PI3K activation is required for PMA-directed activation of cSrc by AFAP-110

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There is significant evidence to indicate the existence of cross talk among PKCα, cSrc, and phosphatidylinositol 3-kinase (PI3K). Activation of each of these kinases results in substantial changes in actin filament integrity and cell morphology concomitantly with increased cell motility and invasive potential (13, 26, 45). Extensive studies by other laboratories have shown that many cancers, including breast, prostate and ovarian, exhibit higher-than-normal levels of cSrc, PKCα, and PI3K (28, 44). The ability of cSrc and PKCα to cross talk was determined by the use of a constitutively active form of cSrc or stable expression of viral Src (vSrc), which will stimulate an increase in PKCα signaling, indicating that PKCα could function downstream of cSrc (52). Alternatively, other studies have demonstrated that PKCα can function upstream and direct activation of cSrc (6, 7). Although PKCα can phosphorylate cSrc, in vitro studies demonstrated that PKCα does not activate cSrc directly (6). Recently, we demonstrated that the PKCα and cSrc binding partner, actin filament-associated protein (AFAP-110), is able to relay signals from PKCα that direct cSrc activation. Expression of myristylated PKCα (myrPKCα) or treatments of cells with phorbol-12-myristate-13-acetate (PMA) induced AFAP-110 to colocalize with cSrc and subsequently activate it (15). The ability of AFAP-110 to colocalize with cSrc was dependent on the integrity of the amino-terminal pleckstrin homology (PH1) domain, while the ability to activate cSrc is contingent on the integrity of its SH3 binding motif, which engages the cSrc SH3 domain. The outcome of AFAP-110-directed cSrc activation is a change in actin filament integrity and the formation of podosomes. Here, we address what cellular signals promote AFAP-110 to colocalize with and activate cSrc, in response to PKCα activation or PMA treatment. Because PH domain integrity in AFAP-110 is required for colocalization, and PH domains are known to interact with both protein and lipid binding partners, we sought to determine whether phosphatidylinositol 3-kinase (PI3K) activation played a role in PMA-induced colocalization between AFAP-110 and cSrc. We show that PMA treatment is able to direct activation of PI3K. Treatment of mouse embryo fibroblast with P3K inhibitors blocked PMA-directed colocalization between AFAP-110 and cSrc and subsequent cSrc activation. PMA also was unable to induce colocalization or cSrc activation in cells that lacked the p85α and β regulatory subunits of PI3K. This signaling pathway was required for migration in a wound healing assay. Cells that were null for cSrc or the p85 regulatory subunits or expressed a dominant-negative AFAP-110 also displayed a reduction in migration. Thus PI3K activity is required for PMA-induced colocalization between AFAP-110 and cSrc and subsequent cSrc activation, and this signaling pathway promotes cell migration.

Phorbol 12-myristate 13-acetate; Src; protein kinase C; AFAP-110; phosphatidylinositol 3-kinase; pleckstrin homology domain

There is significant evidence to indicate the existence of cross talk among PKCα, cSrc, and phosphatidylinositol 3-kinase (PI3K). Activation of each of these kinases results in substantial changes in actin filament integrity and cell morphology concomitantly with increased cell motility and invasive potential (13, 26, 45). Extensive studies by other laboratories have shown that many cancers, including breast, prostate and ovarian, exhibit higher-than-normal levels of cSrc, PKCα, and PI3K (28, 44). The ability of cSrc and PKCα to cross talk was determined by the use of a constitutively active form of cSrc or stable expression of viral Src (vSrc), which will stimulate an increase in PKCα signaling, indicating that PKCα could function downstream of cSrc (52). Alternatively, other studies have demonstrated that PKCα can function upstream and direct activation of cSrc (6, 7). Although PKCα can phosphorylate cSrc, in vitro studies demonstrated that PKCα does not activate cSrc directly (6). Recently, we demonstrated that the PKCα and cSrc binding partner, actin filament-associated protein (AFAP-110), is able to relay signals from PKCα that direct cSrc activation. Expression of myristylated PKCα (myrPKCα) or treatments of cells with phorbol-12-myristate-13-acetate (PMA) induced AFAP-110 to colocalize with cSrc and subsequent activate it (15). The ability of AFAP-110 to colocalize with cSrc was dependent on the integrity of the amino-terminal pleckstrin homology (PH1) domain, while the ability of AFAP-110 to activate cSrc was dependent on the integrity of the proline-rich SH3 binding motif in AFAP-110, which contacts the SH3 domain of cSrc. Thus AFAP-110 is able to integrate signals from PMA or myrPKCα that enable it to colocalize with and subsequently activate cSrc. Previous studies by our laboratory (2, 15) demonstrated that the integrity of the amino-terminal PH1 domain is essential for AFAP-110 to colocalize with cSrc. These self-folding modular domains are known to bind lipids, such as those generated by PI3K (9, 11, 30). In this study, we sought to determine what cellular signals enabled AFAP-110 to colocalize with cSrc in response to PMA. Since colocalization and subsequent cSrc activation were dependent on the integrity of the PH1 domain, and molecular modeling analysis indicated that the PH1 domain of AFAP-110 had the capacity to bind phosphatidylinositol lipids (2), we hypothesized that PI3K activity may play a role in facilitating colocalization between AFAP-110 and cSrc in response to PMA. This hypothesis predicts that, 1) in cells where PI3K activity is blocked or PI3K regulatory subunits are deleted, PMA would fail to direct AFAP-110 to colocalize with cSrc; 2) PMA should be able to direct activation of PI3K; and 3) loss of PI3K protein expression or activity would prevent PMA-induced activation of cSrc and subsequent migration potential.

Materials and Methods

Reagents. DMEM, rhodamine (TRITC)-phalloidin, β-actin, monoclonal and polyclonal anti-FLAG antibodies, and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). Protein A/G PLUS agarose beads and polyclonal cSrc antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). LipofectAMINE reagent was purchased from Invitrogen (Carlsbad, CA). CalPhos mammalian transfection kit, Tet-Off inducible system, and MEF/3T3 cells were purchased from Clontech Laboratories (Mountain View, CA). Doxycycline-HCl, G418, and hygromycin were purchased from Calbiochem (San Diego, CA). DMEM, rhodamine (TRITC)-phalloidin, β-actin, monoclonal and polyclonal anti-FLAG antibodies, and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). Protein A/G PLUS agarose beads and polyclonal cSrc antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). LipofectAMINE reagent was purchased from Invitrogen (Carlsbad, CA). CalPhos mammalian transfection kit, Tet-Off inducible system, and MEF/3T3 cells were purchased from Clontech Laboratories (Mountain View, CA). Doxycycline-HCl, G418, and hygromycin were purchased from Calbiochem (San Diego, CA). 18 U.S.C. Section 1734 solely to indicate this fact.
from Gibco (Invitrogen, Carlsbad, CA). Uncoated glass-bottom plates (35 mm) were purchased from MatTek (Ashland, MA). Supernum
PCR reagent was obtained from Invitrogen, while tSLA29 primers were purchased from IGT. PMA, LY294002, wortmannin, and bisp
dolymaleimide I were obtained from Calbiochem. Monoclonal p85α and p110α antibodies and monoclonal PKα antibody were obtained from
BD Transduction Laboratories (San Diego, CA). The polyclonal AFAP-110 antibody F1 was generated and characterized as previously
described (39). Monoclonal avian cSrc antibody (EC10) and pol
clonal phospho-Src family tyrosine-418 were obtained from Upstate (Charlottesville, VA). Phospho-Src family (Tyr416) antibody was
purchased from Cell Signaling (Beverly, MA). Horseradish pero
dise-conjugated anti-rabbit and anti-mouse IgG secondary antibodies
and [γ-32P]ATP were obtained from Amersham Biosciences (Piscat
away, NJ). Phosphatidylinositol (PI) used in the PI3K kinase assay was purchased from Matreya (Pleasant Gap, PA). All Alexa Fluor
antibodies used were purchased from Molecular Probes (Invitrogen).
The Src family tyrosine kinase inhibitor PPI was purchased from Biomed (Plymouth Meeting, PA). Phosphoinositide 3,4,5-trisphosphate
(PI-3,4,5-P3) monoclonal IgM antibody was obtained from Echelon Biosciences (Salt Lake City, UT). Chemiluminescence reagent was
purchased from Pierce Biochemical (Rockford, IL). All chemicals used throughout this study, except where otherwise stated, were
purchased from J. T. Baker (VWR, West Chester, PA).

**Cell lines and culture.** Mouse embryonic fibroblast, SYF/cSrc and SYF (ATCC), cells were used throughout this study. SYF/cSrc are
derived from a SYF parental cell line that is devoid of the Src family of
non-receptor tyrosine kinase members fyn and c-yes genes but engineered to reexpress c-Src (27). PI3K regulatory subunit knockout
mouse embryoblasts (p85/−/−) derived from Pkk3r2 (encodes p85α genes) and Ptk3r2 (encodes p85β gene) double knockout or
Pkk3r2 (p85α/+/+) single knockout in 129 C57BL/6 mice were a kind gift from Saskia Brachmann (Harvard University, Cambridge, MA).
NHI3T3 mouse embryonic fibroblasts containing the regulatory tetracy
cline plasmid (pTet-Off) were transfected with AFAP-110 within the
tetracycline response plasmid (pTRE2-Hyg) as described below. The
above cell lines, except the Tet-Off cells, were grown at 37°C with 5%
L-glutamine, 100 μM p110α, 1:1,000 p110α, 1:1,000
BSA, 1:1,000 phospho-Src family tyrosine-418 (Upstate) in 5% BSA,
5% nonfat milk, except where indicated: 1:10,000 AFAP-110 pAb
in 1% BSA, 10% fetal calf serum or 100 nM PMA for 5 or 15 min. On completion
of protein A/G PLUS agarose beads (50% slurry) were added
and those cells at 39.5°C were treated with a PKC-specific activator,
100 nM PMA, for 5 or 30 min, alone or in combination with the following: a PKC inhibitor, 6 μM bisindolylmaleimide I, for 6 h: a PI3K inhibitor, 10–20 μM LY294002, for 6 h; 50 nM wortmannin
for 30 min; and/or a Src family inhibitor, 5 μM PP1, for 6 h. For actin
destruction, a 150 μM dilution of TRITC-phalloidin was used, as indicated.
Primary antibody concentrations used were diluted in 5% BSA dis
solved in 1× PBS: EC10 monoclonal antibody (mAb), 1:500; phos
pho-Src family (Y416) polyclonal antibody (pAb), 1:250; anti-PI
-3,4,5-P3 mAb, 1:100; anti-AFAP-110 (F1) pAb, 1:1,000; anti-cSrc
pAb, 1:500; and anti-Flag, 1:10,000. All fluorescent secondary and
phalloidin antibodies were diluted 1:200 in 5% BSA and are labeled
accordingly. Cells were washed and mounted on slides with Fluoro
mount-G (Fisher). A Zeiss LSM-510 confocal microscope was used to scan images ~1 μm thick. To prevent cross-contamination between
the different fluorochromes, each channel was imaged sequentially using the multitrack recording module before merging of the images.
Representative cells are shown (>100 cells examined per image; see Figs. 1–8).

**Immunoblot assay.** All cells were cultured to 70–80% confluence,
serum starved for 16 h, and treated as described above. The cells were
lysed, and Western analysis was performed as previously described
(16). Membranes were probed using the following antibodies diluted in
1× Tris-buffered saline plus 0.1% Tween-20 (TBS-T) containing
5% nonfat milk, except where indicated: 1:10,000 AFAP-110 pAb
(F1), 1:1,000 phospho-Src family tyrosine-416 (Cell Signal) in 5% BSA,
1:1,000, phospho-Src family tyrosine-418 (Upstate) in 5% BSA,
1:500 cSrc (clone N-16), 1:1,000 p85α, 1:1,000 p110α, 1:1,000
PKα, and 1:500 β-actin in 1% BSA. PI3K assay. PI3K activity was determined using in vitro PI3K kinase assay as previously described (23). Cells were serum starved
24 h. Media were changed, and cells were then treated with either 10% fetal calf serum or 100 nM PMA for 5 or 15 min. On completion
of the incubation, the cells were lysed in cold kinase lysis buffer [150
mM NaCl, 100 mM Tris, pH 8.0, 1% Triton-X-100, 5 mM EDTA, and
10 mM NaF plus inhibitors (1 mg/ml leupeptin, 1 mg/ml peptatin,
0.5 M sodium vanadate, 1 mg/ml aprotinin, and 1M DTT)]. Five
hundred micrograms (500 μg) of protein were incubated with mono
clonal p110α antibody (2 μg/μl) overnight at 4°C. Forty microliters
(40 μl) of protein A/G PLUS agarose beads (50% slurry) were added
and incubated for an additional 2 h. The beads were pelleted and washed two times with cold lysis buffer and one time each with fresh
cold TNE buffer (20 mM Tris, pH 7.5, 100 mM NaCl, and 1 mM
EDTA) and 20 mM HEPES, pH 7.5. The pellets were resuspended in
[γ-32P]ATP kinase reaction buffer [20 mM HEPES, pH 7.5, 10 mM
MgCl2, and 0.2 mg/ml PI (in 10 mM) in 10 mM 60 μM ATP, and 0.2
μCi/μl [γ-32P]ATP]. The samples were incubated 15 min on ice, and
the reactions were stopped by adding 1 M HCl. After the addition of
chloroform-methanol (1:1), the samples were vortexed and the beads
pelleted. The lower phase was collected, and the samples were dried,
rehydrated in chloroform, and spotted on a prepared thin layer
chromatography (TLC) plate. The finished plates were then exposed to
radiography film up to 48 h at −70°C.
Fig. 1. LY294002 blocks PMA-induced colocalization between AFAP-110 and cSrc and subsequent cSrc activation. SYF cells transiently coexpressing green fluorescence protein (GFP)-AFAP-110 and cSrc were untreated (A–D), treated with 100 nM PMA for 30 min (E–H), or treated with PMA and either 6 μM bisindolylmaleimide (I–L), 10 μM LY294002 (M–P), or 50 nM wortmannin (Q–T). Fixed cells were immunolabeled with avian-specific cSrc antibody (EC10; 1:500) and phospho-Src family (Tyr416; 1:250) as described in MATERIALS AND METHODS. Secondary antibodies used were Alexa-546 anti-mouse for EC10 and Alexa-647 anti-rabbit for phospho-Src family (Tyr416). PMA treatment resulted in a disruption of actin into punctate structures and an increase in cSrc activation. The majority of active cSrc is localized to these structures; however, a very small population is localized to the membrane (as indicated by arrows in F). A merged image was generated to determine colocalization between AFAP-110 and cSrc. Bars = 20 μm.
Scratch motility assay, SYF, SYF/cSrc, p85Δ−/−, and induced and uninduced AFAP-110 Tet-Off cells were cultured in glass-bottom 35-mm MatTek plates, and a scratch assay was performed as previously described (20). A scratch was made using a 200-μl pipette tip in the confluent monolayer and a gentle wash with 1× PBS to remove dislodged cells. Serum-free CO2 retaining culture medium was added, and wound closure was imaged over 6 h. Images were gathered every 5 min for the 6-h period. Wound widths at time 0 and at the end were measured using Zeiss software. Postwounding cells were treated with 100 nM PMA, 20 μM LY294002, or 100 nM PMA in conjunction with 20 μM LY294002 or 6 μM bisindolylmaleimide I. Images were generated and analyzed using AxioVision 4.4 software with the Axiovert Zoom microscope (Zeiss).

RESULTS

PI3K activity is required for PMA-induced translocation of AFAP-110 to cSrc and subsequent cSrc activation. The ability of AFAP-110 to colocalize with cSrc in response to PMA-directed signals is dependent on the integrity of its PH domain. Deletions in the PH domain will prevent PMA or PKCα from inducing AFAP-110 to colocalize with cSrc and will also block PMA- or PKCα-directed subsequent activation of cSrc (15). There is significant evidence for cross talk between PKCα, cSrc, and PI3K. Furthermore, many PH domains can bind to PI3K-generated lipid products, and the AFAP-110 PH1 domain is predicted to bind as well (2). Thus we sought to determine whether PI3K activity was required for PMA-induced colocalization of AFAP-110 with cSrc and subsequent cSrc activation. We utilized SYF cell lines as a model system, mouse embryo fibroblasts engineered to contain null mutations in the c-src, fyn, and c-yes genes (27). The cells were transiently cotransfected with constructs that encode cSrc and GFP-tagged AFAP-110, as previously described (15). It has been well documented that overexpression of these proteins does not alter their cellular location (37, 41). Coexpression of GFP-AFAP-110 and cSrc in unstimulated SYF cells confirms that wild-type AFAP-110 neither colocalizes with nor activates cSrc (Fig. 1, A–D). PMA treatment caused GFP-AFAP-110 to colocalize with cSrc, and there was evidence for activation of cSrc based on increased immunoreactivity with the anti-phospho-cSrc (Y416) antibody, which recognizes cSrc in its activated state (Fig. 1, E–H). Furthermore, activation of cSrc corresponded with morphological changes associated with the formation of dot-like structures on the ventral membrane, which are consistent with podosomes or invadopodia. Pretreatment of cells with the PKC inhibitor bisindolylmaleimide I (Fig. 1, I–L) or the PI3K inhibitor LY294002 (Fig. 1, M–P) blocked PMA-induced colocalization of AFAP-110 with cSrc, cSrc activation, and associated morphological changes. To confirm the specificity of LY294002, cells were pretreated with the PI3K inhibitor wortmannin, which also blocked colocalization and cSrc activation (Fig. 1, Q–T). These data indicate a potential role for PI3K activation in modulating colocalization between AFAP-110 and cSrc in response to PMA treatment.

To verify the requirement of PI3K, we used p85Δα/β knockout (p85Δ−/−) mouse embryo fibroblasts (4). Work by Yu et al. (51) indicated that the regulatory subunit of PI3K is required to stabilize the p110 catalytic subunit of PI3K. Thus loss of p85α/β would lead to a loss of PI3K activity in the cell. We sought to determine the steady-state levels of expression of endogenous PKCα or the p110 catalytic subunit of PI3K in our MEF model system. Western blot analysis indicates that p85Δ−/− cells contain equivalent endogenous levels of PKCα, AFAP-110, and actin relative to SYF or SYF/cSrc cells (Fig. 2A). Western blot analysis confirmed that the p85Δ−/− cells do not express detectable levels of p85α, which is present in the p85α+/+ and SYF/cSrc control cells (Fig. 2B). Our results also indicate that p85Δ−/− cells lack detectable levels of p110α, while the p110α catalytic subunit is stably expressed when the p85α regulatory subunit of PI3K is present (p85α+/+) in these cells (Fig. 2C). These MEF-derived cells were used as a model system to determine whether PMA could direct activation of cSrc under endogenous protein expression levels. Western blot analysis was performed, which verified the ability of PMA treatment to activate cSrc. As predicted, PMA induced tyrosine-416 phosphorylation of endogenous cSrc in SYF/cSrc and not cells lacking cSrc (SYF) (Fig. 3A). Furthermore, PMA failed to induce endogenous cSrc activation in the mouse embryo fibroblast that lacks the p85 regulatory subunit. The role of the p85 regulatory subunit in PMA-induced cSrc activation was further examined by utilizing the p85αΔ+/+ MEFs, which express only the p85α regulatory subunit of PI3K (Fig. 3B). Both p85Δ−/− and p85αΔ+/+ cells were tran-
Fig. 3. PMA fails to induce cSrc activation in cells that do not express the p85α regulatory subunit. A: SYF, SYF/cSrc, and p85−/− were treated with 100 nM PMA for 15 min, 40 μg of cell lysate were resolved by SDS-PAGE, and Western blot analysis was performed to evaluate phospho-Src family (Y416; 1:1,000) and cSrc (1:500) as described in MATERIALS AND METHODS. β-Actin (1:5,000) was used as a loading control. B: p85 knockout MEFs (p85−/−) and mouse embryo fibroblast cells that express only the p85α isoform of the PI3K regulatory subunit (p85α+/+) were transfected with GFP-AFAP-110 and cSrc, followed by a 30-min treatment with PMA. Fixed cells were immunolabeled with EC10 (1:500) and phospho-Src family (Tyr416; 1:250) as described in MATERIALS AND METHODS. Secondary antibodies used were Alexa-546 anti-mouse for EC10 and Alexa-647 anti-rabbit for phospho-Src family (Tyr416). Images were merged to determine colocalization of cSrc and GFP-AFAP-110. Bars = 20 μm.
Fig. 4. PI3K activity is required for myristylated PKC\(^{\text{myr}}\)-directed cSrc activation. A: SYF/cSrc cells transiently transfected with GFP-AFAP-110 with or without Flag-tagged myrPKC\(^{\text{myr}}\). The cells were fixed and immunolabeled with Flag antibody (1:1,000) and phospho-Src family (Tyr\(^{416}\); 1:250) as described in MATERIALS AND METHODS. Secondary antibodies used were Alexa-546 anti-mouse for Flag and Alexa-647 anti-rabbit for phospho-Src (Tyr\(^{416}\)). A merged image was used to determine colocalization between AFAP-110 and myrPKC\(^{\text{myr}}\). Controls without myrPKC\(^{\text{myr}}\) (untreated; a–d) or treated with 20 \(\mu\)M LY294002 (i–l) showed no change in actin filament integrity. Cells coexpressing GFP-AFAP-110 and myrPKC\(^{\text{myr}}\) showed an increase in cSrc phosphorylation and colocalization between myrPKC\(^{\text{myr}}\) and AFAP-110 (e–h). Treatment of cells treated with 20 \(\mu\)M LY294002 abrogated colocalization and cSrc phosphorylation (m–p).

To verify that the effects observed with PMA treatment were associated with PKC\(^{\text{myr}}\) signaling, SYF/cSrc cells were transiently transfected with GFP-AFAP-110 with or without a constitutively active form of PKC\(^{\text{myr}}\) (myrPKC\(^{\text{myr}}\)) and examined for cSrc activation. Expression of GFP-tagged AFAP-110 in the absence of myrPKC\(^{\text{myr}}\) did not direct an increase in cSrc activation (Fig. 4A, a–d). Coexpression of AFAP-110 with myrPKC\(^{\text{myr}}\) resulted in an increase in cSrc activation concomitantly with significant changes in the localization of AFAP-110 and stress filaments (Fig. 4A, e and h). LY294002 treatment had little effect on AFAP-110 localization in the absence of myrPKC\(^{\text{myr}}\) (Fig. 4A, i–l). Inhibition of PI3K activity by LY294002 blocked the ability of myrPKC\(^{\text{myr}}\) to induce cSrc activation or the formation of ventral membrane structures predicted to be podosomes (Fig. 4A, m–p).

To further evaluate the role of PI3K in PKC\(^{\text{myr}}\)-directed cSrc activation and actin filament disruption, p85\(^{-/-}\) cells were transfected with myrPKC\(^{\text{myr}}\). Expression of myrPKC\(^{\text{myr}}\) failed to direct an increase in cSrc activation or promote disruption of actin filaments (Fig. 4B, e–h) relative to untransfected controls (Fig. 4B, a–d). Collectively, these data indicate that PMA and myrPKC\(^{\text{myr}}\) are unable to direct AFAP-110 to colocalize with or activate cSrc in the absence of PI3K activity. One consequence associated with this is a failure to significantly cotransfect with GFP-tagged AFAP-110 and cSrc. Cells were treated with PMA and evaluated for the ability of AFAP-110 to colocalize with and activate cSrc. PMA induced colocalization of AFAP-110 and cSrc, cSrc activation, and podosome formation in p85\(^{+/+}\) cells (Fig. 3B, e–h). However, PMA failed to induce significant colocalization of AFAP-110 with cSrc, and there was no evidence for cSrc activation or podosome formation in PMA-treated p85\(^{-/-}\) cells (Fig. 3B, m–p). These results further support a role for PI3K in PMA-induced cSrc activation.
PKC ACTIVATION OF cSrc

A

Phospho-cSrc Y⁴¹⁸

cSrc (EC10)

B

SYF: 39.5°C

GFP-AFAP-110  LA29  Actin  Merge

Control

100nM PMA

100nM PMA + 6-β-D-mannopyranoside

SYF: 35°C

GFP-AFAP-110  LA29  Actin  Merge

DMSO

10μM LY294002
form ventral membrane structures that may be podosomes or precursors to invadopodia.

**PI3K is required for PMA-induced translocation of active cSrc to the cell membrane in mouse embryo fibroblasts.** In the quiescent state, ~90% of cSrc is found associated with the perinuclear region of the cell, and this location was shown to correlate with an association with perinuclear vesicles, both in endogenous and overexpression systems (24, 40, 42). On activation, cSrc moves to the cell membrane and stimulates phosphorylation of proteins and downstream signaling cascades that regulate mitogenesis, motility, and invasive potential (42, 44). SYF cells, which are null for endogenous cSrc, were transiently transfected to express avian cSrc. This system allows for efficient detection of avian cSrc with the highly specific avian cSrc antibody (EC10). Cells were treated with PMA for 10 min. The shorter incubation time allowed for evaluation of cSrc translocation to the peripheral membrane and activation before podosome formation (Walker VG and Flynn DC, unpublished observation). Our analysis in quiescent cells confirmed that the vast majority of cSrc is associated with the perinuclear region of the cell, while far less is associated with the cell periphery (Fig. 5A). Activation of PKCo by PMA directed cSrc to translocate from the perinuclear region to the cell periphery (Fig. 5B). This translocation was blocked by pretreatment of cells with either bisindolylmaleimide I (Fig. 5C) or LY294002 (Fig. 5D), where cSrc remained perinuclear. Interestingly, in the absence of PI3K activity, cSrc remained in the perinuclear region in p85+/− cells irrespective of PMA treatment, and these cells were comparable to untreated controls (Fig. 5, E and F).

Because cSrc activation is rapid in response to PMA (<15 min), we sought to design a strategy that would enable us to determine where cSrc and AFAP-110 first colocalize, without inducing cSrc activation and subsequent transit to the membrane. To accomplish this, we utilized the temperature-sensitive form of vSrc, LA29 (43, 47). LA29 exists in perinuclear regions of the cell at nonpermissive temperatures (39.5°C) and moves to the cell periphery at permissive temperature (35°C) where it induces the characteristic cell shape changes associated with transformation, including the formation of motility structures and podosomes (32, 48). We transiently transfected SYF cells with a dual-expression vector encoding both AFAP-110 and LA29 at the nonpermissive temperature of 39.5°C. After 48 h, the cells were left at 39.5°C or transferred to the permissive temperature, 35°C. Western blot analysis demonstrated that LA29 is active at 35°C and not at 39.5°C, based on immunoreactivity with the anti-phospho-Src family (Y418) (Fig. 6A). At the nonpermissive temperature, LA29 is predominantly observed in the perinuclear region of the cell and does not colocalize with AFAP-110 (Fig. 6B, a–d). In cells treated with PMA at 39.5°C, AFAP-110 colocalized strongly with LA29 in the perinuclear region of the cell (Fig. 6B, e–h). Pretreatment of cells with either bisindolylmaleimide (Fig. 6B, i–l) or LY294002 (Fig. 6B, m–p) blocked PMA-induced colocalization between LA29 and AFAP-110. When untreated control cells are shifted to the permissive temperature (35°C) for 6 h, LA29 becomes activated and moves to the cell periphery (Fig. 6B, q–t). In these cells, there was evidence for numerous actin-rich punctate structures on the ventral membrane (as imaged using confocal microscopy and 1-μm-thick sections) that were ~1 μm in diameter, which is characteristic of podosomes (15, 31). Interestingly, treatment of these cells with LY294002 blocked the ability of AFAP-110 to colocalize with LA29, and no disruption in actin filament integrity was observed at the permissive temperature (Fig. 6B, u–x). Collectively, these data indicate that colocalization between AFAP-110 and LA29 occurs in the perinuclear region of the cell and that PI3K activity is required for AFAP-110 colocalization, activation of cSrc, and subsequent translocation of cSrc to the cell periphery.

**PMA treatment induces PI3K activation.** Our data indicated that PI3K activation occurs downstream of PMA treatment and upstream of cSrc activation. Therefore, we predicted that PMA should be able to induce activation of PI3K. Although there are several reports in the literature that indicate PI3K activation can lead to subsequent PKC activation [as a result of 3-phosphoinositide-dependent protein kinase-1 (PDK1) phosphorylation] (3), it is not clear whether PMA and PKCo activation can function as upstream activators of PI3K. To test this, we performed a PI3K lipid kinase activity assay, as previously described (23). SYF/cSrc cells were serum starved and then stimulated with either serum or PMA for 5 or 15 min. The lipid kinase assay demonstrates that there is a significant increase in PI3K activity after 5 or 15 min of treatment with PMA (Fig. 7A). These data indicate that PMA treatment can induce PI3K activity.

To corroborate our results observed in vitro, we stimulated SYF/cSrc cells with PMA and measured the production of phosphatidylinositol 3,4,5-trisphosphate (PtdIns-3,4,5-P3), the predominant lipid product generated by PI3K on activation. PtdIns-3,4,5-P3 production was measured using the anti-PtdIns-3,4,5-P3 antibody (Echelon) for analysis by immunofluorescence and contrasted with stress filament integrity, measured using TRITC-phalloidin as previously described (8, 19). SYF/cSrc cells were left untreated or treated with serum as controls for PI3K activation. Serum was able to direct up-regulation of PtdIns-3,4,5-P3 (25), and the PI3K inhibitor LY294002 was able to block serum-induced PtdIns-3,4,5-P3 production (Fig. 7B, c–f). In addition, SYF/cSrc cells were stimulated with PMA for 5 or 15 min. The data indicate that PMA was able to induce PtdIns-3,4,5-P3 production within 5 min of treatment concomitantly with significant changes in cell

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**Fig. 6.** AFAP-110 colocalizes with LA29 in the perinuclear region of cells. A: SYF cells were transiently transfected with CMV-vLA29 and either maintained at 39.5°C or shifted to 35°C. Cells were lysed, and Western blot analysis was performed. Control lysates of SYF were used to verify expression and activation. Membranes were probed with phospho-cSrc (Y418; 1:1,000). Membranes were stripped and reprobed using avian-specific cSrc antibody (EC10). B: SYF cells were transiently transfected with a cytomegalovirus (CMV) dual-expression vector encoding both AFAP-110 and vLA29, fixed and immunolabeled with anti-AFAP-110 (F1), anti-avian cSrc (EC10), and TRITC-phalloidin. Secondary antibody for anti-cSrc (EC10) was Alexa Fluor-647 anti-mouse and for AFAP-110 was Alexa Fluor-488 goat anti-rabbit IgG. Merged images of AFAP-110 and cSrc were generated using Zeiss confocal software. Cells remaining with LA29, fixed and immunolabeled with anti-AFAP-110 (F1), anti-avian cSrc (EC10), and TRITC-phalloidin. Secondary antibody for anti-cSrc (EC10) was Alexa Fluor-647 anti-mouse and for AFAP-110 was Alexa Fluor-488 goat anti-rabbit IgG. Merged images of AFAP-110 and cSrc were generated using Zeiss confocal software. Cells remaining with LA29, fixed and immunolabeled with anti-AFAP-110 (F1), anti-avian cSrc (EC10), and TRITC-phalloidin. Secondary antibody for anti-cSrc (EC10) was Alexa Fluor-647 anti-mouse and for AFAP-110 was Alexa Fluor-488 goat anti-rabbit IgG. Merged images of AFAP-110 and cSrc were generated using Zeiss confocal software.
shape and actin filament organization (Fig. 7B, g and h). Interestingly, PtdIns-3,4,5-P$_3$ levels were consistently reduced after 15 min of treatment with PMA, although cell shape changes and actin filament reorganization were still apparent (Fig. 7B, i and j). Pretreatment with either bisindolylmaleimide I or wortmannin blocked PMA-induced PtdIns-3,4,5-P$_3$ production (Fig. 7B, k–n), and similar results were observed with LY294002 (data not shown). On the basis of these results, we predicted that PKC$\alpha$ and PI3K signaling were upstream of cSrc in response to PMA treatment. To address this, cells were pretreated with the cSrc inhibitor PP1 before PMA treatment. PP1 did not block PMA-induced PtdIns-3,4,5-P$_3$ production (Fig. 7B, o and p), although PMA-directed changes in stress filaments and cell shape were impeded. Collectively, these data indicate that PMA treatment induces activation of PI3K and that activation is dependent on PKC activity but independent of cSrc activation.

$c$Src, PI3K, and AFAP-110 are required for migration. Several studies have shown that cSrc is required for the ability of cells to migrate into a wound of a scratch assay (27). Therefore, we wanted to address the following question: does PI3K regulate cell migration in our mouse embryo fibroblast system? We developed a Tet-Off system to express both wildtype AFAP-110 and the cSrc SH3 binding mutant AFAP-110$^{11A}$, which we predict acts as a dominant-negative construct. To confirm expression can be induced, Western blot analysis was performed for AFAP-110, demonstrating that removal of doxycycline correlates with induction of AFAP-110 or AFAP-110$^{11A}$ (Fig. 8A). A scratch assay was performed, and the distance migrated over 6 h was measured (Fig. 7, B and C). The average migration was graphed, and a one-way ANOVA ($P < 0.05$) was performed to determine significance. SYF/cSrc cells had the highest rate of migration, and this increase was impeded with inhibition of PI3K activity by LY294002 (Fig. 8B). However, cells that failed to express cSrc (SYF) or the regulatory subunits of PI3K (p85 and p110) displayed a reduced ability to migrate compared with the SYF/cSrc control cells. Interestingly, expression of wild-type AFAP-110 did not promote a significant increase in the distance migrated compared with control (Fig. 8C). However, cells expressing the cSrc SH3 binding mutant did show a significant reduction in migration compared with control. These data indicate that PI3K, cSrc, and AFAP-110 may function in regulating the ability of mouse embryo fibroblast to migrate.

DISCUSSION

Recent evidence indicates that PKC$\alpha$ can direct activation of cSrc, and this activation requires the presence of AFAP-110 (5, 7, 15). On activation, PKC$\alpha$ will bind to, and direct an increase in serine/threonine phosphorylation of, AFAP-110 (35). This interaction affects a conformational change on AFAP-110 that relieves intramolecular interactions that autoinhibit AFAP-110 function (38). In response to these signals, AFAP-110 then colocalizes with and directs activation of cSrc. Colocalization required the integrity of the PH1 domain. Here, we show that colocalization appears to occur in the perinuclear region of the cell where cSrc resides in an inactive state on endosomal vesicle membranes. This is demonstrated by coexpressing AFAP-110 with the temperature-sensitive vSrc construct, LA29, and showing that colocalization does not occur at a nonpermissive temperature; however, when PMA is applied to the cells, colocalization occurs at the perinuclear membrane. In this system, LA29 remains perinuclear even on colocalization with AFAP-110. We predict that LA29 remains inactive regardless of contacting AFAP-110, as mutations rendering it temperature sensitive would be dominant over the ability of AFAP-110 to engage the LA29 SH3 domain and induce activation. These mutations are found in the catalytic domain and likely govern access of ATP to the ATP binding site in the catalytic domain of LA29. Therefore, we predict that PMA directs AFAP-110 to move to cSrc. Thus AFAP-110 and cSrc likely colocalize on the membranes of perinuclear vesicles, where inactive cSrc resides. Colocalization is dependent on the integrity of the PH1 domain, while activation is dependent on the ability of AFAP-110 to bind to the cSrc SH3 domain through its SH3 binding motif. The result of cSrc activation is a change in actin filament integrity and the formation of ventral membrane-associated structures predicted to be either podosomes or precursors to invadopodia (15, 31). In this report, we sought to determine what cellular signals promote colocalization between AFAP-110 and cSrc and subsequent activation of cSrc in response to PMA treatment or PKC$\alpha$ activation. The focus of this study was the role of PI3K in PMA-induced cSrc activation as mediated by AFAP-110. Reasons for this focus were as follows: 1) there is significant evidence for cross talk among PKC, cSrc, and PI3K; 2) the integrity of the PH domain in AFAP-110 is required for colocalization with cSrc; and 3) the PH domain has the capability to bind PI3K-generated phospholipids.

The data presented in this manuscript indicated that PMA, and by extension PKC$\alpha$, has the ability to induce activation of PI3K. The ability of PKC$\alpha$ to direct PI3K activation has been controversial. Although there is much evidence to indicate that PI3K can direct activation of PKC$\alpha$ via its ability to direct activation of PDK1 (12), it has not been clear whether PKC$\alpha$ can, in turn, act upstream and direct PI3K activation. Huang et al. (22) were able to demonstrate that PMA treatment of JB6 epidermal cells stimulated PI3K activation and AP-1 formation, whereas pretreatment with bisindolylmaleimide or LY294002 blocked AP-1 formation, indicating that PKC could contribute to PI3K activation in JB6 epithelial cells. However, further studies using a dominant-negative form of PKC$\alpha$ failed to abrogate the effects of PMA-directed activation of PI3K activation in this system, indicating that perhaps PMA induced...
Fig. 8. Both cSrc and PI3K are required for migration. A: Western blot analysis was performed to determine the expression levels of AFAP-110 of the Tet-Off cells 8–10 days following the removal of doxycycline and reprobing with anti-β-actin. B: SYF/cSrc, SYF, and p85−/− culture media were replaced with carbon dioxide-independent media. Cells were then left untreated or treated with 20 μM LY294002 following a scratch through the confluent monolayer. Distance migrated was measured using AxioVision 4.4 software (Zeiss), and averages were analyzed using Excel. Statistical significance was determined using a 1-way ANOVA (*P < 0.05; ±SD). C: uninduced control Tet-Off and induced (AFAP-110 and AFAP-110\(^{110}\)) Tet-Off clones were cultured in media in the presence (+) or absence (−) of doxycycline. The cells were washed, and media were replaced with carbon dioxide-independent media; then a scratch was made in the confluent monolayer. Distance migrated was measured using AxioVision 4.4 software (Zeiss), and averages were analyzed using Excel. Statistical significance was determined using a 1-way ANOVA (*P < 0.05; ±SD).

activation of PKCε, which in turn activated PI3K (21). Our MEF cells do express low levels of PKCε (35); thus it is possible that PMA-directed activation of PI3K could occur as a result of novel PKC family activation. Thus, although PMA will stimulate activation of PI3K, it is possible that PMA could direct activation of PI3K via another classical or novel PKC family member. To determine whether PMA could activate PI3K in our MEF system, we employed a classic lipid kinase assay, which demonstrated upregulation of PI3K activity. To verify this result, we employed the anti-PtdIns-3,4,5-P\(_3\) antibodies and used immunofluorescence to demonstrate production of PtdIns-3,4,5-P\(_3\) in cells treated with PMA. These data indicated that both serum induction and PMA were able to direct an increase in PtdIns-3,4,5-P\(_3\) production, similar to that observed in the lipid kinase assay. We observed that PtdIns-3,4,5-P\(_3\) production was consistently higher at 5 min compared with 15 min posttreatment. Interestingly, we noticed that serum appeared to induce production of PtdIns-3,4,5-P\(_3\) in both the perinuclear region and along the cytoplasmic membrane, whereas PMA seemed to only induce PtdIns-3,4,5-P\(_3\) production in the perinuclear region of the cell. Serum likely contains growth factors that can activate growth factor receptors as well as other cellular signals, while PMA directs activation of a more narrow spectrum of signaling proteins (14, 18, 46, 49). Thus serum could activate PI3K populations that exist at the cell periphery and along internal membranes, whereas PMA may direct activation of a more limited population of PI3K that might localize to internal membranes. Since inactive cSrc is found primarily along perinuclear vesicles (40), the ability of PtdIns-3,4,5-P\(_3\) to be generated in the perinuclear region of the cell may be consistent with activation of cellular signals that promote cSrc activation. PMA would direct activation of the classical and novel PKC isofoms. An inhibitor of PKC cata-
lytic activity, bisindoylmalide I, also blocked PMA-directed colocalization between AFAP-110 and cSrc, indicating that PMA was sending these signals via its capacity to activate a PKC family member’s catalytic activity. However, within our MEF cell system, PKCo is the only member of the classical and novel PKCs that is expressed that will also bind to AFAP-110 (15, 35). Our data also indicate that, in cells pretreated with the PI3K inhibitors LY294002 and wortmannin, PMA was unable to induce colocalization between AFAP-110 and cSrc. In support of this, PMA failed to direct colocalization between AFAP-110 and cSrc and cSrc activation in MEF cells that lack the p85α and β regulatory subunits of PI3K (4). In the absence of the p85 regulatory subunit, the p110 catalytic subunit is destabilized and degraded, in agreement with our observations (51). These data indicate that the presence of PI3K is required for PMA-directed colocalization between AFAP-110 and cSrc and subsequent activation of cSrc by PMA-110.

The link between PKC and PI3K has also been addressed. Most PKC isoforms can be activated by the PI3K effector PDK1, by phosphorylation of key regulatory serine/threonine residues within the activation loop of PKC, resulting in a confirmation change and subsequent activation (3, 29, 33). Furthermore, the additions of exogenous PI3K-generated lipid products were able to activate PKC by binding the C2 domain, resulting in the formation of motility structures such as lamellipodia (10). Thus we attempted to determine the ability of AFAP-110, cSrc, and PI3K to modulate cell motility using a scratch motility assay. Here, wounding has been shown to activate endogenous PKCs (34); thus we did not treat with PMA, as that would have been redundant. The data presented here indicate that effective migration of mouse embryo fibroblasts into a wound is dependent on both the presence of cSrc and the ability of AFAP-110 to associate with cSrc. Furthermore, cells that fail to express the p85 regulatory subunit exhibited reduced migration, similar to that observed when PI3K activity is inhibited.

These results raised the following question: how does PI3K mediate AFAP-110 and cSrc colocalization in response to PMA treatment? PH domains are self-folding, modular domains that interact with a variety of protein and lipid binding
partners, including PKC isoforms, small G proteins, and lipids (1, 50). Of the latter, many PH domains have been shown to bind phospholipids that are generated on activation of PI3K (e.g., PtdIns-3,4,5-P3). PH domains that interact with phospholipids have a binding groove that contains positively charged amino acids in the base of the groove. In theory, phospholipid binding would be coordinated by interactions between the negatively charged phospho-head group of the phospholipid with positive charges in the base of the groove, and could be further stabilized through hydrophobic interactions between the wall of the groove and the hydrophobic tail(s) of the lipid. The PH1 domain of AFAP-110 is most homologous with the PH domain found in Bruton’s tyrosine kinase, both of which also contain positively charged amino acids positioned to coordinate interactions with negatively charged phospholipid head groups (2). Indeed, we have demonstrated that the PH1 domain can bind to several phospholipids in vitro, including PtdIns-3,4,5-P3 (Jett J, Zet H, and Flynn DC, unpublished data). Work done by our laboratory (15) indicates that deletion of the PH1 domain blocked the ability of PMA to induce colocalization between AFAP-110 and cSrc and subsequent cSrc activation. Thus we hypothesize that the ability of AFAP-110 to colocalize with cSrc (in response to PKCα activation or PMA treatment) might be dependent on interactions with either protein binding partners or lipid binding partners that facilitate translocation to cSrc at perinuclear membranes. Collectively, these data indicate that when PMA directs activation of PKCα, PI3K is concomitantly activated, and these signals work cooperatively to direct AFAP-110 to move to and bind cSrc. Subsequently, cSrc is activated, and cells exhibit podosome formation and an increase in migratory potential. Thus we hypothesize that cSrc, PI3K, and AFAP-110 work together to promote migration of mouse embryo fibroblasts.

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PKC ACTIVATION OF cSrc


