Signaling and Regulation

Prolactin Receptor–Integrin Cross-Talk Mediated by SIRPα in Breast Cancer Cells

Traci Galbaugh, Yvonne B. Feeney, and Charles V. Clevenger

Abstract

The hormone prolactin (PRL) contributes to the pathogenesis of breast cancer in part through its activation of Janus-activated kinase 2 (Jak2)/signal transducer and activator of transcription 5 (Stat5), a PRL receptor (PRLr)–associated pathway dependent on cross-talk signaling from integrins. It remains unclear, however, how this cross-talk is mediated. Following PRL stimulation, we show that a complex between the transmembrane glycoprotein signal regulatory protein-α (SIRPα) and the PRLr, β1 integrin, and Jak2 in estrogen receptor–positive (ER+) and ER– breast cancer cells is formed. Overexpression of SIRPα in the absence of collagen 1 significantly decreased PRL-induced gene expression, phosphorylation of PRLr-associated signaling proteins, and PRL-stimulated proliferation and soft agar colony formation. In contrast, overexpression of SIRPα in the presence of collagen 1 increased PRL-induced gene expression; phosphorylation of Jak2, Stat5, and Erk; and PRL-stimulated cell growth. Interestingly, overexpression of a tyrosine-deficient SIRPα (SIRPα-4YF) prevented the signaling and phenotypic effects mediated by wild-type SIRPα. Furthermore, overexpression of a phosphatase-defective mutant of Shp-2 or pharmacologic inhibition of Shp-2 produced effects comparable with that of SIRPα-4YF. However, the tyrosine phosphorylation of SIRPα was unaffected in the presence or absence of collagen 1. These data suggest that SIRPα modulates PRLr-associated signaling as a function of integrin occupancy predominantly through the alteration of Shp-2 activity. This PRLr-SIRPα-integrin complex may therefore provide a basis for integrin-PRLr cross-talk and contribute to the biology of breast cancer. Mol Cancer Res; 8(10); 1413–24. ©2010 AACR.

Introduction

Prolactin (PRL) is a pituitary hormone required for proliferation and differentiation of normal mammary epithelium and the stimulation of lactogenesis (1-3). The canonical PRL signaling pathway begins with PRL binding, which induces PRL receptor (PRLr) activation of Janus-activated kinase 2 (Jak2). Subsequently, Jak2 activates several downstream signaling proteins involved in proliferation, differentiation, survival, and motility, such as signal transducer and activator of transcription 5 (Stat5), extracellular signal-regulated kinase (Erk), and Akt (1, 4).

Recently, epidemiologic studies have revealed that higher levels of circulating PRL in premenopausal and postmenopausal women increase a woman’s risk of breast cancer (5). It has also been shown that PRL expression in tumors is higher than in normal or hyperplastic epithelium (6) and that the PRLr is expressed in 95% of breast tumors (7-9). In addition, the overexpression of PRL or the PRLr in mice results in enhanced tumor formation (10-12). Integrins have been shown to be critical for PRLr signaling in normal mammary epithelial cells (MEC; ref. 13). One report showed that β1 integrin is necessary for PRL-driven MEC differentiation (14). Furthermore, mice lacking β1 integrin have compromised Stat5 activation and milk production (15). Despite these insights, the mechanism of Stat5 activation and milk production in the context of breast cancer is not fully understood.

SIRPs are transmembrane glycoproteins that were first identified as novel proteins that bind Src homology 2 (SH2)–containing tyrosine phosphatases on phosphorylation of their tyrosine residues (16). There are two subfamilies of SIRPs (SIRPα and SIRPβ) that differ only in the length of their cytoplasmic region. SIRPα contains immunoglobulin-like repeats in its extracellular domain, as well as four tyrosine residues and a proline-rich region in its cytoplasmic domain. SIRPα is ubiquitously expressed in human tissue, and recent studies show that alterations of SIRPα expression may contribute to the pathogenesis of acute and chronic myeloid leukemia and hepatocellular and breast carcinoma (16-19). Meta-analysis of publicly available expression arrays [Oncomine (Compendia Bioscience) was used for analysis and visualization] reveals that SIRPα is significantly overexpressed in triple-negative breast cancer. In addition, unpublished anti-SIRPα immunohistochemistry of primary human breast cancer reveals that SIRPα is overexpressed at
the invasive edge of the tumor. SIRPα binds to CD47 and has been implicated in signaling with integrins (20), growth hormone (GH; ref. 21), insulin, and epidermal growth factor (EGF; ref. 20). The activation of these pathways induces tyrosine phosphorylation of SIRPα and its subsequent association with the SH2 domain-containing protein tyrosine phosphatases Shp-1 and Shp-2. Shp-2 is ubiquitously expressed in vertebrate cells and tissues and plays a critical role in development (22-24). Typically, protein tyrosine phosphatases negatively regulate cellular signaling by opposing the effects of protein tyrosine kinases. Interestingly, it has been shown that Shp-2 can promote the activation of Ras-MAPK (mitogen-activated protein kinase) through various growth factor (25). In contrast, Shp-2 has been shown to negatively regulate Stat family proteins including Stat-1, Stat-3, and Stat-5 in different cell types by the dephosphorylation of Jak family kinases. In the mammary gland, Shp-2 regulates the downregulation of GH receptor (GHr) and its subsequent association with Shp-2. Such interactions seem functionally significant, as they result in the downregulation of GHr/Jak2 signaling (21, 28).

SIRPα has also been shown to be important in integrin-mediated signaling. Integrins are critical extracellular matrix (ECM) receptors that have been identified as important regulators of MEC proliferation, differentiation, survival, apoptosis, and migration (29, 30). The extracellular domains of integrins bind ECM proteins such as collagen, fibronectin, and laminins and activate several salient signaling networks that include Jak2/Stat5, phosphatidylinositol 3-kinase (PI3K), and MAPK (31-33). Integrin-mediated adhesion has been shown to promote SIRPα phosphorylation, which contributes to cell migration primarily through Src family kinases and focal adhesion kinase (Fak; ref. 29). Thus, SIRPα may contribute to integrin-mediated cell migration.

Given the role of SIRPα and Shp-2 in GHr and integrin signaling, it was hypothesized that the SIRPα/Shp-2 complex might play a role in altering PRLr/PRLr-mediated signaling in breast cancer cells. Our findings have shown that PRLr, β1 integrin, and SIRPα interact in both estrogen receptor-positive (ER+) and ER− breast cancer cell lines, whereas SIRPα expression/function is associated with an ECM context-dependent modulation of PRLr signaling. These data reveal a novel role for SIRPα in facilitating cross-talk between the PRLr and β1 integrin and provide a basis for ECM regulation of PRLr action in breast cancer.

Materials and Methods

Cell culture

T47D (American Type Culture Collection) cell lines were grown in complete medium consisting of DMEM (Life Technologies), 10% fetal bovine serum (Sigma-Aldrich), and 1% penicillin-streptomycin (Life Technologies). Defined PRL-deficient medium consisted of phenol-free DMEM, 0.01% bovine serum albumin, and 1% penicillin-streptomycin. MDA-MB-231 (American Type Culture Collection) cell lines were grown in complete medium consisting of DMEM/F12, 5% fetal bovine serum, 1% penicillin-streptomycin, 2 mmol/L glutamine (Life Technologies), 1% sodium pyruvate (Sigma), and 2× nonessential amino acids (Life Technologies).

Stable cell line production

The T47D and MDA-MB-231 breast cancer cell lines were used to generate stable transfectant pools expressing either a control [green fluorescent protein (GFP)], wild-type SIRPα (WT-SIRPα; courtesy of Dr. E. Fuchs, Northwestern University, Chicago, IL), or a SIRPαΔ4YF (described in ref. 34, courtesy of Dr. C. Carter-Su). These stable cell lines were made by inserting each plasmid into a lentiviral vector (pLenti6/V5-Dest, Invitrogen) containing a V5 tag and tested for expression by transient transfection into HeK293 cells. HeK293T cells were transduced with the lentiviral plasmids in addition to three other viral packaging plasmids with LF2000 according to the manufacturer’s protocol (Invitrogen). Virus medium was harvested and used directly to infect the T47D and MDA-MB-231 breast cancer cell lines. Infected cells were grown in the presence of blastocidin (10 μg/mL), and levels of overexpressed proteins were assessed by immunoblot analysis.

Collagen coating and β1 integrin neutralization

Collagen I (Bovine, Cultrex and Rat Tail, Upstate) was diluted to a concentration of 3 mg/mL in deionized H2O and added to tissue culture plates overnight at 4°C. Collagen 1 was aspirated off, and wells were rinsed with deionized H2O and allowed to air dry. Following collagen 1 coating, an anti-β1 integrin (Chemicon) antibody was diluted to 15 μg/mL in 50 mmol/L Tris (pH 8.0) and added to the collagen 1–coated dishes overnight at 4°C. The next day, heat-denatured 3% bovine serum albumin–PBS was added to the tissue culture dishes for 30 minutes at 37°C. This was aspirated off, and cells were then seeded accordingly.

Luciferase assay

T47D transfectant pools were plated in the presence or absence of collagen 1, grown in complete medium, and transiently transfected with 1 μg of pGL4-CISH reporter as well as DN-Shp-2 (courtesy of Dr. E. Fuchs, Northwestern University, Chicago, IL) and 10 ng of pGL4-Renilla according to the LF2000 manufacturer’s protocol (Invitrogen). Twenty-four hours after transfection, the cells were arrested in defined PRL-deficient medium for an additional 24 hours. The cells were stimulated with 250 ng/mL of PRL (courtesy of Dr. A. Kossiakoff, University of Chicago, Chicago, IL) for 24 hours and analyzed for dual luciferase on a Victor3V (Perkin-Elmer). Results were reported as luciferase/Renilla ratio ± SE.

Immunoprecipitation and immunoblotting

T47D, T47D transfectant pools, and MDA-MB-231 cells were grown in complete medium and arrested in

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defined PRL-deficient medium for 24 hours. They were treated with 250 ng/mL of PRL for times indicated. The cells were harvested, lysed in radioimmunoprecipitation assay buffer (35), and immunoprecipitated with either SIRPα (1 μg/sample; Chemicon), Jak2 (1 μg/sample; Cell Signaling), β1 integrin (1 μg/sample; Chemicon), or PRLr (1 μg/sample; Zymed) antibodies as well as 40 μL of a 50% solution of protein A–agarose beads (Invitrogen) overnight at 4°C. For immunoblots, equivalent amounts of protein were separated by SDS-PAGE and transferred to polyvinylidene difluoride filters. The filters were blocked in 0.6% Blotto for 1 hour. Following blocking, the blots were incubated with the appropriate primary antibody overnight at 4°C and then with horseradish peroxidase–conjugated secondary antibody for 1 hour at room temperature. Chemiluminescence was detected with ECL Plus (Amersham), and images were collected on a charge-coupled device camera (Fuji). All blots were quantitated via scanning densitometry (Image Gauge software, Fuji), and numbers reported represent fold decrease or increase compared with the control transfectants.

Proliferation assay

T47D and MDA-MB-231 transfectants were plated in the presence and absence of collagen 1. Twenty-four hours after plating, the transfectants were arrested in defined PRL-deficient medium for 24 hours. The cells were then treated with or without 250 ng/well of PRL for 72 hours, and [3H]Thymidine (0.5 μCi/well) was added for 4 hours. The cells were lysed on a Filtermat Harvester (Perkin-Elmer) and transferred to a Filtermat (Wallac) and read with a Microbeta Luminescence counter (Perkin-Elmer).

Soft agar colony formation

T47D transfectant pools were suspended in 0.3% agar/regular growth medium/PRL mix in the presence or absence of 10% collagen 1. Once solidified, an overlay of regular growth medium/PRL (250 ng/mL) was added and changed every 2 days. Cells were photographed 24 hours after suspension and then, starting on day 7, on Magnafire software (Optronics). Colony size and numbers were counted with Image software (NIH), and results were analyzed by comparing the number and size of colonies of control transfectant pools with those of the WT-SIRPα transfectants.

Statistical analysis

All experiments described were done no less than three times. Statistical analysis was done on GraphPad Prism 4 (GraphPad Software), and results are shown as the means with error bars depicting ±SE. One-way ANOVA statistical tests were done and P values were reported.

Results

SIRPα interacts with the PRLr, β1 integrin, and Jak2 in breast cancer cells

Given literature that indicated that SIRPα could influence GHR signaling (21, 34), we sought to examine if SIRPα could interact and influence PRLr action in the context of breast cancer. To that end, the interaction of SIRPα with the PRLr, β1 integrin, and Jak2 was tested (Figs. 1 and 2). Coimmunoprecipitation analysis revealed endogenous interactions between the PRLr and SIRPα as well as the PRLr and β1 integrin in T47D ER+ breast cancer cells (Fig. 1A) and MDA-MB-231 ER+ breast cancer cells (Fig. 1B). Although SIRPα and the PRLr were shown to interact in T47D cells in the absence of PRL, ligand stimulation enhanced this interaction, which peaked at 15 minutes. In contrast, coimmunoprecipitation revealed that the interaction between the PRLr and β1 integrin was PRL dependent, with their association visualized after 5 minutes of PRL stimulation. The interaction between the PRLr and SIRPα in MDA-MB-231 was comparable with that seen in the T47D cells (Fig. 1B). However, in these cells, unlike T47D, the data suggested that β1 integrin and the PRLr seem to be associated with each other in the absence of PRL, and this interaction was enhanced on PRL stimulation.

To better examine the interactions of SIRPα with β1 integrin and Jak2, three stable T47D transfectant pools (Fig. 2A, B, and E) were created. These three stable transfectant pools were generated to overexpress WT-SIRPα, GFP (control), or a nonphosphorylated mutant SIRPα (SIRPα-4YF), which is inactive in these cells, therefore mimicking the control transfectants with endogenous SIRPα. Overexpression was assessed in WT-SIRPα and SIRPα-4YF transfectant pools by immunoblot analysis. Investigation in these transfectants revealed 2-fold higher SIRPα protein expression than that of the control (Fig. 2E). Coimmunoprecipitation analysis of these transfectants (Fig. 2C) revealed that SIRPα interacted with β1 integrin in the control transfectants as well as in the SIRPα overexpression transfectants within 1 minute of PRL stimulation, and this interaction was maintained through 60 minutes (Fig. 2A). Interestingly, the mutant form of SIRPα also interacted at early time points with β1 integrin, which suggested that the four tyrosine residues in the SIRPα intracellular domain are not required for this interaction. Additionally, coimmunoprecipitation analysis also revealed that SIRPα interacted with Jak2, independent of ligand addition in the SIRPα overexpression transfectants (Fig. 2B). Of importance, each coimmunoprecipitation was done three separate times and reported as fold induction compared with the untreated control sample (Figs. 1A and B, and 2C and D). Taken together, these associations confirmed that SIRPα, the PRLr, β1 integrin, and Jak2 are brought into close proximity by PRL stimulation, opening the possibility for functional cross-talk.

PRL-induced cytokine-inducible SH2-containing protein promoter luciferase activity and protein expression is enhanced by SIRPα in a collagen 1/β1 integrin–dependent manner

Given the requirement for integrins during PRLr signaling (14), PRL-responsive cytokine-inducible SH2-containing
protein (CISH) reporter luciferase activity was used to examine the effects of SIRPα overexpression on PRL-mediated gene expression in the presence and absence of several different ECM substrates (Table 1). Results verified that overexpression of SIRPα inhibited PRL-induced CISH promoter luciferase activity in transfectants when plated on the ECM proteins laminin and fibronectin. These ECM proteins are typically found in the basement membrane niche of the normal ductal-lobular unit. In contrast, SIRPα overexpression transfec-
tant pools plated on collagen 1 (a structural protein of the interstitial space, whose overexpression in the desmo-
plastic stroma of breast cancer contributes to ECM stiff-
ess) showed an enhancement of PRL-induced CISH promoter luciferase activity compared with the control or SIRPα-4YF transfectant pools (Table 1 and Fig. 3A). In contrast, the transfectants overexpressing SIRPα plated in the presence of collagen 1 showed a significant increase in PRL-
induced CISH promoter luciferase activity (Fig. 3B). Similar results were seen with the lactogenic hormone response element, another PRL-responsive luciferase reporter (data not shown). To confirm the results observed by luciferase reporter assay, endogenous PRL-dependent CISH protein expression was also assessed in the presence or absence of collagen 1. These analyses revealed that the transfectant pools overexpressing SIRPα plated in the absence of collagen 1 showed a significant decrease in PRL-induced CISH promoter luciferase activity. This effect was lost in transfectants that overexpressed the control and SIRPα-4YF constructs (Fig. 3A). In contrast, the transfectants overexpressing SIRPα plated in the presence of collagen 1 showed a significant increase in PRL-
induced CISH promoter luciferase activity (Fig. 3B). Similar results were seen with the lactogenic hormone response element, another PRL-responsive luciferase reporter (data not shown). To confirm the results observed by luciferase reporter assay, endogenous PRL-dependent CISH protein expression was also assessed in the presence or absence of collagen 1. As noted in Fig. 3C, endogenous as well as PRL-dependent CISH protein expression was inhibited in the cells overexpressing SIRPα in the absence of collagen 1. In addition, as before, cells overexpressing SIRPα plated in the presence of collagen 1 showed an increase in PRL-dependent CISH protein expression (Fig. 3D). Thus, these data revealed that SIRPα overexpression differentially regulated PRL-induced CISH promoter luciferase activity and endogenous PRL-dependent CISH protein expression.

**FIGURE 1.** Interactions between the PRLr, SIRPα, and β1 integrin in ER+ and ER− breast cancer cells. A and B, T47D (ER+) and MDA-MB-231 (ER−) cells were treated with PRL (250 ng/mL) for times indicated. Whole-cell lysates were immunoprecipitated (IP) and analyzed by Western blotting (IB). All results reported were representative of three individual experiments. Each of the three experimental blots were quantitated. Columns, mean fold increase compared with the control transfectants; bars, SE. *, P < 0.05; **, P < 0.001; ***, P < 0.0001.
To further assess the involvement of β1 integrin on PRL-induced CISH promoter luciferase activity in the presence of collagen 1, a neutralizing anti-β1 integrin antibody was used. Data in Fig. 4 revealed that transfectants overexpressing SIRPα plated in the presence of collagen 1 and anti-β1 integrin showed a reduction in PRL-induced CISH promoter luciferase activity compared with the transfectants overexpressing SIRPα plated in the presence of collagen 1 only. Interestingly, the reduction resembled results observed in the cells overexpressing SIRPα plated in the absence of collagen 1. Consequently, the data revealed that SIRPα-mediated potentiation of PRL-induced CISH promoter luciferase activity is dependent on the activity of β1 integrin in the presence of collagen 1.

Differential regulation of PRLr signaling as a function of SIRPα overexpression

Because SIRPα overexpression modulated PRL-induced gene expression, it was reasoned that this might be due to its modulation of downstream molecules in the PRLr signaling network. Results in Fig. 5 showed that SIRPα overexpression inhibited the phosphorylation of Jak2, Stat5, and Erk in the absence of collagen 1. In contrast, in the presence of collagen 1, SIRPα overexpression enhanced the phosphorylation of Jak2, Stat5, and Erk. As anticipated, the SIRPα-4YF transfectants (data not shown) showed similar results to that of the control transfectants, suggesting that the four tyrosine phosphorylation sites were necessary for the phosphorylation of Jak2, Stat5, and Erk. Taken together, these data indicated that SIRPα overexpression regulated the phosphorylation/activation of proteins involved in cellular proliferation and survival in a collagen 1–dependent manner.

Given the documented role of Shp-2 phosphatase in the regulation of both SIRPα and Jak2 function, it was also hypothesized that Shp-2 might be the signaling switch through which SIRPα mediates its differential effects in the presence or absence of collagen 1. Therefore, the PRL-induced phosphorylation of Shp-2 was also examined. In the absence of collagen 1, SIRPα overexpression resulted in a significant increase in the basal Shp-2 phosphorylation. In contrast, in the presence of collagen 1, Shp-2 phosphorylation was reduced compared with the control transfectants. Furthermore, the tyrosine phosphorylation status of SIRPα in the presence or absence of collagen 1 was examined. Interestingly, the data showed that there was no difference in the tyrosine phosphorylation of SIRPα following PRL stimulation in the presence or absence of collagen 1. These data suggest that the phosphorylation status of Shp-2, but not SIRPα, is associated with the modulation of PRLr signaling.

In addition to signaling, the effect of SIRPα overexpression on PRL-induced cellular proliferation was measured by [3H]thymidine incorporation (Fig. 6). In congruence with the previous data, SIRPα overexpression significantly decreased PRL-dependent cellular proliferation in the
absence of collagen 1 compared with the control or the SIRPα-4YF transfectants (Fig. 6A). In contrast, SIRPα overexpression significantly increased cellular proliferation in the presence of collagen 1 compared with the control or the SIRPα-4YF transfectants (Fig. 6B). This effect on proliferation was not limited to only ER+ cell lines, as SIRPα overexpression in MDA-MB-231 cells also showed similar results (Fig. 6C and D). These data further support the notion that the differential regulation of PRLr signaling by SIRPα extends to the cellular level in breast cancer.

To further assess the effect of SIRPα overexpression on the biology of T47D breast cancer cells, the transfectants overexpressing SIRPα were examined in soft agar. In Fig. 7A, the cells were plated in a 100% soft agar/medium/PRL mixture and allowed to grow for 15 days. In the absence of collagen 1, there was a significant reduction of size and number of colonies in the SIRPα overexpression transfectants compared with the control transfectants (Table 2). In contrast, adding 10% collagen 1 (Fig. 7B) completely reversed the outcome, as SIRPα overexpression resulted in a significant increase in colony number and size when compared with the control or SIRPα-4YF transfectants (Table 2). Thus, comparable with the above results, SIRPα can function as a signaling switch that can increase or decrease soft agar colony formation in a collagen 1-dependent manner.

DN-Shp-2 or SSG reversed the effect of SIRPα overexpression on PRL-induced CISH promoter luciferase activity in the presence or absence of collagen 1

The above results suggested that SIRPα regulation of PRL-mediated signaling could induce the activity of the

![Figure 3](image-url)

**Table 1. Effects of ECM on PRL-induced gene transcription in breast cancer cells overexpressing SIRPα**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>WT-SIRPα</th>
<th>SIRPα-4YF</th>
</tr>
</thead>
<tbody>
<tr>
<td>No ECM</td>
<td>1.0</td>
<td>0.5 (±2.2)*</td>
<td>0.9 (±3.1)</td>
</tr>
<tr>
<td>Laminin</td>
<td>1.0</td>
<td>0.3 (±2.5)*</td>
<td>1.1 (±1.5)</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>1.0</td>
<td>0.5 (±1.5)*</td>
<td>1.0 (±2.7)</td>
</tr>
<tr>
<td>Matrigel</td>
<td>1.0</td>
<td>0.5 (±1.2)*</td>
<td>1.0 (±3.7)</td>
</tr>
<tr>
<td>Collagen 1</td>
<td>1.0</td>
<td>2.5 (±1.4)*</td>
<td>0.9 (±5.0)</td>
</tr>
</tbody>
</table>

NOTE: T47D transfectant pools were plated in the presence or absence of each ECM and transfected with the PRL-responsive luciferase reporter CISH. Cells were treated with PRL (250 ng/mL) for 24 h and analyzed for dual luciferase. Results reported represent fold increase or decrease (±SE) compared with the control transfectants.

*P < 0.001.

†P < 0.01.
Discussion

SIRPα activation and modulation have been well documented in neurons, macrophages, and myeloid cells; however, this is the first report describing its function in breast cancer cells (37, 38). SIRPα has one known ligand, CD47, but can also be activated by various ligand-engaged cell surface receptors including integrins (18, 37). CD47, also named integrin-associated protein, is expressed in a variety of cell types in the immune system and central nervous system, as well as in muscle progenitor cells. One recent report has indicated that CD47 is also expressed in a few breast cancer cell lines, including MCF-7 and MDA-MB-231 (39). This study showed that CD47 mediated apoptosis of breast cancer cells through the inhibition of protein kinase A (39). CD47 has also been shown to regulate intestinal epithelial cell migration primarily through collagen 1–induced cyclooxygenase-2 expression (40). However, the best-characterized function of the CD47-SIRPα signaling complex in the immune system is to inhibit macrophage phagocytosis of RBCs or platelets (19). In addition, the CD47-SIRPα complex negatively regulates the immune system by suppressing both the maturation of immature dendritic cells and the production of cytokines by mature dendritic cells (41).

SIRPα also has been shown to negatively regulate GHr/Jak2 signaling in 3T3 and COS-7 cells (34). In this report, data revealed that this negative regulation was through the ability of SIRPα to associate with the phosphatase Shp-2. In addition, these studies showed that in response to GH stimulation, phosphorylation of the four tyrosine sites on the COOH-terminal tail of SIRPα was required for recruitment of Shp-2, whereas the interaction of Jak2 and SIRPα was independent of the phosphorylation status of SIRPα (21, 34). Because the GHr is the closest PRLr homologue, we hypothesized that SIRPα may affect PRLr signaling in a similar manner. In light of these data, this study sought to examine SIRPα overexpression in the context of PRLr signaling in breast cancer cells.

The PRL/PRLr complex in MECs uses the Jak2/Stat5 as well as the MAPK and PI3K signaling cascade, and this pathway, in part, contributes to the proliferation, differentiation, survival, and invasion of MECs (1, 4, 42). Results in this report showed that in the SIRPα overexpression transfectants, Jak2 is constitutively associated with SIRPα (Fig. 2B), findings that are congruent with published interaction data in COS-7 (34). In addition, the overexpression of SIRPα in the absence of collagen 1 decreased Jak2, Stat5, and Erk phosphorylation through its inhibition of PRLr signaling (Fig. 5). This resulted in downregulation of PRL–induced CISH transcription in the absence of collagen 1, as shown by luciferase and Western experiments (Figs. 3–5). Furthermore, overexpression of SIRPα in the absence of collagen 1 inhibited anchorage-dependent and anchorage-independent growth as shown by proliferation and soft agar colony formation experiments (Figs. 6 and 7).
As shown here, the inhibition of PRL-mediated signaling by SIRPα reveals that in the absence of collagen 1, SIRPα is a negative regulator of PRLr signaling. These results are consistent with results reported in the literature that SIRPα is a negative regulator of GH, EGF, and PI3K signaling pathways in fibroblasts, neurons, and macrophages (18, 34, 37).

Cross-talk between cell surface receptors leads to the modulation of signaling cascades and may alter breast cancer cell growth and progression (12). Data presented in this study shows in both ER+ and ER− breast cell lines that SIRPα, the PRLr, β1 integrin, and Jak2 reside in a partially inducible complex at the cell surface (Figs. 1 and 2). There is current evidence that integrins interact with other cell surface receptors to initiate and drive migration and metastasis (43). For example, integrins can cooperate with the EGF receptor, resulting in a chemotactic response to EGF (44). In addition, integrins have been implicated in facilitating the epithelial to mesenchymal transformation observed in neoplastic cells (43). Given this, and the observation that β1 integrin is required for PRLr signaling, it was hypothesized that SIRPα may function as an intermediary between the PRLr and β1 integrin (45).

Our findings revealed that SIRPα overexpression inhibited PRL-induced CISH promoter luciferase activity in the presence of laminin and fibronectin (Table 1). In the presence of collagen 1, however, SIRPα overexpression enhanced PRL-induced CISH promoter luciferase activity, and the inhibition of β1 integrin in the presence of collagen 1 reversed these effects (Fig. 4). These novel data suggest that collagen 1 (a β1 integrin ligand) promotes a functional

**Figure 5.** SIRPα differentially regulates PRLr signaling in the presence or absence of collagen 1 in T47D cells. T47D transfectant pools were plated in the presence or absence of collagen 1, stimulated with PRL (250 ng/mL) for times indicated, lysed, and analyzed via Western blotting for corresponding protein phosphorylation. Densitometry was done. Columns, mean fold increase compared with the untreated control transfectants; bars, SE. The results reported are representative of three individual experiments. *P < 0.01; **P < 0.001.
switch in the regulation of SIRPα of the PRLr pathway from that of a negative regulator to that of a positive regulator. Taken together, our data in Figs. 1 to 4 and Table 1 highlight a potential role for SIRPα in mediating cross-talk between the PRLr and integrins in breast cancer cells. Such cross-talk could contribute to the aggressive behavior of breast cancer cells once they invade through the basement membrane into the interstitial space of breast tissues.

SIRPα is phosphorylated on its four cytoplasmic tyrosine residues in response to growth factors, hormones,

FIGURE 6. SIRPα differentially regulates proliferation in the presence or absence of collagen 1. T47D (A and B) or MDA-MB-231 (C and D) transfectant pools were plated in the presence or absence of collagen 1 and stimulated with PRL (250 ng/mL) for 72 h and [3H]thymidine for 4 h. Columns, mean [3H]thymidine incorporation compared with the control transfectants; bars, SE. *, P < 0.05; ***, P < 0.001. Results are representative of three individual experiments.

FIGURE 7. SIRPα differentially regulates soft agar colony formation in a PRL- and collagen 1–dependent manner in T47D cells. A, T47D transfectants were suspended in a 0.3% agar-medium mix supplemented with PRL (250 ng/mL) for 10 d. B, T47D transfectants were suspended in a 0.3% agar-medium mix supplemented with 10% collagen 1 and PRL (250 ng/mL) for 10 d. Each condition was photographed under phase-contrast optics.
and integrins (46). The phosphorylation of SIRPα leads to the recruitment, binding, phosphorylation, and activation of Shp-2 as well as Shp-1 (21, 34). There is some debate in the literature as to whether the phosphorylation of Shp-2 confers activity. However, recent studies suggest that the phosphorylation of Shp-2 leaves it in an open conformation, allowing it to act as a phosphatase (47, 48). Several studies have shown that Shp-2 positively affects the MAPK as well as the Jak2/Stat5 signaling cascade (13, 25, 49). Conversely, Shp-2 has also been shown to negatively regulate Stat family proteins, including Stat-1, Stat-3, and Stat-5, in different cell types by the dephosphorylation of upstream Jak family

### Table 2. Quantification of the results of the soft agar colony formation

<table>
<thead>
<tr>
<th>Average number of colonies</th>
<th>Collagen 1 (10%)</th>
<th>PRL (250 ng/mL)</th>
<th>WT-SIRPα</th>
<th>Control</th>
<th>SIRPα-4YF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen 1 (10%)</td>
<td>−</td>
<td>−</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRL (250 ng/mL)</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT-SIRPα</td>
<td>103 (±2.2)</td>
<td>115 (±2.7)*</td>
<td>125 (±3.5)</td>
<td>180 (±3.3)*</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>105 (±4.3)</td>
<td>143 (±4.0)</td>
<td>125 (±5.6)</td>
<td>150 (±928)</td>
<td></td>
</tr>
<tr>
<td>SIRPα-4YF</td>
<td>102 (±5.5)</td>
<td>140 (±6.7)</td>
<td>118 (±3.2)</td>
<td>144 (±2.1)</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Results were reported as average number of colonies per field, represent differences in soft agar colony formation (±SE) compared with the control transfectants, and represent three individual experiments.

*P < 0.01.

![Figure 8](https://example.com/figure8.png)

**Figure 8.** DN-Shp-2 or SSG reversed the effect of SIRPα overexpression on PRL-induced CISH promoter luciferase activity in the presence or absence of collagen 1. A and B, T47D transfectants were plated in the presence or absence of collagen 1 and transfected with a PRL-responsive CISH luciferase promoter and/or a phosphatase-defective mutant of Shp-2 (DN-Shp-2). The cells were treated with PRL (250 ng/mL), harvested, and analyzed for dual luciferase. C and D, T47D transfectants were plated in the presence or absence of collagen 1 and transfected with a PRL-responsive CISH luciferase promoter. The transfectants were treated with SSG (100 ng/mL) for 4 h before PRL stimulation (250 ng/mL), harvested, and analyzed for dual luciferase. Results were reported as luciferase/Renilla ratio. Columns, mean increase or decrease in luciferase activity compared with the control transfectants; bars, SE. ***, P < 0.001. Results are representative of three individual experiments.
kinases. In the mammary gland, Shp-2 regulates PRLr signaling by inhibiting the activity of both Jak2 and Stat5 (21, 26). Our data revealed that in the presence of collagen 1, SIRPα overexpression decreased Shp-2 phosphorylation. However, in the absence of collagen 1, there was an increase in Shp-2 phosphorylation (Fig. 5). This dual regulation may be a direct result of the ability of SIRPα to alter Shp-2 activation in the presence or absence of collagen 1–bound integrins. Given that (a) the tyrosine residues of SIRPα are necessary for its mediation of ECM-modulated, PRL-driven signaling, (b) the tyrosine phosphorylation of SIRPα did not change in the presence or absence of collagen 1 (Fig. 5), and (c) the overexpression of DN-Shp-2 and SSG prevented SIRPα-mediated potentiation of PRLr signaling (Fig. 8A), it was hypothesized that a specific tyrosine kinase or phosphatase exists within the PRLr-SIRPα-integrin complex that directly modulates Shp-2 activity as a function of integrin occupancy. Several candidates such as Jak2, Src, Fak, or Shp-1 may be involved in this alteration of Shp-2. However, this is not the only possible hypothesis that could explain the regulation of Shp-2 activity. Alternatively, the sequestration of Shp-2 by SIRPα in the presence of collagen 1 and PRL may result in its functional inactivation.

The use of the Shp-2 inhibitor SSG resulted in comparable effects to DN-Shp-2. SSG is currently used in the treatment of Leishmania; however, its mechanism of action has been incompletely understood (50). A recent study showed SSG as an effective inhibitor of Shp-2. In addition, this study implied that SSG may be effective in the treatment of acute myeloid leukemia by overcoming cancer cell resistance to IFN treatment (36). In support of this notion, it was shown that using SSG as a single agent in a dose-dependent manner inhibited the growth of prostate, breast, bladder, and colon cancer cells in culture (36). Our data show that SSG blocks the effects of SIRPα overexpression on ECM-modulated, PRL-driven gene expression (Fig. 8C and D). Collectively, our data suggest that the mechanism of action of SIRPα is through the phosphatase Shp-2, and that a protein tyrosine phosphatase inhibitor such as SSG could be used alone or in combination with other anticancer therapies effectively in the treatment of ER+ and ER− breast cancers.

In this report, we sought to elucidate a role for SIRPα in PRLr-integrin–mediated signaling. This is the first report to show that SIRPα facilitates such cross-talk in ER+ and ER− breast cancer cell lines. Importantly, data in this study revealed that SIRPα overexpression in the presence of collagen 1 increased cellular proliferation and soft agar colony formation (Figs. 6 and 7). This regulation of PRL-induced signaling and function was associated with the phosphorylation status and activity of Shp-2. As Shp-2 can be either a negative or a positive regulator of PRLr signaling, we hypothesize that SIRPα facilitates the modification of Shp-2 activity in a collagen 1/PRL–dependent manner. Our data revealed that induced alterations in Shp-2 activity or levels (by SSG or DN-Shp-2, respectively) were associated with altered cell signaling, proliferation, and anchorage-independent growth, processes that contribute to tumor progression. Collectively, these data implicate an important function for SIRPα in breast cancer in its ECM-dependent regulation of PRL-induced function and suggest a potential role for SIRPα as a therapeutic target.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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