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13. ABSTRACT (Maximum 200 Words) The long-term goal of our project is to understand how Syk functions as a tumor suppressor in breast cancer. Syk is expressed in normal breast epithelia and benign breast cancer cells, but is lost from many highly malignant cells. Since, malignant breast cancer cells are characterized by a loss of cell-cell interactions and a corresponding increase in cell-matrix interactions, we hypothesized that Syk would play an important role in the regulation of cellular adhesion. The localization of Syk within breast epithelial cells was studied by the expression of a chimeric protein consisting of Syk fused to enhanced green fluorescent protein (Syk-EGFP). The localization of Syk-EGFP as well as its activity varied depending on the growth properties of the cells as a function of the formation of adherens junctions between neighboring cells and of spreading on surfaces coated with ECM proteins. The expression of Syk modulated both the rate of formation of cell-cell contacts and the motility of breast cancer cells. These studies support a probable role for Syk in the regulation of cellular adhesion.				
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INTRODUCTION

Syk is a 72 kDa, nonreceptor, protein-tyrosine kinase that has been studied extensively as a critical component of the signal transduction machinery that couples multiple immune recognition receptors to numerous intracellular signaling pathways (1). Recent studies have indicated that the tissue distribution of Syk is much more widespread than initially thought (2). For example, Syk is found in epithelial cells, including mammary epithelial cells, where it exhibits a tumor suppressor function (3). Syk is lost from metastatic breast cancer cells and its expression is negatively correlated with several aspects of the malignant cell phenotype including cell motility and invasive growth (3, 4). The mechanisms by which it functions to regulate the growth properties of breast epithelial cells are unknown and are the focus of this project.

Malignant breast cancer cells differ from their non-transformed counterparts in a variety of ways including substantial differences in their adhesive properties, both cell to cell and cell to extracellular matrix. Malignant cells exhibit a reduced capacity to establish cell-cell contacts along with a corresponding enhanced ability to generate and turn-over the contacts with extracellular matrix proteins required for enhanced cell motility and invasiveness. Malignant cells also express a decreased dependency on these contacts for maintenance of cell viability and are resistant to programmed cell death resulting from loss of anchorage. To examine a role for Syk in these processes, we proposed to accomplish three specific objectives: 1) to determine the localization of Syk in malignant and nonmalignant cells through the expression of chimeric proteins consisting of Syk fused to green fluorescent protein (Syk-GFP); 2) to determine how this localization is altered through the clustering of cell surface adhesion receptors and 3) to identify and characterize the cellular proteins with which Syk interacts through the use of biochemical and genetic screens.

BODY

Task 1

Localization of Syk in breast cancer cell lines – In previous reports, we detailed some of the major findings regarding the localization of Syk in breast cancer cells. To summarize, we found that Syk was localized in multiple subcellular compartments in MCF10A, MCF7 and MDA-MB-231 breast epithelial cells including the nucleus, perinuclear region, and cytoplasm. In MCF10A and MCF7 cells, which exhibit substantial cell-cell contacts when grown to confluence, Syk also localized to the plasma membrane at points of contact between cells. The localization of Syk to the membrane was restricted to sites of cell-cell contacts in confluent or subconfluent cultures. The disruption of cell-cell contacts by the chelation of calcium resulted in a loss of adherens junctions, as judged by staining with anti-E cadherin antibodies, and a loss of membrane-associated Syk. Both adherens junctions and the membrane localization of Syk were restored by the reintroduction of calcium to the medium. These results suggested a possible role for Syk in the regulation of cell-cell contacts in breast epithelial cells (see below).

The movement of epithelial cells requires the formation and turnover of focal contacts formed in response to the interactions of cell surface integrins with proteins of the extracellular matrix. We examined the localization of Syk in MCF7 breast cancer cells shortly following their plating on culture dishes coated with the extracellular matrix protein, fibronectin. At this time,

cells begin to form focal contacts and begin to rearrange the cytoskeleton as they spread on the coated surface. Interestingly, Syk moves from the cytoplasm to the membrane at the edges of lamellipodia when cells begin to spread on the fibronectin-coated plate (Fig. 1). It is here that proteins such as Arp2/3 localize to initiate sites of actin polymerization. In fact, Syk co-localizes here with F-actin (not shown). At early time-points, Syk also co-localizes with vinculin, a protein marker for focal adhesions (Fig. 2). Syk does not, however, localize to the mature focal adhesions that form at later time points (Fig. 2). This suggests a possible role for Syk in a cell's initial responses to integrin ligation.

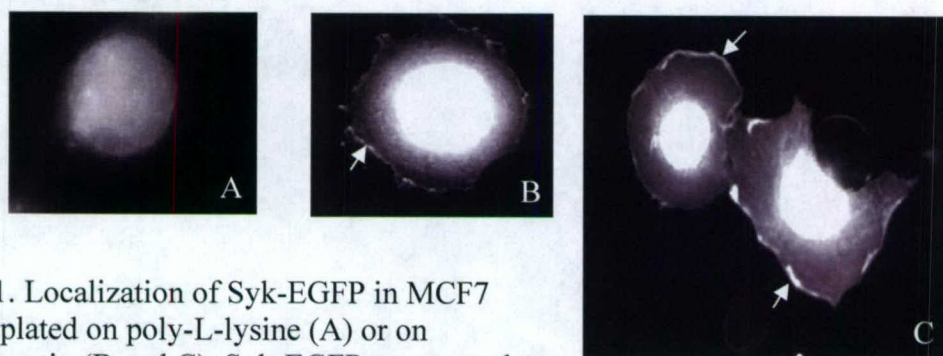


Fig. 1. Localization of Syk-EGFP in MCF7 cells plated on poly-L-lysine (A) or on fibronectin (B and C). Syk-EGFP moves to the edges of the cell membrane during spreading (arrows).

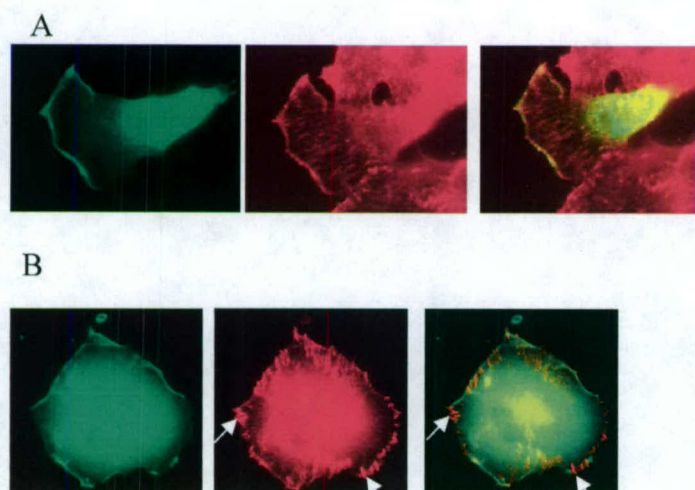


Fig. 2. Co-localization of Syk-EGFP (green) with vinculin (red) at 45 min (A) or 190 min (B) following plating of MCF7 cells on fibronectin. Syk-EGFP co-localizes (yellow) with vinculin at the 45 min time-point, but does not co-localize with vinculin at mature focal adhesions that form at 190 min (arrows).

To determine if Syk could interact with β -integrins at the cell surface, we treated MCF7 cells in suspension with antibodies against β_1 -integrin. Integrin clustering led to a rapid relocation of Syk from its normal distribution throughout the cell to one restricted primarily to the plasma membrane (Fig. 3).



Fig. 3. Localization of Syk-EGFP in MCF7 cells treated without (A) or with (B) anti- β_1 integrin antibodies.

Task 2

Syk and cellular adhesion-Our second task was to characterize the role of Syk in breast cancer cells. In our previous reports, we demonstrated that Syk played a role in the formation and/or stabilization of cell-cell contacts. The expression of Syk enhanced the rate of cell aggregation while the expression of catalytically inactive Syk or the inhibition of Syk inhibited the formation of cell-cell contacts.

The localization of Syk at sites of cell-matrix contacts suggested a possible role for the kinase in the regulation of cell motility. To examine this, we compared the ability of Syk versus catalytically-inactive Syk to alter the motility of a highly invasive breast epithelial cell line, MDA-MB-231. Cells were transiently transfected with EGFP (enhanced green fluorescent protein), Syk-EGFP or KD-Syk-EGFP (kinase-dead or catalytically inactive form of Syk-EGFP) so that transfected cells could be identified by fluorescence. The ability of transfected versus non-transfected cells to migrate through the pores of a polycarbonate filter precoated with matrix proteins was measured. The expression of Syk in the MDA-MB-231 cells consistently inhibited cell migration (Fig. 4). Interestingly, the expression of KD-Syk-EGFP (KD) consistently enhanced cell migration.

We also used this assay to begin to examine some of requirements (in addition to

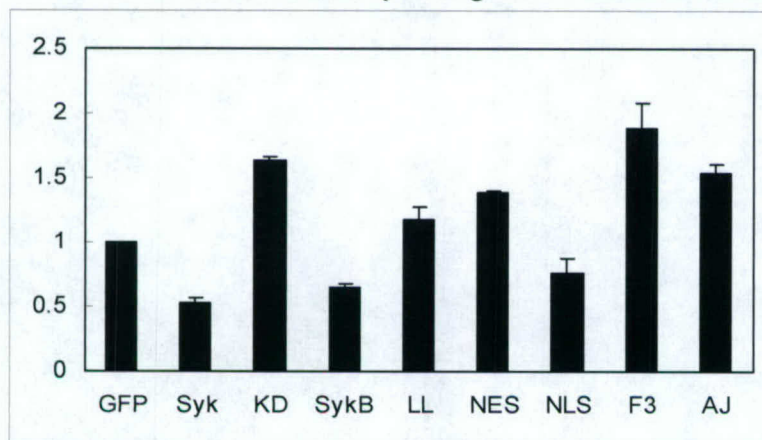


Fig. 4. Effect of Syk on the motility of MDA-MB-231 cells. Cells were transiently transfected with plasmids expressing EGFP alone or each of the indicated Syk-EGFP fusion proteins. Cells were allowed to migrate through membrane pores for 16 h. Cells moving through the membrane were stained with DAPI to detect total cells and examined by fluorescence microscopy to detect transfected cells. The relative ratios of transfected to nontransfected cells were calculated and further normalized to that of EGFP-expressing cells.

The requirement for catalytic activity and for specific tyrosines on Syk for the regulation of cancer cell motility suggests both the activation of the kinase and its phosphorylation on

catalytic activity) for the Syk-dependent inhibition of motility. SykB, an alternatively spliced variant of Syk, retained an ability to inhibit migration. Removal of the entire linker B region (the region between the SH2 domains and the kinase domain) (LL) abrogated Syk's inhibitory activity as did the removal of three sites of tyrosine phosphorylation in the linker B

region at positions 317, 342 and 346 (F3) or the removal of two of these three sites (at 342 and 346) (AJ). Interestingly, addition of a nuclear export signal to the end of Syk-EGFP (NES) also blocked its activity, while addition of a nuclear localization signal (NLS) had much less effect. The basis for the requirement for Syk's ability to translocate into and out of the nucleus is not known.

tyrosine are important. To determine if integrin ligation affected the phosphorylation of Syk, we examined the phosphorylation status of the protein following the treatment of Syk-expressing MCF7 cells with antibodies against β_1 -integrin. As shown in Fig. 5, aggregation of integrins led to an enhancement in the phosphorylation of Syk on tyrosine as detected by Western blotting with antiphosphotyrosine antibodies.

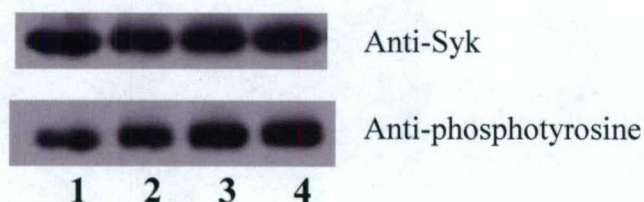


Fig. 5. MCF7 cells expressing Syk were treated with anti-integrin antibodies for 0 (1), 5 (2), 15 (3) or 30 (4) min. Syk was detected by Western blotting with an anti-Syk antibody (upper panel) and phospho-Syk with an anti-phosphotyrosine antibody (lower panel).

We are currently examining candidates for the proteins with which Syk might interact based on our analyses of Syk-interacting proteins in mammary epithelial cells identified in our yeast two-hybrid screen.

Yeast two-hybrid analysis of Syk-interacting proteins—In our previous report, we detailed the results of our yeast two-hybrid analysis of Syk-interacting proteins encoded by a cDNA library derived from human mammary gland. One protein that we identified, tensin2, is a putative cytoskeletal protein related in structure to tensin (5). Tensin is an interesting protein that is recruited to focal adhesions early during their formation. To determine if tensin2 is also a focal adhesion protein in epithelial cells, we examined its localization in cells using vinculin as a marker. As shown in Fig. 6, a portion of tensin2 co-localizes with vinculin in stable focal adhesions. Interestingly, at short times following the plating of MCF7 cells on fibronectin, tensin2 co-localizes at the cell membrane with both F-actin and Syk (not shown). This suggests a possible functional significance between the interactions of Syk and tensin2 in the initial localization of each to the plasma membrane in cells spreading on fibronectin.

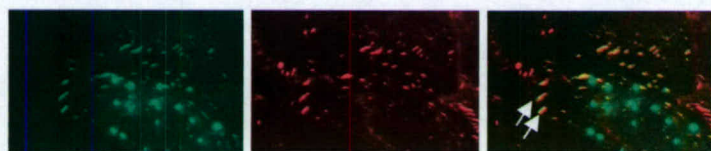
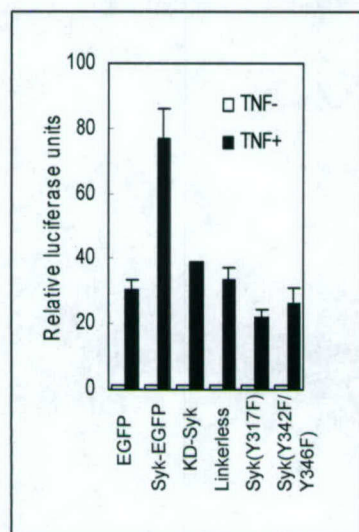


Fig. 6. Co-localization of GFP-tensin2 (green) with vinculin (red) in MCF7 cells. Examples of focal adhesions where both GFP-tensin 2 and vinculin are found (yellow) are indicated by arrows.

A role for Syk in regulation of NF κ B- To examine a role for Syk in TNF-signaling, we examined the ability of ectopically expressed Syk to alter the responses of MCF7 cells to TNF by

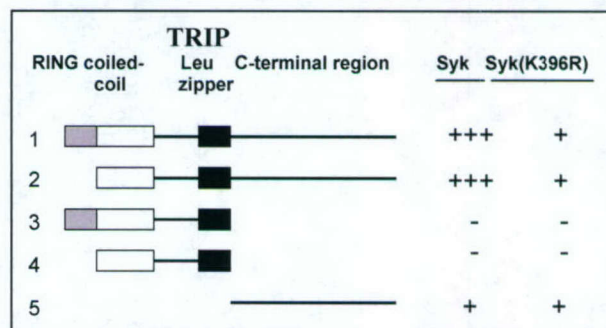
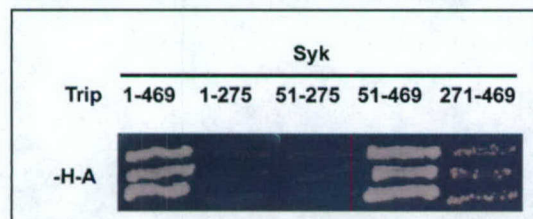


measuring the activation of NF κ B using a luciferase reporter assay. As shown in Fig. 7 at left, the activation of NF- κ B was enhanced more than two-fold by the expression of Syk-EGFP, but was unaffected by transfection with an empty vector (cDNA3.1) or a vector coding for EGFP alone. Enhancement of signaling required Syk's catalytic activity as the expression of Syk(K396R) (KD-Syk) had no significant effect on TNF-signaling to NF- κ B. In preliminary experiments to examine the regions of Syk required to enhance the TNF-dependent activation of NF- κ B, we found that tyrosine residues within the linker B region were required as expression of mutant forms of Syk in which either Tyr-317 or Tyr-342 and -346 were converted to phenylalanines failed to enhance signaling. This suggests that the requirement for Syk's catalytic activity likely reflects a necessity for autophosphorylation followed by the subsequent recruitment of proteins with SH2 or other

phosphotyrosine-binding domains to sites of tyrosine-phosphorylation on Syk.

From a yeast two-hybrid screen using full-length Syk as bait and as prey, proteins coded by a human mammary gland cDNA library, we isolated a cDNA coding for TRIP (Traf-interacting protein), a protein that has been reported to negatively regulate coupling of the tumor necrosis factor (TNF) receptor to the activation of NF κ B (6). A comparison of Syk and Syk(K396R) (catalytically inactive Syk) as baits indicated that, while TRIP interacted strongly with the active kinase, it also interacted weakly with the catalytically inactive enzyme. This is in contrast to control prey plasmids coding for the SH2 domains of Lck or p85, both of which bind to auto-phosphorylation sites within linker B and thus interact only with active Syk.

We have prepared constructs coding for various regions of TRIP and Syk to determine in yeast which of these mediate the interaction between the two proteins. A set of TRIP constructs, shown on the right, was tested for interactions with Syk. Constructs 1 and 2 interacted strongly with Syk, but constructs 3 and 4 failed to interact. Construct 5 did interact, but less robustly than 1 or 2. An example of this screen is shown below. We then screened the same



constructs against Syk(K396R). Constructs 1, 2 and 5 all interacted weakly, but to the same extent, with inactive Syk. Thus, the C-terminal domain is

necessary and sufficient for mediating the interaction of TRIP with Syk or Syk(K396R), but other regions of the protein appear to strengthen the binding of TRIP to the active kinase. The binding of TRIP to TRAF1 or 2 required either the coiled-coil or leucine zipper domains of TRIP (6). It appears possible that TRIP could interact with TRAF and Syk simultaneously.

KEY RESEARCH ACCOMPLISHMENTS

- Syk localizes to multiple subcellular compartments in breast epithelial cells including the plasma membrane at sites of cell-cell contacts. This localization requires the establishment of E cadherin-mediated adherens junctions.
- The expression of Syk in breast epithelial cells enhances the rate of formation of cell-cell contacts.
- In breast epithelial cells freshly plated on fibronectin, a fraction of Syk migrates to the plasma membrane and co-localizes with markers of integrin-mediated focal contacts.
- The expression of Syk in highly motile breast cancer cells decreases cell motility while the expression of catalytically inactive Syk enhances motility. Syk-dependent inhibition of motility requires sites of tyrosine-phosphorylation within the Syk linker B region.
- Syk interacts with multiple proteins from mammary gland as detected by a genetic screen. These include, among others, tensin2 and Trip. Trip binds to Syk via sequences located within its C-terminus.
- The over-expression of Syk in breast epithelial cells enhances the TNF-mediated activation of NF κ B. This enhancement requires sites of tyrosine-phosphorylation located within the linker B region.

REPORTABLE OUTCOMES

Abstracts:

1. Ghate, U., and Geahlen, R.L. (2002) Role of the cytoplasmic tyrosine kinase, Syk, in breast epithelial cells. FASEB J., A1198.
2. Zhang, X., Shrikande, U., Zhou, Q., and Geahlen, R.L. (2004) Localization of Syk in breast cancer cells and a role for the tyrosine kinase in cell-cell adhesion. FASEB J. 18, C107.

Manuscripts:

1. Shrikhande, U., Zhang, X., Hu, J., Zhou, Q., Harrison, M.L., and Geahlen, R.L. (2004) A role for the protein-tyrosine kinase, Syk, in regulating cell-cell adhesion in breast cancer cells. (Submitted for publication).

CONCLUSIONS

In breast epithelial cells, the Syk kinase is localized in multiple subcellular compartments including the cytoplasm and nucleus. In response either to the formation of cell-cell contacts for

of cell-extracellular matrix contacts, a fraction of Syk translocates to the plasma membrane. This suggests possible roles of the kinase in modulating the adhesive properties of epithelial cells. In fact, we observed that the expression of Syk enhances the rate at which cells form cell-cell contacts, while, at the same time, decreasing the rate at which cells are able to migrate on ECM proteins. Decreases in the stability of cell-cell contacts and increases in cell motility are both properties of malignant breast cancer cells and may explain, at least in part, the consequences of the loss of Syk to the growth properties of breast cancer cells.

The molecular mechanisms by which Syk functions to alter these properties of breast cancer cells are incompletely understood. However, we do know that these effects require both the catalytic activity of Syk and its phosphorylation on tyrosine residues located within the linker B region. This suggests a role for SH2 domain-containing, Syk-interacting proteins in the downstream signaling pathways that are modulated by Syk. The initial activation of Syk can occur through the clustering of cell surface integrins and integrins have been implicated in both the formation of cell-cell and cell-ECM contacts.

The regulation of NF κ B is an important factor for the survival of breast cancer cells. Elevated, constitutive activation of NF κ B is commonly associated with malignant cells of many types including breast cancer cells. We have identified a role for Syk in the augmentation of TNF-induced activation of NF κ B in breast epithelial cells. Interestingly, TNF is a survival factor for nonmalignant cells, which are Syk-expressing cells, but is often an apoptotic factor for highly malignant breast cancer cells, which do not express Syk. A clue to the participation of Syk in the TNF-signaling pathway was the identification of Trip, a TRAF-interacting protein, as a binding partner of Syk.

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6. S.Y. Lee, S.Y. Lee, and Y. Choi, TRAF-interacting protein (TRIP): a novel component of the tumor necrosis factor receptor (TNFR)- and CD30-TRAF signaling complexes that inhibits TRAF2-mediated NF- κ B activation. *J. Exp. Med.* 185 (1997) 1275-1285.