v-Src-Induced Modulation of the Calpain-Calpastatin Proteolytic System Regulates Transformation

N. O. Carragher,^{1*} M. A. Westhoff,¹ D. Riley,¹ D. A. Potter,² P. Dutt,³ J. S. Elce,³
P. A. Greer,³ and M. C. Frame^{1,4}

The Beatson Institute for Cancer Research, Cancer Research Campaign Beatson Laboratories, Glasgow G61 1BD,¹ and Institute of Biological and Life Sciences, University of Glasgow, Glasgow G12 8QQ,⁴ Scotland, United Kingdom; Department of Medicine, Division of Hematology/Oncology, Department of Biochemistry and Molecular Biology, and Walther Oncology Center, Indiana University, Indianapolis, Indiana²; and Cancer Research Laboratories, Department of Biochemistry, Queen's University, Kingston, Ontario K7L 3N6, Canada³

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v-Src-induced oncogenic transformation is characterized by alterations in cell morphology, adhesion, motility, survival, and proliferation. To further elucidate some of the signaling pathways downstream of v-Src that are responsible for the transformed cell phenotype, we have investigated the role that the calpain-calpastatin proteolytic system plays during oncogenic transformation induced by v-Src. We recently reported that v-Src-induced transformation of chicken embryo fibroblasts is accompanied by calpain-mediated proteolytic cleavage of the focal adhesion kinase (FAK) and disassembly of the focal adhesion complex. In this study we have characterized a positive feedback loop whereby activation of v-Src increases protein synthesis of calpain II, resulting in degradation of its endogenous inhibitor calpastatin. Reconstitution of calpastatin levels by overexpression of exogenous calpastatin suppresses proteolytic cleavage of FAK, morphological transformation, and anchorage-independent growth. Furthermore, calpastatin overexpression represses progression of v-Src-transformed cells through the G₁ stage of the cell cycle, which correlates with decreased pRb phosphorylation and decreased levels of cyclins A and D and cyclin-dependent kinase 2. Calpain 4 knockout fibroblasts also exhibit impaired v-Src-induced morphological transformation and anchorage-independent growth. Thus, modulation of the calpain-calpastatin proteolytic system plays an important role in focal adhesion disassembly, morphological transformation, and cell cycle progression during v-Src-induced cell transformation.

Oncogenic transformation of cells by v-Src is associated with deregulated growth control, cytoskeletal disassembly, and loss of integrin-linked focal adhesion structures (17, 20, 27, 31). Such alterations contribute to the highly mitogenic and motile phenotype that characterizes v-Src transformation. The precise mechanisms by which v-Src promotes cell transformation remain poorly understood. Previous studies, however, indicate that v-Src-induced morphological transformation occurs by mechanisms independent of gene expression (8, 22), implicating Src kinase activity or other posttranscriptional mechanisms as key mediators of v-Src-induced transformation. Calpain-mediated proteolysis represents a major pathway of posttranslational modification of cellular proteins and has been implicated in diverse cellular processes ranging from apoptosis to cell migration and cell cycle progression (12, 25, 43, 45, 49, 57). We have previously demonstrated that calpain-mediated proteolytic cleavage of the focal adhesion kinase (FAK) and focal adhesion disassembly accompany v-Src-induced morphological transformation. Calpain-mediated disassembly of focal adhesions results in a reduction in the strength of adhesion that transformed cells have to their culture substrate, thereby promoting cell motility (12).

The calpains represent a highly conserved family of nonlysosomal calcium-dependent cysteine proteases comprising two

ubiquitously expressed isoforms, μ -calpain (calpain I) and m-calpain (calpain II), several tissue-specific isoforms, and a small 28-kDa regulatory subunit (calpain 4) (16, 55). Several in vitro studies demonstrate that calpain can be activated by high calcium concentrations. However, the regulation of calpain activity in vivo is less clear because the calcium concentrations required to activate calpain in vitro are significantly higher than physiological levels within cells (28).

The endogenous inhibitor of calpain activity, calpastatin, tightly regulates calpain activity in vivo. Calpastatin is a highly specific inhibitor of calpains and to date has not been demonstrated to inhibit the activity of members of any other protease family (42). Calpastatin is ubiquitously expressed and is translated as several isoforms, including a 110-kDa tissue type and a 70-kDa erythrocyte type (36, 59). The intracellular levels of calpain relative to calpastatin vary between tissues, but generally calpastatin is found at much higher levels than the calpains (9). In addition, each calpastatin molecule can potentially inhibit several calpain molecules (16, 29). Calpain and calpastatin are predominantly cytosolic proteins, indicating that calpain must somehow escape the inhibitory control of calpastatin to become fully activated. It has been suggested that subcellular compartmentalization of either calpain or calpastatin may regulate calpain activity within cells (35, 60). Modulation of the balance between protein levels of calpain relative to calpastatin could also represent a mechanism for regulating calpain activity. In this regard, degradation of calpastatin has been associated with increased calpain activity in a number of in vitro and in vivo scenarios (9, 56).

* Corresponding author. Mailing address: The Beatson Institute for Cancer Research, Cancer Research Campaign Beatson Laboratories, Glasgow G61 1BD, Scotland, United Kingdom. Phone: 44-141-330-3956. Fax: 44-141-942-6521. E-mail: n.carragher@beatson.gla.ac.uk.

The wide substrate specificity of the calpain proteolytic family most likely accounts for proposed roles for calpain in diverse cellular processes, ranging from apoptosis to cell motility and cell cycle progression. Previous studies indicate that calpain can regulate cell cycle progression at distinct points through modulating the protein levels of several cell cycle regulators, such as the tumor suppressor proteins p53, p107, and NF2 (26, 32, 34). In addition, cyclin D1 and the cyclin-dependent kinase (cdk) inhibitor p27^{kip1} are both calpain substrates and so may represent other pathways by which calpain can regulate cell cycle progression (15, 49).

In this study we investigated the mechanism by which v-Src may promote calpain activity during cell transformation and how elevated calpain activity contributes to transformation. Using a conditional, temperature-sensitive v-Src mutant (*ts* LA29 v-Src), we were able to examine both the kinetics of calpain regulation following v-Src activation and the consequences that deregulated calpain activity has on the v-Src-transformed cell phenotype. Here we describe a positive feedback loop whereby activation of v-Src promotes increased synthesis of calpain II, which in turn promotes degradation of the endogenous calpain inhibitor calpastatin, thereby further enhancing calpain activity in v-Src-transformed cells. Replenishing levels of calpastatin by overexpression of an exogenous calpastatin construct suppressed calpain-mediated cleavage of FAK, focal adhesion disruption, morphological transformation, and anchorage-independent growth that normally accompany v-Src transformation. Furthermore, we demonstrate that v-Src-induced morphological transformation is less efficient in calpain 4 (regulatory domain) knockout (KO) fibroblasts. In addition, calpastatin overexpression impaired the progression of v-Src-transformed cells through the G₁ stage of the cell cycle. Calpastatin impairment of cell cycle progression correlated with decreased phosphorylation of the retinoblastoma gene product (pRb) and reduced cyclin A, cyclin D, and cdk2 protein levels. Thus, modulation of the calpain-calpastatin proteolytic system in response to v-Src activation contributes not only to v-Src-induced morphological transformation but also to v-Src-induced cell cycle progression.

MATERIALS AND METHODS

Cell culture and transfection. Primary chicken embryo fibroblasts (CEF) were subcultured as previously described (21). Low-density cultures were transfected with replication-competent avian retroviral constructs, RCAN-v-src, encoding the *ts* LA29 v-Src mutant (62). Transfected CEF were cultured at the permissive temperature of 35°C until cells were uniformly infected and expressing v-Src protein. For analysis of v-Src-induced transformation, CEF were cultured at the restrictive temperature (41°C) and were then examined following a shift to the permissive temperature (35°C). Wild-type and calpain 4 KO mouse embryonic fibroblasts (MEF) were generated by simian virus 40 (SV40) large-T-antigen-mediated immortalization of E10.5 fibroblasts from control or *calpain 4*^{-/-} embryos (1). MEF were transfected with fpGV-1 vector (19) encoding *ts* LA29 v-Src or a previously described control, nontransforming, myristylation-defective v-Src construct (myristylation site at position 2; glycine mutated to alanine), *ts* LA29A2 v-Src (14). Neomycin-resistant MEF stably expressing *ts* LA29 or *ts* LA29A2 v-Src were selected by incubating cell cultures with G418 antibiotic (0.6 mg/ml) (Life Technologies). Full-length human calpastatin cDNA of the form with a deletion of exon 3 provided by Masatoshi Maki (Nagoya University, Nagoya, Japan) was inserted into the neomycin-selectable avian retroviral vector pSFCV (23). CEF were cotransfected with *ts* LA29 v-Src in combination with either empty pSFCV or pSFCV encoding calpastatin. Cells stably expressing calpastatin (SFCV+Calpas.) or empty vector (SFCV) were selected by incubating cell cultures with G418 antibiotic (1 mg/ml) (Life Technologies Ltd.).

K-Ras-transformed Rat-1 fibroblasts, v-Fos^{FBR}-transformed 208F⁺ fibroblasts,

and parental controls were cultured in 1× Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 2 mM L-glutamine. v-Jun (RSV17)- and v-Myc (MC29)-transformed CEF and parental cells were cultured under normal CEF culture conditions as previously described (21).

Antibodies and reagents. Analysis of protein stability in both nontransformed and transformed cells was performed using the protein synthesis inhibitor emetine (10 μM) (Sigma). Calpain inhibitor studies were performed using calpain inhibitor 1 (ALLN) and calpain inhibitor 2 (ALLM) (Calbiochem-Novabiochem Corp.). CEF were preincubated with ALLN or ALLM (50 to 100 μM) for 1 h prior to shift to 35°C and were then subsequently incubated at 35°C in the presence of each inhibitor. Antibodies for Western blot detection and immunocytochemistry included calpain II and calpastatin (Research Diagnostics, Inc.), ²⁻¹⁸N pp125^{FAK}, ⁹⁰³⁻¹⁰⁵⁸C pp125^{FAK} (Santa Cruz Biotechnology, Inc.), ³⁵⁴⁻⁵³⁴N pp125^{FAK}, paxillin (Transduction Laboratories), p27^{kip1} (Oncogene), cyclin A (developed at the Beatson Institute for Cancer Research), cyclin D (Pharmin-gen), and pRb and cdk2 (both from Santa Cruz Biotechnology, Inc.). Anti-mouse and -rabbit peroxidase-conjugated secondary antibodies were purchased from New England Biolabs, Inc.

Immunoblotting. Cells were washed twice with phosphate-buffered saline (PBS) and lysed in low-detergent lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 0.5% NP-40, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.5 mM NaF, 10 mM β-glycerophosphate, 10 mM Na₄P₂O₇, and 100 μM NaVO₃ with the protease inhibitors 1 mM phenylmethylsulfonyl fluoride, 10 μg of leupeptin/ml, and 10 μg of aprotinin/ml). Lysates were clarified by high-speed centrifugation at 4°C, supplemented with sodium dodecyl sulfate (SDS)-sample buffer, separated by SDS-10% polyacrylamide gel electrophoresis, and transferred to a nitrocellulose membrane. Following blocking in 5% milk, membranes were incubated with primary antibody, washed, and incubated with secondary antibody linked to horseradish peroxidase. Protein was detected by enhanced chemiluminescence (Amersham Pharmacia Biotech UK Ltd.). Analysis of pRb phosphorylation was performed by running cell lysates on an SDS-7.5% polyacrylamide gel containing a ratio of acrylamide to bisacrylamide of 30:0.24.

Northern blot analysis. Total RNA was prepared from cells with TRIzol reagent (Life Technologies Ltd.) in accordance with the manufacturer's instructions. Total RNA samples were separated on 1.5% agarose gels containing formaldehyde and were transferred to a nylon membrane (Hybond; Amersham Pharmacia) using standardized procedures. cDNA probes designed for hybridization to chicken calpain II, calpastatin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were generated by reverse transcriptase PCR. All probes were radiolabeled with [α-³²P]dCTP using the oligolabeling kit (Amersham Pharmacia).

Immunocytochemistry. Cells were cultured on permanox plastic chamber slides (Nalge Nunc International). Cells were fixed in 3.7% formaldehyde for 10 min at room temperature; permeabilized in 0.5% NP-40 in PBS for 10 min at room temperature; and washed serially in PBS, 0.15 M glycine-PBS + 0.02% NaN₃, and PBS. Cells were blocked in 10% fetal calf serum-PBS prior to 1 h of incubation at room temperature with a primary antibody, affinity-purified monoclonal antipaxillin (Transduction Laboratories). Primary antibody incubation was followed by several washes in PBS and subsequent incubation with fluorescein isothiocyanate-labeled secondary antibodies (Jackson ImmunoResearch Laboratories). Cells were also incubated with fluorescein isothiocyanate-labeled phalloidin (Sigma). Immunostaining of cells was analyzed by confocal microscopy.

Growth curves, cell cycle analysis, and anchorage-independent growth assays. The rate of cell growth was determined by counting the number of cells at sequential time points following plating of 2 × 10⁵ cells per 60-mm-diameter dish. The cell number was quantified using a Coulter counter. For cell cycle analysis, cells were harvested, fixed in cold 70% ethanol for 2 h on ice, washed once with PBS, and resuspended in PBS containing 0.1% (vol/vol) Triton X-100, 20 mg of DNase-free RNase A (Transgenomic Inc.), and 2 mg of propidium iodide (Sigma). Cell cycle distribution was determined by measuring fluorescence by FACScan (Becton Dickinson) together with the ModFit LT software for Macintosh. Anchorage-independent growth assays were performed as previously described (24). Briefly, 60-mm-diameter bacterial culture dishes were coated with 0.5% base agar supplemented with normal CEF culture medium as described above. CEF expressing *ts* v-Src were preincubated with or without calpain inhibitor ALLN or ALLM (100 μM) for 3 h in suspension prior to the addition of an equal volume of top layer agar consisting of 0.6% agar, double-concentrated CEF growth medium, and ALLN or ALLM (100 μM) where required. CEF expressing *ts* v-Src in combination with calpastatin or empty vector and wild-type and calpain 4 KO MEF were also combined with top layer agar. Cell-agar preparations were added to base agar dishes at 2 × 10⁵ cells per dish and were cultured at *ts* v-Src restrictive or permissive culture temperatures. Following several days in culture, top layer agar was overlaid with base agar

FIG. 1. Following activation of v-Src, increased protein levels of calpain II is coordinated with degradation of calpastatin. Total cell lysates were prepared from *ts* v-Src CEF cultured at the restrictive temperature (41°C) or at sequential time points following shift to the permissive temperature for v-Src activation (35°C). Cell lysates were separated by SDS-10% polyacrylamide gel electrophoresis and immunoblotted with antibodies specific for calpain II and calpastatin. kD, kilodaltons.

supplemented with culture media with or without ALLN or ALLM (100 μ M) where required. The formation of cell colonies was quantified as the number of colonies per high-power field.

RESULTS

Coordinated elevation of calpain II with decreased calpastatin protein levels following v-Src activation. In order to determine the nature of v-Src-mediated regulation of calpain activity, we examined the expression levels of both calpain and its endogenous inhibitor calpastatin in response to v-Src activation. As we have previously reported, we observed a rapid increase in total protein levels of calpain II following v-Src activation. We report here that the increase in calpain II paralleled a decrease in levels of calpastatin (Fig. 1). Two calpastatin antibodies obtained from separate sources (Research Diagnostic Inc.) (Fig. 1) and (Calbiochem) (results not shown) detect an endogenous 70-kDa calpastatin isoform but no apparent 110-kDa isoform in CEF. Following initiation of v-Src-induced transformation by shift of the *ts* L429 v-Src CEF to the permissive temperature (35°C), levels of the endogenous 70-kDa calpastatin isoform decreased in parallel with the generation of a putative 50-kDa proteolytic fragment (Fig. 1).

v-Src-induced protein synthesis of calpain II promotes proteolytic degradation of calpastatin and FAK. Analysis of calpain II and calpastatin mRNA levels by Northern blot analysis demonstrate that calpain and calpastatin are not regulated at the mRNA level and thus must be modified posttranscriptionally following v-Src activation (Fig. 2A). Investigation into calpain and calpastatin protein stability was carried out by treatment of *ts* v-Src CEF with the protein synthesis inhibitor emetine (10 μ M). Protein stability was subsequently determined during a time course of cell culture at the restrictive temperature (41°C) and following shift to the permissive temperature (35°C) (Fig. 2B). Results demonstrate that calpain and calpastatin are highly stable proteins in nontransformed cells. Following v-Src activation, calpain stability remains unchanged, whereas calpastatin becomes highly unstable (Fig. 2B). This data indicates that the increase in calpain II protein levels following v-Src activation is not the result of transcriptional modification or modulation of protein stability. In addition, emetine treatment suppressed the increase in calpain II protein levels normally observed following activation of v-Src.

Thus, v-Src-induced elevation of calpain II is due to increased protein translation, whereas decreased calpastatin is the result of protein degradation.

It has previously been demonstrated that FAK cleavage induced in response to v-Src activation is mediated by calpain activity (12). In this study we demonstrate that the protein stability of FAK is dramatically decreased following v-Src activation in parallel with the decreased stability of calpastatin protein (Fig. 2B). To determine whether proteolytic degradation of calpastatin in v-Src-transformed cells is also mediated by increased calpain activity, we examined v-Src-induced calpastatin cleavage in cells treated with two separate, cell-permeable inhibitors of calpain, ALLN and ALLM. Previous studies indicate that ALLN exhibits inhibitory activity against a range of proteases, including the proteasome complex, whereas ALLM has not been reported to inhibit the proteasome (61). Both inhibitors suppressed the cleavage of endogenous calpastatin following v-Src activation (Fig. 3A).

A previous report demonstrated that the 110-kDa tissue type calpastatin isoform is degraded directly by calpain *in vitro*, resulting in proteolytic fragments of approximate molecular masses of 62 and 35 kDa (56). To determine whether a similar pattern of cleavage could be observed in CEF in response to v-Src activation, we overexpressed the 110-kDa tissue type isoform in *ts* v-Src CEF. Immunoblotting with an anticalpastatin antibody confirmed overexpression of 110-kDa calpastatin in CEF (Fig. 3B). Anticalpastatin antibody also detected the

FIG. 2. v-Src regulates calpain II at the level of protein synthesis and calpastatin at the level of protein stability. (A) Total RNA was extracted from *ts* v-Src CEF cultured at the restrictive temperature (41°C) or at sequential time points following shift to the permissive temperature (35°C). RNA samples were subjected to Northern blot analysis using cDNA probes specific for calpain II and calpastatin. RNA loading was monitored with a probe against GAPDH. (B) Total cell lysates were prepared from *ts* v-Src CEF at sequential time points following culture at 41°C or a shift to 35°C in the presence of protein synthesis inhibitor emetine (10 μ M). Lysates were separated by SDS-10% polyacrylamide gel electrophoresis and immunoblotted with antibodies specific for calpain II, calpastatin, and the N-terminal region of FAK recognizing the 125-kDa (125kd) native protein and a 95-kDa N-terminal proteolytic fragment. T0, time zero.

FIG. 3. Degradation of calpastatin induced in response to v-Src activation is mediated by calpain. (A) Total cell lysates were prepared from *ts* v-Src CEF cultured at the restrictive temperature (41°C) or at the permissive temperature (35°C) in the absence or presence of ALLN and ALLM (100 μ M). (B) Total cell lysates were prepared from CEF coexpressing *ts* v-Src in combination with empty retroviral vector (SFCV) or SFCV encoding 110-kDa calpastatin* (SFCV+calpas.) (C) Total cell lysates were prepared from CEF coexpressing *ts* v-Src and calpastatin cultured at the restrictive temperature (41°C) and at sequential time points (in hours, along top of gels) following shift to the permissive temperature (35°C) in the absence or presence of ALLN. All cell lysates were separated by SDS–10% polyacrylamide gel electrophoresis and immunoblotted with an antibody specific for calpastatin. kD, kilodaltons.

endogenous 70-kDa calpastatin and 50-kDa proteolytic fragment together with an additional putative proteolytic fragment of 35 kDa that was observed exclusively in cells transfected with exogenous 110-kDa calpastatin (Fig. 3B). Upon activation of v-Src, the appearance of the 35-kDa putative fragment increased and was followed by a decrease in levels of the native 110-kDa calpastatin 18 h following v-Src activation (Fig. 3C). Cleavage of exogenous calpastatin and generation of the 35-kDa fragment were suppressed when v-Src was activated in the presence of the calpain inhibitor ALLN (Fig. 3C). These results strongly suggest that a regulatory feedback loop mechanism operates in response to v-Src activation where increased protein synthesis of calpain II promotes degradation of its own inhibitor, calpastatin.

Overexpression of calpastatin results in impaired v-Src-induced proteolytic cleavage of FAK, focal adhesion disassembly, and morphological transformation. To specifically determine the impact that increased calpain activity may have during the process of v-Src-induced oncogenic transformation, we coexpressed *ts* LA29 v-Src protein in combination with 110-kDa calpastatin or vector alone as shown in Fig. 3B. We investigated whether replenishing functional calpastatin levels could counteract calpain-mediated cleavage of FAK induced following v-Src activation (12). As shown in Fig. 4A and B, calpastatin overexpression suppressed FAK cleavage to the

typical 95-kDa N-terminal and 30-kDa C-terminal proteolytic fragments (Fig. 4A and B).

We next monitored the influence that calpastatin overexpression has upon the morphological characteristics of v-Src-induced transformation. Calpastatin overexpression suppresses characteristic features of v-Src-induced cell transformation, such as cell rounding, loss of focal adhesion structures, and disassembly of the actin cytoskeleton at 18 h following v-Src activation (Fig. 4C). However, calpastatin overexpression only delayed the process of v-Src-induced morphological transformation, and by 36 h following v-Src activation, the majority of cells expressing calpastatin exhibited a typical transformed phenotype (results not shown). We speculate that the transient nature of this inhibition is most likely a consequence of calpain-mediated degradation of the exogenously expressed calpastatin protein. Levels of the exogenously expressed native calpastatin are significantly reduced 36 h following v-Src activation (results not shown).

To determine whether calpain-mediated degradation of FAK is unique to Src-induced transformation, we examined FAK cleavage in v-Jun- and v-Myc-transformed CEF and K-Ras-transformed Rat-1 and v-Fos-transformed 208F' fibroblasts (Fig. 5A). Interestingly transformation induced by the oncoproteins v-Myc, K-Ras, and v-Fos but not by v-Jun was accompanied by proteolytic cleavage of FAK. However, in contrast to v-Src-induced transformation (12), proteolytic cleavage of FAK in v-Myc-, K-Ras-, and v-Fos-transformed cells did not result in a significant decrease in levels of native FAK protein (Fig. 5A). Also in contrast to v-Src transformation, treatment with ALLN did not cause the morphology of v-Myc-, K-Ras-, v-Fos-, or v-Jun-transformed cells to revert to a normal phenotype (Table 1) (Fig. 5B, see v-Fos).

Calpain 4 KO fibroblasts exhibit impaired ability to undergo v-Src-induced morphological transformation. To further demonstrate that calpain is critical for v-Src-induced transformation, we overexpressed *ts* v-Src in both wild-type and calpain 4 KO MEF. We have confirmed that MEF are efficiently transformed by *ts* v-Src by comparing morphological transformation induced by *ts* LA29 v-Src with results for cells transfected with a nontransforming mutant *ts* LA29A2 v-Src (Fig. 6). Wild-type MEF stably expressing *ts* LA29 v-Src but not the nontransforming *ts* LA29A2 mutant exhibited a typical v-src-transformed phenotype, characterized by cell rounding and loss of paxillin-containing focal adhesions (Fig. 6A). However, Calpain 4 KO MEF stably expressing *ts* LA29 v-Src exhibit a reduced capacity to disassemble focal adhesion structures (Fig. 6B), and their morphology resembles the relatively flat cells expressing *ts* LA29A2 v-Src (Fig. 6A and B).

Inhibition of calpain activity by overexpression of calpastatin or by treatment with calpain inhibitors suppresses proliferation of transformed fibroblasts. We have previously reported that v-Src activation influences levels of cell cycle regulators and modifies cell cycle progression of Rat-1 fibroblasts (27). Previous studies using pharmacological inhibitors of calpain activity and cells with depleted calpain levels have suggested a role for calpain in promoting cell proliferation (39, 41). More recent studies have identified several key cell cycle regulatory proteins such as p27^{kip1}, cyclin D1, and p107 as targets for calpain proteolytic activity (15, 26, 49). To determine whether enhanced cell proliferation of CEF induced by

FIG. 4. Overexpression of calpastatin inhibits v-Src-induced proteolytic cleavage of FAK and morphological transformation. CEF coexpressing *ts* v-Src in combination with cDNA from empty retroviral vector SFCV or from SFCV + calpastatin were cultured under v-Src-restrictive (41°C) and -permissive (35°C) culture conditions for 18 h. Total cell lysates were prepared, separated by SDS-10% polyacrylamide gel electrophoresis, and immunoblotted with antibodies against the N terminus of FAK (FAK-N) (A) or the C terminus of FAK (FAK-C) (B). (C) CEF coexpressing *ts* v-Src in combination with calpastatin (SFCV + calpastatin) or vector alone (SFCV) were cultured for 18 h at the permissive temperature (35°C) for v-Src activation. Cell morphology was evaluated by phase-contrast microscopy. Focal adhesion structures and the actin cytoskeleton were analyzed by immunocytochemistry utilizing an antipaxillin antibody and fluorescein isothiocyanate (FITC)-labeled phalloidin, respectively. Bar, 25 μ m.

v-Src is mediated by calpain activity, we examined the effect that calpain inhibition has upon proliferation of CEF expressing activated *ts* v-Src. The pharmacological inhibitors of calpain activity, ALLN and ALLM (50 μ M), dramatically inhibited growth of both nontransformed CEF (results not shown) and v-Src-transformed CEF (Fig. 7A). All CEF cultures remained viable following treatment with the calpain inhibitors,

and cell growth recovered following their removal (results not shown). Overexpression of calpastatin also suppressed proliferation of transformed cells when compared with cells expressing the empty vector (Fig. 7B).

To assess the influence that calpain activity has on progression through the cell cycle, we employed flow cytometry analysis to determine the cell cycle profiles for *ts* v-Src CEF cul-

FIG. 5. Proteolytic cleavage of FAK in v-Myc-, v-Fos-, K-Ras-, and v-Jun-transformed cells. (A) Total cell lysates were prepared from v-Jun- and v-Myc-transformed CEF, K-Ras-transformed Rat-1 fibroblasts, and v-Fos-transformed 208F' fibroblasts. Lysates from transformed cells and their parental counterparts were separated by SDS-10% polyacrylamide gel electrophoresis and immunoblotted with antibodies against ³⁵⁴⁻⁵³⁴N-terminal residues of FAK. (B) v-Src-transformed CEF and v-Fos-transformed 208F' fibroblasts were treated with ALLN (50 μ M); cell morphology was evaluated by phase-contrast microscopy.

tured in the absence or presence of ALLN and ALLM (50 μ M). Cell cycle analysis was also performed on *ts* v-Src CEF expressing calpastatin (SFCV + calpastatin) compared with cells expressing empty vector (SFCV). Results indicate that inhibition of calpain activity by cell-permeable calpain inhibitors ALLN and ALLM impaired the progression of v-Src-transformed cells through the G₁ stage of the cell cycle (Fig. 7C). Overexpression of calpastatin also had a similar effect of accumulating v-Src-transformed cells in the G₁ stage of the cell cycle, compared with results for cells coexpressing the empty vector (Fig. 7D).

Calpain promotes progression of v-Src-transformed cells through G₁ stage of the cell cycle in parallel with hyperphosphorylation of pRb and increased cyclin A, cyclin D, and cdk2 levels. To gain insights into the mechanism by which calpain activity may influence cell cycle progression following v-Src activation, we analyzed the protein expression levels of several regulators of G₁ stage progression in substrate-attached CEF. Our results demonstrate that in response to v-Src activation at 35°C, the tumor suppressor protein pRb became hyperphosphorylated (Fig. 8). Protein levels of cyclin A, cyclin D, and

cdk2 are also all increased in v-Src-transformed fibroblasts (Fig. 8). Activation of v-Src in the presence of ALLN (100 μ M) resulted in hypophosphorylation of pRb, with complete loss of the hyperphosphorylated form. Protein levels and phosphorylation status of other Rb family members, p107 and p130, were not significantly altered upon treatment of v-Src-transforming cells with the calpain inhibitor (results not shown). Treatment with the calpain inhibitor also repressed v-Src-induced eleva-

TABLE 1. Effect that calpain inhibition has on morphological transformation induced by various oncoproteins^a

Oncoprotein	Effect on mixtures	
	Without ALLN	With ALLN (50 μ M)
v-Src	+	-
K-Ras	+	+
v-Fos	+	+
v-Myc	+	+
v-Jun	+	+

^a +, transformed phenotype; -, non-transformed phenotype.

FIG. 6. Calpain 4 KO cells exhibit impaired v-Src-induced morphological transformation. Wild-type (calpain^{+/+}) (A) and calpain 4 KO (calpain^{-/-}) (B) MEF were transfected with transforming (*ts LA29*) and defective (*ts LA29A2*) v-Src constructs. Cell morphology and focal adhesion structures were evaluated in cell populations stably expressing v-src constructs by phase-contrast microscopy and immunocytochemistry utilizing antipaxillin antibody. Bar, 25 μ m.

tion of cyclin A and cdk2 levels (Fig. 8) but did not modulate levels of cyclin A, cyclin D, or cdk2 in normal CEF (results not shown). Overexpression of calpastatin also suppressed the hyperphosphorylation of pRb and increase in cyclin A, cyclin D, and cdk2 levels normally observed following v-Src activation (Fig. 8). In contrast, exposure to the cell-permeable ALLN or calpastatin overexpression had no effect on levels of the cdk inhibitor p27^{kip1} (Fig. 8).

Overexpression of calpastatin, treatment with calpain inhibitors, and loss of calpain 4 gene expression in KO cells impairs anchorage-independent growth of v-Src-transformed fibroblasts. A hallmark of the transformed cell phenotype induced by oncogenes such as v-Src is their ability to proliferate in vitro in an anchorage-independent fashion. To test whether calpain activity contributes to anchorage-independent growth of v-Src-transformed cells, we examined the effect that treatment with calpain inhibitors or overexpression of calpastatin has on the ability of v-Src-transformed CEF to form colonies in soft agar. CEF expressing *ts* v-Src occasionally formed a few large colonies when cultured at the restrictive temperature of 41°C. Following shift to the permissive temperature of 35°C, these cell cultures rapidly formed numerous small colonies (Fig. 9A and B). Pretreatment and subsequent incubation with ALLN and ALLM or overexpression of calpastatin dramatically inhibited colony formation of *ts* v-Src CEF cultured under permissive conditions (Fig. 9A and B). To further confirm a direct role for calpain activity in promoting v-Src-induced, anchorage-independent growth, we compared the ability of wild-type and calpain 4 KO MEF expressing activated *ts* v-Src to form colonies in soft agar. This analysis demonstrates that calpain 4 KO cells consistently develop fewer and smaller colonies than do wild-type MEF (Fig. 9A and B).

DISCUSSION

Studies carried out predominantly in vitro demonstrate that the proteolytic activity of the calpain family can regulate the levels of numerous intracellular proteins and influence a vari-

ety of cellular processes, including cell adhesion, migration, proliferation, and apoptosis (25, 39, 51, 57, 63). Calpain activity has also been implicated in vivo in the progression of several pathological diseases, including cataract formation (3), ischemia (5, 9), muscular dystrophy (52, 54), neurodegenerative diseases such as Alzheimer's (6), and tumor invasion (10). We have recently demonstrated a role for calpain in the process of v-Src-induced morphological transformation (12). These recent studies suggest that, in response to v-Src activation, calpain promotes degradation of the focal adhesion component FAK and subsequent disassembly of the focal adhesion signaling complex. Calpain-mediated disassembly of focal adhesions impairs substrate adhesion and increases the motility of transformed cells (12, 13).

In this study we have characterized a positive feedback loop mechanism that promotes calpain proteolytic activity during v-Src-induced oncogenic transformation. Under normal conditions the major calpain isozymes and the endogenous calpain inhibitor calpastatin have been reported to exist as relatively stable, long-lived proteins (4, 64). However, in response to particular stimuli, such as ischemic or reperfusion injury in vivo or elevation of intracellular calcium levels, in vitro calpastatin acts as a substrate for calpain-mediated degradation (9, 40, 44, 46, 56). In response to v-Src activation, we observe an increase in the total protein levels of calpain II in parallel with decreased levels of calpastatin. Analysis of mRNA levels and protein stability allows us to attribute this rise in calpain to increased protein translation in response to v-Src activation. We further demonstrate that the decrease in endogenous calpastatin is the result of calpain-mediated degradation. In addition, an exogenously expressed 110-kDa calpastatin isoform is also cleaved following v-Src activation, giving rise to a 35-kDa product that corresponds in size to a previously identified calpain-mediated cleavage product of the 110-kDa calpastatin isoform (56). Cleavage of both endogenous and exogenous calpastatin forms follows the same kinetics in response to v-Src activation and is blocked in both situations upon treatment with the cell-permeable ALLN. Thus, elevated protein synthe-

FIG. 7. Inhibition of calpain activity by treatment with cell-permeable calpain inhibitors or calpastatin overexpression impairs proliferation and progression of Src-transformed cells through the G_1 stage of the cell cycle. (A) Growth curves were prepared for *ts LA29 v-Src* CEF cultured at 35°C in the absence or presence of ALLN or ALLM ($50\ \mu\text{M}$). (B) Growth curves were prepared for *ts LA29 v-Src* CEF coexpressing calpastatin (SFCV/CALPAS) or empty retroviral vector (SFCV) cultured at 35°C . Flow cytometry analysis was performed on propidium iodide-stained cells to compare the distribution between G_1 and S phase stages of the cell cycle in *ts LA29 v-Src* CEF cultured at 35°C in the absence or presence of ALLN or ALLM ($50\ \mu\text{M}$) (C) or *ts LA29 v-Src* CEF cultured at 35°C coexpressing either calpastatin (SFCV/+calpastatin) or empty retroviral vector (SFCV) (D).

sis of calpain can lead to degradation of its own inhibitor, calpastatin, thereby further enhancing calpain activity in v-Src-transformed cells (Fig. 10).

To demonstrate that proteolytic degradation of calpastatin contributes significantly to v-Src-induced cell transformation, we overexpressed a calpastatin construct encoding the 110-kDa tissue type isoform in CEF also expressing *ts v-Src*. Following activation of v-Src, cells overexpressing calpastatin demonstrated significant impairment and delay in the onset of characteristic features of morphological transformation, such as disassembly of actin stress fibers and focal adhesions and transition to cell rounding. These observations indicate that the loss of calpastatin protein following v-Src activation plays a key role in v-Src-induced morphological transformation. Intracellular translocation of calpain and calpastatin has also been proposed as a mechanism for regulating calpain activity. Intracellular translocation of calpain to the plasma membrane may decrease the calcium sensitivity required to activate calpain function (58). It has further been suggested that translocation

to membranes or focal adhesion attachment sites may serve to transport calpain away from its endogenous inhibitor calpastatin and to promote calpain activity within specific subcellular compartments (7, 30). Conversely, translocation of calpastatin to membranes or intracellular aggregates may also regulate calpain activity (35, 60). Following v-Src-induced transformation, we have observed a decrease in the calpastatin immunostaining intensity in the cytoplasm (results not shown). Decreased levels of cytoplasmic calpastatin were accompanied by calpastatin immunostaining of the nucleus of transformed cells (results not shown). Calpastatin translocation to the nucleus may represent a mechanism for differentially regulating nuclear and cytoplasmic calpain activity during v-Src-induced oncogenic transformation.

To determine whether proteolytic cleavage of FAK is unique to transformation induced by v-Src, we have studied FAK cleavage during transformation induced by other oncoproteins. Proteolytic cleavage of FAK can be detected in K-Ras-, v-Fos-, and v-Myc- but not v-Jun-transformed fibroblasts. The amount

FIG. 8. Inhibition of calpain activity with cell-permeable calpain inhibitors or calpastatin antagonizes v-Src-induced hyperphosphorylation of pRb and elevation of cyclin D, cyclin A, and cdk2 protein levels. Total cell lysates were prepared from *ts* v-Src CEF cultured at the v-Src restrictive temperature (41°C) and the permissive temperatures (35°C) in the absence or presence of ALLN (100 μM). Total cell lysates were also prepared from CEF coexpressing *ts* v-Src and calpastatin (SFCV + calpastatin) or empty retroviral vector (SFCV) cultured at 41 or 35°C. Cell lysates were separated by SDS-polyacrylamide gel electrophoresis and immunoblotted with antibodies against pRb, cyclin D, cdk2, p27, and cyclin A.

of FAK cleavage taking place during v-Src-induced transformation is much greater than that observed during transformation by the other oncoproteins analyzed in this study. In contrast to v-Src-induced transformation, treatment with ALLN did not cause the morphology of v-Myc-, v-Jun-, v-Fos-, or K-Ras-transformed cells to revert to a normal phenotype. Our results suggest that proteolytic cleavage of FAK may be a common feature of transformation induced by many oncogenes. However, the high degree of FAK cleavage taking place during v-Src-induced transformation combined with the inhibitory effects of ALLN suggests that calpain-mediated cleavage of FAK plays a critical role in morphological transformation induced specifically by v-Src.

To further confirm that calpain activity is a critical requirement for v-Src-induced transformation, we have overexpressed *ts* v-Src in calpain 4 KO and wild-type MEF. The calpain 4 gene product represents the small 28-kDa regulatory subunit that is required for the proteolytic activity of ubiquitously expressed calpain I and calpain II isoforms (1, 16). Recent studies indicate that calpain 4 KO cells exhibit impaired cell motility (N. Doourdin, A. Bhatt, P. A. Greer, J. S. C. Arthur, J. S. Elce, and A. Huttenlocher, Abstr. 40th Annu. Meet. Am. Soc. Cell Biol., 2000). Our studies reveal that v-Src-induced morphological transformation was significantly impaired in calpain 4 KO fibroblasts relative to wild-type MEF. Calpain 4 KO cells exhibited a reduced capacity to undergo v-Src-induced focal adhesion disassembly and cell rounding, relative to wild-type MEF expressing v-Src.

Previous studies have demonstrated that v-Src activation in Rat-1 fibroblasts accelerates progression through the G₁ stage of the cell cycle and modulates the levels of cell cycle regulators, such as the cyclins E and A and the cdk inhibitor p27^{kip1} (27). Studies using pharmacological inhibitors have implicated calpain activity as playing a role in promoting cell proliferation

(41, 53, 64). Many of the inhibitors used in these studies, however, cross-react with other proteases, such as the cathepsins and the ubiquitin-proteasome proteolytic pathway, which can influence the stability of numerous cell cycle regulators (47). As calpain 4 KO cells have been immortalized with retrovirus expressing SV40 large T antigen, it is likely that many of the typical signaling mechanisms that regulate cell proliferation are bypassed by the influence that large T antigen has upon cell cycle control proteins such as p53/p21 and pRb (18, 37). Indeed it has previously been reported that SV40 large T antigen can bypass a requirement for Src in promoting platelet-derived growth factor-induced cell proliferation (11). For these reasons we have not utilized calpain 4 KO cells to study the role that calpain plays in v-Src-induced modulation of cell cycle regulator proteins. In this study we have combined the use of calpain inhibitors with overexpression of the highly specific calpain inhibitor calpastatin to determine in detail the role that calpain may play in regulating cell cycle progression during v-Src-induced cell transformation.

Treatment with the cell-permeable ALLN and ALLM or overexpression of calpastatin induced a G₁ stage growth arrest and suppressed the proliferation of both normal and v-Src-transformed cells. Following activation of *ts* v-Src in exponentially growing cells, we observed hyperphosphorylation of the tumor suppressor protein product of the retinoblastoma gene, pRb, and increased protein levels of cyclins A and D and cdk2. Upon treatment with ALLN or overexpression of calpastatin, the hyperphosphorylation of pRb typically observed in response to v-Src activation was either inhibited or suppressed. Similarly v-Src-induced elevation of cyclins A and D and cdk2 was also suppressed by the calpain inhibitors or calpastatin expression. Our data therefore demonstrate that calpain activity is required for normal cell cycle progression and proliferation of nontransformed cells. However, in response to v-Src activation, elevated calpain activity promotes hyperphosphorylation of pRb and increased levels of cyclins A and D and cdk2, thereby further contributing to v-Src-induced acceleration through the G₁ cell cycle stage and enhanced proliferation of v-Src-transformed cells.

The mechanisms by which calpain regulates pRb phosphorylation and levels of cyclins A and D and cdk2 during v-Src transformation remain to be fully elucidated. We can speculate that calpastatin translocation into the nucleus of transformed cells (results not shown) may impair the normal turnover of nuclear calpain substrates such as cyclin D1, subsequently influencing cdk activity and phosphorylation of pRb (15, 38). Previous studies demonstrate that calpain can proteolytically cleave numerous transcription factors (2, 48, 50); therefore, it is possible that calpain may exert an indirect influence on cell cycle regulation by regulating the transcription of genes that are involved in cell cycle control.

A characteristic feature of cell transformation induced by oncoproteins, including v-Src, is the ability to confer anchorage-independent cell proliferation (33). In this study we demonstrate that treatment with cell-permeable calpain inhibitors or overexpression of calpastatin significantly impairs the ability of CEF expressing *ts* v-Src to grow under anchorage-independent culture conditions. In addition calpain 4 KO fibroblasts expressing activated *ts* v-Src also exhibit a defect in anchorage-independent growth compared with wild-type fibroblasts ex-

FIG. 9. Calpastatin overexpression, treatment with calpain inhibitors, or loss of calpain 4 gene expression in KO cells suppresses anchorage-independent cell growth. *ts LA29v-Src* CEF were cultured in soft agar at 41 or 35°C with and without pretreatment with ALLN or ALLM (100 μ M). *ts LA29 v-Src* CEF coexpressing either calpastatin (SFCV+calpastatin) or empty retroviral vector (SFCV) were cultured in soft agar at 35°C. Wild-type (calpain^{+/+}) and calpain 4 KO (calpain^{-/-}) MEF expressing *ts LA29 v-Src* were also cultured in soft agar at 35°C. (A) Phase pictures illustrate colony formation 12 days following cell seeding. (B) Colony formation after 12 days was quantified by counting the number of colonies per high-power field (magnification, $\times 25$).

pressing *ts v-Src*. These studies indicate that regulation of calpain activity during *v-Src*-induced transformation exerts a broad influence on the cell cycle machinery, promoting progression through the G₁ stage of the cell cycle and anchorage-independent growth.

In conclusion, we describe a positive feedback loop mechanism of calpain activation that is initiated in response to activation of the oncogene *v-Src*. This modulation of the calpain-calpastatin proteolytic system contributes significantly to the process of oncogenic transformation induced by *v-Src*. Our previous studies suggest that calpain activity promotes the mi-

gration of transformed cells (12), and in this study we demonstrate that calpain activity also promotes cell cycle progression and the proliferation of *v-Src*-transformed cells even when deprived of substrate attachment. Therefore, regulation of calpain activity appears to serve as a common link that mediates the influence that *v-Src* exerts on both cell migration and proliferation (Fig. 10). Two separate *in vivo* studies suggest a role for calpain activity in the metastases of renal cell carcinoma and an association with the development of some schwannomas and meningiomas (10, 32). Thus, manipulation of the calpain-calpastatin proteolytic system may represent a useful

FIG. 10. Proposed model describing a positive feedback loop mechanism of calpain activation induced by v-Src. Activation of v-Src promotes increased protein synthesis of calpain II. Increased calpain II protein levels overcome the inhibitory action of calpastatin and promote proteolytic degradation of calpastatin, thereby further enhancing calpain activity. Calpastatin relocates to the nucleus in response to v-Src activation (unpublished observations). Increased calpain activity in v-Src-transformed fibroblasts promotes proteolytic cleavage of FAK and subsequent disassembly of focal adhesion complexes, contributing to a loss of cell adhesion and increased cell motility. Enhanced calpain activity also promotes increased proliferation of v-Src-transformed cells. Calpain promotes the progression of transformed cells through the G₁ stage of the cell cycle and is associated with hyperphosphorylation of pRb and increased protein levels of the cyclins D and A and cdk2.

therapeutic approach for inhibition of oncogenic transformation, tumor growth, and invasion.

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