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CAS promotes invasiveness of Src-transformed cells

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CAS ('Crk-associated substrate') is an Src substrate found at sites of integrin-mediated cell adhesion and linked to cell motility and survival. In this study, the involvement of CAS in oncogenic transformation was evaluated through analysis of mouse embryo fibroblast populations expressing an activated Src mutant, either in the presence or absence of CAS expression. CAS was not found to be a critical determinant of either Src-mediated morphologic transformation or anchorage-independent growth. However, CAS had a profound effect on other aspects of oncogenic Src function. CAS expression led to a substantial increase in the phosphotyrosine content of FAK and paxillin, supporting a role for CAS as a positive regulator of Src activity at integrin adhesion sites. Importantly, CAS expression resulted in a striking enhancement of the capacity of Src-transformed cells to invade through Matrigel. The increased invasiveness was associated with increased activation of matrix metalloproteinase MMP-2 and formation of large actin-rich podosomal aggregates appearing as ring and belt structures. Thus, elevated CAS-associated tyrosine phosphorylation signaling events occurring at sites of integrin-mediated cell adhesion can have a major role in the development of an invasive cell phenotype.
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Introduction

CAS (p130Cas, 'Crk-associated substrate') was first recognized as a major tyrosine-phosphorylated protein in cells transformed by viral oncogenes v-*crk* (Mayer *et al*., 1988) and v-*src* (Kanner *et al*., 1990). Molecular cloning revealed CAS to be a 'docking protein' with multiple protein–protein interaction domains including an N-terminal SH3 domain, an Src-binding domain (SBD) near the C-terminus, and an interior substrate domain (SD) (Sakai *et al*., 1994). The SD is characterized by 15 Tyr-X-X-Pro (YxxP) motifs that include the major sites where CAS undergoes tyrosine phosphorylation (Nakamoto *et al*., 1996; Fonseca *et al*., 2004), resulting essentially from the actions of Src or related Src-family kinases (Vuori *et al*., 1996; Sakai *et al*., 1997; Klinghoffer *et al*., 1999; Ruest *et al*., 2001). Src can associate with CAS to phosphorylate the YxxP sites through both a direct binding of the Src SH3 domain to the CAS SBD (Nakamoto *et al*., 1996; Pellicena and Miller, 2001; Ruest *et al*., 2001), and an indirect mechanism involving the interaction of the CAS SH3 domain with focal adhesion kinase (FAK) and the Src SH2 domain with the FAK phosphotyrosine-397 site (Vuori *et al*., 1996; Ruest *et al*., 2001). Both mechanisms appear to make substantial contributions to CAS SD tyrosine phosphorylation in both normal and Src-transformed fibroblasts (Roy *et al*., 2002; Fonseca *et al*., 2004). In untransformed cells, CAS SD phosphotyrosine is localized to sites of integrin-mediated adhesion (Fonseca *et al*., 2004) and has been linked to integrin signaling pathways regulating both cell motility and survival (Klemke *et al*., 1998; Honda *et al*., 1999; Cho and Klemke, 2000; Huang *et al*., 2002). In both cases, recruitment of Crk adaptor proteins to phosphorylated CAS SD YxxP sites has been implicated as a crucial next step in promoting relevant downstream signaling events, including activation of Rac1 and JNK (Klemke *et al*., 1998; Cho and Klemke, 2000).

Studies have also implicated CAS in the process of Src-mediated oncogenic transformation. Hirai and co-workers isolated CAS-deficient mouse embryonic fibroblasts (MEFs) and reported that an activated Src mutant expressed in these cells was unable to induce anchorage-independent growth, full morphologic transformation, and full assembly of actin filaments in podosomes (Honda *et al*., 1998). A subsequent study involving re-expression of CAS mutational variants in CAS−/− MEFs indicated a requirement for the CAS SBD in promoting the anchorage-independent growth of Src-transformed cells (Huang *et al*., 2002). Furthermore, overexpression of the CAS C-terminal region including the SBD promoted soft agar colony formation in murine C3H10T1/2 fibroblasts overexpressing c-Src (Burnham *et al*., 2000). Under these conditions, the

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interaction of the CAS SBD with the Src SH3 domain appears to relieve c-Src autoinhibitory interactions and thereby stimulate Src kinase activity (Burnham *et al*., 2000; Pellicena and Miller, 2001), essentially converting c-Src into an activated oncogenic form.

We have further employed CAS−/− MEFs to study the role of CAS as a regulator of oncogenic Src activity and aspects of cell behavior associated with Src-mediated transformation. Our results did not implicate CAS as a critical determinant of Src-mediated morphologic transformation and anchorage-independent growth, but rather revealed a major role for CAS in promoting an invasive phenotype associated with increased podosome formation and activation of matrix metalloproteinase MMP-2.

Results

CAS is not strictly required for activated Src to promote morphological transformation and anchorage-independent growth

To study the role of CAS in Src transformation, an activated variant of mouse Src (SrcF529, 'SrcF') was expressed in CAS−/− MEFs ('SrcF cells') using an IRES-GFP retroviral vector and sorting for GFP-positive cells. In addition, a drug-selection strategy was used to express wild-type CAS in subpopulations of SrcF cells and parental CAS−/− MEFs. Expression of CAS and SrcF was verified by immunoblotting (Figure 1, upper and middle panels). Regardless of CAS expression, the SrcF cells exhibited greatly elevated cellular phosphotyrosine content on numerous proteins as demonstrated by immunoblotting total cell lysates with an antiphosphotyrosine antibody (Figure 1, lower panel). While CAS expression did not dramatically alter the overall phosphotyrosine profile of the SrcF cells, there was a visible phosphotyrosine increase in the 130 kDa range, presumed to include CAS itself. Whether in the presence or absence of CAS, SrcF expression resulted in morphological transformation to a characteristic fusiform and rounded appearance accompanied by growth to a higher saturation density (Figure 2 and data not shown).

No apparent differences were observed in the monolayer growth rate of SrcF cells in the presence or absence of CAS expression (not shown). To examine the potential role of CAS in anchorage-independent cell growth, a series of soft agar colony formation assays were performed. In initial experiments 30 000 cells per 35 mm dish were plated in suspension and colonies were allowed to form over a 4-week period. Under these conditions SrcF cells formed large visible colonies with high efficiency, while CAS expression had no obvious effect on this property (Figure 3a). As expected, no colonies were detected in either the parental CAS−/− cells or these cells in which only CAS was expressed. To determine if CAS expression affected anchorage-independent growth under other experimental conditions,

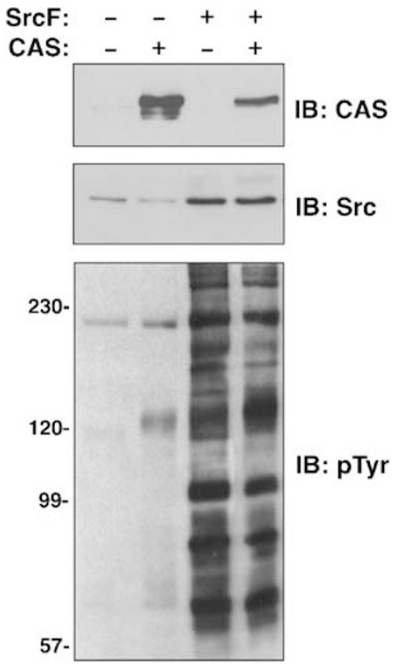

Figure 1 Effects of CAS and SrcF expression on cellular protein phosphotyrosine profiles. Subconfluent cultures of CAS−/− MEFs and derived populations expressing activated SrcF in the presence or absence of CAS were assessed by immunoblot (IB) analysis of total cell lysates containing 15 μg total protein per lane. Replicate blots were used to detect CAS (top panel) using CAS-TL antibody, SrcF (middle panel) using monoclonal antibody 327, and phosphotyrosine (bottom panel) using monoclonal antibody 4G10. Note that the Src antibody detects both endogenous c-Src and SrcF. The apparent reduction in tyrosine phosphorylation of proteins in the ~180 kDa range in cells expressing CAS is not consistently observed

additional soft agar assays were carried out in which cells were plated at two different densities (6000 *vs* 30 000 cells per dish) and colony growth was quantitatively assessed after 2 weeks using an automated colony counter. Again CAS expression did not have a significant effect on colony formation under any of these conditions, although there was a trend toward formation of larger colonies (>40 μm diameter) in the absence of CAS and a greater number of small colonies (<40 μm diameter) in the presence of CAS (Figure 3b). As another measure of anchorage-independent proliferation, growth curves were determined for the cell populations after plating on nonadhesive polyhydroxymethacrylate (poly-HEMA) coated dishes. In this condition, the SrcF cells were observed to proliferate exponentially as nonadherent aggregates until eventually depleting the media nutrients, while the cells lacking SrcF showed no proliferation. Again, CAS expression had no effect on the rate of anchorage-independent proliferation of SrcF cells on poly-HEMA (Figure 3c). Taken together these experiments indicate that, under a number of different experimental conditions, CAS has little or no effect on SrcF529-mediated anchorage-independent growth.

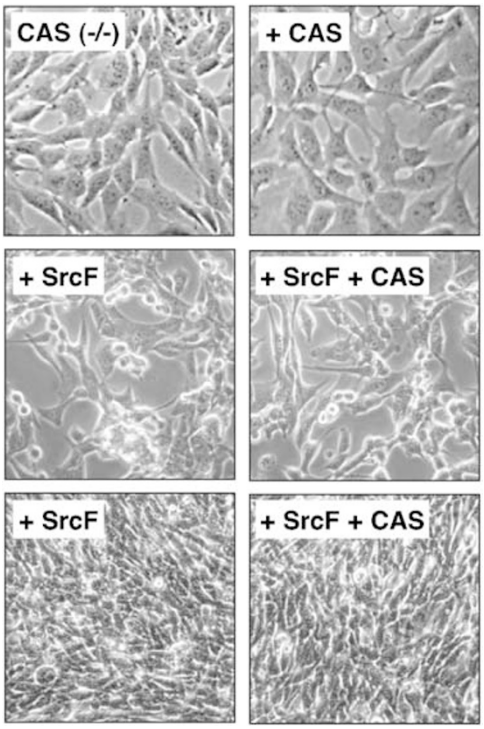

Figure 2 Effect of CAS and SrcF expression on cell morphology. Phase contrast micrographs are shown. Top panels, CAS (−/−) MEF either lacking CAS (left) or re-expressing CAS (right) are shown at subconfluent density. Middle panels, SrcF cells either lacking CAS (left) or after CAS re-expression (right) are shown at subconfluent density. Bottom panels, SrcF cells either lacking CAS (left) or after CAS re-expression (right) are shown at high density

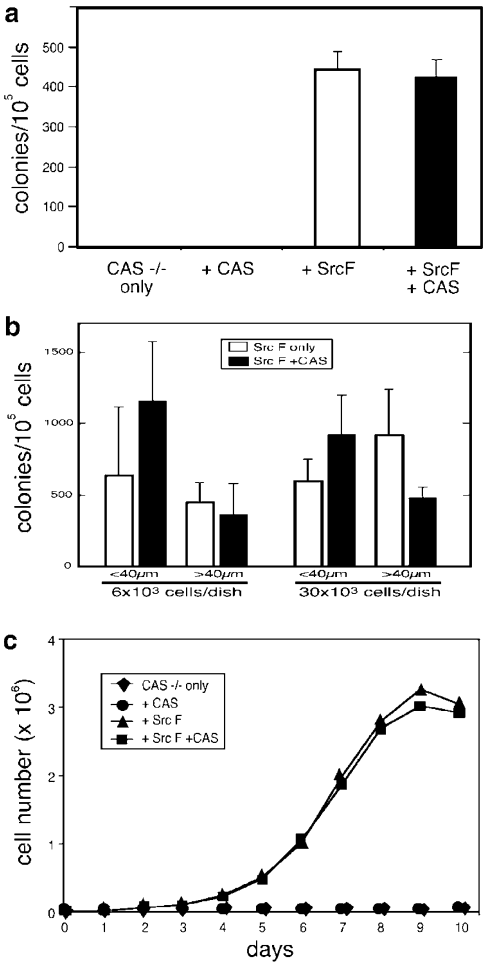

Figure 3 Effect of CAS and SrcF expression on anchorage-independent growth. (**a** and **b**) Soft agar colony formation. (**a**) Visible colonies detected 4 weeks after plating 30 000 cells per 35 mm dish. Histogram bars represent the average from three independent experiments. (**b**) Colonies detected by automated colony counter 2 weeks after plating either 6000 or 30 000 cells per 35 mm dish. Histogram bars represent the average from three replicate plates for each of two independent experiments. Error bars represent 95% confidence intervals. (**c**) Cell growth curves on nonadhesive poly-HEMA dishes. A representative experiment is shown. No colony formation or growth on poly-HEMA was apparent in the absence of SrcF

CAS enhances tyrosine phosphorylation of integrin-associated Src substrates

Although CAS expression did not dramatically alter the overall protein phosphotyrosine profile of the SrcF cells, we wished to more carefully examine the possibility that CAS could act as a scaffold to recruit SrcF to phosphorylate adhesion-related substrates. Since CAS directly associates with FAK, FAK tyrosine phosphorylation was assessed in SrcF cell populations that either expressed or lacked CAS. FAK was immunoprecipitated and analysed by immunoblotting with a general antiphosphotyrosine antibody. Although SrcF expression significantly elevated FAK phosphotyrosine in the absence of CAS, expression of CAS in the SrcF cells resulted in a striking further increase in FAK phosphotyrosine (Figure 4a, top two panels). In the absence of SrcF, CAS expression resulted in only a very minor increase in FAK phosphotyrosine. To further analyse FAK tyrosine phosphorylation under these conditions, replicate blots were also probed with phosphospecific antibodies against major FAK phosphoacceptor tyrosines targeted by Src. While the pY397 antibody against the FAK autophosphorylation site (also an Src site) exhibited increased immunoreactivity resulting from CAS expression in the SrcF cells, more striking increases were observed for Src-specific sites including the kinase domain activation loop sites (Tyr-576/577) and the major site in the C-terminal region (Tyr-861) (Figure 4a, bottom three panels). The capacity for CAS to stimulate tyrosine phosphorylation of paxillin, another major adhesion-associated oncogenic Src substrate, was also examined through immunoprecipitation followed by antiphosphotyrosine immunoblotting. Similar to FAK, CAS expression increased paxillin phosphotyrosine content in the absence of SrcF expression and appeared to also further elevate paxillin phosphotyrosine in the presence of SrcF as indicated by a retarded electrophoretic mobility (Figure 4b, top two panels). Immunoblotting with a phosphospecific antibody directed against a major Src site of paxillin tyrosine phosphorylation (Tyr-118) showed that CAS expression dramatically increased the phosphorylation of this site, both in

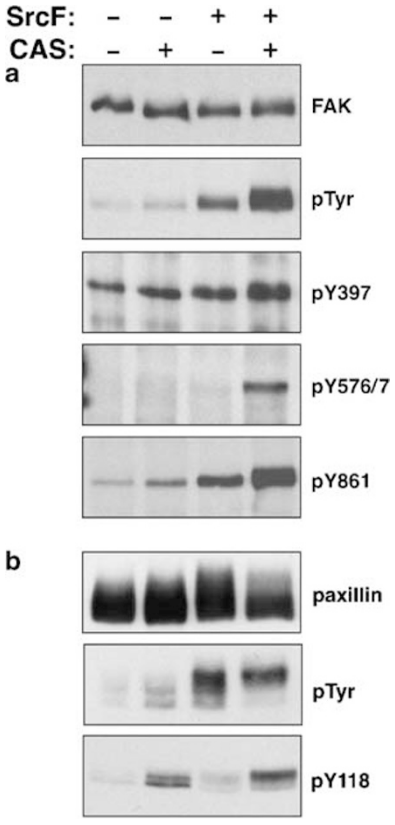

Figure 4 Effect of CAS and SrcF expression on FAK and paxillin phosphorylation. (**a**) FAK immunoprecipitates from the indicated cell populations were analysed by immunoblotting with either FAK C-20 antibody (top panel), general antiphosphotyrosine (pTyr) 4G10 antibody (second panel), or the indicated FAK-phosphospecific antibodies (lower three panels). (**b**) Paxillin immunoprecipitates were analysed by immunoblotting with either antipaxillin antibody (top panel) or 4G10 antibody (middle panel). Equal protein amounts of total cell lysates were analysed by immunoblotting with a phosphospecific antibody against the paxillin Tyr-118 site (bottom panel)

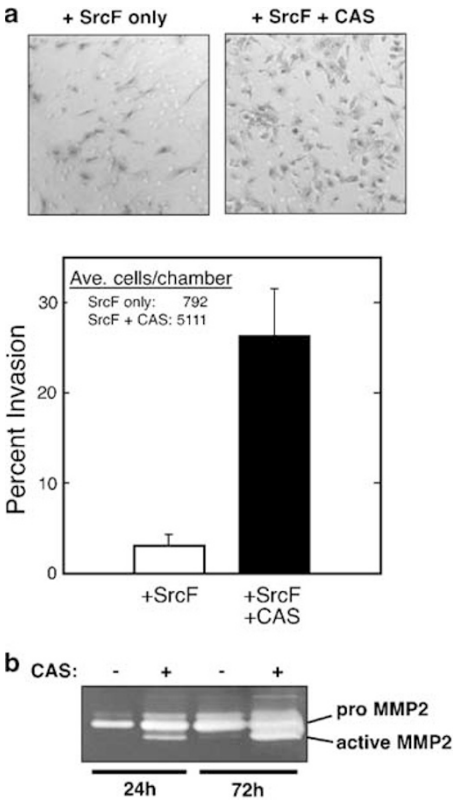

Figure 5 Effect of CAS expression on SrcF-transformed cell invasion and MMP-2 activation. (**a**) Matrigel invasion assay. Top panels are representative photomicrographic fields of the lower surface of Matrigel membranes showing migrating cells stained 48 h after plating on the top surface. The histogram bars represent the mean percent of invasion from six replicate assays, with error bars representing standard deviations. The average number of invading cells per Matrigel chamber is shown in the inset. (**b**) Gelatin zymography assay of conditioned cell media. The result is representative of five independent experiments

the absence or presence of SrcF (Figure 4b, bottom panel). These observations implicate CAS as a major regulator of the activities of both cellular Src family kinases and activated oncogenic Src at integrin adhesion sites.

CAS expression in SrcF-transformed cells promotes Matrigel invasion, MMP-2 activation, and formation of podosome rings and belts

Oncogenic Src expression is also associated with an increased capacity for cell invasion through barriers of extracellular matrix proteins (Chen *et al*., 1985). To examine whether CAS can influence the invasion of SrcF cells, assays were performed using Matrigel invasion chambers. In several independent experiments, CAS expression in the SrcF cells led to a six- to eight-fold enhancement of the invasion capacity quantitated as the 'percent invasion' (the number of cells invading through Matrigel-coated membranes divided by the number of cells migrating through uncoated membranes) over a 48 h period (Figure 5a shows a representative experiment). Under the conditions of this assay, the well-documented capacity of CAS to enhance cell motility was not evident in the control chambers, possibly due to the long time course of the invasion assay and/or overriding effects of SrcF expression.

To invade, cells must degrade the matrix barrier, and oncogenic Src has been shown to promote increased expression of matrix metalloproteinases (Hamaguchi *et al*., 1995; Vincenti *et al*., 1998). To examine whether CAS has a role in regulating the secretion and/or activity of matrix metalloproteinases, gelatin zymography assays were performed on the conditioned media of SrcF cells either expressing or lacking CAS. The major gelatinase activity was detected in the region of the zymograms corresponding to MMP-2 (gelatinase A). Although secreted pro-MMP-2 was observed in the conditioned media of both cell populations, the cleaved activated form of MMP-2 was substantially increased in the cells expressing CAS (Figure 5b).

Src-transformed fibroblasts are also characterized by the presence of podosomes – actin-rich cell/ECM contact structures that represent major sites of ECM degradation (Mizutani *et al*., 2002). Since CAS promoted invasiveness of the SrcF-transformed cells, it was of interest to determine if this was associated with changes in podosome number or distribution. Using a phalloidin probe for F-actin, podosomes were revealed in SrcF cells either expressing or lacking CAS (Figure 6a, b). However, in cells expressing CAS, the podosomes were frequently found organized into much larger aggregates appearing as internal rings, partial rings, and peripheral belts (Figure 6b–d). The ring and belt structures were detected in ~45% of the CAS-expressing SrcF cells but in less than 3% of SrcF cells not expressing CAS (Figure 6e). Podosome rings and belts were also strikingly visualized by staining with a phosphospecific antibody against cortactin Tyr-421 (Figure 6c, d). To determine if CAS SD tyrosine phosphorylation is physically associated with the large podosome rings and belts, antibody 'pCAS-165' that specifically recognizes phosphorylated YxxP tyrosines in the CAS SD (Fonseca *et al*., 2004) was also used to stain the SrcF cells expressing CAS. This antibody also prominently recognized the podosome ring and belt structures (Figure 7a, b), supporting a possible role for

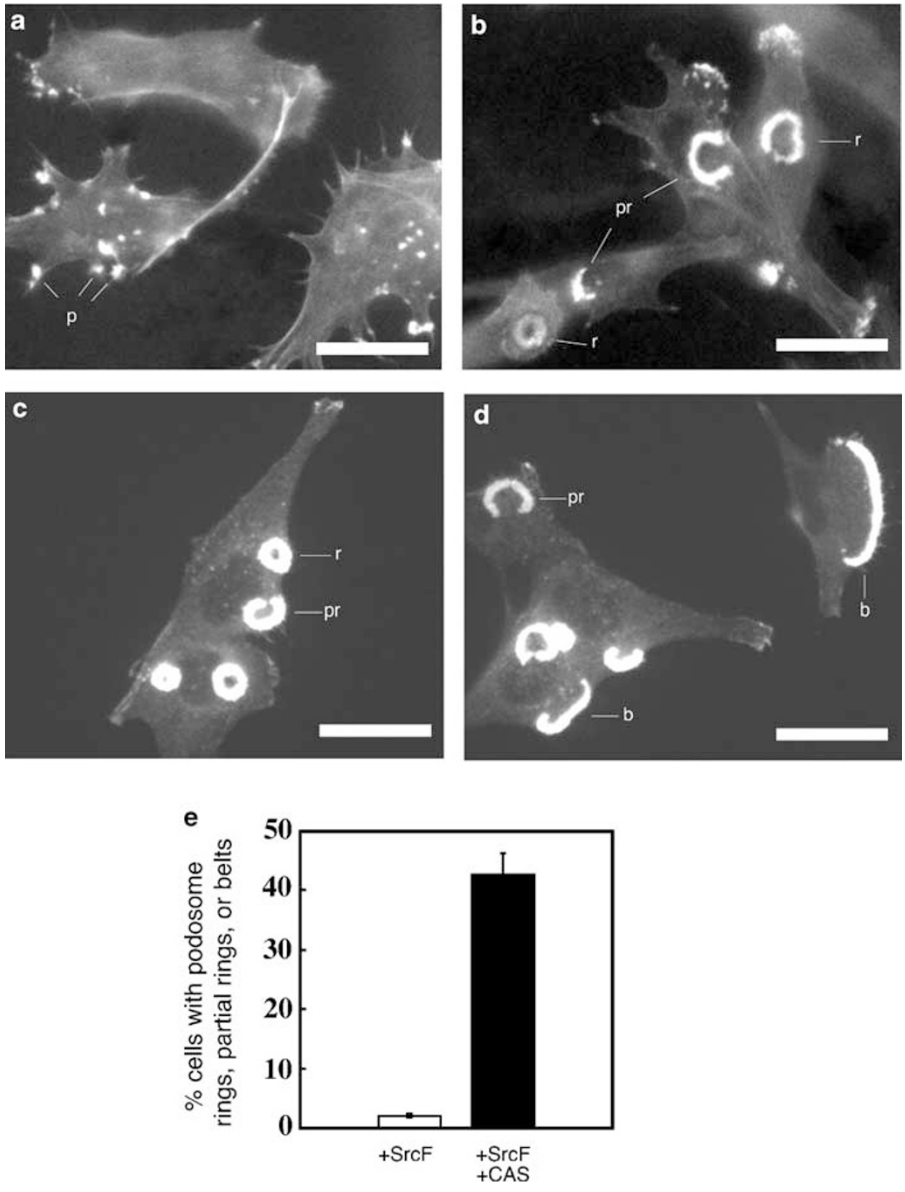

Figure 6 Effect of CAS expression on podosome formation. SrcF-expressing cells, either in the absence (**a**) or presence (**b–d**) of CAS were analysed by fluorescence microscopy using either phalloidin (**a, b**), or cortactin Tyr-421 phosphospecific antibody (**c, d**). Representative examples are marked showing the small punctate podosomes (p) typically seen in cells not expressing CAS, and the large podosome rings (r), partial rings (pr) and belts (b) prominent in CAS-expressing cells. (**e**) Histogram bars represent the percent of SrcF cells having podosome rings, partial rings, or belts (either in the absence or presence or CAS), with error bars representing standard deviations

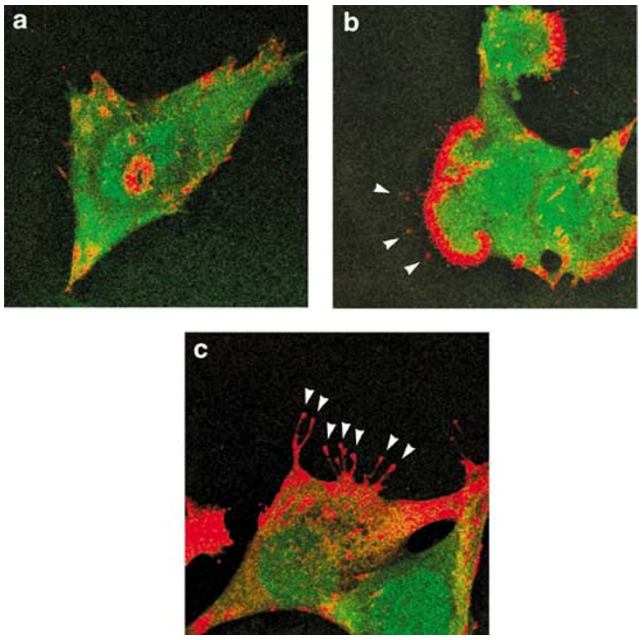

Figure 7 Localization of tyrosine-phosphorylated CAS in SrcF-expressing cells. Confocal sections near the ventral cell surface are shown. The red color indicates staining by the pCAS-165 antibody specific for phosphorylated CAS SD YxxP tyrosines. The green color indicates the general cellular fluorescence from GFP co-expressed with SrcF from the bicistronic transcript. Tyrosine-phosphorylated CAS is detected in podosome rings (panel a), podosome belts (panel b), and in extended filopodia-like plasma membrane protrusions (panels b and c, arrowheads)

CAS signaling in the formation of these structures. In addition, the pCAS-165 antibody revealed the presence of unusual plasma membrane projections, suggestive of highly extended filopodia, that were typically apparent as extensions from the podosome belts (Figure 7b, arrowheads) and also in other cells where belts were not evident (Figure 7c, arrowheads). The pCAS-165 antibody staining was particularly prominent at the tips of the extended membrane protrusions.

Taken together, these data demonstrate a major role for CAS in promoting an invasive phenotype of cells expressing oncogenic Src through a mechanism involving both MMP-2 activation and upregulation of actin polymerization mechanisms involved in podosome assembly and plasma membrane protrusion.

Discussion

To address the potential role of CAS in oncogenic Src-mediated cell transformation, we examined the characteristics and behavior of MEFs expressing the activated mouse Src Phe-529 mutant ('SrcF cells'), either in the presence or absence of CAS expression. While our results did not support a critical role for CAS in either morphological or anchorage-independent growth characteristics, the presence of CAS was found to profoundly affect other aspects of oncogenic Src function. CAS expression in SrcF cells resulted in more extensive tyrosine phosphorylation of major adhesion-associated Src substrates FAK and paxillin, supporting a role for CAS as a positive regulator of SrcF activity. Importantly, CAS expression greatly enhanced the invasive behavior of SrcF cells in association with matrix metalloproteinase MMP-2 activation and the formation of large podosomal aggregates appearing as internal ring and peripheral belt structures.

Our observations that CAS expression did not significantly affect either morphologic or anchorage-independent growth characteristics of Src-transformed cells are in apparent contrast with past reports. Honda *et al*. (1998) first reported that clonal lines of CAS−/− MEFs expressing an activated Src mutant exhibited partial morphologic transformation characterized by a spindle-shaped appearance, but only when CAS was also expressed were clones obtained in which the cells took on a fully rounded refractile morphology and formed colonies in soft agar. Subsequently, Goldberg *et al*. (2003) studied nonclonal populations of the CAS−/− MEFs expressing v-Src and also concluded that CAS co-expression enhanced morphologic transformation and anchorage-independent growth. However, in the Goldberg *et al*. study, many fully rounded cells were evident and significant anchorage-independent growth was achieved even in the absence of CAS. In further contrast, we observed robust morphologic transformation and nonadherent growth upon expression of the activated SrcF mutant protein in CAS−/− MEFs, and these properties were essentially unchanged by CAS expression. Several experimental variables could account for these different outcomes, including differences in the oncogenic Src forms used, their expression levels, genetic changes in the CAS−/− MEF populations, and the use of clonal *versus* whole population selection strategies. Nevertheless, our results indicate that the presence of CAS is not a strict requirement for Src-mediated morphological transformation and anchorage-independent growth. Similar findings that FAK is dispensable for these characteristics (Hauck *et al*., 2002; Roy *et al*., 2002; Moissoglu and Gelman, 2003) further downplay a role for adhesion-associated substrates in these aspects of Src transformation, while anchorage-independent growth has been linked to other oncogenic Src-activated pathways including Ras-MAPK, JAK-STAT, and PI3K-mTOR (Martin, 2001; Frame *et al*., 2002).

Although our study indicated that CAS is not strictly required for morphologic transformation and anchorage-independent growth, we found that CAS plays a major role in other aspects of oncogenic Src function affecting cell behavior. We found an unexpected role for CAS in promoting the ability of SrcF to promote tyrosine phosphorylation of the other major integrin-associated substrates FAK and paxillin. Mechanistically, CAS may act as a scaffold protein in recruiting SrcF to phosphorylate FAK, involving the well-characterized direct interactions of the CAS SBD with SrcF and the CAS SH3 domain with FAK. Since paxillin also interacts with FAK, and could therefore come into indirect contact with CAS, this proposed scaffolding

function could also account for the observed CAS-mediated paxillin tyrosine phosphorylation. Compared to FAK, the CAS scaffolding function appears to be more important in directing the endogenous Src family kinases to paxillin since the effect of CAS expression was also very evident even in the absence of SrcF. This finding is reminiscent of another study (Burnham *et al*. 2000) where overexpression of CAS together with c-Src in COS-1 cells had relatively little effect on FAK phosphorylation but dramatically enhanced the phosphotyrosine content of paxillin, due to the CAS SBD binding to the Src SH3 domain and thereby acting to release c-Src autoinhibition. In the case of the activated SrcF mutant, kinase activity may be further enhanced by SH3 domain displacement (Brábek *et al*., 2002) upon binding to the CAS SBD. In addition to the CAS scaffold mechanism, Src family kinases can also be recruited and activated to phosphorylate FAK, CAS, and paxillin through another mechanism involving direct SH2 binding to the FAK Tyr-397 site (Schaller and Parsons, 1995; Ruest *et al*., 2000; Roy *et al*., 2002). Paxillin also can act as a positive regulator of FAK and CAS phosphorylation by Src (Hagel *et al*., 2002). Thus, the integrin-associated FAK-Src-CAS-paxillin signaling complex can be considered as a functional unit in which all components must be present to achieve optimal output.

A notable cellular consequence of oncogenic Src expression is the acquisition of an invasive behavior (Chen *et al*., 1985) associated with increased expression and secretion of matrix metalloproteinases (Mueller and Chen, 1991; Hamaguchi *et al*., 1995). Recently, Schlaepfer and co-workers showed that FAK-mediated signaling can contribute to the invasive behavior of v-Src transformed fibroblasts and implicated CAS in this process (Hauck *et al*., 2002; Hsia *et al*., 2003). To further test the role of CAS in invasion, we examined the capacity of our SrcF-expressing cell populations to invade through Matrigel. Our results demonstrate that CAS expression results in a strong enhancement of invasion through Matrigel that is independent of its ability to promote cell migration.

To better understand the mechanism by which CAS promotes cell invasion, we carried out zymography assays and observed CAS expression to be associated with a striking increase in the activation of the major matrix-degrading enzyme MMP-2. Past studies have shown MMP-2 to be locally activated to promote ECM degradation at specialized cell surface projections termed 'invadopodia' (Chen, 1989; Monsky *et al*., 1993; Chen and Wang, 1999; Deryugina *et al*., 2001). Invadopodia are important for invasion of many cell types including human melanoma and breast carcinoma cells (reviewed in Chen and Wang, 1999) and correspond to the actin-rich podosomes of Src-transformed cells (Chen, 1989). We found that CAS expression in SrcF cells also resulted in a striking increase in the formation of podosomes, associated with their organization into large aggregates appearing as internal ring and peripheral belt structures. Consistent with this finding, Honda *et al*. (1998) noted that actin filaments are more concentrated in podosomes of oncogenic Src-expressing wild-type cells compared to CAS-deficient cells. Based on our observations, we propose that CAS-related signaling events act to promote MMP-2 activation and invasion, at least in part, by promoting the formation of podosomes/invadopodia.

We showed previously that SrcF expression results in elevated phosphorylation of CAS SD YxxP tyrosines, as revealed by pCAS phosphospecific antibodies against these sites (Fonseca *et al*., 2004). In this study we showed that a pCAS SD antibody prominently stains the podosome rings and belts as well as unusual highly extended plasma membrane protrusions suggestive of invadopodia. Elevated CAS SD tyrosine phosphorylation at cell/ECM adhesion sites could be a driving force in the formation of podosomes in the SrcF cells due to the consequent recruitment of Crk and/or Nck adaptor proteins that possess the signaling capacity to locally activate WASP/WAVE family proteins and the Arp2/3 complex to promote actin polymerization. Aberrant enhanced actin polymerization would then drive the maturation of focal adhesions into the unusual podosome structures associated with the protrusion of ECM-degrading invadopodia. In this context, it has been shown that Arp2/3 localization is an early step required for the formation of podosomes in smooth muscle cells (Kaverina *et al*., 2003), and that dominant-negative mutants of N-WASP can inhibit podosome formation in Src transformed rat fibroblasts (Mizutani *et al*., 2002). However, there is no evidence to date that Crk and Nck proteins are present in podosomes. A second mechanistic possibility involves CAS as a regulator of SrcF activity at adhesion and/or podosome sites. In addition to FAK and paxillin shown in this study, CAS may also direct Src to phosphorylate a number of other podosome-associated Src substrates, including Fish, N-WASP/WASp, cortactin, integrins, tensin, talin, and vinculin (Abram *et al*., 2003; Linder and Aepfelbacher, 2003) in order to effect podosome assembly. The recent finding that FAK can phosphorylate and stimulate the activity of N-WASP (Wu *et al*., 2004) raises the possibility that CAS promotes invasion in part through its ability to promote phosphorylation of the FAK activation loop tyrosines 576/577, which can elevate FAK catalytic activity. Further work addressing these many possibilities will be necessary to reach a full mechanistic understanding of the role of CAS in integrin-related signaling events contributing to podosome formation and invasive cell behavior.

Materials and methods

Cells, cell culture, and antibodies

CAS−/− MEFs were kindly provided by Hisamaru Hirai (U Tokyo) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% nonessential amino acids and 10% fetal bovine serum. The retroviral packaging cell line Phoenix ECO, kindly provided by Gary Nolan (Stanford U.), was maintained in DMEM containing 10% heat-inactivated FBS. Monoclonal antibodies against CAS

(clone 24) and paxillin (clone 349), and horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit IgG were obtained from BD Transduction Laboratories. Anti-Src monoclonal antibody 327 was from Oncogene Research Products. Anti-phosphotyrosine monoclonal antibody 4G10 was from Upstate Biotechnology. Anti-FAK rabbit polyclonal antibody (FAK C-20) was from Santa Cruz Biotechnology. Phosphospecific antibodies against FAK phosphotyrosines 397, 576/577, 861, and cortactin-phosphotyrosine 421 were from Biosource International. Phosphospecific antibodies against CAS phosphotyrosine 165 and paxillin phosphotyrosine 118 were from Cell Signaling Technology. Rabbit anti-mouse IgG antibody was from Jackson ImmunoResearch Labs.

Immunoblotting and immunoprecipitation

Subconfluent cell cultures were washed with phosphate-buffered saline (PBS) and lysed in modified RIPA buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% NP40, 1% sodium deoxycholate, 50 mM NaF, 1% aprotinin and 0.1 mM Na_3VO_4). Protein concentrations in the lysates were determined using the BCA assay (Pierce). Lysates equivalent to 10–30 μg protein were diluted in 2 × SDS–PAGE sample buffer for immunoblot analysis of whole-cell extracts. For immunoblotting, samples were separated on 7% SDS–polyacrylamide gels and transferred onto nitrocellulose membrane. Nonspecific activity was blocked by incubating 1 h at room temperature in Tris-buffered saline containing 0.2% Tween 20 (TBST) and 5% nonfat dry milk (3% for anti-phosphotyrosine antibody). Membranes were then incubated overnight in primary antibody, washed extensively with TBST, and then incubated for 1 h at room temperature with HRP-conjugated secondary antibody. After extensive washing in TBST, the blots were developed by enhanced chemiluminescence (ECL, Amersham Biosciences) and exposed to autoradiographic film. Immunoprecipitations were carried out from 1 ml RIPA lysates containing 500 μg protein. Lysates were incubated 4 h on ice with 1 μg primary antibody (FAK C-20 or anti-paxillin monoclonal antibody 349) and immune complexes were collected by an additional 1 h incubation with protein A-Sepharose (20 μl 50% slurry; Zymed). For paxillin immunoprecipitation, an additional 1 h incubation with 5 μg rabbit anti-mouse IgG was carried out prior to the protein A-Sepharose step. The immunoprecipitates were washed five times with 1 ml ice-cold RIPA buffer, resuspended in 2 × SDS–PAGE sample buffer, and processed for immunoblotting.

Construction of a retroviral vector for expression of activated Src-F529 and generation of cells expressing Src-F529 in the presence or absence of CAS

Initially, plasmid pRc/CMV-SrcF529 carrying the cDNA for activated mouse nonneuronal c-Src-F529 ('SrcF') was constructed by deleting the six amino acid insert of neuronal Src from plasmid pRc/CMV-nSrcF535 (Polte and Hanks, 1997). An *Eco*RI restriction fragment of pRc/CMV-SrcF529 containing the cDNA was then ligated into the *Eco*RI site of the bicistronic retroviral vector pLZRS-MS-IRES-GFP (Ireton *et al*., 2002). The resulting pLZRS-SrcF-IRES-GFP plasmid, after sequence verification, was transfected into Phoenix E cells and LZRS-SrcF-IRES-GFP viral supernatant was prepared and used to infect CAS−/− MEFs as described (Ireton *et al*., 2002). Three successive rounds of infection were carried out to increase both infection rate and expression levels. Cells expressing SrcF were then selected by two rounds of FACS sorting for GFP, encoded by a cDNA downstream of the SrcF cDNA on the bicistronic transcript. Gates were set to collect cells in the top third of the GFP range. After expansion, SrcF expression in the sorted cell populations was estimated to be three- to five-fold over endogenous c-Src levels as determined by immunoblotting. WT mouse CAS was expressed in both the parental CAS−/− MEFs and the SrcF-expressing CAS−/− MEFs using a similar strategy but employing a modified LZRS-IRES vector in which the bicistronic transcript expresses the CAS cDNA upstream of a bleomycin resistance gene. Cells expressing CAS were selected by growth for 3 weeks in media containing 1 mg/ml zeocin.

Analysis of cell morphology and anchorage-independent cell growth

Photomicrographs of cells growing under standard culture conditions were taken using a Zeiss Axiovert 25 microscope equipped with a digital still camera.

For soft agar colony formation assays, the indicated number of cells were suspended in 1 ml DMEM containing 10% FBS and 0.4% SeaPlaque agarose (FMC Bioproducts), then poured into a 35 mm diameter dish over a base layer of 0.8% agarose in DMEM. After allowing the top layer to set at room temperature, the suspension cultures were incubated at 37°C in 5% CO_2 while refeeding every 2 days with fresh DMEM containing 10% FBS. Colonies were either counted by eye after 4 weeks, or counted using a Omnicon 3800 automated colony counter (BioLogics, Inc.) after 2 weeks.

For assay of nonadherent growth on polyhydroxymethacrylate (poly-HEMA, Sigma), 60 mm tissue culture dishes were coated by adding 2.7 ml of 1.2% poly-HEMA in 95% ethanol then allowing complete evaporation to occur by incubation at 37°C in a nonhumidified incubator. The dishes were then washed extensively with PBS, sterilized in UV light, and each plated with 30 000 cells in DMEM containing 10% FBS. The suspension cultures were incubated at 37°C in 5% CO_2. At daily intervals, cells from two dishes were separately harvested, trypsinized to disrupt aggregates, then resuspended in DMEM and counted using a hemacytometer. The average cell number from the two dishes was used for the plotting the growth curves.

Matrigel invasion

Matrigel invasion assays were carried out using Biocoat Matrigel invasion chamber inserts and control uncoated inserts (BD Transduction Laboratories) according to the manufacturer's protocol. Briefly, membranes were rehydrated for 2 h in DMEM and placed in wells containing 5% FBS in DMEM. A total of 50 000 cells suspended in 0.5 ml serum-free DMEM were added to the top of each chamber (Matrigel coated or control chambers), then incubated at 37°C in 5% CO_2. After 48 h, the chambers were washed with PBS and cells were removed from the top side of membranes with a cotton swab. Invading or migrating cells were fixed in 3.7% formaldehyde for 1 h then stained with 2% crystal violet. Representative fields were documented by photomicroscopy, and the numbers of invading or migrating cells was determined from six replicate chambers by counting 10 random areas over a grid pattern. The 'percent 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

In total, 200 000 cells were plated per well of a 24 well dish. After 16 h, cells were washed with PBS and incubated in 300 μl

serum-free media for 24 or 72 h. Aliquots (25 μl) of the conditioned media were loaded for zymography on a 10% SDS–PAGE gel containing 1 mg/ml gelatin as described (Pozzi *et al*., 2000). Briefly, gel proteins were washed for 1 h in 50 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 2.5% Triton X-100, and then incubated at 37°C in 50 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$, 0.02% NaAzide for 17 h. Gels were stained with Coomassie blue and destained in 7% acetic acid/5% methanol.

Cell staining

Cells were grown overnight on fibronectin/gelatin-coated coverslips prepared essentially as described by Chen *et al*. (1994), then fixed in 4% paraformaldehyde in 127 mM NaCl, 5 mM KCl, 1.1 mM NaH_2PO_4, 0.4 mM KH_2PO_4, 2 mM $MgCl_2$, 5.5 mM glucose, 1 mM EGTA, 20 mM PIPES (pH 7.1), and permeabilized with 0.4% Triton-X-100 in PBS. The cells were then stained with either Alexa-594-phalloidin (3.3 nM, Molecular Probes) or antibodies against phosphoCas (1 : 100, pCAS165) or phosphocortactin (1 : 500, anti-PY421), followed by Alexa-594 labeled secondary antibodies (1 : 2000, Molecular Probes). Coverslips were mounted using the Prolong Antifade Kit (Molecular Probes), and imaged on either a Zeiss LSM510 confocal microscope using a $\times 63$ oil immersion PlanApo objective or a Zeiss Axioplan 2 widefield microscope using a $\times 20$ NeoFluor objective and a Hamamatsu Orca ER digital camera. The percent of cells with podosome rings, partial rings, or belts was determined by two independent readers from digital images of cells stained with Alexa-594-phalloidin.

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