Cleavage of Focal Adhesion Kinase by Different Proteases during Src-regulated Transformation and Apoptosis

DISTINCT ROLES FOR CALPAIN AND CASPASES*

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Neil O. Carragher‡, Valerie J. Fincham, Deborah Riley, and Margaret C. Frame

From the the Beatson Institute for Cancer Research, Cancer Research Campaign Beatson Laboratories, Glasgow G61 1BD, United Kingdom

The transforming viral Src gene (v-src) is associated with classic characteristics of oncogenic cell transformation, including deregulated growth control, cell rounding, and substrate detachment resulting from adhesion loss and disruption of the actin cytoskeleton (1–6). The stability of the actin cytoskeleton and adhesive properties of cells are mediated, at least in part, by focal adhesion complexes (7, 8). Dynamic regulation of these adhesive links through assembly of focal adhesions at the leading edge of cells, coordinated with focal adhesion disassembly at the trailing edge, plays a key role in controlling cell migration (9). The mechanisms regulating turnover of focal adhesions are not well understood. However, a recent study suggests that proteolysis of specific components of the focal adhesion complex by the calpain family of proteolytic enzymes promotes disassembly of smooth muscle focal adhesions in response to collagen fragments (10). Furthermore, calpain activity has been implicated in promoting migration of Chinese hamster ovary cells (11). The calpains are defined as a well conserved family of intracellular, nonlysosomal calcium-dependent cysteine proteases consisting of two ubiquitously expressed calpain isozymes, μ-calpain (calpain-I) and m-calpain (calpain-II) and several tissue-specific isoforms (12–14). Colocalization of calpain II with focal adhesion structures (15) and the identification of several focal adhesion proteins as calpain substrates (16–21) suggest that calpains are functional at focal adhesion sites.

Oncogenic transformation of cells by v-Src is associated with an overall loss in abundance of focal adhesions as well as changes in focal adhesion architecture to smaller more condensed structures (22). The precise mechanisms by which v-Src promotes the disassembly of focal adhesions have so far not been elucidated. The focal adhesion kinase (FAK)1 is both a substrate of Src kinase activity and a central component of focal adhesions and may be a strong candidate for mediating v-Src-induced disassembly of focal adhesions. Through multiple protein-binding domains, FAK can interact with and phosphorylate several members of the focal adhesion complex (23). Studies on cells derived from FAK knock-out embryos demonstrate that focal adhesions devoid of FAK are larger and that these cells have a reduced migratory capacity, suggesting that FAK may be required to regulate the turnover of focal adhesion structures (24).

To specifically examine the role of calpain-mediated proteolysis in tyrosine kinase-induced focal adhesion disruption, we have used a temperature-dependent mutant of v-Src (ts LA29 v-Src). Activation of v-Src by shift to the permissive temperature initiates focal adhesion disassembly (5). Our previous work has indicated that this and consequent morphological transformation are dependent on the catalytic activity of v-Src that induces phosphorylation of focal adhesion components, including FAK, followed by dissociation of the Src-FAK complex and degradation of FAK, events that precede focal adhesion loss and cell rounding (5, 6). Despite this characterization of the sequence of events during the initial stages of v-Src-induced cell transformation, we still lack any understanding of the nature of the process of FAK proteolytic cleavage induced

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‡ To whom correspondence should be addressed. Tel.: 44-141-330-3956; Fax: 44-141-942-6521; E-mail: n.carragher@beatson.gla.ac.uk.

1 The abbreviations used are: FAK, focal adhesion kinase; ALLM, N-acetyl-leucyl-leucyl-methional (calpain inhibitor I); ALLN, N-acetyl-leucyl-leucyl-norleucinal (calpain inhibitor II); CEF, chicken embryo fibroblasts; ZVAD-FMK, benzoxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (caspase inhibitor I); ts (temperature sensitive); PBS, phosphate-buffered saline.
by v-Src, and specifically, the identities of the proteases involved have not been elucidated. As mentioned above, calpains represent good candidates; however, FAK has also been demonstrated to be cleaved by caspases as focal adhesions are lost during apoptosis (25, 26). In this study, we have addressed whether FAK cleavage that is triggered prior to focal adhesion disassembly during cell transformation is mediated by members of either the calpain or caspase family of proteolytic enzymes.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection—**Primary chicken embryo fibroblasts (CEF) were subcultured as described previously (5). Low density cultures were transfected with replication-competent avian retroviral constructs, RCAN-v-src, encoding the temperature-sensitive ts LA29 v-Src mutant. 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, cells were cultured at the restrictive temperature (41 °C) and then examined following shift to the permissive temperature (35 °C). Rat 1 fibroblasts expressing ts LA29 v-Src were maintained in DMEM supplemented with 5% newborn calf serum, 1 mM sodium pyruvate, and 2 mM L-glutamine at the permissive temperature (35 °C). Rat 1 cells were serum-starved for 24 h (Dulbecco’s modified Eagle’s medium supplemented with 0.2% newborn calf serum) prior to shift to the restrictive temperature (41 °C).

**Antibodies and Reagents—**Calpain inhibitor studies were performed using calpain inhibitor 1 (ALLN, Calbiochem), calpain inhibitor 2 (ALLM), and the cell-permeable sense and scrambled calpastatin peptides (Calbiochem). Caspase inhibitor studies were carried out with caspase inhibitor 1 (ZVAD-FMK; Calbiochem). CEF were preincubated with ALLN, ALLM, or ZVAD-FMK (50–100 μM) for 1 h prior to shift to 35 °C and subsequently incubated at 35 °C in the presence of ALLN, ALLM, or ZVAD-FMK (50–100 μM) or sense and scrambled calpastatin peptides (50 μM) for the indicated time periods. Serum-starved Rat 1 fibroblasts were preincubated with ZVAD-FMK or ALLN (100 μM) for 1 h prior to shift to restrictive temperature 39.5 °C and then subsequently incubate at this temperature in the presence of the inhibitors. Antibodies for Western blot detection and immunocytochemistry included 2–18N pp125 FAK from Santa Cruz Biotechnology, Inc., 354–534N pp125 FAK (Transduction Laboratories), paxillin (Transduction Laboratories), and calpain-II (Research Diagnostics, Inc.). Anti-mouse and -rabbit peroxidase-conjugated secondary antibodies were purchased from New England Biolabs, Inc.

**Immunocytochemistry—**Nontransformed and transformed ts LA29 CEF were cultured on permox plastic chamber slides (Nalge Nunc International). Cells were fixed in 3% formaldehyde in PBS for 10 min at room temperature, permeabilized in 0.5% Nonidet P-40 in PBS for 10 min at room temperature, and washed serially in PBS, 0.15 M glycine/ PBS plus 0.02% Na3VO4, and PBS. Cells were blocked in 10% fetal calf serum/PBS prior to 1 h incubation at room temperature with primary antibodies, including affinity-purified monoclonal anti-paxillin (Transduction Laboratories) and polyclonal rabbit anti-rabbit antisera (Santa Cruz Biotechnology, Inc.). 304–354N pp125 FAK (Transduction Laboratories), calpain-II (Research Diagnostics, Inc.). Anti-mouse and -rabbit peroxidase-conjugated secondary antibodies were purchased from New England Biolabs, Inc.

**Immunoblotting—**Cells were lysed with TNE buffer containing protease inhibitors, 1% Nonidet P-40, 1 m M EDTA, 1 m M EGTA, 1 m M dithiothreitol, 0.5 m M Na3VO4, 10 m M β-glycerophosphate, 10 m M Na4P2O7, 100 μ M Na4P2O7, or lactacystin (34), had no effect on the characteristics of cell lysates prepared at sequential time points following shift to 35 °C demonstrated a consistent rapid increase in levels of calpain II following activation of v-Src, which subsequently declined at 24 h (Fig. 1A). Calpain I protein was detected at extremely low levels in CEF, and levels of calpain I did not vary during the early stages of transformation (results not shown). Messenger RNA levels of calpain II and the endogenous calpain inhibitor calpastatin are not altered in response to v-Src transformation (results not shown), suggesting that elevation of calpain II protein levels and proteolytic activity following v-Src activation are regulated at a post-transcriptional level.

To analyze the role of the calpain proteolytic enzymes in focal adhesion loss associated with v-Src-induced morphological transformation, we examined protein levels and localization of the ubiquitously expressed calpain II in nontransformed (41 °C) and v-Src-transformed CEF (35 °C). Immunoblotting cell lysates prepared at sequential time points following shift to 35 °C demonstrated a consistent rapid increase in levels of calpain II following activation of v-Src, which subsequently declined at 24 h (Fig. 1A). Calpain I protein was detected at extremely low levels in CEF, and levels of calpain I did not vary during the early stages of transformation (results not shown). Messenger RNA levels of calpain II and the endogenous calpain inhibitor calpastatin are not altered in response to v-Src transformation (results not shown), suggesting that elevation of calpain II protein levels and proteolytic activity following v-Src activation are regulated at a post-transcriptional level.

**RESULTS**

To analyze the role of the calpain proteolytic enzymes in focal adhesion loss associated with v-Src-induced morphological transformation, we examined protein levels and localization of the ubiquitously expressed calpain II in nontransformed (41 °C) and v-Src-transformed CEF (35 °C). Immunoblotting cell lysates prepared at sequential time points following shift to 35 °C demonstrated a consistent rapid increase in levels of calpain II following activation of v-Src, which subsequently declined at 24 h (Fig. 1A). Calpain I protein was detected at extremely low levels in CEF, and levels of calpain I did not vary during the early stages of transformation (results not shown). Messenger RNA levels of calpain II and the endogenous calpain inhibitor calpastatin are not altered in response to v-Src transformation (results not shown), suggesting that elevation of calpain II protein levels and proteolytic activity following v-Src activation are regulated at a post-transcriptional level.

**Immunostaining of nontransformed CEF (41 °C) with an antibody against calpain II was generally weak and diffuse throughout the nucleus and cytoplasm; however, some calpain II immunoreactivity was evident at peripheral focal adhesion sites colocalizing with the focal adhesion protein paxillin (indicated by arrows) (Fig. 1B). Six hours following shift to the permissive temperature (35 °C), immunostaining of ts v-Src-expressing CEF with anti-calpain II was of greater intensity than that of nontransformed cells (Fig. 1C), consistent with the elevated protein levels observed by immunoblotting (Fig. 1A). In v-Src-transformed CEF, calpain II staining often localized at peripheral podosome-like structures (indicated by arrows) that may represent substrate attachment sites consisting of residual focal adhesions (Fig. 1C). Immunostaining of anti-calpain II at these peripheral sites again colocalized with focal adhesion proteins such as paxillin (Fig. 1C).

To determine whether calpain activity was involved in the process of oncogenic transformation, CEF expressing ts v-Src were studied following treatment with pharmacological inhibitors of calpain proteolytic activity as well as a calpastatin peptide highly specific for calpain inhibition. ts v-Src-expressing CEF were shifted from the restrictive temperature (41 °C) to the permissive temperature (35 °C) to induce cell transformation. Characteristic of v-Src transformation, untreated CEF lost focal adhesion structures, exhibited a disorganized actin cytoskeleton, and developed a rounded morphology with many cells having detached from the culture substrate 18 h following shift to 35 °C (Fig. 2B). Treatment of cells cultured at 35 °C with a well characterized pharmacological peptide aldehyde inhibitor of calpain activity, ALLN (100 μ M)/(27–29) suppressed the loss of focal adhesions, cell rounding, and remodeling of the actin cytoskeleton (Fig. 2C). Calpain inhibitor I-ALLN has also been reported to inhibit the lysosomal proteases cathepsin B, cathepsin L, and the ubiquitin-dependent proteasome complex (27, 30, 31). Treatment of ts v-Src-expressing CEF undergoing transformation with ammonium chloride, a reputed inhibitor of lysosomal cathepsins (32, 33), or the specific proteasome inhibitor, lactacystin (34), had no effect on the characteristics of v-Src transformation (results not shown), indicating that the effects of ALLN were most likely due to calpain inhibition. In addition, treatment of ts v-Src-expressing CEF with ALLM (100 μ M), which is inhibitory against calpains, but not the
proteasome, can suppress v-Src-induced loss of focal adhesions and remodeling of the actin cytoskeleton and cell morphology (results not shown). A more specific approach to inhibiting intracellular calpain activity can be achieved by exploiting the characteristics of the naturally occurring endogenous inhibitor of calpain, known as calpastatin. Calpastatin is highly specific for calpain I and calpain II and has not been demonstrated to inhibit other proteases (35, 36). Studies demonstrate that a

![Image](http://www.jbc.org/)

**FIG. 1.** Modulation of calpain II protein levels during v-Src-induced cell transformation of chick embryo fibroblasts. A, total cell lysates were prepared from ts LA29 v-Src CEF cultured at the restrictive temperature (41 °C) or at sequential time points following shift to the permissive temperature (35 °C). Cell lysates were separated by 10% SDS-polyacrylamide gel electrophoresis and immunoblotted with an antibody specific for calpain II. ts LA29 v-Src CEF were cultured at 41 °C for 24 h and then for a further 6 h either at 41 °C (B) or 35 °C (C). Immunocytochemistry was performed with an antibody specific for calpain II or paxillin. Colocalized staining of calpain II with paxillin at focal adhesion attachment sites is indicated by arrows. Bar, 25 μm.

**FIG. 2.** v-Src-induced focal adhesion disassembly, disorganization of the actin cytoskeleton, and cell rounding are prevented by inhibitors of calpain activity. ts LA29 v-Src-expressing CEF were cultured at 41 °C (A) or 35 °C (B) for 18 h in the absence or presence of the following inhibitors: ALLN (100 μM) (C), calpastatin peptide (calpa.P.; 50 μM) (D), scrambled calpastatin peptide (calpa.S.; 50 μM) (E), and ZVAD-FMK (100 μM) (F). Cell morphology was evaluated by phase-contrast microscopy (magnification × 100). Focal adhesion structures and the actin cytoskeleton were analyzed by immunocytochemistry utilizing an anti-paxillin antibody and fluorescein isothiocyanate-labeled phalloidin, respectively. Bar, 25 μm.
alternatively spliced c-terminal isoform, FRNK, is also indicated. FAK and putative cleavage fragments are indicated by the generation of approximately sized 95- and 40-kDa amino-terminal fragments and a 30-kDa C-terminal cleavage product (Fig. 3, A and C). Additionally, 55-, 42-, and 30-kDa fragments of FAK derived from the internal region of the protein can be identified by the \( \text{N}^{354-534} \) pp125FAK antibody (Fig. 3B). The initial cleavage of FAK to approximately 95-kDa amino-terminal and 30-kDa C-terminal fragments is consistent with the pattern of calpain-mediated cleavage described previously for other stimuli (16, 39, 10). Pretreatment and subsequent incubation of \( ts \) v-Src-expressing CEF at 35 °C in the presence of ALLN at 100 \( \mu \)M significantly inhibited cleavage of FAK and generation of all cleavage products associated with v-Src transformation (Fig. 3, A–C), whereas treatment with a caspase inhibitor (ZVAD-FMK; 100 \( \mu \)M) had no effect (results not shown).

To examine the influence calpain inhibition had on the adhesive strength of v-Src-transformed CEF for their culture substrate, \( ts \) v-Src-expressing CEF were cultured for 18 h at 41 °C or at 35 °C in the absence or presence of the calpain inhibitors ALLN and ALLM. The number of cells remaining attached to the substrate after extensive washing was quantified and expressed as the number of cells per high power field (Fig. 4). These results demonstrated that after 18 h at 35 °C there was a significant loss in the number of \( ts \) v-Src-expressing cells remaining strongly adherent to the substrate. Transformation of cells at 35 °C in the presence of the calpain inhibitor ALLN or ALLM (100 \( \mu \)M) resulted in a greater than 2-fold increase in the number of cells remaining attached to the culture substrate. Incubation of CEF at 35 °C in the presence of the general inhibitor of kinase function herbimycin A, which inhibits Src kinase activity, also partially rescued substrate anchorage (Fig. 4). These results indicate that calpain-dependent disassembly of focal adhesions that accompanies v-Src transformation results in a general decrease in the adhesion of transformed CEF to their culture substrate. Thus, treatment of v-Src-transformed CEF with calpain inhibitors stabilized focal adhesion structures and maintained substrate anchorage.

Since we have shown that the proteolysis of FAK during transformation by v-Src is most likely calpain-dependent and since we have shown previously that Src-induced focal adhesion disassembly is required for cell migration (6), we addressed whether inhibitors of calpain could suppress cell migration into a wounded monolayer of v-Src-transformed cells.

![Fig. 3. Calpain-mediated FAK degradation during v-Src transformation of CEF.](Image)

![Fig. 4. Inhibitors of calpain activity limit the loss of cell anchorage that accompanies v-Src transformation.](Image)
After wounding, ts v-Src-expressing CEF cultured at 35 °C for 18 h demonstrated extensive migration into the denuded area (Fig. 5). Incubation of ts v-Src-expressing CEF at 35 °C in the presence of the calpain and proteasome inhibitor (ALLN; 50 μM) inhibited any motility of transformed CEF into the wound (Fig. 5). Treatment of CEF with the inhibitor ALLM (50 μM), which inhibits calpain and not the proteasome complex, also substantially prevented migration. Since ALLN and ALLM have previously been reported to exhibit cathepsin-inhibitory activity (27, 30), we also treated ts v-Src-expressing CEF with the reported inhibitor of lysosomal cathepsins, ammonium chloride, as a negative control and found this had no significant influence on migration (results not shown). Treatment of cells with the caspase inhibitor ZVAD-FMK also has no effect on migration of v-Src-transformed CEF (Fig. 5). These results demonstrate that v-Src-transformed CEF are dependent on calpain activity for optimal migration, most likely as a result of its proposed role in FAK cleavage and focal adhesion turnover.

Our previous studies demonstrate that Rat-1 fibroblasts expressing the ts LA29 v-Src protein undergo apoptosis when cultured in low serum following attenuation of v-Src activity by culture at the restrictive temperature (39.5 °C). Following shift of serum-starved ts LA29 Rat-1 cells from permissive (35 °C) to restrictive temperature (39.5 °C), the cells rapidly round up and become less adherent (40). Proteolytic cleavage of FAK to 85- and 77-kDa N-terminal fragments accompanies these morphological changes (Fig. 6); however, in contrast to v-Src transformation, FAK cleavage in serum-deprived cells switched to the restrictive temperature was not inhibited by ALLN but instead was inhibited by ZVAD-FMK (Fig. 6), indicating that caspases and not calpains are responsible for FAK cleavage that accompanies adhesion loss under these circumstances.

**DISCUSSION**

Cell adhesion to extracellular matrix substrates regulates many aspects of normal cell physiology, including proliferation, migration, and cell survival (9, 41, 42). An integral role for calpain activity in regulating critical aspects of cell behavior has been suggested by previous studies demonstrating that calpain activity can degrade focal adhesion components (39, 4274), mediate substrate detachment at the trailing edge of cells (9), and regulate filipodia and lamellipodia formation and forward protrusion at the leading edge of cells (43). In addition calpain may also contribute to caspase-mediated apoptotic cell death pathways (44–46). However, evidence demonstrating either a physiological or pathological role for calpain-dependent proteolysis of focal adhesion components has not been reported.

In this study, we demonstrate that levels of calpain II protein are regulated at the post-transcriptional level following v-Src transformation of CEF. Furthermore, in response to activation of v-Src, calpain activity promotes proteolytic cleavage of FAK, focal adhesion disassembly, and morphological transformation. Using epitope-specific antibodies, we have identified putative cleavage fragments generated by calpain-mediated proteolysis of FAK taking place during v-Src transformation. FAK cleavage data indicate that a 30-kDa C-terminal fragment containing the focal adhesion targeting sequence and a second proline-rich domain dissociates from a 95-kDa amino-terminal fragment containing the kinase domain. Since the C-terminal domains of FAK are important in targeting FAK to focal adhesions (47), this cleavage event is likely to influence the localization of the kinase domain to focal adhesion sites and may lead to reduced FAK activity at adhesions, something that is known to negatively regulate FAK function and cell motility (48). In addition, the C-terminal cleavage fragment is relatively stable following its generation, so it could potentially act in a dominant negative role, competing with functionally intact full-length FAK for focal adhesion substrates. Calpain-dependent cleavage of FAK would also be expected to impair the ability of FAK to act as an adapter protein, thereby compromising the integrity of the focal adhesion complex. Thus, modulation of FAK function through calpain-dependent cleavage is likely to play a significant role in the process of oncogenic transformation.

The induction of calpain-dependent proteolytic cleavage following v-Src activation appears to be selective for FAK, since other known calpain substrates within focal adhesions, such as paxillin, talin, or Src itself do not undergo calpain-dependent processing during v-Src transformation (results not shown). Although the detailed regulation of calpain-mediated FAK cleavage induced by v-Src remains to be elucidated, we have found that calpain II protein levels are modulated during transformation. In addition, calpain-induced cleavage of FAK may be linked to specific tyrosine phosphorylation of FAK that leads to complex dissociation, perhaps releasing FAK and permitting its cleavage by calpain. Previous studies demonstrate that Src-mediated tyrosine phosphorylation of cortactin and the NR2 subunits of NMDA receptors influences the ability of...
these proteins to be cleaved by calpain (49, 50). It is therefore tempting to speculate that v-Src-dependent phosphorylation of FAK may influence the suitability of FAK to act as a substrate for calpain. We are currently investigating the mechanism by which Src activity controls calpain II protein levels and the influence phosphorylation of specific FAK residues has on its ability to be cleaved by calpain.

FAK cleavage mediated by the caspase family of cysteine proteases has previously been demonstrated in T lymphocytes and endothelial cells undergoing apoptosis (25, 26). Treatment of v-Src-expressing CEF with inhibitors against a broad spectrum of caspase family members (ZVAD-FMK) had no effect on FAK cleavage or morphological transformation. In contrast, FAK cleavage in serum-starved Rat-1 fibroblasts induced to detach and undergo apoptosis after switching off v-Src activity is suppressed by caspase inhibitors, whereas calpain inhibitors had no effect. These data lead us to conclude that proteolysis of FAK is a general phenomenon associated with focal adhesion disassembly but can be mediated by distinct proteases under different biological Src-regulated processes: caspases during apoptosis and calpain (most likely calpain II) during tyrosine kinase-induced cell transformation.

From the data presented in this report, we propose that calpain- and caspase-mediated proteolysis of FAK are important mechanisms for regulating focal adhesion integrity in both viable and apoptotic cells, respectively. During v-Src transformation of CEF, calpain-dependent cleavage of FAK precedes disassembly of the focal adhesion complex and disorganization of the actin cytoskeleton, leading to a loss of substrate anchorage and increased motility. This implicates calpain as a major effector in the process of v-Src-induced oncogenic transformation and Src-dependent cell migration. A recent study examining the expression of calpain I in human renal cell carcinomas demonstrated significantly higher levels of calpain I expression in tumors that presented evidence of metastases to peripheral lymph nodes relative to low expression levels in tumors that apparently had not metastasized (51). This study together with our own data suggests that calpain family proteases could mediate disassembly of focal adhesions and loss of substrate anchorage that may contribute to tumor cell motility and invasion. Targeting the inhibition of calpain activity in transformed cells may provide a useful therapeutic strategy for the prevention of tumor invasion and metastasis.

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