Vascular smooth muscle cell motility is mediated by a physical and functional interaction of CaM Kinase II-δ2 and Fyn

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Running Title: CaMKIIδ2 Interaction with Fyn

Background: Increased vascular smooth muscle cell motility results in neointimal formation.

Results: CaMKIIδ2 and Fyn physically interact and CaMKIIδ2 activity regulates complex formation, Fyn activity, and motility.

Conclusion: CaMKIIδ2 and Fyn regulate VSM cell motility due to their physical and functional interaction.

Significance: Coupling CaMKIIδ2 and Fyn in VSM provides a defined mechanism for increases in intracellular calcium to activate tyrosine kinases required for cell motility.

Summary

In vascular smooth muscle (VSM), CaMKIIδ2 activates non-receptor tyrosine kinases and EGFR, with a SFK (Src Family Kinase) as a required intermediate. SiRNA mediated suppression of Fyn, a SFK, inhibited VSM cell motility. Simultaneous suppression of both Fyn and CaMKIIδ2 was non-additive, suggesting coordinate regulation of cell motility. Confocal immunofluorescence microscopy indicated that CaMKIIδ2 and Fyn, selectively (compared to Src) co-localized with Golgi in quiescent cultured VSM. Stimulation with PDGF resulted in a rapid (<5 min) partial redistribution and co-localization of both kinases in peripheral membrane regions. Furthermore, CaMKIIδ2 and Fyn selectively (compared to Src) co-immunoprecipitated, suggesting a physical interaction in a signaling complex. Stimulation of VSM cells with ionomycin, a calcium ionophore, resulted in activation of
CaMKIIδ2 and Fyn and disruption of the complex. Pretreatment with KN93, a pharmacological inhibitor of CaMKII, prevented activation-dependent disruption of CaMKIIδ2 and Fyn implicating CaMKIIδ2 as an upstream mediator of Fyn. Overexpression of constitutively active CaMKII resulted in the dephosphorylation of Fyn at Tyr527 that is required for Fyn activation. Taken together, these data demonstrate a dynamic interaction between CaMKIIδ2 and Fyn in VSM cells and indicate a mechanism by which CaMKIIδ2 and Fyn may coordinately regulate VSM motility.

Vascular diseases and responses to injury, including restenosis after balloon catheter angioplasty, are characterized by the migration of vascular smooth muscle (VSM) 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) (5) (6).

We and others have identified CaMKIIδ2, a Ca2+/Calmodulin dependent protein kinase, as an important contributor to neointima 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 CaMKIIδ2 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 CaMKIIδ2-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 CaMKIIδ2 may affect to modulate VSM cell migration. Roles for CaMKII in focal adhesion turnover (11) and focal adhesion maturation have also been reported in fibroblasts (12) (13).

Src family kinases (SFK) are multifunctional tyrosine kinases whose activity has also been linked to cell motility through diverse mechanisms. SYF cells (mouse embryonic fibroblasts (MEF) deficient in Src, Yes, and Fyn) showed a reduced ability to migrate in response to the extracellular matrix (ECM) protein fibronectin as compared to wild type MEFs implicating SFKs in focal adhesion maturation and turnover (14). Other studies report that phosphorylation of focal adhesion kinase (FAK) by Src and Fyn is critical for FAK 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 FAK activity (18) and EGFR transactivation (19) (20).

Our previous studies indicated a role for CaMKIIδ2 and downstream SFKs in mediating EGFR transactivation in VSM cells (21) (22). Given this, we hypothesized that CaMKIIδ2-dependent regulation of VSM 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 CaMKIIδ2 interacts selectively with Fyn compared to other SFKs and that CaMKIIδ2 regulates tyrosine phosphorylation events required for Fyn activity. These studies provide a potential mechanism by which CaMKIIδ2 and Fyn coordinately regulate VSM cell motility.

Experimental Procedures:

Antibodies and Materials. Production and specificity of the anti-peptide polyclonal antibody used for the detection of the δ2 specific isoform of CaMKII was described previously (23). Monoclonal antibodies used for Fyn and
CaMKIIδ2 Interaction with Fyn

Src immunoprecipitation and the GST peptide control were from Millipore (Temicula, CA). Polyclonal antibodies for immunoblotting of Src and Fyn and GST fusion proteins (GST-Fyn:SH3 and GST-Lck:SH3) were from Santa Cruz (Santa Cruz, CA). Protein A beads were purchased from Thermo Fisher Scientific (Rockland, Ill) and glutathione beads for GST recovery was purchased from GE Lifesciences (Piscataway, NJ). Purified recombinant CaMKIIδ2 were generous gifts from Dr. Roger Colbran (Vanderbilt University School of Medicine, Nashville, TN). All cell culture media and supplies were from Fisher Scientific (Pittsburgh, PA) unless otherwise specified. Ionomycin was from Calbiochem (La Jolla, CA) and KN-93 from Seikagaku (Falmouth, MA). Smart pools for Fyn (SiFyn) and CaMKIIδ2 (SiCaMKIIδ2) were purchased from Thermo Fisher Scientific (Rockland, Ill).

Cell Culture. VSM cells were enzymatically dispersed from thoracic aortas of 200-300g male Sprague-Dawley rats as previously described (24). Cells were cultured in DMEM/F-12 medium supplemented with 10% fetal bovine serum (FBS) at 37°C and 5% CO₂. Confluent cultures from passages 3–10 were used for each experiment. Prior to experimentation, cells were growth arrested by replacing growth media with DMEM/F-12 medium supplemented with 0.4% FBS for 16 hr at 37°C and 5% CO₂. Reactions were stopped by removal of HBSS, transferring the dishes to ice and adding NP-40 lysis buffer (4°C, 50 mM Tris pH 7.4, 50 mM NaF, 0.1mM NaVO₄, 0.5% NP-40, 0.1 mM phenylmethylsulfonyl fluoride and 0.2U/ml aprotinin) at 1ml/100 mm dish. The lysates were collected into 1.5 ml tubes and cleared by centrifugation at 14,000 rpm at 4°C for 30 min.

For immunoprecipitation and GST pull-down experiments, lysates were precleared with 40 µl protein A beads or 40 µl of glutathione beads, respectively. Cleared lysates were transferred to a fresh 1.5 ml tube and 5 µg of either monoclonal antibody or 5 µg of GST proteins with 40 µl of protein A (immunoprecipitation) or 40 µl glutathione beads (GST pull-downs) was added to each sample. Following incubation overnight at 4°C, the bead complexes were washed 3X in lysis buffer followed by the addition of 30 µl SDS sample buffer.

Lysates and immunoprecipitates were resolved on 8% or 9% SDS-PAGE gels 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% bovine serum albumin (BSA) for blotting of tyrosine phosphorylation. After blocking, the membranes were incubated in primary antibody for 1 hr at 22°C, washed 3X with TBST and incubated with horseradish peroxidase-conjugated secondary antibody (GE Lifesciences) for one hour at 22°C followed by 3 washes with TBST. Membranes were developed using chemiluminescence substrate (GE Lifesciences) and exposed to ECL Hyper film (GE Lifesciences) or visualized using a Fuji LAS4000 (Fujifilm). Analysis of ECL signal intensity in peptide competition experiments was measured using a Fuji LAS4000 and the accompanying Multi Gauge software. All blots shown are representative of at least 3 experiments.

SiRNA. RNA duplexes were transfected into cells using an Amaxa Nucleofector II according to manufacturer’s specifications for primary smooth muscle cells.

Migration Assay and Quantification. Two days post 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 and media containing 10% Fetal Bovine Serum was replaced and cells were allowed to migrate for the appropriate time in a 37°C incubation chamber with 5% CO₂. Images were taken.
with a Leica DM IRB at 10x using brightfield microscopy. The remaining open area of the wound was measured using Adobe Photoshop software. Using this software, open area for an image can be expressed as arbitrary units or normalized to area of the scratch wound immediately after the scratch (0 hr).

**Immunofluorescence.** Cells were plated on collagen coated glass coverslips or cell culture dishes. Cells were fixed using 4% paraformaldehyde for 20 minutes and permeabilized with 0.2% Triton X-100 in PBS for 5 minutes. Nonspecific binding was blocked with 5% fish gelatin in PBS plus 0.1% Triton X-100 followed by a 1 hour incubation at room temperature with the described anti-CaMKII, Fyn, Src or anti-GM130 antibodies diluted to 1:100-1:250 in blocking buffer. This was followed by washes and a 1 hour incubation 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 Hard Mount with DAPI (Vector Laboratories, Inc., Burlingame, CA). Cells were imaged on a Zeiss LSM 510 META confocal microscope (Carl Zeiss, Inc., Thornwood, NY), a Leica DM IRB (Leica Microsystems, Bannockburn, IL) or Total Internal Reflection Fluorescence microscopy (TIRF) using a Zeiss AxioObserverZ-1 with Zeiss Axiovision digital imaging software (Carl Zeiss, Inc., Thornwood, NY).

**Statistical Analysis.** All are expressed as means +/- SEM. Mean values of multiple groups were analyzed by 1- way ANOVA with post hoc comparisons using the Newman-Keuls test (Graphpad Prism). Comparisons between two groups were analyzed using a Students T-Test. For all comparisons, P< 0.05 was considered statistically significant.

**Results:**

**Src family kinases mediate VSM cell migration.** Src family kinases (SFK) 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 pharmacologic inhibitor of SFKs (26), prior to “scratch wounding” a monolayer of VSM cells as described in the “experimental procedures.” Treatment with PP2 significantly attenuated migration of VSM cells into the wound area (Figure 1A). Su6656, another SFK inhibitor (27), had similar effects on VSM migration under the same experimental conditions (data not shown). Although these experiments were carried out in complete medium, the duration (12 hours) 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 as compared to complete medium. Importantly, PP2 treatment prevented VSM cell migration under these conditions as well (Figure 1B).

**CaMKIIδ2 mediates VSM cell migration.** Immunoblotting lysates from cultured VSM cells indicated the expression of the SFK family members Yes, Fyn, and Src (28) (Figure 1C). Further analysis using quantitative PCR with primers selective for Yes, Fyn, and Src confirmed their expression in VSM cells and indicated that mRNA levels of Yes and Fyn are significantly higher than Src, although the significance of this observation at the protein level is not clear. (Figure 1D). Based upon recent studies showing an important role for Fyn in promoting cell migration (16), Fyn’s high expression level in VSM cells, and reports 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 (Figure 2A) resulted in a significant attenuation of VSM cell migration in response to “scratch wounding” (Figure 2B). Similarly, overexpression of a dominant-negative Fyn mutant (K299M) (30) inhibited VSM cell migration in response to scratch wounding (Figure 2C). These results are consistent with the pharmacological approaches implicating SFKs in VSM migration shown in...
Figure 1. It is important to note that these results do not exclude function of other SFK’s in VSM migration, but for the first time demonstrate a significant role for Fyn in VSM cell motility.

Having established that CaMKIIδ (4) and Fyn (Figure 2) are important mediators of VSM cell migration, we suppressed expression of both CaMKIIδ and Fyn simultaneously (Figure 3A,B) and compared their combined effect on VSM cell migration to silencing CaMKIIδ or Fyn expression alone (Figure 3C). Simultaneous suppression of both CaMKII and Fyn had no additive effect to silencing either CaMKII or Fyn alone. The non-additivity suggests that CaMKIIδ and Fyn are in the same pathway and coordinate regulation VSM cell migration.

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

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

**CaMKIIδ 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 and Fyn interact in complexes isolated by immunoprecipitation. Using antibodies specific for Fyn or Src (Figure 6A), a fraction of CaMKIIδ and Fyn was found to coinmunoprecipitate from VSM cell lysates. The lack of CaMKIIδ in Src immunoprecipitations indicate that CaMKIIδ and Fyn interact in situ with some selectivity and confirm the co-localization studies shown in Figure 4B.

CaMKIIδ/Fyn interactions could be direct and/or indirect through interaction with other proteins in a larger complex. CaMKIIδ contains a 21 amino acid C-terminus that is a product of alternative splicing and specific to a subset of δ gene products CaMKII (23;31). This region contains a proline rich sequence from amino acids 539 to 549 (334HRSGSTVPIKPPCIPNGK352) that conforms to multiple consensus SH3 binding domains (P-x-x-P) (32;33). In order to test the hypothesis that the proline rich C-terminus of CaMKIIδ contains the Fyn:SH3 binding domain, a competing peptide spanning the three tandem P-x-x-P consensus SH3 ligand motifs (32;33) (CaMKIIδ2334–352) was generated with a scrambled peptide containing the same 18 amino acids used as a control. As depicted in Figure 6B increasing concentrations of the C-terminal peptide inhibited GST-Fyn:SH3 interaction with CaMKIIδ, 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 has the capacity to act as a ligand for the SH3 domain of Fyn. To further test the selectivity of CaMKIIδ’s interaction with Fyn, we transduced VSM cells with adenoviral constructs containing cDNAs encoding CaMKIIδ, CaMKIIδ, a CaMKIIδ isoform lacking the c-terminal tail (34), or CaMKIIδ. After confirming comparable overexpression of the constructs we carried out Fyn immunoprecipitations. Under
these conditions, CaMKIIδ2 co-immunoprecipitated with Fyn to a greater extent than did either CaMKIIδ2 or γC (Figure 6C).

Stimulation of VSM cells with ionomycin (Iono), a calcium ionophore known to result in transient increases of intracellular [Ca\(^{2+}\)] and robust CaMKIIδ2 activation in VSM cells (25), resulted in a reduced amount of CaMKIIδ2 and Fyn co-immunoprecipitation compared to unstimulated controls (Figure 7A, 7B). The results of these experiments suggest that activation of CaMKIIδ2 is a determinant in its ability to interact with Fyn in a complex.

Consistent with findings shown in figure 6 evaluating endogenous interactions, stimulation of VSM cells with ionomycin prior to lysis decreased the ability of endogenous CaMKIIδ2 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 CaMKIIδ2 following Iono stimulation (Figure 8A). In a similar manner, KN93 treatment prevented the Iono-dependent disruption of endogenous Fyn and CaMKIIδ2 in co-immunoprecipitation studies (Data not shown).

CaMKIIδ2 functionally associates with Fyn in VSM cells

Activation of Fyn is a multistep process requiring dephosphorylation of Tyr527 (35) with subsequent autophosphorylation at Tyr416 residue 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 Tyr527 (the inhibitory phosphorylation site) and Tyr416 (autophosphorylation). Under quiescent conditions, Fyn was predominantly phosphorylated at Tyr527 (Figure 8B). Following ionomycin stimulation Tyr527 phosphorylation decreased and phosphorylation at Tyr416 increased (Figure 8B). Overexpression of an HA-tagged constitutively active CaMKIIδ2 mutant (CaMKIIδ2 T287D) in VSM cells resulted in the loss of Fyn phosphorylation at Tyr527 (Figure 8C). These results indicate that Fyn activity in the CaMKIIδ2/Fyn complex can be regulated in a Ca\(^{2+}\)-dependent manner secondary to activation of CaMKIIδ2.

Discussion

In this study, we identify for the first time physical and functional relationships between the multifunctional serine/threonine protein kinase CaMKIIδ2 and the multifunctional src family kinase, Fyn, in vascular smooth muscle. These studies demonstrate extensive spatial colocalization between Fyn and CaMKIIδ2 which is the primary CaMKII isoform in VSM cells, physical interaction between the kinases in a protein complex, and functional interactions that provide a mechanism whereby CaMKIIδ2 mediates Ca\(^{2+}\) signal-dependent activation of Fyn. These interactions are likely to contribute to CaMKIIδ2- and Fyn-dependent regulation of VSM cell migration.

Expression and function of Fyn in VSM cells has not been previously reported. 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 level of mRNA (Figure 1C). The functional significance of this finding is not clear but studies in other cell types have shown that the proto-oncogene c-CBL induces ubiquitination and proteosomal 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 than Src. In our hands overexpression of Fyn has detrimental effects on VSM proliferation and viability (unpublished data).

Based on previous studies and the fact that Fyn has dual palmitoylation sites on its SH4 domain that targets it directly to lipid rafts (39), Fyn association with Golgi and peripheral membrane as shown in Figure 4 is not surprising. On the other hand, there is no intrinsic structural feature in CaMKIIδ2 which would predict its association with these same membrane structures and co-localization with Fyn. The appearance of endogenous CaMKIIδ2 and Fyn in a protein complex provides a potential mechanism for this pattern of CaMKIIδ2 localization. Our previous study (4) showed that loss of CaMKIIδ2 expression prevented both Golgi orientation and
lamellipodia 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 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 show that CaMKIIδ2 and Fyn are closely associated under quiescent cellular conditions and that this association is disrupted upon CaMKIIδ2 activation. The molecular basis for the re-distribution of Fyn and CaMKIIδ2 to peripheral membrane regions following PDGF stimulation was not examined here. One possibility is the extent of Fyn palmitoylation with mono-palmitoylation 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 CaMKIIδ2/Fyn interaction in Golgi or peripheral membrane compartments are not yet defined, but analysis of Fyn tyrosine phosphorylation indicated that CaMKIIδ2 may mediate Ca²⁺-dependent regulation of Fyn activity. Recently, an interaction between CaMKIIα and Fyn was reported to play an important role in mediating NCAM-stimulated neurite outgrowth (44) (29). Interestingly, these studies implicated CaMKIIα in the activation of PTPα followed by dephosphorylation of Fyn at Tyr527 that results in activation of Fyn. Our data showing that overexpression of constitutively active CaMKIIδ2 resulted in dephosphorylation of Fyn at Tyr527 and that CaMKIIδ2, Fyn, and PTPα co-immunoprecipitate (data not shown) suggests that CaMKIIδ2 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 CaMKIIδ2 and Fyn interact in the context of a caveolae/lipid raft in VSM cells.

The selectivity of the CaMKIIδ2 interaction with Fyn compared to closely related δ6 and γc isoforms, suggests that the alternatively spliced 21 amino acid c-terminus in CaMKIIδ2 may contribute to the interaction. In Figure 6 we demonstrate that a GST:FynSH3 domain construct can interact with CaMKIIδ2 in vitro and is displaced with a CaMKIIδ2 c-terminus 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 CaMKIIδ2 and Fyn compared to other SFKs in situ. While this in vitro experiment demonstrates a direct interaction between the Fyn SH3 domain and CaMKIIδ2, it does not rule out other stabilizing interactions in vivo.

Given the apparent selectivity of the interaction involving Fyn compared to other SFKs and CaMKIIδ2 compared to closely related δ6 and γc isoforms which lack the alternatively spliced c-terminus of CaMKIIδ2, this complex could serve to initiate and/or propagate Ca²⁺-dependent signals in a cell- and/or context-dependent manner. As suggested here by lack of additivity of siRNA- mediated suppression of CaMKIIδ2 and Fyn on VSM cell migration (Figure 3), a specific example of this may be coordinate regulation of VSM cell migration by these protein kinases. Along with our findings implicating Fyn as a mediator of Ca²⁺-dependent cellular functions, several recent studies have reported a role for Fyn as a downstream effector of Ca²⁺ signals and an upstream modulator of Ca²⁺ signals with diverse functional outcomes ranging from fertilization to wound healing (46) (47).

In summary, endogenous CaMKIIδ2 and Fyn interact in VSM cells in a signaling complex and their interaction is modulated by Ca²⁺-dependent stimuli which activate CaMKII. In vitro studies indicate that the Fyn SH3 domain is capable of mediating a direct interaction with a proline-rich domain in the CaMKIIδ2 c-terminus, although other more complex mechanisms involving adapters such as GIT family proteins (48) cannot yet be ruled out. Co-localization of CaMKIIδ2 and Fyn in Golgi and peripheral plasma membrane compartments are consistent with their function to coordinate regulate VSM cell motility.
Acknowledgements:
We would like to 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.

This work was supported by the AHA-Northeast Affiliate Predoctoral Training Grant 0215300T to Paul J. Pfleiderer and an NIH Predoctoral Training Grant (T32-HL-07194) and grants to Dr. Harold A. Singer from the National Heart, Lung and Blood Institute (RO1-HL49426 and RO1-HL092510).

Reference List

CaMKIIδ Interaction with Fyn


Figure Legends

**Figure 1.** Src family kinases mediate VSM cell migration. (A) VSM cells were grown to confluence and treated with either DMSO (vehicle control) or 3 μM PP2 for 30 min before a “scratch” was made as described in the “Experimental Procedures”. The area of the wound after the initial scratch (0 hr) was measured in pixels and identified as 100%. The area was measured after 12 hr and expressed as a % of the time 0 wound area. The right panel is a representative micrograph depicting the “scratch wound” at 0 and 12 hr. (B) VSM cells were growth-arrested by incubation in media containing 0.4% FBS and treated as described in (A). For (A) and (B) * P< 0.05; n=3 separate experiments. Statistical analysis was performed using a 1-way ANOVA and a Neuman-Keuls post hoc comparison. (C) VSM cell lysates were resolved on 8 % SDS-PAGE and transferred to nitrocellulose membrane and immunoblotted (IB) with antibodies specific for Yes, Fyn, and Src, respectively. GAPDH was immunoblotted to confirm equivalent levels of protein loading. (D) mRNA levels of Fyn, pp60Src (Src), and Yes were determined using quantitative PCR and normalized against GAPDH message levels. The graph represents the means ± S.E.M. n=3 separate qPCR reactions.

**Figure 2.** Fyn promotes VSM migration. (A) VSM cells were transduced with siRNA targeting Fyn and loss of Fyn protein expression was confirmed by immunoblotting for Fyn (IB:Fyn). Immunoblotting for Src (IB:Src) and Yes (IB:Yes) was performed to insure specificity of the Fyn siRNA. The right panel shows quantification of immunoblots. Values are means ±S.E.M., n= 4 separate experiments. (B) Scratch wound in VSM cells transduced with either control siRNA or siRNA targeting Fyn. Wound areas were quantified as described in Figure 1. The graph represents the quantification of three separate experiments. The right panel is a representative micrograph depicting the scratch wound at 0 and 10 hours after scratch. * P< 0.05 by 1-way ANOVA followed by a Neuman-Keuls post hoc comparison. (C) VSM cells were transfected with expression plasmid expressing dominant negative Fyn (dnFyn) and overexpression of dnFyn was confirmed by immunoblotting for Fyn (IB:Fyn) (Right Panel) followed by scratch wounding and area analysis after 10 hr as described in Figure . * P< 0.05, n=3 separate experiments. Analysis is by 1-way ANOVA followed by a Neuman-Keuls post hoc comparison.

**Figure 3.** CaMKIIδ2 and Fyn coordinately regulate VSM cell migration (A) VSM cells were transfected with siRNA targeting Fyn, CaMKIIδ2, or both and loss of Fyn and CaMKIIδ2 protein expression was confirmed by immunoblotting for Fyn (IB:Fyn) and CaMKIIδ2, respectively. (B) Quantification of 4 separate experiments as depicted in (A). (C) A “scratch” wound was performed as described in Figure 1 and analyzed at 5 and 10 hr. The graph represents the quantification of three separate experiments. * P<0.05 by 1-way ANOVA followed by a Neuman-Keuls post hoc comparison.

**Figure 4.** Co-localization of CaMKIIδ2 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. CaMKIIδ2 or Fyn were localized by indirect immunofluorescence microscopy. Decreased signal in siRNA treated cells validated specificity of CaMKIIδ2 or Fyn antibodies used in subsequent experiments. The cells were labeled with Dapi to visualize the nucleus. (B) VSM cells were processed for immunofluorescence using antibodies to CaMKIIδ2 and Fyn (a-c) or CaMKIIδ2 and Src (d-f), followed by the fluorescent secondary antibodies Alexa Fluor 488 and Alexa Fluor 594. Confocal microscopy indicated CaMKIIδ2 and Fyn perinuclear co-localization (inset). CaMKIIδ2 and Src failed to colocalize. Each of these micrographs are representative of three separate experiments. Bar, 20μm.

**Figure 5.** CaMKIIδ2 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 antibodies for CaMKIIδ2 (a,d) or Fyn (b,c) with the secondary antibodies
CaMKIIδ2 Interaction with Fyn

Alexa Fluor 488 and Alexa Fluor 594, respectively. Assessment by TIRF microscopy indicates that CaMKIIδ2 and Fyn do not co-localize under quiescent conditions (c) but do co-localize at the leading edge after PDGF stimulation (f). The micrographs are representative of 4 slides from two separate experiments.

**Figure 6. CaMKIIδ2 and Fyn complexes.** (A) Fyn (left panel) or Src (right panel) were immunoprecipitated from quiescent VSM cells. Immunoprecipitates were immunoblotted for Fyn or Src, respectively, and CaMKIIδ2 (IB:CaMKIIδ2). The immunoblots are representative of three separate experiments. (B) VSM cell lysates were incubated with GST-Fyn:SH3 and assayed for interaction with CaMKIIδ2 by immunoblotting proteins in the pull down with an antibody specific for CaMKIIδ2 (IB: CaMKII). Addition of increasing concentrations of a peptide that contains the putative SH3 binding domain (amino acids 534 to 552) in the unique c-terminus of CaMKIIδ2 (C-terminal Peptide) resulted in dissociation of CaMKIIδ2 binding to the SH3 domain of Fyn. As a control, a peptide that contained the same amino acids in a scrambled sequence (Scrambled Peptide) showed no competitive displacement at concentrations up to 1 mM. The immunoblot is representative of three separate experiments. The 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 a 1 way ANOVA followed by a Neuman-Kuels post hoc comparison. (C) VSM cells were transduced with adenovirus encoding wild type (wt) CaMKIIδ2, CaMKIIδ6, or CaMKIIγ respectively. An immunoblot with a pan-CaMKII antibody was performed to monitor levels of CaMKII overexpression (Pre-IP lysate). Fyn was immunoprecipitated from these cells and immunoblotted for CaMKII and Fyn.

**Figure 7. Ca2+ and CaMKII-dependent phosphorylation of Fyn.** CaMKIIδ2 was immunoprecipitated from VSM cells stimulated with 0.5 μM Iono for 2 min. (A) Pre-immunoprecipitation lysate was immunoblotted with antibody specific for threonine287 autophosphorylated (activated) CaMKII (IB: P-CaMKIIδ2), total CaMKII (IB: CaMKIIδ2), and Fyn (IB:Fyn). (B) CaMKIIδ2 (left panel) or Fyn immunoprecipitates (right panel) were immunoblotted for Fyn (IB:Fyn) and CaMKIIδ2 (IB:CaMKIIδ2). Micrographs are representative 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 unpaired Students T- Test.

**Figure 8.** (A) VSM cells were pretreated with 30 μM KN93 for 30 min and then stimulated with 0.5 μM ionomycin 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 for CaMKIIδ2 (IB:CaMKIIδ2) (right panel). The micrograph is representative of 3 separate experiments quantified in the graph on the right. *P>0.05 by 1-Way ANOVA and Neuman-Keuls post hoc analysis. (B) VSM cells were stimulated with 0.5 μM ionomycin for 2 min and pre-IP lysates were immunoblotted for activated CaMKII (IB:P-CaMKII). Fyn was immunoprecipitated and the pull down immunoblotted for CaMKIIδ2 (IB:CaMKIIδ2), total Fyn (IB:Fyn), phosphorylated Fyn (tyrosine527) (IB:P-FynY527), and phosphorylated Fyn (tyrosine416) (IB:P-FynY416). The immunoblots shown are representative of three separate experiments. (C) VSM cells were transduced with control adenovirus or adenovirus encoding HA-tagged CaCaMKIIδ2 (constitutively active CaMKIIδ2). Pre-IP lysates were immunoblotted for HA (IB:HA) and total CaMKIIδ2 (IB: CaMKIIδ2) (top panel). Fyn was immunoprecipitated from the remaining lysate and immunoblotted for P-FynY527, P-FynY416 and total Fyn (lower panel). The immunoblots shown are representative of three separate experiments.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
**Figure 7**

**CaMKIIδ2 Interaction with Fyn**

**A**
- **Pre-IP lysate**
  - IB: P-CaMKIIδ2
  - IB: CaMKIIδ2
  - IB: Fyn

**B**
- **IP: CaMKIIδ2**
  - IB: Fyn
  - IB: CaMKIIδ2

**C**
- Graph showing (Lono) vs. Fyn/CaMKIIδ2 interaction
  - Control (-Iono)
  - Treatment (+Iono)

- Graph showing CaMKIIδ2/Fyn interaction
  - Control (-Iono)
  - Treatment (+Iono)
Figure 8
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J. Biol. Chem. published online September 3, 2013

Access the most updated version of this article at doi: 10.1074/jbc.M113.477257

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