Growth factor dependent AKT activation and cell migration requires the function of c-K(B)-Ras versus other cellular Ras isoforms.
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Running title: Cell migration requires K(B)-Ras and Calmodulin

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K-Ras negative fibroblasts are defective in their steady-state expression of MMP-2. This occurs through c-K(B)-Ras dependent regulation of basal levels of AKT activity. In this report, we have extended those studies to demonstrate that in the absence of K-Ras expression, PDGF-BB fails to induce significant AKT activation, though this was not the case in N-Ras negative cells. This phenotype was directly linked to PDGF-dependent cell migration. All of the independently immortalized K-Ras negative cells failed to migrate upon addition of PDGF. Only ectopic expression of c-K(B)-Ras, not c-K(A)-Ras or oncogenic N-Ras could restore both PDGF-dependent AKT activation and cell migration. Since most Ras binding partners can interact with all Ras isoforms, the specificity of PDGF-dependent activation of AKT and enhanced cell migration suggests these outcomes are likely to be regulated through a c-K(B)-Ras specific binding partner. Others have published that of the four Ras isoforms, only K(B)-Ras can form a stable complex with calmodulin (CaM). Along those lines, we provide evidence that (1) PDGF addition results in increased levels of a complex between c-K(B)-Ras and CaM and (2) the biological outcomes that are strictly dependent on c-K(B)-Ras (AKT activation and cell migration) are blocked by CaM antagonists. The PDGF dependent activation of ERK is unaffected by the absence of K(B)-Ras and presence of CaM antagonists. This is the first example of a linkage between a specific biological outcome, cell migration, and the activity of a single Ras isoform, c-K(B)-Ras.

Cell migration is the process through which cells detach from their extracellular substratum and move in a directional fashion, usually toward a specific chemoattractant or to fill a wound. This generally involves polarization of the cell and orientation toward the direction of cell migration. Following polarization, cells put out lamellipodia and filopodia forming new focal contacts in the direction of movement. These focal contacts then engage the intracellular actin network, which has been reorganized to accommodate cell movement. The connection between focal contacts and the intracellular actin network provide the traction necessary for movement. This is then accompanied by release of the ‘rear’ of the cells from the extracellular matrix. The molecular events involved in many of these steps have been well-characterized and are regulated by members of the Ras superfamily, those being the Rho family of GTPases, Rac, Rho and Cdc42. (1-3).

Reports suggest that PI-3 kinase activation is upstream of the Rho family of GTPases in the PDGF-dependent pathway that regulates cell motility (4,5). Other reports place PI-3 kinase activation downstream of these GTPases (6). These reports can be reconciled by the idea put forth by Weiner et al. Their data suggest the existence of a positive feedback loop between PtdInsP3 and Rho in which PtdInsP3 and the Rho GTPase function both upstream and downstream from one another. In their model, increasing the levels of PtdInsP3 may serve to both polarize cells and regulate Rho activity, which in turn may result in the activation of an additional subcellular PI-3 kinase pool (7). It is clear, however, that activation of both PI-3 kinase and AKT are critical events in the migratory machinery (8-14). Though the mechanism of AKT activation is incompletely understood, growth factor dependent activation of AKT is thought to occur through the Ras-dependent activation of PI-3 kinase, followed by the activation of at least PDK1 (15,16).

The Ras family of GTPases (Ha-, N-, K(A)- and K(B)-Ras) control many cellular processes, inclusive of those involved in
proliferation, transformation, differentiation, metastasis, and apoptosis. Ras mutations occur in approximately 30% of human tumors (17-19). Ras proteins can directly interact with, and therefore have the potential to regulate, at least 22 different proteins, including Raf-1, A-Raf, B-Raf, p110 isoforms (the catalytic subunits of PI-3 kinase) α, γ, and δ, RaLGDS, RGL and RGL2 (20). Expression of oncogenic Ha-Ras increases the speed of endothelial cell motility in the absence of a directional component (21). Inhibition of Ras function, by microinjection of neutralizing antibodies or the expression of dominant-negative forms, results in cells that are severely defective in directional movement (21,22). Oncogenic K-Ras maintains the cytoskeletal disruption necessary for decreased adhesion to the extracellular matrix, which contributes toward increased migration of HCT-116 colon carcinoma cells (23). The contribution of each cellular Ras isoform in the migratory machinery has yet to be clearly defined.

The identification of a second, regulatory Ras-GTP binding site on SOS has raised the possibility of Ras isoform cooperativity. This second Ras binding site on SOS is distal to the exchange site. This distal site binds only Ras-GTP and results in increased SOS-dependent exchange activity (24). This opens up the possibility that a single activated Ras molecule can trigger other Ras isoforms through its upregulation of SOS activity. Therefore, it is essential that we have a better understanding of each cellular Ras isoform so that we may appropriately assign function(s) to both the cellular and oncogenic gene products in regulating specific biological outcomes that contribute to oncogenicity. We have found that plasma membrane-associated c-N-Ras is in a single signaling module that regulates Raf-1/MEK/ERK cascade responses to acute extracellular signals (25). e-K(B)-Ras regulates the steady-state expression of MMP-2 through a PI-3 kinase/AKT dependent pathway, (26). These two observations support the notion that each cellular Ras isoform is likely to regulate specific arms of the Ras-signaling pathways. In the present report, we extend those studies and demonstrate that growth-factor dependent cell migration and activation of AKT depend solely on the function of c-K(B)-Ras. We also demonstrate that oncogenic N-Ras is unable to substitute for c-K(B)-Ras function in the PDGF-dependent activation AKT and increasing cell motility. This observation supports the concept that Ras isoforms regulate each other, possibility through the distal Ras-GTP binding site on SOS.

Materials and Methods:

Cell lines: Stable mouse fibroblast cell lines were generated from K-Ras knockout and control embryo fibroblasts as previously described (26,27). All cell lines were maintained in DMEM plus 10% fetal calf serum.

Materials: Recombinant PDGF-BB was obtained from Oncogene Research Products. LipofectAMINE and PLUS reagents were from Invitrogen. Transwell plates were purchased from Costar. Vectashield mounting medium with DAPI was from Vector Laboratories, Inc. CHAPS and BSA were from U.S. Biochemical Corp.

Pharmacological inhibitors: W12, W13, Calmidazolium, BAPTA/AM, LY 294002, PD 98059 and AKT inhibitor were from Calbiochem. U73122 and U73343 were obtained from Biomol. Treatments with pharmacological inhibitors were performed 30 min before PDGF-BB challenge.

Antibodies: Antibodies against ERK, pERK, K-Ras, N-Ras, Anti-v-H-Ras antibody (Y13-
pPDGFR-β (Tyr751), PLCγ-1, pPDGFR-β (Tyr1021) and PDGFR-β were obtained from Santa Cruz. Anti-Hsp90 antibody was obtained from BD Transduction Laboratories. Tubulin antibody was obtained from Sigma-Aldrich, St. Louis, MO. Anti-AKT and pAKT(Ser473) antibodies were obtained from Cell Signaling Technology, Inc. Anti-Calmodulin antibody was from Upstate.

Transfection and selection: All transfections were performed with LipofectAmine and PLUS reagents as per the manufacturers recommendations (Invitrogen). After transfection, G418 resistant clones were selected for growth in 800 µg/ml G418. The expression of target proteins was detected by immunoblotting.

Cell migration Assay: The surfaces of the multi-porous membrane of transwell inserts (Corning) were coated with 200 µg collagen to help cell attachment. The liquid was allowed to dry overnight under a laminar flow hood. The wells were washed with PBS before use. Cells were trypsinized and suspended in DMEM without FBS and washed once with DMEM+0.1%BSA. 2x10⁵ cells were suspended in 200 µl DMEM+0.1%BSA and loaded onto the top of each insert membrane. 800 µl DMEM+0.1%BSA was then added to each well and the cells were cultured in the absence of serum for the following 6 hours. Cells were stimulated with PDGF-BB to induce cell migration by adding PDGF-BB basolaterally at the indicated concentrations. After an overnight incubation, the cells remaining on the top of the multi-porous membrane were carefully removed with cotton swabs. The migrated cells on the bottom surface of the membrane were stained using Vectashield mounting medium with DAPI and observed under a fluorescent microscope. Images were taken and the number of cells in 3 random fields were quantitated using Image Pro Plus software.

Preparation of lysates: After treatment, cells were immediately submerged in excess (2-4L) ice-cold PBS. Cells were scraped and collected by centrifugation (500xg for 5 minutes). The cell pellet was resuspended in 1Xp21 lysis buffer (20mM MOPS, 5mM MgCl₂, 0.1mM EDTA, 200mM sucrose [pH 7.4] containing 1% CHAPS, phosphatase and protease inhibitors (30 mM β-glycerophosphate, 5mM p-nitrophenyl phosphate, 1mM each phosphoserine and phosphothreonine, 0.2mM phosphotyrosine, and 100 µM sodium orthovanadate, 25µg/µl aprotinin, leupeptin pepstatin A and 1mM PMSF). The cell lysates were centrifuged at 13,000 x g for 10 min to remove nuclei and cell debris. The supernatant solution was retained for further experiments. The protein concentration was determined by the method of Bradford (28).

Immunoblotting: Lysates containing equal amounts of protein (100µg unless otherwise specified) were separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane (PVDF) (Hybond P, Amersham/Pharmacia Biotech.). The membrane was blocked with Blocker Casein in PBS (Pierce Chemical Co.) containing 2% newborn calf serum (Life Technologies, Inc.-Invitrogen). The washed blots were incubated with primary antibodies (1:1000) for 2 to 3 h at room temperature or overnight at 4°C. Following washing in TBS-0.1% Tween, the blots were incubated with horseradish peroxidase labeled secondary antibodies (1:1,000) for 1 h at room temperature. After washing, the signals were detected using standard enhanced chemiluminescence (ECL) techniques. The signals were quantitated using a Microtek scanner and NIH Image 1.60b7 and normalized to untreated controls.

Immunoprecipitation: The cells were lysed in 1Xp21 lysis buffer containing 100nM CaCl₂. The indicated antibodies (5µg) were
pre-coupled to Protein G/A Sepharose (PG/AS) beads for 1 hour. The pre-coupled PG/AS beads were incubated with cell lysate (800 µg) for 3 hours (4°C). The beads were washed 3 times with 1x p21 lysis buffer containing 100nM CaCl₂ and 15mM MgCl₂. 2x sample buffer was added (80µl), the samples heated (100°C, for 15 minutes) and analyzed by SDS-PAGE and immunoblotting. **AKT activity assay:** The AKT activity in each sample was determined using the AKT activity assay kit from Cell Signaling Technology. Briefly, cells were lysed in 1x Cell Lysis Buffer. An overnight immunoprecipitation (800 µg) of AKT was performed with a mouse anti-AKT antibody. An in vitro kinase assay was performed using GSK-3 Fusion Protein as the substrate. The amount of phosphorylated GSK-3 was detected by immunoblotting for Phospho-GSK-3alpha/beta (Ser21/9) using the included specific antibody. **Migration assay with siRNA treatment:** Cells were transfected in OptiMEM (Gibco/Invitrogen) with 300nM SMARTpool siRNA against PLC-γ1 (Dharmacon) or 300nM negative control #1 siRNA (Ambion) using siPORT NeoFX transfection reagent (Ambion) as directed by the manufacturer. Transfected cells were incubated for 24 hours. Cells for the migration assay were trypsanized and plated in the apical chamber of migration wells, as described above, with the addition of 300nM SMARTpool siRNA against PLC-γ1 or negative control #1 siRNA using siPORT NeoFX transfection reagent. The migration assay was performed as described above. Cells used for protein analysis were transfected a second time with 300nM SMARTpool siRNA against PLC-γ1 or negative control #1 siRNA using the siPORT NeoFX transfection reagent. Following an additional 24 hour incubation, cells were harvested by scraping and protein levels of PLC-γ1 were determined by western blot as described above.

**Results:**

K-Ras negative fibroblasts show defects in PDGF-BB-induced activation of AKT but not ERK.

Previous data from this laboratory documented that c-N-Ras is the preferential binding partner of Raf-1, even in the presence of an oncogenic Ha-Ras protein. Downregulation of c-N-Ras resulted in the parallel decrease in ERK activation in Ha-Ras transformed fibroblasts (25,29,30). These observations suggested to us that other c-Ras isoforms might regulate growth factor dependent activation of non-Raf-1 dependent signaling mechanisms. To explore this possibility, we tested whether K-Ras negative (expressing c-N-Ras, not c-Ha-Ras, c-K(A)-Ras nor c-K(B)-Ras (26)) cells were defective in the growth factor-dependent activation of AKT. In the absence of c-K-Ras, murine fibroblasts demonstrated a significantly decreased phosphorylation of AKT compared to wild-type cells when challenged with either PDGF-BB or EGF (Figures 1A and B). This appears to be an isoform-specific event, given that N-Ras negative cells activated AKT in a manner identical to the control cells when exposed to PDGF-BB (Figure 1A) and EGF (data not shown). All the cell lines possess roughly equivalent amounts of total AKT (Figure 1A). The data shown in Figure 1A suggest that not only do the K-Ras negative cells only activate AKT to a minimal degree upon a growth factor challenge, they also possess significantly reduced levels of steady-state AKT activity. This supports our previous work demonstrating a specific K-Ras requirement to generate MMP-2 through the steady-state regulation of AKT activity (26). Control experiments revealed normal levels of PDGFR phosphorylation and activation of the ERK pathway in the K-Ras negative cells (Figures 1C & D). These data suggest that the PDGFR tyrosine phosphorylated docking

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sites and the PDGF-dependent activation of ERK, through Raf-1, are intact in the K-Ras negative fibroblasts.

The data in Figure 1 demonstrate that the PDGF-BB and EGF-dependent phosphorylation of AKT requires the presence of at least one isoform of c-K-Ras. To identify the isoform, we challenged wild-type fibroblasts (K+/+), K-Ras negative fibroblasts (K-/-), and fibroblasts restored with either myc-tagged c-K(A) or myc-tagged c-K(B) or those expressing an oncogenic N-Ras (K61N), with 2ng/ml PDGF-BB for 5 minutes (Figure 1E). Phosphorylation of AKT was determined by western analysis of cell lysates. Neither the K-Ras negative, c-K(A) restored or the K-Ras negative cells expressing an oncogenic N-Ras produced robust phosphorylation of AKT. The c-K(B) restored cell lines, however, phosphorylated AKT similar to the control cells upon PDGF addition. These data demonstrate that c-K(B)-Ras is necessary for PDGF-BB-dependent phosphorylation of AKT. These data also demonstrate that oncogenic N-Ras does not substitute for c-K-Ras in promoting PDGF-induced AKT activation, suggesting that the expression of an oncogenic Ras protein does not necessarily mimic the functions of all other Ras isoforms.

**K-Ras knockout mouse fibroblasts showed defects in PDGF-BB-dependent cell migration.**

Activation of AKT is an essential component in the cell migration machinery (8-14). We designed experiments to test whether PDGF-dependent cell migration specifically required c-K(B)-Ras function. We first set out to optimize a migration assay based on the addition of PDGF to quiescent cells. We used a standard Boyden chamber assay, as described in the Materials and Methods section. Cells were plated on collagen coated wells in the morning in serum-free media with PDGF added at the indicated concentrations in late-afternoon. The following day, the non-migrated cells were removed and the migrated cells DAPI stained and quantitated microscopically. The data shown in Figure 2A and B demonstrate the number of migrating cells increases linearly with respect to increasing PDGF to approximately 10ng/ml. Using this optimized migration assay, we tested whether PDGF-dependent cell migration required the function of c-K(B)-Ras. Addition of PDGF-BB increased the number of control cells that migrated, without a significant effect on the number of migrating K-Ras negative cells. This was apparent in all independently derived, control and K-Ras negative cell lines tested. N-Ras negative cell lines, developed in parallel with the K-Ras negative cells, possessed levels of migration consistent with the normal range observed in the control K-Ras expressing cell lines (Fig. 2C). These data suggest that at least one of the c-K-Ras isoforms is a necessary component of the cell migration machinery. Stable cell lines expressing N-terminally myc-tagged c-K(A)-Ras, c-K(B)-Ras or an oncogenic N-Ras (K61) were tested for their ability to migrate upon a PDGF challenge (Figure 2D). Only those cells expressing myc-c-K(B)-Ras were able to migrate in response to PDGF addition. The data presented in Figures 2C and D indicate that the basal rates of migration vary between the different immortalized cell lines and within the context of each given experiment. The important issue is whether the addition of PDGF increases migration compared to its absence for each specific cell line, not whether the migration of each cell line is different in the absence of PDGF. PDGF stimulates at least a 2 fold increase, and in most cases 3-4 fold, in the number of migrating cells in all the K+/+ cell lines, the N/-/- cell line and the K/-/- restored with c-K(B)-Ras. In all the other cell lines, PDGF was unable to stimulate a significant increase.
in the number of migrating cells compared to the basal rate of migration in the absence of PDGF. The data support the hypothesis that growth factor dependent signal transduction bifurcates at the level of cellular Ras isoforms, with c-K(B)-Ras being directly coupled to AKT activation and the biological outcome of cell migration.

**PDGF stimulation enhances the formation of a CaM-K-Ras complex that requires calcium.**

The selectivity for c-K(B)-Ras in both PDGF-dependent activation of AKT and enhanced cell migration suggests that c-K(B)-Ras signals these events through a unique binding partner. Others have shown that each Ras isoform is able to bind any of the well-characterized putative target molecules (20). We have also shown that overexpression of oncogenic Ha-Ras can promiscuously signal through Raf-1, even though c-N-Ras is constitutively associated with Raf-1 at the plasma membrane (25,29,30). The inability of oncogenic N-Ras to substitute for c-K(B)-Ras function suggests that c-K(B)-Ras regulates AKT activation and cell migration through a unique target that fails to bind to N-Ras. c-K(B)-Ras and CaM (calmodulin) form a stable, GTP-dependent complex (31-33). Other Ras isoforms are unable to form a similar complex with CaM (31), making the interaction between c-K(B)-Ras and CaM a likely candidate for the target that mediates PDGF-dependent AKT activation and cell migration through c-K(B)-Ras.

We then tested, therefore, for a PDGF-enhanced stable complex between endogenous K-Ras and CaM by co-immunoprecipitation (Figure 3). We used the rat monoclonal antibody, Y13-238, that has been reported to immunoprecipitate K-Ras without reacting with endogenous N-Ras (32). Because these cells do not express detectable levels of c-Ha-Ras (26), Y13-238 becomes a K-Ras specific immunoprecipitating antibody. In addition to Y13-238, we also used the mouse monoclonal antibody specific for K-Ras (clone F234) to test for the presence of K-Ras:CaM complex. Cells were challenged with PDGF-BB for 5 minutes, lysed and the soluble lysate immunoprecipitated with the indicated antibodies. The data shown in Figures 3A & B demonstrate that acute stimulation of cells with PDGF-BB results in the formation of a complex between K-Ras and CaM. This is the first demonstration of a growth factor-mediated complex formation between endogenous CaM and c-K-Ras. The data shown in Figures 3A and B, while providing different interpretations of the unstimulated situation, both demonstrate a clear increase in the CaM signal associated with the K-Ras immunoprecipitates following a PDGF challenge. This might result from the two antibodies recognizing different epitopes within c-K(B)-Ras and the possibility that there are significant changes in the constituents of the complex between unstimulated and stimulated. For instance, the data with the mouse K-Ras specific antibody suggests that there is a K-Ras:CaM complex in the quiescent cell population, similar to our reported detection of a steady-state complex between c-N-Ras and Raf-1(25). It is possible that the epitope recognized by the rat monoclonal Y13-238 is inaccessible within this complex and becomes available following PDGF stimulation which might recruit other proteins, such as PI-3 kinase, to the K-Ras:CaM complex.

Others have shown that the association between CaM and K(B)-Ras-GTP is calcium dependent in *in vitro* experiments (31). We tested whether increased intracellular calcium was required for the PDGF-dependent increase in abundance of the CaM:K-Ras complex. Cells were preincubated with either BAPTA/AM or the PI-3 kinase inhibitor LY294002, challenged with PDGF and tested for the presence of CaM in K-Ras specific immunoprecipitates (Figure 3C). As in
Figures 3A and B, challenging cells with PDGF resulted in increased abundance of CaM in the K-Ras immunoprecipitates. This increase was virtually completely blocked by treatment with BAPTA/AM, though no differences were seen with the LY294002 pretreatment. These data suggest that increased intracellular calcium contributes to the PDGF-dependent enhancement of a complex between CaM and K-Ras, though being independent of PI-3 kinase activity. This is the first demonstration that growth-factor dependent increases in intracellular calcium contribute to the association between Ras and any of its known binding partners.

**Growth factor dependent activation of AKT is blocked by CaM antagonists.**

Our data suggest a coincident link between c-K(B)-Ras, CaM and the PDGF-dependent activation of AKT. We tested, therefore, whether AKT activation by PDGF-BB and EGF required CaM. We first tested whether the well-characterized CaM inhibitor W13 could selectively block AKT activation. ERK activation, which is resistant to the absence of K-Ras isoforms was used as the internal, negative control. We confirmed, by both Western analysis (Figure 4A) and an AKT activity assay (Figure 4B), that W13 potently inhibited PDGF-BB-dependent AKT activation without altering the level of ERK phosphorylation (Figure 4C). The chemically-related, but much less potent, W12 was ineffective in decreasing PDGF-BB-dependent AKT activation (Figure 4A & B). The involvement of CaM in the activation of AKT is consistent with previous reports that CaM can directly regulate the activation of PI-3 kinase (34). Treatment of cells with W13 did not alter the levels of PDGFR phosphorylation, including that of Tyr751, the docking site for the p85 regulatory subunit of PI-3 kinase (Figure 4C). This places CaM function downstream of receptor-mediated tyrosine phosphorylation of the PDGFR. Similar experiments revealed that EGF-dependent activation of AKT required CaM function (Figure 4D). The PI-3 kinase inhibitor, LY294002, was used as a positive control. As expected, pretreatment with LY294002 completely blocked EGF-dependent AKT activation without altering EGF-dependent ERK activation (Figures 4D and E). W13 and an unrelated CaM inhibitor, calmidazolium (CMZ) both effectively blocked EGF-dependent AKT activation, paralleling the data obtained with PDGF-dependent AKT activation.

**Growth factor dependent activation of AKT requires intracellular calcium and phospholipase Cγ.**

The results described above suggest that growth factor dependent activation of AKT requires CaM function. We tested, therefore whether growth-factor dependent activation of AKT also requires intracellular calcium and phospholipase Cγ activity. Pretreating cells with BAPTA/AM, a cell permeable calcium chelator or U73122 (a phospholipase Cγ inhibitor) was sufficient to block PDGF-dependent AKT activation (Figures 5A and B). The chemically-related inactive U73343 did not affect the ability of PDGF to increase AKT phosphorylation (data not shown). Treating cells with either U73122 or BAPTA/AM did not affect the activation of ERK by either PDGF or EGF (data not shown). These data suggest that the activation of AKT by growth factors requires K(B)-Ras, CaM and intracellular calcium, likely generated through enhanced phospholipase Cγ activity.

**PDGF-dependent cell migration is abrogated by CaM, calcium and phospholipase Cγ antagonists.**
The data presented to this point implicate a K(B)-Ras:CaM dependent process that results in growth factor dependent activation of AKT. We then tested whether blocking PDGF dependent CaM activity, which blocks AKT activation, also inhibited cell migration. Since these assays extend over greater than a 16 hour time frame, we performed preliminary experiments to reduce any possible inhibitor toxicity. The levels of each specific inhibitor used in the migration assay were found to not be toxic over the time frame of the migration assay (data not shown). In some cases, such as the phospholipase Cγ inhibitor, these concentrations were significantly lower than those used to examine the acute stimulation of AKT by PDGF. In all cases, however, migration was significantly retarded by the presence of W13, BAPTA/AM and U73122 (Figure 6A). In separate experiments, neither of the inactive analogs, W12 or U73343, reduced the number of migrating cells compared to the PDGF alone samples (data not shown). To confirm the involvement of PLCγ in cell migration, we down-regulated PLCγ-1 expression using siRNA as described in the Materials and Methods section. Preliminary dose-response experiments demonstrated that 300nM siRNA was necessary to acquire sufficiently reduced protein levels of PLCγ-1 (data not shown). Control K-Ras expressing cells were then tested for their ability to migrate in response to PDGF in the absence of PLCγ-1 expression (Figure 6B). The data demonstrate that under the conditions of the migration assay and presence of the PLCγ-1 specific siRNA, there is significantly reduced levels of PLCγ-1 protein (see inset). In the absence of PLCγ-1, PDGF was unable to induce significant cell migration, while treatment with the negative control siRNA had no statistically significant affect on PDGF dependent cell migration. These data confirm the role of PLCγ PDGF-dependent cell migration. Others have previously shown that blocking AKT reduces cell migration, an observation that was also true in our PDGF-induced fibroblast model (Figure 6C). Migration of control cells and K-Ras negative cells was unaffected by the presence of W13 in the absence of PDGF (data not shown). These data suggest that the contribution of CaM function to cell migration is only apparent in the presence of c-K(B)-Ras expression.

**Discussion:**

The distribution of Ras isoforms within specific micro-domains of the plasma membrane is not uniform. Ha-Ras is most commonly associated with the cholesterol-enriched caveolar structures, though K-Ras is generally associated with the less organized structures of the plasma membranes (35). N-Ras appears to distribute between caveolar and non-caveolar plasma membrane structures (36). There is now ample published data to suggest that Ras proteins exist in subcellular membrane compartments other than the plasma membrane (37-40). These data suggest that each Ras isoform is likely to have multiple functions, dependent on their distribution within specific microdomains and the availability of specific binding partners within these domains. The identification of Ras isoforms within endomembrane structures also suggests that each Ras isoform is expected to possess a number of unique binding partners that are likely to be defined by their co-localization to Ras-containing endomembrane structures. Previous work from this laboratory identified a steady-state complex between c-N-Ras, Raf-1 and PKCε at the plasma membrane (25). This complex appears to regulate the activation of Raf-1 and ERKs through receptor mediated tyrosine kinase activity and was the first specific Ras signaling module identified (25,30). c-N-Ras distributed to other subcellular locations is likely to possess functions unrelated to the activation of Raf-1
and ERKs (Wolfman et al, unpublished data). These observations suggest that each subcellular Ras isoform specific pool is predicted to provide a unique function contributing to specific biological outcomes.

Our data support this prediction. Cells failing to express c-K-Ras are completely defective in their migratory responses to PDGF-BB addition. K-Ras negative cells failed to migrate and activate AKT when challenged with either PDGF or EGF (data not shown). Migratory activity was restored by the expression of c-K(B)-Ras and not c-K(A)-Ras or oncogenic N-Ras, demonstrating that this isoform-specific pathway is necessary for cell motility. The absence of c-K-Ras expression did not compromise PDGF-BB dependent activation of ERK 1/2, suggesting that c-K-Ras function is not involved in regulating ERK activation. This observation is predictable based on our previous work demonstrating an N-Ras-specific steady-state complex with Raf-1. We also established that c-N-Ras was required for the constitutive activation of Raf-1 and ERKs by the expression of an oncogenic Ha-Ras (29). Not only does c-K(B)-Ras regulate the steady-state level of activated AKT (26), it also appears to be the sole c-Ras isoform that is responsible for the activation of AKT upon a PDGF-BB or EGF challenge.

One of the most interesting and novel aspects of the work described in this reports is the inability of oncogenic N-Ras to substitute for c-K(B)-Ras in the PDGF-dependent activation of AKT and increased cell migration. Work by others demonstrates that the majority of Ras binding partners can interact with each of the individual Ras isoforms (20). These experiments were performed in the context of both in vitro and transient overexpression assays. Our previous work documented that c-N-Ras is the preferential target for Raf-1 at the plasma membrane, through overexpression of oncogenic Ha-Ras did form promiscuous complexes with Raf-1 (25,29). Taken together, these data suggest that overexpression of an oncogenic Ras isoform can signal through common Ras targets. The inability of oncogenic N-Ras, in this case, to substitute for c-K(B)-Ras function, suggested that c-K(B)-Ras utilizes a unique binding partner that does not interact with N-Ras. The only Ras binding partner that fits this description is CaM (20,31). The parallel inhibition of both AKT activation and cell migration by inhibitors of CaM function are consistent with the hypothesis that a c-K(B)-Ras:CaM complex is the driving force behind growth-factor-dependent AKT activation. CaM function appears to be unnecessary for cell migration in the absence of PDGF (data not shown). Therefore, the only CaM dependent interaction present in the control cells compared to the K-Ras negative cells is that between K(B)-Ras and CaM. This supports the idea that the K(B)-Ras:CaM complex is the missing ingredient in the migratory machinery in the K-Ras negative cells. We do not, however, interpret this to suggest that, following an acute activation of quiescent cells, all the c-K(B)-Ras is complexed with CaM and functions only to activate AKT. We feel it is more likely that only a small fraction of the c-K(B)-Ras-GTP binds to CaM. Other pools of newly activated c-K(B)-Ras probably have other binding partners that are dependent on their specific micro-environments. The specificity defined by their micro-environments is likely to reflect not only differences in membrane structure (caveolae versus bulk plasma membrane), but also co-localization of each Ras isoform and their putative binding partners to similar spatial regions within endomembrane structures.

Others have identified a high affinity CaM target sequence conserved in the C-terminal region of all the p110 isoforms (the catalytic subunit of PI-3 kinase), distal from the N-terminal Ras binding domain within
each p110 subunit (41). This would suggest the formation of a complex between c-K(B)-Ras, CaM and PI-3 kinase, similar to the ternary complex we previously identified between c-N-Ras, Raf-1 and PKCε (25). Another possibility is presented by the reported that CaM itself can dimerize through a domain that is external to its calcium dependent interaction with target proteins (42). These two possibilities might reconcile the observation that CaM can directly activate PI-3 kinase (34) with our data that a K(B)-Ras:CaM complex is involved in the activation of AKT. It is also possible that the CaM dependent activation of AKT and its role in cell migration might be completely independent of the function of c-K(B)-Ras in these processes.

The ability of Ras isoforms to ‘talk to each other’ by the up-regulation of SOS activity through its distal, regulatory Ras-GTP binding sites has added complexity in assigning Ras isoform-specific binding partners and biological outcomes (24). Ras-GTP regulation of SOS activity opens the possibility that expression of an activated Ras-isoform, in an attempt to dissect its isoform-specific signaling pathways, could result in crosstalk to endogenous c-Ras proteins, thereby complicating any experimental interpretation. In addition to SOS, the Ras-GRF family of exchange factors can be activated through a calcium/CaM dependent interaction (43,44). In a paradigm parallel to that described for SOS, the formation of a PDGF-BB-induced complex between K-Ras and CaM raises the possibility that an effector bound Ras-GTP molecule might upregulate the activity of a Ras-exchange factor, thereby propagating a ‘Ras-GTP wave’.

Overexpression of oncogenic N-Ras is unable to substitute for K(B)-Ras in the regulation of cell migration (Figure 2D) and MMP-2 expression (data not shown). This report also demonstrates that expression of oncogenic N-Ras in the K-Ras negative cells also does not restore the ability of these cells to activate AKT upon a PDGF challenge (Figure 1E). These observations highlight the specificity of specific signaling pathways with each Ras isoform, whether cellular or oncogenic. Expression of a single oncogenic Ras isoform, however, does result in the generation of a fully transformed phenotype, including increased cell migration, AKT and ERK activity, systems we have documented to be regulated through the actions of distinct Ras isoforms (K(B)-Ras and N-Ras). Since we have also provided data suggesting overexpression of oncogenic N-Ras does not substitute for c-K(B)-Ras, enhanced cell migration in cells transformed by either an oncogenic Ha-Ras or N-Ras is likely to arise from activation of c-K(B)-Ras induced by the expression of the alternative oncogenic Ras isoform.

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**Figure legends:**

**Figure 1:** K-Ras expression is required for PDGF-BB-dependent phosphorylation of AKT. **A:** The indicated cell lines were tested for their ability to generate phosphorylated AKT following a 5-minute challenge with PDGF-BB. Cells cultures were incubated overnight in the absence of serum. Following a 5-minute incubation with the indicated concentration of PDGF-BB, cells were lysed and analyzed for the presence of pAKT (473) and total AKT by immunoblot analysis. **B:** The indicated cell lines were tested for the ability to generate phosphorylated AKT and ERK as described above following a 5 minute stimulation with EGF (10ng/ml). **C:** The indicated cell lines were challenged with PDGF-BB and the levels of pERK and total ERK determined as described in the Methods section. **D:** The indicated cell lines were challenged for 5 minutes with PDGF-BB and analyzed for the presence of phosphorylated PDGFR (Tyrosine 1021) and total levels of PDGFR. **E:** The indicated cell lines were tested for their ability to generate phosphorylated AKT following a 5-minute challenge with PDGF-BB, as in Figure 1A. K+/+, wild-type fibroblasts; K-/-, K-Ras knockout fibroblasts; N-/-, N-Ras knockout fibroblasts; K-/- myc-K(A), K-/- fibroblasts expressing myc-K(A); K-/- myc-K(B), K-/- fibroblasts expressing myc-K(B); K-/- K61N, K-/- fibroblasts expressing oncogenic N-Ras (K61 mutation). The levels of the myc-tagged K-Ras proteins and the K61N-Ras are shown in the westerns on the right of the figure.

**Figure 2:** K-Ras negative cells fail to migrate upon addition of PDGF-BB. **A:** Control K-Ras expressing murine fibroblasts, seeded in transwell migration inserts as described in the methods section, were challenged with the indicated concentrations of PDGF-BB. Following an overnight incubation, non-migrating cells were removed and the migrated cells stained with DAPI. The images are representative of the entire field. Three random fields of each condition were quantitated using Image Pro Plus software. The number of migrating cells was found to increase linearly to a dose of 10ng/ml PDGF. **B:** The number of migrated cells from at least three separate chambers in A were counted as described in the Materials and Methods section. **C:** Individually immortalized control (K+/+), K-Ras negative (K-/-) and N-Ras negative (N-/-) cell lines were tested for PDGF-BB-dependent migration (2ng/ml) as described in the methods section. Each condition was performed in triplicate. The data were quantitated using Image Pro Plus software and displayed ± standard deviation. The data shown are representative of at least 3 separate determinations. Students T-Tests were performed (2-tailed, paired). * Treated versus Control, p < 0.01. **D:** Wild-type (K+/+), K-Ras knockout (K-/-), and K-/- fibroblasts restored with K61N-Ras (K-/- K61), myc-tagged K(A) (K-/-KAmyc), or myc-tagged K(B) (K-/-KBmyc), were stimulated to migrate in the presence of 0, 5, or 10ng/ml PDGF-BB. Following an overnight incubation, non-migrating cells were removed, migrated cells were stained with DAPI and quantitated using Image Pro Plus software ± SE. Each point was performed in triplicate. Students T-Tests were performed (2-tailed, paired). * Treated versus Control, p < 0.01.

**Figure 3:** PDGF enhances the formation of a calcium-dependent complex between CaM and K(B)-Ras. Control K-Ras expressing cells were challenged with PDGF-BB (2ng/ml) for 5 minutes, lysed and immunoprecipitated with the either a K-Ras specific mouse monoclonal (A) or the rat monoclonal Y13-238 (B) antibodies as described in the Methods section. The immunoprecipitates were thoroughly washed and specifically associated proteins analyzed by SDS-PAGE (15%) and immunoblotted for the presence of CaM. Fifty micrograms of lysate was separated by SDS-PAGE and used as an internal standard to mark the migration of CaM. This is representative of at least 3
separate experiments. C. Control K-Ras expressing cells were pretreated with either 50µM BAPTA-AM or 20µM LY294002 30 minutes prior to the addition of PDGF-BB (2ng/ml). Cells were harvested after 5 minutes, lysates generated and immunoprecipitated with Y13-238 as previously described. The immunoprecipitates were washed and analyzed for the presence of CaM and SDS-PAGE and immunoblotting.

**Figure 4:** CaM inhibitors block PDGF-BB-dependent migration and AKT activation. A: Quiescent cultures of control K-Ras expressing cells were pretreated for 30 minutes with the indicated concentrations of the potent CaM inhibitor W13 or its less potent analog W12. The cultures were challenged with PDGF-BB and analyzed for the generation of PDGF-BB-dependent phosphorylation of AKT. The immunoblots were quantitated using NIH image and expressed as a percent of the PDGF signal. The data represent the average of two separate ± standard deviation. B: Cells pre-treated with 15µg/mL W13 or W12 were challenged with PDGF-BB and assayed for AKT activity as described in the Methods section. The data shown are representative of 3 independent experiments. C: Control K-Ras expressing cells were pretreated with the indicated CaM antagonists, lysed and analyzed for the presence of the indicated signaling components. Immunoblotting for Hsp90 was used as a loading control. D. Quiescent control K-Ras expressing cells were treated with 10µM LY294002, 15µg/ml W13, 10µM calmidazolium, and 10µM BAPTA/AM. Cells were challenged with EGF (10ng/ml) and analyzed for the presence of pAKT and AKT as described in the Materials and Methods section. The data are representative of 2 separate experiments. E. Identical samples as those described for D were analyzed for the presence of p-ERK and ERK.

**Figure 5:** Growth factor dependent AKT activation requires intracellular calcium and phospholipase Cγ activity. A: Serum-starved control K-Ras expressing cells were pretreated with the indicated concentrations of BAPTA/AM. Cells were challenged with PDGF-BB (2ng/ml for 5 minutes) and analyzed for the PDGF-BB-dependent increase in pAKT as described in the Methods section. Equal amounts of protein (100µg) were loaded into each lane. Hsp90 was used as a loading control. B: Serum-starved control K-Ras expressing cells were pretreated for 30 minutes with the indicated concentrations of the phospholipase C inhibitor U73122. Cells were challenged with PDGF-BB (2ng/ml for 5 minutes) and the level of phosphorylated AKT determined by immunoblot analysis. Equal amounts of protein (100µg), as in A, were loaded into each lane.

**Figure 6:** PDGF-dependent cell migration requires intracellular calcium, CaM and phospholipase Cγ activity. A: Control K-Ras expressing cells were left untreated or treated with either PLCγ-1 specific siRNA or a negative control siRNA as described in the Materials and Methods section. Migration assays were performed and the number of migrated cells quantitated as previously described. The level of PLCγ-1 protein was analyzed by immunoblot analysis as shown in the inset. The data represent the mean of 3 separate determinations ± SD. B: PDGF-dependent migration was assessed in the presence of the AKT inhibitor (10µM) as described in Figure 2A. C: Cells were plated as previously described for the migration assay. 30 minutes prior to the addition of PDGF, the inhibitors at the indicated concentrations were added. PDGF was then added and cells were allowed to migrate overnight. The number of migrating cells was determined as previously described. The data are averages of 6 individual samples ± standard deviation. A
student T-test was performed and the migration of samples in the presence of each of the inhibitors was significantly reduced (*p<0.05) compared to those in the presence of PDGF alone.
Figure 1:

A:

|       | K+/+(1) | K+/+(2) | K-/- (1) | K-/- (2) | N-/- |
|-------|---------|---------|----------|----------|------|
| -     | -       | -       | -        | -        | -    |
| +     | +       | +       | +        | +        | +    |

- PDGF (2ng/mL)
- pAkt
- Akt

B:

|       | K+/+ | K-/- |
|-------|------|------|
| -     | -    | -    |
| +     | +    | +    |

- EGF (10ng/ml)
- pAKT
- pERK

C:

|       | K+/+(1) | K+/+(2) | K-/- (1) | K-/- (2) |
|-------|---------|---------|----------|----------|
| -     | -       | -       | -        | -        |
| +     | +       | +       | +        | +        |

- PDGF (2ng/mL)
- pERK
- ERK

D:

|       | K+/+(1) | K+/+(2) | K-/- (1) | K-/- (2) |
|-------|---------|---------|----------|----------|
| -     | -       | -       | -        | -        |
| +     | +       | +       | +        | +        |

- PDGF (2ng/mL)
- pPDGFRβ
- PDGFRβ

E:

|       | K+/+(1) | K+/+(2) | K-/- (1) | K-/- (2) |
|-------|---------|---------|----------|----------|
| -     | -       | -       | -        | -        |
| +     | +       | +       | +        | +        |

- Vector
- myc-K(A)-Ras
- myc-K(B)-Ras
- K-/-
- K-/- (K61N-Ras(1))
- K-/- (K61N-Ras(2))

- pAKT
- anti-myc IB
- anti-N-Ras IB
Figure 2:

A:

B:

C:

D:
Figure 3:

A:

| mouse IgG | mouse anti-K-Ras | Lysate |
|-----------|------------------|--------|
| -         | +                | -      |
|           |                  | +      |

PDGF
- Light chain
- CaM

IB: CaM

B:

| Lysate | Rat IgG | Y13-238 |
|--------|---------|----------|
| -      | +       |          |

PDGF
- Light chain
- CaM

IB: CaM

C:

| PDGF | BAPTA/AM | LY294002 |
|------|----------|----------|
| -    | +        | +        |
|      |          |          |

Lysate

Light chain
CaM
Figure 4:
A.

B.

C.

D.

E.
Figure 5:

A.

B.

PDGF - + + + + 50 25 10 5 BAPTA/AM (μM)
pAkt

Hsp90

PDGF - + + + + 100 50 20 10 U73122 (μM)
pAkt
Figure 6:

A:

B:

C.
Growth factor dependent AKT activation and cell migration requires the function of c-K(B)-Ras versus other cellular Ras isoforms
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