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PRINCIPAL INVESTIGATOR: Mollianne J. McGahren
Khandan Keyomarsi, Ph.D.

CONTRACTING ORGANIZATION: The University of Texas
M.D. Anderson Cancer Center
Houston, TX 77030

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6. AUTHOR(S)
Mollianne J. McGahren
Khandan Keyomarsi, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Texas M.D. Anderson Cancer Center
Houston, TX 77030

E-Mail: mollianne.j.mcgahren@uth.tmc.edu

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13. ABSTRACT (Maximum 200 Words)

Cyclin E is a positive regulator, which controls the transition of the G1 to S phase of the cell cycle. When associated with CDK2, it is responsible for cells passing through the restriction point, which is the barrier between G1 and S. This commits the cell to complete one round of cell division. Previous findings by this laboratory have found that overexpression of cyclin E and the presence of lower molecular weight isoforms (LMW) are found more often in breast tumors and cancer cell lines when compared to normal tissues and cells. Also, tumor cells, but not normal cells have the mechanisms to proteolytically cleave the full length cyclin E into these LMW forms. An altered cyclin E may contribute to the deregulation of the G1 to S checkpoint and lead to tumorigenesis. Our laboratory has also identified through mutational and biochemical analysis, the region of cyclin E that is proteolyticaly cleaved to generate the LMW forms. Critical phosphorylation sites of cyclin E are responsible for the appearance of the LMW forms of cyclin E. To investigate the possible role of phosphorylation in the processing of cyclin E into these lower forms, two approaches have been employed. First, incubation of breast cancer cell line extracts expressing the LMW forms with phosphatases was examined via western blot analysis. Visualization of cyclin E showed downward shifts in both the full length and lower forms in the presence of active dephosphorylation. Second, full length and truncated cyclin E cDNAs were mutated at critical phosphorylation residues via site-directed mutagenesis. Transfection of these mutants into tumor cells capable of LMW form processing followed by western analysis indicates that at least one of the mutations results in the loss of 2 lower forms of cyclin E. However, assays for kinase activity in the transfected tumor cells demonstrate no change activity of the mutants deficient in these phosphorylation sites.

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Introduction:

One of the first steps in the multi-step process of tumorigenesis is the deregulation of the cell cycle, which can cause the cells to replicate uncontrollably. Many cancers have been associated with the abnormal expression of proteins involved in the regulation of the cell cycle. Alterations of cyclin E, a positive regulator of the G1 to S phase transition, have been found in several types of cancer, including breast carcinomas. Furthermore, in breast cancer patients, there is a correlation with overexpression of cyclin E and the lower molecular weight (LMW) forms and poor patient prognosis. Also, tumor cells, but not normal cells have the mechanisms to proteolytically cleave the full length cyclin E into these LMW forms. An altered cyclin E may contribute to the deregulation of the G1 to S checkpoint and lead to tumorigenesis. Our laboratory has also identified through mutational and biochemical analysis, the region of cyclin E that is proteolyticaly cleaved to generate the LMW forms. Critical phosphorylation sites of cyclin E are responsible for the appearance of the LMW forms of cyclin E. Characterizing the processing of the LMW forms and exploring the processing mechanism will address their role if any, in breast cancer tumorigenisis.

Body:

To determine the post-translational changes which give rise to the LMW doublet forms of cyclin E found in breast tumors (figure 1 is a representative example of cycE LMW forms), cyclin E constructs were mutated at key phosphorylation sites and analyzed via western blotting. FLAG-tagged Cyclin E expression plasmids which give rise to the different lengths of cyclin E observed in breast and other tumors were mutated and transfected into a processing-competent cell line, 293T. Site directed mutagenesis was used to alter Thr 395 and Thr 77 [1] for each of the three plasmids; a double T77A/T395A mutant was also

EL1: full length cyclin E

EL2/3: Doublet formed; proteolytic processing in residues 40-45 (45/44kD)

EL4: Formed via alternate start site Met46 (365aa, 40kD)

EL5/6: Doublet formed via proteolytic processing at aa 70 (346aa, 35/33 kD)

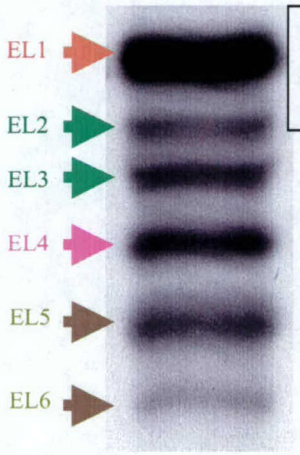

Figure 1. *Cyclin E LMW forms in the breast cancer cell line MDA-MB231.*

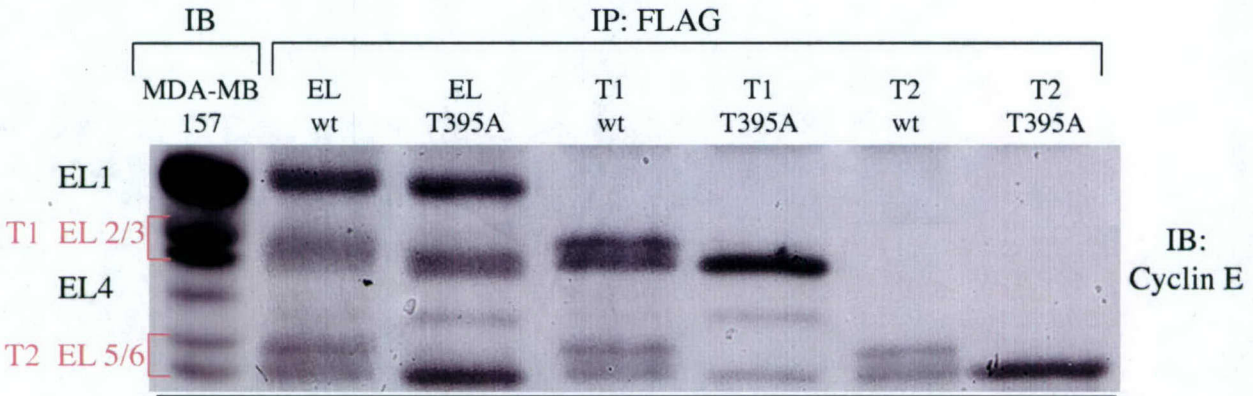

Figure 2. *293T cells transfected with mutated phosphorylation cyclin E sites.*

generated for each construct. Successful mutation reactions were confirmed via direct sequencing. Transfected cells were harvested after 24 hours, and 150 µg of each sample was subjected to immunoprecipitation using anti-FLAG antibody and protein G. The samples were then separated via SDS-PAGE and western blotted. The blot was probed with anti-cyclin E to demonstrate any effects on the appearance of the doublet LMW forms of cyclin E at EL2/3 and EL 5/6. While mutation at T77 had no effect, the T395A mutation demonstrated a loss of

both the EL2 and EL5 forms, leaving only the lower band of each doublet (figure 2). This was also seen in the T77A/T395A double mutant (data not shown). We therefore conclude that phosphorylation, specifically at Thr 395, is responsible for the appearance of the LMW doublets of cyclin E seen in breast tumors.

This data correlates with the was previously shown in our MC□ paper [2], in which it was demonstrated that the LMW forms are generated through a class of enzymes known as elastase. There are two consensus sequences (A66 to P71□AVCADP generates EL 5/6 and K32 to K4□□VFL□DP generates EL2/3) in cyclin E for the elastase class of serine proteases. To demonstrate that cyclin E proteolysis is through elastase, cyclin E

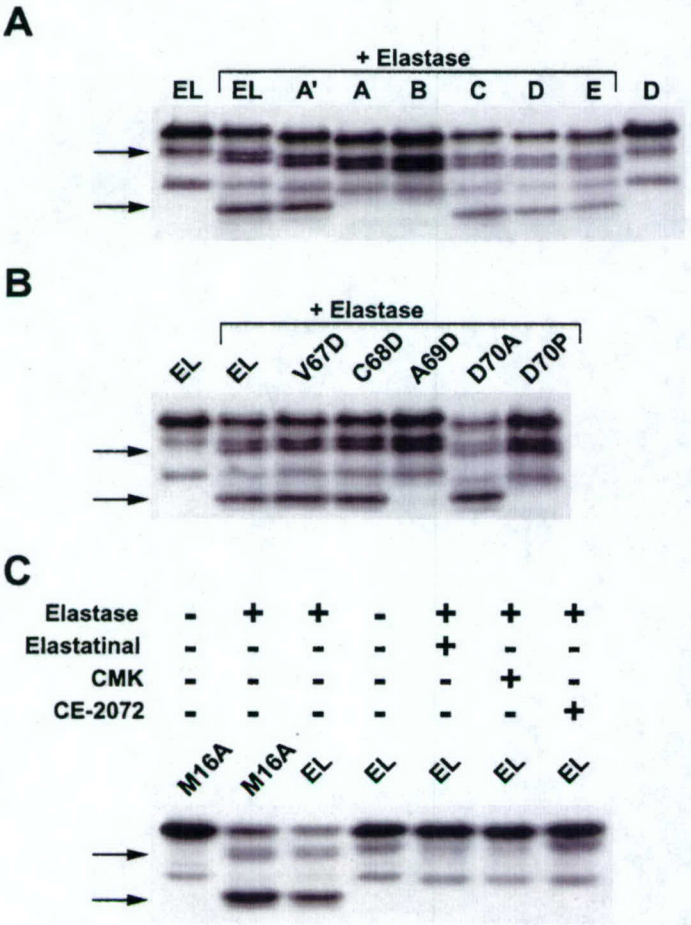

Figure 3. *Elastase digestion of in vitro-translated cyclin E.* (A and B) Plasmids containing full-length cyclin EL and its block deletions and point mutations were used to synthesize cyclin E using the TNT reticulocyte lysate system. After digestion with porcine pancreatic elastase, the proteins were separated by SDS-PAGE and analyzed by Western blotting with a FLAG antibody. (C) M16A and EL TNT protein products were incubated in the presence of no elastase, elastase alone, or elastase plus the indicated elastase inhibitors. CMK, methoxysuccinyl-Ala-Ala-Pro-Val-chloromethylketone; the arrows point to the elastase-mediated cleavage sites at EL3 (top arrow) and EL6 (bottom arrow).

was synthesized through in vitro translation using rabbit reticulocyte lysate and then partially digested with porcine pancreatic elastase (figure 3—originally figure 6 in MCB paper). This figure demonstrates that in vitro proteolysis of cyclin E is similar to the proteolysis of cyclin E using elastase (figure 3 A and B). This resulted in the generation of EL3 and EL6 (figure 3A lanes 1 and 2). Using block deletions, EL6 was completely removed in A and B (figure 3A lanes 4 and 5). However, the other block deletions were incapable of deleting EL6. Figures 3B and 3C demonstrate that elastase preferentially cleaves at two sites and the EL2/3 doublet is distinct from the EL5/6 doublet. Finally, figure 3C demonstrates that the proteolysis is due entirely by elastase through the use of specific elastase inhibitors. The three different inhibitors of elastase used in this study were elastatinol, methoxysuccinyl-Ala-Ala-Pro-Val-chloromethylketone (MPCMK), and α-ketooxadiazole (CE-2072).

Key Research Accomplishments:

- Established phosphatase treatment causes a shift in the wild type and LMW forms of cyclin E
- Demonstrated that mutation of T395->A caused loss of the E2 and E5 forms of cyclin E.
- Verified that the LMW forms of cyclin E are generated through elastase specific proteolysis.

Reportable Outcomes:

Manuscripts in progress

Conclusions:

The lower molecular weight (LWM) isoforms of cyclin are only expressed in tumor cells and such overexpression of these forms is indicative of the stage of the disease. Cyclin E could be used as a strong prognostic indicator of correlation with overexpression of cyclin E and the LMW forms and poor patient prognosis. Furthermore, there is evidence that overexpression of the T2-form of cyclin E results in a decrease in the levels of the full length, wild type form of cyclin E, possibly indicating a role for the LMW forms in further proteolytic cleavage or degradation of the full length cyclin E. Phosphatase treatment of cycE causes a shift in full-length and LMW forms. Mutation of T395 results in loss of doublet LMW forms (E2 and E5), however, mutation of T77 results in no mobility change. The loss of either or both phosphorylation sites has no effect upon kinase activity/substrate binding. Furthermore, it was demonstrated that the origin if these doublets is through elastase specific proteolysis.

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