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PRINCIPAL INVESTIGATOR: Ulrike Lorenz, Ph.D.

CONTRACTING ORGANIZATION: University of Virginia
Charlottesville, Virginia 22906

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6. AUTHOR(S)
Ulrike Lorenz, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Virginia
Charlottesville, Virginia 22906

E-MAIL:
ul4q@virginia.edu

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13. ABSTRACT ***(Maximum 200 Words)***

Purpose: To test the working hypothesis that SHP-1 is essential for controlling growth and differentiation of mammary epithelial cells and that its dysregulation contributes to the development of breast cancer.

Scope: To biochemically and functionally characterize SHP-1 in human breast cancer cell lines and to define its biological function in normal epithelial cells.

Major Findings: We have shown that SHP-1 localizes to the lipid-rafts. Moreover, our data indicate a functional difference between rafts- and non-rafts-associated fractions of SHP-1. While these experiments have not yet been performed in human breast cancer cells, we expect to learn about SHP-1 from similar studies in epithelial cells. In addition, we have created a transgenic mouse expressing SHP-1 under its own hematopoietic promoter. Although this mouse shows only low expression levels it is able to partially rescue the *motheaten* phenotype. .

Significance: Based on the systems we have set up and the reagents we generated, we expect to have the necessary tools to gain a better understanding of SHP-1's role in epithelial cells. Moreover, we expect not only to deepen our knowledge of SHP-1's role in epithelial cells but also to learn how a dysregulated SHP-1 is potentially involved in the onset/progression of breast cancer.

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INTRODUCTION

While a strong correlation between the development of breast cancer and expression of certain protein tyrosine kinases (PTKs), such as members of the ErbB family of receptor kinases or the cytoplasmic c-Src kinase, has been observed, little is known about the role of protein tyrosine phosphatases (PTPs) in breast cancer. We have hypothesized, that PTPs would balance the PTK activities and thereby counteract their tumor-promoting actions or unbalanced PTPs could be tumor-promoting themselves. SHP-1 is a cytoplasmic tyrosine phosphatase expressed exclusively in epithelial cells and the hematopoietic lineage. We chose to study SHP-1 as a possible mediator in the onset/progression of breast cancer for the following reasons: (1) In the hematopoietic system, the role of SHP-1 as a negative regulator has been well established. It is conceivable that SHP-1 has a similar role in epithelial cells, and its dysregulation could contribute to neoplasms arising in breast epithelial cells. Biochemical and functional characterization of SHP-1 in normal and transformed epithelial cells are being addressed as part of Tasks 1, 2 and 3. (2) In our preliminary studies, mice which lack one of the wild type SHP-1 alleles have a high incidence of breast tumors, suggesting a role for this phosphatase in the onset/progression of breast cancer. This hypothesis will be addressed in Tasks 3 and 4. The goal of this proposal is to rigorously examine the involvement of SHP-1 in the development of breast cancer in mice as a model system and in human primary breast tumors and cell lines.

BODY

Task 1 and 2 (Characterization and defining the function of SHP-1 in human breast cancer lines and normal epithelial cells)

As proposed in our original application, we have tried to identify substrates of SHP-1 in epithelial cells and in particular, in human breast cancer cells. As reported last year, we had observed an EGF stimulation-dependent association of SHP-1 with several tyrosyl phosphorylated proteins, one of which co-migrates with the EGF receptor. These data indicated that the EGFR and other, yet unidentified proteins, might be direct binding partners and/or substrates of SHP-1. However, it has turned out more difficult than we anticipated to conclusively identify the associated proteins. One of the reasons for these technical difficulties might be that the amount of co-precipitated protein is very low and some of the available antibodies not sensitive enough. We are in the process of repeating these experiments using more material as well as different commercially available antibodies.

In addition, we have started to focus our characterization of SHP-1 on its localization to specialized membrane microdomains, the so-called lipid-rafts. Recently, the importance of the subcellular localization of the involved proteins has been re-emphasized for early signaling events. In particular, the critical role of lipid rafts has been recognized [reviewed in (1-4)]. Cell membranes are composed of proteins and lipids, such as cholesterol and various glycophospholipids and sphingolipids, that form microdomains within the membrane. Based on their biophysical properties, glycophospholipids tend to display a mobile fluid phase, whereas sphingolipids show a more tightly packed higher organization [reviewed in (1)]. Moreover, gaps between the fatty-acyl chains of the sphingolipids are filled with cholesterol, thereby forming a closely-packed lateral lipid cluster, the so-called lipid rafts, in an unsaturated glycophospholipid environment [reviewed in (4, 5)]. Due to their biophysical properties, these cholesterol/sphingolipid rafts are insoluble in non-ionic detergent at 4°C and can be isolated as low-density complexes in sucrose gradients. They have also been referred to as detergent-insoluble glycolipid-enriched complexes (DIGs) (6), low-density Triton-insoluble fraction (LDTI) (7), or glycolipid-enriched membrane domains (GEMs) (8). Since during the last two years a number of studies have focused on lipid-rafts and their role in early TCR-signaling [reviewed in (9-11)], we decided to also use T cells for our initial studies and to optimize the conditions for rafts isolations and characterization. For example, several key players in early signal transduction pathways downstream of the TCR, such as the ζ chain of the TCR/CD3 complex, Lck, Fyn, ZAP-70, Shc, LAT, SLP-76 and PLCγ1, have been shown to localize either constitutively or upon stimulation to the rafts fraction (8, 12-14). However, SHP-1 has not been analyzed for its subcellular localization. Using the BYDP T cell hybridoma line, we have now shown that about 30-40 % of total SHP-1 is localized to the rafts fraction before and after TCR plus CD4 stimulation. In addition, we have observed that the rafts-associated fraction of SHP-1 is hypo-phosphorylated compared to the non-rafts fraction indicating a functional difference between these two subcellular pools of SHP-1 indicating functional diffferences between these two pools. We have also generated mutants of SHP-1 to address the mechanism of SHP-1's association with the rafts fraction since SHP-1 does not contain any post-translationally modifications that have been shown to cause rafts association. Once we have the conditions established in the well-characterized system of T cells, we will perform similar analyses in epithelial cells. We expect that results obtained from these studies will provide indications about the place of action for SHP-1, potential up-stream players, such as kinases phosphorylating SHP-1, SHP-1's localization and regulation through other proteins and overall help to gain a better understanding of SHP-1's mechanism of action in epithelial cells.

Task 3 (Defining the biological function of SHP-1 in normal epithelial cells)

We had proposed to generate a transgenic mouse expressing SHP-1 under the control of its hematopoietic promoter (Fig. 1). As described in the last progress report, we obtained two female founder mice carrying the transgene. However, only one of the mice delivered off-spring carrying the transgene. By further breeding, we generated a stock of these transgenic mice. Mice carrying the transgene are viable and, at least based on what we have observed so far, seem normal.

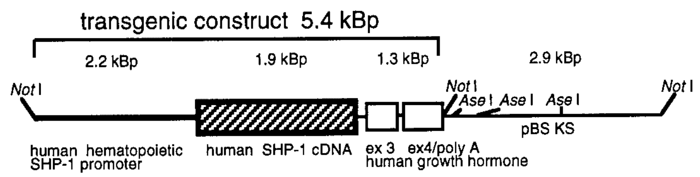

Figure 1: *Map of DNA construct used to generate transgenic mouse.* Human SHP-1 cDNA (without its polyA tail) was cloned under the control of the human growth hematopoietic SHP-1 promoter. The polyA tail was provided by a genomic DNA fragment of the human growth hormone (starting with the third exon). pBS KS served as a vector backbone but was removed befor einjection into pronuclear zygotes (by *Not*I digest of the construct).

One of the reasons, we have generated this transgenic mouse, was to cross it into the *motheaten* background with the hypothesis to thereby by generate a partially rescued *motheaten* mouse, which would allow us to study SHP-1-deficient epithelial cells in an otherwise "normal" background. When we tested the transgenic mouse for expression of transgenic mRNA, we were unable to detect the transgene by Northern Blots or from hematopoietic tissue, such as macrophages. We crossed the transgenic mouse into the *motheaten* background despite the discouraging Northern Blot results since we thought that we might

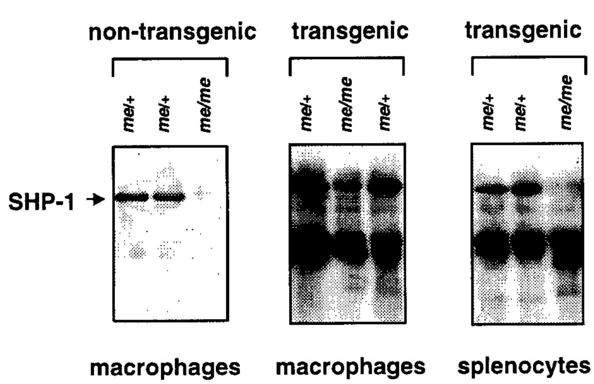

Figure 2: *Expression levels of exogenoyus SHP-1 in transgenic mouse.* SHP-1 was immunoprecipitated from 0.5 mg of the indicated cell lysate s(bone marrow-derived macrophages or total splenocytes derived from mice of the indicated genotypes. The immunoprecipitated proteins were separated by 8% SDS-PAGE, and analyzed for SHP-1 levels by anti-SHP-1 immunoblotting.

have very low expression , therefore not detectable in Northern Blots, but maybe enough to rescue, at least partially, the *motheaten* phenotype. Indeed, we observed that *me/me* mice carrying the transgene live for up to 10 weeks compared to the average *me/me* life-span of 3-4 weeks. They transgenic *me/me* mice eventually die of the same macrophage-induced symptoms as the non-transgenic. We believe that the transgene might be expressed in a mosaic pattern in a subset of the hematopoietic cells, not uncommon for transgenic mice, and the non-expressing cells expand until they overtake and cause the death. Interestingly, while we had been unable to detect the transgenic message, we could detect protein in the *motheaten* background (Fig. 2), which together with the expanded life-span was encouraging. We are now in the process of analyzing various tissues from these mice and in particular epithelial cells. We expect this mouse together with their littermate controls to provide us with a system that allows comparison of SHP-1 expressing and non-expressing epithelial cells.

Task 4 (Analysis of breast tumors in *me*/+ mice)

In our preliminary studies, we had observed that retired *me*/+ female mice display an unusual high frequency of breast tumors. As a control, we observed more closely +/+ mice of the same C3HeB/FeJLe-*a/a* strain. At this point, we have not observed a similar high frequency of breast tumors in these +/+ mice, whereas we continue to observe breast tumor formation in the *me*/+ mice. We are continuing this study to get greater numbers of animals developing tumors and thereby better statistics about the time and frequency of breast tumor on-set.

Key Research Accomplishments

- SHP-1 localizes to lipid rafts. (Task 1 and 2)
- Generation of transgenic founder mouse carrying cDNA for SHP-1 under the control of its hematopoietic promoter. Cross of transgene into *motheaten* background. Prolonged lifespan in transgenic *motheaten* mice compared to non-transgenic (Task 3)
- Increased frequency of breast tumors in C3HeB/FeJLe-*a/a* female *me*/+ mice compared to +/+ mice is observed. (Task 4)

Reportable Outcomes ---- not yet finished

Conclusions

During the last year, we have obtained data about SHP-1's localization to the lipid-rafts that indicate a functional difference between rafts-associated and non-associated fractions of SHP-1. While these studies have been performed in T cells to optimize the experimental conditions, we expect to gain a better understanding of SHP-1's mechanism of action from similar studies in epithelial cells. In addition, we have created a transgenic mouse carrying a gene for SHP-1 under its hematopoietic promoter. Although this mouse shows only low expression levels it is able to partially rescue the *motheaten* phenotype with respect to longevity upon crossing into the *motheaten* background. .

"So what": In our original grant application, we had proposed as a working hypothesis that SHP-1 is essential for controlling growth and differentiation of mammary epithelial cells and that dysregulation of SHP-1 contributes to the development of breast cancer. Based on the systems we have set up and the reagents we generated, we expect to have the necessary tools to gain a better understanding of SHP-1's role in epithelial cells. Moreover, we expect not only to deepen our knowledge of SHP-1's role in epithelial cells but also to learn how a dysregulated SHP-1 is potentially involved in the onset/progression of breast cancer. Moreover, the knowledge of SHP-1's dysfunction and its consequences in certain breast tumors might allow us to use it as a diagnostic and/or a prognostic marker. This might also have implications for possible future therapies.

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