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TITLE: Functional Analysis of the Beclin-1 Tumor Suppressor Interaction with hVps34 (Type-III PI3'-kinase) in Breast Cancer Cells

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14. ABSTRACT: Macroautophagy is associated with type II programmed cell death. Beclin 1 regulates macroautophagy. Overexpression of Beclin promotes autophagy and inhibits tumorigenesis in breast carcinoma cells, and conversely, heterozygous disruption of the Beclin gene can promote tumorigenesis in mice. In Year-1 we established that Beclin associates with the human type-III phosphatidylinositol 3-kinase (PI3K), hVps34, but not with another putative partner, Bcl-2. The lipid product of Vps34, PI(3)P, is required not only for autophagy, but also for assembly of proteins involved in endocytosis and trafficking of enzymes from the trans-Golgi network to the lysosomes. Our studies indicated that Beclin is required for hVps34 to function in autophagy, but is dispensable for hVps34 to function in endocytosis. In Year-2 we have generated a stable MCF7 breast cancer cell line with expression of FLAG-tagged Beclin under the control of an inducible promoter. Using this cell line, we purified the FLAG-Beclin-Vps34 complex and performed mass spectrometry to identify other protein components present in the complex. We established for the first time that p150, a regulatory subunit of type-III PI3K, associates with Beclin. We generated a Beclin mutant that fails to associate with p150, but remains competent to interact with Vps34. In Year-3 we have generated valuable MCF7 breast cancer cell lines that are essentially deficient in expression of both Beclin-1 and p150. We have also determined that increased autophagy precedes apoptosis in MCF7 cells treated with tamoxifen. Using the Beclin and p150 knockdown cells, we will now extend our studies to determine definitively if autophagy is a protective mechanism or a cause of cell death					
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Table of Contents

Cover.....	
SF298	
Table of Contents.....	
Introduction.....	Pg. 4
Body.....	Pgs. 4-10
Key Research Accomplishments.....	Pg.10-11
Reportable Outcomes.....	Pg. 11-12
Conclusions.....	Pg. 12
References.....	Pg. 12-13

Introduction

The general goal of this study has been to define at the molecular level the function of Beclin-1 in breast cancer. We have shown that the primary intracellular partner for Beclin in human cells is a class-III phosphatidylinositol 3'-kinase termed hVps34¹. We hypothesize that Beclin, through its interaction with hVps34 and perhaps other proteins in a larger complex, plays a key role in regulating macroautophagy. Macroautophagy (hereafter referred to simply as autophagy) is a process whereby cytoplasmic proteins and organelles are incorporated into vacuoles termed autophagosomes, and subsequently are degraded when these structures fuse with lysosomes^{2,3}. Some types of cells use autophagy as a short-term survival strategy in response to stress or nutrient deprivation. However, autophagy has also been described as a hallmark feature of a non-apoptotic form of cell death, often referred to as type-II programmed cell death⁴. Type-II cell death has been reported to occur during the regression of hormone-dependent breast cancer cells treated with tamoxifen and related compounds^{5,6}. However, it remains unclear whether the increased autophagy under these circumstances is a direct cause of cell death or instead represents a survival strategy design to rid the tumor cells of damaged organelles or misfolded proteins^{7,8}. By understanding the molecular details of how Beclin regulates autophagy, and how autophagy relates to cell death in tamoxifen-treated breast cancer cells, we hope to identify components of the autophagy pathway that can be manipulated to sensitize malignant cells to anti-estrogen therapy.

Body

This progress report will follow the outline of the Objectives listed in the Statement of Work described in the original grant proposal. For each objective I will summarize what was done in Years 1 and 2, and provide a more detailed description of the work completed during Year-3. We recently requested, and were granted, permission to continue this project for a fourth year with no additional costs. This should allow sufficient time to complete some of the studies that remain “in progress” due to unanticipated technical problems or changes in personnel.

Objective 1: Test the hypothesis that Beclin-1 acts through Vps34 to mediate autophagic cell death in cultured breast carcinoma cells.

Task 1. Suppression of Beclin-1 expression in MCF7 cells by siRNA-mediated gene silencing and evaluation of the effects of this manipulation on the development of autophagosomes and autophagic cell death in response to tamoxifen or nutritional deprivation. (In Progress)

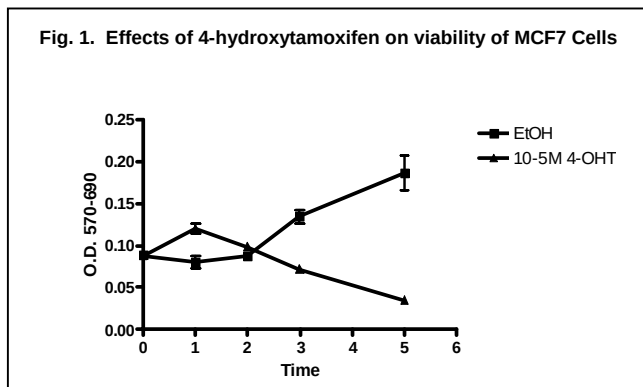
Progress:

Year-1 Summary: In the first year of the project we used siRNA-mediated gene silencing to deplete Beclin in cultured cells and determine whether or not Beclin selectively affects the function of hVps34 in autophagy *versus* normal trafficking. Since we were unable to obtain efficient Beclin knockdown in breast carcinoma cells, we performed the initial mechanistic studies with U251 glioma cells. The results indicated that Beclin is important for regulating the function of hVps34 in autophagy, but that Beclin is dispensable for the function of hVps34 in endocytosis and trafficking of proteins to the lysosomal compartment⁹. In contrast, knocking down the expression of hVps34 caused significant perturbation of trafficking in the late endosome compartment¹⁰.

Year 2 Summary: During the second year of this project we made several attempts to generate stable Beclin knockdown cell lines from MCF7, ZR75-1 and T47D breast carcinoma cells. These studies used retrovirus or AMAXA nucleofection technology to introduce pSuper vectors harboring double-stranded hairpin RNAi constructs into the cells. After selection with puromycin, the MCF7 and T47D cell lines had levels of Beclin that were reduced approximately 60%, making them unsuitable for studies aimed at elucidating the role of

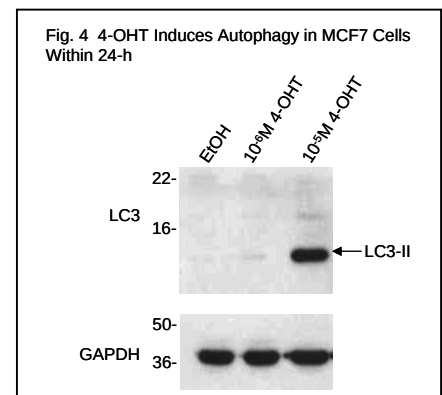
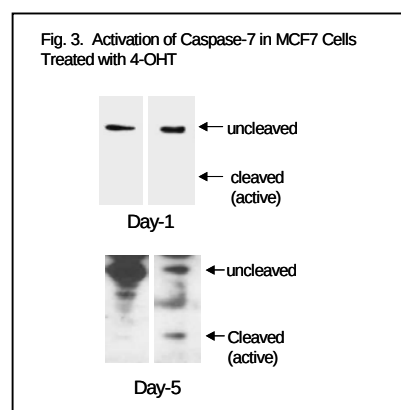
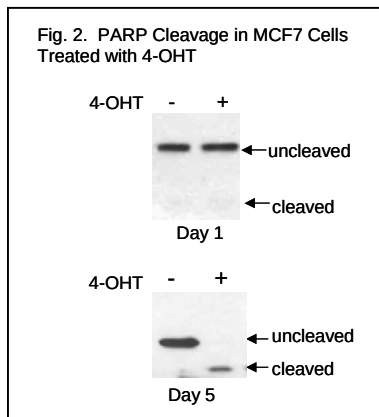
Beclin in controlling autophagy during tamoxifen treatment. We had better results with the ZR-75 cell line, achieving >90% knockdown of Beclin expression.

Year-3 Progress Report: We have devoted considerable effort in Year-3 to characterizing the autophagy and cell death response of MCF7 cells to tamoxifen treatment, in preparation for assessing the effects of Beclin



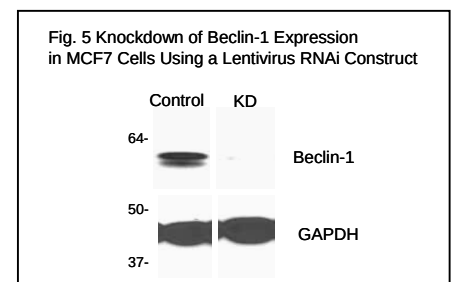
knockdown on these processes. As shown by the MTT cell viability assay in Fig. 1, MCF7 cells treated with 10 μ M 4-hydroxytamoxifen (4-OHT) begin to die after three days of anti-estrogen treatment and show greatly reduced viability compared to matched controls after 5 days. Cell death is accompanied by signs of apoptosis, such as cleavage of the caspase substrate, PARP (Fig.2). MCF7 cells do not contain caspase-3, but the alternative caspase-7 is activated in connection with cell death (Fig. 3). In separate studies we examined the effects of tamoxifen on induction of autophagy, using the widely accepted autophagosome

marker LC3-II¹¹ to monitor autophagosome accumulation in the cells. As shown in Fig. 4, the amount of LC3-II increased dramatically within 24 h after tamoxifen treatment a 10⁻⁵M.



Increased autophagy was noted well before evidence of caspase activation was detected (Fig. 2). This raises the key question: Is the induction of autophagy an attempt by the cell to counteract the effects of tamoxifen and prolong survival, or is it the first step in a series of events that leads to activation of apoptosis pathways?

To address this question we have re-initiated our attempts to generate a Beclin knockdown in MCF7 cells so that we can block autophagy and then see if tamoxifen treatment causes apoptosis earlier than it would in cells with an intact autophagy response. To do this we have taken advantage of a lentivirus delivery vehicle to obtain high-efficiency infection of MCF7 cells with Beclin-targeted RNAi. After screening several different target sequences, we have identified one that appears to yield >90% knockdown of Beclin-1 in MCF7 cells, determined by western blot analysis (Fig. 5). Similar results were obtained with a second Beclin target sequence (not shown). The Control cells were infected with virus containing a non-specific RNAi sequence that does not match any known cellular mRNA sequence. The knockdown and control cells have been selected in medium containing puromycin to eliminate any residual non-infected cells and generate stable MCF7 control and Beclin KD cell lines that can be used to determine if autophagy is a pro-death or pro-survival response. During the no-cost extension period (Year-4) we will also use the lentivirus RNAi approach to generate MCF7 cell lines that are deficient in other autophagy proteins (e.g., Atg7) to further establish the relationship between autophagy and tamoxifen-induced cell death.



Task 2. Over-expression of wild-type Beclin or Vps34-interaction-deficient Beclin (Δ 80-107) and evaluation of the effects of this manipulation on the development of autophagosomes in other estrogen receptor positive (T47D, ZR-75) and negative (MDA-MB231) breast cancer cell lines. (In Progress)

Progress:

Year-1 Summary: We generated a stable MCF7 breast cancer cell line that over-expresses FLAG-tagged wild-type Beclin under control of a tetracycline-responsive promoter (tet-off).

Year-2 Summary: We made use of the inducible MCF7 cell line to isolate proteins associated with FLAG-Beclin (Objective 3). These studies led to the identification of p150 as a component of the Beclin/Vps34 complex (discussed later) by immunoprecipitation and mass spectroscopy.

Year-3 Progress Report. We have conducted pull-down studies of wild-type FLAG Beclin compared with FLAG-Beclin ($\Delta 80-107$), expressed in MCF7 breast cancer cells. The latter studies have demonstrated that the $\Delta 80-107$ mutant form of Beclin fails to interact with the p150 component of the Vps34 complex, but remains competent to interact with the catalytic component of the PI 3-Kinase, Vps34. This finding was unexpected, as we had originally thought the Beclin mutant would fail to associate with Vps34. Based on this observation and the finding that p150 is a subunit of the Beclin/Vps34 complex, we opted to postpone our planned studies with this mutant in favor of learning more about the role of p150 through siRNA knockdown studies.

Task 3. Examination of the effects of Beclin gene-silencing on autophagy in other estrogen receptor positive (T47D, ZR-75) and negative (MDA-MB231) breast cancer cell lines with higher (compared to MCF7) Beclin expression. (In Progress).

Progress:

Year-1 Summary: While the studies on the basic cell biology of Beclin in protein trafficking were being conducted, we continued to experiment with siRNA-mediated suppression of Beclin in estrogen receptor-positive breast cancer cell lines, so that we could come back to the question of how Beclin depletion might affect the response of these cells to anti-estrogens. In T47D cells we were able to obtain a 60-65% suppression of Beclin expression. In ZR-75 breast carcinoma cells, we were able to obtain a 90-95% knockdown of Beclin expression.

Year-2 Summary: A report from Codogno and colleagues¹² suggested that tamoxifen's ability to induce type-II cell death in MCF7 cells is mediated through an accumulation of the intracellular sphingolipid, ceramide, which is known to be a potent inducer of autophagy. Based on these observations, we decided to begin our assessment of the effects of Beclin knockdown by examining the responsiveness of wild-type and Beclin knockdown ZR-75 breast cancer cells to C2-ceramide. As reported in the Year-2 progress report, suppression of Beclin in ZR-75 breast cancer cells blunts the induction of autophagy that normally occurs when these cells take up C2-ceramide. In these studies, autophagy was measured by quantifying the expression of the autophagosome-associated marker protein, LC3-II¹¹. There have been some suggestions that autophagy may be increased as a pro-survival response in cells that incur damage to organelles (e.g., mitochondria), to enable cells to degrade these organelles before they can trigger apoptosis. With this in mind, we hypothesized that ZR-75 cells treated with ceramide, but lacking Beclin necessary to initiate autophagy, might accumulate increased numbers of mitochondria with membrane damage, compared with cells that can mount an autophagic response. This was tested by measuring mitochondrial membrane potential with the dye, JC-1. The results suggested that ceramide caused mitochondrial damage (permeability transition) in ZR-75 cells after 24 h, but there was no significant difference between the Beclin knockdown cells and the controls. This indicates that although ceramide stimulates autophagy, the increased autophagic activity does not play a major role in eliminating mitochondrial damage.

Year-3 Progress Report: We have not conducted any additional studies with the ZR-75 Beclin knockdown cells in year-3. However, we are planning to study these cells alongside the MCF7 Beclin knockdown cells in relation to the effects of Tamoxifen in Year-4. Specifically, we will determine whether the presence of an intact Beclin-mediated autophagy pathway renders the ZR-75 and MCF7 cells more or less sensitive to the effects of tamoxifen on cell viability. Since tamoxifen is known to induce cell death in some types of tumors, independent of its interaction with the estrogen receptor, we plan to extend our studies of tamoxifen-induced autophagy and cell death to estrogen-receptor negative cells such as MDA-MB231. Having successfully generated a lentivirus vector that yields >90% knockdown of Beclin expression in MCF7 cells, we anticipate that the same vector will be useful to suppress Beclin and block autophagy in MDA-231 cells as well.

Objective 2. Define the mechanism of Beclin action by determining whether Beclin regulates the catalytic activity or membrane recruitment of hVps34 PI-3'-kinase (Mostly Completed).

Task 1. Studies of the effects of recombinant Beclin on the activity of hVps34 *in vitro*. (This task is no longer necessary. The goal was achieved by methods described under Task-2)

Progress:

Year –1 Summary: Efforts to produce sufficient quantities of recombinant Beclin in *E. coli* were hampered by the tendency of this protein to form insoluble aggregates when overexpressed in bacterial systems.

Year-2 Summary: In light of the difficulties in purifying recombinant Beclin and hVps34 for Task-1, we explored the alternative of refining our ability to pull-down the FLAG-Beclin/Vps34 complex from stable MCF7 breast cancer cells. This technique succeeded to the point where we were able to address the central question posed under Objective 2 by working with cell extracts instead of recombinant proteins. Since completion of Task-1 in its original form is no longer essential to completing Objective 2, we requested permission to delete Task-1 from the experimental plan in the Year-2 progress report. Instead we proposed to focus attention on the more productive approach of working with the proteins in MCF7 cells (Task-2).

Task 2. Assess the effects of Beclin on the activity of hVps34 in cultured cells (Completed).

Progress:

Year-1 Summary: We generated an inducible (Tet-off) MCF7 breast cancer cell line that expresses a moderate amount of Beclin in the presence of doxycycline (Dox) and a robust over-expression of Beclin when Dox is removed. The parental MCF7 cell line exhibits very low levels of Beclin expression due to monoallelic deletion of the Beclin gene.

Year-2 Summary: We used the Beclin Tet-off cell line and wild-type MCF7 cells to test the hypothesis that Beclin functions as a molecular chaperone that regulates the subcellular partitioning of Vps34 between soluble and membrane compartments. When cells with graded levels of Beclin expression were fractionated into soluble and particulate components, we found no major differences in the distribution of Vps34, despite huge differences in Beclin expression. As indicated under Objective 1, we are continuing to work on obtaining a more complete knockdown of endogenous Beclin in MCF7 cells, so that we can definitively determine if a complete absence of Beclin may have any effect on Vps34 membrane targeting. In the meantime, we have tentatively concluded that Beclin must affect the function of Vps34 in autophagy by some mechanism other than directing its membrane targeting. This left us with two alternative possibilities: 1) Beclin acts as a protein scaffold to help recruit or assemble other essential proteins required to form an active regulatory complex in autophagy, or 2) Beclin interaction with Vps34 modulates the catalytic activity of the PI 3-Kinase.

Year-3 Progress: Studies aimed at determining if the presence or absence of Beclin affects the catalytic activity of the Vps34 complex have been hampered by technical factors. Because of the presence of other classes of PI 3-kinases in mammalian cells, Class-III PI 3-Kinase cannot be assayed in whole cell extracts. The available antibodies against human Vps34 have proven to be very poor as precipitating reagents, delaying our completion of Task-2 (originally scheduled for months 12-16). To remedy the situation, we have tried to transfect cells with vectors encoding Myc-tagged or His6-tagged hVps34, but have not been able to achieve high-efficiency transfections with these constructs. In the meantime, a study published by Backer's group¹³ has essentially answered the question we were trying to address. Specifically, they showed that the amount of Vps34 associated with Beclin-1 does not change when MCF7 cells are subjected to nutrient deprivation, a potent stimulus for autophagy. At the same time, they found that the activity of Vps34 in the Beclin pool decreases substantially. Therefore, it is unlikely that association with Beclin *per se* affects the catalytic activity of Vps34. This has prompted us to concentrate on the alternative possibilities that Beclin-mediated regulation of autophagy is related to the ability of the Beclin to target Vps34 to nascent autophagosomes or to the possible interaction of Beclin/Vps34/p150 with the mTOR complex (discussed under Objective 3).

Objective 3: Elucidation of the nature of the Beclin-Vps34 complex by determining whether the interaction between Beclin and hVps34 is direct or indirect (Tasks 1 & 2), and identifying other proteins that may be part of the complex (Tasks 3-5). In particular, we will test the hypothesis that interaction of Beclin with hVps34 is mediated by p150, a known Vps34 partner (Completed).

Task 1. Complete studies of the physical interaction between recombinant Beclin and hVps34 in vitro (Completed via alternative approach described in Task 3)

Task 2. Assess the effects of recombinant p150 on the interaction between Beclin and hVps34. (Completed via alternative approach described in Task 3)

Year 1 Summary: Size exclusion chromatography analyses of the endogenous cytosolic complexes containing Beclin and Vps34 in MCF7 breast cancer cells and U-251 glioma cells indicated that the protein complexes migrate at a much larger size than predicted by the individual molecular masses of Beclin (60 kDa) and Vps34 (105 kDa). This led us to believe that the regulatory subunit of type-III PI 3-kinase, termed p150, or other unidentified proteins, could be components of the Beclin complex.

Year-2 Summary: Because of the aforementioned difficulty of expressing and purifying recombinant Beclin (Objective-2, Task-1), we decided to approach Objective 3 through the use of the FLAG-Beclin MCF7 cell line, which allows us to isolate intact Beclin complexes under native conditions. Essentially, these are the studies discussed in connection with Tasks 3-5, below.

Task 3. Perform FLAG-Beclin affinity isolation to identify other proteins that may be functional components of the Beclin/hVps34 complex. (Completed)

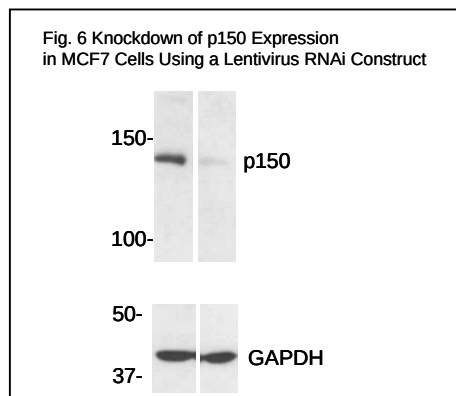
Progress:

Year-1 Summary: In the Year-1 progress report we proposed a minor modification of the experimental approach for Task 3, using FLAG-Beclin immunoaffinity interaction instead of GST-Beclin interaction to capture cellular Beclin interacting proteins and characterize them by mass spectrometry.

Year-2 Summary: We completed Task-3 ahead of schedule. Our approach was to grow large batches of MCF7 cells side-by-side with MCF7 (tet-off) cells expressing low levels of FLAG-Beclin. By keeping the expression levels of FLAG-Beclin low (through the inclusion of low concentration of Doxycycline in the culture medium), we hoped to capture physiologically relevant Beclin protein complexes. Cells were collected and lysed in non-denaturing buffer and mixed with anti-FLAG affinity beads. FLAG-Beclin was eluted from the beads under mild conditions using an excess of FLAG peptide, and the proteins eluted together with FLAG-Beclin were run on an SDS gel. As a control for non-specific protein interactions with the anti-FLAG beads, the lysates from regular MCF7 cells were treated in an identical manner. After staining the parallel SDS-gels with silver stain or colloidal blue, the control and FLAG-Beclin gels were compared side-by-side to identify any unique bands that were present only in the FLAG-Beclin pull-downs. These unique bands were excised and subjected to tryptic digestion. The resulting peptides were separated by liquid chromatography and analyzed by tandem mass spectrometry by our collaborator, Dr. Basrur, in our proteomics core lab. Based on the amino acid sequences, two proteins were identified as specific Beclin partners. The first was the anticipated hVps34. The second was p150¹⁴, the human homolog of Vps15, and a proposed regulatory adapter subunit for Vps34. These interactions were confirmed independently by immunoblot analysis of the proteins co-eluted with FLAG-Beclin from the FLAG affinity beads. This represents the first demonstration that p150 is present in the complex with Beclin and Vps34. When we repeated the pull-down analysis with MCF7 cells expressing a mutant form of FLAG-Beclin(Δ 80-107), we found that the mutant was markedly defective in its interaction with p150, but was still able to pull-down Vps34. This observation favors a tripartite model wherein Beclin makes contact with *both* Vps34 and p150.

Year-3 Progress Report: In fractionation studies of MCF7 cells, we have found that p150 can be detected in FLAG-Beclin complexes isolated from both the soluble and membrane fractions, despite earlier assumptions that p150 (a myristylated protein) is localized exclusively in membranes. As mentioned earlier, studies from other laboratories have recently identified several additional proteins that are associated with the Beclin complex. Almost nothing is known about how these proteins may influence the subcellular localization of

Beclin or its role in autophagy. Hence, we propose to focus on the role of p150 during the Year-4 extension of this project. In preparation for these studies, we devoted significant effort to the task of developing stable MCF7 knockdown cells with markedly reduced expression of p150. As shown in Fig. 6, we were able to



complete this task by using lentiviral RNAi vectors targeted to p150. We are now poised to use these cell lines in Year-4 to determine if p150 is essential for Beclin to promote autophagy in response to 4-OHT. We will also be able to determine if the depletion of p150 from the Beclin complex affects the subcellular localization of Beclin and Vps34.

Task 4. Perform yeast two-hybrid screen with Beclin as the bait to identify other proteins that may be functional components of the Beclin/hVps34 complex. (Unnecessary, due to success of Task-3 and work performed in other labs resulting in the identification of Beclin-interacting proteins)

Progress:

Year-1 Summary: Because of the labor-intensive nature of the yeast two-hybrid approach and the high probability of obtaining false positives, we proposed to defer this task until we evaluated the FLAG-pull down + mass spectrometry approach as a means to identify Beclin partners (Task 3).

Year-2 Summary: Having completed Task-3, we have achieved the goal of identifying key protein components of the Beclin complex.

Year-3 Progress Report: Attempts to identify additional protein components of the Beclin complex by mass spectroscopy were hindered by the departure of our collaborator, Dr. Basrur, the director of our proteomics core lab. A national search for a new director is in progress. In the meantime, several reports have appeared in the literature describing additional components of the Beclin complex, identified by yeast two-hybrid or other methods. The proteins include Bcl-2^{15,16}, UVRAG¹⁷, and the mammalian homolog of yeast Atg14, termed p70 (reported by T. Yoshimori at the recent Keystone Conference on Autophagy in Health and Disease). To date the roles of these proteins in the Beclin complex remain unknown. Therefore, we believe that continuation of studies of the functional importance of p150 will be more likely to yield new insights than repetition of yeast two hybrid screens aimed at identifying additional Beclin interacting proteins.

Task 5. Begin to address the functional significance of any newly identified Beclin interacting proteins for the formation of the Beclin-hVps34 complex and the initiation of autophagy in MCF7 cells. (In Progress).

Progress: Although this task was originally planned for Year-3 of the project, our progress was delayed due to the technical difficulty of developing a p150 knockdown cell line. The difficulties included the lack of a good antibody to monitor the expression of p150 and the task of screening for an appropriate target sequence to obtain good p150 silencing. As described in Fig. 6 above, we have now overcome these obstacles and generated MCF7 p150 knockdown cells. There are two current models for how Beclin may regulate the Vps34 PI 3'-kinase and control autophagy. The first model is based on the concept that Vps34 generates PI(3)P, which is essential for recruitment of regulatory and structural proteins to the autophagosome membrane¹⁸. According to this model, induction of autophagy should be accompanied by an *increase* in the activity of Vps34. In this model, the role of Beclin might be to stimulate the activity of Vps34, at least in the specific compartments involved in autophagosome biogenesis. More recently, a very different alternative model has been proposed by Byfield et al.¹³ who found an unexpected link between Vps34 and regulation of mTOR. In this model *decreasing* the activity of Vps34 triggers autophagy by inhibiting the activity of mTOR. This suggests that Vps34 may normally inhibit autophagy by activating mTOR, and that downregulation rather than stimulation of Vps34 is necessary for increased autophagic activity. In this model, the role of Beclin and/or p150 might be to suppress the activity of Vps34. Preliminary studies conducted during Year-3 suggested that MCF7 breast cancer cells with increased levels of Beclin expression show reduced phosphorylation of p70 S6 kinase by mTOR.

It remains entirely unclear what role, if any, the p150 component of the Beclin/Vps34 complex plays in autophagy triggered by Tamoxifen or other stimuli. Thus the studies with the p150 knockdown cells proposed

for Year-4 are likely to provide new insights into the molecular mechanisms for the regulation of autophagy and cell death in breast cancer cells.

Key Research Accomplishments

Year-1

1. Vps34 PI 3-kinase is an interacting partner for Beclin in human MCF7 and U-251 cells
2. Vps34 co-elutes with Beclin in a broad peak suggestive of a 500-600 kDa complex, indicating that there are other proteins in this complex besides these two proteins.
3. To study the role of Beclin in relation to the function of Vps34, we attempted to suppress the expression of Beclin in MCF7 breast carcinoma cells using siRNA. Although this approach was successful, the extent of Beclin suppression was incomplete. Similar problems were encountered with another breast cancer line (T47D)
4. To facilitate basic cellular studies of Beclin and Vps34, we turned to U-251 glioma cells, where we were able to obtain a much more extreme suppression of Beclin and/or Vps34 expression.
5. In U251-cells the apparent molecular mass of the cytosolic Vps34 complex is reduced to 200-300 kDa when Beclin expression is ablated in the Beclin knockdown cells. This suggests that Beclin is essential for the formation of the cytosolic Vps34 complex.
6. Beclin plays an essential role in Vps34-dependent macroautophagy induced by nutrient deprivation or treatment with C2-ceramide.
7. Knockdown of Vps34 expression demonstrates that this PI 3-kinase plays an important role in maintenance of late endosome morphology and trafficking of proteins to the lysosome.
8. Beclin is not required for Vps34 to function in lysosomal enzyme sorting and endocytic protein trafficking.
9. Beclin's role as a tumor suppressor is most likely related to its specific role in regulating Vps34's function in macroautophagy.
10. A stable ZR-75 breast cancer cell line was established with Beclin expression suppressed by more than 90%. This cell line will be used for future studies to determine whether Beclin plays a role in the induction of autophagic cell death in response to treatment with anti-estrogens.
11. Stable MCF7 breast cancer cell lines expressing FLAG-tagged wild-type and Vps34-binding-deficient forms of Beclin have been established. These will be used to isolate novel Beclin interacting partners that comprise the Vps34 complex.

Year-2

1. In light of the fact that tamoxifen induces type-II cell death in breast cancer cells through an accumulation of the intracellular sphingolipid, ceramide, we evaluated the effects of Beclin knockdown on the responsiveness of ZR-75 breast cancer cells to C2-ceramide. The results showed that suppression of Beclin expression (90%) in ZR-75 cells prevents the induction of autophagy that normally occurs when these cells take up C2-ceramide.
2. JC-1 mitochondrial membrane potential assays showed that elimination of Beclin-mediated autophagy in ZR-75 breast cancer cells did not increase the amount of mitochondrial damage, compared to cells with normal autophagic response.
3. Using Tet-inducible MCF7 cells lines with graded levels of Beclin over-expression, we found that the subcellular distribution of the Beclin partner, Vps34 PI 3'-kinase, does not vary in conjunction with the level of Beclin expression. This suggests that Beclin may affect the function of Vps34 in autophagy by some mechanism other than directing its membrane targeting.
4. Using anti-FLAG affinity beads, we have isolated the Beclin complex from MCF7 cells and have used mass spectrometry to identify two specific proteins that associate with Beclin; hVps34 and the p150 ser/thr kinase. This represents the first identification of p150 as a component of the Beclin complex.
5. Immunoblot methods were used to confirm the identities of the Beclin interacting proteins and to establish that p150 is present in both soluble and membrane-associated Beclin complexes.

6. Using FLAG pull-down assays, we have established that the mutant form of Beclin, (Δ 80-107), fails to associate with p150 but remains capable of interacting with Vps34. This supports a model of a trimeric complex wherein Beclin interacts directly with *both* Vps34 and p150.

Year-3

1. We have obtained evidence suggesting that increasing levels of Beclin expression may stimulate autophagy by inhibiting mTOR and reducing phosphorylation of p70 S6 kinase in MCF7 breast cancer cells. Studies planned for Year-3 with Beclin and p150 knockdown cells will establish if this effect is dependent on p150.
2. We have use lentiviral RNAi vectors to generate stable MCF7 p150 knockdown cell lines.
3. We have used lentiviral RNAi vectors to generate stable MCF7 Beclin knockdown cell lines with >90% suppression of Beclin expression.
4. We have established the timing of autophagy versus apoptosis in MCF7 cells treated with 4 hydroxy-tamoxifen. These studies demonstrate that autophagy (monitored by accumulation of LC3-II) in an early response, preceding caspase-7 activation, PARP cleavage.
5. We have initiated the development of MCF7 cell lines in which another key autophagy protein, Atg7, will be depleted by siRNA-mediated gene silencing.
6. The preceding studies set the stage for key experiments during the no-cost extension period, where we will determine if the Beclin, p150 and Atg7 knockdowns inhibit the autophagic response to 4-OHT. Once this is established, we will be able to use these cell lines to determine definitively if autophagy is a pro-survival or pro-death mechanism in breast cancer.

Reportable Outcomes

Manuscripts Published:

Zeng, X., Overmeyer, J.H., and Maltese, W.A. (2006) Functional specificity of the mammalian Beclin-Vps34 PI 3'-kinase complex in macroautophagy versus endocytosis and lysosomal enzyme sorting. *J. Cell Sci.* **119**, 259-270.

Johnson, E.E. Overmeyer, J. H., Gunning, W.T. and Maltese, W.A. (2006) Specific function of mammalian Vps34 phosphatidylinositol 3-kinase in late *versus* early endosomes revealed by siRNA-mediated gene silencing. *J. Cell Sci.* **119**, 1219-1232.

Presentations:

Xuehuo Zeng and William A. Maltese (2004) Formation of a Beclin complex with Vps34 is required for autophagy but not endocytic trafficking. The American Society for Cell Biology 44th Annual Meeting, Washington, DC.

Johnson, E.E, Gunning, W.T., and Maltese W.A. (2004) Endosomal and lysosomal perturbations caused by siRNA-mediated silencing of Vps34 PI 3'-kinase expression in glioblastoma cells. The American Society for Cell Biology 44th Annual Meeting, Washington, DC.

Johnson, E.E., Gunning, W.T., and Maltese, W.A. (2004) Endosomal and lysosomal perturbations caused by siRNA-mediated silencing of Vps34 PI 3'-kinase expression in glioblastoma cells. Michigan Microscopy and Microanalysis Society Fall Meeting, Michigan State University, Lansing, MI

Maltese, W.A., Zeng, X., Johnson, E.E. and Overmeyer, J.H. (2005) Beclin 1 is required for cellular macroautophagy but not endocytic trafficking. Department of Defense "Era of Hope" Breast Cancer Meeting, Philadelphia, PA.

Koterba, K. and Maltese, W.A. (2007) Regulation of autophagy and cell death in breast carcinoma cells. Case Comprehensive Cancer Center Symposium of Signaling in Cancer, Cleveland, Ohio.

Conclusions

Several lines of evidence support the idea that Beclin is a key component required for the accumulation of autophagosomes observed in conjunction with Type II non-apoptotic cell death. The latter type of cell death is associated with the demise of estrogen receptor positive breast cancer cells treated with tamoxifen or similar compounds. However, it remains unclear whether autophagy is a cellular tactic to prolong survival under adverse conditions, or a direct cause of cell death. The work completed during Year-1 provided important new insights into the basic mechanisms whereby two key proteins, Beclin and hVps34, function in autophagy and late endosomal protein trafficking. Whereas Vps34 plays a dual role in both autophagy and endosomal protein trafficking, our findings support the hypothesis that Beclin functions selectively to regulate hVps34 PI 3-kinase in the autophagic pathway. An alternative role for Beclin as an essential chaperone or adapter for hVps34 in normal vesicular trafficking has been ruled out by our work. These findings are important because they shed new light on the molecular mechanism whereby Beclin may function as a tumor suppressor. The studies completed in Year-2 resulted in the identification of another protein, the p150 serine/threonine kinase, as a component of the Beclin/Vps34 complex. It appears that p150 interacts directly with Beclin in addition to Vps34. In Year-3 we obtained evidence that Beclin and Vps34 may act to control autophagy through effects on the activity of mTOR. We hypothesize that p150 may play a key role in downregulation of mTOR by the Beclin/Vps34 complex, thereby triggering autophagy. If this proves to be true, it will highlight a novel kinase pathway that may be amenable to drug targeting, with the goal of stimulating autophagic Type-II cell death in breast cancers. In Year-3 we have also generated valuable MCF7 breast cancer cell lines that are essentially deficient in expression of both Beclin-1 and p150. These cell lines will enable us to extend our studies to determine definitively if autophagy is a protective mechanism or a cause of cell death in breast cancer cells treated with Tamoxifen. Our basic investigations into the details of how Beclin regulates autophagosome biogenesis may lay the groundwork for eventual manipulations of Beclin-dependent pathways to enhance the response of breast tumor cells to anti-estrogens.

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