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14. ABSTRACT This project focuses on the novel finding from our group that the serine-threonine kinase Akt1 partitions into specialized membrane microdomains, termed lipid rafts, and that this localization event strongly influences the nature of Akt1 signaling. Lipid rafts are cholesterol-enriched membrane microdomains that serve as signal transduction platforms by sequestering and excluding signaling proteins and by harboring multi-protein complexes. Evidence was presented in the original proposal that in prostate cancer cells critical cell survival cues are processed via lipid rafts, which are dependent on cholesterol for signal transduction. This is a significant finding because the Akt1 kinase is a central signaling protein that is frequently activated in prostate cancer. I have hypothesized in this project that cholesterol accumulation in prostate cancer cells may promote oncogenesis by altering the nature of Akt1 signals that flow through lipid raft microdomains. The purpose of this project is to identify the mechanism of Akt1 recruitment to cholesterol-rich microdomains and to explore the biological consequences for regulation of this important kinase. Several new lines of evidence consistent with my hypothesis have been produced in year 1 and are described and summarized in this report.					
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“Membrane Heterogeneity in Akt Activation in Prostate Cancer”

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Progress report for 02/15/2007 - 02/14/2008

INTRODUCTION

This project focuses on the novel finding from our group that the serine-threonine kinase Akt1 partitions into specialized membrane microdomains, termed lipid rafts, and that this localization event strongly influences the nature of Akt1 signaling. Lipid rafts are cholesterol- and sphingolipid-enriched membrane microdomains that serve as signal transduction platforms by sequestering and excluding signaling proteins and by harboring pre-formed multi-protein complexes. Evidence was presented in the original proposal that in prostate cancer cells critical cell survival cues are processed via lipid rafts, which are dependent on cholesterol for signal transduction. This is a significant finding because the Akt1 kinase is a central signaling protein that is frequently activated in prostate cancer. I have hypothesized in this project that cholesterol accumulation in prostate cancer cells may promote oncogenesis by altering the nature of Akt1 signals that flow through lipid raft microdomains. The purpose of this project is to identify the mechanism of Akt1 recruitment to cholesterol-rich microdomains and to explore the biological consequences for regulation of this important kinase.

BODY

In year 1, I have made significant progress on Tasks 1 and 2 and on preliminary studies for Task 3.

Task 1 and 2. Determine the unique functional consequences of Akt recruitment to lipid rafts for signaling processes in prostate cancer cells.

I have now completed Task 1. i) and Task 2. i) and tested the lipid raft distribution of the already available Akt-T308D/S473D version (construct #1), which represents activated Akt1 that resides in the cytoplasm. I included other Akt1 mutants in this study to see if a combination of various mutations would have any impact on the lipid raft distribution.

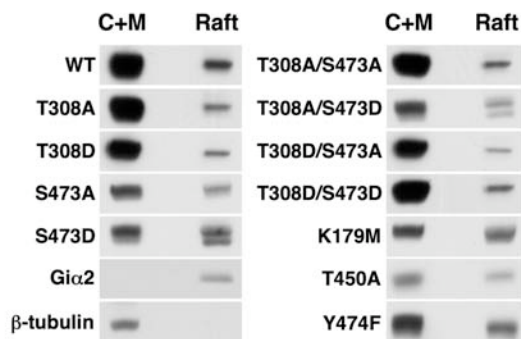


Figure 1: Lipid rafts contain a population of Akt1 kinases. Comparison of cytoplasmic/non-raft membrane fractions (C+M) and raft fractions after differential extraction reveals that partitioning of Akt1 into the lipid raft compartment is not influenced by mutating the 2 major regulatory sites of Akt1- threonine 308 and serine 473. Note that generation of a phosphomimetic at position 473 alone leads to increased accumulation of Akt1 in the lipid raft compartment. Interestingly, the kinase-dead mutant of Akt1 (K179M) is predominantly present in lipid rafts.

As seen in Fig. 1, all mutants including the constitutively active Akt-T308D/S473D were successfully expressed in HEK293 cells. The specific subcellular localization of each mutant was verified by differential extraction with Triton X-100 and octylglucoside detergents and revealed that all Akt1 mutants partitioned into lipid raft microdomains as demonstrated by the prevalence of G-protein subunit α -2 and the absence of the non-raft marker β -tubulin. The phosphomimetic double mutant Akt-T308D/S473D does not show a lipid raft distribution that is different from the wild type and will consequently serve as the proposed activated control construct. Interestingly, the Akt-S473D phosphomimetic mutant displayed altered distribution with increased accumulation of the kinase in the lipid raft compartment. Moreover, the kinase-dead mutant of Akt1 (K179M), which is unable to bind ATP in the active site, accumulated predominantly in lipid rafts. These are unexpected and very interesting findings that point to an important function of serine 473 and lysine 179 in regulating the admission of Akt1 to the lipid raft signaling platform.

I have also completed cloning of the five proposed Akt1 variants that will serve to understand how the lipid raft compartment changes Akt1 signaling (Task 1. ii) and iii)). Of these constructs, the most promising pair turned out to be a N-terminal fusion of the Akt1 PH-domain to the transmembrane domain of the Fc γ RIIb receptor (TMFc γ RIIb-Akt1 and TMFc γ RIIbT₂₃₂-Akt, constructs #2 and #3 in the research proposal). After raft fraction isolation using our established SDEM protocol to study subcellular localization (Task 2. i), I was able to demonstrate that TMFc γ RIIb-Akt1 was clearly enriched in raft membranes, which leads me to believe that this construct can serve as a tool to investigate the signals that originate from membrane rafts. By introduction of a single amino acid substitution into the transmembrane domain of TMFc γ RIIb-Akt1 by site-directed mutagenesis (TMFc γ RIIbT₂₃₂-Akt, construct #3), I further demonstrated virtually complete exclusion of this Akt1 version from the lipid raft compartment.

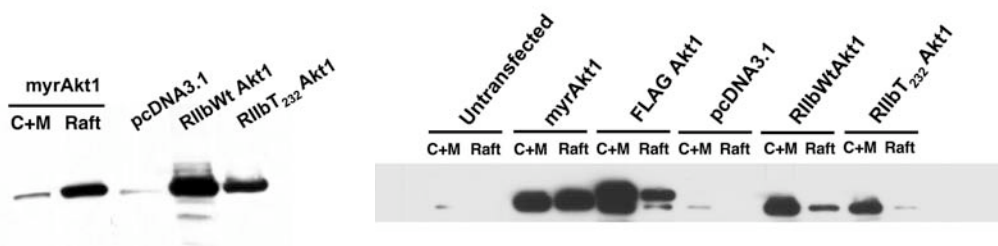


Figure 2: Generation of raft-resident and raft-excluded variant of Akt1. Left: Expression of a raft-targeted version of Akt1 (RIIbWtAkt1) and a raft-excluded version of Akt1 (RIIbT₂₃₂Akt1) in HEK293 cells. Right: Verification of subcellular localization by differential extraction shows that the myristoylated form of Akt1 is highly enriched in lipid rafts and that FLAG-tagged wild-type Akt1 partitions into the raft compartment. The transmembrane domain of the Fc γ RIIb receptor is able to target Akt1 to lipid rafts and a single point mutation at position 232 abolishes this effect.

With these two constructs, one of which will target Akt1 to the raft compartment, whereas the other will exclude Akt1 from the rafts, I am now able to perform the proposed experiments to elucidate how the lipid raft environment affects Akt1 signaling. Consequently, the results meet the requirements for completion of Task 1. i)- iii). Unfortunately, the proposed alternative approach to create a prenylated, raft-excluded Akt1 version (prenAkt, construct #6) turned out to be very difficult to realize. The prenylation signal derived from K-Ras turned out not to be functional when fused to the PH-domain

of Akt1. Fusion of this motif to the Akt1 C-terminus resulted in very low expression levels in my hands and interference with Akt1 kinase activity.

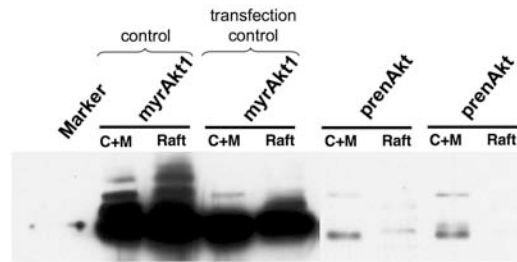


Figure 3: Differential extraction of a raft-excluded version of Akt1. Addition of a prenylation sequence to Akt1, which is predicted to exclude Akt1 from rafts results in very low expression levels in comparison to the raft-targeted Akt1 version.

I have also started to address the question how signals transmitted from raft-resident Akt affect discrete cellular behaviors (Task 2. ii)). To this end, we have generated stably transfected LNCaP prostate cancer cells, which express a myristoylated form of Akt1 that significantly enriches Akt1 in rafts (myrAkt, construct #5). Sequences encoding LacZ (control) or myrAkt1 were cloned into pLenti6/V5-DEST and transfected into HEK293T cells for virus production. Lentiviruses were collected and used for infection of LNCaP prostate cancer cells. After selection of blasticidin-resistant cells, stable transformed clones were isolated and further analyzed. With these stably transfected cell lines we have now developed and optimized an assay to determine the biological consequences of membrane heterogeneity of Akt1 activation.

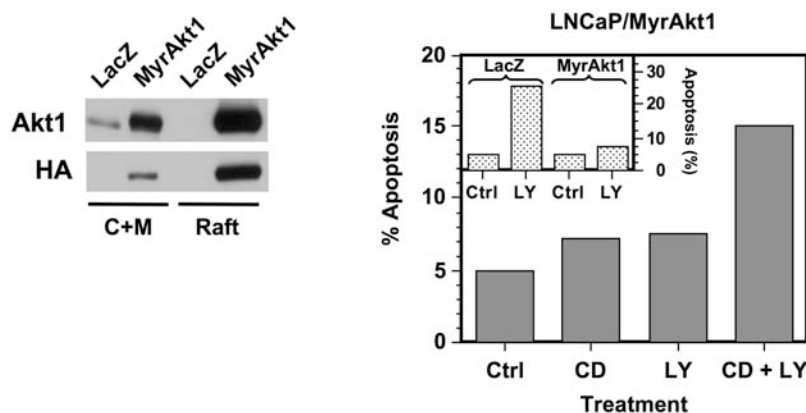


Figure 4: Stable expression of myristoylated Akt1 in lentivirus-transfected LNCaP cells shows considerable enrichment of Akt1 in lipid rafts in comparison to the control (LacZ). LNCaP cells stably expressing myrAkt1 or control cells expressing LacZ (inset) were treated without or with LY294002 for 24 hours and the extent of apoptosis determined by flow cytometry. LNCaP/myrAkt1 cells were treated without (Ctrl) or with 5mM cyclodextrin (CD) for 1 hour, 10uM LY294002 (LY) for 24 hours or both agents (CD + LY) and harvested for flow cytometry. Data are presented as apoptotic cells (sub-G1-peak) expressed as a percentage of the total cell population and are representative of two independent trials.

As shown in Figure 4, enrichment of myrAkt1 within lipid rafts was confirmed in the isolated clones, which were then chosen to further elucidate whether raft-resident Akt1 could function to promote cell survival. Stably transformed LNCaP/myrAkt1 and control cells expressing LacZ were exposed to the PI3K inhibitor LY294002 for 24 hours and its apoptotic effect on LNCaP cells was assessed by flow cytometry. Surprisingly, LNCaP/myrAkt cells were almost completely insensitive to PI3K inhibition, in contrast to LNCaP/LacZ cells that displayed significant induction of apoptosis (inset). However, the cytoprotective effect of myrAkt1 was diminished by depletion of membrane cholesterol prior to treatment with LY294002, suggesting that anti-apoptotic signals are transmitted, at least in part, by the raft-resident population of Akt1.

Based on these results, I am now in a position to test the differentially targeted variants of Akt1 for their impact on cell proliferation and cell survival as proposed in Task 2. ii).

Task 3 and 4. Determine the functional role of HSP90 family members in Akt1 activity and recruitment to lipid rafts in prostate cancer cells.

To elucidate the underlying molecular mechanism of Akt1 recruitment to rafts, I have begun to generate FLAG epitope tagged versions of HSP90 and HSP89 α DN as proposed in Task 3. i). Total RNA from LNCaP, PC-3 and DU145 prostate cancer cells was isolated and RT-PCR with gene specific primers was subsequently performed to isolate the HSP89 α DN coding sequence. A PCR product in the expected size range was generated in LNCaP cells (band A) and DU145 (band B, C) cells but was not detected in PC-3 cells. TA-cloning and sequencing of the amplified fragment revealed that band A from LNCaP cells and band B from DU145 cells both mapped to a different isoform of HSP90 on chromosome 3 instead of chromosome 14. Band C turned out to be a non-specific amplification artifact.

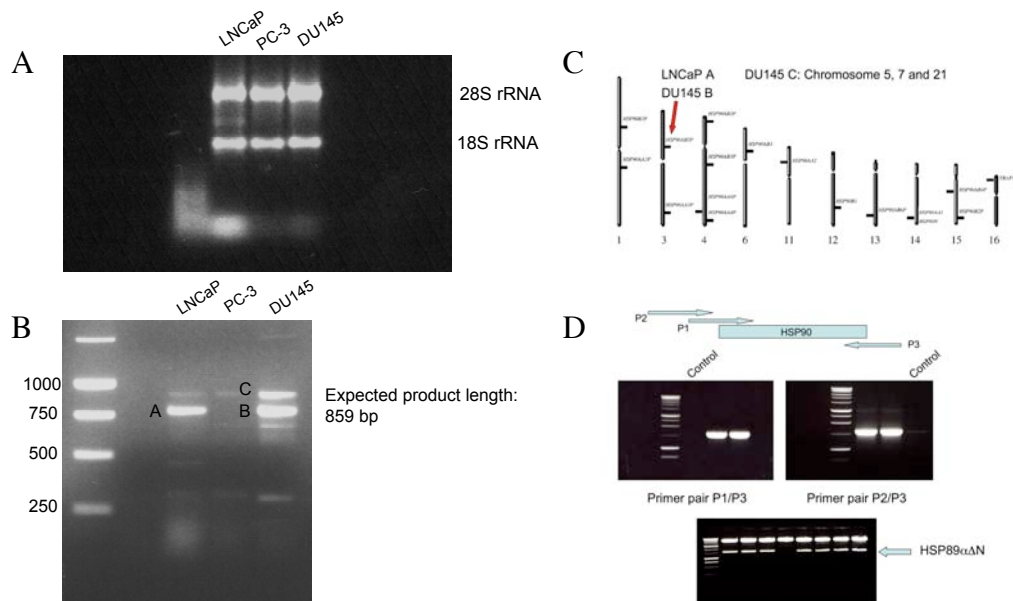


Figure 5: Cloning of HSP89 α DN. (A) Isolation of total RNA from LNCaP, PC-3 and DU145 cells shows the regular signal pattern of abundant 28S and 18S rRNA. (B) RT-PCR experiments with gene-specific primers for the HSP89 α DN gene result in a reproducible product in LNCaP and DU145 cells, which does not map to the HSP89 α DN gene but to another HSP90 isoform on chromosome 3 (C). (D) Cloning strategy for HSP89 α DN with schematic overview of the primer positions used in the nested PCR approach.

Due to the difficulties I encountered with retrieving the cDNA of HSP89 $\alpha\Delta$ N, I decided to clone this gene with a nested primer strategy. To this end, I designed two overlapping 5' PCR primers (Fig. 5, P1/P2) that together encode the entire N-terminal domain of HSP89 $\alpha\Delta$ N and prime with the conserved part of the HSP90 gene that is identical to HSP89 $\alpha\Delta$ N. Together with the HSP90 gene-specific 3' primer P3, the inner 5' primer (P1) was used in a RT-PCR reaction to amplify the HSP90 gene from total cDNA. Subsequently, the PCR product from this reaction served as a template to amplify the complete HSP89 $\alpha\Delta$ N gene in a PCR reaction using primer pair P2/P3. As shown in Figure 5, control restriction digest with BamHI/HindIII demonstrated a fragment of correct molecular weight that was confirmed by sequencing. Consequently, I have now generated all the tools that enable me to proceed in the second year with Task 3 and 4 of the proposal.

KEY RESEARCH ACCOMPLISHMENTS

- In year 1 I have demonstrated that it is indeed possible to generate differentially targeted variant of Akt1 that are either raft-targeted or raft-excluded.
- I have shown that myristoylated Akt1, which is an oncogene, is over-represented in lipid raft fractions in comparison to wild type Akt1 and that mutations at important regulatory sites of Akt1 alter its lipid raft distribution.
- Raft-resident Akt1 was identified as an important signaling molecule that confers a cytoprotective effect.
- This is the first evidence that cholesterol is a direct regulator of Akt-dependent signaling in prostate cancer cells.

CONCLUSION

In summary, I have obtained evidence that signals emanating from raft-resident Akt1 provide important cell survival cues. These signals are sensitive to cholesterol depletion suggesting that anti-apoptotic signals derive from Akt1 when localized to rafts. Furthermore, these findings suggest a direct mechanistic link between cholesterol and cell survival signaling in tumor cells and may be functionally relevant to the reported chemopreventive benefit of long-term use of cholesterol-lowering drugs in certain cancers.