanism(s) to be involved in this process.

Keywords: Pragmin, Morphological changes, EPIYA motif, Src, PP2 inhibitor, Adenocarcinoma

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Introduction

Pragmin (also was known Sgk223) was originally identified as a partner of Rnd2 that stimulates RhoA to induce cell contraction in neural cells.[1] Moreover, Pragmin was the first mammalian protein, which contains a single functional Glu-Pro-Ile-Tyr-Ala (EPIYA) motif.[2] Among mammalian proteins, there are many proteins with EPIYA (or alike) motifs and the function(s) of EPIYA motif in most of these proteins are still unknown.[3] In fact, EPIYA motifs were originally discovered in CagA *Helicobacter pylori*.[4,5] All *H. pylori* strains from the world, dependent on geographic area, contain two types of CagA oncoprotein: Western type and East Asian type (Japan, Korea, and China). Both types contain EPIYA-A and EPIYA-B motifs. The presence of EPIYA-D segments instead of EPIYA-C segments in the East Asian type of CagA was shown.[6] In this regard, the presence of EPIYA (or alike) motifs in several pathogenic bacterial effectors was shown to be important to manipulate cell signal transduction to better colonization in their cell host and spread infection. These pathogenic bacterial effectors include Anaplasma phagocytophilum AnkA,[7,8] enteropathogenic *Escherichia coli* Tir,[9] *Citrobacter rodentium* Tir,[10] *Chlamydia trachomatis* Tarp,[11] *Haemophilus ducreyi*

LspA, [12] and *Bartonella henselae* BepD, BepE, and BepF.[13] These bacterial EPIYA effectors are delivered into host cells via type III or IV secretion system, where they undergo tyrosine phosphorylation at the EPIYA motif by host kinases. Also, it was shown that after tyrosine phosphorylation at EPIYA (or alike) motifs, bacterial effector proteins containing EPIYA (or alike) motifs can interact with a huge number of the SH2 domain containing proteins in their host cells perturbing cell signaling functions.[14] It was proposed that long-time co-evolution of pathogenic bacteria with mammalian hosts provide special advantages for them. Importantly, it was demonstrated that pathogenic bacterial effectors such as *H. pylori* CagA correlate with carcinogenesis.[15] Furthermore, it was exhibited that infection (or transfection) of host cells with CagA *H. pylori* induced characterized cell morphological changes known as hummingbird phenotype. Some studies were demonstrated that CagA inducing hummingbird phenotype was dependent on tyrosine phosphorylation at EPIYA motifs.[16-18] However, related mechanisms are not still fully understood.

Among the mammalian proteins, two mammalian proteins have been reported to contain functional EPIYA motif (or like) motifs.[2,19] It was found that Pragmin was phosphorylated by Src family kinases (SFKs), in response to epidermal growth factor (EGF) stimulation, and C-terminal Src kinase (CSK) at EPIYA motif and it specifically interacted with CSK.[2,20] CSK is a unique inhibitor of Src and Pragmin/CSK interaction sequestrated CSK in the cytoplasm and thereby, it generates a positive feedback loop to induce Src activity. Interestingly, it was shown that the cells, which transiently transfected by Pragmin showed an elongated morphology (or invasive form).[2] Recently, it was reported that Pragmin-CSK axis increases CSK activity and it was involved in cell-morphological changes.[20] Some studies reported that tyrosine phosphorylation at EPIYA motif induced an invasive form of cells contributing to tumor invasion and metastasis.[21,22] Therefore, it was important to elucidate more details of the mechanism(s) that mediate in inducing elongated morphology of cells by tyrosine phosphorylation at EPIYA (or alike) motifs. This information will provide insights into the better understanding of why the EPIYA (or like) motifs are important sequences in carcinogenesis and why these critical motifs were exploited by several pathogenic bacteria among many available sequences in the host cells during long time co-evolution. In the present study, we aimed to investigate the role(s) of SFKs activity to induce elongated cell morphology in Pragmin-transfected AGS cells.

Methods

Expression Vectors

A pCMV-based mammalian expression vector for rat Pragmin was kindly provided by Dr. M. Negishi, (Kyoto University, Japan). Myc-tagged Pragmin- Y391F, in which tyrosine-391 was substituted with phenylalanine, was generated from the rat Pragmin cDNA by the use of the Chameleon site-directed mutagenesis kit (Stratagene).[2] pSP65Srα mammalian expression vectors were also used as control (empty vector).

Antibodies and Reagents

Anti-Actin polyclonal antibody C-11 (Santa Cruz Biotechnology), anti-Myc monoclonal antibody 9E10 (Santa Cruz Biotechnology), and anti-phosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology) were used as primary antibodies for immunoblotting. Src family protein kinase inhibitor PP2 was purchased from Calbiochem.

Cell Culture and Transfection

AGS (human gastric epithelial cells, ATCC), were cultured in RPMI 1640 medium (10% fetal bovine serum (FBS), 37°C, 5% CO2 humidified atmosphere). Cells were transiently transfected with expression vectors using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Then, the cells were harvested at 36 hours after transfection.

Cell Treatment

AGS cells were cultured and transfected with lipofectamine 2000 reagent (Invitrogen). On the other hand, 30 µg expression vectors were totally transfected into 1.5×10^6 AGS cells in a 100-mm dish. After 24 hours after transfection, the medium was changed and the cells were cultured in starved condition (medium without serum). Before harvesting cells, we treated AGS cells by 100 nM PP2 for 6 hours. Finally, we evaluate morphological changes of cells under the light microscope (Nikon, Tokyo, Japan).

SDS-Page and Western Blot

AGS cells were cultured. 36 hours after transfection, cells were harvested and lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2 mM Na_3VO_4, 1 mM PMSF). Total cell lysates (TCL) was subjected to SDS-PAGE. Proteins transferred to PVDF membrane filters (Millipore) were soaked in solutions containing primary antibody (1:1000, 90 minutes at room temperature) and secondary antibody (1:10,000, 45 minutes at room temperature). Western blot chemiluminescence reagent (PerkinElmer Life Sciences) was applied to visualize the bands. Intensities of chemiluminescence on the immunoblotted filters were quantitated by using a luminescence image analyzer (LAS-4000, Fuji Film).

Statistical Analysis

Statistical analyses were carried out by Student's *t* test using SPSS software version 16. $P<0.05$ was considered to be statistically significant.

Results

Downregulation of Src Activity in Pragmin Transfected Cells by Using a PP2 Inhibitor

AGS cells were cultured and transfected by Myc-Pragmin vector. pSP65Srα mammalian expression vectors were used as a control. To inhibit tyrosine kinase activity, AGS cells were incubated with 100 nM PP2 for 6 h. After 36 hours, AGS cells were harvested and the all sample were subjected to SDS-PAGE followed by immunoblotting using specific antibodies. Our results showed that in the presence of PP2, tyrosine phosphorylation of Pragmin was reduced. We used actin as a loading control (Figure 1).

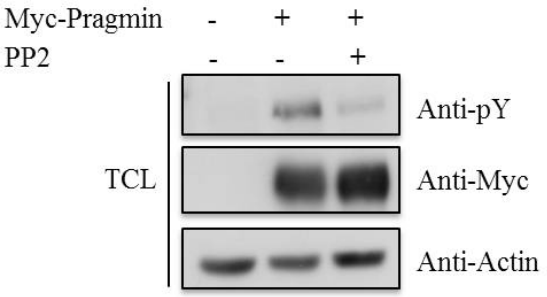

Figure 1. Effect of 100 nM PP2 on Tyrosine Phosphorylation of Pragmin. AGS cells were treated with 100 nM PP2 for 6 h before harvest. Totall cell lysates (TCLs) were subjected to immunoblotting with an Anti-pY: anti-phosphotyrosine antibody, anti-Myc antibody, and anti-Actin antibody.

Morphological Changes in Transfected AGS Cells

AGS cells were cultured and then AGS cells were transfected with Pragmin and Y391F vectors in the presence of PP2 (Pragmin +, Y391F +) and in the absence of PP2 (Pragmin-, Y391F-). By using a microscope, we enumerated morphological changes of cells in random fields (Figure 2A). Next, we compared a number of elongated cells (Figure 2B). Mean percentage of elongated cells was 55, 70, 420, 100 and 350 in control (pSP65Srα), Y391F-, Pragmin-, Y391F+ and Pragmin+ transfected cells, respectively (Figure 2B). Our results demonstrated that there is no significant difference between the number of elongated cells which were transfected with Pragmin in the presence or in the absence of PP2.

Discussion

Src tyrosine kinases are key molecules in the regulation of cell motility and invasion and, deregulation of these kinases was reported in many human cancers.[23] In addition, the importance of EPIYA motif in carcinogenesis was previously shown in several pathogenic bacterial effectors and two mammalian proteins. Our previous study exhibited that SFKs are capable of Pragmin tyrosine phosphorylation at EPIYA motif and Pragmin is able to elevate Src activity in AGS cells.[2] Based on these observations, we investigated the role of Src tyrosine kinase activity in induction of morphological changes

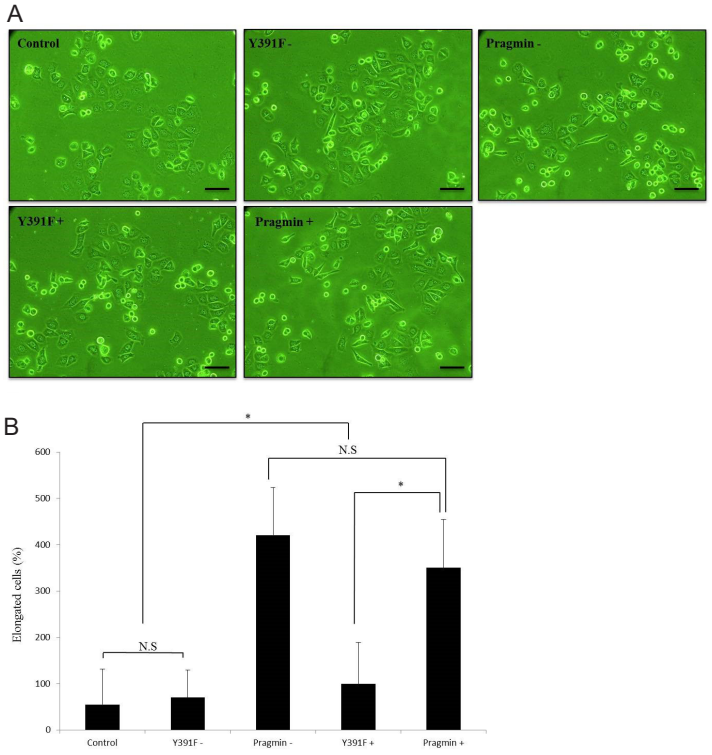

Figure 2. Evaluation of a Number of Elongated Cells in the Presence of PP2 (+) or in the Absence of PP2 (-). A: Morphology of transfected cells was analyzed by microscope. (Scale bars, 10 μm.). AGS cells were transfected with a Myc-Pragmin or Myc-Pragmin-Y391F vector. To inhibit tyrosine kinase activity, AGS cells were incubated with 100 nM PP2 for 6 h. B: Percentage of elongated cells was shown. Error bars represent means ± SD. * indicates significance with a $P < 0.05$, N.S: not significance (Student's *t* test).

in Pragmin-transfected AGS cells compared to either Pragmin mutant (Y391F)- transfected cells or control (empty vector). Our results showed that down- regulation of Src tyrosine kinase activity by using PP2 had no effect on the number of Pragmin-transfected AGS cells with elongated morphology. Therefore, it seems that other Src activity- independent mechanism(s) to be involved in the induction of elongated cell morphology in Pragmin transfected cells.

Recently, it was demonstrated that CSK is also capable of Pragmin tyrosine phosphorylation at Y238, Y343, and Y391 (the latter one is a major site for tyrosine phosphorylation in Pragmin).[20] After tyrosine phosphorylation at EPIYA motif of Pragmin, SH2 domain of CSK interacts with Pragmin EPIYA motif and CSK activity is amplified by CSK/Pragmin complex formation. Also, it was reported that CSK/Pragmin interaction (and no single expression of CSK or Pragmin) has a major role to induce invasive shape of cells in the MKN7 human gastric epithelial cell line. Importantly, formation and disassembly of focal adhesions play a key role in cell migration.[24] In this regards, it was reported that CSK/Pragmin complex was co-localized with a focal adhesion component(s), vinculin, and it seems to participate in cell motility in MKN7 cells.[20] In fact, Pragmin is initially tyrosine phosphorylated at EPIYA motif by SFKs in response to EGF stimulation, and after formation of CSK/Pragmin complex, CSK kinase activity is amplified. Therefore, it seems that CSK has a key role to regulate morphological changes of cells.[2,20] In this regards, it was previously shown that in HeLa cells, overexpressed CSK co-localized with focal adhesions and caused disruption of focal adhesion spots, redistribution of integrins, and inhibition of cell adhesion.[25] Taken together, these findings suggested that cell elongation morphology in Pragmin infected cells by using recombinant lentiviruses is dependent on CSK/Pragmin complex formation, activation of CSK, and co-localization of CSK/Pragmin complex formation on focal adhesion components.

Interestingly, *H. pylori* CagA molecule can induce hummingbird phenotype, as well as polygonal and needle forms. In this regards, it was reported that CagA oncoprotein was tyrosine phosphorylated by SFKs and then c-Abl kinase at EPIYA motifs.[26,27] Next, Src-mediated CagA phosphorylation is followed by a rapid inactivation of Src kinase activity by CSK/CagA complex formation. Inhibition of Src kinase activity results in dephosphorylation of adaptor target proteins (i.e. vinculin, ezrin, and cortactin) involved in the organization of actin and focal adhesions. Furthermore, the activation of SHP-2 phosphatase activity has consequently been reported to inactivate FAK (focal adhesion kinase) in cells that ectopically express CagA.[29] Inactivated FAK is not recruited to focal adhesions which may in some extent contribute to the formation of elongated cell phenotype.

Conclusion

Finally, it is not clear whether the other EPIYA containing proteins including bacterial effector proteins and mammalian proteins are able to induce elongated morphology of cells and thereby, more studies will be required to elucidate the mechanism (s) involved in these processes. Such results will be helpful to design new drugs to inhibit cell invasion and metastasis as anticancer therapeutics approach.

Ethical Approval

Not applicable.

Competing Interests

Authors declare that they have no competing interests.

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References

1. Tanaka H, Katoh H, Negishi M. Pragmin, a novel effector of Rnd2 GTPase, stimulates RhoA activity. J Biol Chem. 2006;281(15):10355-10364. doi:10.1074/jbc.M511314200
2. Safari F, Murata-Kamiya N, Saito Y, Hatakeyama M. Mammalian Pragmin regulates Src family kinases via the Glu-Pro-Ile-Tyr-Ala (EPIYA) motif that is exploited by bacterial effectors. Proc Natl Acad Sci U S A. 2011;108(36):14938-14943. doi:10.1073/pnas.1107740108
3. Safari F. EPIYA (or -like) motifs in mammalian proteins. Journal of King Saud University - Science. 2014;26(4):276-284. doi:10.1016/j.jksus.2014.05.001
4. Covacci A, Rappuoli R. Tyrosine-phosphorylated bacterial proteins: Trojan horses for the host cell. J Exp Med. 2000;191(4):587-592.
5. Backert S, Selbach M. Tyrosine-phosphorylated bacterial effector proteins: the enemies within. Trends Microbiol. 2005;13(10):476-484. doi:10.1016/j.tim.2005.08.002
6. Hatakeyama M. Oncogenic mechanisms of the Helicobacter pylori CagA protein. Nat Rev Cancer. 2004;4(9):688-694. doi:10.1038/nrc1433
7. JW IJ, Carlson AC, Kennedy EL. Anaplasma phagocytophilum AnkA is tyrosine-phosphorylated at EPIYA motifs and recruits SHP-1 during early infection. Cell Microbiol. 2007;9(5):1284-1296. doi:10.1111/j.1462-5822.2006.00871.x
8. Lin M, den Dulk-Ras A, Hooykaas PJ, Rikihisa Y. Anaplasma phagocytophilum AnkA secreted by type IV secretion system is tyrosine phosphorylated by Abl-1 to facilitate infection. Cell Microbiol. 2007;9(11):2644-2657. doi:10.1111/j.1462-5822.2007.00985.x
9. Campellone KG, Giese A, Tipper DJ, Leong JM. A tyrosine-phosphorylated 12-amino-acid sequence of enteropathogenic Escherichia coli Tir binds the host adaptor protein Nck and is required for Nck localization to actin pedestals. Mol Microbiol. 2002;43(5):1227-1241.
10. Deng W, Vallance BA, Li Y, Puente JL, Finlay BB. Citrobacter

rodentium translocated intimin receptor (Tir) is an essential virulence factor needed for actin condensation, intestinal colonization and colonic hyperplasia in mice. Mol Microbiol. 2003;48(1):95-115.
11. Clifton DR, Fields KA, Grieshaber SS, et al. A chlamydial type III translocated protein is tyrosine-phosphorylated at the site of entry and associated with recruitment of actin. Proc Natl Acad Sci U S A. 2004;101(27):10166-10171. doi:10.1073/pnas.0402829101
12. Deng K, Mock JR, Greenberg S, van Oers NS, Hansen EJ. Haemophilus ducreyi LspA proteins are tyrosine phosphorylated by macrophage-encoded protein tyrosine kinases. Infect Immun. 2008;76(10):4692-4702. doi:10.1128/iai.00513-08
13. Schulein R, Guye P, Rhomberg TA, et al. A bipartite signal mediates the transfer of type IV secretion substrates of Bartonella henselae into human cells. Proc Natl Acad Sci U S A. 2005;102(3):856-861. doi:10.1073/pnas.0406796102
14. Selbach M, Paul FE, Brandt S, et al. Host cell interactome of tyrosine-phosphorylated bacterial proteins. Cell Host Microbe. 2009;5(4):397-403. doi:10.1016/j.chom.2009.03.004
15. Ohnishi N, Yuasa H, Tanaka S, et al. Transgenic expression of Helicobacter pylori CagA induces gastrointestinal and hematopoietic neoplasms in mouse. Proc Natl Acad Sci U S A. 2008;105(3):1003-1008. doi:10.1073/pnas.0711183105
16. Bourzac KM, Botham CM, Guillemin K. Helicobacter pylori CagA induces AGS cell elongation through a cell retraction defect that is independent of Cdc42, Rac1, and Arp2/3. Infect Immun. 2007;75(3):1203-1213. doi:10.1128/iai.01702-06
17. Segal ED, Cha J, Lo J, Falkow S, Tompkins LS. Altered states: involvement of phosphorylated CagA in the induction of host cellular growth changes by Helicobacter pylori. Proc Natl Acad Sci U S A. 1999;96(25):14559-14564.
18. Selbach M, Moese S, Hurwitz R, Hauck CR, Meyer TF, Backert S. The Helicobacter pylori CagA protein induces cortactin dephosphorylation and actin rearrangement by c-Src inactivation. Embo j. 2003;22(3):515-528. doi:10.1093/emboj/cdg050
19. Repetto D, Aramu S, Boeri Erba E, et al. Mapping of p140Cap phosphorylation sites: the EPLYA and EGLYA motifs have a key role in tyrosine phosphorylation and Csk binding, and are substrates of the Abl kinase. PLoS One. 2013;8(1):e54931. doi:10.1371/journal.pone.0054931
20. Senda Y, Murata-Kamiya N, Hatakeyama M. C-terminal Src kinase-mediated EPIYA phosphorylation of Pragmin creates a feed-forward C-terminal Src kinase activation loop that promotes cell motility. Cancer Sci. 2016;107(7):972-980. doi:10.1111/cas.12962
21. Leroy C, Fialin C, Sirvent A, et al. Quantitative phosphoproteomics reveals a cluster of tyrosine kinases that mediates SRC invasive activity in advanced colon carcinoma cells. Cancer Res. 2009;69(6):2279-2286. doi:10.1158/0008-5472.can-08-2354
22. Tactacan CM, Phua YW, Liu L, et al. The pseudokinase SgK223 promotes invasion of pancreatic ductal epithelial cells through JAK1/Stat3 signaling. Mol Cancer. 2015;14:139. doi:10.1186/s12943-015-0412-3
23. Kim LC, Song L, Haura EB. Src kinases as therapeutic targets for cancer. Nat Rev Clin Oncol. 2009;6(10):587-595. doi:10.1038/nrclinonc.2009.129
24. Webb DJ, Parsons JT, Horwitz AF. Adhesion assembly, disassembly and turnover in migrating cells -- over and over and over again. Nat Cell Biol. 2002;4(4):E97-100. doi:10.1038/ncb0402-e97
25. Bergman M, Joukov V, Virtanen I, Alitalo K. Overexpressed Csk tyrosine kinase is localized in focal adhesions, causes reorganization of alpha v beta 5 integrin, and interferes with HeLa cell spreading. Mol Cell Biol. 1995;15(2):711-722.
26. Poppe M, Feller SM, Romer G, Wessler S. Phosphorylation of Helicobacter pylori CagA by c-Abl leads to cell motility. Oncogene. 2007;26(24):3462-3472. doi:10.1038/sj.onc.1210139
27. Tammer I, Brandt S, Hartig R, Konig W, Backert S. Activation of Abl by Helicobacter pylori: a novel kinase for CagA and crucial mediator of host cell scattering. Gastroenterology. 2007;132(4):1309-1319. doi:10.1053/j.gastro.2007.01.050
28. Selbach M, Moese S, Backert S, Jungblut PR, Meyer TF. The Helicobacter pylori CagA protein induces tyrosine dephosphorylation of ezrin. Proteomics. 2004;4(10):2961-2968. doi:10.1002/pmic.200400915
29. Tsutsumi R, Takahashi A, Azuma T, Higashi H, Hatakeyama M. Focal adhesion kinase is a substrate and downstream effector of SHP-2 complexed with Helicobacter pylori CagA. Mol Cell Biol. 2006;26(1):261-276. doi:10.1128/mcb.26.1.261-276.2006