Cyclin E Both Regulates and Is Regulated by Calpain 2, a Protease Associated with Metastatic Breast Cancer Phenotype

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Abstract

Overexpression of cyclin E in breast tumors is associated with a poor response to tamoxifen therapy, greater genomic instability, more aggressive behavior, and a poor clinical prognosis. These tumors also express low molecular weight isoforms of cyclin E that are associated with higher kinase activity and increased metastatic potential. In the current study, we show that cyclin E overexpression in MCF7 cells transactivates the expression of calpain 2, leading to proteolysis of cyclin E as well as several known calpain substrates including focal adhesion kinase (FAK), calpastatin, pp60src, and p53. In vivo inhibition of calpain activity in MCF7-cyclin E cells impedes cyclin E proteolysis, whereas in vivo induction of calpain activity promotes cyclin E proteolysis. An analysis of human breast tumors shows that high levels of cyclin E are coincident with the expression of the low molecular weight isoforms, high levels of calpain 2 protein, and proteolysis of FAK. Lastly, studies using a mouse model of metastasis reveal that highly metastatic tumors express proteolysed cyclin E and FAK when compared to tumors with a low metastatic potential. Our results suggest that cyclin E–dependent deregulation of calpain may be pivotal in modifying multiple cellular processes that are instrumental in the etiology and progression of breast cancer. (Cancer Res 2005; 65(23): 10700-8)

Introduction

Cyclin E, the regulatory component of the cyclin/cyclin-dependent kinase (CDK) complex, is overexpressed in different tumor types and is often associated with a poor clinical outcome (1–9). The contribution of cyclin E to tumorigenesis has been best studied in breast cancers where overexpression is apparent in 10% to 25% of breast cancers, is a strong predictor of endocrine therapy failure (10), and an accurate predictor of a poor clinical outcome (9, 11). Studies of cyclin E–overexpressing breast tumors and tumor-derived cell lines found that cyclin E is also proteolytically processed into more active low molecular weight (LMW) isoforms (12) that are resistant to inhibition by the CDK inhibitors p21 and p27, and hence are associated with higher CDK kinase activity (13). Analyses using an in vivo melanoma model suggest that the LMW isoforms are more effective than the full-length cyclin E in enhancing angiogenesis and metastasis (14), thus establishing a link between the expression of cyclin E LMW isoforms and metastasis.

Although studies substantiate the role of cyclin E LMW isoforms in tumorigenesis, the mechanisms involved in cyclin E proteolysis are unclear. An analysis of LMW isoforms expressed in a cyclin E–overexpressing cell line suggests that elastase is instrumental in cyclin E proteolysis (12). Another study indicates that cyclin E can be cleaved by calpain to generate LMW isoforms that are similar in size to the isoforms present in tumors (15). Therefore, at present, the protease(s) that generate the LMW isoforms in breast tumors and tumor-derived cell lines are not clearly delineated.

Calpains are calcium-dependent thiol proteinases that have been implicated in many cellular processes. Calpain activity is tightly regulated by levels of expression, calcium requirement, autoproteolysis, phosphorylation, intracellular distribution, and the endogenous inhibitor calpastatin (16). In general, calpains cleave proteins at a limited number of sites to generate large polypeptide fragments (16). Over 100 calpain substrates have been identified, and many are cytoskeleton/membrane attachment proteins including focal adhesion kinase (FAK; ref. 17), α-spectrin (18), talin (19), paxillin (20), E-cadherin (21), and β-catenin (22). Calpain also cleaves proteins that are involved in signal transduction and transcriptional regulation such as pp60src (23), p53 (24, 25), and fos (26). Therefore, deregulation of calpain activity would affect proliferation, migration, apoptosis, and metastasis.

We previously reported that cyclin E overexpression in MCF7 and T47D tumor–derived breast cells confers partial tamoxifen resistance (27). We also noted that cyclin E–overexpressing cell lines have increased levels of the LMW isoforms. The current study shows that in MCF7 cells, cyclin E transactivates calpain 2 transcription, thus, positively regulating its own proteolysis as well as the proteolysis of other calpain substrates. An analysis of human mammary tumors identified a correlation between cyclin E expression, calpain 2 levels, and calpain-dependent proteolysis. Such modifications are consistent with the aggressive behavior and poor clinical prognosis that is evident in cyclin E–overexpressing breast tumors.

Materials and Methods

Cell culture. HepG2 cells, obtained from American Type Culture Collection (Manassas, VA) and the Hct116 cell line, a gift from Dr. K. Kaplan, University of California, Davis, Davis, CA, were propagated in DMEM (Life Technologies, Rockville, MD) supplemented with 10% fetal bovine serum, penicillin/streptomycin at 37°C and 5% CO2. The generation of the MCF7 and T47D cell lines has been previously described (27, 28).

Microarray analysis. Labeling of samples, hybridization to U133A GeneChips (Affymetrix, Santa Clara, CA), staining, and scanning were done as described in the Affymetrix Expression Analysis Technical Manual. Fluorescence intensity values (.CEL files) generated from hybridized, stained GeneChips were analyzed with R statistical software (v.2.0.1, and “affy” BioConductor package) and BRB Array Tools to identify genes that were differentially expressed. The settings used for Robust Multichip Analysis in R included Microarray Suite 5.0-based background correction.
quantile normalization, and Robust Multichip Analysis–based algorithms for calculation of expression values using perfect match only fluorescence intensities. A detection \( P \leq 0.05 \) and a mean fold change of \( \geq 1.5 \)-fold (\( P \leq 0.01 \)) were used as criteria for filtering genes for clustering analyses. Hierarchical clustering and comparative fold-change analysis were used to identify and group similar patterns of gene regulation. Assignment of genes to functional categories was done by annotation of gene lists with the database. For Annotation, Visualization, and Integrated Discovery (http://apsk.liaid.nih.gov/david) and literature-based classification was done by hand. Statistically overrepresented (Fishers exact probability score <0.05) biological processes within clusters were identified using Expression Analysis Systematic Explorer v.1.0 analysis software (29).

**In vivo calpain induction.** MCF7 cells were plated in six-well plates and cultured overnight. Cells were pretreated with 20 \( \mu \)M/L of the calpain inhibitor calpeptin (Calbiochem, La Jolla, CA) or DMSO for 15 minutes and then treated with 10 \( \mu \)M/L ionomycin (Calbiochem) for 20 minutes, harvested, washed with ice-cold PBS, and lysed in radioimmunoprecipitation assay buffer.

**In vivo calpain inhibition.** MCF7 cells were plated in six-well plates and cultured overnight. Cells were treated with DMSO, 5 or 20 \( \mu \)M/L of calpeptin, and 2.5 or 23 \( \mu \)M/L of N-acetyl- Leu-Leu-methionyl (ALLM; Calbiochem) for 24 or 48 hours, at which time they were washed with ice-cold PBS and harvested.

**Transfection.** Cells were transfected with Superfect reagent (Qiagen, Valencia, CA) and 5 \( \mu \)g of pBLCMV-cyclin E or pBLCMV plasmid DNA following the manufacturer's recommendation. For the promoter studies, cells were transfected with FuGene 6 (Roche Applied Science, Indianapolis, IN) and capn-\( \beta \)-hu2, SV\( \gamma \)-gal, pBLCMV-cyclin E\(^{380}\), pBLCMV, or p21 plasmids. Forty-eight hours after transfection, cells were harvested and subjected to analysis as previously described (28). pBLCMV-cyclin E\(^{380}\) harbors a site-directed mutation converting the tyrosine 380 residue to alanine, thus enhancing cyclin E stability. The mutation was generated using the following primers: 5'CCCATGGTGCCCGGCCCACAGAC3' and 5'GCTGGTGCGGCGGGGAGGACACGG3'.

**Northern blot analysis.** Total RNA was isolated using the Trizol reagent (Life Technologies) following the manufacturer's recommendation. Total RNA (10 \( \mu \)g) was electrophoresed on a 1% agarose gel and transferred to Duralon-UV membrane (Stratagene, La Jolla, CA). The following primers were used to generate calpain 2–specific RT-PCR products: 5'AGGATGAG-GATGAGACGAGGAGGAT3' and 5'TTTTACGAAAAAGCAGCGATTG3'. The RT-PCR products were labeled with a\(^{32}\)P-dCTP. The hybridization and wash conditions have been previously described (27).

**Western immunoblot analysis.** Cyclin E overexpression MCF7 mammary cell lines were generated to define cyclin E–dependent alterations in gene expression. Two clones, MCF7-CycE1 and MCF7-CycE3F, that expressed intermediate and high levels of cyclin E, and had elevated levels of cyclin E–dependent kinase activity were chosen for further studies (Fig. L4 and B). Notably, MCF7-CycE1 and MCF7-CycE3F cells had abundant amounts of the LMW isoforms of cyclin E that have been previously detected in cyclin E–overexpressing tumors (12, 15), whereas MCF7 control cells expressed only trace amounts of these isoforms. Overexpression of cyclin E in ZR75, LNCaP, PC3, HEK293, and T24 cells also resulted in the expression of the same-sized LMW isoforms (data not shown). These observations raised the possibility that cyclin E, when overexpressed, might promote its own processing.

**Cyclin E overexpression transactivates genes involved in catabolism.** To determine the effects of cyclin E overexpression in MCF7 cells, microarray analysis was used to analyze changes in gene transcription. The analysis was conducted in triplicate for each line. Statistical analysis of the microarray data resulted in identification of 645 genes that were differentially expressed (fold change \( \geq 1.5 \); \( P \leq 0.01 \)) in cyclin E–overexpressing MCF7 cells. As expected, MCF7-CycE1 and MCF7-CycE3F expressed increased levels of cyclin E, as well as decreased levels of genes that we previously found to be repressed in cyclin E–overexpressing cells—Bcl-2 (28) and BB.\(^4\) Hierarchical clustering of differentially expressed genes revealed similar patterns of regulation in both cyclin E–overexpressing cell lines (Fig. 2A). Interestingly, one subcluster of genes up-regulated by cyclin E expression are genes involved in catabolism (Fig. 2B). This subcluster contained calpain 2, which was previously implicated in cyclin E proteolysis.

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\(^4\) B. Robinson and M. Madryj, unpublished data.
Calpain 2 expression is elevated in cyclin E–overexpressing MCF7 cells. The elevated expression of calpain 2 in cyclin E–overexpressing MCF7 cells was confirmed by Northern blot analysis of mRNA isolated from MCF7 control and cyclin E–overexpressing cells (Fig. 1C). Western immunoblot analysis established that calpain 2 protein levels were elevated in cyclin E–overexpressing cells (Fig. 3A). GAPDH and β-actin served as gel loading controls.

To determine if cyclin E directly transactivates calpain 2 transcription, we cloned the human calpain 2 promoter into the pGL3 luciferase expression vector. The 456 bp promoter contained sequences from −25 to −481 of the translational start site. Previous analyses found that this promoter has multiple transcriptional start sites, and harbors Sp1, Ap–1, Ap-2, E-box, and E2F-binding sites (31). The capnP-luc reporter construct was transfected into MCF7 cells along with the cyclin E expression plasmid or with a vector control plasmid (pBcCMV). As shown in Fig. 1D, cyclin E transactivates luciferase expression. To determine if CDK kinase activity was instrumental in promoter transactivation, cells were transfected with the capnP-luc plasmid, the cyclin E expression plasmid, and the p21 CDK inhibitor expression plasmid. Inclusion of p21 resulted in decreased promoter activity, arguing that cyclin E–dependent kinase activity is required for calpain 2 transactivation.

Proteolysis of known calpain substrates in cyclin E–overexpressing cells. Calpain cleaves a number of cellular substrates to generate LMW isoforms; therefore, detection of these cleaved forms would be indicative of elevated calpain activity. Previous studies have shown that FAK, a 120 kDa protein, is cleaved by calpain to a 90 kDa form (17). MCF7 control cells were transfected with the capnP-luc plasmid, the cyclin E expression plasmid, and the p21 CDK inhibitor expression plasmid. Inclusion of p21 resulted in decreased promoter activity, arguing that cyclin E–dependent kinase activity is required for calpain 2 transactivation.

Calpain 2 expression is elevated in cyclin E–overexpressing MCF7 cells. Whereas calpastatin is encoded by a single gene, multiple calpastatin isoforms were detected in MCF7 control cells (Fig. 3A). In contrast, calpastatin was barely detected in MCF7-CycE1 cells and was undetectable in MCF7-CycE3F cells. In this system, an increase in cyclin E causes a drastic decrease in calpastatin expression.

Another calpain substrate, pp60c-src, is proteolyzed by calpain to a 48 kDa form in platelets (23). This truncated protein has a deletion of the NH2-terminal region which harbors the myristoylation site as well as a deletion of part of the SH3 domain. As shown in Fig. 3A, the MCF7 control and MCF7-CycE1 cells expressed low, but detectable levels of the 48 kDa isoform. However, MCF7-CycE3F cells expressed equal amounts of the full-length and the truncated 48 kDa form of pp60c-src. Therefore, whereas pp60c-src is cleaved by calpain in vitro, it is not as sensitive to calpain-dependent proteolysis as calpastatin or FAK.

In vitro studies showed that human and mouse p53 are calpain substrates (24, 25). Human p53 is cleaved to a 48 kDa NH2-terminally truncated isoform. The full-length, but not the LMW isoform, can be detected by an antibody directed against the NH2-terminal domain of the molecule (DO-1). An antibody generated against the full-length p53 detects the full-length and the calpain-cleaved isoform of p53. As shown in Fig. 3A, the NH2-terminal antibody detected the p53 protein in MCF7 control and MCF7-CycE1 cells. MCF7-CycE3F cells seemed to have very low levels of p53. To detect the truncated p53, the blot was stripped and reanalyzed with an antibody directed against the full-length p53 protein. This analysis revealed that MCF7-CycE3F expressed p53, but most of the protein was cleaved to the NH2-terminally truncated form. Also, the amount of p53 was reduced in the MCF7-CycE3F cells, suggesting that the truncated form of the protein may be more labile. This result supports the hypothesis that calpain 2 expression is elevated in cyclin E–overexpressing MCF7 cells.

Calpastatin, an endogenous inhibitor of calpain is a very sensitive calpain substrate (32). Whereas calpastatin is encoded by a single gene, multiple calpastatin isoforms were detected in MCF7 control cells (Fig. 3A). In contrast, calpastatin was barely detected in MCF7-CycE1 cells and was undetectable in MCF7-CycE3F cells. In this system, an increase in cyclin E causes a drastic decrease in calpastatin expression.

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that calpain cleavage of p53 may be an alternative mechanism of p53 degradation (24).

Cyclin E overexpression transactivates calpain 2 in other cell lines. T47D (breast), HCT116 (colon), and HepG2 (hepatocellular carcinoma) cells were used to determine if cyclin E overexpression in other cellular backgrounds resulted in a similar transactivation of calpain 2. Cyclin E–overexpressing T47D clones (T47D-CycE4 and T47D-CycE18) expressed higher amounts of the cyclin E LMW isoforms, higher levels of calpain protein, and a dose-dependent decrease in FAK expression (Fig. 3B). Transient expression of cyclin E in HCT116 resulted in increased expression of the cleaved isoform of calpain 2 and the complete proteolysis of FAK to the 90 kDa form (Fig. 3B). HepG2 transiently transfected with the cyclin E plasmid expressed the LMW cyclin E isoforms and had elevated levels of calpain 2. Cyclin E–overexpressing HepG2 cells had very low levels of FAK, suggesting that the protein may be completely proteolyzed (Fig. 3B). Previous studies found that the 90 kDa FAK isoform is further degraded to 45 and 40 kDa fragments and ultimately is completely degraded (17). This analysis suggests that cyclin E–dependent transactivation of calpain may be a feature of other tumors.

Cyclin E is a calpain substrate in vitro. To confirm that cyclin E can be cleaved by calpain, cellular extracts prepared from MCF7 control cells were treated with increasing amounts of CaCl2. Figure 4A shows a CaCl2 dose-dependent increase in cyclin E proteolysis to the LMW isoforms in MCF7 cells (lanes 1–4). Additionally, the cyclin E–overexpressing clones expressed LMW isoforms of similar mass (lanes 5 and 6). To confirm that calpain activity was activated by the inclusion of CaCl2, the immunoblot was stripped and reprobed with a FAK-specific antibody. Increasing concentrations of CaCl2 also resulted in the cleavage of FAK. The same analysis was used to detect the sensitivity of calpastatin to calpain–dependent proteolysis. C-rel, a protein that has not been described as a calpain substrate, served as a negative control. Increasing concentrations of CaCl2 had no effect on c-rel, therefore, the degradation of cyclin E, FAK, and calpastatin are calpain-specific and not due to a general degradation of the cellular extracts.

An alternative method was used to confirm that cyclin E is a calpain 2 target. MCF7 control cellular extracts were treated with increasing concentrations of purified calpain 2. As shown in Fig. 4B, calpain 2 addition resulted in a dose-dependent proteolysis of cyclin E into the same-size LMW isoforms detected in cyclin E–overexpressing MCF7 cells. The two complementary analyses show that, in vitro, cyclin E is a calpain substrate.

In vivo activation and inhibition of cyclin E proteolysis. Although previous studies reported calpain-dependent cyclin E cleavage in vitro (15), it was unknown if calpain-dependent proteolysis occurred in vivo. To address this issue, proliferating MCF7 control cells that expressed only trace amounts of the LMW isoforms were treated with the calcium ionophore, ionomycin. Previous studies have shown that ionomycin activates endogenous calpain (21). As shown in Fig. 5A, ionomycin treatment resulted in

Figure 2. Hierarchal cluster analysis of gene transcription in MCF7-RecMV, MCF7-CycE1, and MCF7-CycE3F cells. Hierarchal clustering of the microarray data was used to identify similar patterns of altered transcription in cyclin E–overexpressing cells. Clusters containing functionally related genes revealed several biological processes including catabolism that were deregulated in cyclin E–overexpressing cells. B, a representative list of up-regulated catabolic genes in cyclin E–overexpressing cells includes calpain 2.
cleavage of cyclin E into LMW isoforms. Inclusion of the cell-permeable calpain inhibitor, calpeptin, prevented cyclin E proteolysis. To confirm that calpain was activated, the immunoblot was stripped and reprobed with a FAK-specific antibody. This shows that cyclin E is a calpain substrate \textit{in vivo}.

If cyclin E cleavage in MCF7-CycE3F cells is indeed due to calpain, then \textit{in vivo} inhibition of calpain activity would prevent cyclin E proteolysis. To test this hypothesis, MCF7-CycE3F cells were treated with the vehicle DMSO or with two different concentrations of the calpain inhibitors calpeptin or ALLM for 24 or 48 hours. As shown in Fig. 5b, calpeptin treatment effectively prevented cyclin E cleavage in a dose-dependent manner. ALLM inhibited cyclin E proteolysis, but to a lesser extent than calpeptin. The immunoblot was stripped and reprobed with FAK antibodies. Inhibition of FAK cleavage served as a positive control for the inhibition of calpain activity. Our ability to modulate the expression of cyclin E LMW isoforms by activation or inhibition of calpain activity \textit{in vivo} showed that in MCF7 cells, the LMW isoforms were generated by calpain-dependent proteolysis.

Previous reports indicate that the cyclin E LMW forms are associated with higher cyclin E–dependent kinase activity. To test this hypothesis \textit{in vivo}, cellular extracts prepared from MCF7-CycE3F treated with 20 μmol/L calpeptin or DMSO for 24 hours were used to detect cyclin E–associated kinase activity. Cyclin E–dependent kinase activity was reduced 1.8-fold by calpeptin treatment, arguing that the LMW isoforms are associated with higher kinase activity (data not shown).

**Cyclin E overexpression, calpain 2 levels, and focal adhesion kinase proteolysis in human breast tumors.** Cyclin E is overexpressed in 10% to 25% of human breast tumors and these tumors also express the LMW isoforms of the protein (9, 11). Therefore, we asked if there is a correlation between cyclin E overexpression, calpain 2 levels, and calpain-dependent proteolysis of a known calpain substrate. Ten frozen mammary tumor samples and one control (normal mammary tissue) were used in the analysis. Equal amounts of protein extracts were analyzed for the expression of cyclin E. GAPDH was used to confirm equal protein loading. As shown in Fig. 6a, one tumor, 3440, expressed high levels of cyclin E, whereas tumors 571 and 3040 expressed a moderate amount of cyclin E. Tumor 3440 also expressed cyclin E LMW isoforms. Tumor 571 had a small amount of one LMW isoform. The MCF7-CycE3F extract served as marker for the expression of the LMW cyclin E isoforms. Consistent with the hypothesis that cyclin E transactivates calpain 2 expression, tumors 3440, 3040, and 571 had elevated levels of calpain 2 when compared with normal mammary tissue (1827). FAK was almost completely proteolyzed to the 90 kDa form in tumors 3440, 3040, and 571, indicative of elevated calpain activity. In contrast, tumor 2385 had low levels of calpain 2 and expressed abundant levels of full-length FAK. This tumor analysis supports the hypothesis that

**Figure 3.** Cyclin E overexpression in MCF7 cells results in increased calpain 2 protein expression and activity. \textit{A}, calpain 2 protein levels are increased in cyclin E–overexpressing cells (lanes 2 and 3). The FL and LMW forms of cyclin E are marked. Calpain substrates FAK, calpastatin, p60src, and p53 are proteolytically processed in cyclin E–overexpressing (lanes 2 and 3), but not in control cells (lane 1) indicating an increase in calpain activity. Arrows, different isoforms of calpain 2, FAK, c-src, and p53. \textit{B}, calpain substrate FAK is proteolytically processed in cyclin E–overexpressing (lanes 2, 3, 5 and 7), but not in control cells (lanes 1, 4 and 6).
Figure 4. Activation of calpain promotes proteolytic cleavage of cyclin E and FAK. A, treatment of MCF7-RcCMV control cellular extracts with increasing concentrations of Ca2+ causes progressively greater cleavage of cyclin E (lanes 1–4). The proteolytic fragments are identical in size to the forms present in cyclin E–overexpressing MCF7 clones (lanes 5 and 6). Cleavage of calpain substrates FAK and calpastatin served as positive controls, whereas c-rel served as negative control. B, addition of increasing amounts of purified calpain 2 to MCF7-RcCMV cellular extracts (lanes 4–9) promoted proteolysis of cyclin E to the same-size LMW isoforms that are expressed in cyclin E–overexpressing MCF7 clones (lanes 1 and 2).

LMW isoforms of cyclin E are associated with increased calpain-dependent proteolysis.

Cyclin E and focal adhesion kinase proteolysis in non-metastatic and metastatic tumors. Many calpain substrates are cytoskeleton/membrane attachment proteins. These proteins are important for cellular adhesion, a process that is modified by calpain proteolysis. Alterations in cellular adhesion may have a role in tumor invasion and/or metastasis. Therefore, a mouse breast tumor metastasis model (30, 33) was used to determine if (a) calpain may have a role in this process and (b) if cyclin E proteolysis is associated with metastasis. The study employed four related polyomavirus-middle T (PyV-mT) transgenic mouse mammary carcinoma transplant lines that display differences in metastatic potential (33). Importantly, PyV-mT has been used as a surrogate for Her2/neu overexpression and interacts with and activates the 85 kDa regulatory phosphoinositide-3-kinase (PI3K) subunit. The Met line, derived from transgenic mice constructed with the wild-type PyV-mT line, develops rapid mammary carcinoma in all animals with 100% pulmonary metastasis. The Db7 line is derived from animals with double site-directed mutations of the PyV-mT that decouple it from the PI3K pathway. The Db7 model has 100% penetration of mammary tumor but exhibits significantly fewer pulmonary metastases (9%). The PD5 and PD6 transplantable tumor lines are derived from rare metastases of the Db7 line. The order of metastatic potential from least metastatic to most metastatic is Db7, PD6, PD5, and Met. Extracts prepared from these tumors were used to detect expression of FAK, calpain 2, and cyclin E. β-Actin and GAPDH served as gel loading controls. As shown in Fig. 6B, FAK is poorly cleaved in the Db7 tumor, intermediate cleaved in PD6 and PD5, and greatly cleaved in the Met tumor. Calpain 2 autolysis results in a more active, but less stable, form of calpain 2 (34). The Met tumor expressed a smaller form of calpain 2, further suggesting that this tumor had higher calpain activity. Finally, the Met tumor, but not the Db7 tumor, expressed LMW isoforms of cyclin E, demonstrating that cyclin E proteolysis correlated with calpain activity. These results suggest that calpain-dependent proteolysis is associated with metastasis.

In summary, this study supports the hypothesis that cyclin E transactivates calpain 2 expression thus regulating its own proteolysis as well as the proteolysis of additional calpain substrates. This conclusion is substantiated by studies of human mammary tumors. Further analysis using a mouse mammary tumor model suggests that calpain-dependent proteolysis may have a role in mammary metastasis.

Discussion

This study identifies a link between cyclin E overexpression and enhanced expression and activity of calpain 2, indicating that cyclin E both regulates and is regulated by calpain 2. Similar autoregulatory loops have been described for cyclin E and the CDK inhibitor p27, as well as cyclin E and the E2F/RB transcription factor complex (35–38). The cyclin E–dependent activation of calpain 2 results in increased calpain-mediated proteolysis of cyclin E into LMW isoforms that are refractory to inhibition by p21 and p27, resulting in higher cyclin E–associated kinase activity. Moreover, the increase in calpain 2 expression affects proteolysis of the calpain inhibitor and substrate calpastatin. Calpastatin proteolysis further shifts the calpain/calpastatin homeostasis in favor of increased calpain activity, thus leading to the proteolysis of additional calpain substrates.

Calpain-dependent proteolysis has been implicated in signal transduction, adhesion and metastasis. Calpain’s proteolytic activity is distinct from other proteases because calpains have a limited and complex site specificity (16) that is determined by both the sequence and conformation of the polypeptide (39). The sequence specificity is not dependent on an exact sequence, but rather by preferred amino acids in distinct sites (39). This rather nebulous definition of calpain cleavage sites makes it difficult to predict substrate specificity in the absence of experimental data. Because calpain specificity is dependent in part upon conformation, posttranslational modifications such as phosphorylation, significantly affect the rate of
proteolysis (40, 41). Therefore, the cleavage of specific calpain substrates in a cell may be dependent on posttranslational modifications of the substrates as well as the rates of calpain cleavage.

Increased calpain 2 activity promotes proteolysis of FAK, which is cleaved to a readily detectable 90 kDa form (17). We found that FAK is a sensitive calpain substrate and the extent of FAK proteolysis is proportional to cyclin E expression. Therefore, FAK proteolysis is a useful measure of calpain activity. FAK is thought to coordinate cell adhesion and migration. Previous studies in platelets reported that proteolysis of FAK resulted in a reduction in its autokinase activity and led to its dissociation from the cytoskeleton. Interestingly, cells devoid of FAK exhibit reduced in vitro mobility and an increase in the number of focal adhesions, suggesting that FAK may have a role in focal adhesion turnover.

Figure 5. In vivo induction of calpain activity promotes cyclin E and FAK cleavage, whereas in vivo inhibition of calpain activity prevents cleavage. A, to induce calpain activity, MCF7-RcCMV control cells were treated with the calcium ionophore ionomycin (lanes 3 and 4) in the presence or absence of the calpain inhibitor calpeptin for 20 minutes. Control cells were treated with the vehicle DMSO (lane 1) or calpeptin alone (lane 2). Ionomycin treatment of cells resulted in cyclin E and FAK cleavage, whereas inclusion of calpeptin inhibited cleavage. B, calpain activity was inhibited by treating MCF7 cyclin E–overexpressing cells with two different concentrations of calpeptin (lanes 2, 3, 7 and 8) or ALLM (lanes 4, 5, 9 and 10) for 24 (lanes 1-5) or 48 hours (lanes 6-10). Calpeptin was very effective in preventing cyclin E and FAK cleavage, indicating that cyclin E proteolysis in MCF7 cyclin E–overexpressing cells is dependent on calpain activity.
(42). A recent study reported a novel role for FAK that is independent of its kinase activity—the recruitment of calpain into focal adhesions where it might potentially cleave other focal adhesion proteins (43), modifying cell adhesion and migration.

Cells overexpressing high, but not intermediate levels of cyclin E, exhibited proteolysis of pp60src to a 48 kDa form. The interaction between c-src and calpain is complex because studies have shown that c-src is involved in increasing calpain levels (44). Calpain-dependent proteolysis of pp60src to a 48 kDa isoform has been previously described in platelets and the cleavage site was localized to the NH2-terminal region of the molecule. We found a consensus calpain site at amino acids 125 to 135 which would generate a 48 kDa polypeptide. This 48 kDa protein would have a deletion of the myristylation site and part of the SH3 domain, but would retain the SH2 and catalytic domains, suggesting that the kinase activity of this molecule would not be abolished. However, deletion of the myristylation site may alter subcellular localization (23), and deletion of part of the SH3 domain may modify pp60src kinase activity as well as interactions with specific proteins including paxillin (45).

Calpain also cleaves proteins that are instrumental in transcriptional regulation including p53. In vitro studies found that calpain cleaves p53 at the NH2-terminal end to generate a 48 kDa protein. Removal of the NH2-terminal region would promote p53 degradation in a ubiquitin-independent manner, thus diminishing the levels of this tumor suppressor (24). The current study is the first to report the detection of the 48 kDa p53 isoform in vivo. Cells overexpressing high levels of cyclin E expressed only trace amounts of the full-length p53, but the 48 kDa form was readily detectable. However, the total amount of p53 was reduced, supporting the hypothesis that calpain proteolysis promotes p53 degradation. A reduction in the levels of p53 would facilitate the transversal of DNA damage checkpoints, contribute to genome instability, modify apoptotic responses, and hence, promote tumorigenesis.

Several studies have suggested a link between increased calpain activity and metastasis (21, 46, 47). The mechanisms by which calpain may be involved in this process is poorly explored. Using a mouse model of mammary tumor metastasis (30, 33), that in part mimics Her2/neu overexpression, we found that calpain cleavage of FAK and cyclin E is coincident with increased metastatic potential. The metastatic phenotype of the Met cells is dependent on the PyV-mT protein initiating a signaling cascade through an interaction with the PI3K pathway (33). How is this pathway linked to calpain activity? Sonenshein and coworkers found that activation of the PI3K pathway by overexpression of the HER2/neu leads to a calpain-dependent proteolysis of I-catenin and subsequent activation of NFκB-dependent transcription (48). The exact mechanism involved in calpain activation is unknown. Studies have also shown that extracellular signal-regulated kinase, a kinase that is instrumental in growth factor–initiated signal transduction can directly phosphorylate and activate calpain 2 (49). HER2/neu is overexpressed in ~20% to 30% of all breast cancers and, like cyclin E overexpression, is predictive of a poor clinical outcome (50). Therefore, signaling pathways that are perturbed in breast cancer and are associated with a poor clinical outcome may share a common feature—an augmentation of calpain activity.

The expression of calpain and calpastatin in human mammary tumors has been largely unexplored. One study indicates that calpain activity is higher in human breast tumors than in normal mammary tissue (51). Studies of other tumor types have provided evidence that increased calpain expression is associated with tumorigenesis. Calpain 2 expression is elevated in prostate tumors (21) and in colorectal adenocarcinomas (32). Calpain 1 expression is increased in renal cell carcinomas (53). There are no studies on calpastatin expression in human breast tumors, but the expression of calpastatin is decreased in nasopharyngeal carcinomas and in colorectal adenocarcinomas (52, 54). These studies suggest that a deregulation of the calpain/calpastatin equilibrium may have a role in tumor progression, but further studies are needed to clearly define the role of these proteins in mammary tumorigenesis.

In conclusion, this study shows that cyclin E–overexpressing MCF7 cells, human mammary tumors overexpressing cyclin E, and mammary metastatic tumors derived from mouse PyV-mT expressing cells exhibit an increase in calpain-dependent proteolysis. Together, these results suggest that cyclin E–dependent deregulation of calpain 2 may be the pivotal feature that confers a cellular phenotype of aggressive behavior and genomic instability associated with cyclin E–overexpressing tumors.

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