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Research article

A p130^{Cas} tyrosine phosphorylated substrate domain decoy disrupts v-Crk signaling

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Abstract

Background: The adaptor protein p130^{Cas} (Cas) has been shown to be involved in different cellular processes including cell adhesion, migration and transformation. This protein has a substrate domain with up to 15 tyrosines that are potential kinase substrates, able to serve as docking sites for proteins with SH2 or PTB domains. Cas interacts with focal adhesion plaques and is phosphorylated by the tyrosine kinases FAK and Src. A number of effector molecules have been shown to interact with Cas and play a role in its function, including c-crk and v-crk, two adaptor proteins involved in intracellular signaling. Cas function is dependent on tyrosine phosphorylation of its substrate domain, suggesting that tyrosine phosphorylation of Cas in part regulates its control of adhesion and migration. To determine whether the substrate domain alone when tyrosine phosphorylated could signal, we have constructed a chimeric Cas molecule that is phosphorylated independently of upstream signals.

Results: We found that a tyrosine phosphorylated Cas substrate domain acts as a dominant negative mutant by blocking Cas-mediated signaling events, including JNK activation by the oncogene v-crk in transient and stable lines and v-crk transformation. This block was the result of competition for binding partners as the chimera competed for binding to endogenous c-crk and exogenously expressed v-crk.

Conclusion: Our approach suggests a novel method to study adaptor proteins that require phosphorylation, and indicates that mere tyrosine phosphorylation of the substrate domain of Cas is not sufficient for its function.

Background

Metastasis of tumor cells is critically dependent on the ability of cancer cells to adhere and migrate. The cell surface expressed integrins can control this process by physically interacting with the extracellular matrix proteins and other cell surface proteins on endothelial cells lining the blood vessel wall [1]. These integrins signal adhesion and migration by communicating with several tyrosine kinases inside the cell, including the Focal Adhesion Kinase (FAK) and Src family kinases [1,2]. Src kinases control the activation of FAK, as well as the tyrosine phosphorylation of critical substrates that regulate adhesion and migration [3]. Indeed, colon cancer cells with high metastatic potential have elevated levels of Src activity or activating mutations in the Src gene [4,5]. One Src substrate that is involved in regulating an important signaling node in this process is the adaptor protein p130^{cas} (Cas) [6–10]. Cas appears to play a central role in the transformation process by several oncogenes including ras, ornithine decarboxylase (ODC), v-Src, v-crk, and Bcr-Abl, as these tumors all have elevated levels of tyrosine phosphorylated Cas [6,11–13]. Cells from mice that lack Cas have much reduced migration and are resistant to transformation by v-Src, while expression of Cas anti-sense RNA in cells transformed with ras, v-Src or ODC result in reversion of the transformed phenotype [11,14,15]. Furthermore, increased expression of Cas can rescue cell migration and adhesion in cells expressing the tumor suppressor PTEN, and can enhance cell migration and adhesion in normal cells, with a major role being played by the substrate domain [16,17].

Cas is a protein which migrates with an apparent mass of approximately 130 kDa, with a N-terminal SH3 domain, a central substrate domain (SD) with up to 15 tyrosines which can be phosphorylated, a C-terminal region with a proline-rich region, and two tyrosine phosphorylation sites capable of binding Src kinases (Src binding domain (SBD)) [6,18]. The latter SBD domain regulates the ability of Src to activate the serum response element, as well as in the phosphorylation of the SD of Cas by Src [18–20]. The substrate domain controls adhesion and migration and the majority of the tyrosines within this domain are of the sequence YQXP (5) or YDXP (10), which when phosphorylated, bind to the adaptors c-crk, nck and AND-34 [16,21–23]. The importance of these molecules in Cas function is unclear, however c-crk seems to be critical in the regulation of adhesion and migration mediated by Cas. In addition, the SH3 domain of Cas mediates an interaction with FAK, C3G, CMS, and the zinc finger containing protein CIZ, while nephrocystin interacts with Cas via its SH3 domain and proline rich sequences in Cas [24–30]. The SH3 domain of Cas can also interact with the tyrosine phosphatase PTP1B, and expression of PTP1B in transformed cells reverts the phenotype, in part by dephosphorylating Cas [31,32].

Tyrosine phosphorylation of Cas is regulated by adhesion, such that adherent and migrating cells have high levels of tyrosine phosphorylated Cas, whereas cells in suspension have reduced levels of Cas phosphorylation [10,33]. In addition, as mentioned above, transformed cells that are able to grow in an anchorage independent manner show increased tyrosine phosphorylation of Cas [10,33]. These data suggest that tyrosine phosphorylation of Cas in part regulates a switch from adhesion dependent growth to adhesion independent growth. Whether mere tyrosine phosphorylation or localization of Cas is more important for its regulation of adhesion and migration is unclear. Tyrosine phosphorylation of adaptor molecules such as Cas is dependent on upstream signals that lead to the phosphorylation of the adaptor, and with multiple pathways leading from these upstream signals it is difficult to tease out the events resulting from the phosphorylated adaptor itself. We have tried to address this problem by constructing a chimeric Cas molecule (Src*/Cas(SD)) that is phosphorylated independently of upstream signals by fusing the SD of Cas to a Src kinase domain that had been attenuated by mutating the activation tyrosine (Y^{416}) to phenylalanine. Such a mutant is inactive against exogenous substrates [34–36]. We found that this Src*/Cas(SD) molecule is constitutively tyrosine phosphorylated when expressed either transiently or stably in cells, and acts as a dominant negative mutant, attenuating v-crk mediated transformation when expressed in cells transformed by this oncogene. We also observed a reduction in basal JNK activity in normal cells expressing this chimera and in v-crk mediated activation of JNK in cell expressing both v-crk and the chimera. By contrast, tyrosine phosphorylation of endogenous Cas and FAK was unaffected by the Cas chimera, indicating that these processes lie upstream of the tyrosine phosphorylated Cas SD. Finally, we show that this chimera can act as a decoy to selectively disrupt signaling events downstream of the tyrosine phosphorylated SD of endogenous Cas, acting as a dominant negative mutant. We suggest that this approach and modified versions of this approach can be a general method to determine the role of tyrosine phosphorylated domains without regard to upstream signals.

Results

Creation of a dominantly tyrosine phosphorylated Cas substrate domain

The adaptor protein Cas has a N-terminal SH3 domain, a large number of tyrosines that serve as potential kinase substrates within its substrate domain and a C-terminal Src binding domain (Fig. 1). Understanding the function of this substrate domain and the tyrosines within it could lead to a better understanding of the signaling pathways

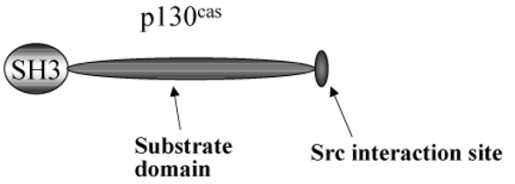

Figure 1
Structure of Cas. The SH3 domain, substrate domain and Src interaction sites are indicated.

regulated by this protein. Previously, investigators have created dominant negative versions of this protein lacking its substrate domain and shown that it is required for cell adhesion and migration. However, these studies depend on upstream signals and so it is not clear which signals are directly emanating from the substrate domain itself, as this domain requires the activation of upstream kinases for its phosphorylation and function. We have tried to address this problem by attempting to create a dominant active substrate domain of Cas, i.e. a Cas substrate domain that is constitutively tyrosine phosphorylated and able to send signals independently of upstream signals. We did this by fusing the SD of Cas to a Src kinase domain that had been attenuated by mutating the activation tyrosine (Y^{416}) to phenylalanine (Src*/Cas(SD), Fig. 2). This Src mutant is inactive against exogenous substrates [34–36]. We also generated fusions of the Cas SD with a Src kinase inactive mutant (K295M) that has no enzymatic activity (Src^{KM}/Cas(SD)), fusions of either the attenuated kinase domain or kinase inactive domain to the 5' or 3' regions of the Cas SD (Src*/Cas(5'SD), Src*/Cas(3'SD), Src^{KM}/Cas(5'SD), Src^{KM}/Cas(3'SD)), as well as the attenuated Src kinase (Src*) alone without the attached Cas SD (Fig. 2). Transient expression of the Src*/Cas(SD) in Cos-7 cells followed by analysis of total cell lysates using anti-phosphotyrosine antibodies demonstrated that while the attenuated Src kinase domain alone did not increase total cellular tyrosine phosphorylated proteins as expected, expression of the Src*/Cas(SD) fusion resulted in tyrosine phosphorylation of a protein of the expected size of the chimera, as well as other proteins (Fig. 3a, 3b). Expression of the Cas(SD) or the Src^{KM}/Cas(SD), fusion did not result in any increase in total phosphotyrosine containing proteins nor in the tyrosine phosphorylation of the Cas SD (data not shown and see Fig. 4).

The C-terminus of the Cas SD is involved in the interaction with v-crk

Investigators have shown that v-crk interacts via its SH2 domain with the substrate domain of Cas [6]. Association of v-crk with endogenous Cas is believed to protect the phosphorylation status of this molecule due to protection from phosphatases [12]. Expression of an unphosphorylated Cas SD along with v-crk should therefore result in stable tyrosine phosphorylation of the Cas SD if they interact. The Cas SD has two sets of potential SH2 interacting tyrosines, the N-terminal subdomain containing 4 YQXP motifs located between a.a. L^{157} to A^{324} and the C-terminal subdomain containing, 9 YDXP motifs located between a.a. S^{325} to R^{516}. The v-crk SH2 domain interacts with YXXP motifs upon phosphorylation, suggesting that both of these regions may be able to interact with v-crk [37]. Fusions of the kinase inactive Src kinase domain and either the full Cas SD, the 5' region of the Cas SD (Cas(5'SD), containing 4 YQXP motifs) or the 3' of the Cas SD (Cas(3'SD), containing 9 YDXP motifs), are not stably tyrosine phosphorylated when expressed alone in cells (data not shown and see Fig. 4b). We therefore used these fusions to determine which region of the Cas SD interacts with v-crk. Initially, we expressed the Src^{KM}/Cas(SD) (entire Cas SD) in Cos-7 cells alone, or along with wild type (WT) v-crk, or v-crk SH2 or SH3 mutants and assayed for tyrosine phosphorylation and association by co-immunoprecipitation (Fig. 4a). Figure 4a demonstrates that while the chimera was not stably tyrosine phosphorylated when expressed alone, expression of the Src^{KM}/Cas(SD) chimera along with either WT or an SH3 domain mutant of v-crk resulted in its stable tyrosine phosphorylation and co-immunoprecipitation with v-crk (Fig. 4a, lanes 2 & 4). However, co-expression of the Src^{KM}/Cas(SD) chimera along with a SH2 mutant of v-crk demonstrated that an intact v-crk SH2 domain is required for association with the tyrosine phosphorylated Src^{KM}/Cas(SD) (Fig. 4a, lane 3). These data suggest that the kinase inactive fusion with the Cas SD is either transiently tyrosine phosphorylated in cells and v-crk protects them from being dephosphorylated by interacting with them via its SH2 domain, or alternatively, that v-crk induces their tyrosine phosphorylation.

We then used the 5' and 3' fusions of the Cas SD to determine which motif, YQXP or YDXP, interacts with the v-crk SH2 domain. Expression of the Src^{KM} fused to the full length Cas SD, 5' N-terminal YQXP motifs or 3' C-terminal YDXP motifs alone demonstrated that these fusions were not stably tyrosine phosphorylated in cells (Fig. 4b, lanes 1–3), although they can be stably tyrosine phosphorylated when fused to the attenuated Src kinase domain (data not shown and see Fig. 7a). By contrast, co-expression of these fusions along with v-crk results in stable tyrosine phosphorylation of the 3' C-terminal YDXP motifs

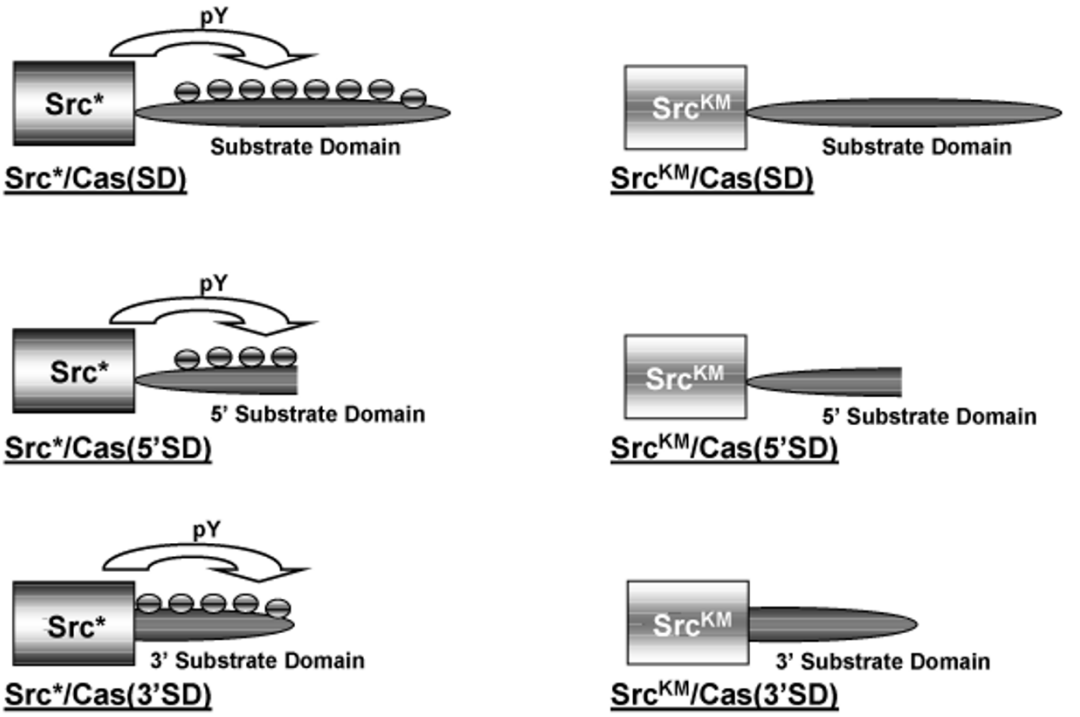

Figure 2
Structure of attenuated Src kinase domain/Cas chimeras. The Src/Cas(SD) chimeras were constructed as described in the materials and methods. Src* is the isolated attenuated Src kinase domain (Y416F). Src*/Cas(SD) is a fusion of the attenuated Src kinase domain to a.a. L^{157} to R^{516} of the Cas substrate domain. Src*/Cas(5'SD) is a fusion of the attenuated Src kinase domain to the 5' portion of the Cas substrate domain (a.a. L^{157} to A^{324}). Src*/Cas(3'SD) is a fusion of the attenuated Src kinase domain to the 3' portion of the Cas substrate domain (a.a. S^{325} to R^{516}). Src^{KM} is the isolated inactive kinase domain (K295M). Src^{KM}/Cas(5'SD) is a fusion of the inactive Src kinase domain to the 5' portion of the Cas substrate domain (a.a. L^{157} to R^{516}). Src^{KM}/Cas(3'SD) is a fusion of the inactive Src kinase domain to the 3' portion of the Cas substrate domain (a. a. S^{325} to R^{516}).

and the full length Cas SD (carrying both motifs), but not the 5' N-terminal YQXP motifs (Fig. 4b, lanes 4–6). These data indicate that the v-crk SH2 domain interacts with the C-terminal YDXP motifs, and as suggested by Birge et al, v-crk may be able to protect them from dephosphorylation and/or induces their phosphorylation [12]. They also suggest that the YQXP motifs may be involved in binding to other intracellular signaling molecules.

Suppression of v-crk mediated transformation by tyrosine phosphorylated Cas SD

As our tyrosine phosphorylated Cas SD chimera can interact with v-crk, we decided to find out if the chimera would affect cell growth and/or transformation in normal cells and those transformed with v-crk. NIH3T3 cells or those transformed with v-crk were stably transfected with the Cas SD chimera (Fig. 5). Several independent clones were obtained for each set of transfections suggesting that expression of the tyrosine phosphorylated Cas SD domain did not negatively affect cell growth (data not shown). Figure 5a demonstrates that stable expression of the Src*/Cas(SD) chimera resulted in constitutive tyrosine phosphorylation of this protein in NIH3T3 cells. Analysis of the effect of this chimera in non-transformed NIH3T3 cells indicates that these cells were not transformed and had normal morphology, although they grew slightly slower than vector-transfected cells (data not shown). Analysis of the growth of either non-transformed or v-crk transformed NIH3T3 cells in soft agar confirmed that expression of tyrosine phosphorylated Cas SD chimera did

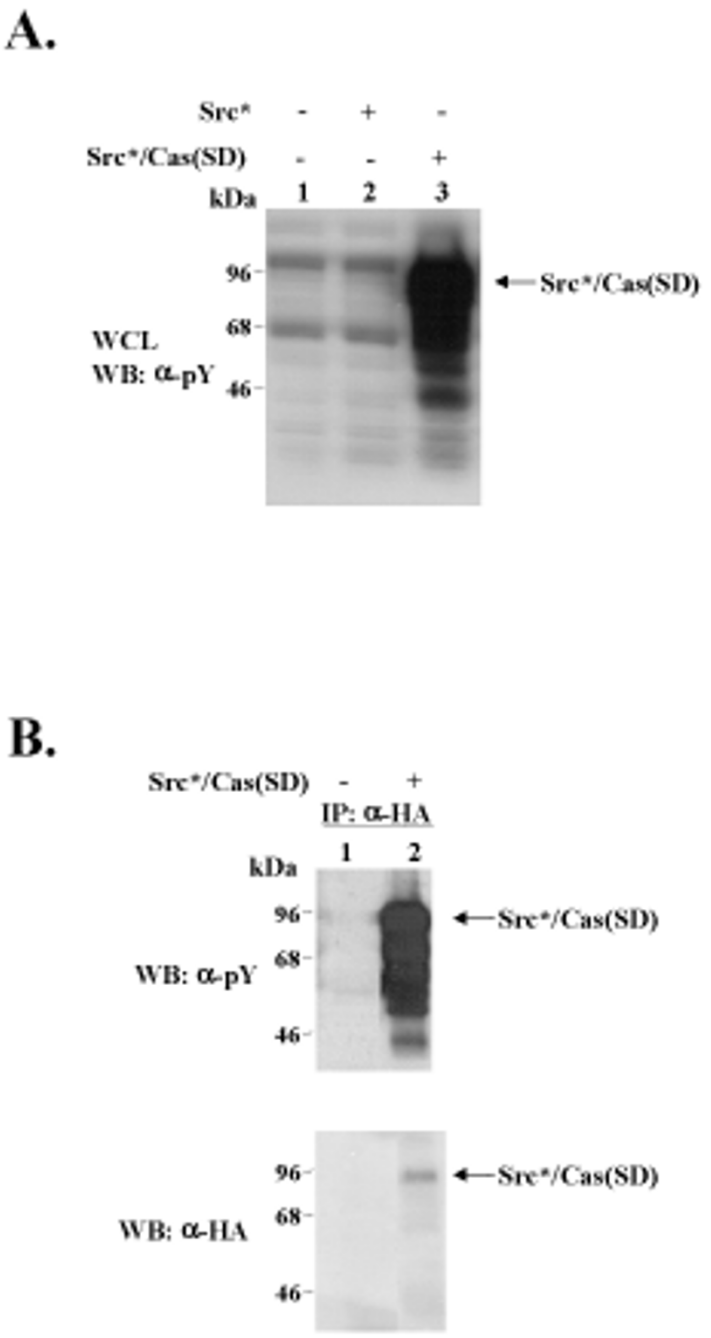

Figure 3
The Src*/Cas(SD) chimera is highly tyrosine phosphorylated when expressed in cells and associates with tyrosine phosphorylated proteins. **a)** Cos-7 cells were transfected with vector alone (lane 1), plasmid coding for the isolated attenuated Src kinase domain (lane 2) or the Src*/Cas(SD) chimera (lane 3). Cell lysates were probed with antibodies to phosphotyrosine. The arrow indicates the Src*/Cas(SD) chimera. **b)** Cos-7 cells were transfected with vector alone (lane 1) or plasmid coding for the Src*/Cas(SD) chimera (lane 2). The Src*/Cas(SD) chimera was then immunoprecipitated with antibodies to HA and probed with anti-phosphotyrosine (top panel). The blot was then stripped and then probed with antibodies to HA to detect the Src*/Cas(SD) chimera (bottom panel). The arrow indicates the Src*/Cas(SD) chimera.

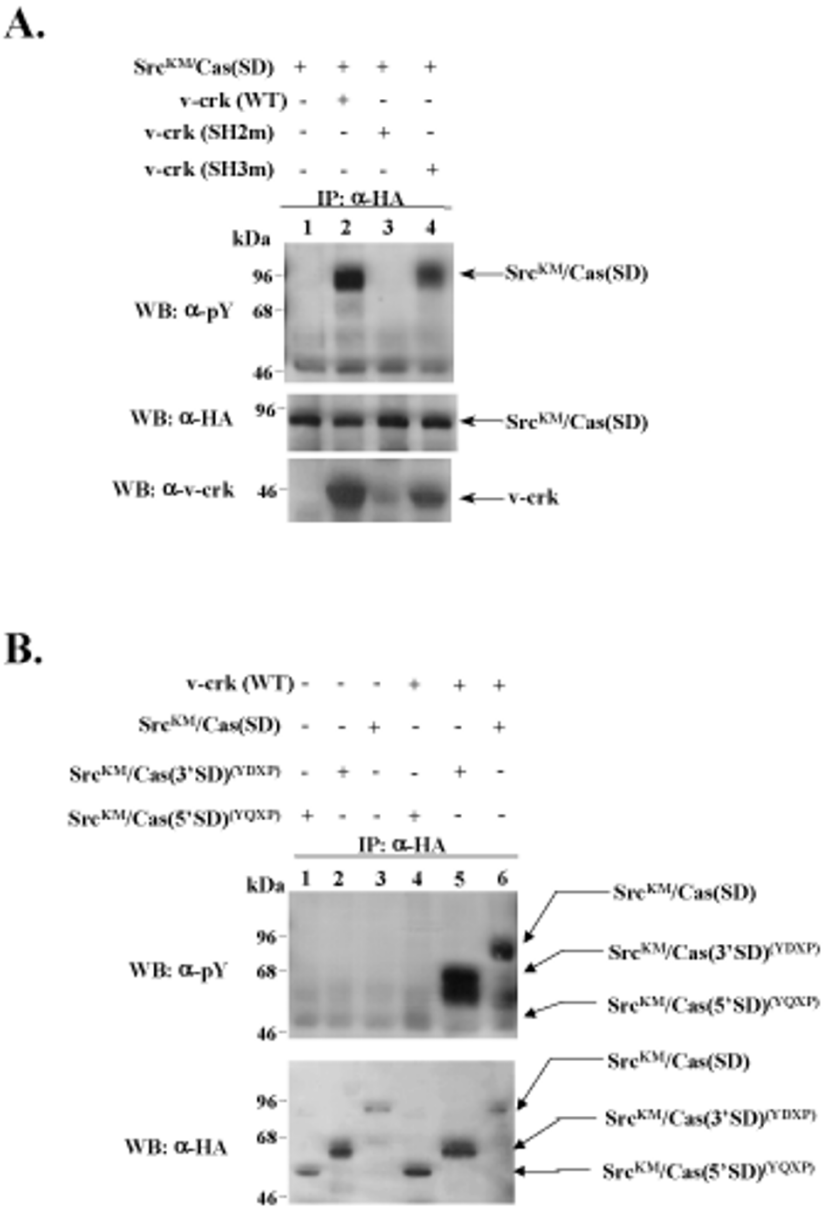

Figure 4

v-crk specifically interacts with and enhances the tyrosine phosphorylation status of the 3' portion of the Cas SD chimera in cells (Src^{KM}/Cas(SD)).**a)** Cos-7 cells were transfected with vector coding for the Src^{KM}/Cas(SD) chimera alone (lane 1), or along with mutants of v-crk, WT (lane 2), SH2 mutant (lane 3) or SH3 mutant (lane 4). The Src^{KM}/Cas(SD) chimera was then immunoprecipitated with antibodies to HA and probed with anti-phosphotyrosine (top panel). The blot was then stripped and then probed with antibodies to HA to detect the Src^{KM}/Cas(SD) chimera (middle panel). The bottom panel shows the expression of the v-crk mutants in the transfection as determined by anti-gag blotting. Arrows point to the Src^{KM}/Cas(SD) chimera and v-crk in the top, middle and bottom panels respectively. **b)** Cos-7 cells were transfected with the following plasmids, and immunoprecipitated with anti-HA antibodies. Src^{KM}/Cas(5'SD) chimera (lane 1), Src^{KM}/Cas(3'SD) chimera (lane 2), and Src^{KM}/Cas(SD) chimera (lane 3) alone, or along with WT v-crk (lanes 4–6). The top panel was probed with antibodies against phosphotyrosine, and the bottom panel with antibodies against HA to detect the chimeras. Probing whole cell lysates with antibodies to v-crk (anti-gag) demonstrated that cells leading to lanes 4–6 expressed equivalent levels of v-crk (data not shown).

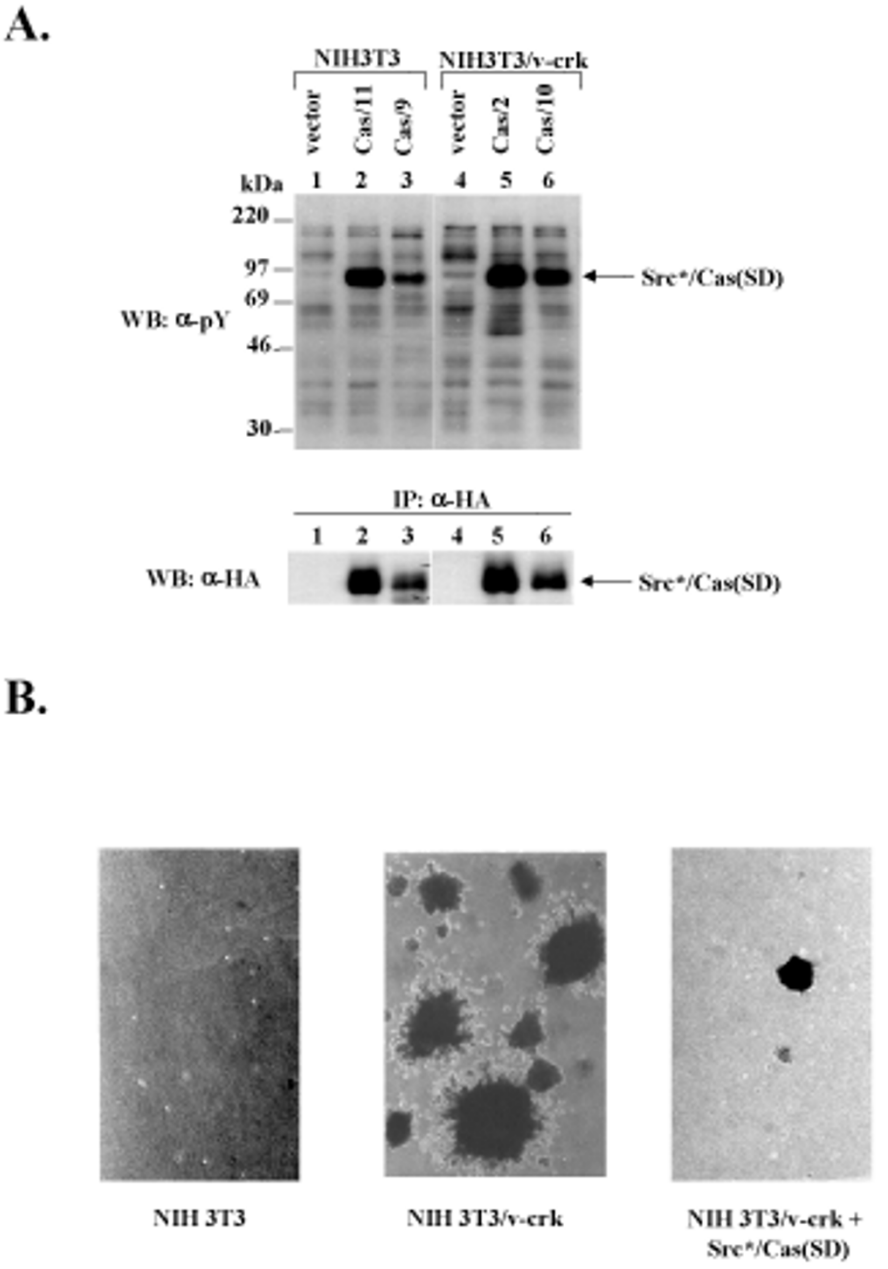

Figure 5
Tyrosine phosphorylated substrate domain of Cas blocks v-crk mediated transformation. **a)** NIH3T3 cells or those transformed with v-crk were either transfected with vector alone or with vector carrying the Src*/Cas(SD) chimera. Whole cell extracts (WCL) from cells were probed with anti-phosphotyrosine (top panel) or with anti-HA antibody (bottom panel). **b)** NIH3T3 cells stably transfected with vector alone (left panel), with v-crk (middle panel) or v-crk and the Src*/Cas(SD) chimera were assayed for growth in soft agar. Representative sections are shown. Colony numbers per view field were as follows: 26.1 (+/- 4.2) for v-crk alone vs. 2.9 (+/- 1.4), statistically significant ($p = 7.76 \times 10^{-8}$, t-test).

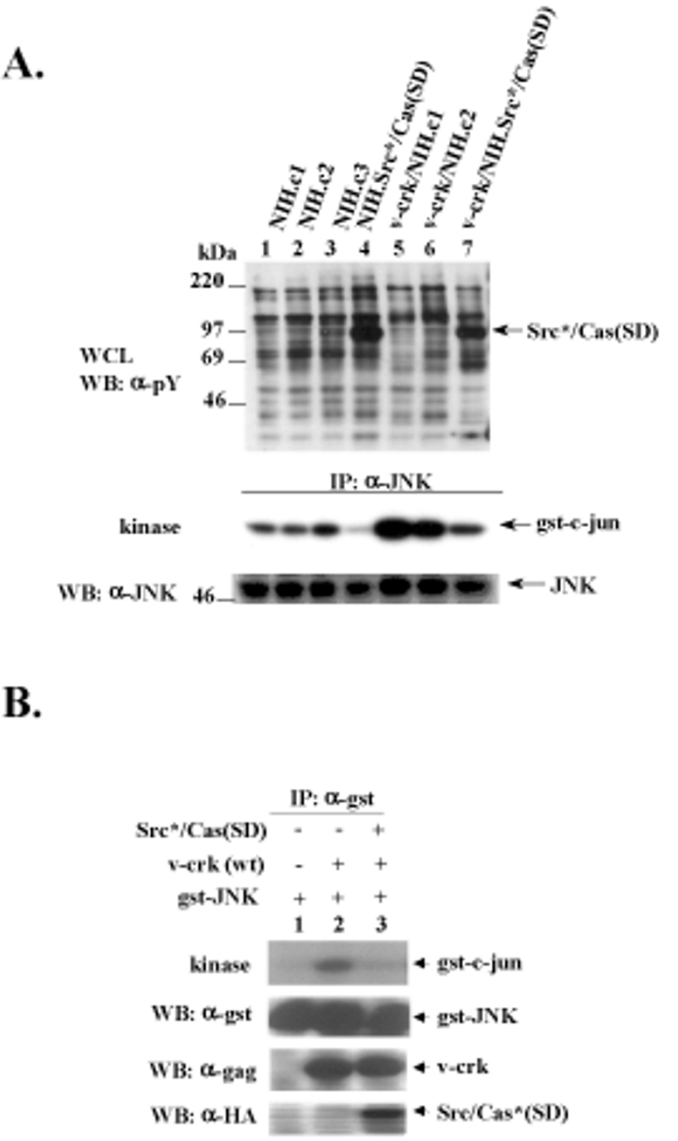

Figure 6
Tyrosine phosphorylated Cas substrate domain blocks activation of JNK. **a)** NIH3T3 cells stably transfected with vector alone, the Src*/Cas(SD) chimera alone, v-crk alone or v-crk plus the Src*/Cas(SD) chimera were assayed for total cellular tyrosine phosphorylation (top panel). JNK was immunoprecipitated and assayed for kinase activity against gst-c-jun in vitro (middle and bottom panels). Lanes 1, 2 and 3, NIH3T3 vector transfected clones; lane 4 NIH3T3 transfected with the Src*/Cas(SD) chimera; lanes 5 and 6, NIH3T3 transfected with v-crk; lane 7, NIH3T3/v-crk cells transfected with the Src*/Cas(SD) chimera. Arrows are pointing to the Src*/Cas(SD) chimera (top panel), gst-c-jun (middle panel) and JNK (bottom panel). **b)** Cos-7 cells were transiently transfected with gst-JNK alone, or along with v-crk, or v-crk plus the Src*/Cas(SD) chimera. gst-JNK was immunoprecipitated and assayed for kinase activity against gst-c-jun in vitro (first panel). The expression levels of the gst-JNK, v-crk and the Src*/Cas(SD) chimera were determined as indicated. Arrows are pointing to the gst-c-jun substrate (first panel), gst-JNK (second panel), v-crk (third panel) and the Src*/Cas(SD) chimera (fourth panel).

not transform NIH3T3 cells as no colonies of these cells grew in soft agar (data not shown). Instead, expression of tyrosine phosphorylated Cas SD chimera resulted in significant reduction in the number of v-crk/NIH3T3 soft agar colonies as well as their size (Fig. 5b), suggesting that the Src*/Cas(SD) chimera blocked the ability of v-crk to transform these cells.

Tyrosine phosphorylated Cas SD blocks JNK activation by v-crk

The JNK pathway is regulated by interaction of c- or v-crk with Cas via the substrate domain [38]. In determining which pathways are affected in our stable cell lines generated, we determined if JNK was activated in the v-crk transformed cells, and if the tyrosine phosphorylated Cas chimera was affecting this activation. JNK was immunoprecipitated and tested for activity from control NIH3T3 cells, those expressing the Cas chimera and those expressing the Cas chimera and v-crk (Fig. 6a). We found that while JNK activity was elevated in v-crk transformed NIH3T3 cells, expression of the tyrosine phosphorylated Cas chimera resulted in reduced activity of JNK (Fig. 6a, cf. lanes 1, 5 and 7). Interestingly, JNK activity was also lower in the NIH3T3 cells expressing the tyrosine phosphorylated Cas chimera than the vector transfected cells, suggesting that this chimera also affected JNK activity in those cells (Fig. 6a, cf. lanes 1 and 4). These data suggest that the tyrosine phosphorylated Cas chimera perturbs signaling via v-crk and probably via endogenous c-crk to affect the activation of the JNK pathway.

We also verified that the tyrosine phosphorylated Cas chimera could affect the ability of v-crk to activate the JNK pathway in transient transfection experiments (Fig. 6b). Transfection of v-crk along with gst-JNK into Cos-7 cells resulted in activation of the co-transfected JNK (Fig. 6b, cf. lanes 1 and 2). However, co-transfection of v-crk and gst-JNK with the tyrosine phosphorylated Cas chimera blocked the activation of JNK induced by v-crk (Fig. 6b, cf. lanes 2 & 3), confirming that tyrosine phosphorylated Cas SD can inhibit the ability of v-crk to activate the JNK pathway. Transient expression of the Src* increased JNK activity slightly while expression of the Src^{KM}/Cas(SD) fusion did not affect JNK activity (data not shown).

To determine if the tyrosine-phosphorylated Cas SD chimera affected other signaling pathways, we tested whether the association between c-crk and C3G was affected in NIH3T3 cells expressing the chimera (Fig. 7a). Immunoprecipitating endogenous c-crk and probing for associated C3G demonstrated that C3G association with c-crk was reduced in the presence of the tyrosine phosphorylated chimera (Fig. 7a, cf. lanes 3, 4 & 5). In addition, we determined if tyrosine phosphorylation of endogenous FAK and endogenous Cas was affected by the presence of the chimera. This was not the case. Figure 7b demonstrates that there was no difference in tyrosine phosphorylation or association with tyrosine phosphorylated proteins of either FAK or endogenous Cas upon stable expression of the tyrosine phosphorylated Cas SD chimera.

Tyrosine phosphorylated Cas SD acts as a decoy for v-crk and endogenous c-crk

As the tyrosine phosphorylated Cas substrate domain acts as a dominant negative for v-crk mediated transformation and JNK activation, and can also interact with v-crk and c-crk (data not shown), we wanted to determine if it could compete for binding of v- and c-crk to endogenous Cas. We transfected v-crk alone or along with the chimera of the attenuated Src kinase domain fused to the C-terminal crk interacting region of the Cas substrate domain (Src*/Cas(3'SD), carrying the YDXP motifs) into Cos-7 cells and immunoprecipitated either the chimera or v-crk and assayed for interacting tyrosine phosphorylated proteins (Fig. 8a). Our analysis showed that expression of this chimera resulted in expression of the highly tyrosine phosphorylated Cas 3'SD chimera (Fig. 8a, lanes 2 (whole cell lysates) or lane 3, anti-chimera immunoprecipitate). Expression of v-crk alone resulted in its co-immunoprecipitation with endogenous tyrosine phosphorylated Cas (Fig. 8a, lane 4). By contrast, co-expression of v-crk with the chimera resulted in reduction in the association of v-crk with endogenous Cas and in induced association of v-crk with the tyrosine phosphorylated Cas 3'SD (Fig. 8a, lane 5). To determine if the tyrosine phosphorylated chimera could compete with endogenous Cas for binding to endogenous c-crk, we transfected either the isolated attenuated Src kinase domain as control, or the tyrosine phosphorylated Cas substrate domain (Src*/Cas(SD)) into Cos-7 cells, and immunoprecipitated endogenous c-crk (Fig. 8b).

Immunoprecipitation of endogenous c-crk showed association with endogenous Cas (Fig. 8b, lane 1), while co-expression of the tyrosine phosphorylated Cas substrate domain resulted in reduced association of endogenous c-crk with endogenous Cas and association with the tyrosine phosphorylated chimera (Fig. 8b, lane 2). These data suggest that the tyrosine phosphorylated substrate domain of Cas acts as a decoy for binding to v- and c-crk thereby interfering with signaling to JNK activation and transformation.

Discussion

Adaptor proteins that regulate tyrosine kinase signaling pathways fall into two major categories, those primarily with protein/protein interaction domains such as SH2, SH3, PTB, and PH domains, exemplified by the adaptor proteins c-crk, nck and grb-2, and those that are primarily tyrosine phosphorylated, exemplified by proteins such as

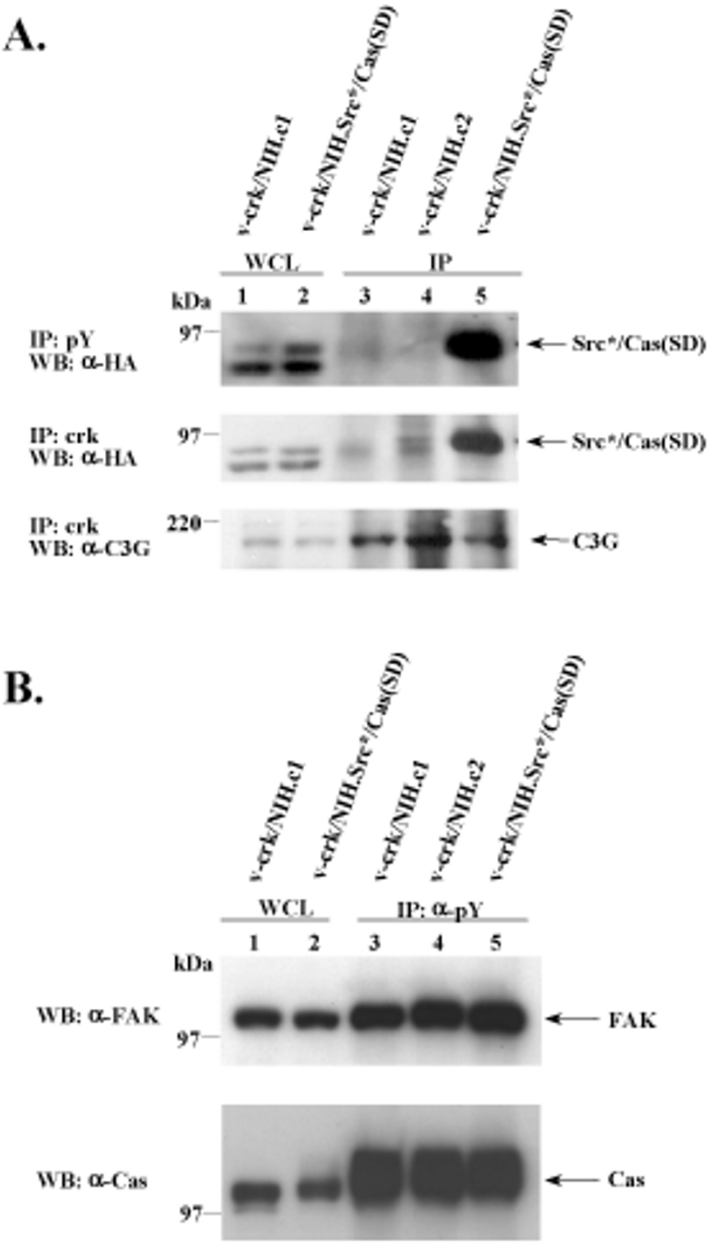

Figure 7
Tyrosine phosphorylated Cas substrate domain affects c-crk interaction with C3G, but not endogenous FAK or Cas tyrosine phosphorylation.**a)** Lysates from NIH3T3 cells stably carrying vector alone or expressing the Src*/Cas(SD) chimera were immunoprecipitated with anti-phosphotyrosine (top panel), or anti-crk (middle and bottom panels) and probed with anti-HA to detect the Src*/Cas(SD) chimera (top and middle panels), or C3G (bottom panel). Lanes 1 and 2, vector and the Src*/Cas(SD) chimera transfected NIH3T3 cells respectively, whole cell extract; lanes 3 and 4, vector transfected NIH3T3 cells immunoprecipitated with anti-phosphotyrosine antibody; lane 5, NIH3T3 cells expressing the Src*/Cas(SD) chimera immunoprecipitated with anti-phosphotyrosine antibody. Arrows indicate the Src*/Cas(SD) chimera and C3G in the top, middle and bottom panels. **b)** A duplicate blot to that shown in (a) was probed with anti-Fak antibodies (top panel) or with anti-Cas antibodies (bottom panel). Arrows indicate FAK and Cas.

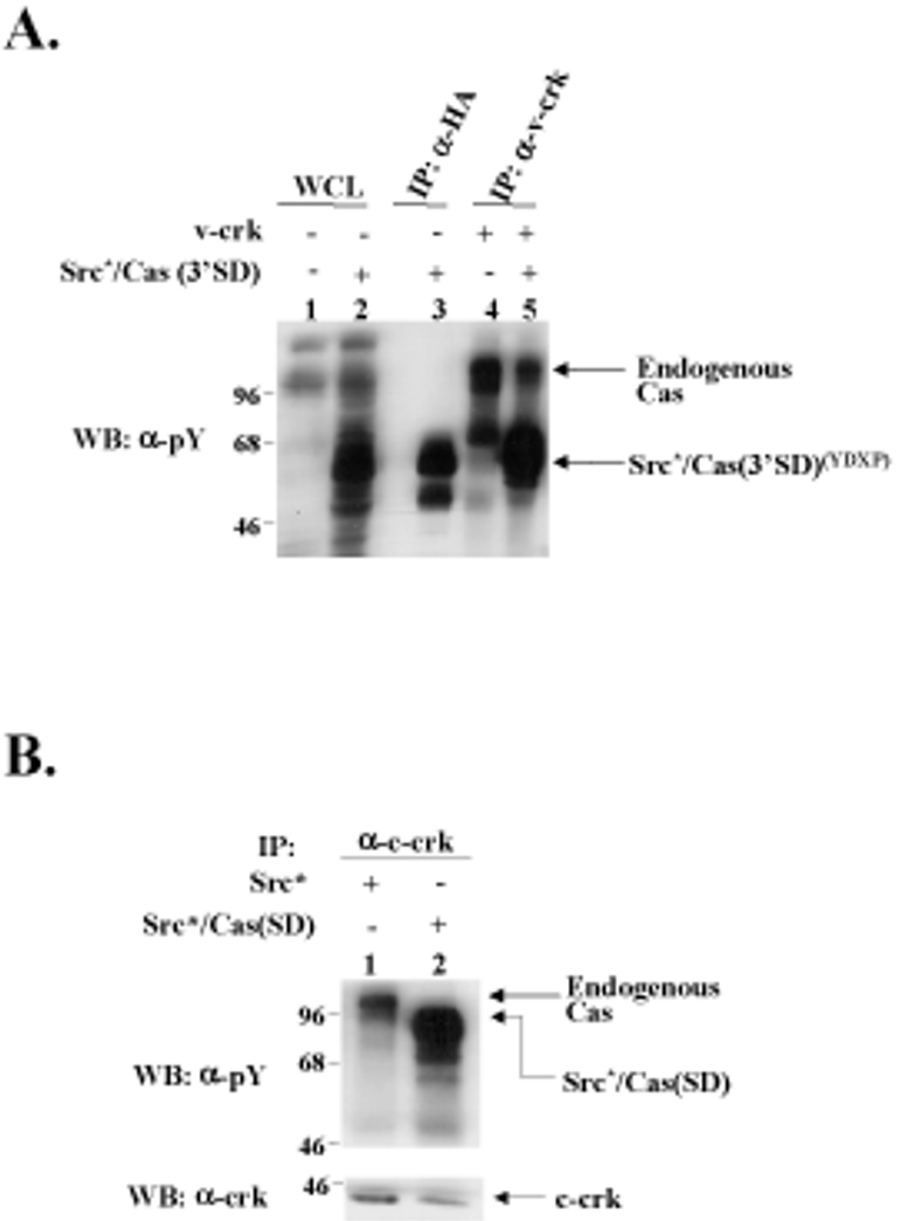

Figure 8
The tyrosine phosphorylated Cas substrate domain competes with endogenous Cas for binding to v- and c-crk. **a)** Cos-7 cells were transfected with vector alone (lane 1), the Src*/Cas(3'SD) chimera alone (lanes 2 and 3), v-crk alone (lane 4) or the Src*/Cas(3'SD) chimera along with v-crk (lane 5). The Src*/Cas(3'SD) chimera or v-crk was then immunoprecipitated and probed for phosphotyrosine. Note that v-crk normally associates with more Cas when transfected alone compared when transfected with the Src*/Cas(3'SD) chimera. **b)** Cos-7 cells were transfected with vector (lane 1) or the Src*/Cas(SD) chimera (lane 2). Endogenous c-crk was then immunoprecipitated and probed with anti-phosphotyrosine (top panel) or with anti-c-crk (bottom panel).

Cas, Dok, IRS-1 and 2, Slp-76, LAT, TRIM or SIT. The latter class may also have protein/protein interaction domains. The former class of adaptor proteins form complexes with downstream effectors in a constitutive fashion via their SH3 domains. Signals are initiated by tyrosine phosphorylation of upstream effectors that recruit these adaptors to assemble larger protein complexes by bringing downstream effectors to the distinct cellular compartments for activation. Thus c-crk interacts constitutively with downstream effectors such as C3G via its SH3 domains, and interacts in a phosphotyrosine dependent fashion with tyrosine phosphorylated receptors using its SH2 domain [39]. To analyze downstream signals regulated by these adaptor proteins, investigators have developed methods that target them to distinct cellular membrane compartments by using heterologous targeting sequences. This leads to the generation of constitutive signals by the adaptor proteins in the absence of other upstream signals [40].

The second class of phosphotyrosine dependent adaptors exemplified by proteins such as Cas, Dok, IRS-1 and 2 or Slp-76, as cytosolic adaptors, or LAT, TRIM or SIT as membrane anchored adaptors, also require the activation of upstream signals by tyrosine kinases for their function. This second class of adaptor proteins is characterized by a substrate domain with multiple tyrosines for phosphorylation by upstream kinases. Phosphorylation of these sites leads to recruitment of other proteins, including the first class of adaptor proteins with SH2 or PTB domains, and subsequent signal transmission (see [41] for review). Analysis of the specific signals emanating from the second class of adaptor proteins has been difficult due to their dependence on upstream kinases for tyrosine phosphorylation of the substrate domains for subsequent assembly of downstream effectors. While these upstream kinases lead to tyrosine phosphorylation of the adaptor and activation of downstream effectors, they usually also activate other signaling pathways that can confound analysis of the specific role of the tyrosine phosphorylated adaptor protein.

We describe a novel chimeric molecule approach that allows the analysis of signaling events regulated by adaptor proteins that require tyrosine phosphorylation by upstream signals for which we used the Cas protein as a model. The adaptor protein Cas has several domains that are important for its function, an SH3 domain, a central substrate domain with up to 15 tyrosines that can serve as tyrosine kinase substrates for upstream kinases such as Src and Abl [19,42,43], and a C-terminal Src binding domain with PXXP motifs for binding to the SH3 domain of Src, and two tyrosines providing docking sites for the Src SH2 domain upon phosphorylation [6]. The substrate domain of Cas has two major types of tyrosine motifs that can be phosphorylated and bind downstream effectors, YQXP (4 copies) and YDXP motifs (9 copies), suggesting that at least two classes of downstream effectors, one capable of binding to YDXP motifs and the other capable of binding to YQXP motifs, are assembled by this adaptor protein. Tyrosine phosphorylation of the substrate domain leads to the recruitment of other downstream adaptors such as crk and nck, as well as other downstream effectors possessing SH2 domains, and signaling events attributed to this domain include activation of the JNK MAPK pathway and control of adhesion and migration of cells [6,10,16,38,44]. We constructed a chimera between a kinase that phosphorylates Cas *in vivo* and the substrate domain of Cas, in order to determine events regulated by this domain. We constructed chimeras by placing the Cas SD at either the N-terminus or C-terminus of the Src kinase domain. However, we found that placing the Cas SD at the N-terminus of the Src kinase domain was inferior to placing it at the C-terminus with regards to tyrosine phosphorylation of the Cas SD. Subsequently, in all our experiments reported, we used chimeras of the Cas SD fused to the C-terminus of the Src kinase domains. Rather than the WT kinase domain of Src, we used an attenuated Src kinase domain (Y416F) as the fusion partner for our chimera to avoid phosphorylation and activation of other Src substrates that would occur with a WT kinase domain (data not shown, [35]). The attenuated Src kinase domain has reduced kinase activity and does not phosphorylate exogenous substrates [35]. We also used a kinase inactive Src kinase domain as a control fusion protein. The chimera of the Cas SD domain fused to the attenuated Src kinase domain was highly tyrosine phosphorylated in cells, and could bind known Cas binding proteins in cells. In contrast, the chimera containing the kinase inactive domain was not stably phosphorylated in cells. These findings indicated that this approach would be useful in analyzing the events that occur following tyrosine phosphorylation of the Cas SD in the absence of upstream signals that may activate other signaling pathways. Our initial experiments were designed to determine whether tyrosine phosphorylation of the SD of Cas would lead to the assembly of protein complexes and activation of downstream effectors.

Using different regions of the Cas SD containing either the YDXP or the YQXP motifs, we could demonstrate that v-crk specifically interacts with the C-terminal YDXP motifs when tyrosine phosphorylated. Moreover, in the absence of stable tyrosine phosphorylation as seen in the Src kinase inactive fusions, v-crk could either induce their phosphorylation, or protect transiently phosphorylated YDXP motifs from dephosphorylation through yet unknown mechanisms. Thus while both sets of motifs in the Cas SD have been thought to interact with the v-crk SH2 domain, we demonstrated that this domain only interacts with the YDXP motifs. Since crk is the major protein reported to bind to the Cas SD, our data suggest that the YQXP motifs

may interact with other yet to be identified downstream effectors.

We also found that while downstream effectors assemble onto the tyrosine phosphorylated Cas SD domain, the downstream signals were not activated. In particular, we found that the tyrosine phosphorylated Cas SD domain blocked v-crk mediated activation of the JNK pathway. In addition, we found that expression of the tyrosine phosphorylated Cas SD domain in v-crk transformed cells reduced their ability to grow in soft agar, a measure of their transformation. Furthermore, we found that the tyrosine phosphorylated Cas SD competed effectively with the endogenous Cas for binding to v-crk and endogenous c-crk, while not affecting tyrosine phosphorylation of proposed upstream proteins such as Fak and endogenous Cas. Thus, our data suggest that the tyrosine phosphorylated Cas SD acts as a decoy for endogenous Cas binding proteins and blocks signaling via crk to the activation of JNK, as well as transformation of cells by the oncogene v-crk. The ability of the tyrosine phosphorylated Cas SD to act as a decoy suggests that while tyrosine phosphorylation of the substrate domain of Cas may be important for its function in assembling downstream signals, specific localization of Cas may also be critical for its downstream functions. Indeed, phosphorylated endogenous Cas is localized to FAK and the focal adhesions via its SH3 domain and/or its Src binding domain in its distal C-terminus, both were excluded from our chimera, suggesting that proper localization of the tyrosine phosphorylated substrate domain may be required for Cas activity [30,45].

Conclusions

A number of proteins have been identified that bind to the substrate domain of Cas, however the large number of tyrosines and potential binding sites for downstream effectors suggest that there may be more. We propose that our chimeric approach can be used to identify Cas binding proteins in mammalian cells in the absence of specific upstream signals normally required for tyrosine phosphorylation of the Cas substrate domain. We can also use this approach to ask which tyrosines within the SD of Cas can act as decoys for specific downstream signals attributed to the SD of Cas. Other uses for such a chimera include screening approaches such as the yeast two-hybrid screen to select proteins that interact with the tyrosine phosphorylated Cas SD. Finally, we can theoretically target this tyrosine phosphorylated chimera to specific areas of the cell to determine which cellular localization will recover the activation of downstream Cas functions. This chimeric approach can also be extended to other adaptor proteins that require upstream signals for tyrosine phosphorylation, creating tyrosine phosphorylated decoys or targeting tyrosine phosphorylated substrate domains or full length adaptor proteins in transgenic cellular and animal systems. We also propose that similar approaches could be used for analyzing proteins that are phosphorylated on serine or threonine and assemble complexes, such as 14-3-3, for analysis of their downstream signals or identification of binding proteins.

Materials and Methods

Materials

Antibodies against the following proteins were obtained from the following suppliers or individuals: ant-phosphotyrosine, RC20-HRP (Transduction Laboratories, Lexington, KY); anti-HA (Boehringer Mannheim, Indianapolis, IN); anti-gag (Dr. R. Birge, New Jersey Medical School, Newark, NJ); anti-JNK (NEB, Beverley, MA); anti-c-crk (Santa Cruz Biotechnology, Santa Cruz, CA); anti-C3G (Dr. Beatrice Knudsen, Weill Medical College of Cornell University, NY, NY); anti-FAK and anti-Cas (Transduction Laboratories, Lexington, KY).

Cloning and chimera construction

The kinase domains of the c-Src Y416F mutant (kind gift of Dr. Joan Brugge, Harvard Medical School, Boston, MA) or the kinase inactive c-Src (K295M, [46]), were amplified by PCR and cloned in pJ3H and pJ3M, two vectors for HA- and Myc-tagging at the N-terminus of the protein (kind gifts from Dr. J. Chernoff, Fox Chase Cancer Center, Philadelphia, PA, [47]). The pEBG-JNK was a kind gift of Dr. Bruce Mayer (Harvard Medical School, Boston, MA). The substrate domain of Cas (Fig. 1, a.a. L^{157} to R^{516}) was amplified by PCR and fused in frame to the C-terminus of the attenuated SrcY416F kinase domain (Src*), or to the C-terminus of the inactive SrcK295M kinase domain (Src^{KM}). Subfragments of the Cas substrate domain were generated in the same fashion, with the N-terminal subdomain (a.a. L^{157} to A^{324}, 1 YKVP motif and 4 YQXP motifs) and the C-terminal subdomain (a.a. S^{325} to R^{516}, 9 YDXP motifs) of Cas amplified. Experiments used the chimeras expressed either via the pJ3H vector, or the HA-tagged chimera subcloned into the expression vector pMEXneo [48]. The oncogene v-crk and its mutants cloned into pMEXneo have been described [49,50].

Cell culture and transfection and soft agar assays

Cos-7 and NIH3T3 cells were grown in DMEM containing 10% FCS and antibiotics at 37°C with 5% CO_2. Cos-7 cells were transfected by calcium phosphate method, while the NIH3T3 cells were transfected using Lipofectin (Gibco, BRL). Growth of cells in soft agar was performed as described [51].

Cell Lysis, Immunoprecipitation and Kinase Assays

Cell lysis, Immunoprecipitation, SDS-PAGE, and Western blotting were performed exactly as described [48]. Tyrosine phosphorylation of chimeras and other substrates were determined following immunoprecipitation using

the indicated antibodies and probing with RC20-HRP anti-phosphotyrosine antibodies. Expression of the chimeras was determined with anti-HA antibodies. Western blots were developed using ECL+ (Amersham Pharmacia).

Author's contributions

AA carried out the construction of the Src/Cas chimeras, and characterized the chimeras with the help of MK. AA also performed JNK assays in transient transfections, analyzed the association of the chimeras with crk and drafted the manuscript. KHK generated stable cell lines carrying the chimeras, and characterized these lines in soft agar transformation assays, activation of JNK, tyrosine phosphorylation of FAK, endogenous Cas and association of C3G with endogenous crk. AA conceived of the study and HH provided intellectual input. The manuscript was written with the input of all the authors and all authors read and approved the final version of the manuscript.

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