Blockade of v-Src-stimulated tumor formation by the Src homology 3 domain of Crk-associated substrate (Cas)

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Received 30 September 2003; revised 3 December 2003; accepted 6 December 2003

First published online 29 December 2003

Edited by Masayuki Miyasaka

Abstract Crk-associated substrate (Cas) is highly phosphorylated by v-Src and plays a critical role in v-Src-induced cell transformation. In this study, we found that the Src homology (SH) 3 domain of Cas blocked v-Src-stimulated anchorage-independent cell growth, Matrigel invasion, and tumor growth in nude mice. Biochemical analysis revealed that the Cas SH3 domain selectively inhibited v-Src-stimulated activations of AKT and JNK, but not ERK and STAT3. Attenuation of the AKT pathway by the Cas SH3 domain rendered v-Src-transformed cells susceptible to apoptosis. Inhibition of the JNK pathway by the Cas SH3 domain led to suppression of v-Src-stimulated invasion. Taken together, our results indicate that the Cas SH3 domain has an anti-tumor function, which severely impairs the transforming potential of v-Src.

Key words: Src; Crk-associated substrate; Src homology 3 domain; Tumor; Anoikis; Invasion

1. Introduction

The *v-src* oncogene, which was first identified from the transforming gene of Rous sarcoma virus, encodes a constitutively activated form of c-Src non-receptor tyrosine kinase [1–3]. Cell transformation by v-Src results in a wide variety of phenotypic changes, including morphological transformation, increase in cell migration or invasiveness, acquisition of anchorage and growth factor independence, and tumorigenicity [4]. Activation of several intracellular signal transduction pathways has been shown to be associated with transforming potential of activated Src, such as the Ras/ERK [5], phosphatidylinositol 3-kinase (PI3K)/AKT [6], and STAT3/Myc [7] pathways. Considerable evidence now indicates that elevated expression and/or activity of Src is associated with human cancers [8].

Crk-associated substrate (Cas) was originally identified and cloned as a highly tyrosine-phosphorylated protein in cells transformed by v-Src [9] or v-Crk [10]. It contains an NH_2-terminal Src homology 3 (SH3) domain, a substrate domain that consists of 15 YXXP motifs, and a COOH-terminal Src binding domain [11,12]. The SH3 domain of Cas could bind proline-rich sequences of various signaling molecules such as focal adhesion kinase (FAK [13]), guanine nucleotide exchange factor C3G [14], and tyrosine phosphatase PTP-PEST [15]. Cas-deficient mouse embryonic fibroblasts showed impaired actin bundling and cell migration and were refractory to transformation by activated Src [16]. These phenotypes were restored after re-expression of Cas, suggesting that Cas plays essential roles in the control of the actin cytoskeleton organization, cell migration, and transformation [16,17].

Cas has been demonstrated to act downstream of FAK to promote cell migration and cell survival [18,19]. The expression of the Cas SH3 domain alone was found to inhibit FAK-promoted cell functions by competing with endogenous Cas for FAK binding [18,19]. Recently, we showed that the Cas SH3 domain is able to reverse the transformed cell phenotype induced by the synergistic effect of FAK overexpression and hepatocyte growth factor stimulation [20]. In this study, we have examined whether the Cas SH3 domain has an inhibitory effect on v-Src-stimulated cell transformation.

2. Materials and methods

2.1. Materials

Matrigel was purchased from Collaborative Biomedical Products (Bedford, MA, USA). The 24-well transwell chamber for the invasion assay was purchased from Costar (Cambridge, MA, USA). The monoclonal anti-FAK, anti-phosphotyrosine, anti-STAT3, anti-Bcl-2, anti-Bad, and anti-Bcl-X_L antibodies were purchased from BD Transduction Laboratories (Lexington, KY, USA). The rabbit polyclonal anti-Cas (C-20), anti-Crk (C-18), anti-Src (N-16), anti-ERK (K-23), and anti-JNK (C-17) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The rabbit polyclonal anti-phosphoERK (Thr202/Tyr204), anti-phosphoJNK (Thr183/Tyr-

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Abbreviations: Cas, Crk-associated substrate; SH, Src homology; PI3K, phosphatidylinositol 3-kinase; FAK, focal adhesion kinase; HA, hemagglutinin; polyHEMA, poly-hydroxyethylmethacrylate; PYK2, proline-rich tyrosine kinase 2; MMP, matrix metalloproteinase

0014-5793/03/$30.00
doi:10.1016/S0014-5793(03)01501-1

185), anti-phospho-c-Jun (Ser63), anti-phosphoSTAT3 (Tyr705), anti-AKT, anti-phosphoAKT (Ser473), anti-phosphoFKHR (Ser256), anti-phosphoBAD (Ser136), anti-cleaved caspase 3 antibodies were purchased from Cell Signaling Technology. (Beverly, MA, USA). The rabbit polyclonal anti-phosphoSrc (Tyr416) antibody was purchased from Biosource (Camarillo, CA, USA). The monoclonal anti-hemagglutinin (HA) epitope was purchased from Roche. The mouse ascites containing monoclonal anti-Src (peptide 2–17) antibody produced by the hybridoma (ATCC, CRL-2651) was collected in our laboratory. The plasmid pKH3-Cas SH3 was described previously [19,20].

2.2. Cell lines and biological assays

NIH3T3 cells and v-Src-transformed NIH3T3 cells were described previously [21] and were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. To generate NIH3T3 cells or v-Src-transformed NIH3T3 cells stably expressing HA-CasSH3, NIH3T3 cells or v-Src-transformed NIH3T3 cells were grown on 60-mm dishes and co-transfected with 2 μg of pKH3-CasSH3 and 0.2 μg of pREP3 using 10 μl of lipofectamine following the manufacturer's instructions. Clones were selected in growth medium containing 0.5 mg/ml G418 and 100 U/ml hygromycin and screened by immunoblotting with anti-HA and in vitro Src activity assay.

Soft agar colony formation assays and Matrigel invasion assays were performed as described previously [20]. For gelatin zymography, serum-free 36-h-conditioned medium was harvested from sub-confluent cultures. Volumes representing equivalent cell numbers were separated on a 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) containing 0.1% gelatin. The gel was washed several times in Tris buffer (50 mM Tris, pH 7.4) containing 2.5% (v/v) Triton X-100 at room temperature, followed by further incubation in substrate buffer (50 mM Tris, pH 7.4, 2.5% Triton X-100, 10 mM $CaCl_2$, 150 mM NaCl) at 37°C for 15 h. The gel was fixed in 10% methanol and 10% acetic acid for 10 min and gelatinolytic activity was visualized following Coomassie blue staining.

For the anoikis assay, 10-cm culture dishes were coated with 4 ml of 1.2% poly-hydroxyethylmethacrylate (polyHEMA) in 95% ethanol. The dishes were dried at room temperature and washed once in phosphate-buffered saline and twice in serum-free medium. 3×10^6 cells in serum-free medium were seeded on a polyHEMA-coated plate for 48 h. The cell survival rate was determined by trypan blue exclusion, as described previously [22]. Each experiment was performed in duplicate.

2.3. Tumorigenicity in nude mice

Female 5–6-week-old nude mice were subcutaneously injected in the flank with v-Src-transformed NIH3T3 cells or those stably expressing HA-CasSH3. Each animal received 5×10^5 cells suspended in 0.2 ml of phosphate-buffered saline. Tumor volumes were measured every other day with calipers. When tumors reach a volume of 2500–3000 mm^3, mice were killed and tumors were surgically removed.

2.4. Immunoprecipitations, immunoblotting, and in vitro kinase assay

Immunocomplexes and immunoblotting were performed as described previously [20]. To measure Src or JNK activity, anti-Src or anti-JNK immunoprecipitates were washed three times with 1% NP-40 lysis buffer and once in 20 mM Tris buffer. In vitro kinase reactions were carried out in 40 μl of kinase buffer (50 mM Tris–HCl, pH 7.5, 10 mM $MnCl_2$) containing 10 μCi [γ-^{32}P]ATP (3000 Ci/mmol, NEN) and 5 μg acid-denatured enolase (for Src) or GST-Jun (for JNK) for 20 min at 25°C. Reactions were terminated by the addition of SDS sample buffer and proteins were resolved by SDS–PAGE.

2.5. Immunofluorescent staining

Cells were grown on glass coverslips for 24 h prior to fixation in phosphate-buffered saline containing 4% paraformaldehyde at room temperature for 30 min. Samples were permeabilized with phosphate-buffered saline containing 0.3% Triton X-100 for 30 min. Coverslips were stained with primary antibody for 60 min and followed by goat anti-mouse or anti-rabbit tetramethylrhodamine isothiocyanate-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) at 4 μg/ml for 60 min. Polyclonal anti-Cas (Santa Cruz Biotechnology) and monoclonal anti-paxillin (Transduction Laboratories) antibodies were diluted 1:200 before use. Fluorescein isothiocyanate (FITC)-conjugated phalloidin (Sigma-Aldrich) at 2 μM was used to stain actin filaments. Coverslips were mounted in anti-fading solution and viewed using a Zeiss LSM laser-scanning confocal microscope.

3. Results

3.1. Expression of the Cas SH3 domain inhibits v-Src-stimulated tyrosine phosphorylation of Cas and the JNK pathway

HA epitope-tagged Cas SH3 domain was stably expressed

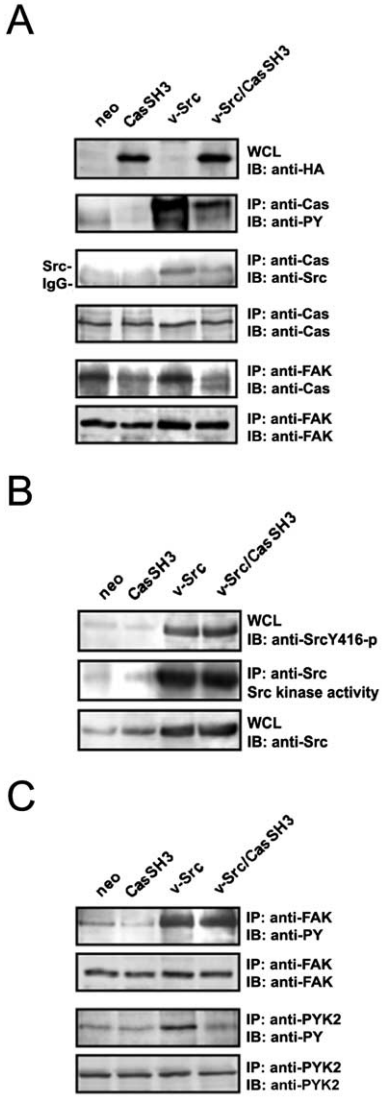

Fig. 1. The Cas SH3 domain inhibits v-Src-stimulated phosphorylation of Cas. The cell lines are designated *neo* for neomycin-resistant control cells; *CasSH3* for the cells expressing the Cas SH3 domain; *v-Src* for the v-Src-transformed cells; *v-Src*/*CasSH3* for the v-Src-transformed cells expressing the Cas SH3 domain. A: Equal amounts of whole cell lysates (*WCL*) were analyzed by immunoblotting (*IB*) with anti-HA to detect the expression of the Cas SH3 domain. The tyrosine phosphorylation of Cas and its associations with Src and FAK were analyzed. B: The phosphorylation (Y416), activity, and expression of Src were analyzed. C: The tyrosine phosphorylation and expression of FAK and PYK2 were analyzed. The result shown is representative of four experiments using two clones for each cell line.

in v-Src-transformed or normal NIH3T3 cells. To avoid clonal variation, several cell clones that expressed similar levels of v-Src and/or the Cas SH3 domain were selected for analysis. In consistency with its function as a dominant-negative version of Cas [18,19], the expression of the Cas SH3 domain in v-Src-transformed cells markedly inhibited v-Src-stimulated phosphorylation of Cas and its associations with Src and FAK (Fig. 1A) without affecting the activity and expression of Src (Fig. 1B). The v-Src-stimulated tyrosine phosphorylation of FAK was not affected by the Cas SH3 domain, but the v-Src-stimulated tyrosine phosphorylation of proline-rich tyrosine kinase 2 (PYK2), another member in the FAK family, was inhibited by the Cas SH3 domain (Fig. 1C).

Tyrosine-phosphorylated Cas is known to recruit the binding of the adapter protein Crk and activate the JNK pathway [23,24]. In v-Src-transformed 3T3 cells, increased Cas phosphorylation coincided with increases in Crk phosphorylation, JNK activation, and c-Jun phosphorylation (Fig. 2A). Conversely, inhibition of v-Src-stimulated Cas phosphorylation by the Cas SH3 domain was concomitant with down-regulation of those, indicating an inhibitory effect of the Cas SH3 domain on the Cas-JNK signaling pathway. In contrast, ERK and STAT3, both of which have been shown to be important for v-Src's transforming potential [25], were not affected by the Cas SH3 domain (Fig. 2B).

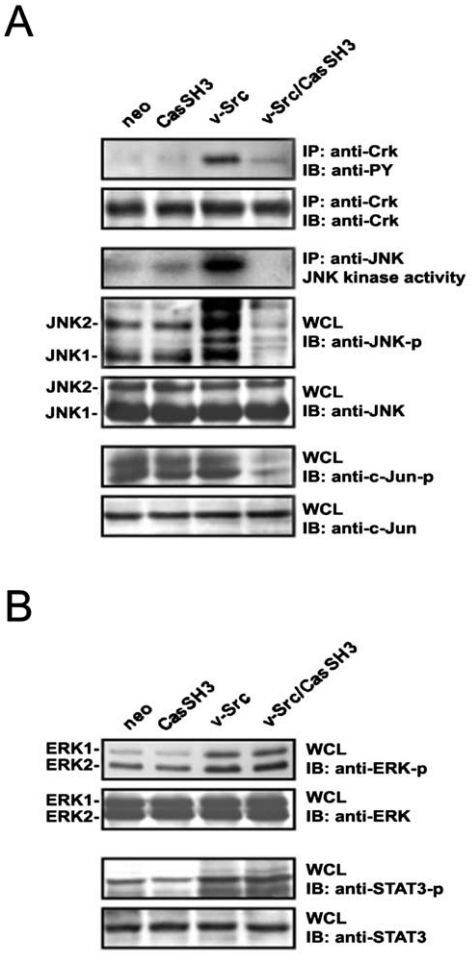

Fig. 2. The Cas SH3 domain inhibits v-Src-stimulated activation of the JNK pathway. A: The tyrosine phosphorylation of Crk, the phosphorylation and activity of JNK, and the phosphorylation of c-Jun were analyzed. B: The phosphorylation and expression of ERK and STAT3 were analyzed. The result shown is representative of four experiments using two clones for each cell line.

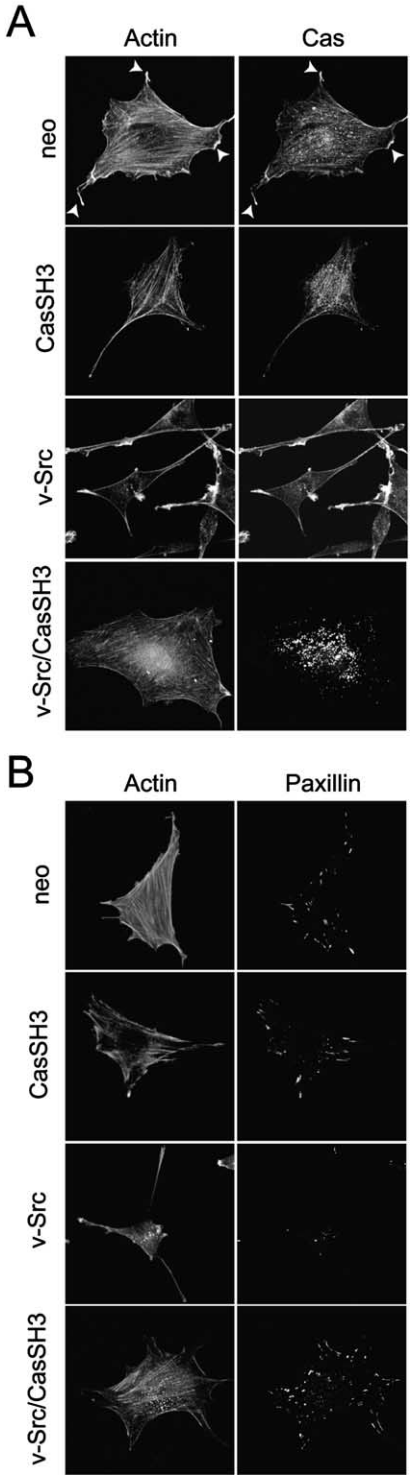

Fig. 3. Effects of the Cas SH3 domain on actin cytoskeleton, Cas localization, and focal contacts. A: Cells were stained for actin with FITC-phalloidin and for Cas with polyclonal anti-Cas. B: Cells were stained for actin with FITC-phalloidin and for paxillin with monoclonal anti-paxillin. Paxillin was stained to mark the location of focal contacts. The result shown is representative of three experiments using two clones for each cell line.

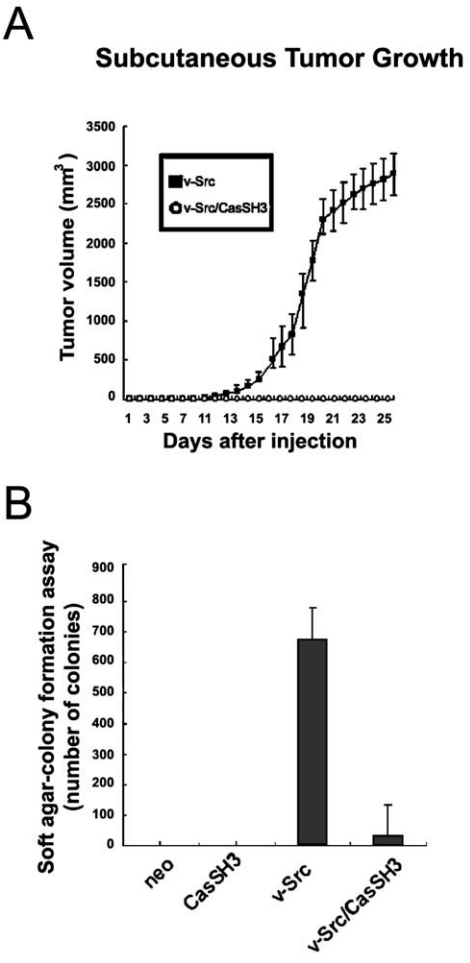

Fig. 4. The Cas SH3 domain blocks v-Src-stimulated tumor growth in vivo and anchorage-independent cell growth in vitro. A: Cells (5×10^5) from v-Src cells or v-Src/CasSH3 cells were subcutaneously injected into nude mice (v-Src, $n=6$; v-Src/CasSH3, $n=9$) to measure their tumorigenicity. Two v-Src cell clones and three v-Src/CasSH3 cell clones were used in the assay. Each clone was injected into three mice. The size of tumor (length × width × height) was measured every other day with calipers. All of the mice injected with v-Src cells formed tumors. In contrast, none of the mice injected with v-Src/CasSH3 cells did. B: Cells (10^4) were subjected to soft agar colony formation assay. One month later, cells were stained and the number of colonies per 60-mm dish was counted. The values (means ± S.E.M.) are from six data points from two experiments using three clones for each cell line.

3.2. The Cas SH3 domain reverses the long spindle morphology of the v-Src-transformed cells into a more spread cell shape

The actin cytoskeleton, localization of Cas, and focal contacts were examined by fluorescent staining (Fig. 3). Paxillin, a protein localized in focal contacts, was stained to mark the location of focal contacts. In control NIH3T3 cells, Cas was localized in focal contacts and organized into long parallel filaments with a similar distribution as actin stress fibers. v-Src-transformed cells exhibited a long spindle shape and had prominent membrane ruffles where Cas was accumulated. Actin stress fibers and focal contacts were hardly detected in those cells. The expression of the Cas SH3 domain in 3T3 cells had little effect on the formation of actin stress fibers and focal contacts, but it led to disappearance of Cas from focal contacts. Importantly, the Cas SH3 domain reversed the long spindle morphology of the v-Src-transformed cells into a more spread cell shape, concomitant with more organized actin cytoskeleton and increased formation of focal contacts. Nevertheless, Cas failed to be localized in focal contacts and showed a punctate distribution in the center of the cells (v-Src/CasSH3) expressing both v-Src and the Cas SH3 domain.

3.3. The Cas SH3 domain blocks v-Src-stimulated tumor growth in vivo and anchorage-independent cell growth in vitro

To analyze the tumorigenicity of the cells, they were sub-

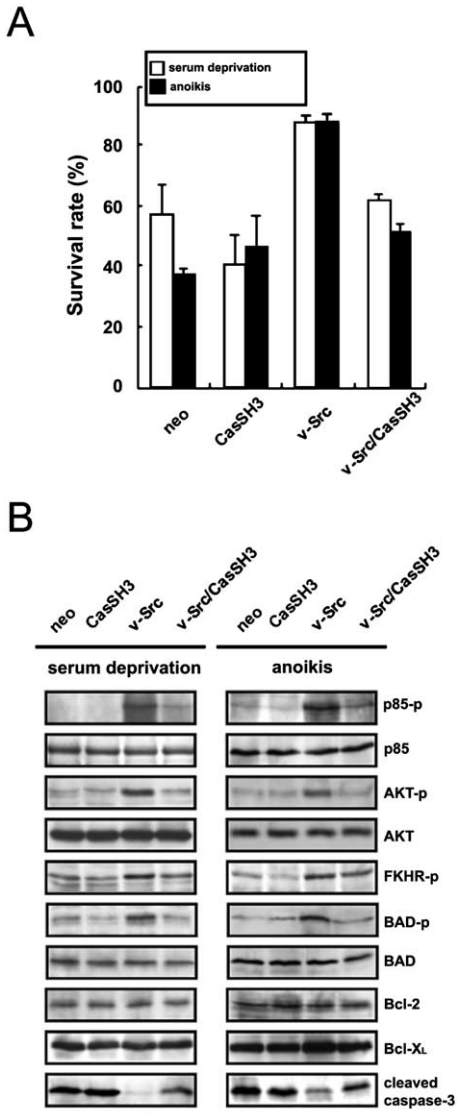

Fig. 5. The Cas SH3 domain suppresses v-Src-stimulated resistance to apoptosis. A: Cells were grown on culture dishes in serum-free medium or on polyHEMA-coated dishes in the medium with 10% serum to analyze their resistance to serum deprivation and anoikis, respectively. After 48 h, cells were collected and their survival rates were measured by trypan blue exclusion. The values (means ± S.E.M.) are from six data points from three experiments using three clones for each cell line. B: Under the same conditions as described for A, 48 h later, cells were lysed and equal amounts of cell lysates were analyzed by immunoblotting with antibodies as indicated. The result shown is representative of three independent experiments.

cutaneously injected into athymic nude mice. Our results (Fig. 4A) showed that 100% of the mice ($n = 6$) injected with v-Src-transformed cells formed in situ solid tumors, whereas none of the mice ($n = 9$) injected with the v-Src/CasSH3 cells did. Therefore, even though the catalytic activity and the expression of v-Src were not affected by the Cas SH3 domain (Fig. 1B), its tumorigenicity was completely abolished by the Cas SH3 domain. Next, the effect of the Cas SH3 domain on anchorage-independent cell growth, a hallmark of cell transformation, was analyzed by soft agar colony formation assay (Fig. 4B). Unlike v-Src cells, v-Src/CasSH3 cells hardly formed colonies in soft agar, suggesting that the Cas SH3 domain may render v-Src-transformed cells susceptible to anoikis, a type of apoptosis induced by the loss of cell–matrix adhesions.

3.4. Inhibitory effect of the Cas SH3 domain on v-Src-stimulated resistance to apoptosis

To examine whether the Cas SH3 domain modulates the sensitivity of the cells to apoptosis, the cells were grown on culture dishes in serum-free medium or on polyHEMA-coated dishes in the medium with 10% serum (Fig. 5A). PolyHEMA is known to prevent matrix deposition and cell attachment [26] and has been shown to induce anoikis [27]. After 48 h, the cells were harvested and analyzed for their survival. Our results showed that the resistance of v-Src-transformed 3T3 cells to serum deprivation or anoikis could be suppressed by the Cas SH3 domain to a level similar to that of the control cells.

The PI3K/AKT pathway has been shown to transmit survival signals for a variety of extracellular survival factors [28,29]. We found that this pathway was constitutively activated in v-Src-transformed cells, manifested by the activation of AKT and the phosphorylation of AKT substrates such as FKHR and BAD. Importantly, the Cas SH3 domain apparently inhibited the v-Src-stimulated activation of the pathway (Fig. 5B). In addition, v-Src suppressed the activation of caspase 3 in response to apoptotic stimuli from serum deprivation or anoikis, which was partially restored by the Cas SH3 domain (Fig. 5B). The results in Fig. 5 together suggest that the Cas SH3 domain may render v-Src-expressed cells susceptible to low serum and anoikis by inhibiting v-Src-stimulated activation of the PI3K/AKT pathway.

3.5. The Cas SH3 domain blocks v-Src-stimulated Matrigel invasion in vitro

Increased invasiveness is another characteristic of v-Src-transformed cells. Matrigel invasion assay was employed to measure the invasiveness of the cells in vitro (Fig. 6A). As expected, the ability of the v-Src/CasSH3 cells to invade Matrigel was significantly (~65%) lower than that of v-Src-transformed cells. Recently, FRNK, a dominant-negative version of FAK, was shown to block v-Src-stimulated invasion by decreasing matrix metalloproteinase-2 (MMP-2) expression through inhibition of the Cas/JNK pathway in NIH3T3 cells [30]. Similarly, we found that the v-Src/CasSH3 cells exhibited decreased MMP-2 expression in comparison with v-Src-transformed cells (Fig. 6B). It is possible that inhibition of v-Src-stimulated activation of the JNK pathway by the Cas SH3 domain may account for its inhibition of invasion. Consistent with this assumption, we found that the specific JNK inhibitor SP600125, but not the MEK inhibitor PD98059 and the PI3K inhibitor LY294002, suppressed the invasiveness of v-Src-transformed cells (data not shown).

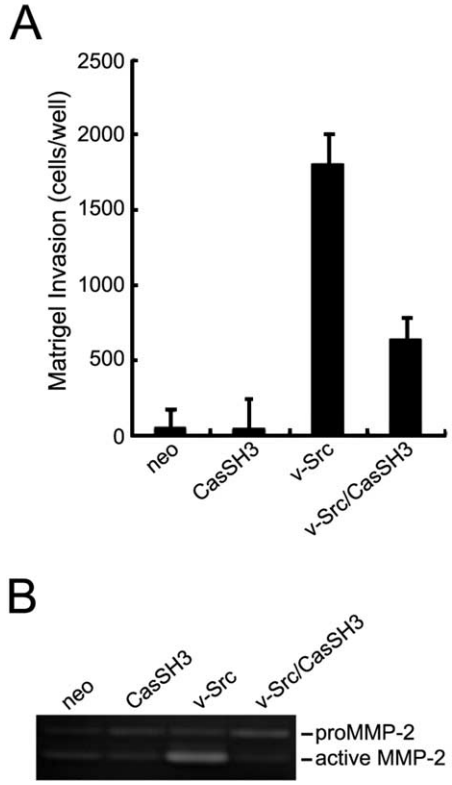

Fig. 6. The Cas SH3 domain blocks v-Src-stimulated invasion in vitro. A: Cells (5×10^4) were subjected to Matrigel invasion assay. The values (means ± S.E.M.) are from nine data points from three experiments using three clones for each cell line. B: Conditioned media collected from the indicated cells were analyzed by gelatin zymography. The positions of proMMP-2 and active MMP-2 are indicated. The result shown is representative of three independent experiments using two clones for each cell line.

4. Discussion

In this study, we have examined the effect of the Cas SH3 domain on v-Src's transforming potential by stably expressing the Cas SH3 domain in v-Src-transformed NIH3T3 cells. Our results demonstrate that although the Cas SH3 domain did not affect the activity and expression of v-Src (Fig. 1), it severely impaired the transforming potential of v-Src at least on two aspects – tumorigenicity (Fig. 4) and invasion (Fig. 6). We demonstrated that inhibition of the JNK pathway by the Cas SH3 domain or the specific JNK inhibitor blocked v-Src-stimulated invasion. On the other hand, inhibition of the AKT pathway rendered v-Src-transformed cells susceptible to apoptosis (Fig. 5). Our results therefore suggest that the Cas SH3 domain selectively inhibits at least two crucial signaling pathways (i.e. JNK and AKT), thereby blocking the transforming potential of v-Src. It is worth noting that although the Cas SH3 domain had a profound impact on the JNK and AKT pathways, it had no effects on v-Src-stimulated activation of ERK and STAT3 (Fig. 2), suggesting the activation of ERK and STAT3 may not be sufficient for v-Src to induce cell transformation. Nevertheless, our results do not exclude the necessity of both in v-Src-stimulated cell transformation.

Cas-deficient mouse embryonic fibroblasts are refractory to full transformation by activated Src, which show fewer podosomes and more organized actin filaments than those in the control counterparts [16]. In our study, we found that the Cas SH3 domain consistently reverses the long spindle morphology of the v-Src-transformed cells into a more spread cell shape in separate experiments, concomitant with more organized actin cytoskeleton and increased formation of focal contacts (Fig. 3B). It is already known that the Cas SH3 domain can function as a competitor for endogenous Cas to bind FAK [18,19], thereby leading to less Cas localized in focal contacts. Whether the displacement of endogenous Cas from focal contacts by the Cas SH3 domain, as shown in Fig. 3, is responsible for the reversion of transformed phenotypes is unclear. However, our results strongly suggest that the Cas SH3 domain may have a positive effect on the actin cytoskeleton and that the reversion of v-Src-transformed cell morphology may underscore observed changes in cell functions. In fact, Cas has been reported to be important for the formation of the actin stress fibers [16]. If the Cas SH3 domain indeed has a positive effect on the actin cytoskeleton, it could suppress cell transformation induced by *v-src* or other oncogenes through its general effect on the cytoskeleton. Answers to these questions will help us not only to understand the function of the Cas SH3 domain on the cytoskeleton but also to evaluate its potential use for therapeutic purposes.

Cas is highly phosphorylated in v-Src-transformed cells and plays an essential role in v-Src-induced cell transformation [16]. In this study, we found that Cas was prominently accumulated in the membrane ruffles of v-Src-transformed cells (Fig. 3A). The expression of the Cas SH3 domain induced a translocation of Cas from membrane ruffles to the center of the cells (Fig. 3A), concomitant with a marked (~70%) reduction in the v-Src-stimulated phosphorylation of Cas (Fig. 1A). This reduction is likely partial because of the inhibitory effect of the Cas SH3 domain on the association of Cas with v-Src and FAK (Fig. 1A). FAK is known to form stable complexes with v-Src [21]. The displacement of Cas from focal contacts by the Cas SH3 domain may account for decreased Cas–FAK association, thereby rendering Cas less likely to be phosphorylated by v-Src.

Hauck et al. [30] reported that expression of FRNK, a dominant-negative version of FAK, blocked v-Src-stimulated invasion and metastasis by inhibiting the v-Src-stimulated JNK pathway in v-Src-transformed NIH 3T3 cells. They demonstrated that the v-Src-stimulated expression of MMP-2 is essential for invasion, which is largely dependent on the activation of the JNK pathway. Similar to FRNK, the Cas SH3 domain blocks v-Src-stimulated invasion by decreasing MMP-2 expression likely through its inhibition in the JNK pathway (Fig. 6). More recently, JNK was found to directly phosphorylate paxillin and promote cell motility [31], which may facilitate invasion as well. Unlike the Cas SH3 domain, FRNK can only block v-Src-stimulated invasion without effects on the ability of v-Src to promote cell growth in soft agar and tumor growth in nude mice [30]. This could be because FRNK is a specific inhibitor for FAK, and has a more specific effect on intracellular signaling pathways than the Cas SH3 domain does. In fact, in addition to FAK and PYK2, several other cellular proteins have been reported to associate with Cas through its SH3 domain, including two protein tyrosine phosphatases PTP1B and PTP-PEST and the guanine nucleotide exchange factor C3G [14,15]. Whether the Cas SH3 domain blocks v-Src-stimulated cell transformation through its binding to any of these molecules remains to be examined.

Acknowledgements: This work was supported by the National Science Council, Taiwan (Grants NSC-92-2311-B-005-035 and NSC-92-2320-B-005-007), Taichung Veterans General Hospital, and National Chung Hsing University (Grants 91VH-13 and TCVGH-NCHU-927613).

References

[1] Stehelin, D., Varmus, H.E., Bishop, J.M. and Vogt, P.K. (1976) Nature 260, 170–173.
[2] Shalloway, D., Zelenetz, A.D. and Cooper, G.M. (1981) Cell 24, 531–541.
[3] Parker, R.C., Varmus, H.E. and Bishop, J.M. (1984) Cell 37, 131–139.
[4] Thomas, S.M. and Brugge, J.S. (1997) Annu. Rev. Cell Dev. Biol. 13, 513–609.
[5] Wyke, A.W., Frame, M.C., Gillespie, D.A., Chudleigh, A. and Wyke, J.A. (1995) Cell Growth Differ. 6, 1225–1234.
[6] Johnson, D., Agochiya, M., Samejima, K., Earnshaw, W., Frame, M. and Wyke, J. (2000) Cell Growth Differ. 7, 685–696.
[7] Bowman, T., Broome, M.A., Sinibaldi, D., Wharton, W., Pledger, W.J., Sedivy, J.M., Irby, R., Yeatman, T., Courtneidge, S.A. and Jove, R. (2001) Proc. Natl. Acad. Sci. USA 98, 7319–7324.
[8] Frame, M.C. (2002) Biochim. Biophys. Acta 1602, 114–130.
[9] Reynolds, A.B., Kanner, S.B., Wang, H.C. and Parsons, J.T. (1989) Mol. Cell. Biol. 9, 3951–3958.
[10] Matsuda, M., Mayer, B.J., Fukui, Y. and Hanafusa, H. (1990) Science 248, 1537–1539.
[11] Sakai, R., Iwamatsu, A., Hirano, N., Ogawa, S., Tanaka, T., Mano, H., Yazaki, Y. and Hirai, H. (1994) EMBO J. 13, 3748–3756.
[12] Nakamoto, T., Sakai, R., Ozawa, K., Yazaki, Y. and Hirai, H. (1996) J. Biol. Chem. 271, 8959–8965.
[13] Polte, T.R. and Hanks, S.K. (1995) Proc. Natl. Acad. Sci. USA 92, 10678–10682.
[14] Kirsch, K.H., Georgescu, M.M. and Hanafusa, H. (1998) J. Biol. Chem. 273, 25673–25679.
[15] Garton, A.J., Burnham, M.R., Bouton, A.H. and Tonks, N.K. (1997) Oncogene 15, 877–885.
[16] Honda, H., Oda, H., Nakamoto, T., Honda, Z., Kakai, R., Suzuki, T., Saito, T., Nakamura, K., Nakao, K., Ishikawa, T., Katsuki, M., Yazaki, Y. and Hirai, H. (1998) Nat. Genet. 19, 361–365.
[17] Honda, H., Nakamoto, T., Sakai, R. and Hirai, H. (1999) Biochem. Biophys. Res. Commun. 262, 25–30.
[18] Cary, L.A., Han, D.C., Polte, T., Hanks, S.K. and Guan, J.-L. (1998) J. Cell Biol. 140, 211–221.
[19] Chan, P.-C., Lai, J.-F., Cheng, C.-H., Tang, M.-J., Chiu, C.-C. and Chen, H.-C. (1999) J. Biol. Chem. 274, 26901–26906.
[20] Chan, P.-C., Liang, C.-C., Yu, K.-C., Chang, M.-C., Ho, W.L., Chen, B.-H. and Chen, H.-C. (2002) J. Biol. Chem. 277, 50373–50379.
[21] Guan, J.-L. and Shalloway, D. (1992) Nature 358, 690–692.
[22] Cheng, C.-H., Hsieh, C.-L., Shu, K.-H., Chen, Y.-L. and Chen, H.-C. (2002) FEBS Lett. 516, 191–196.
[23] Dolfi, F., Garcia-Guzman, M., Ojaniemi, M., Nakamura, H., Matsuda, M. and Vouri, K. (1998) Proc. Natl. Acad. Sci. USA 95, 15394–15399.
[24] Kiyokawa, E., Hashimoto, Y., Kobayashi, S., Sugimura, H., Kurata, T. and Matsuda, M. (1998) Genes Dev. 12, 3331–3336.
[25] Odajima, J., Matsumura, I., Sonoyama, J., Daino, H., Kawasaki, A., Tanaka, H., Inohara, N., Kitamura, T., Downward, J., Nakajima, K., Hirano, T. and Kanakura, Y. (2000) J. Biol. Chem. 275, 24096–24105.
[26] Folkman, J. and Moscona, A. (1978) Nature 273, 345–349.
[27] Frisch, S.M. and Francis, H. (1994) J. Cell Biol. 124, 619–626.

[28] Franke, T., Kaplan, D.R. and Cantley, L.C. (1997) Cell 88, 435–437.
[29] Khwaja, A., Rodrigues-Viciana, P., Wennstrom, S., Warne, P.H. and Downward, J. (1997) EMBO J. 16, 2783–2793.
[30] Hauck, C.R., Hsia, D.A., Puente, X.S., Cheresh, D.A. and Schlaepfer, D.D. (2002) EMBO J. 21, 6289–6302.
[31] Huang, C., Rajfur, Z., Borchers, C., Schaller, M. and Jacobson, K. (2003) Nature 424, 219–223.