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14. ABSTRACT Class IA PI3Ks are regarded the most important in regulating cell proliferation and tumorigenesis. The p55y protein is a regulatory subunit of class IA PI3K. In vitro study has demonstrated that the NH2-terminal of p55y is sufficient to bind the cell cycle regulatory protein pRb. Association between calmodulin and p55y in 293T cells has been demonstrated by calmodulin sepharose beads pull-down assay in the previous report. We also demonstrated that p55y stabilized the interaction between calmodulin and Rb. We aim to uncover the cell cycle and growth regulation effect of p55y protein by overexpression and RNA interference analysis. Here we confirmed the co-immunoprecipitation of Rb with p55y protein. However, by overexpression of full-length p55y in MCF-7 cells or knocking down of p55y in AU565 cells, we could not demonstrate a significant effect on the cell cycle or cell growth. Using SK-Br-3 cells as a model for breast cancer cells, we found a shift in composition among the PI3K regulatory subunits once p55y was manipulated. Other PI3K regulatory subunits such as p85α and p50α increased when p55y was knocked down, and p85α decreased when p55y was overexpressed. This could account for the marginal effect we observed when we expressed or down-regulated p55y in breast cancer model systems.					
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Introduction:

Polypeptide growth factors and steroid hormones regulate cell proliferation, cell growth, and cell survival, and are important in both the etiology of breast cancer and as targets of its therapy. Central to the actions of both growth factors and steroids are control of cellular phospholipid metabolism, Ca^{++} metabolism, and protein kinase/phosphatase cascades. Interaction of Ca^{++} metabolism with phospholipids and protein kinases is a complex, understudied area, but also one with promise for new avenues of cancer therapy, since Ca^{++} is required for cell cycle progression.

Phosphatidylinositol 3-kinases (PI3Ks) are lipid kinases that generate second messengers upon receptor tyrosine kinase activation, through phosphorylation of phosphatidylinositols at the 3' position of the inositol ring. These second messengers govern cell morphology in addition to cellular activities including proliferating, survival and motility (Bader et al, 2005). The PI3K family contains three classes with multiple subunits and isoforms. Among the three classes, class IA PI3K proteins are regarded the most important in regulating cell proliferation and tumorigenesis. Class IA catalytic subunits, with p110 α being the most widely studied, are constitutively associated with an adaptor subunit to form a heterodimeric complex (Cantley et al, 2002).

Each of the regulatory subunits of PI3K has three major functions. Each binds and stabilizes the catalytic subunit, induces lipid kinase activity upon insulin stimulation, and confers inhibitory effects on the p110 kinase activity in the basal state (Chen et al, 2004). The p55 γ protein is a regulatory subunit of class IA PI3K, encoded by *pik3r3* gene. When expressed in Chinese Hamster Ovary (CHO) cells, p55 γ showed both basal and insulin-stimulated tyrosine phosphorylation (Pons et al, 1995). Human p55 γ binds IGF1R, IR-1 and IRS-1 upon receptor activation in NIH3T3 cells (Dey et al, 1998). Its N-terminal 30 amino acids are unique among PI3K regulatory subunits. It contains an YXXM sequence, which is a putative SH2-binding motif; this motif may contribute to the specificity of its function (Dey et al, 1998). *In vitro* study showed that NH₂-terminal 24 amino acid of p55 γ is sufficient to bind the cell cycle regulatory protein pRb (Xia et al, 2003). The speculated function of the overexpressed 24 amino acid residue was to serve as a dominant-negative module, blocking the endogenous p55 γ -Rb binding in MCF-7 cells, and leading to cell cycle arrest (Xia et al, 2003). It is worth mentioning that Rb also binds HA-tagged p55 α expressed in monkey kidney COS-7 cells and mouse NIH3T3 cells (Xia et al, 2003), however, the association between Rb and p85 α , or Rb and p50 α was not detected in the same system. So far there is no evidence on the direct interaction between p55 α and CaM, and we are more interested in p55 γ because among the regulatory subunits, p55 γ is encoded by a distinct gene, whereas p55 α is encoded by the same gene as p85 α and p50 α .

The retinoblastoma (*rb*) gene was the first tumor suppressor gene identified by its involvement in hereditary retinoblastoma. Accumulating evidence from *in vitro* and *in vivo* studies have confirmed that its gene product, a 105 KDa protein, is implicated in cellular functions including cell proliferation, differentiation, and apoptosis (Classon et al, 2002). The major function of Rb is as a cell cycle inhibitor; cells undergo arrest in the G1 phase of the cell cycle upon the activation of Rb. Rb directly binds and inhibits the transcriptional activity of E2F family members by recruitment of several chromatin-remodeling complexes to promoter regions, and results in chromatin condensation and transcription inhibition (Harbour et al, 2000). The function of Rb depends on its

phosphorylation status. The hypophosphorylated Rb is the active form, which has growth suppressive activity, and phosphorylated Rb is inactive and unable to bind E2F transcription factors (Goodrich et al, 1991).

p55 γ selectively binds hypophosphorylated Rb in MCF-7 cells. Stronger binding of p55 γ was selectively detected when MCF-7 cells were treated with Heregulin, a differentiation factor, compared to serum-starved, quiescent cells (Xia et al, 2003). Addition of mitogens disrupts the interaction, and the time course of complete dissociation of the p55 γ -Rb complex is consistent with the time course of Rb phosphorylation, suggesting that there is a mechanistic connection between the two events (Xia et al, 2003).

Calmodulin senses the cellular calcium concentration, binds to and then relays the growth factor-dependent signals from epidermal growth factor receptor (Li et al, 2004a) and the Her2/Neu/ErbB2 receptors (Li et al, 2004b). Although calcium-independent calmodulin signaling also exists in the cells, here we will at least emphasize initially the calcium-dependent activation pathway, because calcium/calmodulin plays an important role in regulating cell cycle transitions. In late G1, it functions *via* cyclin D1/cdk4 to hyperphosphorylate Rb, thus releasing the E2F transcription factor (Kahl and Means, 2003). Direct association between Ca⁺⁺/calmodulin and the p85 regulatory subunit of PI3K has been demonstrated by co-immunoprecipitation and affinity chromatography (Jayal et al, 1997). Ca⁺⁺/calmodulin directly interact with SH₂ domains of p85, resulting in activation of p110. The NH₂-terminal and COOH terminal of p55 γ SH₂ domains are 89% and 81% identical with that of p85 α , respectively. However, direct interaction between p55 γ and Ca⁺⁺/calmodulin has been understudied and was not proven so far.

Body:

Overall Hypothesis:

We hypothesize that calmodulin forms a ternary complex with p55 γ and retinoblastoma protein (Rb), such that activated calmodulin modulates Rb phosphorylation and cell cycle progression through p55 γ .

Working Hypothesis 1: P55 γ directly associates with both CaM and Rb, forming a ternary complex.

In order to test the first working hypothesis, I first investigated whether p55 γ overexpressed in human embryonic kidney (HEK) 293 cells indeed binds to calmodulin. The major features of the HEK 293 cell system as compared to previously proposed insect cell system are (i) the human cell expression system which likely expresses p55 γ as its native endogenous form and (ii) well-documented high transfection efficiency and protein expression. The association between p55 γ and calmodulin has been further confirmed in the human breast cancer AU565 cells, using a calmodulin pull-down assay.

The biological mechanisms underlying how p55 γ binds to CaM are important. If we identify specific binding domains, novel therapeutics which inhibits tumor cell progression by blocking interaction of p55 γ and CaM could be developed in treating human breast cancer. Using a computer search, putative CaM binding sites have been identified in the SH₂ domains of p55 γ sequence, which are conserved among p55 γ , p85 α ,

and p85 β (Fig. 2). Furthermore, literature indicated a direct, calcium-dependent binding between SH2 domains of p85 and CaM (Joyal et al, 1997), which enabled us to propose a direct binding between SH2 domains of p55 γ and CaM. Interestingly, the laboratory of Dr. David Sacks used a GST pull-down assay, and confirmed that the major CaM binding site on p85 was located in the C-SH2 domain, while the N-SH2 domain binds with less strength (Joyal et al, 1997). This result was contradictory to the prediction of CaM Target Database, which predicted the strongest binding site to be located at the N-SH2 domain, and the weaker site to be located at the C-SH2 domain. A third binding was predicted in between the p110 binding site and the C-SH2 domain. We are using two different approaches: (i) we have generated synthetic peptides from the predicted N-SH2 domain-binding site to block the CaM-p55 γ interaction in the CaM pull-down assays; (ii) we also generated the plasmids expressing p55 γ deletion mutants with the coding sequences of the three predicted binding sites deleted separately. These mutated constructs will be used in immunoprecipitation experiments to determine the possible calmodulin binding sites on p55 γ .

Methods

1. Construction of plasmids—full-length cDNA encoding human p55 γ was purchased from NIH Mammalian Gene Collection (MGC) (Invitrogen). All full-length and truncated constructs were generated *via* PCR amplification. The forward primer used for generation of N-terminal Flag tagged p55 γ contains the sequence coding for an additional DYKDDDK prior to the start codon of *pik3r3*. The truncated constructs are generated by two-step PCR. The PCR products were cloned into pcDNA3.1/V5-His TOPO TA (Invitrogen). For the constructs expressing a V5-epitope and His-tag, the stop codon at 3' of *pik3r3* is mutated. All constructs were verified by DNA sequencing.

2. Cell culture and transfection--- Human embryonic kidney (HEK) 293T cells were maintained in Dulbecco's modified Eagle's Medium (GIBCO) with 10% heat-inactivated fetal bovine serum. Human breast cancer AU565 cells were maintained in RPMI 1640 (GIBCO) supplemented with 10% heat-inactivated fetal bovine serum. Human breast cancer MCF-7 cells were maintained in IMEM medium (GIBCO) supplemented with 10% heat-inactivated fetal bovine serum. For 293T and MCF-7 cells, DNA transfections were carried out using FuGENE 6 (Roche Applied Science), according to manufacture's specifications. For AU565 and SK-Br-3 cells, nucleofection (Amaxa) was performed with 1.5 μ g of siRNA (Dharmacon).

3. Calmodulin pull-down assay and Immunoblotting---293T cells were transfected with pcDNA3.1-Flag55, or non-tagged p55, or a control vector that expresses an N-terminal Flag tagged protein (pFlagSOX9). Cells were serum-starved for 24 hrs before lysis with proteinase-inhibitor cocktail-supplemented lysis buffer. Fifty μ l of CaM-conjugated sepharose beads (BIOMOL) were incubated with 500 μ l of cell lysate in the presence of or absence of calcium for 2hrs at 4°C, glutathione sepharose beads were used as non-CaM-conjugated control for beads. Beads were boiled in reducing buffer after extensive wash with calcium-containing or calcium-free buffer to elute the bound proteins. The proteins were resolved with 12% NuPAGE gel (Invitrogen) and transferred to a PVDF membrane. The membranes were blotted with anti-flag (gift from Dr. Chen-yong Lin), anti-Rb (Cell Signaling), or anti-actin antibody (Sigma).

4. Immunoprecipitation and Immunoblotting---293T cells were transfected with p55 γ constructs expressing V5 epitope tag. V5 is an epitope tag composed of a 14 residue peptide, GKPIP NPLLGLDST, derived from the P/V proteins of paramyxovirus SV5. Transfection of mutated p55 γ will be used to identify the interacting site of CaM on p55 γ by disrupting the CaM-p55 γ interaction. Cell lysates were prepared as described, and lysates were precleared with protein A-agarose before incubation with anti-V5 antibody or control mouse IgG overnight at 4°C. Then bound proteins were captured with 50 μ l of protein A-agarose for 2hr at 4°C. The protein were resolved, transferred and blotted with anti-CaM, anti-Rb, and anti-p55 γ antibody.

Results

CaM pull-down assay showed that Flag-tagged p55 γ overexpressed in 293T cells interacts with calmodulin, when calcium is added to the reaction, more p55 γ was pulled down by CaM beads, suggesting these proteins interact in a calcium-dependent manner. Compared to the cells expressing p55 γ , CaM pulls down more Rb protein than control Flag-tagged protein-expressing cell lysates, suggesting the formation of the CaM-p55 γ -Rb protein complex. CaM beads do not pull down Flag-tagged control protein, suggesting that the interaction is specific. The control sepharose beads do not bind the Flag-p55 γ , or the Rb (see 2006 annual report).

Anti-V5 immunoprecipitation showed that Rb was detected in the immunocomplex from anti-V5 antibody in p55-V5 expressing 293T cells, which confirmed the results from the other research group (Xia et al., 2003), although some basal level of Rb protein was detected from the control IgG immunoprecipitants (Figure 1.). However, CaM was not detected in the anti-V5 immunoprecipitants (data not shown). Since we couldn't confirm the interaction between wild-type p55V5 and CaM, proposed experiments using the deletion mutations to narrow down the CaM-binding site were not done.

Task 2. Investigate using microscopic live image, if p55 γ binds CaM in the erbB2/HER2 receptor-expressing AU565 and SKBr3 breast cancer cells. (Not done)

We obtained the eYFP and eCFP vectors from our collaborator, however, the supplementary vector map and information required for cloning were not attached, we searched the internet and found these vectors have been removed from the product list. After careful discussion we decided to work on some other aspect of the experiment first. Currently we are studying the localization of p55 γ protein using a GFP-p55 γ construct.

Summary for Hypothesis 1.

We confirmed the p55 γ -Rb-CaM interaction using CaM pull down and p55 γ -Rb interaction in anti-V5 immunoprecipitation. We didn't detect CaM in anti-V5 immunocomplex. It is possible that the interaction between p55 γ and CaM was not strong enough to be detected in our experimental settings, or the His-V5 tag at the C-terminal of p55 γ was masking the CaM-binding site. For future directions we will use synthesized proteins at different ionic strength in a BIACORE study to confirm if there is any direct interaction between p55 γ , CaM and Rb.

Hypothesis 2: Activated calmodulin, by binding p55pik, phosphorylates Rb and causes cell cycle progression.

Task 3. Determine if overexpression of p55 γ results in cell cycle progression in breast cancer cells with activated CaM.

- a. Transfect MCF-7 cells with plasmid pCDNA3.1, containing the cDNA of human p55pik. Select stable expressing cell lines using the G418 selection method. (Months 1-3) ---Completed
- b. Screen positive clones with anti-p55 γ antibodies. (Months 4-5)--- Completed.
- c. Pool the positive clones and synchronize the cells, stimulate cells with growth factors and analyze cell cycle progression profile with flow cytometry (Months 6-12)---Completed
- d. Detect the activation status of Rb in these cell lysates using anti-phospho Rb antibodies (Cell Signaling, Beverly, MA). (Months 13-18)---Completed

Preliminary experiments using HEK293 cells stable expressing N-terminal Flag-tagged p55 γ showed a cell cycle profile distinct from that of HEK293 cells expressing vehicle vector (2006 report). The similar cell cycle promoting effect was also confirmed in His/V5 tagged p55 γ transfected A1N4 cells (2006 report).

Methods

1. Double thymidine blocking to synchronize MCF-7 cells

MCF-7 cells were cultured in standard IMEM+10%FBS to about 40% confluency. Then thymidine was added to a final concentration of 2.5mM in the media. The culture was incubated at 37°C for 12-16hrs. The media was removed and the cells were washed three times with PBS. Fresh media without thymidine was added and incubated for 8-10 hrs at 37°C. Thymidine was added again to a concentration of 2.5mM and the culture was incubated for 12-16 hrs at 37°C. The cells were washed with PBS and incubated with fresh media.

2. Estrogen Deprivation to synchronize MCF-7 cells

MCF7 are ER-positive human breast cancer cells. Phenol-red, a pH indicator in tissue culture media bears a structural resemblance to some nonsteroidal estrogens. Phenol red binds to the estrogen receptor of MCF-7 human breast cancer cells with an affinity 0.001% that of estradiol ($K_d = 2 \times 10^{-5}$ M). It stimulates the proliferation of estrogen receptor-positive MCF-7 breast cancer cells in a dose-dependent manner (Y. Berthois et al., Proc. Natl. Acad. Sci. USA, 83:2496-2500, 1986). In cell culture, serum provides a wide variety of macromolecular proteins, low molecular weight nutrients, carrier proteins for water – insoluble components, and other compounds necessary for in vitro growth of cells, such as hormones and attachment factors. Serum also adds buffering capacity to the medium and binds or neutralizes toxic components. Attempts to replace serum entirely with serum-free medium have met only with limited success. Charcoal-dextran stripping method removes the hormones from the serum.

MCF-7 cells were synchronized by estrogen deprivation method. Briefly, cell monolayers grown to 70% were withdraw from estrogen by changing to an estrogen-depleted medium consisting of phenol red-free modified IMEM supplemented with charcoal stripped calf serum. To remove residual estrogen the monolayer was washed every hour for 5h with phenol red-free IMEM and cultured in fresh medium in between washes. The cells were

then cultured overnight and then trypsinized with phenol red-free trypsin. The cells were incubated with estrogen (E2) at concentrations of 0, 1E-12, 3E-12, 1E-11, 3E-11, 1E-10, 3E-10, 1E-9 M, and then stained with crystal violet at 3, 5, 7 days after E2 addition.

3. Crystal Violet staining to measure the cell proliferation

Cells were washed two times with ice-cold 1X PBS, and then fixed with ice-cold methanol for 10 minutes. Methanol was then aspirated from plates and enough 0.5% crystal violet solution (made in 25% methanol and stored at room temperature) was added. Cells were incubated at room temperature for 10 minutes. Plates were then rinsed in ddH₂O until color no longer coming off in rinse. Plates were allowed to dry at room temperature and absorbance measured at OD 550nm.

Results

To confirm the pooled positive clone expression, MCF-7 cells as well as HEK293 cells stable expressing p55 γ protein were validated by western blot analysis (Figure 2). To determine the effect of Flag-tagged p55 γ overexpression on cell cycle progression, we synchronized the MCF-7 stable cells by double thymidine blocking method. Upon releasing from the arrest, p55 γ -expressing MCF-7 cells went through the S phase faster than the control vector expressing cells. This effect was manifested by monitoring the distance between the peak of G0/G1 phase and the peak of the S phase in the DNA distribution histograms (Figure 3).

Since a previous study showed that upon estrogen stimulation, mRNA level of p55 γ is 21-fold higher in ER-positive MCF-7 than in ER-negative MDA-MB-231 cells, indicating that p55 γ expression might be involved with estrogen response of breast cancer cells (Kuang et al., 1998). To synchronize the MCF-7 cells and to investigate whether p55 γ overexpression could promote cell growth in case of estrogen deprivation, estrogen (phenol red) were stripped from MCF-7 cells stable expressing p55 γ or control vector, and the synchronized cells were stimulated with estrogen at increasing concentrations. The result showed no significant difference between the p55 γ overexpressing cells and the control cells (Figure 4). It indicates that overexpression of p55 γ protein neither increased the cells' tolerance for estrogen deprivation, nor provided the cells with a significant growth advantage upon estrogen stimulation.

Task 4. Determine if knockdown of p55 γ protein expression by RNA interference leads to cell cycle arrest in breast cancer cells with activated CaM.

a. Pilot study for the optimal transfection efficiency of four individual siRNAs against p55 γ using real-time PCR and anti-Np55 γ antibodies. (Months 1-6)---Completed

b. Transfect the selected siRNAs into AU565 cells and analyzes the interference of p55 γ protein expression, Rb phosphorylation status, and the apoptosis status of the cells. (Months 7-24)---Completed

Knockdown protein expression using small interfering RNA ---Four pairs of small interfering RNAs directed against *pik3r3* were designed and synthesized at Dharmacon (Chicago, IL). AU565 breast cancer cells expressing endogenous p55 γ were transfected with 1.5 μ g of siRNA against *pik3r3*. Non-targeting siRNA was transfected as control.

Seventy-two hrs post-transfection, cells were lysed and the lysates were resolved, transferred and blotted with anti- p55 γ or anti-actin. For future studies, the apoptosis status of these cells will be detected using Apo-ONE Homogeneous Caspase-3/7 Assay (Promega Corporation, Madison, WI), and PARP cleavage assay (Calbiochem).

Preliminary study showed that p55 γ protein expression was successfully knocked down with the proper siRNA combination in AU565 cells (report 2006). In a previous study of mammary involution using an immortalized mammary epithelial cell line (KIM-2), when p55 α and p50 α was overexpressed, Akt phosphorylation was down-regulated (Abell et al, 2005). The inhibitory function of these PI3K regulatory subunits seems to contribute to this effect. Therefore, an inhibitory effect of p55 γ on Akt activation is expected at basal level. No data are currently published concerning the Rb mutation status in AU565 cells, so we presume that functional Rb exists in AU565 cells. During serum starvation, p55 γ binds to hypophosphorylated Rb in quiescent cells; we hypothesize that upon growth factor stimulation, p55 γ facilitates the hyperphosphorylation of Rb by Ca⁺⁺/CaM activation, most probably through Cyclin D/cdk4. If this hypothesis is proven, when p55 γ is down regulated, Rb phosphorylation should be down regulated. In a preliminary experiment, we did not observe a significant decrease in phosphoRb signal compare to total Rb at 20 minutes time point after serum stimulation (Data not shown), possibly because we missed the time point for detection of Rb phosphorylation. We now conduct careful time course experiments post stimulation.

Cell cycle analysis by flow cytometry---MCF-7 cells transfected with p55 γ siRNA will be synchronized and subjected to flow cytometry for cell cycle profile analysis. Cells are grown on 60mm dishes with growth medium and synchronized by double thymidine blocking, cells are released by addition of fresh complete medium and harvested with trypsin-EDTA. After one to ten hours of stimulation, cells are washed in PBS and pelleted. After removing the wash buffer, the pellets are vortexed and resuspended in 0.1 ml of citrate/DMSO buffer (250 mM sucrose, 40 mM Na₃C₆H₅O₇ 2H₂O, 5% DMSO, pH 7.60). The pellets are then sent to cell cycle analysis by flow cytometry.

Results

Transfection of siRNA against p55 γ in AU565 cells was repeated. Cells were harvested at different time points after growth factor stimulation. At different time points after stimulation, I couldn't detect a decrease in phosphorylation of Rb in p55 γ knockdown cells, nor did the percentage of cells in S phase cell cycle change (data not shown). Since no significant apoptotic event was observed in cell culture, no further apoptotic assay was conducted.

We investigated the relative expression level of endogenous p55 γ in a serial of breast cancer cell lines by western blot analysis (Figure 5). The endogenous p55 γ in MCF-7 cells was a lot lower than in AU565 cells, and was undetectable with our specific antibody (Figure 5A), which made it difficult to monitor the efficiency of siRNA knockdown in MCF-7 cells using western blot. We also found from the cell line screening data (Figure 5B) that SK-Br-3 cells contain higher endogenous p55 γ protein level than AU565 cells, although they were both originated from the same patient. We used SK-Br-3 cells in the following siRNA experiments for more significant knockdown efficiency.

Summary for hypothesis 2:

Using flow cytometry-based cell cycle analysis, XTT based proliferation assay and western blot analysis; we confirmed that within the detection range of our system, we could not detect any significant effect of p55 γ overexpression or knockdown in human breast cancer cells.

Since this result deviated from our previous hypothesis, we went on to investigate why this would occur. In non-stimulated SK-Br-3 cells we found that when p55 γ is down-regulated, the other PI3-kinase regulatory subunits such as p85 α and p50 α are up-regulated by 25~30% and ≥ 10 times, respectively (Figure 6a). This could have off-set p55 γ protein's effect, leaving one of the PI-3kinase downstream target, a cyclin-dependent kinase inhibitor p27^{pik}, unchanged at protein level (Figure 6a). Meanwhile, we detected an increase of more than 50% in phosphorylated Akt after p55 γ knockdown, in accordance with the surge in other regulatory subunits and p55 γ 's inhibitory effect on PI3K (Figure 6b). On the other hand, we also found that when p55 γ was overexpressed, p85 α protein level was noticeably decreased (Figure 7). These results suggest that when p55 γ was manipulated in breast cancer cells, the molecular balance among PI3K regulatory subunits were shifted, and the compensatory up-regulation or down-regulation of other regulatory subunits, especially the more potent p85 α protein has made the effect of p55 γ undetectable.

Key research accomplishments:

- The composition shift between PI3-kinase regulatory subunits, especially between p85 α and p55 γ was confirmed in breast cancer cell line, which could account for the marginal effect of p55 γ overexpression or knockdown on cell cycle profile and cell proliferation.

Reportable Outcomes:

- Poster presentation at The 46th Annual Meeting of the American Society for Cell Biology at San Diego, CA
- Poster presentation at 9th Annual Lombardi Research Fair at Georgetown University Lombardi Comprehensive Cancer Center

Conclusion and Discussion:

We initially proposed that by serving as a coordinate protein between CaM and Rb, the overexpression or knockdown of p55 γ would influence the cell cycle progression and cell proliferation because our preliminary data suggested that CaM binds to p55 γ but not to p85 α , and the literature also stated that p55 γ binds to Rb with its amino-terminal portion. However, the results from our detection system did not find sufficient evidence to fully support this hypothesis. With further investigation on breast cancer SK-Br-3 cells, we concluded that the composition shift among regulatory subunits, especially that between

p55 γ and p85 α , might be the major reason we couldn't detect a significant effect of p55 γ overexpression or knockdown, since p85 α is a potent PI3K subunit that activates downstream cell cycle and cell proliferation target proteins. Although p85 α does not interact directly with Rb, it could change the phosphorylation status of Rb through other PI3K targets, such as Akt/PKB.

We learnt valuable information on the composition shift for the PI-3kinase regulatory subunits in knockout mice and embryonic stem cells. The gene products of *pik3r1* (p85 α , p55 α , and p50 α) and *pik3r2* (p85 β) are the predominant isoforms in insulin-sensitive tissues. The tissue extracted from *Pik3r1*^{-/-} mice shows 70% reduction of regulatory activity; *Pik3r1*^{+/-} shows 40% reduction and *Pik3r2*^{-/-} shows 25% reduction (Ueki et al., 2003). These data suggests that when the p55 γ encoding gene *pik3r3* was knocked down, the reduction in regulatory activity upon insulin stimulation will probably be minor and insignificant. The effect of knocking down p85 α , p55 α and p50 α has been documented in *Pik3r1*^{-/-} mice, it was shown that p85 β increases in the liver and muscle, and the PI3K activity associated with p55 γ was increased by four-to five folds in liver tissues and slightly increased in muscle homogenates (Fruman DA et al., 2000). In complementary to these findings, in p55 α ^{-/-}, p50 α ^{-/-} mice the levels of p85 α associated with IRS-2 were enhanced; insulin-stimulated Akt phosphorylation was enhanced in muscle homogenates, and Akt kinase activity was increased by 40% (Chen D et al., 2004).

So far there has been no report on a p55 γ ^{-/-} mice, one possibility is that there is no reportable significant phenotype manifested by these mice, the reason is as we have discussed previously, the p55 γ knockdown effect has been compensated by overexpression of other regulatory subunits. On the other hand, the other reason for the unavailability of the p55 γ ^{-/-} mice might be that since p55 γ was highly expressed in the fetal brain and testis tissue, it is essential for the mice in embryonic development so that knocking out of this protein has made the animal unviable.

Ongoing experiments:

A p55 γ construct with an EGFP coding sequence upstream of p55 γ was generated. This construct was transiently transfected into HEK293 cells and the localization of the fluorescent protein was detected under an immunofluorescent microscope. My preliminary results showed that EGFP-fusion p55 γ is undergoing foci formation upon growth factor stimulation (data not shown), similar to those published for EGFP-p85 α (Luo, et al., 2005). So far there is no report on the localization of p55 γ after the stimulation of growth factor.

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Appendices:

Abstract presented during this report period

1. Youhong Wang, Tushar Deb, Michael Johnson. Title: Phosphatidylinositol-3 Kinase Regulatory Subunit P55 γ Interacts with Rb and Regulates Cell Cycle. The 46th Annual Meeting of the American Society for Cell Biology. Place: San Diego, CA, Date: December 9-13, 2006. Category: A02 Signal Transduction.
2. Youhong Wang, Tushar Deb, Michael Johnson. Title: Phosphatidylinositol-3 Kinase Regulatory Subunit P55 γ Interacts with Rb and Regulates Cell Cycle. The 9th Annual Lombardi Research Fair, Place: Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington, DC. Date: February 20-23, 2007

Supporting Data:

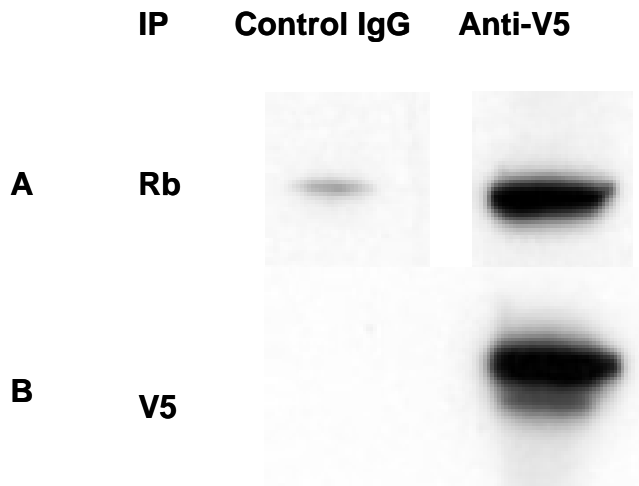


Figure 1. Rb co-precipitates with V5-tagged p55 γ in transiently transfected 293T cells. Lysates from transfected 293T cells were immunoprecipitated with goat anti-V5 antibody (Bethyl) or control IgG, the protein complex were detected with (A) mouse anti-Rb (Cell Signaling) and (B) mouse anti-V5 antibody (Invitrogen).

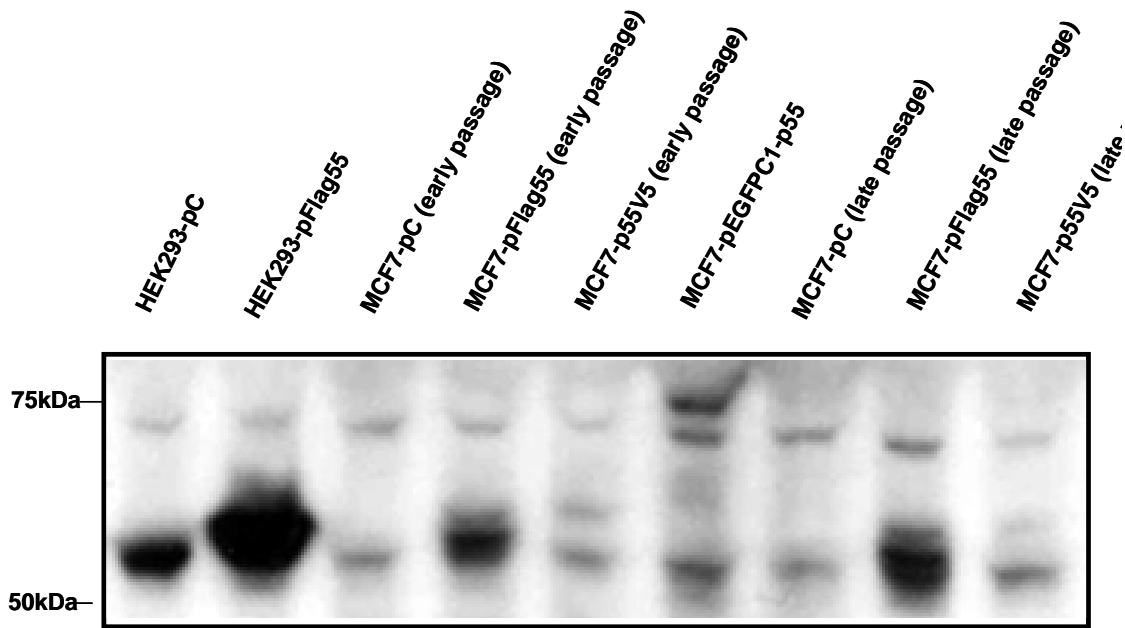


Figure 2. Verification of p55 γ expression in HEK293 and MCF-7 stable cell lines. Whole cell lysates from the stables were analyzed by western blot (90 μ g/lane). IB: anti-p55 γ .

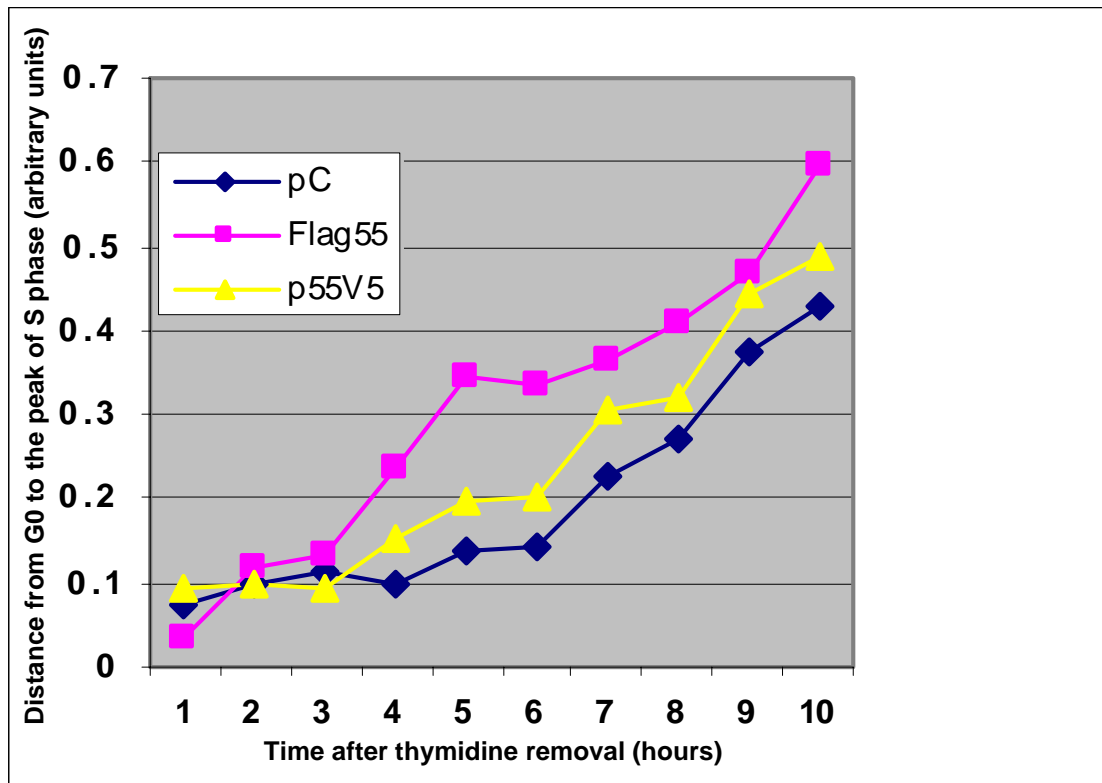


Figure 3. MCF-7 cells stable expressing p55 γ are going through the S phase

Faster than control cells. MCF-7 stables were synchronized by double thymidine blocking method and released by thymidine removal. At each hour after thymidine removal, the cells were subjected to cell cycle analysis and the distance between G0/G1 peak and the S peak was recorded.

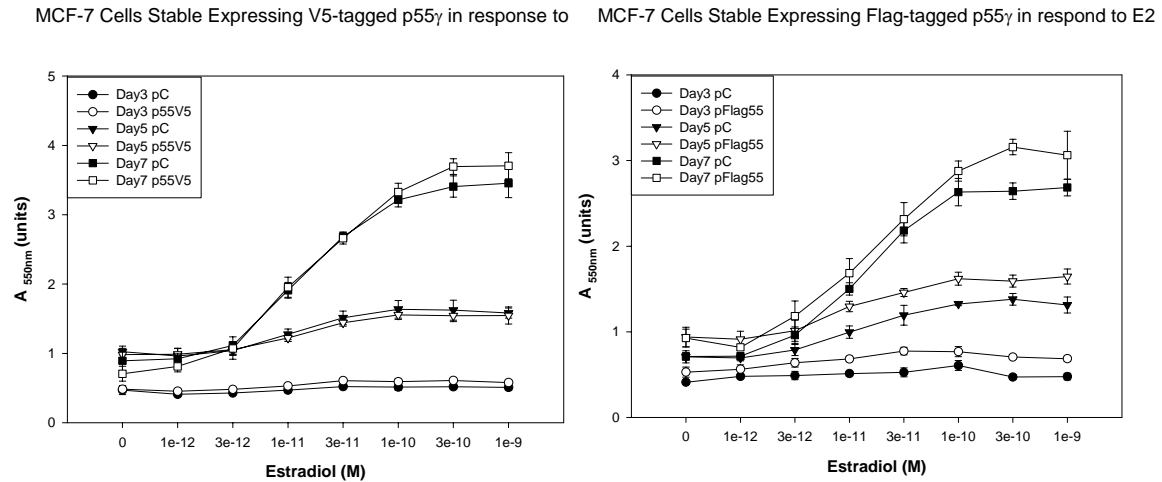


Figure 4. p55 γ overexpression showed no significant growth advantage for MCF-7 cells upon estrogen deprivation. MCF-7 cells stable expressing p55 γ were synchronized by estrogen deprivation and stimulated with increasing amounts of estrogen, cells were stained with crystal violet at 3,5,7 days after estrogen addition and OD550nm absorbance was plotted as shown.

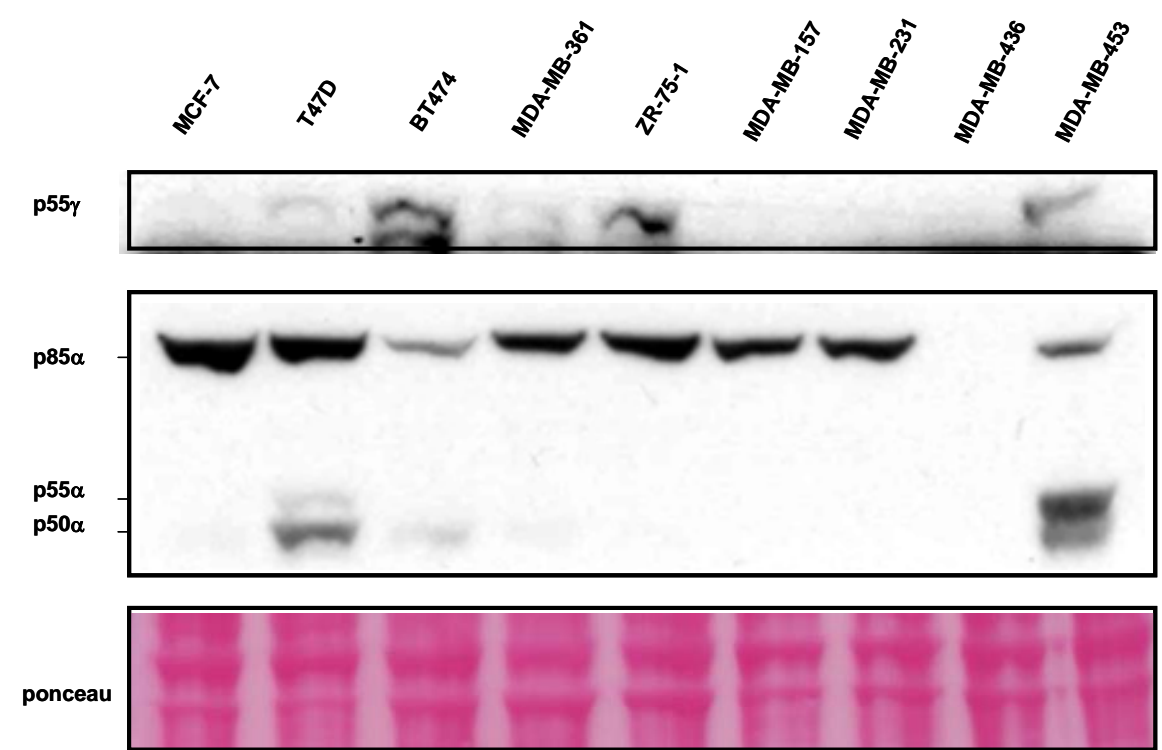


Figure 5A. Screening of a serial of human breast cancer cell lines. Sixty micrograms of whole cell lysates were resolved and blotted with anti-p55 γ (top panel), anti-p85 α (middle panel, recognize p85 α , p55 α and p50 α), and stained with ponceu for protein loading control (bottom panel).

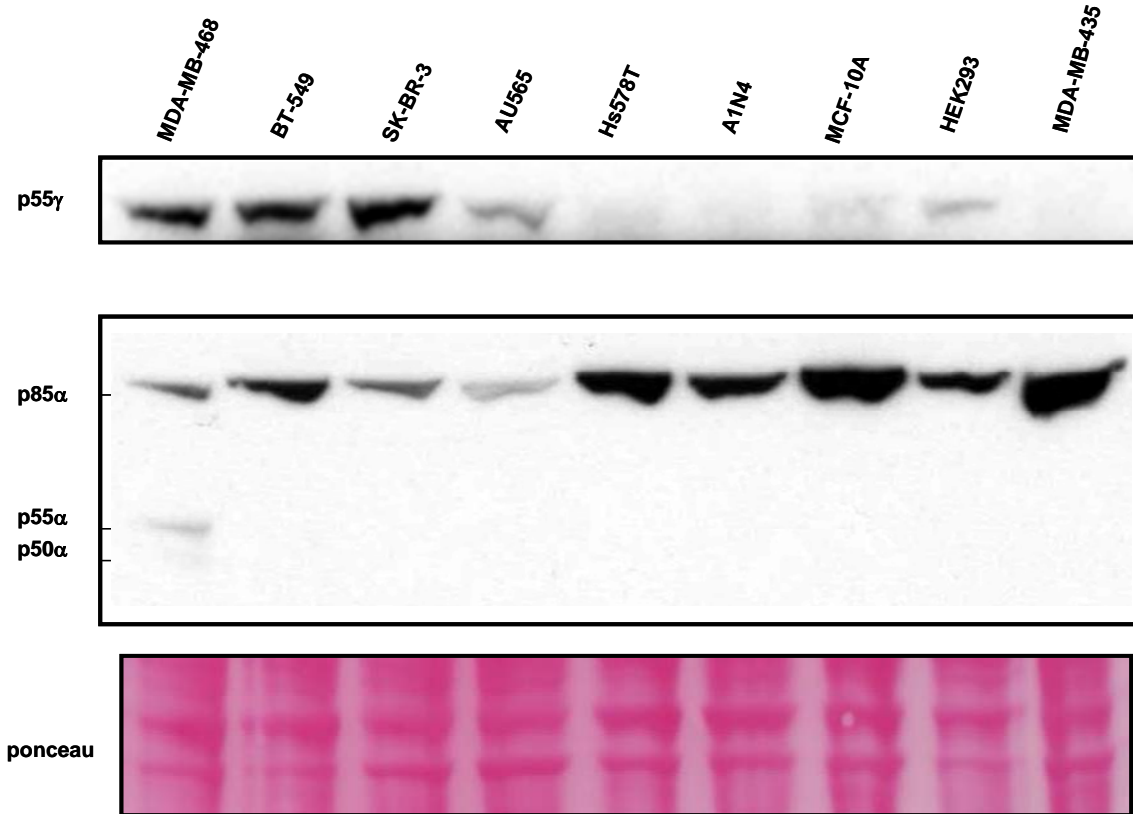


Figure 5B. Screening of a serial of human breast cancer cell lines. Sixty micrograms of whole cell lysates were resolved and blotted with anti-p55 γ (top panel), anti-p85 α (middle panel, recognize p85 α , p55 α and p50 α), and stained with ponceu for protein loading control (bottom panel).

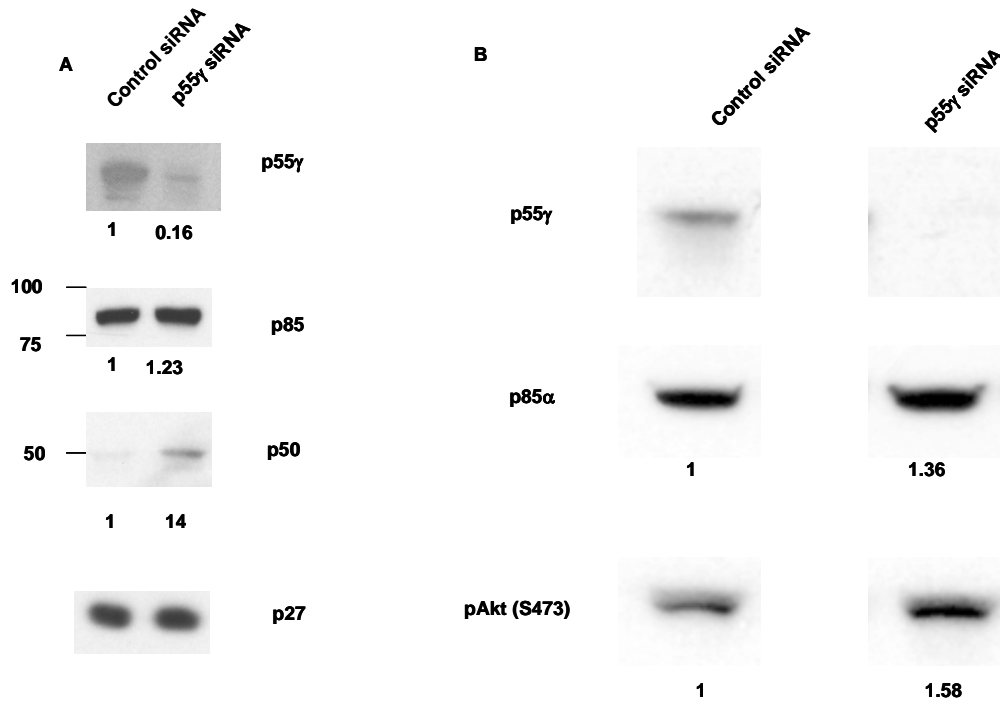


Figure 6. Knocking down p55 γ in SK-Br-3 cells results in increased p85 α , p50 α , and increased phosphorylated Akt (S473), the accumulating effect on p27 was not significant. SK-Br-3 cells were transfected with siRNA against p55 γ or control siRNA, Seventy-two hours after transfection, the cell lysates were assayed for p55 γ (A. and B. top panel) for efficiency of RNA interference. The membrane were also blotted for p85 α (A. middle two panels; B. middle panel), and anti-p27 (A. bottom panel), or anti-pAkt at Ser473 (B. bottom panel). The density of each band was evaluated by densitometry and the calculated numbers were presented below each band. Each blot was the representative of three repeated experiments.

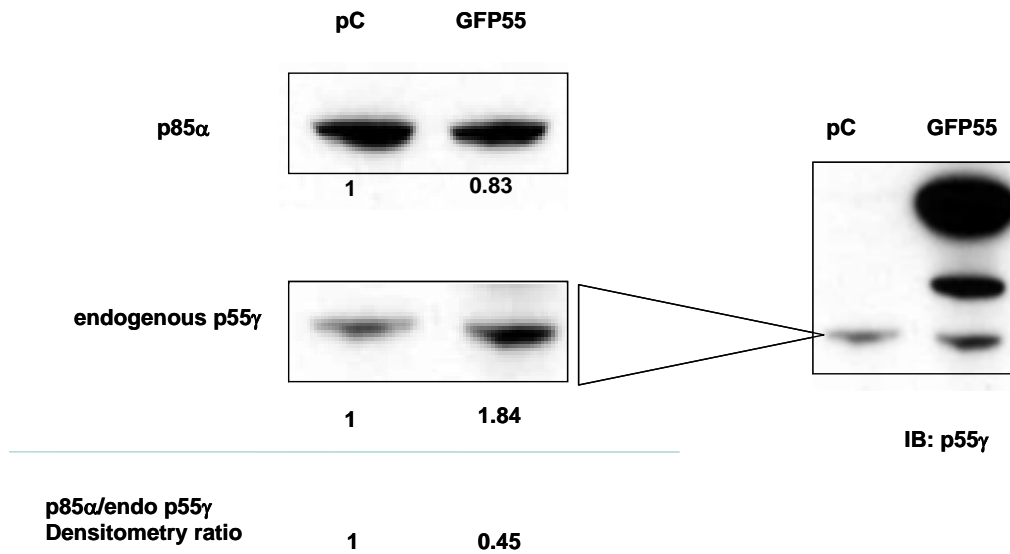


Figure 7. p85 α protein decreased in SK-Br-3 cells overexpressing p55 γ . SK-Br-3 cells were transfected with the construct expressing GFP fusion p55 γ (GFP55) or control vector pcDNA 3.1 (pC), and lysates were assayed 48 hours post transfection. The membrane were blotted with anti-p85 α (top left panel), and p55 γ (right panel). The increase in p85 α was shown as the density of each p85 band normalized by the density of the endogenous p55 γ protein.