THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 288, NO. 41, pp. 29703–29712, October 11, 2013

Vascular Smooth Muscle Cell Motility Is Mediated by a Physical and Functional Interaction of Ca^{2+}/Calmodulin-dependent Protein Kinase IIδ_2 and Fyn*

Received for publication, April 15, 2013, and in revised form, August 27, 2013 Published, JBC Papers in Press, September 3, 2013, DOI 10.1074/jbc.M113.477257

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Background: Increased vascular smooth muscle cell motility results in neointimal formation.
Results: CaMKIIδ_2 and Fyn physically interact, and CaMKIIδ_2 activity regulates complex formation, Fyn activity, and motility.
Conclusion: CaMKIIδ_2 and Fyn regulate the motility of VSM cells due to their physical and functional interaction.
Significance: Coupling CaMKIIδ_2 and Fyn in VSM cells provides a defined mechanism for increases in intracellular calcium to activate tyrosine kinases required for cell motility.

In vascular smooth muscle (VSM) cells, Ca^{2+}/calmodulin-dependent protein kinase IIδ_2 (CaMKIIδ_2) activates non-receptor tyrosine kinases and EGF receptor, with a Src family kinase as a required intermediate. siRNA-mediated suppression of Fyn, a Src family kinase, inhibited VSM cell motility. Simultaneous suppression of both Fyn and CaMKIIδ_2 was non-additive, suggesting coordinated regulation of cell motility. Confocal immunofluorescence microscopy indicated that CaMKIIδ_2 and Fyn selectively (compared with Src) co-localized with the Golgi in quiescent cultured VSM cells. Stimulation with PDGF resulted in a rapid (<5 min) partial redistribution and co-localization of both kinases in peripheral membrane regions. Furthermore, CaMKIIδ_2 and Fyn selectively (compared with Src) co-immunoprecipitated, suggesting a physical interaction in a signaling complex. Stimulation of VSM cells with ionomycin, a calcium ionophore, resulted in activation of CaMKIIδ_2 and Fyn and disruption of the complex. Pretreatment with KN-93, a pharmacological inhibitor of CaMKII, prevented activation-dependent disruption of CaMKIIδ_2 and Fyn, implicating CaMKIIδ_2 as an upstream mediator of Fyn. Overexpression of constitutively active CaMKII resulted in the dephosphorylation of Fyn at Tyr-527, which is required for Fyn activation. Taken together, these data demonstrate a dynamic interaction between CaMKIIδ_2 and Fyn in VSM cells and indicate a mechanism by which CaMKIIδ_2 and Fyn may coordinately regulate VSM cell motility.

Vascular diseases and responses to injury, including restenosis after balloon catheter angioplasty, are characterized by the migration of vascular smooth muscle (VSM)[3] cells into the lumen of the blood vessel, where they proliferate, forming a neointimal plaque (1). Angiogenesis during embryonic development and during vascularization of tumors also requires the directed migration of VSM cells (2, 3). The mechanisms that mediate VSM cell migration require the coordinated regulation of multiple cellular proteins, including protein kinases, structural proteins, and focal adhesion proteins (4–6).

We and others have identified Ca^{2+}/calmodulin-dependent protein kinase IIδ_2 (CaMKIIδ_2) as an important contributor to neointimal formation after balloon catheter angioplasty in rats (1) and carotid ligation in mice (7). Early studies established a function for CaMKII in regulating VSM cell migration *in vitro* in response to PDGF and FGF (8). Studies from our laboratory have focused on potential mechanisms and identified a role for CaMKIIδ_2 in mediating VSM cell adhesion and spreading, important early components of cell migration, through regulation of focal adhesion proteins and the ERK1/2 signaling pathway (9). We have also reported that CaMKIIδ_2-dependent regulation of VSM cell migration involves activation of Rac1, a Rho family protein (4). Recently, CaMKIIδ-dependent regulation of VSM cell migration through post-transcriptional stabilization of MMP9 mRNA levels was reported (10). This study, which used genetic models to delete the CaMKIIδ gene, not only confirmed earlier studies but also highlighted the multiplicity of direct and indirect mechanisms that CaMKIIδ_2 may affect to modulate VSM cell migration. Roles for CaMKII in focal adhesion turnover (11) and focal adhesion maturation (12, 13) have also been reported in fibroblasts.

Src family kinases (SFKs) are multifunctional tyrosine kinases whose activity has also been linked to cell motility

* This work was supported, in whole or in part, by National Institutes of Health Predoctoral Training Grant T32 HL07194 and Grants R01 HL49426 and R01 HL092510 (to H. A. S.) from NHLBI. This work was also supported by American Heart Association Northeast Affiliate Predoctoral Training Grant 0215300T (to P. J. P.).

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[3] The abbreviations used are: VSM, vascular smooth muscle; CaMKIIδ_2, Ca^{2+}/calmodulin-dependent protein kinase IIδ_2; SFK, Src family kinase; ANOVA, analysis of variance.

through diverse mechanisms. SYF cells (mouse embryonic fibroblasts deficient in Src, Yes, and Fyn) show a reduced ability to migrate in response to the extracellular matrix protein fibronectin compared with wild-type mouse embryonic fibroblasts, implicating SFKs in focal adhesion maturation and turnover (14). Other studies have reported that phosphorylation of focal adhesion kinase by Src and Fyn is critical for its activation and ability to mediate focal adhesion maturation (15, 16). Src has also been reported to positively mediate endothelial cell migration through regulation of p38 MAPK (17). In VSM cells, Src has an important role in PDGF-dependent chemotaxis through regulation of focal adhesion kinase activity (18) and EGF receptor transactivation (19, 20).

Our previous studies indicated a role for CaMKIIδ$_2$ and downstream SFKs in mediating EGF receptor transactivation in VSM cells (21, 22). Given this, we hypothesized that CaMKIIδ$_2$-dependent regulation of VSM cell migration might be mediated, at least in part, via activation of a SFK. In this study, we demonstrate that the SFK Fyn positively regulates VSM cell migration. We also show by co-localization and immunoprecipitation that CaMKIIδ$_2$ interacts selectively with Fyn compared with other SFKs and that CaMKIIδ$_2$ regulates tyrosine phosphorylation events required for Fyn activity. These results provide a potential mechanism by which CaMKIIδ$_2$ and Fyn coordinately regulate VSM cell motility.

EXPERIMENTAL PROCEDURES

Antibodies and Materials—The production and specificity of the anti-peptide polyclonal antibody used for detection of the δ$_2$-specific isoform of CaMKII were described previously (23). Monoclonal antibodies used for Fyn and Src immunoprecipitation and the GST peptide control were from Millipore. Polyclonal antibodies for immunoblotting of Src and Fyn and GST fusion proteins (GST-Fyn SH3 and GST-Lck SH3) were from Santa Cruz Biotechnology. Protein A beads were purchased from Thermo Fisher Scientific, and glutathione beads for GST recovery was purchased from GE Healthcare. Purified recombinant CaMKIIδ$_2$ was a generous gift from Dr. Roger Colbran (Vanderbilt University School of Medicine, Nashville, TN). All cell culture media and supplies were from Fisher unless specified otherwise. Ionomycin was from Calbiochem, and KN-93 from Seikagaku America Inc. (Falmouth, MA). SMARTpool siRNAs for Fyn and CaMKIIδ$_2$ were purchased from Thermo Fisher Scientific.

Cell Culture—VSM cells were enzymatically dispersed from thoracic aortas of 200–300-g male Sprague-Dawley rats as described previously (24). Cells were cultured in DMEM/F-12 medium supplemented with 10% FBS at 37 °C in 5% CO_2. Confluent cultures from passages 3–10 were used for each experiment. Prior to experimentation, cells were growth-arrested by replacing the growth medium with DMEM/F-12 medium supplemented with 0.4% FBS for 16 h at 37 °C in 5% CO_2.

Cell Lysates, Immunoprecipitation, GST Pulldown Experiments, and Immunoblotting—30 min prior to experimentation, the growth arrest medium was removed and replaced with Hanks' balanced salt solution supplemented with Ca^{2+}/Mg^{2+} and 10 mM HEPES (pH 7.4). Preincubation for 30 min with 30 μM KN-93 was used to inhibit CaMKII activation, conditions shown previously to provide optimal effects in these cells (25). Cells were maintained at 37 °C in 5% CO_2 during the pretreatment. Reactions were stopped by removing the Hanks' balanced salt solution, transferring the dishes to ice, and adding Nonidet P-40 lysis buffer at 4 °C (50 mM Tris (pH 7.4), 50 mM NaF, 0.1 mM $NaVO_4$, 0.5% Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride, and 0.2 units/ml aprotinin) at 1 ml/100-mm dish. The lysates were collected into 1.5-ml tubes and cleared by centrifugation at 14,000 rpm for 30 min at 4 °C.

For immunoprecipitation and GST pulldown experiments, lysates were precleared with 40 μl of protein A beads or 40 μl of glutathione beads, respectively. Cleared lysates were transferred to a fresh 1.5-ml tube, and either 5 μg of monoclonal antibody or 5 μg of GST proteins with 40 μl of protein A (immunoprecipitation) or 40 μl of glutathione beads (GST pulldown experiments) was added to each sample. Following incubation overnight at 4 °C, the bead complexes were washed three times with lysis buffer, followed by the addition of 30 μl of SDS sample buffer.

Lysates and immunoprecipitates were resolved on 8 or 9% SDS-polyacrylamide gel and transferred to nitrocellulose. The membranes were blocked in Tris-buffered saline containing 0.2% Tween 20 (TBST) and either 5% nonfat dry milk or 3% BSA for blotting of tyrosine phosphorylation. After blocking, the membranes were incubated with primary antibody for 1 h at 22 °C, washed three times with TBST, and incubated with horseradish peroxidase-conjugated secondary antibody (GE Healthcare) for 1 h at 22 °C, followed by three washes with TBST. Membranes were developed using chemiluminescence substrate (GE Healthcare) and exposed to Hyperfilm ECL (GE Healthcare) or visualized using a Fujifilm LAS 4000 system. Analysis of ECL signal intensity in peptide competition experiments was measured using the LAS 4000 system and the accompanying MultiGauge software. All blots shown are representative of at least three experiments.

siRNA—RNA duplexes were transfected into cells using Amaxa Nucleofector II system (Lonza) according to the manufacturer's specifications for primary smooth muscle cells.

Migration Assay and Quantification—2 days after siRNA treatment, an artificial wound was made in the monolayer by scraping a 10-μl pipette tip across the bottom of the dish. The wound was extensively washed, the medium containing 10% FBS was replaced, and cells were allowed to migrate for the appropriate time in a 37 °C incubation chamber with 5% CO_2. Images were taken with a Leica DM IRB microscope at ×10 using bright-field microscopy. The remaining open area of the wound was measured using Adobe Photoshop software. Using this software, the open area for an image can be expressed as arbitrary units or normalized to the area of the scratch wound immediately after the scratch (0 h).

Immunofluorescence—Cells were plated on collagen-coated glass coverslips or cell culture dishes. Cells were fixed using 4% paraformaldehyde for 20 min and permeabilized with 0.2% Triton X-100 in PBS for 5 min. Nonspecific binding was blocked with 5% fish gelatin in PBS plus 0.1% Triton X-100, followed by a 1-h incubation at room temperature with the described anti-CaMKII, anti-Fyn, or anti-Src antibodies diluted 1:100–1:250 in blocking buffer. This was followed by washes and a 1-h incu-

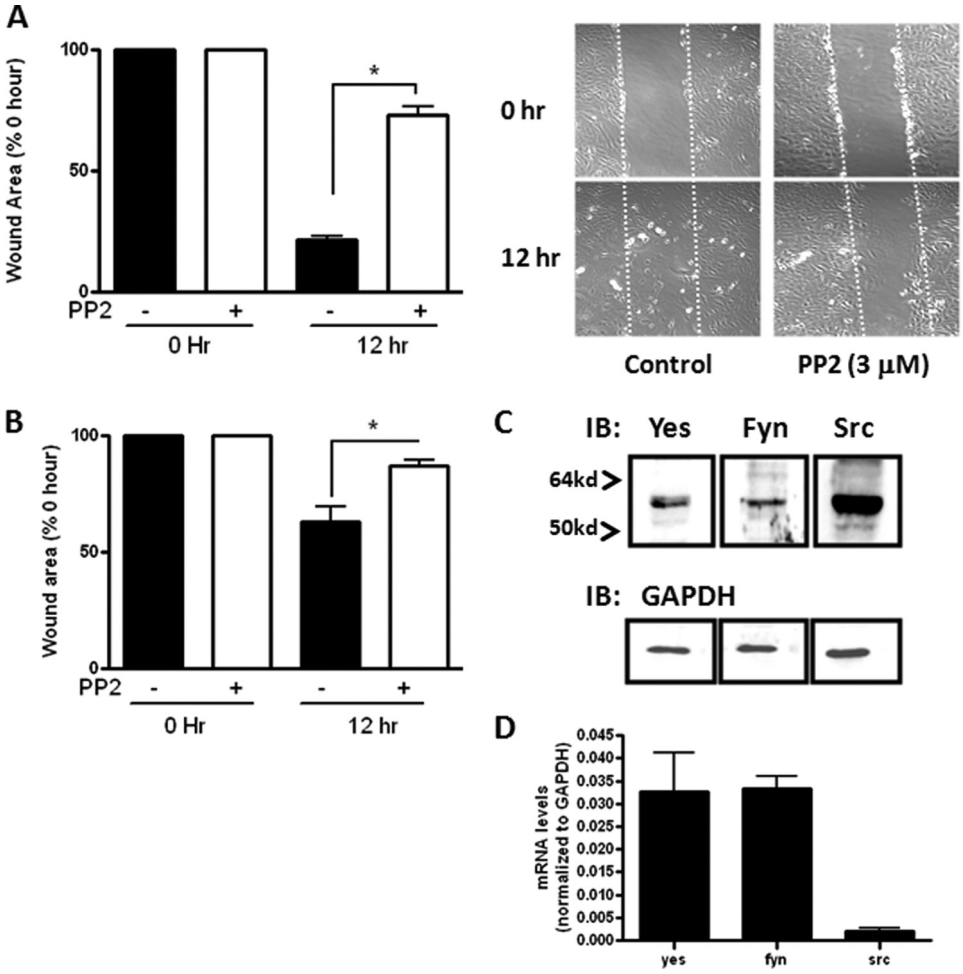

FIGURE 1. **SFKs mediate VSM cell migration.** *A*, VSM cells were grown to confluence and treated with either dimethyl sulfoxide (vehicle control) or 3 μM PP2 for 30 min before a scratch was made as described under "Experimental Procedures." The area of the wound after the initial scratch (0 h) was measured in pixels and identified as 100%. The area was measured after 12 h and expressed as a percentage of the time 0 wound area (*left panel*). A representative micrograph depicting the scratch wound at 0 and 12 h is shown (*right panel*). *B*, VSM cells were growth-arrested by incubation in medium containing 0.4% FBS and treated as described for *A*. *, $p < 0.05$ (n = three separate experiments). Statistical analysis was performed using a one-way ANOVA and a Newman-Keuls post hoc comparison. *C*, VSM cell lysates were resolved by 8% SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted (*IB*) with antibodies specific for Yes, Fyn, and Src. GAPDH was immunoblotted to confirm equivalent levels of protein loading. *D*, mRNA levels of Fyn, pp60^{Src} (*src*), and Yes were determined by quantitative PCR and normalized against GAPDH message levels. The data represents the means ± S.E. (n = three separate quantitative PCRs).

bation at room temperature with the appropriate fluorochrome-conjugated secondary antibodies. F-actin was labeled using rhodamine- or FITC-conjugated phalloidin diluted 1:250. Coverslips were mounted onto slides using VECTASHIELD HardSet mounting medium with DAPI (Vector Laboratories). Cells were imaged on a Zeiss LSM 510 META confocal microscope, on a Leica DM IRB microscope, or by total internal reflection fluorescence microscopy using a Zeiss Axio Observer Z1 microscope with Zeiss Axiovision digital imaging software.

Statistical Analysis—All data are expressed as means ± S.E. Mean values of multiple groups were analyzed by one-way analysis of variance (ANOVA) with post hoc comparisons using the Newman-Keuls test (GraphPad Prism). Comparisons between two groups were analyzed using Student's t test. For all comparisons, $p < 0.05$ was considered statistically significant.

RESULTS

SFKs Mediate VSM Cell Migration—SFKs are multifunctional non-receptor tyrosine kinases that have been implicated in various cell functions, including migration (14). To confirm a general function of SFKs in VSM cell migration, VSM cells were treated with 3 μM PP2, a pharmacological inhibitor of SFKs (26), prior to scratch wounding a monolayer of VSM cells as described under "Experimental Procedures." Treatment with PP2 significantly attenuated migration of VSM cells into the wound area (Fig. 1*A*). SU6656, another SFK inhibitor (27), had similar effects on VSM cell migration under the same experimental conditions (data not shown). Although these experiments were carried out in complete medium, the duration (12 h) was designed to minimize cell proliferation as a major contributing factor to wound closure. As an alternative approach to minimize effects of cell proliferation, these experiments were carried out in low serum medium. Under these conditions, VSM cells still filled the wound area, although at a reduced rate compared with complete medium. Importantly, PP2 treatment prevented VSM cell migration under these conditions as well (Fig. 1*B*).

The SFK Fyn and CaMKIIδ$_2$ Mediate VSM Cell Migration—Immunoblotting lysates from cultured VSM cells indicated the

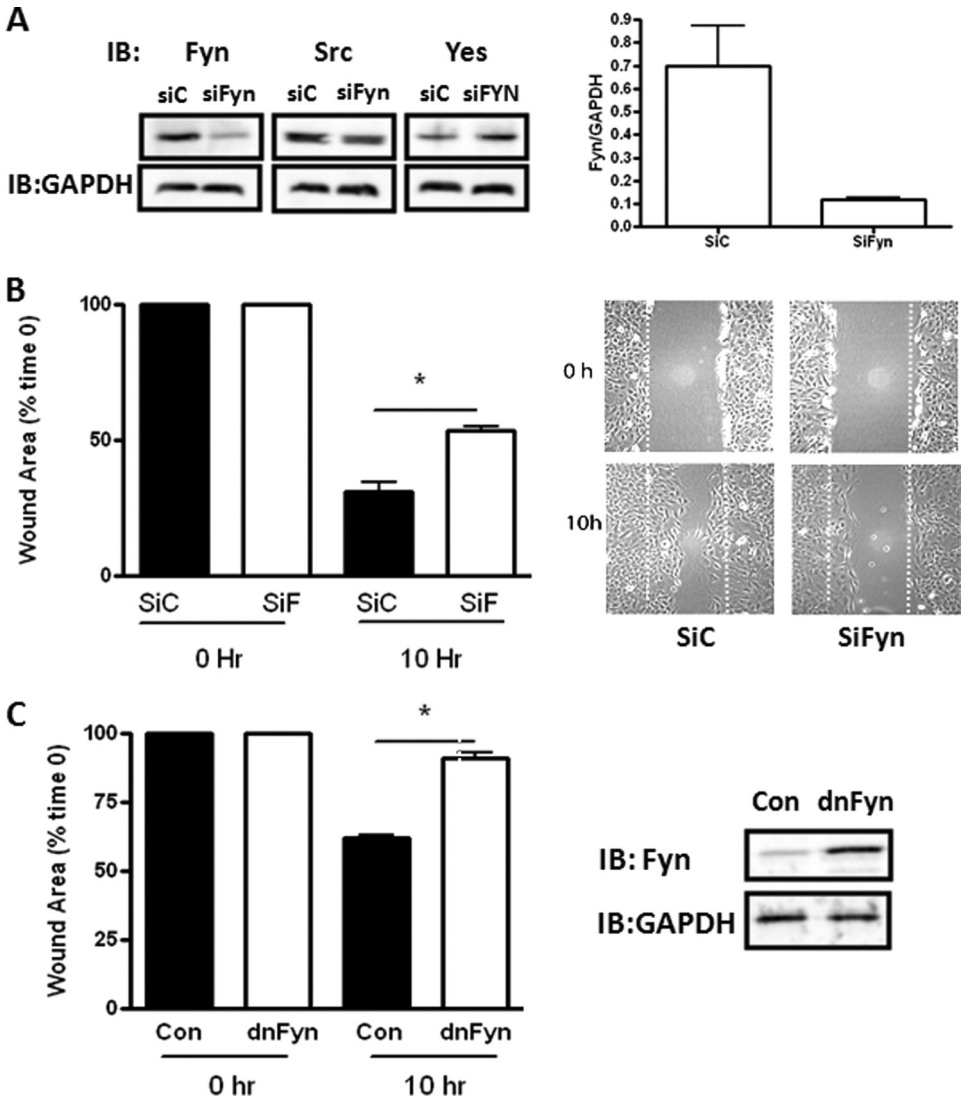

FIGURE 2. **Fyn promotes VSM cell migration.** *A*, VSM cells were transduced with siRNA targeting Fyn (*siFyn*), and loss of Fyn protein expression was confirmed by immunoblotting (*IB*) for Fyn (*left panel*). Immunoblotting for Src and Yes was performed to ensure specificity of the Fyn siRNA. Quantification of the immunoblots is shown (*right panel*). Values are means ± S.E. (n = four separate experiments). *B*, scratch wound in VSM cells transduced with either control (*siC*) or Fyn siRNA. Wound areas were quantified as described in the legend to Fig. 1. The graph (*left panel*) represents the quantification of three separate experiments. A representative micrograph depicting the scratch wound at 0 and 10 h after scratching is shown (*right panel*). *, $p < 0.05$ by one-way ANOVA, followed by a Newman-Keuls post hoc comparison. *C*, VSM cells were transfected with expression plasmid expressing dominant-negative Fyn (*dnFyn*), and overexpression of dominant-negative Fyn was confirmed by immunoblotting for Fyn (*right panel*), followed by scratch wounding and area analysis after 10 h as described in the legend to Fig. 1. *, $p < 0.05$ by one-way ANOVA, followed by a Newman-Keuls post hoc comparison (n = three separate experiments). *Con*, control.

expression of the SFK family members Yes, Fyn, and Src (Fig. 1*C*) (28). Further analysis using quantitative PCR with primers selective for Yes, Fyn, and Src confirmed their expression in VSM cells and indicated that the mRNA levels of Yes and Fyn are significantly higher than those of Src (Fig. 1*D*), although the significance of this observation at the protein level is not clear. On the basis of a recent study showing an important role for Fyn in promoting cell migration (16), the high expression level of Fyn in VSM cells, and the report that Fyn is regulated in a CaMKII-dependent manner (29), we hypothesized that Fyn may be a selective downstream mediator of CaMKII-dependent VSM cell migration.

Selective suppression of Fyn expression using siRNAs introduced by electroporation (Fig. 2*A*) resulted in a significant attenuation of VSM cell migration in response to scratch wounding (Fig. 2*B*). Similarly, overexpression of a dominant-negative Fyn mutant (K299M) (30) inhibited VSM cell migration in response to scratch wounding (Fig. 2*C*). These results are consistent with the pharmacological approaches implicating SFKs in VSM cell migration shown in Fig. 1. It is important to note that these results do not exclude function of other SFKs in VSM cell migration but, for the first time, demonstrate a significant role for Fyn in VSM cell motility.

Having established that CaMKIIδ$_2$ (4) and Fyn (Fig. 2) are important mediators of VSM cell migration, we suppressed expression of both CaMKIIδ$_2$ and Fyn simultaneously (Fig. 3, *A* and *B*) and compared their combined effect on VSM cell migration with silencing CaMKIIδ$_2$ or Fyn expression alone (Fig. 3*C*). Simultaneous suppression of both CaMKII and Fyn had no additive effect to silencing either CaMKII or Fyn alone. The

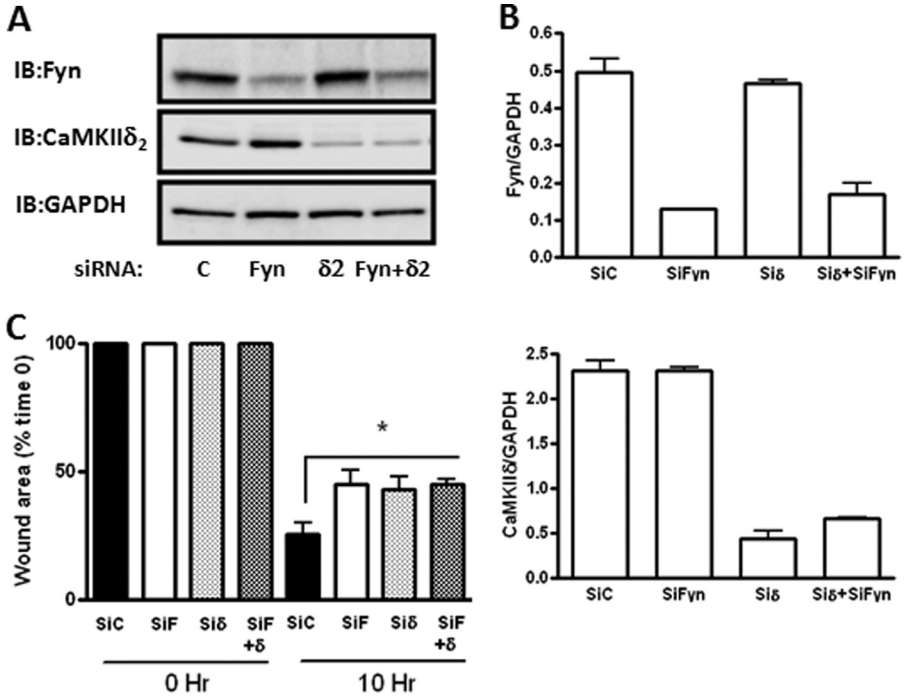

FIGURE 3. **CaMKIIδ$_2$ and Fyn coordinately regulate VSM cell migration.** *A*, VSM cells were transfected with siRNA targeting Fyn, CaMKIIδ$_2$, or both, and loss of Fyn and CaMKIIδ$_2$ protein expression was confirmed by immunoblotting (*IB*) for Fyn and CaMKIIδ$_2$, respectively. *C*, control. *B*, quantification of four separate experiments as depicted in *A*. *SiC*, control siRNA; *SiFyn*, Fyn siRNA; *Siδ*, CaMKIIδ$_2$ siRNA. *C*, scratch wounding was performed as described in the legend to Fig. 1 and analyzed at 10 h. The graph represents the quantification of three separate experiments. *, $p < 0.05$ by one-way ANOVA, followed by a Newman-Keuls post hoc comparison.

non-additivity suggests that CaMKIIδ$_2$ and Fyn are in the same pathway and coordinately regulate VSM cell migration.

CaMKIIδ$_2$ and Fyn Co-localize in Cultured VSM Cells—The intracellular distribution of CaMKIIδ$_2$ was analyzed and compared with that of Fyn using double-label indirect immunofluorescence and confocal microscopy. In control experiments, the efficacies of the anti-CaMKIIδ$_2$ and anti-Fyn antibodies for immunofluorescence localization experiments were validated by comparing signals in cells transduced with control siRNAs or siRNAs targeting the protein kinases (Fig. 4*A*). In quiescent cells, both protein kinases displayed a strong perinuclear localization. (Fig. 4*B*, *panels a–c*). Interestingly, the intracellular localization of Src and CaMKIIδ$_2$ did not overlap in this region (Fig. 4*B*, *panels d–f*), indicating a selective association between CaMKIIδ$_2$ and Fyn.

To determine whether localization of CaMKIIδ$_2$ and/or Fyn is dependent on activation conditions, VSM cells were stimulated with PDGF (100 ng/ml), a stimulus known to activate CaMKII and to stimulate migration in VSM cells (4). Within minutes after the addition of PDGF, Fyn and, to a lesser extent, CaMKIIδ$_2$ localized at cell margins. In cell border regions where CaMKIIδ$_2$ was localized, there was extensive co-localization with Fyn (Fig. 5). These results under conditions of PDGF stimulation further establish the proximity between CaMKIIδ$_2$ and Fyn in VSM cells and support the hypothesis that CaMKIIδ$_2$ and Fyn could be positioned to coordinately regulate leading edge dynamics required for VSM cell migration.

CaMKIIδ$_2$ and Fyn Form Activation State-dependent Complexes—Because the formation of protein complexes is a common mechanism for regulating intracellular signaling pathways and subsequent cell functions, we tested the hypothesis that CaMKIIδ$_2$ and Fyn interact in complexes isolated by immunoprecipitation. Using antibody specific for Fyn or Src (Fig. 6*A*), a fraction of CaMKIIδ$_2$ and Fyn was found to co-immunoprecipitate from VSM cell lysates. The lack of CaMKIIδ$_2$ in Src immunoprecipitations indicates that CaMKIIδ$_2$ and Fyn interact *in situ* with some selectivity and confirms the co-localization studies shown in Fig. 4*B*.

CaMKIIδ$_2$/Fyn interactions could be direct and/or indirect through interaction with other proteins in a larger complex. CaMKIIδ$_2$ contains a 21-amino acid C terminus that is a product of alternative splicing and that is specific to a subset of δ-gene products of CaMKII (23, 31). This region contains a proline-rich sequence from amino acids 539 to 549 (^{534}HRSGS**P**TV**P**IK**PP**CI**P**NGK^{552}) that conforms to multiple consensus SH3-binding domains (P*XX*P) (32, 33). To test the hypothesis that the proline-rich C terminus of CaMKIIδ$_2$ contains the Fyn SH3-binding domain, a competing peptide spanning the three tandem P*XX*P consensus SH3 ligand motifs (CaMKIIδ$_2$(534–552)) (32, 33) was generated with a scrambled peptide containing the same 18 amino acids used as a control. As depicted in Fig. 6*B*, increasing concentrations of the C-terminal peptide inhibited GST-Fyn SH3 interaction with CaMKIIδ$_2$, whereas the scrambled peptide at the same concentrations had no effect on the interaction. These results indicate that the C-terminal proline-rich region of CaMKIIδ$_2$ has the capacity to act as a ligand for the SH3 domain of Fyn. To further test the selectivity of the CaMKIIδ$_2$ interaction with Fyn, we transduced VSM cells with adenoviral constructs containing cDNAs encoding CaMKIIδ$_2$, CaMKIIδ$_6$ (a CaMKIIδ isoform lacking the C-terminal tail) (34), or CaMKIIγ$_C$. After confirming comparable overexpression of the constructs, we carried

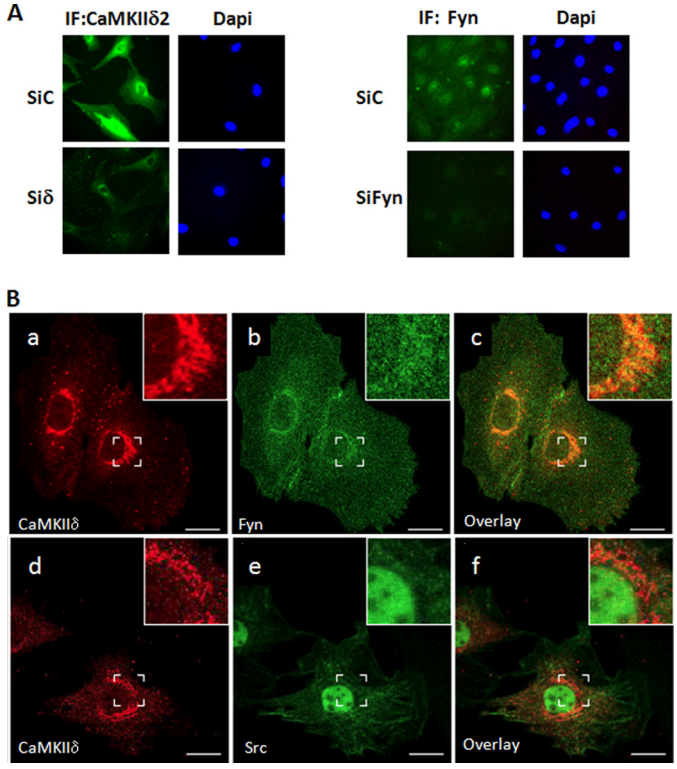

FIGURE 4. **Co-localization of CaMKIIδ$_2$ and Fyn in cultured vascular smooth muscle cells.** *A*, VSM cells were transduced with control siRNA (*SiC*) or siRNA targeting CaMKIIδ (*Siδ*) or Fyn (*SiFyn*) and plated onto glass coverslips. CaMKIIδ$_2$ or Fyn were localized by indirect immunofluorescence microscopy (*IF*). A decreased signal in siRNA-treated cells validated the specificity of the anti-CaMKIIδ$_2$ or anti-Fyn antibody used in subsequent experiments. The cells were labeled with DAPI to visualize the nucleus. *B*, VSM cells were processed for immunofluorescence using antibodies to CaMKIIδ$_2$ and Fyn (*panels a–c*) or CaMKIIδ$_2$ and Src (*panels d–f*), followed by Alexa Fluor 488- and Alexa Fluor 594-conjugated fluorescent secondary antibodies. Confocal microscopy indicated CaMKIIδ$_2$ and Fyn perinuclear co-localization (*insets*). CaMKIIδ$_2$ and Src failed to co-localize. Each of these micrographs is representative of three separate experiments. *Scale bars* = 20 μm.

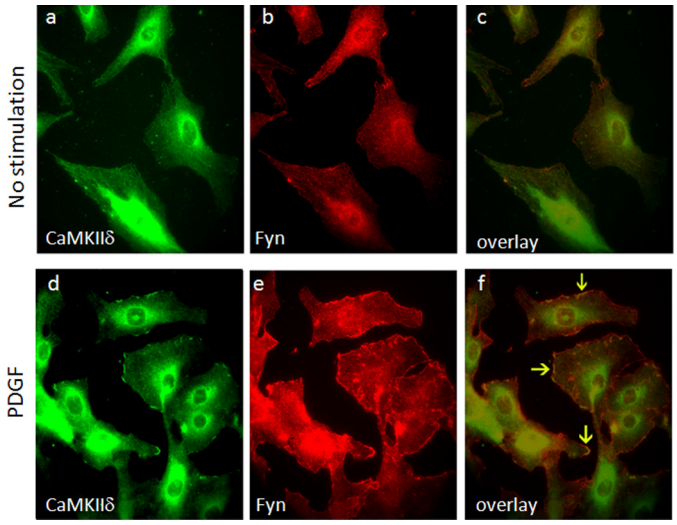

FIGURE 5. **CaMKIIδ$_2$ and Fyn co-localize at the leading edge of VSM cells.** VSM cells cultured under growth conditions were stimulated with 100 ng/ml PDGF for 4 min and processed for immunofluorescence using anti-CaMKIIδ$_2$ (*a* and *d*) or anti-Fyn (*b* and *e*) antibody with Alexa Fluor 488- and Alexa Fluor 594-conjugated secondary antibodies, respectively. Assessment by total internal reflection fluorescence microscopy indicated that CaMKIIδ$_2$ and Fyn did not co-localize under quiescent conditions (*c*) but did co-localize at the leading edge after PDGF stimulation (*f*). The micrographs are representative of four slides from two separate experiments.

out Fyn immunoprecipitations. Under these conditions, CaMKIIδ$_2$ co-immunoprecipitated with Fyn to a greater extent than did either CaMKIIδ$_6$ or CaMKIIγ$_C$ (Fig. 6*C*).

Stimulation of VSM cells with ionomycin, a calcium ionophore known to result in transient increases in intracellular [Ca^{2+}] and robust CaMKIIδ$_2$ activation in VSM cells (25), resulted in a reduced amount of CaMKIIδ$_2$ and Fyn co-immunoprecipitation compared with unstimulated controls (Fig. 7, *A* and *B*). The results of these experiments suggest that activation of CaMKIIδ$_2$ is a determinant in its ability to interact with Fyn in a complex.

Consistent with the findings shown in Fig. 6 evaluating endogenous interactions, stimulation of VSM cells with ionomycin prior to lysis decreased the ability of endogenous CaMKIIδ$_2$ to interact with exogenous GST-Fyn SH3. Pretreating the VSM cells with the selective CaMKII inhibitor KN-93 prevented the loss of GST-Fyn SH3 interaction with CaMKIIδ$_2$ following ionomycin stimulation (Fig. 8*A*). In a similar manner, KN-93 treatment prevented the ionomycin-dependent disruption of endogenous Fyn and CaMKIIδ2 in co-immunoprecipitation studies (data not shown).

CaMKIIδ$_2$ Functionally Associates with Fyn in VSM Cells—Activation of Fyn is a multistep process requiring dephosphorylation of Tyr-527 (35) with subsequent autophosphorylation at Tyr-416 and activation of the kinase (36). Fyn immunoprecipitates from quiescent VSM cells or cells stimulated with ionomycin were immunoblotted with antibodies that specifically detect Fyn phosphorylation at Tyr-527 (the inhibitory phosphorylation site) and Tyr-416 (autophosphorylation). Under quiescent conditions, Fyn was predominantly phosphorylated at Tyr-527 (Fig. 8*B*). Following ionomycin stimulation Tyr-527 phosphorylation decreased, and phosphorylation at Tyr-416 increased (Fig. 8*B*). Overexpression of a HA-tagged constitutively active CaMKIIδ$_2$ mutant (T287D) in VSM cells resulted in the loss of Fyn phosphorylation at Tyr-527 (Fig. 8*C*). These results indicate that Fyn activity in the CaMKIIδ$_2$-Fyn complex can be regulated in a Ca^{2+}-dependent manner secondary to activation of CaMKIIδ$_2$.

DISCUSSION

In this study, we have identified for the first time physical and functional relationships between the multifunctional serine/threonine protein kinase CaMKIIδ$_2$ and the multifunctional SFK Fyn in VSM. Our results demonstrate extensive spatial co-localization between Fyn and CaMKIIδ$_2$ (the primary CaMKII isoform in VSM cells), physical interaction between the kinases in a protein complex, and functional interactions that provide a mechanism whereby CaMKIIδ$_2$ mediates Ca^{2+} signal-dependent activation of Fyn. These interactions are likely to contribute to CaMKIIδ$_2$- and Fyn-dependent regulation of VSM cell migration.

Expression and function of Fyn in VSM cells has not been reported previously. It was interesting and somewhat surprising to find that Fyn is highly expressed relative to the more widely studied Src kinase, at least at the mRNA level (Fig. 1*C*). The functional significance of this finding is not clear, but stud-

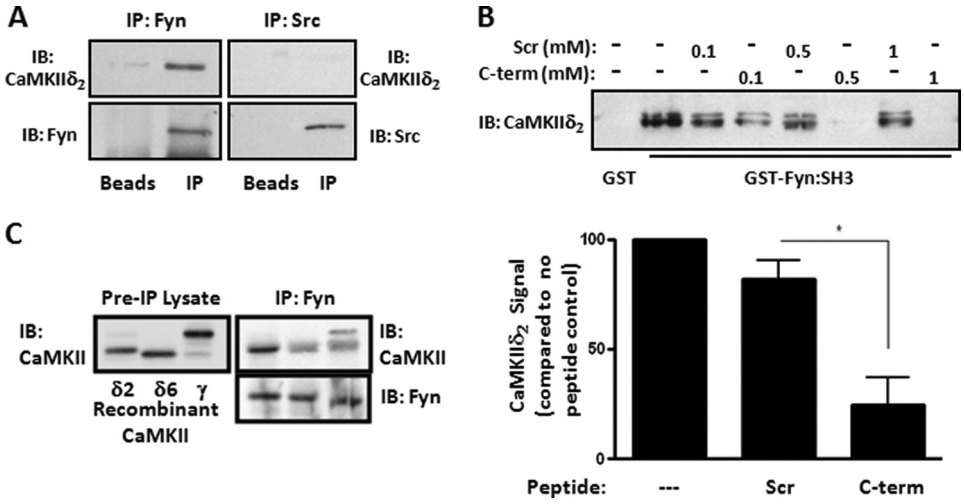

FIGURE 6. **CaMKIIδ$_2$ and Fyn complexes.** *A*, Fyn (*left panels*) and Src (*right panels*) were immunoprecipitated (*IP*) from quiescent VSM cells. Immunoprecipitates were immunoblotted (*IB*) for Fyn ands Src, respectively, and CaMKIIδ$_2$. The immunoblots are representative of three separate experiments. *B*, VSM cell lysates were incubated with GST-Fyn SH3 and assayed for interaction with CaMKIIδ$_2$ by immunoblotting proteins in the pulldown with an antibody specific for CaMKIIδ$_2$ (*upper panel*). The addition of increasing concentrations of a peptide that contains the putative SH3-binding domain (amino acids 534–552) in the unique C terminus of CaMKIIδ$_2$ (C-terminal peptide (*C-term*)) resulted in dissociation of CaMKIIδ$_2$ binding to the SH3 domain of Fyn. As a control, a peptide that contained the same amino acids in a scrambled sequence (*Scr*) showed no competitive displacement at concentrations up to 1 mM. The immunoblot is representative of three separate experiments. The graph (*lower panel*) represents quantification of samples treated with either 1 mM scrambled peptide or 1 mM C-terminal peptide. *, $p < 0.05$ as determined by one-way ANOVA, followed by a Newman-Keuls post hoc comparison. *C*, VSM cells were transduced with adenovirus encoding wild-type CaMKIIδ$_2$, CaMKIIδ$_6$, or CaMKIIγ. Immunoblotting with anti-pan-CaMKII antibody was performed to monitor the levels of CaMKII overexpression (*Pre-IP Lysate*). Fyn was immunoprecipitated from these cells and immunoblotted for CaMKII and Fyn.

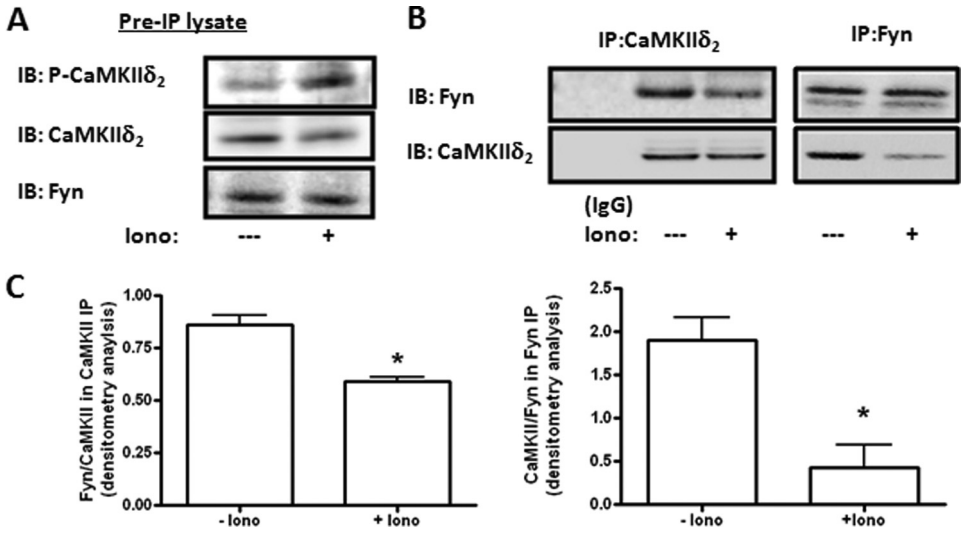

FIGURE 7. **Ca^{2+}- and CaMKII-dependent phosphorylation of Fyn.** CaMKIIδ$_2$ was immunoprecipitated from VSM cells stimulated with 0.5 μM ionomycin (*Iono*) for 2 min. *A*, the pre-immunoprecipitation (*Pre-IP*) lysate was immunoblotted (*IB*) with antibody specific for Thr-287-autophosphorylated (activated) CaMKIIδ$_2$ (*P-CaMKIIδ$_2$*), total CaMKIIδ2, and Fyn. *B*, CaMKIIδ$_2$ (*left panels*) and Fyn (*right panels*) immunoprecipitates were immunoblotted for Fyn and CaMKIIδ$_2$. Micrographs are representative of three separate experiments. *C*, quantification of three separate experiments depicting Fyn levels in CaMKII immunoprecipitates (*left panel*) or CaMKII levels in Fyn immunoprecipitates (*right panel*). *, $p < 0.05$ as determined by Student's unpaired *t* test.

ies in other cell types have shown that the proto-oncogene c-*cbl* induces ubiquitination and proteasomal degradation of Fyn and Lyn as part of a negative feedback mechanism (37, 38). It is possible that Fyn protein expression levels are more tightly regulated compared with Src. In our hands, overexpression of Fyn had detrimental effects on VSM proliferation and viability.[4]

Based on previous studies and the fact that Fyn has dual palmitoylation sites in its SH4 domain that target it directly to lipid rafts (39), Fyn association with the Golgi and peripheral membrane as shown in Fig. 4 is not surprising. On the other hand, there is no intrinsic structural feature in CaMKIIδ$_2$ that would predict its association with these same membrane structures and co-localization with Fyn. The appearance of endogenous CaMKIIδ$_2$ and Fyn in a protein complex provides a potential mechanism for this pattern of CaMKIIδ$_2$ localization. Our previous study (4) showed that loss of CaMKIIδ$_2$ expression prevents both the Golgi orientation and lamellipodial formation needed for VSM cells to migrate in a directed manner (40). Based on the results presented in this study, it is reasonable to speculate that Fyn may also regulate VSM cell migration through an effect on Golgi function or organization. Consistent

[4] R. Ginnan, X. Zou, P. J. Pfleiderer, M. Z. Mercure, M. Barroso, and H. A. Singer, unpublished data.

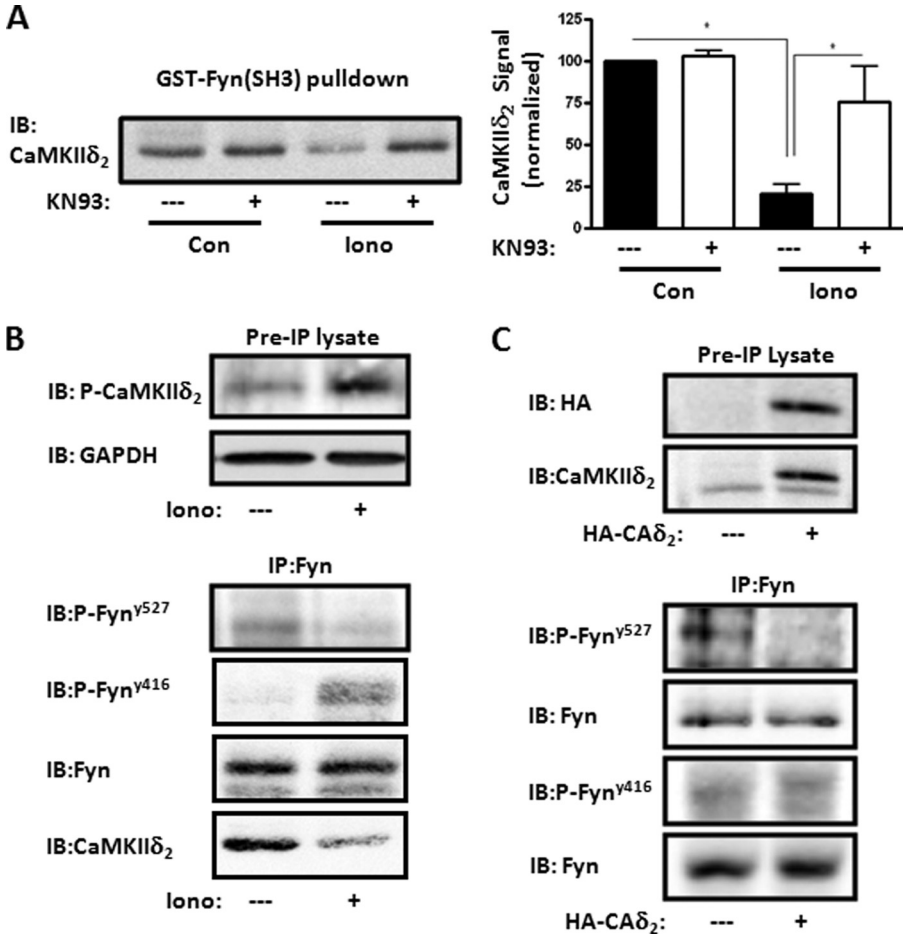

FIGURE 8. *A*, VSM cells were pretreated with 30 μM KN-93 for 30 min and then stimulated with 0. 5 μM ionomycin (*Iono*) for 1 min. The cell lysates were incubated with the GST fusion protein containing the SH3 domain of Fyn, resolved by SDS-PAGE, and immunoblotted (*IB*) for CaMKIIδ_2 (*left panel*). The micrograph is representative of three separate experiments quantified in the graph (*right panel*). *, $p > 0.05$ by one-way ANOVA, followed by Newman-Keuls post hoc analysis. *Con*, control. *B*, VSM cells were stimulated with 0.5 μM ionomycin for 2 min, and pre-immunoprecipitation (*Pre-IP*) lysates were immunoblotted for activated CaMKIIδ_2 (*P-CaMKIIδ$_2$*) (*upper panels*). Fyn was immunoprecipitated (*IP*), and the pulldown was immunoblotted for CaMKIIδ_2, total Fyn, Tyr-527-phosphorylated Fyn (*P-Fyn*y527), and Tyr-416-phosphorylated Fyn (*P-Fyn*y416) (*lower panels*). The immunoblots shown are representative of three separate experiments. *C*, VSM cells were transduced with control adenovirus or adenovirus encoding HA-tagged CaMKIIδ_2 (constitutively active CaMKIIδ_2 (*HA-CAδ$_2$*). Pre-immunoprecipitation lysates were immunoblotted for HA and total CaMKIIδ (*upper panels*). Fyn was immunoprecipitated from the remaining lysate and immunoblotted for Tyr-527-phosphorylated Fyn, Tyr-416-phosphorylated Fyn, and total Fyn (*lower panels*). The immunoblots shown are representative of three separate experiments.

with this speculation is a study in SYF cells that reported a perturbed Golgi apparatus and disruption of the normal protein trafficking due to lack of SFKs (41).

Both the biochemical and confocal microscopy analyses showed that CaMKIIδ_2 and Fyn are closely associated under quiescent cellular conditions and that this association is disrupted upon CaMKIIδ_2 activation. The molecular basis for the redistribution of Fyn and CaMKIIδ_2 to peripheral membrane regions following PDGF stimulation was not examined here. One possibility is the extent of Fyn palmitoylation, with monopalmitoylation favoring Golgi localization and dipalmitoylation favoring direct membrane association (42). Alternatively, the complex may traffic from the Golgi to plasma membrane via the secretory pathway (43).

The functional implications of a dynamic CaMKIIδ_2/Fyn interaction in the Golgi or peripheral membrane compartments are not yet defined, but analysis of Fyn tyrosine phosphorylation indicated that CaMKIIδ_2 may mediate Ca^{2+}-dependent regulation of Fyn activity. Recently, an interaction between CaMKIIα and Fyn was reported to play an important role in mediating neural cell adhesion molecule-stimulated neurite outgrowth (29, 44). Interestingly, these studies implicated CaMKIIα in activation of protein-tyrosine phosphatase α, followed by dephosphorylation of Fyn at Tyr-527, which results in activation of Fyn. Our data showing that overexpression of constitutively active CaMKIIδ_2 results in dephosphorylation of Fyn at Tyr-527 and that CaMKIIδ_2, Fyn, and protein-tyrosine phosphatase α co-immunoprecipitate (data not shown) suggest that CaMKIIδ_2 may mediate Fyn activity in VSM cells through a similar mechanism. This type of protein kinase regulation is a hallmark of caveolae/lipid rafts (45) and may indicate that CaMKIIδ_2 and Fyn interact in the context of a caveola/lipid raft in VSM cells.

The selectivity of the CaMKIIδ_2 interaction with Fyn compared with the closely related CaMKIIδ_6 and CaMKIIγ_C isoforms suggests that the alternatively spliced 21-amino acid C terminus in CaMKIIδ_2 may contribute to the interaction. In Fig. 6, we demonstrated that a GST-Fyn SH3 domain construct can

interact with CaMKIIδ$_2$ *in vitro* and is displaced with a CaMKIIδ$_2$ C-terminal peptide. However, additional structural studies are needed to define the exact nature of this *in vitro* interaction and subsequently whether or not it accounts for the selectivity of interaction between CaMKIIδ$_2$ and Fyn compared with other SFKs *in situ*. Although this *in vitro* experiment demonstrated a direct interaction between the Fyn SH3 domain and CaMKIIδ$_2$, it did not rule out other stabilizing interactions *in vivo*.

Given the apparent selectivity of the interaction involving Fyn compared with other SFKs and CaMKIIδ$_2$ compared with the closely related CaMKIIδ$_6$ and CaMKIIγ$_C$ isoforms, which lack the alternatively spliced C terminus of CaMKIIδ$_2$, this complex could serve to initiate and/or propagate Ca^{2+}-dependent signals in a cell- and/or context-dependent manner. As suggested here by the lack of additivity of siRNA-mediated suppression of CaMKIIδ$_2$ and Fyn on VSM cell migration (Fig. 3), a specific example of this may be the coordinated regulation of VSM cell migration by these protein kinases. Along with our findings implicating Fyn as a mediator of Ca^{2+}-dependent cell functions, several recent studies have reported a role for Fyn as a downstream effector of Ca^{2+} signals and an upstream modulator of Ca^{2+} signals, with diverse functional outcomes ranging from fertilization to wound healing (46, 47).

In summary, endogenous CaMKIIδ$_2$ and Fyn interact in VSM cells in a signaling complex, and their interaction is modulated by Ca^{2+}-dependent stimuli that activate CaMKII. *In vitro* studies indicated that the Fyn SH3 domain is capable of mediating a direct interaction with a proline-rich domain in the CaMKIIδ$_2$ C terminus, although other more complex mechanisms involving adapters such as GIT family proteins (48) cannot yet be ruled out. Co-localization of CaMKIIδ$_2$ and Fyn in the Golgi and peripheral plasma membrane compartments are consistent with their function to coordinately regulate VSM cell motility.

Acknowledgments—We acknowledge the technical assistance of Ginny Foster and Diane Singer in the preparation and maintenance of cell cultures and thank Wendy Vienneau for assistance in submitting this manuscript.

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