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Fyn is Induced by Ras/PI3K/Akt Signaling and is Required for Enhanced Invasion/Migration

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Abstract

Src family kinases (SFKs) are frequently over-expressed and/or activated in human cancers, and play key roles in cancer cell invasion, metastasis, proliferation, survival and angiogenesis. Allosteric activation of SFKs occurs through well-defined post-translational mechanisms, however the SFK member Fyn is over-expressed in multiple human cancers (prostate, melanoma, pancreatic, glioma, chronic myelogenous leukemia) and the mechanism of increased Fyn expression is unclear. Since activation of Ras oncogenes is a common oncogenic event leading to the activation of multiple effector pathways, we explored if Ras could induce Fyn expression. Retroviral transduction of the human keratinocyte cell line HaCaT with oncogenic H-Ras dramatically up-regulated Fyn mRNA (>100-fold, $p < 0.001$), protein, and kinase activity without affecting Src levels or activity. Activation of Akt, but not MAPK or EGFR, was necessary and sufficient for induction of Fyn by H-Ras. Expression of active Fyn was sufficient to increase HaCaT cell migration and invasion, and the enhanced migration and invasion induced by H-Ras could be significantly blocked (70% reduction, $p < 0.01$) by knockdown of Fyn with a specific siRNA or inhibition of SFKs with PP2. In addition, expression of Fyn in MDA-MB-231 breast cancer cells was dependent on PI3K activity and was involved in their invasive phenotype. Thus, the Ras/PI3K/Akt pathway can account for Fyn over-expression in cancers, and Fyn is a critical mediator of the Ras-stimulated invasive cell phenotype. These results support the development of therapeutic strategies targeting Akt/Fyn pathway to block migration and invasion of tumor cells.

Keywords

Src-Family Kinases; Proto-Oncogene Proteins c-Fyn; Proto-Oncogene Proteins c-Akt; Genes; ras; Tumor Cell Invasion; Squamous Cell Carcinoma

INTRODUCTION

Members of the Src family of non-receptor tyrosine kinases are oncogenic and promote neoplastic progression when activated or over-expressed (1). Many human cancers have elevated SFK activity, and small molecule inhibitors of SFKs have enjoyed clinical success against multiple cancer types (2). SFKs are key signaling intermediates for receptor tyrosine kinases, cytokine receptors, and integrin extracellular matrix receptors. SFKs are activated by receptors primarily via protein-protein interactions involving their SH2 domains, and are negatively regulated by phosphorylation on a conserved carboxyl-terminus tyrosine by Csk

(3). SFKs are required for a variety of biological processes, especially cancer cell migration and invasion due to their role regulating cell-matrix adhesion, cell-cell adhesion and actin reorganization. Numerous targets for Src kinases substrates are implicated in cell-matrix adhesion and migration, including focal adhesion kinase (FAK) and other cytoskeleton regulatory proteins (2).

In addition to being activated by growth factor receptor/integrin signaling, some SFKs are over-expressed in human cancers. For example, Fyn is up-regulated at the mRNA level in prostate cancer, glioma, melanoma and imatinib-resistant chronic myelogenous leukemia (4–7). The mechanisms of SFK over-expression in cancer are poorly understood, but are important since they may represent novel therapeutic targets in tumors which have acquired resistance to SFK inhibitors. Activation of Ras oncogenes by mutation or amplification is one of the most common dominant oncogenic events in human cancers (8). In addition, activation of upstream components of the Ras signaling pathway by growth factor or growth factor receptor over-expression, or growth factor receptor mutation/truncation further increase the frequency of Ras pathway activation in human cancers. Multiple effector pathways of Ras have been identified and characterized, including Raf/MEK/ERK MAPK, PI3K/Akt, Ral-GEFs, and phospholipase C ϵ (9). These effector pathways are responsible for the diverse effects of active Ras on cell transformation, neoplastic growth, migration and invasion (9,10).

Activation of SFKs is common in cutaneous squamous cell carcinomas as well as pre-cancerous actinic keratoses, and Fyn protein levels are elevated in human squamous cell carcinoma (11,12). Fyn is essential for keratinocyte migration and carcinoma invasion, and ectopic expression of active Fyn in the epidermis of transgenic mice elicits spontaneous actinic keratoses and squamous cell carcinomas (12,13). Squamous cell carcinomas of the skin frequently harbor activating Ras mutations and have activation of PI3K/Akt signaling, a major Ras effector pathway (14). In this study, we found that oncogenic H-Ras induces a dramatic up-regulation of Fyn expression in a human keratinocyte cell line in a PI3K/Akt-dependent manner. The increased Fyn activity was required for H-Ras-enhanced cell migration and invasion. PI3K/Akt signaling was also important for Fyn expression, and Fyn was involved in invasion of MDA-MB-231 human breast cancer cells which harbor mutant K-Ras (15). Thus, Fyn is a critical node in the Ras/Akt effector pathway involved in tumor cell invasion.

MATERIALS AND METHODS

Cell culture, reagents and retroviral infections

All cell lines used were maintained in DMEM with 10% FBS. HaCaT and HaCaT Ras II-4 cells were a gift from Dr Norbert Fusenig (German Cancer Research Center, Heidelberg, Germany), and MDA-MB-231 cells were a gift from Dr. Clodia Osipo (Loyola University Chicago, Maywood, IL). HaCaT and HaCaT-Ras II-4 cells were authenticated as keratinocytes by cytokeratin staining. Retrovirus production and transductions were performed as described previously (16). Active H-Ras(G12V) was expressed from the LZRS retroviral vector, and the active Fyn (I338T) cDNA was a gift from Dr. Tadashi Yamamoto (University of Tokyo, Tokyo, Japan), and cloned into the pMV7 retroviral vector. Constitutively active Akt (Myr-Akt) lentiviral vector was kindly provided by Dr. Maria Soengas (Spanish National Cancer Research Centre, Madrid, Spain). PP2 (Invitrogen, Carlsbad, CA), AG1478 (Calbiochem, San Diego, CA), U0126 (Cell Signaling, Danvers, MA) and LY294002 (Enzo Biochem Inc., Farmingdale, NY) were purchased from the indicated companies. Fyn siRNA was obtained from Santa Cruz Biotechnology Inc (Santa Cruz, CA). SiRNA transfections were carried out using Lipofectamine transfection reagent (Invitrogen) as per manufacturer instructions.

Immunoblotting

Western blotting was performed as described previously (16), except that proteins were detected using the Odyssey Infrared Imaging System (Li-COR Biosciences, Lincoln, NE). Antibodies against c-Fyn (sc-16 and sc-434), c-Src, Akt1, FAK, and P~FAK were obtained from Santa Cruz Biotechnology Inc. Antibodies against P~ERK1/2, P~Akt1 (S473), P~EGFR (Y1068) were obtained from Cell Signaling Technology. Anti- β -Actin (ICN Biochemical Inc., Costa Mesa, CA), Anti- α -Tubulin (Upstate, Charlottesville, VA) and Anti-Ras (Upstate) antibodies were obtained from the indicated companies.

Reverse transcriptase

PCR Total RNA was isolated by Trizol (Gibco, Chagrin Falls, OH). Complementary DNA was synthesized by reverse transcription of total RNA (Superscript First Strand Synthesis, Invitrogen). Quantitative RT-PCR for Fyn and GAPDH was performed using a GeneAmp 5700 sequence detection system (Applied Biosystems, Carlsbad, CA) with Platinum SYBR Green PCR reagents (Invitrogen). The GAPDH was used to normalize the expression levels. Relative mRNA expression was calculated using the $\Delta\Delta C_t$ method. The primers used were as follows: Fyn Forward: 5' CTCAGCACTACCCAGCTTC-3', Fyn Reverse: 5'-ATCTCCTTCCGAGCTGTTCA-3', GAPDH Forward: 5'-GCACCGTCAAGGCTGAGAAC-3', GAPDH Reverse: 5'-GCCTTCTCCATGGTGGTGAA-3'.

Migration and invasion assays

Migration and invasion assays were performed using 24-well chambers with 8 μ m FluoroBlok cell culture inserts (BD Biosciences, Bedford, MA). For invasion assays, inserts were pre-coated with Matrigel (1:3 dilution, BD Biosciences). Cells were pretreated with CFDA-SE fluorescence tracer (Molecular Probes, Eugene, OR) for 30 minutes at 37° C and 2.5×10^5 cells were seeded in serum-free DMEM on the upper compartment of the FluoroBlok chambers. DMEM with 10% FBS was added to the lower compartment. Fluorescence of the cells in the lower side of the insert was measured using a POLARstar Omega microplate reader (BMG Labtech, Offenburg, Germany) at 0, 24 and 48 hours. Values shown are the mean $\pm$ SD.

RESULTS

Regulation of Fyn by Ras

To determine if active Ras could up-regulate c-Fyn expression, we transduced the immortalized human keratinocyte HaCaT cell line with an active H-Ras(G12V) retrovirus and measured c-Fyn levels by western blotting. Figure 1A shows that both HaCaT-Ras cells and the independently generated HaCaT Ras II-4 cells had elevated levels of Fyn protein relative to HaCaT cells. HaCaT-Ras cells also had elevated Fyn enzymatic activity, as determined by immunoprecipitation kinase assays (Supplemental Figure S1A). H-Ras did not increase the activity of Src (Supplemental Figure S1B), indicating that the effects of H-Ras were relatively selective for Fyn.

Mechanism of Fyn induction

We evaluated if either of two major Ras effector pathways (Raf/MEK/ERK, PI3K/Akt) were involved in the induction of Fyn by H-Ras. Figure 1B shows that HaCaT-Ras cells had elevated P~ERK1/2 and P~Akt1 relative to HaCaT cells. The over-expression of Fyn in HaCaT-Ras cells was inhibited by the PI3K inhibitor LY294002, but not the MEK1/2 inhibitor U0126 (Figure 1C). Oncogenic H-Ras also mediates some of its effects by activating EGFR via autocrine EGFR ligand production (17). H-Ras induced a slight

increase in P~EGFR, however, the EGFR inhibitor AG1478 was not able to reduce Fyn levels in HaCaT-Ras cells despite inhibiting P~EGFR levels. Note the PI3K and MEK1/2 inhibitors also inhibited P~EGFR, suggesting a role for these Ras-effector pathways in autocrine EGFR ligand production. Thus, the induction of Fyn by H-Ras appears to require PI3K/Akt signaling.

Activation of PI3K/Akt signaling is required for induction of Fyn mRNA

We further explored the mechanism of Fyn induction by examining Fyn mRNA levels. Figure 2A shows that Fyn mRNA was undetectable in HaCaT cells, but was strongly up-regulated in HaCaT-Ras cells. The PI3K inhibitor LY294002 was able to inhibit the over-expression of Fyn in HaCaT-Ras cells at both the protein and mRNA level, and inhibited H-Ras-induced P~Akt1 (S473) levels (Figures 1C and 2A). Quantitation of Fyn mRNA by qRT-PCR in Figures 2B and 2C showed dramatic induction of Fyn mRNA levels by H-Ras and >97% inhibition by LY294002. Transduction of HaCaT cells with a constitutively active Akt virus induced Fyn mRNA levels (Figure 2C). Taken together, these results demonstrate that PI3K/Akt signaling is necessary and sufficient for induction of Fyn expression by H-Ras.

Role of Fyn in Ras-mediated increased migration and invasion

Many oncogenes, especially SFKs such as Fyn, are able to promote tumor cell migration and invasion. We therefore measured migration and invasion through Matrigel of HaCaT cells and HaCaT cells transduced with either H-Ras or active Fyn. Over-expression of Fyn was confirmed in both HaCaT-Ras and HaCaT-Fyn cells by western blot (Figure 3A). Figures 3B and 3C show that both H-Ras and Fyn induced significant migration and invasion of HaCaT cells over 24–48 hours. To test if Fyn was required for the increased migration and invasion of HaCaT-Ras cells, Fyn was knocked-down with siRNA (Figure 4B). Figure 3D show that both migration and invasion were significantly ($p<0.01$) inhibited by Fyn knock-down. The SFK inhibitor PP2 also significantly ($p<0.01$) inhibited migration of HaCaT-Ras and HaCaT-Fyn cells (Supplemental Figure S2).

Fyn is necessary and sufficient for FAK activation by Ras

FAK is located at cell-matrix adhesions and plays a key role in cell migration and proliferation (18). FAK is over-expressed in many cancers including human SCCs and is activated by SFKs (19). Upon activation by SFK, FAK undergoes auto-phosphorylation at Tyrosine 397 (18). We explored if FAK is over-expressed and/or activated in HaCaT-Ras cells by analyzing total FAK and pY397-FAK protein levels. Interestingly, we found FAK is activated, but not overexpressed in HaCaT-Ras cells compared to HaCaT cells (Figure 4A). Furthermore, we evaluated if Fyn was responsible for the activation of FAK by H-Ras. FAK became auto-phosphorylated (Y397) in both HaCaT-Ras and HaCaT-Fyn cells (Figure 4A), indicating that Fyn is sufficient for FAK activation in HaCaT cells. Furthermore, inhibition of SFK activity with PP2 or knockdown of Fyn with siRNA inhibited FAK auto-phosphorylation by H-Ras (Figure 4B). These results indicate that Fyn is necessary and sufficient for activation of FAK by active-H-Ras.

PI3K regulation of Fyn in human tumor cells with active K-Ras

We also explored whether Ras/PI3K/Akt signaling was involved in Fyn expression in human tumor cell lines with activated Ras. We analyzed Fyn mRNA levels by qRT-PCR in MDA-MB-231, a well characterized human breast cancer line with activated K-Ras (15), and found that inhibition of PI3K activity reduced expression of Fyn mRNA (Figure 5B). In addition, the invasive capacity of MDA-MB-231 cells was significantly inhibited by Fyn

siRNA knockdown, indicating that Fyn is involved in invasion of these human tumor cells harboring active K-Ras (Figure 5C).

DISCUSSION

While the over-expression and oncogenic activity of Fyn in human and experimental tumors is well-documented, the mechanism of how Fyn is over-expressed in cancers is less clear (4,5,12,20–22). Here we found that oncogenic H-Ras dramatically induced the expression of Fyn through the PI3K/Akt Ras effector pathway. Src was not activated or up-regulated (data not shown) by H-Ras transduction, indicating some specificity of this effect among SFKs for Fyn. The induction of Fyn by Ras is highly significant since Ras genes are among the most commonly mutated oncogenes in human cancers, and multiple growth factor receptor pathways activate Ras and PI3K/Akt signaling in tumors, even tumors with wild-type Ras alleles (8).

Fyn is rather unique among SFKs since it is up-regulated at the mRNA level in multiple cancers, including glioblastoma, head and neck squamous cell carcinoma, melanoma, chronic myelogenous leukemia, and during prostate cancer progression (20,21). Fyn mRNA is up-regulated by Bcr-Abl1-induced oxidative stress in chronic myelogenous leukemia cells, and this transcriptional mechanism involves the redox-sensitive Egr1 transcription factor (23). Akt activation also induces oxidative stress, and thus a similar mechanism may be responsible for Fyn induction in other cancers with elevated Fyn expression (24,25). We also found elevated Fyn kinase activity in HaCaT-Ras cells (Supplemental Figure 1), most likely due to the large increase in Fyn expression. Fyn protein levels can also be down-regulated by the Src-activating and signaling molecule Srcasm, but since Srcasm does not influence Fyn mRNA levels (26), it cannot account for regulation of Fyn by active Ras we observed.

We found that PI3K/Akt signaling was involved in Fyn induction by H-Ras, and active Akt was sufficient to induce Fyn expression (Figures 1 and 2). The Ras effector pathway ERK was not required for Fyn induction. Ras binds directly to the p110 α catalytic subunit of PI3K to activate Akt (27). In addition, Akt is activated via phosphatidylinositol-3,4,5-trisphosphate generated by PI3K in response to growth factor/receptor tyrosine kinase activation or loss/repression of the PTEN dual-specificity phosphatase (28). Direct phosphorylation by PDK1 also activates Akt in response to growth factors (28). Activation of these signaling pathways are common in human cancers, making Akt a major survival mechanism active in most tumor cells (29).

None of the well characterized Akt effectors, including mammalian target of rapamycin, GSK3, and FOXO, have been described as being able to regulate Fyn (28). Akt can repress Egr1, the transcription factor shown to induce Fyn in chronic myelogenous leukemia cells, but this cannot explain the induction of Fyn by Akt (23). It is possible that steady-state Fyn mRNA levels are elevated by active Ras/Akt signaling due to mRNA stabilization, but this was not investigated. We also demonstrated that Fyn expression in active K-Ras expressing MDA-MB-231 cells was dependent on PI3K (Figure 5). Thus the role of the Akt/Fyn pathway we described in the HaCaT-Ras model is functional in other human cancers, although the detailed molecular mechanism requires further investigation.

We also demonstrated that the enhanced migration and invasion in H-Ras-transduced HaCaT cells was due to induction of Fyn (Figure 2), and that Fyn was sufficient to enhance HaCaT cell migration and invasion (Figure 3B and Supplemental Figure 2). Fyn was also important for invasion of MDA-MB-231 cells (Figure 5). These findings are consistent with the established role of SFKs in integrin and growth factor receptor signaling resulting in

FAK activation, actin cytoskeleton reorganization, and enhanced cell migration. We also demonstrated that both Ras and Fyn can increase the level of phospho-FAK (active), and that Fyn was important for the elevated phospho-FAK in HaCaT-Ras cells (Figure 4). H-Ras activates multiple effectors capable of promoting cell migration and invasion, including p190 Rho-GAP and AF6 (8), and our findings that Fyn is required for enhanced invasion in oncogenic Ras-expressing cells is noteworthy. In addition, SFKs have been implicated as a potent inducer of tumor angiogenesis, and thus play multiple roles in neoplastic progression (2).

SFKs are over-expressed in many cancers, including human squamous cell carcinomas, and these studies provide mechanistic insights into Fyn induction (11,12). Over-expression and activation of Fyn has a dominant function in tumor cells, and Fyn selective inhibitors should have a good therapeutic window and be useful in a wide range of human cancers. For example, the phytochemical myricetin inhibits Fyn activity and suppresses UVB-induced skin cancer in mice (30). Orally available kinase inhibitors which target SFKs are effective against a variety of cancers, and currently numerous clinical trials are underway to evaluate their efficacy in additional cancer types (2). Our identification of Fyn as a key mediator of Ras/Akt oncogenic signaling provides additional rationale for developing and characterizing SFK-targeted therapeutics. Directly targeting Ras has proven difficult, and Akt inhibitor development has had to deal with metabolic side-effects due to the central role of Akt in energy metabolism (24). Thus selective targeting of Fyn may prove to be especially effective given the role of Fyn in tumor progression (invasion, metastasis) (20).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

SFK Src Family Kinases

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FIGURE 1. Active H-Ras up-regulates Fyn protein levels

A, Fyn protein levels were examined in HaCaT, HaCaT-Ras, and HaCaT Ras II-4 cells using western blotting (sc-16 antibody). Tubulin levels are shown as a loading control. **B**, HaCaT and HaCaT-Ras cells were evaluated for induction of indicated Ras effector pathways by western blotting. Increases in P~ERK1/2, P~Akt1 (S473) and P~EGFR (Y1068) are shown. **C**, HaCaT-Ras cells were treated with the PI3K inhibitor LY294002 (20 μ M), MEK inhibitor (U0126, 10 μ M) and EGFR inhibitor (AG1478, 5 μ M) for 48 hours and Fyn protein levels were examined by western blotting. Protein levels of Fyn, P~Akt1 (S473), total Akt, P~EGFR (Y1068), and Actin are shown.

FIGURE 2. PI3K/Akt signaling is required for the induction of Fyn mRNA by Ras

A, HaCaT-Ras cells were treated with the PI3K inhibitor LY294002 (20 μ M) for 48 hours and Fyn mRNA levels were examined by RT-PCR. Total Akt1 and P~Akt1 (S473) levels were also examined by western blotting after 48 hours of LY294002 treatment. B, Akt activation is necessary and sufficient for Fyn mRNA induction. HaCaT and HaCaT-Ras cells were transduced with either constitutively active Akt or treated with LY294002 (20 μ M). After 48 hours, Fyn mRNA levels were analyzed by qRT-PCR normalized to GAPDH. Data is represented as mean $\pm$ SD from a representative experiment performed in triplicate. The numbers over each bar represent relative Fyn mRNA levels relative to untreated HaCaT cells.

FIGURE 3. Fyn is necessary and sufficient for Ras-induced migration and invasion of HaCaT cells

A, Fyn protein levels in HaCaT, HaCaT-Ras and HaCaT-Fyn cells were determined by western blotting (sc-434 antibody). Protein levels of α -Tubulin are shown as a loading control. B and C, The indicated cells were pretreated with CFDA fluorescence tracer and plated on FluoroBlok inserts without (B) or with (C) Matrigel coating. Migration and invasion were measured at 0, 24 and 48 hours, and all data was normalized to the zero time point to clearly show the percentage increase with time. T-test was performed on the indicated groups (*, #), $p < 0.01$. D, HaCaT-Ras cells were transfected with either control or Fyn specific siRNA, and migration and invasion were measured after 24 and 48 hours, respectively. All data was normalized to the migration/invasion in control siRNA cells to clearly show the percentage decrease. Data is represented as mean $\pm$ SD from a representative experiment performed in triplicate. T-test was performed on the indicated groups (*, #), $p < 0.01$.

FIGURE 4. Fyn is necessary and sufficient for FAK activation by Ras

A, Cells were treated with the Src family kinase inhibitor (PP2, 10 μ M) for 48 hours and P~FAK (Y397) and total FAK levels examined by western blotting. Levels of Actin are shown as a loading control. **B**, HaCaT-Ras cells were transfected with control or Fyn-specific siRNA. After 48 hours, levels of Fyn, P~FAK (Y397), and total FAK were examined by western blotting. Actin is shown as a loading control.

FIGURE 5. Fyn regulation and role in invasion in MDA-MB-231 cells

A, Expression of Fyn in MDA-MB-231 cells in PI3K dependent. Cells were treated with LY294002 (20 μ M). After 48 hours, Fyn mRNA levels were analyzed by qRT-PCR normalized to GAPDH. Data is represented as mean $\pm$ SD from a representative experiment performed in triplicate. **B**, MDA-MB-231 cells were transfected with either control or Fyn specific siRNA (dotted line) or treated with SFK inhibitor PP2 (5 μ M), and invasion was measured after 48 hours. Data is represented as mean $\pm$ SD from a representative experiment performed in triplicate. T-test was performed on the indicated groups (*, #), $p < 0.01$. **C**, Model of Fyn induction. Fyn expression is induced by Ras via activation of the PI3K/Akt signaling pathway. Induction and activation of Fyn is required for FAK activation and increased migration/invasion by active Ras.