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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 raft. Moreover, our data indicate a functional difference between rafts- and non-rafts-associated fractions of SHP-1. While most of these studies have been performed in T cells and primary thymocytes, in initial experiments using human breast cancer cells, we have observed that limited amounts of SHP-1 localize to lipid rafts before and after EGF stimulation. In addition, we have obtained additional data showing that a transgenic mouse expressing SHP-1 under its own hematopoietic promoter is able to partially rescue the *motheaten* phenotype.

Significance: We expect to gain a better understanding of SHP-1's role in epithelial cells and thereby to learn how a dysregulated SHP-1 is potentially involved in the onset/progression of breast cancer.

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Our progress during the last year was significantly slowed down due to lack of personnel. In particular, one individual who had planned to join the lab decided last minute not to come to the University of Virginia for personal reasons. Therefore, I have asked and was granted a no cost extension for another year. This September, a student has joined the lab who is working full time on this project.

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)

Based on the preliminary results we had obtained the previous year, we have continued 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 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, a pre-TCR line and primary thymocytes, 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. We have also generated fusion proteins between SHP-1 and the Green Fluorescence Protein (GFP) and have shown localization of SHP-1-GFP to lipid rafts using confocal microscopy. Interestingly, we have observed that the rafts-associated fraction of SHP-1 is hypo-phosphorylated compared to the non-rafts fraction in response to TCR/CD4 stimulation. Surprisingly, even one of the strongest tyrosine phosphorylation-inducing agents (pervanadate treatment) failed to promote tyrosine phosphorylation of the raft-associated fraction of SHP-1 while the non-raft associated fraction was highly phosphorylated. Using the cholesterol-depleting drug methyl-β-cyclodextrin (MβCD), we have shown that induced tyrosine phosphorylation of the non-raft associated fraction of SHP-1 is still raft-dependent. Taken together, our data suggest functional differences between the raft-associated and the non-raft-associated fractions of SHP-1. A manuscript describing the results derived from this study has been submitted for review (for figures and further details see Appendix). Using mutants of SHP–1 fused to either an HA tag (for biochemical analyses) or GFP (for confocal microscopy), we are trying to elucidate the mechanism of SHP-1's association with lipid rafts as well as the functional properties of the raft-associated vs. the non-raft-associated fractions of SHP-1.

Currently, we are addressing SHP-1's localization to lipid rafts in epithelial cells. Our initial experiments have shown a limited amount of SHP-1 localizing to lipid rafts in human breast tumor cell lines before and after EGF stimulation. However, we are still in the process of optimizing the biochemical purification of lipid rafts in epithelial cells since due to the presence of caveolae, the membrane composition of epithelial cells differs dramatically from the composition of lymphocytes. We expect that better knowledge of the subcellular localization of SHP-1 will enhance the possibility to find binding partners as well as substrates of SHP-1 in epithelial cells. It will be informative to know whether the indicated functional differences between raft-associated and non-raft-associated fraction of SHP-1 observed in lymphocytes will be present 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 previous progress reports, we obtained two female founder mice carrying the transgene. However, only one of the mice delivered offspring 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.

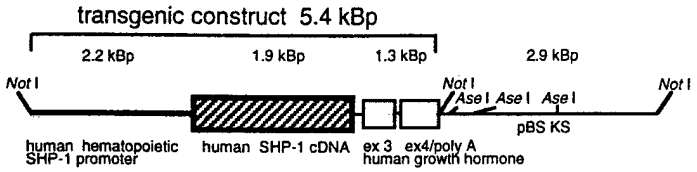

Figure 1: *Map of DNA construct used to generate transgenic mouse.* Human SHP-1 cDNA (without its polyA tail) was cloned under the control of the human growth hematopoietic SHP-1 promoter. The polyA tail was provided by a genomic DNA fragment of the human growth hormone (starting with the third exon). 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. 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.

As mentioned in the beginning of this report, our progress was less than anticipated due to lack of personnel. Therefore, we have been unable to perform a detailed analysis of individual tissues derived from these mice. Instead, we have increased our breeder colony and acquired additional data about the longevity of the transgenic *me/me* mice further supporting our initial finding that these mice live on average 10 weeks. Analyses of various tissues from these mice should give us further insight in the extent of rescue. 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. This study is on-going to acquire 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. Indication that raft-associated and non-raft-associated fractions of SHP-1 show functional differences (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 ---- submitted manuscript and 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 and primary thymocytes 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 preliminary studies using human breast tumor cell lines, we observed a limited amount of SHP-1 in the lipid rafts before and after EGF stimulation. 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 believe to have the necessary tools to gain a better understanding of SHP-1's role in epithelial cells. 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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The tyrosine phosphatase SHP-1 localizes to lipid rafts in T lymphocytes

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Running title: Localization of SHP-1 to lipid rafts

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SUMMARY

Recently, the importance of the subcellular localization of individual proteins for the successful transduction of signaling events downstream of the T cell receptor (TCR) has been re-emphasized. In particular, formation of signaling centers in microdomains of the plasma membrane, in the so-called lipid rafts, has been recognized as critical for mediating productive signaling. A number of signaling molecules have been shown to localize either constitutively or in a stimulus-dependent manner to lipid rafts. However, less is known about the localization of negative regulators or phosphatases. We and others have previously shown that the tyrosine phosphatase SHP-1 functions as a negative regulator of TCR-mediated signaling. Here, we describe that in a T cell hybridoma line as well as in primary thymocytes, a fraction of SHP-1 localizes basally and upon T cell activation to the lipid rafts. Interestingly, upon T cell activation, the raft-associated fraction is hypo-phosphorylated compared to the non-raft fraction of SHP-1, indicating functional differences between these two subpopulations of SHP-1. Our data further indicate that the raft-associated fraction of SHP-1 is catalytically active suggesting a possible role for SHP1- in dephosphorylating substrates in the lipid rafts.

INTRODUCTION

Activation of T cells via the T cell receptor (TCR)/CD3 complex induces tyrosine phosphorylation of numerous intracellular proteins and evokes a signaling cascade that, depending on the differentiation state of the T lymphocyte, promotes proliferation, differentiation or cell death. The TCR is composed of antigen-specific α and β chains along with several invariant chains of the CD3 complex. When the α:β TCR binds to the antigen presented on major histocompatibility complex (MHC) molecules, intracellular signals are propagated via the cytoplasmic tails of the CD3 chains [reviewed in (1-3)]. MHC molecules also serve as ligands for the co-receptors CD4 and CD8 on T cells, thereby activating the associated tyrosine kinase Lck. Current models of T cell activation suggest that many of the CD3 chains, most notably the ζ chain, become heavily tyrosyl-phosphorylated upon receptor engagement by antigen plus MHC. A similar effect can be evoked by crosslinking of the TCR/CD3 complex with antibodies. Recent studies have shown that in addition to the TCR/CD3 chains and the associated kinases, adaptor proteins, such as LAT, SLP-76, GADS and Shc, are essential for TCR signaling [reviewed in (4-7)].

Recently, the importance of the subcellular localization of the signaling molecules has been re-emphasized for the process TCR-mediated activation. In particular, the critical role for one type of membrane microdomains, often referred to as lipid rafts, has been recognized [reviewed in (8-10)]. These microdomains are enriched in cholesterol and glycosphingolipids thereby forming a tightly packed higher organization within the more fluid plasma membrane [reviewed in (11)]. 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. Defined by these properties, they have also been referred to as detergent-insoluble

glycolipid-enriched complexes (DIGs) (12), low-density Triton-insoluble fraction (LDTI) (13), or glycolipid-enriched membrane domains (GEMs) (14). In addition to the biochemical techniques, various microscopic techniques have allowed the visualization of individual components of the rafts and provided further evidence that some molecules, such as GPI-linked proteins and the *src* family kinases are tightly anchored in lipid rafts, while other proteins move in and out in a regulated manner in response to stimuli (10, 14-16).

In early signal transduction pathways downstream of the TCR, several key players, such as the ζ chain of the TCR/CD3 complex, Lck, Fyn, ZAP-70, Shc, LAT, SLP-76, GADS and PLCγ1, have been shown to localize either constitutively or upon stimulation to the raft fraction (14, 16-18), while other molecules, such as the transmembrane phosphatase CD45 appear to be excluded from the lipid rafts following activation [(19) and reviewed in (8, 20)]. Several studies have shown that disruption of lipid rafts abolishes TCR/CD3 induced signaling events, emphasizing the importance of lipid rafts as signaling centers (16, 21, 22). Moreover, the localization to the lipid rafts has been shown to be critical for both LAT- and SLP-76-mediated signaling (17).

Although much is known about the initiation of TCR-mediated signaling events, relatively less is known about the mechanism(s) by which TCR signaling is negatively controlled or terminated. Dephosphorylation of tyrosyl phosphorylated cellular substrates is considered as an important part of this process. Several lines of data obtained by us and others suggest a negative regulatory role for the tyrosine phosphatase SHP-1 in T cell signaling [reviewed in (23)]. For example, thymocytes derived from SHP-1-deficient *motheaten* mice display a hyper-responsiveness to TCR stimulation, as evidenced by hyper-proliferation, prolonged activation of the kinases Lck and Fyn and increased phosphorylation of several intracellular substrates*

compared to normal thymocytes (24, 25). Moreover, negative regulation of TCR signaling by SHP-1 has also been demonstrated in T cell lines and T cell hybridomas (26, 27). We therefore asked whether SHP-1 would localize to lipid rafts and whether its localization would be influenced by the activation state of the TCR/CD3 complex.

We found that in a murine T cell hybridoma cell line as well as in primary thymocytes, a fraction of SHP-1 basally localizes to the lipid rafts. There was no detectable change upon TCR/CD3 plus CD4 stimulation. Interestingly, upon T cell activation, the raft-associated fraction is hypo-phosphorylated compared to the non-raft fraction of SHP-1, indicating functional differences between these two subpopulations of SHP-1. Our data further indicate that the raft-associated fraction of SHP-1 is catalytically active suggesting a possible role for SHP1- in dephosphorylating substrates in the lipid rafts.

EXPERIMENTAL PROCEDURES

Cell culture, generation of cell lines, and primary thymocytes

Parental BYDP (28) and transfectants stably expressing GST-SHP-1 (27) or SHP-1-GFP fusion proteins were grown in RPMI medium supplemented with 10% Fetal Bovine Serum (FBS), $5x10^{-5}$ M 2-mercaptoethanol, 2 mM L-glutamine, 10 units/ml penicillin, and 10 µg/ml streptomycin (complete medium). The previously described GST-SHP-1-expressing clones of BYDP cells used in this study over-express the GST-fusion protein 2-3-fold.

Transfecting BYDP cells with pEGFP-N1 (Clontech Lab., Palo Alto) or a pEGFP-N1 construct, in which SHP-1 had been inserted, generated stable cell lines expressing GFP alone or SHP-1-GFP. The stop codon at the 3' end of SHP-1 was mutated into a *Not*I restriction site using the polymerase chain reaction (PCR) technique (5' **TGA** GCG GTG C 3' -> 5' **TGC** GCG GCCG C 3') and this version of SHP-1 was subcloned from pBS/KS-SHP-1 [*Eco*RI and *Not*I] into pEGFP-N1 [*Eco*RI and *Bsp*120I]. This construct expresses SHP-1 with the green fluorescence protein (GFP) fused to its carboy-terminus. Clones were selected for G418 resistance (1.5 mg/ml) and screened for expression of SHP-1-GFP or GFP alone by fluorescence and immunoblotting. Five to ten independent clones were isolated for each construct and analyzed for GFP expression and localization. Localization of SHP-1-GFP and GFP alone was very reproducible between clones, and data derived from representative clones are shown.

Murine thymi were obtained from 4-8 week old C3HeB/FeJLe mice and a single cell suspension was prepared, as described previously (24). Isolated thymocytes were rested for 7 hours at 37°C in complete medium prior to stimulation.

T cell stimulation and lipid raft isolation

6.5 x 10^7 BYDP cells or murine thymocytes were used per time point and stimulated as described previously (24). Briefly, cells were co-stimulated through CD3ε using 1 μg/ml of 145-2c11 (Southern Biotechnology Associates, Inc., Birmingham, AL), and anti-CD4 antibody, followed by secondary antibody crosslinking via the addition of 10 μg/ml goat anti-mouse Ig (Southern Biotechnology Associates, Inc., Birmingham, AL). Following incubation at 37°C for the indicated times, cells were lysed in 1 ml TNE lysis buffer (25 mM Tris-HCL pH7.6, 150 mM NaCl, 5 mM EDTA) containing 0.5% Triton X-100, phosphatase inhibitors (5 mM NaF, 1 mM Na_3VO_4, 30 mM β-glycerophosphate), and protease inhibitors (10 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 μg/ml antipain, and 20 μg/ml PMSF). Cell lysates were mixed with an equal volume of 80% sucrose solution. The resultant 40% sucrose solutions containing the lysates were overlaid with 2 ml 30% sucrose solution, and 1 ml 5% sucrose solution. All sucrose solutions were made up in TNE buffer, supplemented with phosphatase inhibitors. Following ultracentrifugation at 200,000 g for 18 hours at 4°C, eleven equal fractions were harvested from each gradient.

For disruption of lipid rafts by drug treatment, BYDP cells (2 x 10^7/ml) were incubated in complete medium containing 10mM methyl-β-cyclodextrin (MβCD) at 37°C for 60 minutes prior to stimulation. Viability was assessed before and after drug treatment by trypan blue exclusion.

Confocal microscopy

SHP-1-GFP- and GFP alone-expressing BYDP cells (10^6/condition) were washed in PBS supplemented with 1% FBS and 0.1% NaN_3 before they were fixed with 2% paraformaldehyde for 30 minutes on ice. After 2 washes in supplemented PBS, cells were resuspended in 50 μl

Vectashield (Vector Lab. Inc., Burlingame, CA) and attached to poly-L-Lysine-coated slides (Sigma, St. Louis, MO). Microscopy was performed with an Olympus confocal microscope using a 60x objective lens.

Immunoprecipitation and immunoblotting

Aliquots of each fraction were analyzed by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent immunoblotting. Raft fractions and detergent-soluble fractions were distinguished by the presence of LAT (14). 3.75 % and 37.5 % aliquots of the combined fractions were used for analysis of total fractions and immunoprecipitations, respectively. For SHP-1 immunoprecipitations, 2μg rabbit polyclonal IgG antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were added to pooled raft and non-raft fractions for 1.5 hours at 4°C. Based on an initial titration of the antibody, 2μg anti-SHP-1 antibody was able to clear SHP-1 out of the lysate. Immunoprecipitations and immunoblottings were performed as described previously (27) using the following antibodies for immunoblotting: monoclonal anti-phosphotyrosine (4G10 at 0.5/ml), polyclonal rabbit anti-SHP-1 (1:1000), polyclonal rabbit anti-LAT antibodies (1:500 dilution) (Upstate Biotechnology, Lake Placid, NY) and monoclonal anti-transferrin receptor antibodies (1:1,000 dilution, (29)).

To quantitate the relative amounts of SHP-1 localized to raft vs. detergent soluble fraction and the relative tyrosine phosphorylation of SHP-1, exposures of immunoblots that were in the linear range based on a range of exposures were analyzed by densitometry using ImageQuant. Intensities measured at the perimeter of each band were quantitated and subtracted as background.

RESULTS

SHP-1 localizes to the lipid rafts before and after TCR stimulation

During the last two years, a number of studies have recognized the importance of lipid rafts in TCR signaling [reviewed in (8, 10)]. Several proteins involved in the transmission of early signaling events downstream of the TCR have been shown to localize either constitutively or upon stimulation to the raft fraction (14, 16-18). However, less is known about the raft localization of negative regulators of TCR-initiated signaling and, in particular, about the localization of non-transmembrane tyrosine phosphatases. Since the tyrosine phosphatase SHP-1 has been identified by us (27) and others (25, 26) as a negative regulator of signaling events downstream of the TCR, we asked whether it might also localize to the lipid rafts and whether this localization might be TCR-regulated. In our previous studies, we have used the BYDP T cell hybridoma line, which mimics various aspects of thymocyte signaling (28), to characterize SHP-1 biochemically (30) and functionally (27), and we chose to initially analyze SHP-1's subcellular localization in this cell line following TCR stimulation.

Lipid rafts are biochemically defined by their insolubility in non-ionic detergents at 4°C. To establish optimal conditions for the purification of lipid rafts from BYDP cells, we used increasing amounts of Triton X-100 (0.1%, 0.5%, 1.0%) in the lysis buffer. Cell lysates of unstimulated BYDP cells were fractionated via a sucrose step gradient ultracentrifugation, and each fraction was analyzed for the presence of SHP-1. At 0.5% Triton X-100, we could detect SHP-1 in the lipid raft fraction (Fig. 1A). In contrast at 1% Triton X-100, the majority of this fraction of SHP-1 moved into the intermediate fractions (fractions 5-8) between the raft and detergent-soluble fractions. Such sensitivity to higher Triton X-100 concentrations with respect to raft localization is not uncommon, as it has been described for other signaling molecules (15,

31). Based on these results, we chose 0.5% Triton X-100 as the optimal detergent concentration for the subsequent studies.

While the above experiment had shown that SHP-1 was basally associated with the lipid rafts, we asked whether there was any change upon TCR/CD4 stimulation of the cell. When BYDP cells were stimulated with anti-CD3 alone or anti-CD3 plus anti-CD4 and lipid raft and detergent-soluble fractions were isolated, a readily detectable fraction of SHP-1 (roughly 30%) localized to the raft fraction. This observation was independent of the length or type of stimulation, such as TCR alone (data not shown) or TCR plus CD4 stimulation (Fig. 1B). LAT was used throughout these studies as a marker for lipid rafts and ≈ 30%-50% of LAT localized to the lipid rafts, as has been previously published (14). To control for contamination of membrane- but not raft-localized proteins, we re-probed each blot for the presence of the TfR, a transmembrane protein that has been shown to be excluded from the lipid rafts (29). TfR was absent from the raft fractions at all tested detergent concentrations while it was readily detectable in the detergent-soluble fractions.

Co-staining of SHP-1 and lipid rafts

As an additional approach for the evaluation of SHP-1's raft association, we chose confocal microscopy. While data obtained from this approach are less informative for quantitative analyses, they are detergent-independent and are therefore complementary to the biochemical studies. To analyze SHP-1's localization without having to permeabilize the membrane and thereby potentially destroying the organization of membrane microdomains, we generated a fusion protein between SHP-1 and the green fluorescent protein (GFP). BYDP cells

were stably transfected with an expression vector driving the expression of SHP-1-GFP or GFP alone.

To assess whether SHP-1-GFP protein localizes to lipid rafts similar to the endogenous protein, cell lysates from SHP-1-GFP expressing cells were fractionated and analyzed for SHP-1 expression. Although SHP-1-GFP was about 4-5 fold over-expressed compared to endogenous SHP-1, the distribution between lipid raft and detergent-soluble fractions was comparable between endogenous and exogenous transfected proteins. As observed in the parental BYDP cells, a fraction of SHP-1 localized to the lipid rafts (Fig. 2A). Confocal microscopy showed that SHP-1-GFP mostly localized to the cytoplasm and the plasma membrane, which was detectable as a ring around the cell (Fig. 2B). In contrast, GFP alone localized mostly to the nucleus. Upon staining of the cells with fluorescence-labeled cholera toxin B (CTB) as a marker for lipid rafts, some of the membrane-associated SHP-1-GFP appeared to co-localize with CTB while there was no co-localization between GFP alone and CTB (data not shown). While the shape of the CTB staining changed upon TCR/CD3 plus CD4 activation, the co-staining with SHP-1-GFP was not affected, consistent with our biochemical data.

SHP-1 is hypo-phosphorylated in rafts fraction

We have previously shown that SHP-1 becomes tyrosyl phosphorylated at its C-terminus upon stimulation of TCR plus CD4 (30). Mapping of the phosphorylation sites as well as analysis of primary thymocytes and several T cell lines had indicated that this phosphorylation was Lck-dependent. Since Lck preferentially localizes to lipid rafts (due to its myristoylation/palmitoylation moieties), we asked whether there were differences in the tyrosyl phosphorylation status of SHP-1 between raft and the detergent-soluble fractions. BYDP cells

were stimulated via TCR/CD3 plus CD4, and SHP-1 was immunoprecipitated from the raft and detergent-soluble fractions. Surprisingly, we found that there was very limited tyrosyl phosphorylation of SHP-1 in the raft fraction upon TCR/CD4 stimulation while its phosphorylation in the detergent-soluble fraction was readily detectable, suggesting a hypo-phosphorylation of the raft-associated fraction of SHP-1 (two independent experiments are shown in Fig. 3 A and B). Quantitation of the relative tyrosine phosphorylation of SHP-1 in the raft vs. the detergent soluble fractions showed that there was on average at least a two-fold higher relative phosphorylation of SHP-1 associated with the detergent-soluble fraction compared to the raft-localized SHP-1. This was not due to a general absence of tyrosine-phosphorylated proteins in the lipid rafts, since a number of phosphoproteins were detectable upon TCR/CD3 plus CD4 stimulation (Fig. 3 C). As has been observed previously, different sets of phosphoproteins appeared to be associated with the raft and the detergent-soluble fractions (16, 18).

Hypo-phosphorylation of SHP-1 in lipid raft fractions depends on its enzymatic activity

To our surprise, we had observed a hypo-phosphorylation of the lipid raft-associated SHP-1 compared to the detergent-soluble fractions. There are several possible explanations: SHP-1 could become mostly phosphorylated outside the lipid rafts and there is very limited movement of SHP-1 between lipid rafts and non-rafts. Alternatively, SHP-1 could get phosphorylated in the lipid rafts and then either rapidly move out of the rafts or partially remain in the lipid rafts but preferentially lose its phosphorylation when localized there. In the latter case, SHP-1 could lose its phosphorylation in the raft fraction either by auto-dephosphorylation or through another phosphatase. We have previously shown that wild type SHP-1 is able to auto-dephosphorylate (30). To test whether auto-dephosphorylation plays a role in the hypo-

phosphorylation of raft-associated SHP-1, we analyzed stable BYDP transfectants expressing an enzymatically inactive mutant of SHP-1 (SHP-$1_{\Delta P}$) (27). Our hypothesis was that this mutant would be hyper-phosphorylated compared to wild type SHP-1. As shown in Fig. 4A, although only a very small fraction of GST-SHP-$1_{\Delta P}$ localized to the lipid rafts compared to the endogenous SHP-1, the intensity of its phosphorylation was comparable to that of endogenous SHP-1. The relative tyrosine phosphorylation of SHP-$1_{\Delta P}$ was therefore much higher than that of wild type SHP-1 (Fig. 4 A). Moreover, SHP-$1_{\Delta P}$'s phosphorylation was sustained (time points 20 and 30 minutes), further indicating that auto-dephosphorylation is, at least in part, causing the loss of phosphorylation on wild type SHP-1. Quantitation of the relative tyrosine phosphorylation revealed that in the rafts, GST-SHP-$1_{\Delta P}$ showed a 10-20-fold increased phosphorylation in response to TCR/CD4 stimulation compared to the endogenous SHP-1. This value increased to >50-fold for the later time points (20 and 30 minutes). In contrast, similarly GST-tagged wild type SHP-1 (GST-SHP-1_{wt}) did not show this strong relative increase in phosphorylation but lacked detectable phosphorylation in the raft fraction (Fig. 4 B). Both GST-SHP-$1_{\Delta P}$ and GST-SHP-1_{wt} showed inducible phosphorylation in the detergent-soluble fraction. Interestingly, although both the wild type and the phosphatase inactive mutant of SHP-1 showed a relative increase in phosphorylation compared to the endogenous protein, their phosphorylation still followed the same pattern of induction, which peaked at 15 minutes and is to a great extent lost after 30 minutes. This indicates that another phosphatase, and not SHP-1, is most likely the main phosphatase responsible for dephosphorylating SHP-1 in the detergent-soluble fraction.

Although our previous data had suggested that Lck is the major tyrosine kinase phosphorylating SHP-1 upon TCR/CD4 stimulation (30), we detected the majority of phosphorylated SHP-1 in the non-raft detergent-soluble fraction. Therefore, we asked whether

intact lipid rafts are necessary for the TCR-induced phosphorylation of SHP-1. To deplete the membrane of cholesterol and thereby destabilize the lipid rafts, BYDP cells were treated with methyl-β-cyclodextrin (MβCD) and the overall tyrosine phosphorylation as well as phosphorylation of SHP-1 were analyzed in the remaining lipid rafts and in the detergent-soluble fractions. Overall, there was very limited TCR/CD4-inducible tyrosine phosphorylation detectable following MβCD treatment (Fig. 5). This was not due to viability of the cells, since based on Trypan Blue exclusion, since over 85% of the cells were viable after treatment. SHP-1 was not inducibly phosphorylated upon TCR/CD4 stimulation following the MβCD treatment indicating that SHP-1's phosphorylation, although mostly observed in the non-raft fraction, was still dependent on intact lipid rafts. In some experiments, we observed an increased basal phosphorylation of SHP-1 in the lipid rafts upon MβCD treatment. This unregulated phosphorylation was most likely caused by *src* kinases, such as Lck and Fyn, that were activated due to the loss of regulation normally coordinated by the lipid rafts. A similar increased basal phosphorylation in response to disruption of lipid rafts has been reported previously for other proteins (22).

Lipid rafts localization and hypo-phosphorylation of raft-associated SHP-1 in primary thymocytes

We also analyzed the subcellular localization of SHP-1 in primary murine thymocytes. Lipid rafts were isolated from TCR/CD3 plus CD4 stimulated thymocytes and overall tyrosine phosphorylation, distribution of SHP-1 and its tyrosine phosphorylation were assessed. As observed in the BYDP cells, there was inducible tyrosine phosphorylation in the raft and detergent-soluble fractions with different patterns of overall phosphorylation. In addition,

consistent with the data obtained from the BYDP cells, 20-30% of SHP1 localized to the lipid rafts and this fraction of SHP-1 was hypo-phosphorylated compared to the detergent-soluble fraction (Fig. 6). In fact, the difference in inducible relative tyrosine phosphorylation of SHP-1 between the raft and detergent-soluble fractions was even more pronounced in the primary thymocytes. On average, the detergent-soluble fraction of SHP-1 showed an over 10-fold higher relative phosphorylation than the raft-associated fraction.

DISCUSSION

Recently, it has been recognized that the subcellular localization of individual proteins and in particular, formation of signaling centers in microdomains of the plasma membrane, in so-called lipid rafts, play a critical role in the successful transduction of signaling events downstream of the TCR [reviewed in (8-10)]. A number of signaling molecules, such as the tyrosine kinases Lck, Fyn and Zap-70 as well as the adaptor proteins LAT, SLP76 and GADS, have been shown to localize either basally or upon TCR stimulation to the lipid rafts. In contrast, little is known about the localization of negative regulator or phosphatases. We therefore asked where the tyrosine phosphatase SHP-1 localizes to the rafts before and after TCR/CD4 stimulation.

Using biochemical as well as microscopic studies, we observed that a fraction of SHP-1 localizes to lipid rafts basally and that the same amount of SHP-1 can be found in lipid rafts following TCR or TCR plus CD4 stimulation. This is in contrast to data obtained for the transmembrane phosphatase CD45, which has been implicated in keeping basal phosphorylation of the *src* family kinases down and thereby preventing non-specific activation. While CD45 is not localized to lipid rafts it is thought that it is in close enough proximity to dephosphorylate raft-associated substrates. However following TCR activation, CD45 is excluded and has no access to raft-associated proteins [reviewed in (8, 20)]. It is well established that SHP-1 is a negative regulator of TCR signaling by turning signals off following receptor activation [reviewed in (7)]. Based on studies using T lymphocytes derived from the SHP-1-negative *motheaten* mice (24, 25) and cell lines over-expressing dominant negative mutants of SHP-1 (26, 27), SHP-1 does not seem to affect basal tyrosine phosphorylation and therefore likely exists in an inactive state in the lipid rafts. Following TCR stimulation, SHP-1 becomes activated by a yet

unknown mechanism and acts on its potential targets, such as Lck (24) and Zap-70 (26). Interestingly, it has recently been shown that the activating auto-phosphorylation site of Lck serves as a good substrate for SHP-1 (32). SHP-1's constant presence in the lipid rafts would suggest that there is a secondary mechanism to control its activity. Since it is known that SHP-1's engagement of its SH2 domains leads to its activation, it is possible that one or more of the proteins in the lipid rafts that are phosphorylated on tyrosine following TCR stimulation act as binding partners of SHP-1 and thereby cause its activation.

Since we had previously suggested Lck as a likely candidate for SHP-1's tyrosine phosphorylation upon TCR/CD4 activation, and Lck is activated in the lipid rafts, we initially hypothesized that the lipid raft-associated fraction of SHP-1 may be hyper-phosphorylated. However, we observed that there is very little tyrosine phosphorylation of SHP-1 in the lipid rafts. Compared to the non-raft detergent-soluble fraction, the raft-associated fraction of SHP-1 was hypo-phosphorylated. There are several possible explanations for this finding. (i) SHP-1 could become phosphorylated in the non-raft compartments and remain there in a phosphorylated state. However based on our previous data suggesting Lck as the primary kinase phosphorylating SHP-1, this seems unlikely. (ii) SHP-1 could become phosphorylated in the lipid rafts and phosphorylation provides a signal to become excluded from the rafts with the concurrent influx of unphosphorylated SHP-1. Or (iii) SHP-1 could become phosphorylated within the rafts and there is an exchange between raft- and non-raft-associated SHP-1. However, SHP-1 might lose its phosphorylation within the rafts than outside faster than in the non-raft regions.

To distinguish between these possibilities, we made use of an enzymatically inactive mutant of SHP-1, SHP-$1_{\Delta P}$, that is unable to auto-dephosphorylate. In cell lines stably expressing this mutant we observed an increased and sustained phosphorylation of SHP-$1_{\Delta P}$ in the lipid rafts

compared to SHP-1_{wt}. In contrast, the phosphorylation pattern of the detergent-soluble fraction of SHP-$1_{\Delta P}$ was similar to the one of SHP-1_{wt}. These data suggest that SHP-1, which resides within the lipid rafts, is catalytically active and auto-dephosphorylates, thereby losing its phosphorylation. This is consistent with previous data where we had shown that SHP-1 is very potent in dephosphorylating its own phospho-tyrosine *in vitro* (30). Our data further suggest that the fraction of SHP-1 residing outside the lipid rafts does not undergo auto-dephosphorylation, either because it is in an enzymatically inactive state or the phosphorylated tyrosine is inaccessible for SHP-1. During the time course following TCR/CD4 stimulation, phosphorylation on non-raft SHP-1 did decrease. We would like to propose that this dephosphorylation is most likely due to another phosphatase since there is no difference between wild type and mutant SHP-1 in their pattern of dephosphorylation.

To address whether the induced tyrosine phosphorylation of SHP-1 outside the rafts requires intact lipid rafts, we treated cells with the raft-disrupting agent MβCD and found that any inducible tyrosine phosphorylation of SHP-1 was abolished. This indicated that although SHP-1 is mostly in the non-raft fraction in a phosphorylated state, its either becomes phosphorylated outside the rafts by a still raft-dependent tyrosine kinase or it becomes phosphorylated within the rafts and this phosphorylated SHP-1 may then move out. Our data suggest that such an exchange would not be driven by the tyrosine phosphorylation since we find the highly phosphorylated SHP-$1_{\Delta P}$ mutant in the rafts, consistent with it being phosphorylated there but unable to auto-dephosphorylate.

It is unclear how SHP-1 localizes to the lipid rafts. It lacks any of the post-translational modifications, such as acylation or palmitoylation, that have been described for other raft-targeted proteins (33). Therefore a protein-protein or a protein-lipid interaction most likely

mediates SHP-1's localization to lipid rafts. It has been reported that SHP-1 can bind via its SH2 domains and its carboxyl-terminus to phospholipids, such as phosphatidic acid, and can be activated by this binding *in vitro* (34, 35). However, while phosphatidylinositol 4,5-bisphosphate is enriched in detergent-resistant membrane fractions (36), phospholipids are in general thought to locate to the more fluid phase of the membrane surrounding the raft islands. Lipids are therefore unlikely to confer SHP-1's enrichment in the rafts. Instead, SHP-1 might localize to the lipid rafts via a protein-protein interaction. For example, it has recently been reported that in human T cells, SHP-1 stably associates with the Leukocyte-Associated Ig-Like Receptor 1 (LAIR-1) (37, 38). At this point, there is no murine homologue known and it remains to be seen whether and how a putative murine LAIR-1 will play a role in SHP-1 targeting to the lipid rafts.

Recently, it was reported that in Jurkat cells, SHP-1 localizes basally and during the first 20-30 min. of anti-CD3 stimulation exclusively to the detergent-soluble fraction when cells were lysed in 1% Triton X 100 (39, 40). We believe this discrepancy could be due to the higher detergent concentration used by both groups or due to cell type differences. While Su et al.'s and Kosugi et al.'s studies were performed in the human T cell line Jurkat, we observe localization of SHP-1 to lipid rafts in primary thymocytes and in a murine T cell hybridoma. In addition, we have obtained the same results from another T cell line (SL-12β.12, (41) (Johnson and Lorenz, unpublished observation). However, the different results are more likely due to the higher detergent concentration used in Su et al.'s study since we observe that at 1% Triton X 100 most of the raft-associated fraction of SHP-1 localizes to the intermediate fractions.

Our data imply that SHP-1 may act as a negative regulator of TCR-mediated signaling by dephosphorylating substrates in the lipid rafts. Further studies will address whether all of

SHP–1's function is mediated in the lipid rafts or whether the non-raft-associated phosphorylated fraction of SHP-1 also plays a role in TCR signaling.

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FIGURE LEGENDS

Figure 1: SHP-1 localizes to lipid rafts. [A] BYDP cells (6.5 x 10^7) were lysed in 0.5 or 1% Triton X-100 lysis buffer and fractionated via a sucrose step gradient. 8.75% of each fraction were separated by 8% SDS-PAGE and subjected to anti-SHP-1 immunoblotting. The second band migrating slightly above SHP-1 corresponds to the recently identified splice variant of SHP-1, SHP-1L (42), which differs in its carboxyl-terminus from SHP-1. [**B**] BYDP cells (6.5 x 10^7) were stimulated for the indicated times with anti-CD3 plus anti-CD4, lysed in 0.5% Triton X-100 and fractionated. 3.75% of fractions 2-4 and 9-10 were combined as raft and detergent-soluble fractions respectively and separated by 8% SDS-PAGE followed by immunoblotting for the presence of SHP-1, LAT and TfR. All pairs of raft and detergent-soluble fractions were run on the same gel and exposed for the same times.

Figure 2: Localization studies of a SHP-1-GFP fusion. [A] BYDP cells (6.5 x 10^7), stably transfected with a SHP-1-GFP-expressing plasmid were lysed and fractionated. 8.75% of each fraction were separated by 8% SDS-PAGE and analyzed by immunoblotting for the presence of endogenous SHP-1 and exogenous SHP-1-GFP. Raft and detergent-soluble non-raft fractions are marked. [**B**] BYDP cells stably expressing SHP-1-GFP or GFP alone. Images were taken with an Olympus confocal microscope using a 60x lens.

Figure 3: SHP-1 is hypo-phosphorylated in lipid rafts. BYDP cells (6.5 x 10^7) were stimulated with anti-CD3 and anti-CD4 for the indicated times. 0.5% Triton X-100 cell lysates were fractionated and fractions 2-4 and 9-10 were combined as rafts and detergent-soluble respectively. [**A** and **B**] SHP-1 was immunoprecipitated from the combined raft and non-raft fractions (37.5% each) and analyzed for its tyrosyl phosphorylation by immunoblotting (top

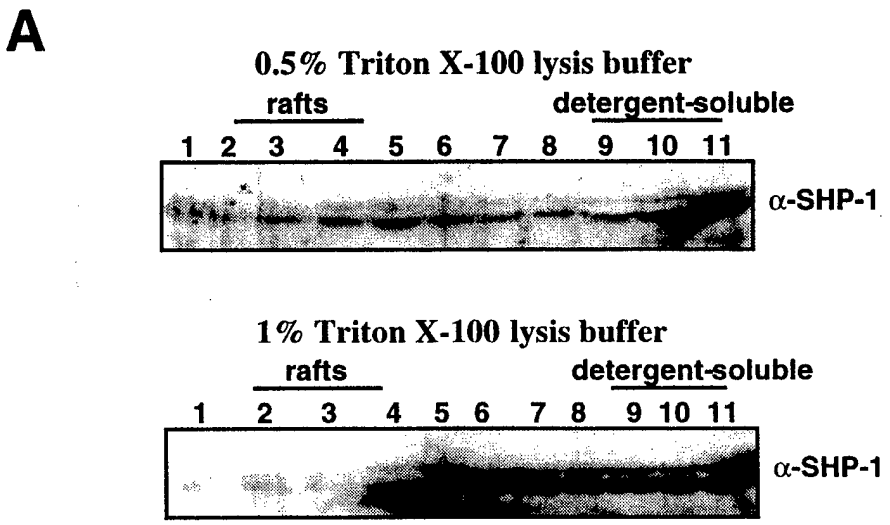
panels). The immunoblots were stripped and re-probed for SHP-1 (bottom panels). The ≈70 kDa band migrating above SHP-1 in the detergent-soluble fractions is Zap-70 and is due to the presence of stimulating antibodies (anti-CD3 plus anti-CD4) rather than a direct interaction with SHP-1 (Johnson and Lorenz, unpublished observation). It is not clear why we see most of Zap-70 in the detergent-soluble fractions and only very limited amounts in the lipid raft fractions. [C] 3.75% of the combined raft and detergent-soluble fractions of the experiment shown in B were separated by 8% SDS-PAGE and analyzed for their tyrosyl phosphorylation by immunoblotting. All pairs of raft and detergent-soluble fractions were run on the same gel and exposed for the same times.

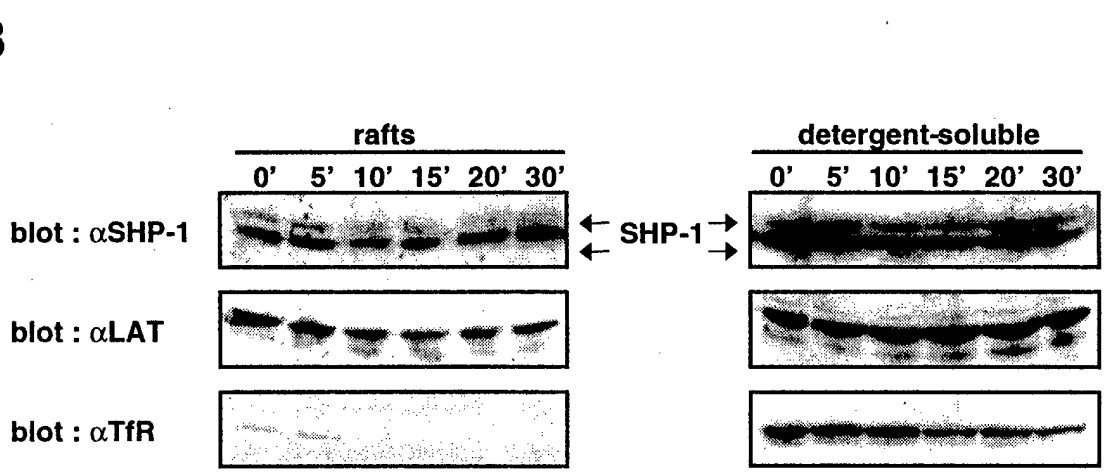
Figure 4: Catalytically inactive mutant of SHP-1 shows increased phosphorylation in lipid rafts compared to endogenous SHP-1. GST-SHP-$1_{\Delta P}$ [A] or GST-SHP-1_{wt} [B] expressing BYDP cells were stimulated for the indicated times with anti-CD3 plus anti-CD4. 0.5% Triton X-100 cell lysates were fractionated and 37.5% of combined fractions 2-4 (rafts) and 37.5% of combined fractions 9-10 (detergent-soluble) were immunoprecipitated using anti-SHP-1 antibodies, followed by immunoblotting with anti-pTyr antibodies. The immunoblots were stripped and reprobed for SHP-1. Bands corresponding to endogenous SHP-1 and exogenous GST-SHP-1 fusion proteins are marked. All pairs of raft and detergent-soluble fractions were run on the same gel and exposed for the same times.

Figure 5: TCR-induced phosphorylation of SHP-1 is dependent on intact lipid rafts. BYDP cells (6.5×10^7) treated with 10mM MβCD or vehicle (PBS) were stimulated with anti-CD3 plus anti-CD4 for the indicated times followed by fractionation of cell lysates. [A] 3.75% of the raft (fractions 2-4) and detergent-soluble (fractions 9-10) fractions were combined and analyzed for their phosphotyrosine content by immunoblotting. [B] 37.5% of the combined raft and

detergent-soluble fractions were immunoprecipitated using anti-SHP-1 antibodies, followed by immunoblotting with anti-pTyr antibodies. The immunoblots were stripped and reprobed for SHP-1. All pairs of raft and detergent-soluble fractions were run on the same gel and exposed for the same times. It is unclear why we detect reduced levels of SHP-1 in the MβCD-treated cells.

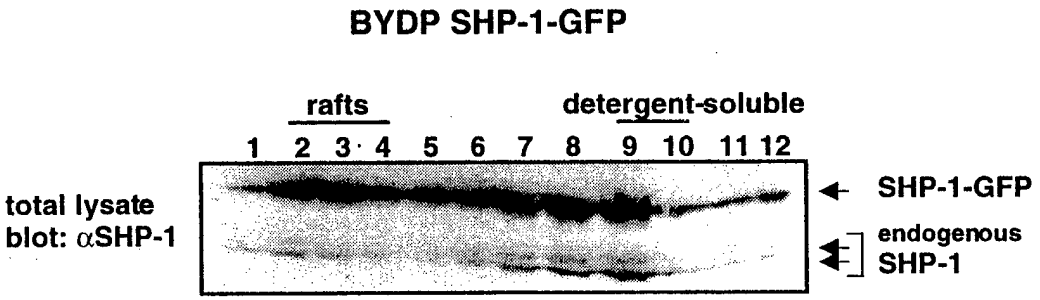
Figure 6: SHP-1 localizes to lipid rafts in primary thymocytes. Primary murine thymocytes (6.5×10^7) were stimulated for the indicated times with anti-CD3 and anti-CD4 followed by 0.5% Triton X-100 cell lysis and fractionation. 3.75% of the combined fractions 2-4 (rafts) and of the combined fractions 9-10 (detergent-soluble) were separated by 8% SDS-PAGE and analyzed for their tyrosyl phosphorylation by immunoblotting. 37.5% of the combined raft and detergent-soluble fractions were immunoprecipitated using anti-SHP-1 antibodies, followed by immunoblotting with anti-pTyr antibodies. The immunoblots were stripped and reprobed for SHP-1. All pairs of raft and detergent-soluble fractions were run on the same gel and exposed for the same times.





Johnson et al.
Figure 1

A



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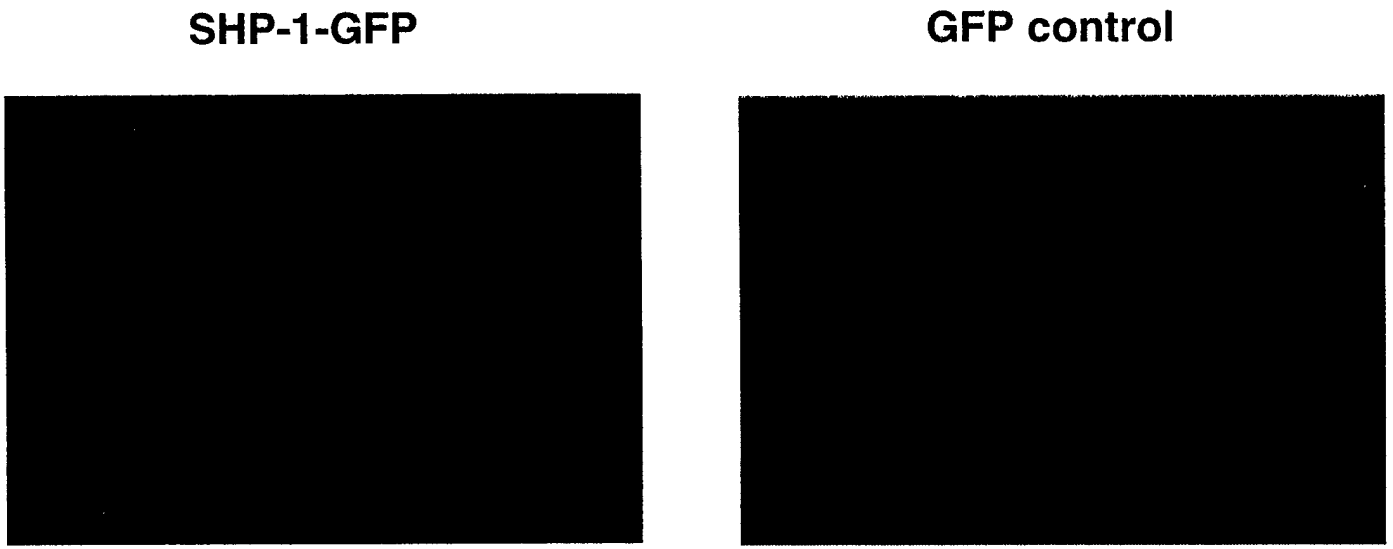
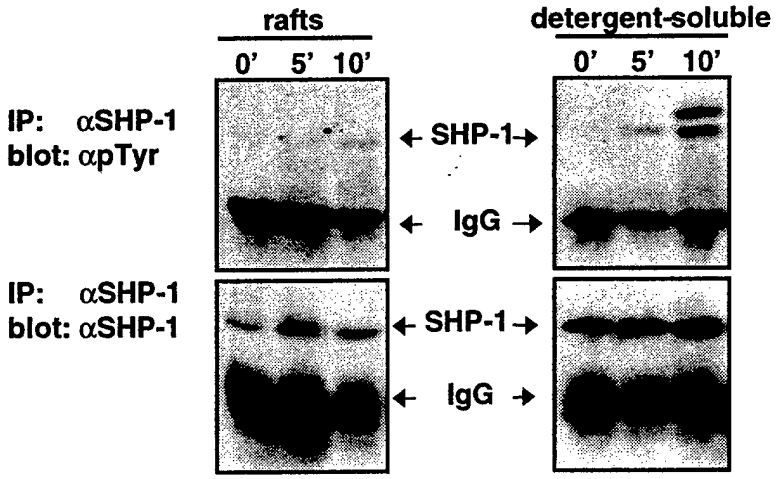


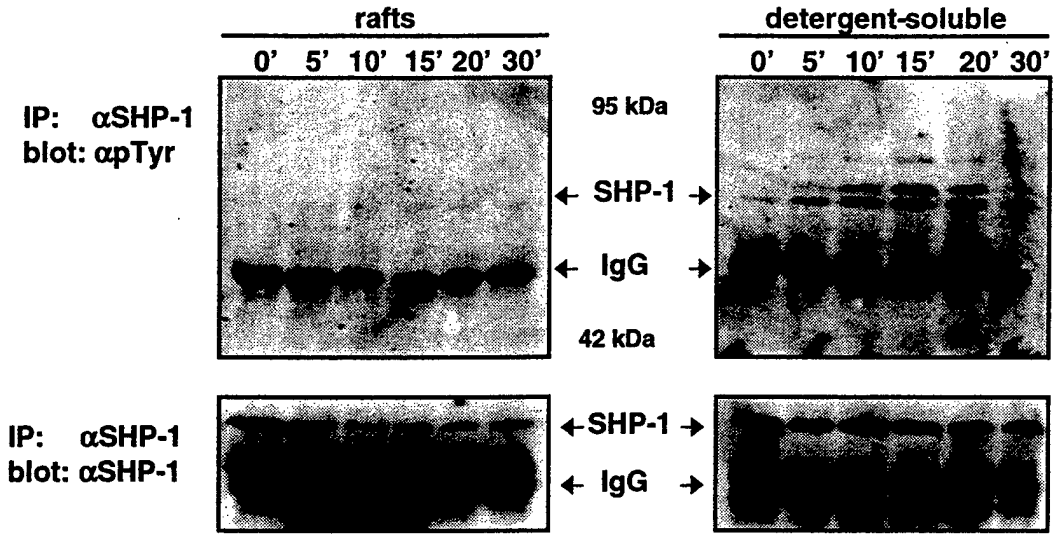
Johnson et al.
Figure 2

A



Johnson et al.
Figure 3A

B



C

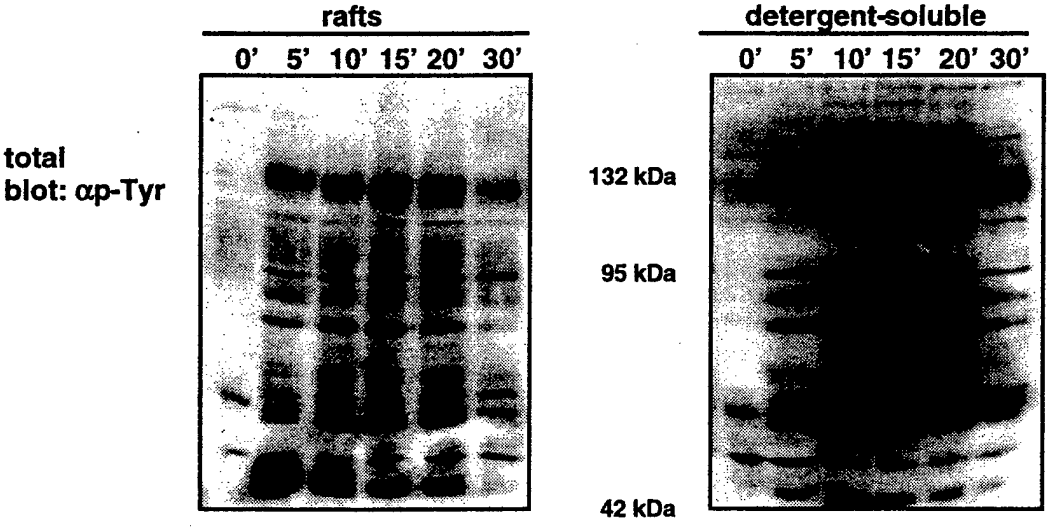


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Fig. 3 B and C

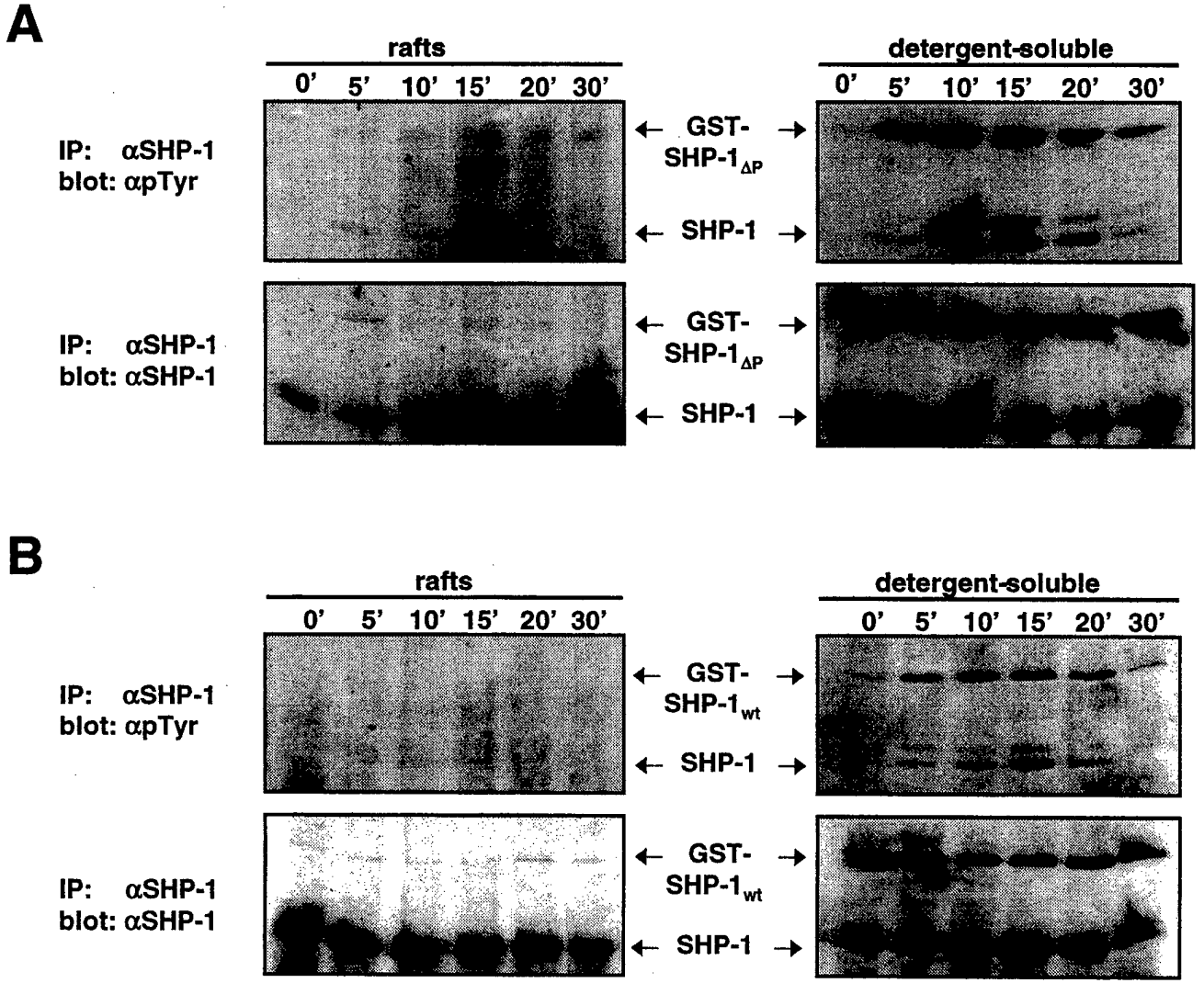

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Figure 4

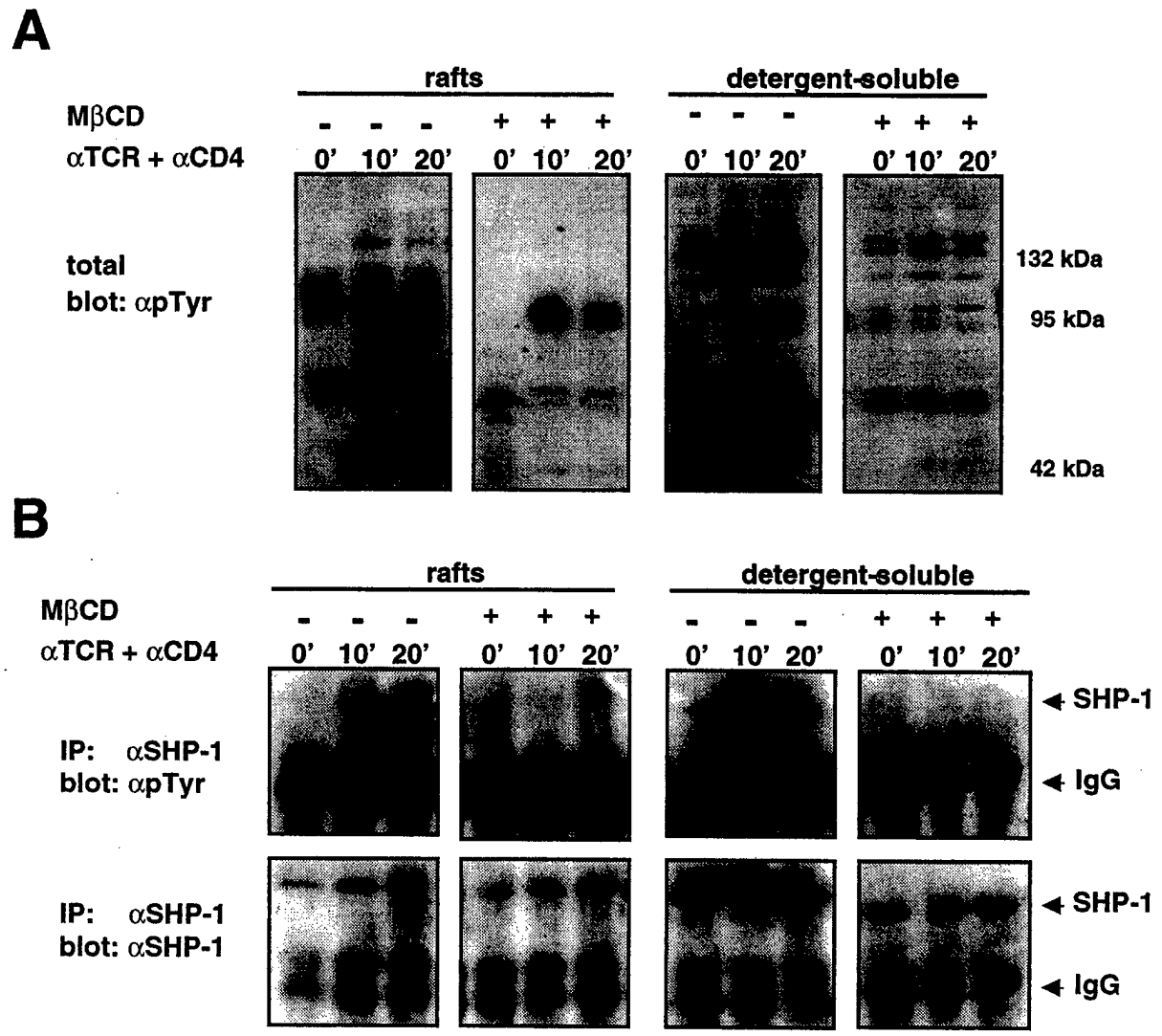

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Figure 5

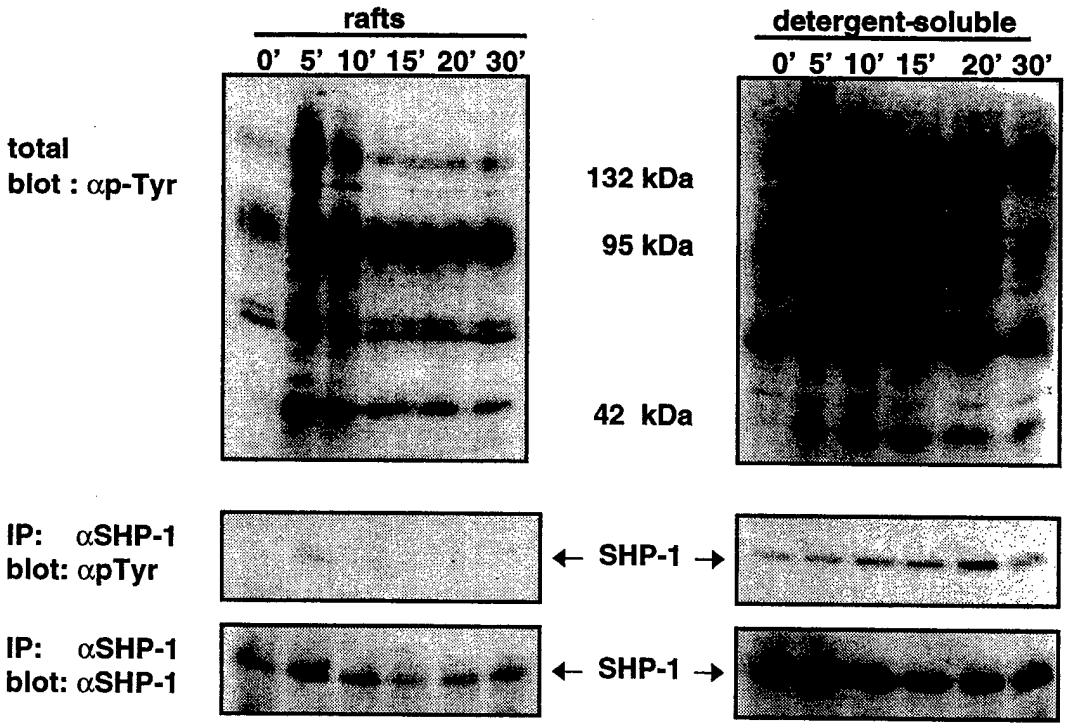

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Figure 6