Downregulation of Src-family tyrosine kinases by Srcasm and c-Cbl: A comparative analysis

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Abstract:

AIM: Elevated Src-Family tyrosine kinase (SFK) activity drives carcinogenesis in vivo and elevated SFK activity is found ubiquitously in human cancers. Although human squamous cell carcinomas (SCCs) demonstrate increased SFK activity, in silico analysis of SCCs demonstrates that only 0.4% of lesions contain mutations that could potentially increase SFK activity; similarly, a low frequency of activating SFK mutations is found in other major cancers. These findings indicate that SFK activation in cancers likely is not due to activating mutations but alternative mechanisms. To evaluate potential alternative mechanisms, we evaluated the selectivity of c-Cbl and Srcasm in downregulating native and activated mutant forms of SFKs.

MATERIALS AND METHODS: We co-transfected native and activated forms of Src and Fyn with c-Cbl and Srcasm into HaCaT cells and monitored the ability of Srcasm and c-Cbl to downregulate native and activated forms of SFKs by Western blotting. The mechanism of downregulation was probed using mutant forms of Srcasm and c-Cbl and using proteosomal and lysosomal inhibition.

RESULTS: The data indicate that Srcasm downregulates native Fyn and Src more effectively than c-Cbl, whereas c-Cbl preferentially downregulates activated SFK mutants, including Fyn Y528F, more effectively than Srcasm. Srcasm downregulates SFKs through a lysosomal-dependent mechanism while c-Cbl utilizes a proteosomal-dependent mechanism.

CONCLUSION: Given the rarity of activating SFK mutations in human cancer, these data indicate that decreasing Srcasm level/function may represent a mechanism for increasing SFK activity in SCC and other human tumors.

Keywords: Cancer, Cbl, Srcasm, Src-family kinases

Introduction

Src-family tyrosine kinases (SFKs) are the largest family of nonreceptor tyrosine kinases and are the key regulators of cell growth and differentiation.[1] As such, cellular SFK activity is tightly regulated, and increased levels of kinase activity, as may be seen with activating mutations or impaired downregulation, promote oncogenesis.[1,2] A majority of human carcinomas, including colonic, breast, pancreatic, and cutaneous squamous cell demonstrate the increased levels of SFK activity compared to adjacent nontumorigenic tissue.[3,7] Potential mechanisms leading to increased SFK activity in tumors could include activating mutations and/or impaired down-regulatory mechanisms. However, activating mutations in SFKs are rarely detected in human carcinomas; typically, only 1%–3% of tumors of a specific type contain potentially activating mutations in SFKs (TCGA, https://www.cancer.gov/)

tca). Therefore, to achieve broad activation of SFKs in human cancer, impaired downregulation of activated SFKs would be a plausible mechanism.

The proto-oncogene Cbl is a platform molecule and ubiquitin ligase that serves as a negative regulator of tyrosine kinase signaling.\[^{8,9}\] Notably, previous studies have shown that Cbl can downregulate activated epidermal growth factor (EGF) receptor and SFKs.\[^{8,10,11}\] Regarding Cbl-dependent SFK downregulation, Cbl can target activated mutant forms of Src and Fyn for ubiquitin mediated proteosomal degradation.\[^{8,10,11}\] However, the selectivity of Cbl for targeting native vs. activated mutated forms of SFKs for proteosomal degradation remains an open question. The mechanism of Cbl-dependent SFK downregulation appears to require SFK activity, the SH2 and SH3 domain of Fyn, and the C-terminal half of Cbl.\[^{12}\] This region of Cbl contains three potential phosphorylation sites, Y700, Y731, and Y774.\[^{12}\] Prior work has shown that Cbl Y731 appears to be the preferred phosphorylation site for Fyn and potentially could play a role in Cbl-mediated downregulation of SFKs.\[^{13}\]

Src-Activating and Signaling Molecule (Srcasm, aka TOM1L) is an SFK substrate, which once phosphorylated on specific tyrosines targets activated SFKs and EGF receptor (EGFR) for degradation in a lysosomal-dependent manner.\[^{2,14}\] Srcasm is phosphorylated on at least four tyrosine that constitute docking sites for the SH2 domains of Grb2, the p85 subunit of PI-3K and SFKs; phosphorylation of Srcasm is promoted by EGF/TGF-α stimulation.\[^{15-17}\] Previous work in transgenic mice demonstrates that Srcasm can downregulate Fyn in a phosphorylation-dependent manner.\[^{3}\] Other studies have shown that Srcasm levels are decreased in cutaneous human squamous cell carcinomas (SCCs) that exhibit increased SFK activity indicating that Srcasm may function as an anti-oncogene.\[^{2,16,18}\]

Given that Cbl and Srcasm are the negative regulators of SFKs, we will determine if either of these molecules exhibits selectivity for native vs. activated mutant kinases. We hypothesize that Cbl and Srcasm, both negative regulators of SFKs, display selectivity for native and/or mutant forms of SFKs. Since activating mutations of SFKs are rarely encountered in human tumors, Srcasm may play an important role in regulating the levels of activated native SFKs. Therefore, decreased Srcasm levels may be an important feature of human carcinogenesis. To test this hypothesis, we determine the selectivity of Cbl and Srcasm for SFKs using co-transfection studies. In this way, we provide new insights into the mechanisms driving human oncogenesis.

Materials and Methods

Expression vectors

All constructs used the pcDNA3.1 ± vector (Invitrogen/Thermo Fisher Scientific). All mutations were confirmed by sequencing. The pcDNA 3.1 + c-Cbl and c-Cbl Y731F plasmids were kindly provided by Dr. Steven M. Anderson (San Diego, CA).\[^{13}\] pcDNA 3.1 + expression vectors containing murine Fyn and murine HA-Srcasm were used as previously described.\[^{17,19}\] The Src expression vector was generated by cloning a BamH1-HindIII cDNA fragment containing the coding region of murine Src into the corresponding sites of pcDNA 3.1+. The murine Fyn Y528F isoform coding region (EcoR1-Not1) and the murine Src Y527F coding region (HindIII-EcoR1) were excised using the indicated restriction sites from a parent vector and directionally cloned into the corresponding sites in pcDNA 3.1. The Srcasm Y392F mutant was used as previously described.\[^{17}\] The 279PTAP to 178AAAA/309PSAP to 309AAAA mutations in Srcasm to eliminate the TSG101 binding sites were introduced by the polymerase chain reaction into the 3.1 HA-Srcasm vector and confirmed by sequencing.

Antibodies

For the Western blotting, the following antibodies were used at the specified dilutions: α-c-Cbl (Cell Signaling, #2747, 1:1000), α-Fyn (Santa Cruz Biotechnology, SC-16, 1:1000), and α-Src (Santa Cruz Biotechnology, SC-19, 1:500), α-phospho-Tyr-416 (Cell Signaling, #2101, 1:1000), α-β-actin (Abcam, Ab 6276, 1:10,000). The α-Srcasm antibody used for Western blotting was a polyclonal antibody, generated by incubating rabbits with three purified glutathione S-transferase fusion proteins spanning murine Srcasm (aa 1–200, aa 150–400, and aa 389–474) was used as described previously.\[^{15}\] The secondary antibody for all but β-actin was goat α-rabbit multi-HRP at 1:20,000 (Amersham). For β-actin, sheep α-mouse IgG-HRP (Amersham) was used at 1:10,000.

Transfection studies

COS-7 (ATCC) cells were obtained in 1999 and frozen as low passage number stocks p11-16. These stocks were thawed, and cultures were established using recommended culture conditions. These cells have not been re-authenticated. The COS7 cells were transfected with the indicated expression vectors, using Lipofectamine as previously described.\[^{15,17}\] For lysosomal inhibition studies, transfected cells were subjected to bafilomycin A (20 μM, Sigma-Aldrich) or ammonium chloride (10 mM, Sigma-Aldrich) for 1 or 3 h before lysis. For proteasomal inhibition studies, transfected cells were pre-incubated with a combination of lactacystin (10 μM, Calbiochem), MG132 (0.1 mM, Calbiochem), and ALLN (0.1 mM, Calbiochem) for 1 or 3 h before lysis.
Immunoblotting and immunoprecipitation

The cells were lysed as previously described. Protein concentration was determined using the MicroBCA protein assay kit (Pierce Chemical Co.). Equivalent amounts of protein were loaded and subjected to the Western blotting with the indicated antibodies and the western blots were developed using an enhanced chemiluminescence kit per the manufacturer’s instructions (Lumilight Plus, Roche Molecular Biochemicals). Both gradient gels and non-gradient gels were used for SDS-PAGE accounting for the subtly different banding patterns.

Results

Srcasm downregulates native Fyn and Src more efficiently than c-Cbl. Co-transfection experiments were performed to compare the capacity of Srcasm and c-Cbl to downregulate native Fyn and Src. Two levels of Srcasm plasmid (low: 0.5 µg and high: 1 ug) were transfected together with Fyn (0.5 µg) or Src (0.5 µg) expression plasmids. The lower level of Srcasm plasmid yields Srcasm levels that efficiently downregulate Fyn levels compared to either amount of c-Cbl plasmid [Figure 1a]. The high amount of Srcasm plasmid yields the higher levels of protein that do not efficiently downregulate Fyn indicating that Fyn downregulation by Srcasm likely requires additional molecules that may be diluted by the elevated levels of Srcasm.

To determine if Srcasm and c-Cbl downregulate Src, analogous co-transfection experiments were performed using Src, c-Cbl, and Srcasm expression vectors [Figure 1b]. At the lower level of Srcasm plasmid, Src protein levels remained stable [Figure 1b]. However, at the higher level of Srcasm, Src levels diminished, but not to the same level compared to Srcasm-dependent Fyn downregulation [Figure 1a]. Both levels of c-Cbl did not downregulate levels of Src. Together, these data show that Srcasm more effectively downregulates native Fyn and Src more effectively than c-Cbl; c-Cbl has mild downregulatory effects on native Fyn.

Srcasm downregulates both native and activated mutant Fyn, while c-Cbl selectively downregulates activated mutant but not native Fyn. Srcasm has been shown to downregulate native Fyn. In addition, Srcasm can downregulate Fyn Y528F in transgenic models; however, the relationship between Srcasm levels and Fyn Y528F downregulation is not well characterized. For further elucidation, the downregulatory selectivity of Srcasm for these forms of Fyn, three levels of Srcasm expression vector were co-transfected with a constant level of Fyn or Fyn Y528F expression vector [Figure 2]. Increasing dosages of Srcasm plasmid were cotransfected with constant amounts of Fyn or Fyn Y528F plasmids. The data show that Srcasm downregulates Fyn most effectively at lower Srcasm levels than at higher levels [Figure 2a]. In contrast, Srcasm downregulates Fyn Y528F levels in a proportional manner with the greatest downregulation occurring with the highest Srcasm levels [Figure 2a].

Previous work in 293T cells showed that c-Cbl can negatively regulate the levels of both native Fyn and Fyn Y528F. We evaluated the selectivity of c-Cbl for downregulating these Fyn molecules further in COS cells using co-transfection studies [Figure 2b]. Increasing levels of c-Cbl expression vector were co-transfected with a constant amount of Fyn or Fyn Y528F plasmid. Increasing c-Cbl levels did not significantly downregulate Fyn levels [Figure 2b]. In contrast, increasing c-Cbl levels markedly downregulates Fyn Y528F [Figure 2b]. These data suggest that c-Cbl preferentially downregulates the activated mutant Fyn but is less selective for native Fyn in some cell types.

Activated Src Y527F mutant is downregulated by Cbl but not Srcasm. To better define the specificity of Srcasm and c-Cbl for downregulating activated mutant Src tyrosine

Figure 1: Srcasm downregulates native Fyn and Src more efficiently than c-Cbl. (a) COS cells were transfected with Fyn (0.5 µg) alone or with either Srcasm or c-Cbl at 0.5 µg (l) or 1.0 µg (h). After 16 h, cells were lysed and lysates were subjected to SDS-PAGE and analyzed by western blot to detect Fyn, Srcasm, c-Cbl, and β-actin. At both doses, Srcasm downregulates Fyn more effectively than c-Cbl and most effectively at the lower dose. (b) COS cells were transfected with Src (0.5 µg) alone or with either Srcasm or c-Cbl at 0.5 µg (l) or 1.0 µg (h). Cells were lysed after a 16-h incubation and lysates were subjected to SDS-PAGE and blotted for Src, Srcasm, c-Cbl, and β-actin. Similarly, Src is downregulated more effectively by Srcasm than c-Cbl. Data representative of three experiments.
kinase, COS cells were transfected with various levels of Srcasm and c-Cbl plasmids and with low or high doses of Src Y527F plasmid [Figure 3]. Consistent with data from Figure 1, c-Cbl downregulates the levels of Src Y527F while both low and high levels of Srcasm were not effective in downregulating Src Y527F. Higher levels of c-Cbl (lanes 9 and 10) downregulated both low and high levels of Src Y527F. Comparatively, the low and high doses of Srcasm plasmid co-transfected with varying doses of Src Y527F (lanes 7 and 8) demonstrate less downregulation of Src kinase compared to c-Cbl.

Differential sensitivity of Srcasm-dependent and c-Cbl-dependent downregulation of Fyn to lysosomal and proteosomal inhibitors. To further characterize the biological features of the Fyn kinase downregulation by Srcasm and c-Cbl, cotransfection experiments were conducted using lysosomal or proteosomal inhibitors [Figure 4]. COS cells were transfected with either Srcasm or Fyn [Figure 4a]; and in parallel experiments, COS cells were transfected with Fyn Y528 and c-Cbl [Figure 4b]. Cells were pre-treated for 1 or 3 h with bafilomycin or ammonium chloride, both inhibitors of lysosomal function, or a cocktail of three proteosomal inhibitors (lactacystin, ALLN and MG132).

The data show that Srcasm-dependent Fyn downregulation is not inhibited by proteosomal inhibitors; while bafilomycin inhibited Srcasm-dependent Fyn downregulation prominently at the 1-h time point while ammonium chloride inhibited downregulation more prominently at the 3-h time point [Figure 4a]. In contrast, downregulation of Fyn Y528F by c-Cbl is inhibited by proteosomal inhibitors, while the lysosomal inhibitors, bafilomycin, or ammonium chloride did not inhibit c-Cbl-mediated downregulation of Fyn Y528F [Figure 4b]. Therefore, Srcasm appears to target Fyn for degradation through a pathway requiring lysosomal function, while c-Cbl-mediated downregulation requires proteosomal activity.

Impaired Srcasm/Tsg101 interaction enhances downregulation of Fyn and Src, while phosphorylation of c-Cbl on Tyrosine 731 is not required for Fyn downregulation. Studies have shown that Srcasm contains conserved PTAP/PSAP amino acid motifs that represent canonical binding sites for TSG101, a regulator of the multi-vesicular body (MVB)/endosomal protein sorting pathway. Based on prior studies, Srcasm is known to downregulate native Fyn and Src by targeting these proteins to the endosomal/lysosomal pathway. Therefore, we hypothesized that Srcasm-mediated downregulation of Fyn or Src may be regulated by its ability to interact with TSG101. Since Srcasm binds Grb2 in a phosphorylation-dependent manner and this interaction is required for the downregulation of SFKs and EGFR, we utilized the Srcasm Y392F mutant as a loss-of-function control for this experiment.

To evaluate this hypothesis, native Srcasm and a PTAP to AAAA/PSAP to AAAA Srcasm mutant with both the PTAP and PSAP sequences mutated were cotransfected with Fyn, Src or SrcY527F. COS cells transfected with the Srcasm PTAP to AAAA/PSAP to AAAA Srcasm mutant demonstrated enhanced downregulation of both Fyn and Src compared to cells transfected with native Srcasm [Figure 5]. However, cells transfected with the PTAP/PSAP double mutant did not downregulate Src Y527F as efficiently as native Src. Of note, neither Srcasm nor the gain-of-function PTAP/PSAP mutant is able to downregulate Src Y527F [Figure 5b].

To further clarify the potential mechanisms for downregulation of Fyn Y528F by c-Cbl, we compared native c-Cbl and a c-Cbl Y731F mutant which eliminates a tyrosine residue that is a known Fyn phosphorylation site associated with c-Cbl binding to the p85 subunit of PI3 kinase. Native c-Cbl and the c-Cbl Y731F mutants were cotransfected with native Fyn and activated Fyn Y528F [Figure 5c]. These cotransfection studies show that both native c-Cbl and c-Cbl Y731F downregulate Fyn Y528F but not native Fyn [Figure 5c]. Therefore, Fyn Y528F downregulation by c-Cbl is not dependent on phosphorylation at Y731.
Discussion

This manuscript provides new data regarding the specificity of Srcasm- and c-Cbl-mediated degradation of SFKs. The data show that Srcasm downregulates native forms of Fyn and Src more efficiently than the activated mutant form of either kinase in co-transfection models [Figures 1, 3 and 6]. In addition, Srcasm is more effective in downregulating native SFKs than c-Cbl. In contrast, c-Cbl downregulates the activated mutant forms of Fyn and Src more efficiently than the native forms of either kinase [Figure 6]. Given these findings, Srcasm may be an important downregulator of native SFKs in cells where the elevated level of SFK activity is derived from the activity of endogenous native Fyn or Src.

The significance of these observations is reinforced by the low frequency activating mutations of SFKs in human tumors. For example, squamous cell carcinomas of cutaneous, cervical, esophageal/stomach, head and neck or lung origins showed that approximately 0.4% (15/3567) of all tumors had either a missense or

Figure 3: Src Y527F mutant is downregulated by c-Cbl but not Srcasm. COS cells were transfected with either 0.5 µg (L) or 1.0 µg (H) of Src Y527F and either low or high doses of c-Cbl (2.0 and 2.5 µg) or Srcasm (1.0 and 1.5 µg). After a 16-h incubation, cells were lysed and lysates were subjected to SDS-PAGE and analyzed by western blot. At both low and high doses, c-Cbl downregulates Src Y-F while Srcasm does not. Data representative of three experiments. In this figure, the image of the lane for c-Cbl was cropped at both ends denoted by black rectangles

Figure 4: Differential sensitivity of Srcasm-dependent and c-Cbl-dependent Fyn downregulation to lysosomal and proteosomal inhibitors. (a) COS cells were transfected with expression vectors containing Fyn and Srcasm indicated and incubated for 16 h. Cells were then incubated with lysosomal inhibitors, NH4Cl or Bafilomycin (Baf.) for 1 or 3 h. Some cells were incubated with a combination of proteosomal inhibitors (Prot.): lactacystin, MG132, and ALLN for 1 or 3 h. Cells were lysed using RIPA and analyzed by western blot. Data representative of two experiments. (b) COS cells were transfected with expression vectors containing Fyn Y528F and c-Cbl as indicated and incubated for 16 h. Cells were then treated as in A. Data representative of two experiments

Figure 5: Srcasm Y178PTAP to Y178AAAA Y309PSAP to Y309AAAA Srcasm double mutant enhances downregulation of Fyn and Src; while c-Cbl tyrosine 731 is not required for downregulating Fyn Y528F. (a) COS cells were transfected with 0.5 µg of Fyn and either 1.5 µg of Src or Srcmutants, PTAP/PSAP and Y392F. After a 16-h incubation, cells were lysed. Lysates were subjected to SDS-PAGE and analyzed by western blot. Fyn levels decreased with Srcasm double mutant PTAP/PSAP, but not with Y392F. (b) COS cells were transfected with 0.5 µg of either Src or Src Y527F and 1.0 µg of either Srcasm or Srcasm double mutant PTAP/PSAP. After a 16-h incubation, cells were lysed. Lysates were then subjected to SDS-PAGE and analyzed by western blot. Double mutant Srcasm PTAP/PSAP downregulates Src more effectively than Srcasm while Src levels remain stable with Src Y527F. Data representative of three experiments. (c) COS cells were transfected with Fyn or Fyn Y528F plasmids (0.5 µg) with or without plasmids expressing (2.0 µg) of c-Cbl or c-Cbl Y731F. After a 16-h incubation, cells were lysed using RIPA buffer. Lysates were then subjected to SDS-PAGE and analyzed by western blot. Data representative of three experiments
truncating mutation in an SFK (TCGA WES data 2020). Interestingly, none of these mutations were the classic C-terminal Y to F mutation that produces a constitutively activated SFK. Therefore, the elevated SFK activity in human SCCs and other human cancers is principally secondary to the increased activity of native SFKs. These data indicate that Srcasm represents an anti-oncogene that needs to be downregulated in human cancers so that elevated levels of SFK activation, a key driver of neoplasia, may be sustained.

Together, these data demonstrate unique characteristics of the Srcasm and c-Cbl downregulatory pathways for SFKs related to specificity. c-Cbl has been shown to downregulate SFKs, including Fyn and Fyn Y528F through a proteosomal-dependent mechanism. However, these studies did not conclude that c-Cbl exhibits a preference for downregulating activated mutant Fyn Y528F over native Fyn.

It was noted that the higher levels of c-Cbl were associated with elevated Src levels compared with controls [Figure 1b]. In addition, Src levels appear to downregulate the levels of c-Cbl as compared to controls [Figure 1b]; this observation supports prior observations that implicated c-Src in the proteosomal-dependent downregulation of c-Cbl.

However, significantly elevated levels of Srcasm do not downregulate Fyn suggesting that the larger pool of Srcasm may dilute the impact of other molecules involved in Fyn downregulation. These data are consistent with our previous data which revealed an inverse relationship between Srcasm and Fyn. A small increase in Srcasm levels can produce a prominent decrease in cellular Fyn levels, but higher levels of Srcasm dilute the downregulatory effect.

The data show that Srcasm downregulates SFKs through a lysosomal, not proteosomal-dependent mechanism [Figures 4 and 6]. Since TSG-101 is a key regulator of protein sorting in the endosomal-lysosomal pathway and that Srcasm contains conserved, canonical-binding sites for TSG-101, we determined if the Srcasm/TSG-101 interaction regulated Srcasm’s ability to downregulate SFKs. To address this hypothesis, we generated a PTAP to AAAA/PSAP to AAAA
Srcasm mutant which ablates the TSG-101 binding motifs. The Srcasm PTAP/PSAP double mutant demonstrates enhanced downregulation of Fyn in the cotransfection model. These data suggest that TSG101 modulates Srcasm’s ability to target SFKs to the lysosome for destruction.

Collectively, we show that Srcasm and Cbl both target activated mutants of Fyn and Src for degradation. However, Srcasm preferentially downregulates the native form while Cbl does not. Phosphorylation of Cbl tyrosine 731 is not required for Cbl-dependent downregulation of activated SFKs. These data suggest that Srcasm may preferentially regulate the activated forms of native SFKs more effectively than c-Cbl. Future studies on the mechanism for preferential downregulation of native SFKs could inform the development of a novel therapeutic target for a wide variety of cancers.

**Conclusion**

In summary, c-Cbl and Srcasm are the key regulators of SFK activity that target activated kinases for degradation using different cellular pathways with different, yet overlapping, specificities for SFKs. The preference of Srcasm for downregulating native SFKs indicates that it may play an important role in oncogenesis.

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**Conflicts of interest**

There are no conflicts of interest.

**References**