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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 plays a pivotal role in type II programmed cell death. Beclin 1 regulates macroautophagy. Over expression of Beclin has been reported to promote autophagy and inhibit 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. 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 generated stable MCF7 breast cancer cells 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 in the complex. We established that p150, a regulatory subunit of type-III PI3K, and associates with Beclin. In Year-3 we generated stable Beclin knockdown cell lines in ZR-75 and MCF7 breast cancer lines. During the extension year we have used these lines to explore the relationship of autophagy to cell death in breast cancer cells treated with tamoxifen and rottlerin. These studies have revealed that Beclin is not required for initiation of autophagic cell death in breast cancer cells and that under some circumstances autophagy can occur independent of Beclin-1. These studies challenge the accepted notion that Beclin is an essential regulator of autophagy in breast cancer.					
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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 proteins⁷⁻⁹. By understanding the molecular details of how Beclin regulates autophagy, and how autophagy relates to cell death in drug-treated breast cancer cells, we hope to assess the feasibility of manipulating the autophagy pathway as a means to sensitize malignant cells to chemotherapy.

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, 2, 3, and the no-cost extension, Year-4.

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.

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 initially unable to obtain efficient Beclin knockdowns in breast carcinoma cells, we performed the first 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 compartment¹⁰. In contrast, knocking down the expression of hVps34 caused significant perturbation of trafficking in the late endosome compartment¹¹.

Year 2 Summary: During the second year of this project we made several attempts to generate Beclin knockdown stable cell lines from MCF7, ZR75-1 and T47D breast carcinoma cells, using AMAXA nucleofection technology to introduce pSuper vectors harboring double-stranded hairpin RNAi constructs into the cells, followed by selection with puromycin. Unfortunately, the MCF7 and T47D cell lines had levels of Beclin that were reduced by only 60-70%, making them unsuitable for studies of the role of Beclin in controlling autophagy in response to tamoxifen. The ZR75-1 cells had a 90% Beclin knockdown.

Year-3 Summary: We devoted considerable effort in Year-3 to characterizing the autophagy and cell death response of MCF7 cells to tamoxifen, in preparation for assessing the effects of Beclin knockdown. As shown by the MTT cell viability assay in Fig. 1, MCF7 cells treated with 10 μ M 4-hydroxytamoxifen (4-OHT) began to die after three days of anti-estrogen treatment and show greatly reduced viability compared to matched controls after 5 days. Cell death was 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-II¹² 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 was started, well before evidence of caspase activation was detected (Fig. 2).

Fig. 1. Effects of 4-hydroxytamoxifen on viability of MCF7 Cells

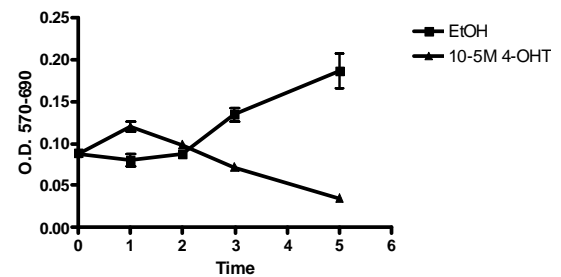


Fig. 2. PARP Cleavage in MCF7 Cells Treated with 4-OHT

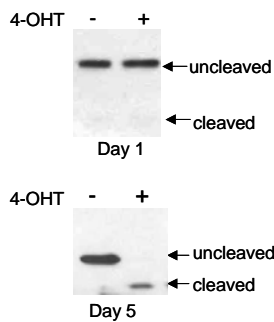


Fig. 3. Activation of Caspase-7 in MCF7 Cells Treated with 4-OHT

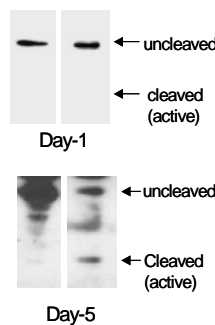
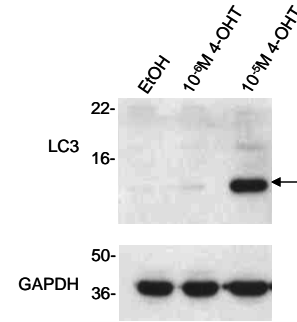


Fig. 4 4-OHT Induces Autophagy in MCF7 Cells Within 24-h



Year-4 Summary: The foregoing studies raised the 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 cell death? To address this question, our goal for Year-4 was to generate breast cancer cell lines that would be incapable of inducing autophagy, so that we could determine if autophagy influences the cell death program in cells exposed to therapeutic agents. Toward this end, we generated new Beclin knockdown lines in ZR75-1 and MCF7 cells. This was accomplished by using a lentivirus vector to obtain high-efficiency infection of the cells with Beclin-targeted RNAi. Using this approach we have successfully generated stable cell lines that exhibit >90% knockdown of Beclin-1, determined by western blot analysis. (Figs. 5) During the no-cost extension period (Year-4) we also used the lentivirus RNAi approach to generate ZR75-1 cell lines that are deficient in other autophagy regulatory proteins, Atg7 and Atg5 (Figs. 6 & 7).

Fig. 5. Western blot showing knockdown of Beclin-1 in ZR-75-1 breast cancer cells Relative to GAPDH (loading control)



Fig. 6. Western blot showing knockdown of Atg-7 in ZR-75-1 breast cancer cells Relative to GAPDH (loading control)

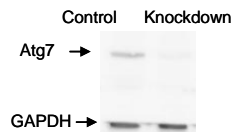
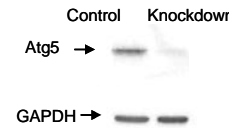


Fig. 7. Western blot showing knockdown of Atg-5 in ZR-75-1 breast cancer cells Relative to GAPDH (loading control)



Task 2. Over-expression of wild-type Beclin or Vps34-interaction-deficient Beclin ($\Delta 80-107$) and evaluation of the effects of this manipulation on the development of autophagosomes in breast cancer cell lines.

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 excellent 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 Summary: We 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 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. This observation, coupled with the finding that p150 is a subunit of the Beclin/Vps34 complex, prompted us to defer our planned studies with this mutant in favor of learning more about the role of p150 through siRNA knockdown studies (see Objective 3)

Task 3. Examination of the effects of Beclin gene-silencing on autophagy in breast cancer cell lines.

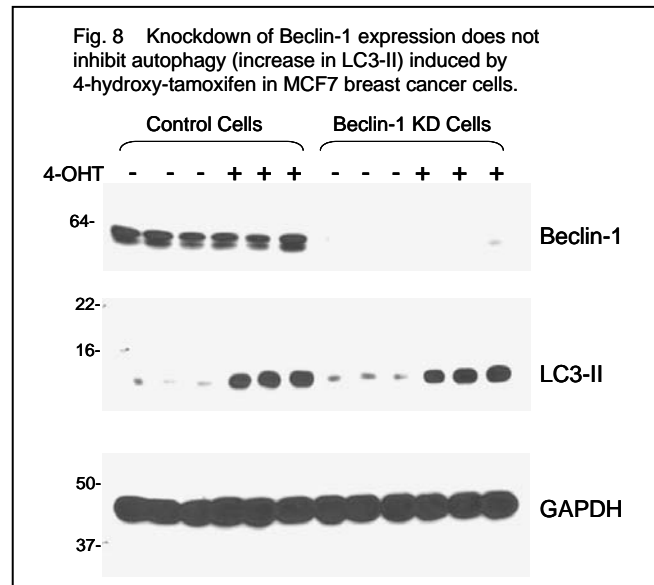
Progress:

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. However, as noted earlier, we had difficulty obtaining MCF7 and T47D breast cancer cell lines with sufficiently high knockdown of Beclin to enable us to initiate these studies. We did, however, obtain >90% knockdown in ZR75-1 cells

Year-2 Summary: A report from Codogno and colleagues¹³ 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-II¹². 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 Summary: We did not conduct any studies with the ZR-75 Beclin knockdown cells in year-3.

Year-4 Summary: In the no cost extension year we completed a series of studies to determine how knockdown of Beclin-1 affects autophagy triggered by tamoxifen treatment in breast cancer cells. Surprisingly, we found that near complete knockdown of Beclin-1 did not suppress tamoxifen-induced autophagy, as measured by the increase in an autophagy-specific marker LC3-II (Fig. 8). This finding suggested that autophagy induced by tamoxifen in breast cancer cells is independent of regulation by Beclin-1. We have recently extended these studies to examine the effects of another drug that is a potent stimulator of autophagy in breast cancer cells (rottlerin, a PKC δ inhibitor), and found the same thing; i.e., autophagy was insensitive to loss of Beclin. In contrast, knockdown of Atg5 blocked the induction of autophagy. We have also found that Beclin- knockdown has no effect on survival of breast cancer cells exposed to rottlerin. These studies are consistent with recent reports that have described non-canonical Beclin-independent pathways for induction of autophagy in MCF7 cells treated with resveratrol¹⁴, and neuronal cells treated with the parkinsonian neurotoxin, MPP⁺¹⁵. We are planning to conduct further studies to investigate the basis for the Beclin-independent autophagy observed in breast cancer cells, but these studies are beyond the scope of the work proposed for this project.



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

Task 1. Studies of the effects of recombinant Beclin on the activity of hVps34 *in vitro*. (Completion of this task was not necessary because the same 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, so 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 was 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 immunoprecipitated proteins isolated from MCF7 cells (Task-2).

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

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. These results suggest that levels of endogenous Beclin in MCF7 cells do not modulate Vps34 membrane targeting. Thus, Beclin must affect the function of Vps34 in autophagy by some mechanism other than directing its membrane targeting. Two alternative possibilities are: 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 Summary: Studies aimed at determining if the presence or absence of Beclin affects the catalytic activity of the Vps34 complex were 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 were very poor as precipitating reagents, delaying our completion of Task-2 (originally scheduled for months 12-16). To remedy the situation, we tried to transfect cells with vectors encoding Myc-tagged or His6-tagged hVps34, but we were unable to achieve high-efficiency transfections. While these studies were in progress, a study published by Backer's group¹⁶ 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 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 the association of Beclin with Vps34 *per se* affects the catalytic activity of Vps34.

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.

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)

Progress:

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.

Progress:

Year-1 Summary: We developed 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 made very significant progress on Task-3 in this year and completed the work 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 collected from 10 large dishes were 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 the 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 p150¹⁷, 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 represented 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 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 Summary: In fractionation studies of MCF7 cells, we 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 below, 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 decided to develop a stable MCF7 knockdown cell line with markedly reduced expression of p150. As shown in Fig. 9 we were able to complete this task by using lentiviral RNAi vectors targeted to p150. These cell lines will be extremely useful in future studies aimed at determining how p150 affects the assembly and function of the Beclin complex.

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 deferred 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 Summary: 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. In the meantime, several reports appeared in the literature describing additional components of the Beclin complex, identified by yeast two-hybrid or other methods. The proteins include Bcl-2^{18,19}, UVRAG²⁰, 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.

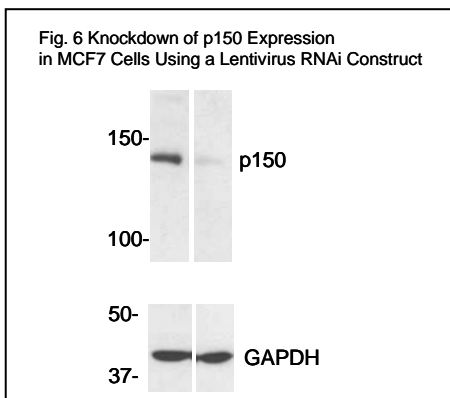
Task 5. Begin to address the functional significance of any newly identified Beclin interacting proteins for the formation of the Beclin-hVps34 complex and the initiation of autophagy in MCF7 cells.

Progress:

Years 1 & 2 Summary: This task was not intended to be addressed in the first two years of the project.

Year 3 Summary: We originally planned to examine the functional significance of p150 for the activity of the Beclin-1/Vps34 complex in Year-3. However, progress was slowed by technical problems with generating good Beclin and p150 knockdown breast cancer cell lines. 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 Figs. 8 & 9, we have now overcome these obstacles and generated MCF7 cells with Beclin and p150 knockdown. 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 membrane²¹. 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.¹⁶ 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.

Year-4 Summary We had originally planned to spend part of year-4 trying to determine what role, if any, the p150 component of the Beclin/Vps34 complex plays in autophagy triggered by Tamoxifen or other stimuli. For



these studies we had planned to use the Beclin and p150 knockdown cells. However, other results obtained during the no-cost extension year-4 (Objective-1, Task 3, Year-4) indicated that the induction of autophagy in breast cancer cells treated with tamoxifen and other drugs does not depend on the Beclin/Vps34 complex. Hence, the molecular details of how Beclin and Vps34 regulate autophagy and mTOR activity during nutrient deprivation seem not to apply to circumstances where cells are treated with therapeutic agents like tamoxifen. In light of these findings, we decided that detailed studies of the role of the Vps34 subunit, p150, would be unlikely to provide new insights into the molecular mechanisms for the regulation of autophagy and cell death in breast cancer cells treated with tamoxifen.

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. A stable ZR-75 breast cancer cell line was established with Beclin expression suppressed by more than 90%.
10. Stable MCF7 breast cancer cell lines expressing FLAG-tagged wild-type and Vps34-binding-deficient forms of Beclin were established.

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 isolated the Beclin complex from MCF7 cells and 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 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 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.
2. We used lentiviral RNAi vectors to generate stable MCF7 p150 knockdown cell lines.
3. We used lentiviral RNAi vectors to generate stable MCF7 Beclin knockdown cell lines with >90% suppression of Beclin expression.
4. We 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.

Year-4 (no cost extension)

1. We developed ZR-75-1 cell lines in which key autophagy proteins, Atg7 and Atg5, are depleted by siRNA-mediated gene silencing.
2. We determined that autophagy induced by tamoxifen or rottlerin treatment is not suppressed in breast cancer cells where Beclin expression is knocked down.
3. Knockdown of Atg5 in rottlerin-treated breast cancer cells *does* inhibit autophagy.
4. Viability of breast cancer cells is equally reduced by rottlerin regardless of whether Beclin is depleted or not.
5. Taken together, the studies from year-4 indicate that drug-induced autophagy in breast cancer cells is regulated independent of regulation by Beclin-1/Vps34.

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.

Koterba, K. and Maltese, W.A. (2008) Rottlerin induces cell death in breast cancer cells through a novel mechanism involving ER stress, disruption of lysosomal pathways and induction of Beclin-independent autophagy. Manuscript in preparation.

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.

Conclusions

Several lines of evidence support the idea that Beclin is a key component required for the accumulation of autophagosomes in Type II non-apoptotic cell death. The latter type of cell death appears to be particularly important for the demise of estrogen receptor positive breast cancer cells treated with tamoxifen or similar compounds. Therefore, our basic investigations into the details of how Beclin regulates autophagosome biogenesis were initiated with the goal of determining if manipulation of Beclin-dependent pathways might be a feasible approach to enhance the response of breast tumor cells to therapeutic agents like tamoxifen. 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 have 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. Our Beclin overexpression and knockdown studies suggest that Beclin does not play a major role in directing the subcellular localization of Vps34. This raises the likely possibility that Beclin affects the activity of Vps34 by directing its interaction with specific downstream molecular targets. In the final two years of this project we used Beclin knockdown cell lines to determine if the Beclin/Vps34 complex plays an essential role in regulating autophagy and cell survival when breast cancer cells are treated with tamoxifen or other agents like rottlerin. Both of these drugs induce a dramatic increase in autophagy in MCF7 and ZR75-1 breast cancer cells. However, in contrast to Beclin/Vps34-dependent autophagy induced by nutrient deprivation of C2-ceramide, the autophagy induced by tamoxifen and rottlerin is not dependent on the presence of Beclin. These findings suggest that Beclin may not be a suitable target for manipulating autophagic activity in conjunction with chemotherapeutic treatment of breast cancer.

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