RAFTK/Pyk2 Activation Is Mediated by Trans-acting Autophosphorylation in a Src-independent Manner*

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The related adhesion focal tyrosine kinase (RAFTK), also known as Pyk2, undergoes autophosphorylation upon its stimulation. This leads to cascades of intracellular signaling that result in the regulation of various cellular activities. However, the molecular mechanism of RAFTK autophosphorylation is not yet known. Using various RAFTK constructs fused with two different tags, we found that the autophosphorylation of RAFTK was mediated by a trans-acting mechanism, not a cis-acting mechanism. In addition, overexpression of kinase-mutated RAFTK inhibited wild type RAFTK autophosphorylation in a dose-dependent manner by a trans-acting interaction. Trans-acting autophosphorylation was also observed between endogenous and exogenous RAFTK upon potassium depolarization of neuroendocrine PC12 cells. Using immunoprecipitation and affinity chromatography, we detected RAFTK self-association that was not affected by deletion of a single region or domain of RAFTK. Furthermore, RAFTK autophosphorylation occurred only at site Tyr402 in a Src kinase activity-independent manner. However, Src significantly enhanced RAFTK-mediated paxillin phosphorylation, suggesting a key role for Src in RAFTK activation and phosphorylation of downstream substrates. Our results indicate that the activation of RAFTK occurs in several steps. First, upon stimulus, RAFTK trans-autophosphorylates Tyr402. Second, phosphorylated Tyr402 recruits and activates Src kinase that in turn phosphorylates RAFTK and enhances its kinase activity. Lastly, the enhanced RAFTK activity induces the activation of downstream signaling molecules. Taken together, these studies provide insights into the molecular mechanism of RAFTK autophosphorylation and the specific role of Src in the regulation of RAFTK activation.

The related adhesion focal tyrosine kinase (RAFTK)1 (1), also known as Pyk2 (2), CAK-β (3), and CADTK (4), is a nonreceptor tyrosine kinase related to focal adhesion kinase (FAK). RAFTK transduces key extracellular signals through tyrosine phosphorylation, leading to various cellular responses, and is also implicated in the regulation of numerous cellular activities (5, 6). FAK and RAFTK exhibit ~48% amino acid identity (65% similarity) and have a similar domain structure: a unique N terminal, a centrally located protein tyrosine kinase domain, and two proline-rich regions at the C terminus (6, 7). Neither kinase contains SH2 and SH3 domains. Analyses of FAK activation events have shown that phosphorylation of FAK occurs at six sites in vivo: two sites (Tyr397 and Tyr407) within the FAK N-terminal region; two sites (Tyr576 and Tyr577) within the kinase domain activation loop; and two sites (Tyr861 and Tyr895) within the C-terminal region. Four FAK tyrosine phosphorylation sites (Tyr397, Tyr576, Tyr577, and Tyr895) are conserved at analogous positions in RAFTK (Tyr402, Tyr407, Tyr409, and Tyr411). Among these four tyrosine phosphorylation sites, Tyr402 is known to be autophosphorylated (2). It is well established that RAFTK autophosphorylation plays a key role in various cellular signaling processes, such as mitogen-activated kinase kinase activation mediated by G protein-coupled receptors (8), the activation of T cells through T cell antigen receptor cross-linking (9), nephrinoidin signaling in a subset of renal epithelial cells (10), integrin-mediated osteoclast motility (11), association of RAFTK with nerve terminals (12), cardiac remodeling mediated by the reorganization of focal adhesion contacts (13), and adhesion-induced osteoclast spreading and bone resorption (14). Upon stimulation, RAFTK is known to autophosphorylate Tyr402, which recruits Src through binding of the Src-SH2 domain to the RAFTK-Tyr402 site, leading to the activation of Src (6, 7). The activated Src, in turn, phosphorylates RAFTK at Tyr417 and Tyr419, which enhances the activity of RAFTK (6, 15, 16). Although the autophosphorylation of RAFTK is reported to play an important role in various systems, the detailed mechanism of this event, such as the nature and sites of its autophosphorylation, are unknown.

In this study, we focused on the following questions: 1) Is the autophosphorylation of RAFTK a cis- or trans-acting event? 2) What are the essential components needed for RAFTK autophosphorylation? 3) Is Tyr402 the only autophosphorylation site? 4) Is RAFTK self-associated similar to the heterodimerization of other receptor-type tyrosine kinases? Better understanding of the initial steps of RAFTK activation will provide insights into RAFTK-mediated cellular functions, such as cell adhesion and migration, stress response, and induction of neuronal long term potentiation.

HEK, human embryonic kidney; KM, kinase mutant; SYF, Src‘/Yes’/Fyn‘ cells; SYF + Src, Src‘/Yes’/Fyn‘ cells; WT, wild type.

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1 The abbreviations used are: RAFTK, related adhesion focal tyrosine kinase; FAK, focal adhesion kinase; GFP, green fluorescent protein;
Experimental Procedures

Cells and Cell Culture—Rat pheochromocytoma PC12 cells were obtained from ATCC and maintained in Dulbecco's modified Eagle's medium (Invitrogen) with 10% (v/v) heat-inactivated horse serum. Serotonin (5-HCA) and the reverse primer 5'-TGGTCCGAGCCCC and the reverse primer 5'-H11002 were plated on poly-D-lysine-coated plates a day before transfection and then transiently transfected with various cDNA constructs using Lipofectamine 2000 reagent according to the manufacturer's instructions (Invitrogen). The cell lysates were prepared with modified RIPA buffer and immunoprecipitated with specific antibodies, followed by immunoblotting as described below.

Preparation of Cell Lysates, Immunoprecipitations, and Immunoblotting—The cells were lysed in modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, and 1 mM EDTA) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin) and phosphatase inhibitors (1 mM NaF, and 1 mM Na3PO4). Immunoprecipitations were performed with the indicated antibodies, followed by protein G incubation. The immunoprecipitates were separated by either 8% SDS-PAGE or 7% Tris-acetate-NTPAGE (Novex, San Diego, CA) under reducing conditions, electrophoretically transferred to Immobilon polyvinylidene difluoride (Millipore, Bedford, MA), and probed using immunoblotting with the enhanced chemiluminescence technique (Amersham Biosciences). Levels of tyrosine phosphorylation, Tyr402-specific phosphorylation, and the amounts of RAFTK protein were quantified using a software program for densitometry analysis (Un-Scan-It, Silk Scientific Corp.). Each band was normalized to the total RAFTK level in the respective sample.

In Vitro Pull-down Assay—The fast protein liquid chromatography column was prepared with mouse anti-FLAG M2 antibody-attached agarose beads according to the manufacturer's instructions (Sigma). HEK 293 cells were co-transfected with 2 μg of FLAG-tagged WT RAFTK and 2 μg of GFP-tagged WT RAFTK, followed by preparation of whole cell lysates with modified RIPA lysis buffer. 4 ml of the cell lysates (1 mg/ml) were loaded on anti-FLAG affinity chromatography columns. After extensive washing with Tris-buffered saline buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.4), each 1 ml fraction of elutes was collected after loading 5 ml of FLAG peptide (100 μg/ml in Tris-buffered saline buffer; Sigma) for competitive elution according to the manufacturer's instructions (Sigma). The collected fraction samples were resolved on 8% SDS-PAGE followed by immunoblotting.

Results and Discussion

Autophosphorylation of RAFTK Requires Both Intact RAFTK Kinase Activity and the Tyrosine Residue at 402—RAFTK, a FAK subfamily member, is an intracellular tyrosine kinase that mediates tyrosine phosphorylation in various signaling pathways (6). Upon extracellular stimulation, such as by cell adhesion, intracellular Ca2+ increase, or growth factors, RAFTK undergoes autophosphorylation followed by subsequent molecular interaction and the activation of downstream signaling cascades. To analyze the molecular mechanism of RAFTK autophosphorylation, various deletion and point mutations of RAFTK were constructed (Fig. 1A). HEK 293 cells have no endogenous expression of RAFTK. These cells were transfected with the RAFTK constructs and analyzed for the tyrosine phosphorylation status of RAFTK by immunoblotting with anti-phosphotyrosine (4G10) and autophosphorylation at Tyr402 by immunoblotting with phospho-specific anti-Tyr(P)402-RAFTK antibody. As shown in Fig. 1B, although wild type RAFTK (WT-FLAG and WT-GFP) was highly phosphorylated, a kinase mutant of RAFTK (KM-GFP) and RAFTK with a point mutation (tyrosine to phenylalanine) of the autophosphorylation site at Tyr402 were not phosphorylated. The pan RAFTK tyrosine phosphorylation was correlated with the RAFTK Tyr402 phosphotyrosine. This demonstrates that intact kinase activity and the tyrosine residue 402 were essential for RAFTK autophosphorylation. These data were further supported by the expression of an N terminus deletion mutant (del N-FLAG), which showed no phosphorylation because of the lack of the Tyr402 site, although it contained an intact kinase domain. Interestingly, autophosphorylation was detected in a C terminus deletion mutant (del C-FLAG) that contained the kinase domain and the autophosphorylation site at Tyr402, suggesting that the C terminus region was not essential for RAFTK autophosphorylation at residue 402.

RAFTK Autophosphorylation Was Mediated by a Trans-acting Mechanism Not by a Cis-acting Mechanism—Autophosphorylation of a kinase can be accomplished by two different mechanisms. One is intramolecular cis-autophosphorylation and the other is trans-acting autophosphorylation. One is intramolecular cis-autophosphorylation and the
other is intermolecular trans-autophosphorylation. We hypoth-
thesized that if autophosphorylation can be mediated by a trans-
acting mechanism, the interaction between RAFTK kinase ac-
tivity and the tyrosine residue at 402 present in different
RAFTK molecules would mediate the autophosphorylation of
RAFTK. However, if the autophosphorylation is mediated by a
cis-acting mechanism, the two components, RAFTK kinase ac-
tivity and the tyrosine residue at 402, should be present in a
single RAFTK molecule. To investigate this hypothesis, we
utilized co-expression of two RAFTK constructs differentially
fused with either FLAG epitope (an eight-amino acid peptide,
DYKDDDDK) or GFP. As shown in Fig. 2A, a GFP-tagged
kinase mutant RAFTK (KM-GFP) was phosphorylated at
Tyr402 by co-expressed FLAG-tagged wild type RAFTK (WT-
FLAG), suggesting the existence of trans-acting autophospho-
ylation of RAFTK. Confirming the trans-autophosphorylation
of RAFTK, co-expression of Y402F-FLAG with KM-GFP
showed that intact kinase domain-containing Y402F-FLAG
phosphorylated KM-GFP (Fig. 2A), although neither of the
constructs could be autophosphorylated alone (Fig. 1B). Based
on the tyrosine phosphorylation level of KM-GFP, the trans-
autophosphorylation activity of WT-FLAG was much greater
than that of Y402F-FLAG (Fig. 2A).

Next, we examined whether trans-autophosphorylation is
the only autophosphorylation mechanism or whether cis-auto-
phosphorylation also plays a role in RAFTK autophosphory-
ation. We reasoned that if the autophosphorylation of RAFTK
was mediated by a trans-acting mechanism, overexpression of
KM-GFP would inhibit the autophosphorylation of WT-FLAG
through competition. Therefore, the level of WT-FLAG auto-
phosphorylation could be determined by the relative amounts
of the kinase-inactive molecule (KM-GFP) upon their co-ex-
pression. However, if the autophosphorylation occurs in a sin-
gle molecule through a cis-acting mechanism, the autophospho-
ylation of WT-FLAG would be preserved at a certain level of
phosphorylation regardless of KM-GFP co-expression. To this
end, we co-transfected WT-FLAG and KM-GFP in various
doses to examine the potential competition between the two
constructs. As shown in Fig. 2B, KM-GFP inhibited the auto-
phosphorylation of WT-FLAG at Tyr402 in a dose-dependent
manner, and overexpression of KM-GFP almost completely
abolished the autophosphorylation of WT-FLAG, indicating
that RAFTK autophosphorylation is a trans-acting event not a
cis-acting event. As a control, we examined the effect of the del
N-FLAG RAFTK construct on WT-FLAG autophosphorylation
upon their co-expression. As shown in Fig. 2C, del N-FLAG did
not inhibit the autophosphorylation of WT-FLAG at Tyr402,
indicating that inhibition of WT-FLAG autophosphorylation
was a specific process dependent on the kinase-defective KM-
GFP. To confirm that the inhibition of RAFTK wild type auto-
phosphorylation by the RAFTK kinase mutant was through a
trans-acting interaction and not through inhibition of RAFTK
subcellular localization by the focal adhesion targeting domain
at its C terminus, we examined the effects of KM-GFP on the
autophosphorylation of del C-FLAG that lacks a C terminus
region (Fig. 1A). In support of the trans-acting interaction of

FIG. 1. Autophosphorylation of various RAFTK constructs. A, a
schematic illustration of various RAFTK constructs. Various FLAG- or
GFP-tagged wild type (WT-FLAG or WT-GFP), autophosphorylation
site Y402F mutant (Y402F-FLAG), kinase-mutated K457A mutant
(KM-GFP or KM-FLAG), and deletion mutants of RAFTK cDNA were
cloned into pCDNA3 (Invitrogen) or pEGFP-C3 (Clontech) vectors.
FLAG-tagged cloned pCDNA3 (Vector-FLAG) was used as the vector
control. Transient transfection of various RAFTK constructs into HEK
293 cells was performed using the calcium phosphate precipitation
method. N, N-terminal region; K, kinase domain; C, C-terminal region.
B, autophosphorylation of various RAFTK constructs. 2 μg of various
RAFTK cDNA constructs as labeled in the panel were used for each
100-mm dish of HEK 293 cells. Empty vector cDNA was used as a
transfection control. After 48 h of transfection, the cells were harvested.
The cell lysates were prepared with modified RIPA buffer and immu-
noprecipitated (IP) with specific antibodies against FLAG (goat anti-
FLAG from Santa Cruz Biotechnology) or against GFP (mouse anti-
GFP antibody from MBL). The proteins in the immunoprecipitates were
resolved by SDS-PAGE and immunoblotted (IB) with mouse phospho-
tyrosine-specific antibody (4G10; Upstate Biotechnology Inc.), mouse
anti-Tyr(P)402-RAFTK antibody (Upstate Biotechnology Inc.), mouse
anti-GFP antibody (MBL), or mouse anti-FLAG (M2; Sigma) after mem-
brane stripping.
**Molecular Mechanism of RAFTK/Pyk2 Autophosphorylation**

**Fig. 2.** RAFTK auto-phosphorylation was mediated by trans-autophosphorylation. **A**, HEK 293 cells were co-transfected with 1 μg of GFP-tagged kinase-mutated K457A (KM-RAFTK) RAFTK and 2 μg of FLAG-tagged wild type (WT-FLAG) or Y402F mutant (Y402F-FLAG) RAFTK, followed by immunoprecipitation (IP) and immunoblotting (IB) with specific antibodies as indicated. **B and C**, KM-GFP inhibited the autophosphorylation of WT-FLAG in a dose-dependent manner. HEK 293 cells were co-transfected with 0.2 μg of FLAG-tagged wild type (WT-FLAG) RAFTK plus various amounts of GFP-tagged kinase-mutated K457A (KM-GFP) RAFTK (**B**) or FLAG-tagged N terminus deletion mutant RAFTK (del N-FLAG) (**C**), followed by immunoprecipitation with mouse anti-FLAG (M2; Sigma) or goat anti-RAFTK (N-19; Santa Cruz Biotechnology). After resolving on gel electrophoresis, the proteins were immunoblotted with specific antibodies, as indicated. Immunoblotting of GFP antibody with whole cell lysates (WCL) was performed to show the expression of GFP-tagged WT RAFTK or KM RAFTK cDNA (2 μg) using the calcium phosphate method. Empty vector pEGFP-C3 (V-GFP) cDNA was used as a control. After 24 h of incubation, the cells were unstimulated or stimulated with KCl (60 mM, 5 min). Whole cell lysate preparation, immunoprecipitation, and immunoblotting procedures were the same as described above (for A). The phosphotyrosine ratio (pY/RAFTK or Tyr(P)402/RAFTK) demonstrates the relative amounts of trans-autophosphorylation of each tyrosine- or Tyr402-specific phosphorylation in the samples.

RAFTK, KM-GFP inhibited the autophosphorylation of del C-FLAG at 402 (Fig. 2D), suggesting that the KM-mediated inhibition of autophosphorylation is not dependent on the C terminus, which controls the subcellular localization of RAFTK to focal adhesion sites. Although we as well as other groups have reported that the RAFTK kinase mutant worked as a dominant negative (2, 8, 18–20), its detailed mechanism of action is unknown. The intermolecular trans-phosphorylation of RAFTK provides a molecular basis for the observation that the RAFTK kinase mutant dominant-negatively inhibits wild type RAFTK autophosphorylation. Overexpression of exogenous RAFTK might produce clustering of the protein independent of subcellular localization. It may also explain why overexpression of RAFTK by transient transfection or viral infection leads to a high level of RAFTK phosphorylation. However, under physiological conditions, extracellular stimuli may provide a signal that results in the clustering of RAFTK and trans-autophosphorylation. For example, potassium depolarization of neuroendocrine PC12 cells induced a distinct redistribution of RAFTK at point contacts, which serve as activation centers for the recruitment of various signaling molecules, such as Src, paxillin, and Grb2.

**Fig. 3.** Trans-acting autophosphorylation of endogenous RAFTK upon potassium depolarization in PC12 cells. **A**, PC12 cells were transfected with GFP-tagged WT RAFTK or KM RAFTK cDNA (4 μg) using LipofectAMINE 2000 (Invitrogen). Empty vector pEGFP-C3 (V-GFP) cDNA was used as a control. After 24 h incubation, the cells were unstimulated or stimulated with KCl (60 mM, 5 min). The cells were harvested with RIPA buffer followed by immunoprecipitation (IP) with mouse anti-GFP antibodies (MBL) and immunoblotting (IB) with specific antibodies, as indicated. The phosphorylation of endogenous RAFTK was examined by immunoprecipitation with rabbit anti-RAFTK antibody and immunoblotting with specific antibodies, as indicated. **B**, HEK 293 cells were transfected with GFP-tagged WT RAFTK or KM RAFTK cDNA (2 μg) using the calcium phosphate method. Empty vector pEGFP-C3 (V-GFP) cDNA was used as a control. After 24 h of incubation, the cells were unstimulated or stimulated with KCl (60 mM, 5 min). Whole cell lysate preparation, immunoprecipitation, and immunoblotting procedures were the same as described above (for A). The phosphotyrosine ratio (pY/RAFTK or Tyr(P)402/RAFTK) demonstrates the relative amounts of phosphorylation at each tyrosine- or Tyr402-specific phosphorylation in the samples.

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*S.-Y. Park and S. Avraham, unpublished data.*
epitopes. A, HEK 293 cells were co-transfected with 0.2 μg of FLAG-tagged wild type (WT-FLAG) RAFTK with various amounts of GFP-tagged RAFTK (WT-GFP) RAFTK, followed by immunoprecipitation (IP) with goat anti-RAFTK antibody (Santa Cruz Biotechnology) and immunoblotting (IB) with mouse anti-RAFTK antibody as indicated. The percentage of GFP/FLAG demonstrates the relative amounts of differentially tagged RAFTK in the samples. B, in vitro pull-down assay using fast protein liquid chromatography affinity chromatography with anti-FLAG antibody-conjugated agarose beads. 2 μg each of RAFTK WT-FLAG and WT-GFP cDNA constructs were used for co-transfection on each 100-mm dish of HEK 293 cells. After 48 h of transfection, the cells were harvested with modified RIPA buffer. 4 mg of whole cell lysates were loaded on the column with anti-FLAG antibody-conjugated agarose beads. Eluates of each fraction were resolved by gel electrophoresis and probed with mouse anti-RAFTK antibody to show the association of two RAFTK molecules can be detected during trans-autophosphorylation. To confirm the association between differentially tagged RAFTKs, we analyzed this association of two RAFTK molecules can be detected during trans-autophosphorylation using an in vitro pull-down assay using affinity chromatography with anti-FLAG antibody-conjugated agarose. As shown in Fig. 4B, WT-GFP was specifically associated and eluted with WT-FLAG. This supports the co-immunoprecipitation result (Fig. 4A). The relative amount of co-immunoprecipitated WT-GFP with WT-FLAG was very small, suggesting that the self-association of RAFTK was weak and transient.

Analysis of RAFTK Regions Mediating Self-association—To further investigate the self-association region(s) in RAFTK, we analyzed the association of WT-GFP with various deletion mutants using immunoprecipitation. Both the N- and C-terminal deletion mutants associated with wild type RAFTK (WT-GFP), albeit at a significantly decreased level as compared with the WT-FLAG and KM-FLAG, indicating that the N- or C-terminal region was partially responsible for the association with WT-GFP (Fig. 5). Even deletion of the N terminus and Kinase domain (del NK) or of the kinase domain alone (del NC) showed some association. This suggests the possibility of multiple association regions. Because many receptor-type tyrosine kinases demonstrate a formation of stable dimers and trans-acting autophosphorylation (21), we attempted to detect the formation of stable dimers using nondenatured native gel electrophoresis, a sucrose density gradient, and chemical coupling with various cross-linkers. However, we did not observe the formation of a stable complex using any of these approaches (data not shown). Although RAFTK induces trans-acting autophosphorylation as shown in receptor-type tyrosine kinases (21), our data indicate that the trans-interaction of RAFTK does not form stable dimers. Thus, this suggests that RAFTK self-association is a transient event with more dynamic spatial and temporal responses.
The Trans-autophosphorylation of RAFTK Is a Src Kinase-independent Process—Autophosphorylated RAFTK induces the activation of Src kinase through the association of RAFTK phosphotyrosine at 402 and the Src-SH2 domain (8). Activated Src, in turn, mediates the phosphorylation of RAFTK at several tyrosine residues in the kinase domain and the C terminus region of RAFTK, which leads to increased RAFTK kinase activity and the recruitment of signaling molecules, such as Grb2 (6, 8). To analyze whether Src also plays a role in RAFTK trans-autophosphorylation through either increased RAFTK kinase activity or direct phosphorylation of Tyr\(^{402}\), we utilized two cell lines: a murine embryonic fibroblast cell line lacking Src family kinases (SYF) and a c-Src-reintroduced cell line (SYF + Src). First, we examined the autophosphorylation of various RAFTK constructs upon single transfection. As shown in Fig. 6A (first panel with 4G10 antibody), in SYF cells only the WT-FLAG and del C-FLAG showed the tyrosine phosphorylation, whereas in SYF + Src cells the KM-GFP, Y402F-FLAG, and del N-FLAG as well as WT-FLAG and del C-FLAG showed significant tyrosine phosphorylation. However, Src did not affect the autophosphorylation level of RAFTK at Tyr\(^{402}\) (Fig. 6A, second panel with anti-Tyr(P)\(^{402}\)-RAFTK-specific antibody), although Src significantly increased the tyrosine phosphorylation of RAFTK. These data suggest that Src does not affect RAFTK autophosphorylation either directly or indirectly. To examine the expression of Src in these two cell lines, whole cell lysates were analyzed by immunoblotting with Src antibody. Lack of Src expression was observed in the SYF cells, and positive expression of Src was found in the SYF + Src cells (Fig. 6A, fifth panel). Interestingly, immunoblotting with anti-phosphospecific Tyr(P)\(^{418}\)-Src antibody against the active form of Src kinase demonstrated that Src is constitutively activated in the SYF + Src cells (Fig. 6A, sixth panel). Because c-Src-reintroduced SYF + Src cells were immortalized by simian virus 40 large T antigen from murine embryonic fibroblasts, SV40 large T antigen might activate Src and lead to the phosphorylation of RAFTK in the absence of interactions with Tyr\(^{402}\) of RAFTK. This possibility is consistent with another report investigating the interaction of v-Src with FAK, which showed that the FAK Tyr\(^{972}\)-autophosphorylation site was not required for v-Src to stably interact with and activate FAK (22). In comparison, because HEK 293 cells do not have constitutively activated Src, only autophosphorylated RAFTK can activate Src, which is followed by Src-mediated phosphorylation of the Tyr\(^{577/580}\) residues (Fig. 1B). It is important to note that in the SYF + Src cells, overexpression of Src induced the tyrosine phosphorylation of KM-GFP, Y402F-FLAG, and del N-FLAG other than at site Tyr\(^{402}\) (compare first and second panels).

Fig. 6. Role of Src in RAFTK autophosphorylation. Various FLAG- or GFP-tagged RAFTK cDNA constructs were transfected into SYF or SYF + Src cells with LipofectAMINE 2000 (Invitrogen). Empty vector pEGFP-C3 (A and C, Vector-GFP) or FLAG-tagged cloned pcDNA3 (B, Vector-FLAG) cDNAs were used as transfection controls. After 48 h, the cells were harvested with RIPA buffer, followed by immunoprecipitation (IP) and immunoblotting (IB) with specific antibodies, as indicated. A, autophosphorylation of various RAFTK constructs in SYF or SYF + Src cells. The cell lysates (500 μg) were immunoprecipitated with either goat anti-FLAG antibody (for del C-FLAG) or rabbit anti-RAFTK antibody (Transduction Laboratory) to check Y402F-FLAG protein levels. C, co-transfection of Y402F-FLAG with various FLAG- or GFP-tagged RAFTK cDNA constructs. Y402F-FLAG proteins were immunoprecipitated with anti-FLAG antibodies, followed by immunoblotting with 4G10 antibody (Upstate Biotechnology Inc.) to check Tyr\(^{402}\) phosphorylation, and mouse anti-RAFTK antibody (Transduction Laboratory) to check Y402F-FLAG protein levels. The percentage of phosphotyrosine ratio (Tyr(P)\(^{402}\)/pY, %) demonstrates the relative amounts of Tyr\(^{402}\)-specific phosphorylation to pan tyrosine phosphorylation in the samples.
of RAFTK-KM at Tyr\(^{402}\), Tyr\(^{578/580}\), and Tyr\(^{881}\) in primary osteoclast-like cells, suggesting the possible involvement of another tyrosine kinase(s), given our data showing that activated Src phosphorylated RAFTK independent of the Tyr\(^{402}\) site (Fig. 6B) and that endogenous RAFTK can phosphorylate RAFTK-KM in a trans-acting mode, the suggestion that another kinase(s) may phosphorylate RAFTK Tyr\(^{402}\) is not convincing.

**Role of Src in RAFTK-mediated Paxillin Phosphorylation**—Because RAFTK can exhibit several different levels of phosphorylation status, it is of interest to examine which state is critical for the phosphorylation of substrates, such as Paxillin. Paxillin is a focal adhesion molecule and is well known as an *in vivo* substrate of RAFTK. We investigated whether autophosphorylation of RAFTK would be sufficient to phosphorylate Paxillin or whether Src-mediated RAFTK phosphorylation would be required. We examined RAFTK-mediated paxillin phosphorylation in SYF as compared with SYF + Src cell lines using co-expression of various RAFTK constructs with paxillin. As shown in Fig. 7, RAFTK-mediated paxillin phosphorylation was significantly enhanced in the SYF + Src cells. These data suggest that autophosphorylation of RAFTK is not required for paxillin phosphorylation and that Src plays an important role in RAFTK-mediated paxillin phosphorylation. KM-GFP showed some paxillin phosphorylation in the SYF + Src cells, which might be mediated by Src activity (Fig. 7). Interestingly, in both cell lines, paxillin was significantly phosphorylated by del C-FLAG that lacks the RAFTK C-terminal region including the paxillin-binding focal adhesion targeting domain (Fig. 7), suggesting an alternative pathway for RAFTK to phosphorylate Paxillin. Although RAFTK autophosphorylation is an initial step in RAFTK activation leading to the recruitment and activation of Src, activated Src, in turn, plays an important role in RAFTK-mediated paxillin phosphorylation as shown in Fig. 7. This result suggests an interdependent loop-like activation mechanism involving RAFTK and Src. This concerted activation mechanism of RAFTK and Src may provide a crucial step for tight regulation upon physiological stimuli.

Based on our study, the activation of RAFTK occurs in several steps: 1) upon its stimulation, RAFTK trans-phosphorylates the autophosphorylation site 402, which induces RAFTK activation; 2) the RAFTK phosphorylated 402 then recruits and activates Src kinase, which in turn phosphorylates the kinase domain of RAFTK and enhances its kinase activity; and 3) the enhanced RAFTK activity induces the activation of downstream signaling pathways.

Our results may provide important clues for the pharmacological application of RAFTK. Because RAFTK activity and downstream signaling heavily depend on RAFTK autophosphorylation of 402 through Src recruitment, blocking the Tyr\(^{402}\) autophosphorylation of RAFTK can be a good therapeutic target to inhibit RAFTK function.

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