

Crk-Associated Substrate Tyrosine Phosphorylation Sites Are Critical for Invasion and Metastasis of Src-Transformed Cells

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Abstract

Crk-associated substrate (CAS, p130Cas) is a major tyrosine phosphorylated protein in cells transformed by *v-crk* and *v-src* oncogenes. We recently reported that reexpression of CAS in CAS-deficient mouse embryo fibroblasts transformed by oncogenic Src promoted an invasive phenotype associated with enhanced cell migration through Matrigel, organization of actin into large podosome ring and belt structures, activation of matrix metalloproteinase-2, and elevated tyrosine phosphorylation of the focal adhesion proteins FAK and paxillin. We have now extended these studies to examine the mechanism by which CAS achieves these changes and to evaluate the potential role for CAS in promoting *in vivo* tumor growth and metastasis. Whereas the presence or absence of CAS did not alter the primary growth of subcutaneous-injected Src-transformed mouse embryo fibroblasts, CAS expression was required to promote lung metastasis following removal of the primary tumor. The substrate domain YxxP tyrosines, the major sites of CAS phosphorylation by Src that mediate interactions with Crk, were found to be critical for promoting both invasive and metastatic properties of the cells. The ability of CAS to promote Matrigel invasion, formation of large podosome structures, and tyrosine phosphorylation of Src substrates, including FAK, paxillin, and cortactin, was also strictly dependent on the YxxP tyrosines. In contrast, matrix metalloproteinase-2 activation was most dependent on the CAS SH3 domain, whereas the substrate domain YxxP sites also contributed to this property. Thus multiple CAS-mediated signaling events are implicated in promoting invasive and metastatic properties of Src-transformed cells. (Mol Cancer Res 2005;3(6):307–15)

Introduction

Crk-associated substrate (CAS, p130Cas) is a major tyrosine phosphorylated protein in cells transformed by *v-crk* and *v-src* oncogenes. In nontransformed cells, CAS localizes to focal adhesions and undergoes tyrosine phosphorylation in response to integrin-mediated adhesion (reviewed in refs. 1, 2). Studies using kinase-deficient cells indicate that CAS tyrosine phosphorylation is primarily achieved by Src family kinases (3–5). Primary structure analysis indicates that CAS is a “docking” protein containing multiple domains and motifs for potential interactions with other signaling proteins (6). CAS deficiency in mice results in embryonic lethality associated with growth retardation and cardiovascular abnormalities, whereas CAS^{−/−} mouse embryo fibroblasts exhibit disorganized actin cytoskeleton and motility defects (7, 8).

The distinguishing feature of CAS is a large substrate domain characterized by 15 Tyr-X-X-Pro (YxxP) motifs. *In vitro* mapping studies showed that Src directly phosphorylates CAS essentially on the substrate domain YxxP tyrosines, and that 14 of the YxxP tyrosines (all but the most amino-terminal site) are subject to phosphorylation by Src (9, 10). In Src-transformed fibroblasts, CAS exhibits elevated substrate domain phosphorylation (11), and in this circumstance at least six of the YxxP tyrosines are phosphorylated (12). Recruitment of Src to CAS can occur by either direct binding of the Src SH3 domain to a Pro-X-X-Pro motif in the Src-binding domain (13, 14) or by indirect binding achieved by the scaffolding function of focal adhesion kinase (FAK), whereby FAK simultaneously interacts with the CAS SH3 domain (15) and the Src SH2 domain (16). Both mechanisms seem to substantially contribute to CAS substrate domain tyrosine phosphorylation (11, 13, 17, 18). The CAS SH3 domain also interacts with tyrosine phosphatases PTP1B (19) and PTP-PEST (20), indicating a possible role for this domain in both positive and negative regulation of CAS substrate domain tyrosine phosphorylation.

In nontransformed mouse embryo fibroblasts, CAS substrate domain YxxP site phosphorylation is detected predominantly at focal adhesions of stationary cells and is enriched at the cell periphery in nascent integrin adhesion sites and/or membrane ruffles of actively spreading and migrating cells (11). The phosphorylated YxxP motifs serve as docking sites for SH2-mediated binding to adaptor proteins of the Crk family (10, 13, 17, 21) and other SH2-containing downstream signaling effectors (22, 23). Phosphorylation of CAS substrate domain YxxP sites has been linked to integrin signaling pathways controlling both cell motility and cell survival (8, 10, 21,

Received 2/15/05; revised 5/9/05; accepted 5/9/05.

Grant support: National Institute of General Medical Sciences/NIH grant R01 GM49882 (S.K. Hanks), National Cancer Institute/NIH grant R01 CA94849-01 (A. Pozzi), and a pilot project funding from Specialized Programs of Research Excellence in Gastrointestinal Cancer grant 5P50 CA95103-04.

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24, 25). In both processes, CAS/Crk coupling is implicated as a critical step in promoting relevant downstream signaling events, including activation of the small GTPase Rac1 and the protein kinase c-Jun-NH₂-kinase (21, 24, 26).

CAS has also been implicated in human cancer progression. The human ortholog of CAS (termed BCAR1, breast cancer antiestrogen resistance) was identified in a functional screen for genes involved in resistance of breast cancer cells to antiestrogenic drugs (27). In breast cancer patients, high CAS/BCAR1 levels are associated with early disease recurrence, poor response to tamoxifen treatment, and lower overall survival (28, 29). Increased CAS levels also correlate with an invasive phenotype in feline and canine mammary tumors (30). Further supporting a possible role for CAS in cancer progression to malignancy, we recently reported that CAS reexpression in CAS^{-/-} mouse embryo fibroblasts transformed by oncogenic Src resulted in a striking enhancement of Matrigel invasiveness associated with increased activation of matrix metalloproteinase-2 (MMP-2) and formation of large podosomal structures containing tyrosine-phosphorylated CAS (31). In addition, CAS expression in the Src-transformed mouse embryo fibroblasts led to increased tyrosine phosphorylation of focal adhesion proteins FAK and paxillin, indicating a novel role for CAS as a positive regulator of Src activity at integrin adhesion sites (31).

The objectives of this study were to further characterize the role of CAS substrate domain YxxP tyrosines and other functional domains in CAS-stimulated invasiveness of Src-transformed cells, and also to examine the functional requirements for CAS in *in vivo* tumor growth and metastasis. Our results revealed that CAS plays a major role in promoting metastasis of Src-transformed cells and that CAS YxxP tyrosines are critical for both *in vitro* invasiveness and *in vivo* metastasis.

Results

CAS Substrate Domain YxxP Tyrosines Are Required for Enhanced Invasiveness

To better understand the mechanism by which CAS promotes invasiveness, signaling-deficient CAS mutational variants were evaluated, including "15F," where all 15 of the YxxP tyrosine residues in the substrate domain were changed to phenylalanine; "ΔSH3," where the SH3 domain was deleted; and "mPR," where the SH3 domain binding site in the Src-binding domain was disrupted (Fig. 1A). Each variant was stably expressed in CAS^{-/-} mouse embryo fibroblasts that had previously been transformed by expressing oncogenic murine Src Y529F (SrcF cells). The CAS variants were expressed to similar levels (Fig. 1B, top). As expected, the 15F variant was not recognized by a mixture of "pCAS" antibodies specific for phosphorylated substrate domain YxxP sites (Fig. 1B, middle two panels). In comparison to wild-type (WT) CAS, both the ΔSH3 and mPR variants showed reduced immunoreactivity toward the pCAS antibodies (Fig. 1B, middle two panels), indicating that both the SH3 domain and Src-binding domain have roles in directing Src to phosphorylate CAS. The more striking loss of CAS substrate domain phosphorylation observed for the mPR mutant suggests that the direct binding of the SrcF SH3 domain to the CAS Src-

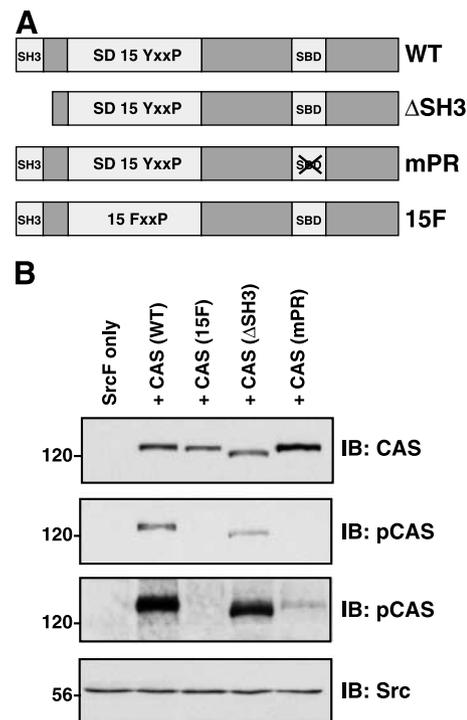


FIGURE 1. Expression and tyrosine phosphorylation of CAS variants in Src-transformed CAS^{-/-} mouse embryo fibroblasts (SrcF cells). **A.** Depiction of the CAS variants used in this study (see text for details). **B.** SrcF cells either without CAS (SrcF only) or expressing one of the four CAS variants were assessed by immunoblot (IB) analysis of total cell lysates containing equal amount of total protein per lane. Replicate blots were used to detect total CAS, using CAS clone 24 antibody (top); phosphorylated CAS substrate domain YxxP tyrosines, using equimolar mixture of phosphospecific antibodies pCAS-165, pCAS-249, and pCAS-410 (middle two panels, representing two different exposures of the same blot); and Src using monoclonal antibody 327 that detects both endogenous c-Src and activated SrcY529F (bottom). Numbers indicate molecular mass markers.

binding domain may be major mechanism of recruitment. However, SrcF indirectly bound to CAS via FAK could have a more prominent role in promoting substrate domain tyrosine phosphorylation than apparent from analysis of the ΔSH3 variant, because CAS SH3 interacts with tyrosine phosphatases in addition to the FAK/Src complex and, therefore, may function as a switch regulating CAS substrate domain phosphorylation and dephosphorylation.

The CAS variants were then analyzed for their ability to promote cell invasion through Matrigel (Fig. 2A). As previously reported (31), WT CAS expression resulted in a striking ~6-fold increase of the capacity of SrcF cells to invade through Matrigel, which was independent of overall migration capacity. Notably, the ability to promote invasion was entirely lost in the 15F variant, indicating a critical requirement for the substrate domain YxxP tyrosines. The ΔSH3 and mPR variants showed a similar partial defect in invasion-promoting activity. Because both the SH3 domain and Src-binding domain can function in recruiting Src to phosphorylate the YxxP tyrosines, this observation is also consistent with a critical role for Src-mediated CAS substrate domain phosphorylation in the invasion response. However, the observation that the ΔSH3 variant was

much more effective than the mPR variant in promoting substrate domain phosphorylation (Fig. 1) suggests that the CAS SH3 domain could have other invasion-related functions. The observed differences in invasion are unlikely due to proliferative differences as the cell populations expressing the four different CAS variants had similar growth curves (Fig. 2B).

CAS Expression, Requiring Substrate Domain YxxP Tyrosines, Promotes *In vivo* Metastasis

To examine the role of CAS and the substrate domain YxxP tyrosines in primary tumor growth and metastasis, we used an *in vivo* lung metastasis model. Athymic nude mice were injected subcutaneously with 5×10^5 SrcF cells that either lacked CAS expression, expressed WT CAS, or expressed the 15F CAS mutant. After 2 weeks, primary tumors were evident at the injection sites for all three populations and there were no significant differences in average tumor volume (Fig. 3A). No tumors were formed from control populations lacking SrcF (not shown). Thus, consistent with our past observations on anchorage-independent growth in soft agar (31), oncogenic Src expression is critical for tumor growth *in vivo*, whereas CAS expression is not an important determinant of this process.

The SrcF cell populations express cytoplasmic green fluorescent protein (GFP) as a selection marker, and this property was exploited to assess their metastatic capacity. As removal of primary tumors can often lead to lung metastasis (32, 33), we evaluated the incidence of lung metastases 2 weeks after surgical removal of the subcutaneous tumors by scoring the percentage of mice with GFP-positive metastases visible on and within the lung parenchyma. Whereas metastases were not observed for any of the mice injected with cells expressing SrcF in the absence of CAS, remarkably 10 of 11 mice injected with SrcF cells expressing WT CAS developed multiple lung metastases (Fig. 3B and C). In contrast, only one of seven mice injected with SrcF cells expressing the 15F CAS mutant developed a single lung metastasis. Thus, the capacity of the SrcF cell populations to invade through Matrigel *in vitro* strongly correlates with their capacity to invade and metastasize *in vivo*, and the CAS substrate domain tyrosine phosphorylation sites are critical for both processes.

The Substrate Domain YxxP Tyrosines Are Required for CAS to Promote Formation of Large Podosome Structures

A cellular attribute contributing to the invasive behavior of Src-transformed cells is the capacity to form podosomes, which are sites where cells locally degrade extracellular matrix and project membrane protrusions or “invadopodia” (34). We previously observed that expression of WT CAS in SrcF cells resulted in increased organization of podosomes into large aggregates appearing as ring- and belt-like structures (31). To gain mechanistic insight into how CAS regulates podosome organization and to explore the relationship between podosome organization and invasive/metastatic behavior, SrcF cells expressing the CAS variants were costained for the podosomal markers F-actin and cortactin-phosphotyrosine 421. Depending upon which CAS variant was expressed, significant differences were observed in the fraction of cells containing large podosome structures (Fig. 4G). Approximately one half of the cells

expressing either WT CAS or the Δ SH3 variant exhibited large podosomal structures, typified by the cell shown in Fig. 4D-F (arrows). In contrast, only ~20% of the cells expressing the 15F and mPR variants exhibited such large podosome aggregates, a value similar to what was observed for SrcF cells expressing no CAS. These latter populations mainly consisted of cells with podosomes appearing as smaller punctate structures, typified by the cell shown in Fig. 4A-C, and occasionally retained F-actin organization as stress fibers (not shown). Overall, the capacity of the CAS variants to promote the formation of the larger podosome structures is strongly correlated with their capacity to undergo phosphorylation of substrate domain YxxP tyrosines (see Fig. 1). Thus, phosphorylation of substrate domain YxxP tyrosines may be the critical signaling event leading to CAS effects on podosome organization.

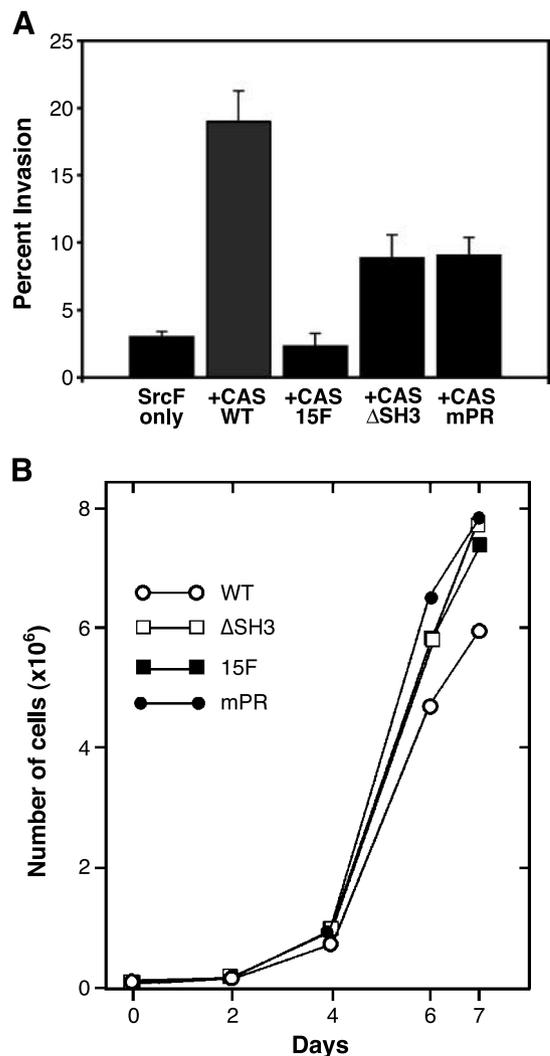


FIGURE 2. Effects of CAS variants on invasion and anchorage-dependent growth. **A.** Matrigel invasion. SrcF cells either without CAS (SrcF only) or expressing one of the CAS variants (WT, 15F, Δ SH3, or mPR) were evaluated for their capacity to invade through Matrigel over a 45-hour period. Columns, mean percentage of invasion from three independent assays, each done in triplicate; bars, SD. **B.** Anchorage-dependent growth. The growth curves were obtained for the indicated cell populations as described in Materials and Methods.

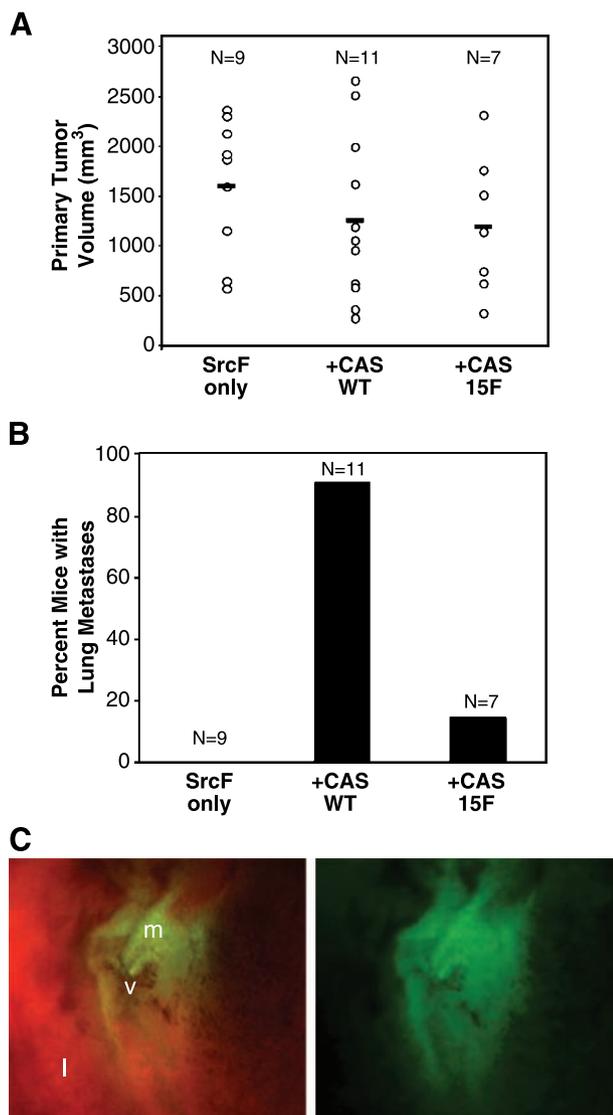


FIGURE 3. CAS substrate domain YxxP tyrosines contribute to experimental lung metastasis. **A.** Primary tumor volume. Tumors were measured 2 weeks after subcutaneous injection of GFP-positive SrcF cells that either lacked CAS expression, expressed WT CAS, or expressed the CAS 15F variant. Individual (○) and mean (bar) tumor volumes are shown. Mean values (SD) were as follows: SrcF only, 1,596 mm³ (677); SrcF + WT CAS, 1,241 mm³ (835); SrcF + CAS 15F, 1,187 mm³ (699). **B.** Lung metastasis. GFP-positive nodules grown on and/or within the lung parenchyma were analyzed by fluorescence microscopy 10 to 14 days after primary tumor removal. Columns, percentage of mice with visible lung metastases. **C.** Example of lung metastasis derived from SrcF cells expressing WT CAS. Left, light field image. Right, same image in dark field. m, GFP-positive metastasis; v, vessels; l, lung parenchyma.

The SH3 Domain Plays a Major Role in CAS-Mediated Enhancement of MMP-2 Activity

In addition to its effects on podosome organization, WT CAS expression in SrcF cells promotes activation of the major matrix-degrading enzyme MMP-2 (31). To explore the mechanism by which CAS promotes MMP-2 activation and the relationship of this property to invasive/metastatic behavior, gelatin zymography assays were done on SrcF cell populations expressing the different CAS variants. Whereas comparable

quantities of secreted pro-MMP-2 were found in conditioned media of all SrcF cell populations, all three CAS mutational variants were found to be deficient in their ability to promote the appearance of the cleaved activated form of MMP-2 (Fig. 5). Notably, the relative effects of the three mutational variants on MMP-2 activation were quite distinct from what was observed for podosome organization. Thus, the ΔSH3 variant was most deficient in MMP-2 activation while being most efficient in promoting large podosome structures. Furthermore, whereas the 15F variant was least able to promote large podosome structures, it was best able to promote MMP-2 activation. Thus, distinct CAS-mediated signaling events are implicated in podosome organization and MMP-2 activation.

The Substrate Domain YxxP Tyrosines Are Required for CAS to Promote Phosphorylation of Integrin/Cytoskeleton-Associated Src Substrates

WT CAS expression in SrcF cells also leads to elevated tyrosine phosphorylation of the integrin-associated Src substrates, FAK and paxillin (31). Because these signaling effects could also contribute to an invasive/metastatic phenotype, it was of interest to determine the capacity of the CAS variants to promote FAK and paxillin tyrosine phosphorylation. Thus, immunoblot analyses of whole cell lysates were carried out. For FAK, replicate blots were probed with either total FAK antibody (Fig. 6A, top) or phosphospecific antibodies against major sites of FAK tyrosine phosphorylation by Src, including Tyr³⁹⁷ (also a FAK autophosphorylation site), Tyr⁵⁷⁶/Tyr⁵⁷⁷ in the kinase domain activation loop, and Tyr⁸⁶¹ in the carboxyl-terminal region (Fig. 6A, bottom three panels). The 15F CAS variant was greatly deficient compared with WT CAS in promoting FAK tyrosine phosphorylation at these major Src sites, whereas the two other mutational variants designed to inhibit FAK/Src or Src binding (ΔSH3 and mPR, respectively) exhibited only partial defects in this property. Similar results were obtained for a major paxillin site of phosphorylation by Src, Tyr¹¹⁸ (Fig. 6B). In addition, we found that a third Src substrate, cortactin, exhibited a significant increase in phosphorylation at the major Tyr⁴²¹ site upon WT CAS expression and this also was greatly decreased by the F15 mutation and partially decreased by the ΔSH3 and mPR mutations (Fig. 6C). Thus, the ability of CAS to promote tyrosine phosphorylation of various adhesion/cytoskeleton-associated Src substrates is dependent on the substrate domain YxxP tyrosine phosphorylation sites.

Discussion

Oncogenic Src promotes the capacity of cells to invade through extracellular matrices and form experimental metastases in mice (35-37), and observations linking elevated Src activity to malignant progression of various human cancers (reviewed in refs. 38-40) heightens interest in understanding the mechanisms by which Src promotes invasive/metastatic behavior. We recently reported that WT CAS reexpression in Src-transformed CAS^{-/-} mouse embryo fibroblasts enhanced Matrigel invasiveness associated with formation of large podosome structures, activation of MMP-2, and elevated tyrosine phosphorylation of FAK and paxillin (31). We now

show that CAS is also a critical determinant of the capacity of these cells to undergo an experimental metastasis in mice. We also show that the CAS substrate domain YxxP tyrosines, the major sites of Src phosphorylation, have critical roles in conferring the capacity of the cells to invade through Matrigel, form large podosome structures, and metastasize. The YxxP sites were also critical for CAS to promote tyrosine phosphorylation of the focal adhesion proteins FAK and paxillin, as well as the actin-associated Src substrate cortactin. However, MMP-2 activation was most dependent on the CAS SH3 domain, rather than the YxxP tyrosines, implicating multiple CAS-mediated signaling events in promoting invasive properties of Src-transformed cells.

A likely mechanism by which CAS substrate domain YxxP tyrosines function to promote invasive/metastatic behavior is through activating signaling events leading to podosome

assembly. Upon phosphorylation, these tyrosines promote high-affinity binding of Crk family adaptor proteins that in turn can activate Rac via DOCK180/ELMO (reviewed in ref. 2). Rac may then activate the WAVE/Arp2/3 complex to promote actin polymerization that, under normal circumstances, would contribute to the protrusion step of cell motility (reviewed in ref. 41). Aberrant up-regulation of this pathway by oncogenic Src could subjugate this machinery to drive assembly on the ventral cell surface of the actin-rich podosome structures that promote the invasive properties of cells (34), presumably by clustering proteases involved in matrix degradation. Thus, the larger podosome structures we observed, which correlate well with the capacity for CAS YxxP phosphorylation, could reflect enhanced Arp2/3-driven podosome assembly. Consistent with this hypothesis are observations that CAS YxxP sites are phosphorylated in

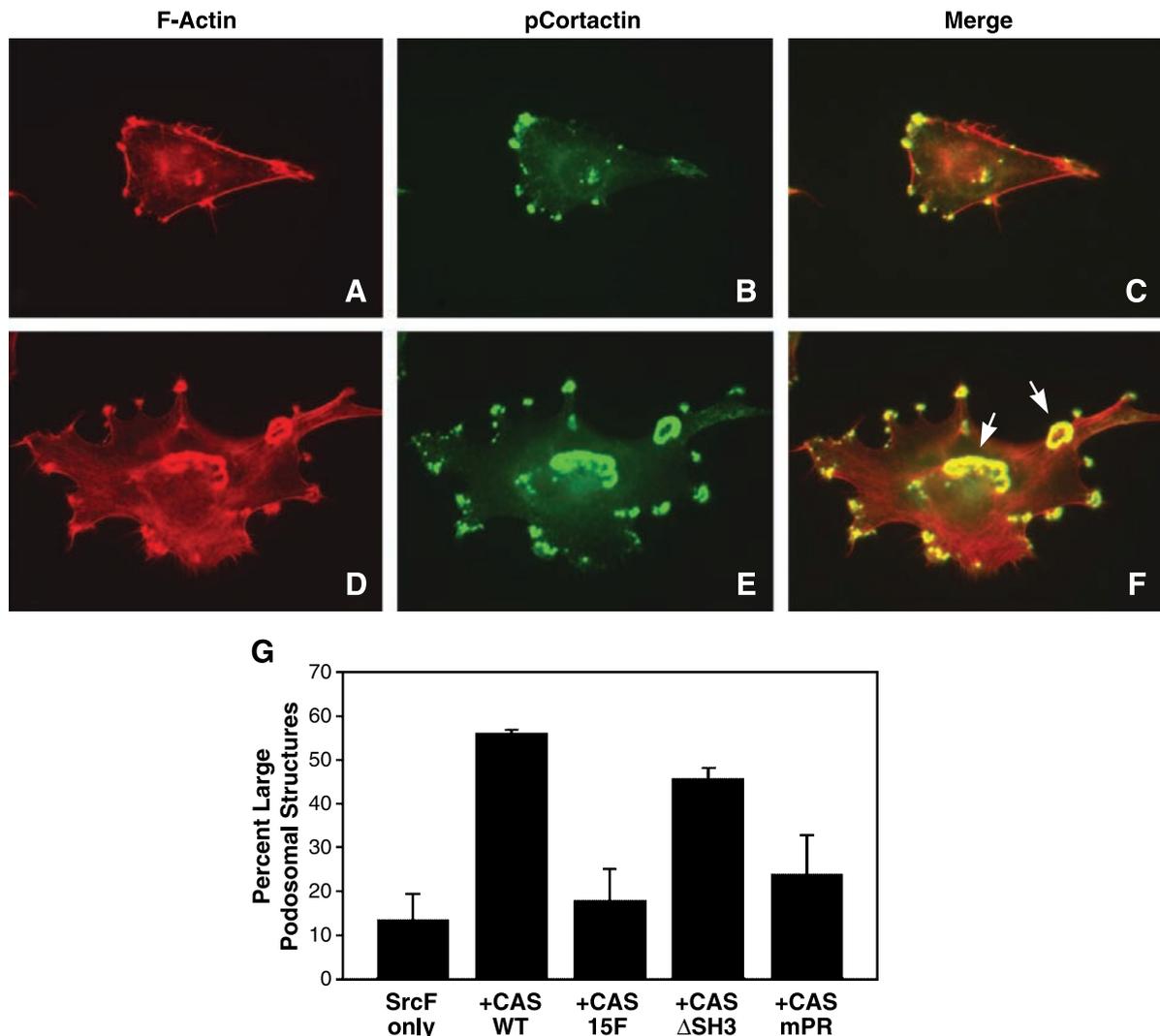


FIGURE 4. CAS substrate domain YxxP tyrosines promote formation of larger podosome structures. SrcF cells either without CAS (*SrcF only*) or expressing one of the CAS variants (*WT*, *15F*, Δ *SH3*, or *mPR*) were analyzed by fluorescence microscopy after costaining for F-actin using Alexa 594–conjugated phalloidin (*red*) and phosphorylated cortactin Tyr⁴²¹ to visualize podosome structures. **A to C.** Example of a cell with smaller punctate podosomes typical of cells lacking CAS or expressing the 15F and mPR CAS variants. **D to F.** Example of a cell with larger podosome structures (*arrows*) more commonly seen in cells expressing WT CAS or the Δ SH3 variant. **G.** Columns, average percentage of cells with larger podosomal structures determined in three independent experiments; bars, SD.

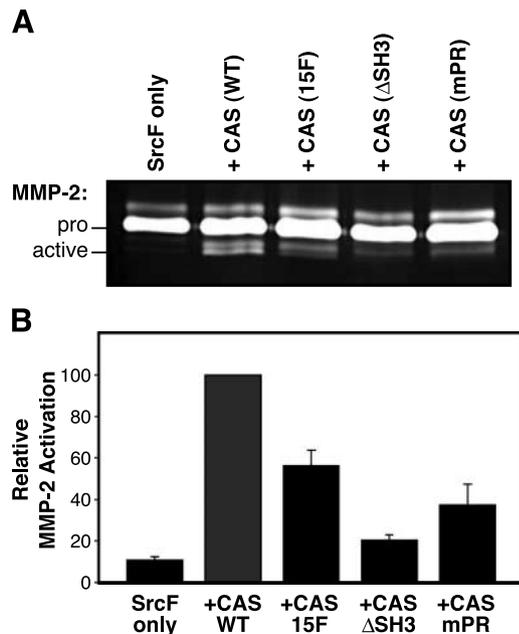


FIGURE 5. Effect of CAS variants on MMP-2 activation. **A.** Representative gelatin zymography assay of conditioned cell media (*pro*, non-cleaved MMP-2 proenzyme; *active*, activated cleaved MMP-2). **B.** Columns, relative amount of active MMP-2 averaged from three independent experiments; bars, SD.

podosomes (31), recruitment of Crk family member CrkL to podosomes is diminished in the absence of CAS (42), activated Rac promotes invasion of Src-transformed fibroblasts (43) and invasion and metastasis of murine melanoma cells (44), and localization of the Arp2/3 complex is required for formation of smooth muscle cell podosomes (45).

We observed that CAS substrate domain YxxP tyrosines are also important for promoting tyrosine phosphorylation of other Src substrates, such as FAK, paxillin, and cortactin. The CAS substrate domain, and presumably the YxxP sites, was also recently shown to be important for enhanced FAK tyrosine phosphorylation associated with overexpression of Crk family member CrkII (46). Thus, CAS does not seem to function in this regard simply as a scaffold protein acting to bind and recruit Src to these proteins. Rather, signaling events downstream of the CAS/Crk > Rac pathway may act to somehow direct Src to these substrates. This is consistent with the finding that Rac1 promotes cortactin tyrosine phosphorylation associated with cortactin targeting to actin networks (47). We also found that the CAS mPR and Δ SH3 variants were also partially defective in their ability to stimulate phosphorylation of FAK, paxillin, and cortactin. For mPR, defective in the Src SH3 binding site, this could be explained by the reduced level of CAS substrate domain YxxP tyrosine phosphorylation observed for this variant. However, it should also be noted that a carboxyl-terminal fragment of CAS containing the Src SH3 binding site, but lacking the substrate domain, can activate c-Src leading to increased tyrosine phosphorylation of FAK, paxillin, and cortactin (48). If YxxP site phosphorylation were the sole determinant driving phosphorylation of these other substrates, one might expect the Δ SH3 variant that undergoes

substantial YxxP phosphorylation to be nearly as effective as WT CAS. However, although the Δ SH3 variant was indeed better able than mPR to promote FAK Tyr⁸⁶¹ phosphorylation, this was not the case for the other FAK, paxillin, and cortactin sites examined. Thus, the CAS SH3 domain also seems to contribute to this response by an unknown mechanism.

The enhanced tyrosine phosphorylation of FAK, paxillin, and cortactin at adhesion/podosome sites could contribute to the invasive and metastatic characteristics conferred by CAS. In particular, recent evidence implicates FAK-mediated signaling events in invasion and metastasis. Dominant-negative inhibition of FAK in v-Src-transformed NIH 3T3 fibroblasts inhibits their ability to invade through Matrigel and metastasize in mice in association with decreased expression and secretion of MMP-2 (49). FAK inhibition also decreases cell invasiveness of human adenocarcinoma (50) and chondrosarcoma (51) cells. In studies of FAK^{-/-} mouse embryo fibroblasts, FAK kinase activity, autophosphorylation, and CAS binding sites are all required for v-Src-stimulated Matrigel invasion, in this case associated with both increased MMP-2 activation and increased MMP-9 secretion (43). Further implicating CAS as a key downstream signaling effector of FAK-stimulated invasiveness, CAS overexpression in the absence of FAK was sufficient to promote invasiveness of v-Src-transformed FAK^{-/-} mouse embryo fibroblasts (43). These observations, combined with our own results, suggest that CAS substrate domain signaling may act both upstream and downstream of FAK to promote invasive behavior.

In our studies, the major MMP-related change associated with CAS-mediated invasion was increased MMP-2 activation. Unlike the other invasion-associated properties bestowed by CAS, the 15F variant was still able to promote considerable MMP-2 activation, whereas it was the CAS SH3 domain that was most critical for this response. The FAK interaction mediated by the CAS SH3 domain may be important for the mechanism leading to MMP-2 activation. Because the CAS Δ SH3 variant was most efficient in promoting large podosome structures, the signaling events leading to MMP-2 activation and podosome assembly do not seem to have a strict mechanistic link. Efficient MMP-2 activation involving CAS SH3 interactions and enhanced podosome assembly involving CAS substrate domain tyrosine phosphorylation are both likely to be important for CAS-mediated invasiveness. This is consistent with the observed partial rescue of Matrigel invasion by the CAS mPR variant with greatly reduced substrate domain phosphorylation but an intact SH3 domain, and the CAS Δ SH3 variant with substantial substrate domain phosphorylation but no SH3 domain.

Our findings raise the prospect that elevated CAS substrate domain signaling may also play an important and widespread role in the malignant progression of human cancers. Of interest for future work will be studies aimed at reaching a fuller mechanistic understanding of the CAS-mediated signaling events leading to podosome formation and MMP-2 activation in Src-transformed fibroblasts and evaluating the possible role of CAS and CAS substrate domain phosphorylation in the invasive/metastatic behavior of colorectal, breast, prostate, and other cancers where high Src activity is associated with disease progression.

Materials and Methods

Materials

Monoclonal antibodies against CAS (clone 24) and paxillin (clone 349), and horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG were obtained from BD Transduction Laboratories (Franklin Lakes, NJ). Anti-Src monoclonal antibody 327 was from Oncogene Research Products (San Diego, CA). Anti-FAK rabbit polyclonal antibody (FAK C-20) was from Santa Cruz Biotechnology (Santa Cruz, CA). Phosphospecific antibodies against FAK phosphotyrosines 397, 861, and cortactin-phosphotyrosine 421 were from Biosource International (Camarillo, CA). Anticortactin rabbit polyclonal antibody and phosphospecific antibodies against FAK phosphotyrosines 576/577; CAS phosphotyrosines 165, 249, and 410; and paxillin phosphotyrosine 118 were from Cell

Signaling Technology (Beverly, MA). Alexa 594-conjugated phalloidin was from Molecular Probes (Eugene, OR) and FITC-conjugated anti-rabbit IgG was from Jackson Immunoresearch Laboratories (Westgrove, PA).

Src-Transformed Mouse Embryo Fibroblast Populations Expressing CAS Variants

CAS^{-/-} mouse embryo fibroblast populations expressing cytoplasmic GFP and mouse Src Y529F (SrcF cells) constitutive active mutant, prepared using the LZRS-MS-IRES-GFP retroviral vector and the Phoenix E packaging line, were previously described (31). CAS variants were subsequently expressed in the SrcF cells using LZRS-MS-IRES-Zeo as described for WT CAS (31). In addition to WT CAS, mutational variants expressed included 15F, in which all 15 YxxP tyrosine residues in substrate domain were changed to phenylalanine (10); Δ SH3, in which the SH3 domain was deleted (18); and mPR, in which the RPLPSP SH3 binding site in the Src-binding domain was functionally inactivated by changing to RAAASPP (18). Cells expressing CAS mutants were selected by growth for 3 weeks in media containing 1 mg/mL Zeocin. The selected cell populations were finally sorted by high GFP expression (31) to ensure comparable SrcF expression. All cells were maintained in DMEM containing 10% fetal bovine serum.

Immunoblotting

Subconfluent cell cultures were washed twice with PBS and lysed in modified radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 5 mmol/L EDTA, 1% NP40, 1% sodium deoxycholate, 50 mmol/L NaF, 1% aprotinin, and 0.1 mmol/L Na₃VO₄]. Protein concentrations in the lysates were determined using the DC Protein Assay (Bio-Rad Laboratories, Hercules, CA). Lysates equivalent to 10 to 20 μ g protein were diluted in 2 \times SDS-PAGE sample buffer for immunoblot analysis of whole cell extracts. Samples were separated on 7% SDS-polyacrylamide gels and transferred to nitrocellulose membrane. Nonspecific activity was blocked by incubating for 1 hour at room temperature in TBS containing 0.05% Tween 20 and 3% nonfat dry milk. Membranes were then incubated overnight in primary antibody, washed extensively with TBS containing 0.05% Tween 20, and then incubated for 1 hour at room temperature with horseradish peroxidase-conjugated secondary antibody. After extensive washing in TBS containing 0.05% Tween 20, the blots were developed by enhanced chemiluminescence (ECL, Amersham Biosciences, Chicago, IL) and exposed to autoradiographic film.

Growth Curves and Matrigel Invasion

Growth curves were obtained after plating cells harvested from nonconfluent growing cultures into multiple 60 mm tissue culture dishes at 30,000 cells per dish. The cells were incubated at 37°C with DMEM containing 10% fetal bovine serum with the medium replaced every other day. At days 2, 4, 6, and 7, the cells were counted from two dishes using a Z1 Coulter Particle Counter (Beckman Coulter, Inc., Fullerton, CA) and the mean cell number was plotted.

For Matrigel invasion assays (31), representative fields were documented by photomicroscopy, and numbers of invading or

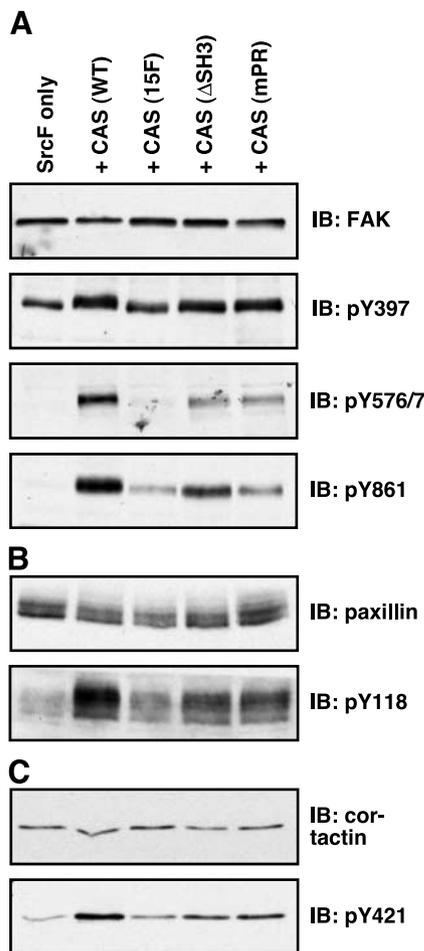


FIGURE 6. CAS substrate domain YxxP sites promote tyrosine phosphorylation of FAK, paxillin, and cortactin. Total cell lysates from the indicated cell populations were analyzed by immunoblotting. **A.** FAK analysis. Total FAK protein was detected with C-20 antibody (*top*), and tyrosine-phosphorylated FAK was detected with phosphospecific antibodies against either Tyr³⁹⁷, Tyr⁵⁷⁶ and Tyr⁵⁷⁷, or Tyr⁸⁶¹ as indicated (*bottom three panels*). **B.** Paxillin analysis. Total paxillin protein was detected with a general antipaxillin antibody (*top*), and tyrosine-phosphorylated paxillin was detected with a phosphospecific antibody against the Tyr¹¹⁸ site (*bottom*). **C.** Cortactin analysis. Total cortactin protein was detected with a general anticortactin antibody (*top*), and tyrosine-phosphorylated cortactin was detected with a phosphospecific antibody against the Tyr⁴²¹ site (*bottom*).

migrating cells per chamber was determined after 45 hours by counting 10 random areas over a grid pattern (at least 15 for invasion and 5 for migration chambers). Data were collected from three independent experiments done in triplicate invasion chambers. The percentage of invasion was calculated as the number of cells invading through Matrigel-coated membranes divided by the number of cells migrating through uncoated membranes.

Gelatin Zymography

Gelatin zymography assays were carried out essentially as described previously (31). The developed gels were scanned using Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE) and bands representing activated MMP-2 were quantified using Odyssey analysis software.

Cell Staining

Cells were plated onto glass coverslips coated with 10 $\mu\text{g}/\text{mL}$ human fibronectin (Sigma, St. Louis, MO) in DMEM containing 10% fetal bovine serum. After overnight culture, attached cells were fixed for 15 minutes in 4% paraformaldehyde in 127 mmol/L NaCl, 5 mmol/L KCl, 1.1 mmol/L NaH_2PO_4 , 0.4 mmol/L KH_2PO_4 , 2 mmol/L MgCl_2 , 5.5 mmol/L glucose, 1 mmol/L EGTA, and 20 mmol/L piperazine-1,4-bis(2-ethanesulfonic acid; pH 7.1), then permeabilized for 10 minutes in 0.4% Triton X-100 in PBS. After extensive washing in PBS, nonspecific binding sites were blocked for 1 hour in PBS containing 1% bovine serum albumin. The coverslips were then incubated overnight at 4°C with polyclonal antibody to phosphocortactin pY421 (1:500 dilution). After extensive washing in PBS, the coverslips were incubated with FITC-conjugated anti-rabbit secondary antibody, again washed extensively in PBS, then incubated 45 minutes in 3.3 nmol/L Alexa 594-conjugated phalloidin. The coverslips were then washed in PBS, mounted on glass slides with ProLong Antifade kit (Molecular Probes), and imaged on a Zeiss Axiophot microscope. The percentage of cells with large podosomal structures was determined from digital images of phosphocortactin staining. At least 100 cells were scored for each cell type, and the experiment was done three independent times.

Primary Tumor Growth and Lung Metastasis in Nude Mice

All experiments were done according to institutional animal care guidelines. Athymic nude mice (BALB/c; Harlan, Madison, WI; $n = 7$ -11 per group) received a single subcutaneous injection of 500,000 GFP-positive cells (200 μL of suspension in PBS) expressing either SrcF alone, SrcF and WT-CAS, or SrcF and CAS 15F mutant. Two weeks after the injection, the visible primary tumors were measured with a caliper and their volumes calculated using the following formula: tumor volume (mm^3) = length (mm) \times width (mm)² / 2 (52). For analysis of lung metastasis, subcutaneously grown tumors were surgically removed and the mice sacrificed 10 to 14 days after removal. Immediately at sacrifice, the lungs were removed, washed in PBS, and viewed under a fluorescence microscope. GFP-positive metastases grown on the surface, as well as within the lung parenchyma, were subsequently counted and imaged.

Acknowledgments

We thank Larisa Ryzhova for technical support and the Vanderbilt Diabetes Research Training Center, the Vanderbilt Digestive Disease Research Center, and the Vanderbilt-Ingram Cancer Center for support with the use of the Flow Cytometry Resource Center and the DNA Sequencing core facilities.

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Mol Cancer Res 2005;3:307-315.

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