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

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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 crosstalk is mediated. Following PRL stimulation, we show that a complex between the transmembrane glycoprotein signal regulatory protein- α (SIRP α) and the PRLr, β_1 integrin, and Jak2 in estrogen receptor–positive (ER⁺) and ER[–] breast cancer cells is formed. Overexpression of SIRP α 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 SIRP α 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 SIRP α (SIRP α -4YF) prevented the signaling and phenotypic effects mediated by wild-type SIRP α . Furthermore, overexpression of a phosphatase-defective mutant of Shp-2 or pharmacologic inhibition of Shp-2 produced effects comparable with that of SIRP α -4YF. However, the tyrosine phosphorylation of SIRP α was unaffected in the presence or absence of collagen 1. These data suggest that SIRP α modulates PRLr-associated signaling as a function of integrin occupancy predominantly through the alteration of Shp-2 activity. This PRLr-SIRP α -integrin complex may therefore provide a basis for integrin-PRLr cross-talk and contribute to the biology of breast cancer.

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

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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 β_1 integrin is necessary for PRL-driven MEC differentiation (14). Furthermore, mice lacking β_1 integrin have compromised Stat5 activation and milk production (15). Despite these insights, the mechanism of β_1 integrin and PRLr cross-talk 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 (SIRP α and SIRP β) that differ only in the length of their cytoplasmic region. SIRP α contains immunoglobulin-like repeats in its extracellular domain, as well as four tyrosine residues and a proline-rich region in its cytoplasmic domain. SIRP α is ubiquitously expressed in human tissue, and recent studies show that alterations of SIRP α 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 SIRP α is significantly overexpressed in triple-negative breast cancer. In addition, unpublished anti-SIRP α immunohistochemistry of primary human breast cancer reveals that SIRP α is overexpressed at the invasive edge of the tumor. SIRP α 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 SIRP α 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 PRLr signaling by inhibiting the activity of both Jak2 and Stat5 (21,26). In addition, the conditional deletion of Shp-2 suppresses Stat5 activation and lobuloalveolar outgrowth and lactation (27). Like the PRLr, GH receptor (GHR) is also inhibited by the actions of Shp-2. Recent studies have shown that GH regulates the tyrosine phosphorylation of SIRP α and its subsequent association with Shp-2. Such interactions seem functionally significant, as they result in the downregulation of GHR/Jak2 signaling (21,28).

SIRP α 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 collagens, 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 SIRP α phosphorylation, which contributes to cell migration primarily through Src family kinases and focal adhesion kinase (Fak; ref. 29). Thus, SIRP α may contribute to integrin-mediated cell migration.

Given the role of SIRP α and Shp-2 in GHR and integrin signaling, it was hypothesized that the SIRP α /Shp-2 complex might play a role in altering PRL/PRLr-mediated signaling in breast cancer cells. Our findings have shown that PRLr, β_1 integrin, and SIRP α interact in both estrogen receptor-positive (ER⁺) and ER⁻ breast cancer cell lines, whereas SIRP α expression/function is associated with an ECM context-dependent modulation of PRLr

signaling. These data reveal a novel role for SIRP α in facilitating cross-talk between the PRLr and β_1 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 $\times$ 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 SIRP α (WT-SIRP α ; courtesy of Dr. C. Carter-Su, University of Michigan, Ann Arbor, MI), or a SIRP α -4YF (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 transfected 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 blasticidin (10 μ g/mL), and levels of overexpressed proteins were assessed by immunoblot analysis.

Collagen coating and β_1 integrin neutralization

Collagen1 (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- β_1 integrin (Chemicon) antibody was diluted to 15 μ g/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 μ g 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). Cells treated with sodium stibogluconate (SSG; 100 ng/mL; courtesy of Dr. E. Eklund, Northwestern University, Chicago, IL) were pretreated for 4 hours before PRL stimulation. Results were reported as luciferase/*Renilla* ratio $\pm$ SE.

Immunoprecipitation and immunoblotting

T47D, T47D transfectant pools, and MDA-MB-231 cells were grown in complete medium and arrested in 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 SIRP α (1 μ g/sample; Chemicon), Jak2 (1 μ g/sample; Cell Signaling), β_1 integrin (1 μ g/sample; Chemicon), or PRLr (1 μ g/sample; Zymed) antibodies as well as 40 μ L 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 [3 H]Thymidine (0.5 μ Ci/well) was added for 4 hours. The cells were lysed on a Filtermat Harvester (Perkin-Elmer) and transferred to a Filtermat (Wallac) and read on 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 ImageJ software (NIH), and results were analyzed by comparing the number and size of colonies of control transfectant pools with those of the WT-SIRP α 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 $\pm$ SE. One-way ANOVA statistical tests were done and *P* values were reported.

Results

SIRP α interacts with the PRLr, β_1 integrin, and Jak2 in breast cancer cells

Given literature that indicated that SIRP α could influence GHr signaling (21,34), we sought to examine if SIRP α could interact and influence PRLr action in the context of breast cancer. To that end, the interaction of SIRP α with the PRLr, β_1 integrin, and Jak2 was tested (Figs. 1 and 2). Coimmunoprecipitation analysis revealed endogenous interactions between the PRLr and SIRP α as well as the PRLr and β_1 integrin in T47D ER $^+$ breast cancer cells (Fig. 1A) and MDA-MB-231 ER $^-$ breast cancer cells (Fig. 1B). Although SIRP α 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 β_1 integrin was PRL dependent, with their association visualized after 5 minutes of PRL stimulation. The interaction between the PRLr and SIRP α 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 β_1 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 SIRP α with β_1 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-SIRP α , GFP (control), or a nonphosphorylated mutant SIRP α (SIRP α -4YF), which is inactive in these cells, therefore mimicking the control transfectants with endogenous SIRP α . Overexpression was assessed in WT-SIRP α and SIRP α -4YF transfectant pools by immunoblot analysis. Investigation in these transfectants revealed 2-fold higher SIRP α protein expression than that of the control (Fig. 2E). Coimmunoprecipitation analysis of these transfectants (Fig. 2C) revealed that SIRP α interacted with β_1 integrin in the control transfectants as well as in the SIRP α overexpression transfectants within 1 minute of PRL stimulation, and this interaction was maintained through 60 minutes (Fig. 2A). Interestingly, the mutant form of SIRP α also interacted at early time points with β_1 integrin, which suggested that the four tyrosine residues in the SIRP α intracellular domain are not required for this interaction. Additionally, coimmunoprecipitation analysis also revealed that SIRP α interacted with Jak2, independent of ligand addition in the SIRP α 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 SIRP α , the PRLr, β_1 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 SIRP α in a collagen 1/ β_1 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 SIRP α overexpression on PRL-mediated gene expression in the presence and absence of several different ECM substrates (Table 1). Results verified that overexpression of SIRP α 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, SIRP α overexpression transfectant 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 stiffness) showed an enhancement of PRL-induced CISH promoter luciferase activity compared with the control or SIRP α -4YF transfectant pools (Table 1 and Fig. 3B). Comparable results were obtained with two independent preparations of collagen 1 (data not shown). These data suggested that SIRP α may differentially regulate PRL-induced CISH luciferase activity depending on ECM occupancy.

In light of the data in Table 1, PRL-induced CISH (Fig. 3A and B) promoter luciferase activity was examined in transfectant pools overexpressing SIRP α plated in the presence or absence of collagen 1. These analyses revealed that the transfectant pools overexpressing SIRP α 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 SIRP α -4YF constructs (Fig. 3A). In contrast, the transfectants overexpressing SIRP α 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 SIRP α in the absence of collagen 1. In addition, as before, cells overexpressing SIRP α plated in the presence of collagen 1 showed an increase in PRL-dependent CISH protein expression (Fig. 3D). Thus, these data revealed that SIRP α over-expression differentially regulated PRL-induced CISH promoter luciferase activity and endogenous PRL-dependent CISH protein expression.

To further assess the involvement of β_1 integrin on PRL-induced CISH promoter luciferase activity in the presence of collagen 1, a neutralizing anti- β_1 integrin antibody was used. Data in Fig. 4 revealed that transfectants overexpressing SIRP α plated in the presence of collagen 1 and anti- β_1 integrin showed a reduction in PRL-induced CISH promoter luciferase activity compared with the transfectants overexpressing SIRP α plated in the presence of collagen 1 only. Interestingly, the reduction resembled results observed in the cells overexpressing SIRP α plated in the absence of collagen 1. Consequently, the data revealed that SIRP α -mediated potentiation of PRL-induced CISH promoter luciferase activity is dependent on the activity of β_1 integrin in the presence of collagen 1.

Differential regulation of PRLr signaling as a function of SIRP α overexpression

Because SIRP α 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 SIRP α over-expression inhibited the phosphorylation of Jak2, Stat5, and Erk in the absence of collagen 1. In contrast, in the presence of collagen 1, SIRP α overexpression enhanced the phosphorylation of Jak2, Stat5, and Erk. As anticipated, the SIRP α -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 SIRP α over-expression 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 SIRP α and Jak2 function, it was also hypothesized that Shp-2 might be the signaling switch through which SIRP α 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, SIRP α 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 SIRP α in the presence or absence of collagen 1 was examined. Interestingly, the data showed that there was no difference in the tyrosine phosphorylation of SIRP α following PRL stimulation in the presence or absence of collagen 1. These data suggest that the phosphorylation status of Shp-2, but not SIRP α , is associated with the modulation of PRLr signaling.

In addition to signaling, the effect of SIRP α overexpression on PRL-induced cellular proliferation was measured by [3 H]thymidine incorporation (Fig. 6). In congruence with the previous data, SIRP α overexpression significantly decreased PRL-dependent cellular proliferation in the absence of collagen 1 compared with the control or the SIRP α -4YF transfectants (Fig. 6A). In contrast, SIRP α overexpression significantly increased cellular proliferation in the presence of collagen 1 compared with the control or the SIRP α -4YF transfectants (Fig. 6B). This effect on proliferation was not limited to only ER $^+$ cell lines, as SIRP α 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 SIRP α extends to the cellular level in breast cancer.

To further assess the effect of SIRP α overexpression on the biology of T47D breast cancer cells, the transfectants overexpressing SIRP α 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 SIRP α overexpression transfectants compared with the control transfectants (Table 2). In contrast, adding 10% collagen 1 (Fig. 7B) completely reversed the outcome, as SIRP α overexpression resulted in a significant increase in colony number and size when compared with the control or SIRP α -4YF transfectants (Table 2). Thus, comparable with the above results, SIRP α 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 SIRP α overexpression on PRL-induced CISH promoter luciferase activity in the presence or absence of collagen 1

The above results suggested that SIRP α regulation of PRL-mediated signaling could induce the activity of the Shp-2 phosphatase (Fig. 5). To test this hypothesis, two approaches were used to investigate the contribution of Shp-2 in the actions of SIRP α : overexpression of DN-Shp-2 or the use of the Shp-2 inhibitor SSG. As seen in Fig. 8A, DN-Shp-2 coexpression in SIRP α overexpression transfectants prevented SIRP α -mediated inhibition on PRL-induced CISH promoter luciferase activity in the absence of collagen 1. In contrast, introduction of DN-Shp-2 in SIRP α overexpression transfectants plated on collagen 1 prevented SIRP α -mediated potentiation of CISH promoter luciferase activity, restoring levels to that of the control or SIRP α -4YF transfectants (Fig. 8B). As such, these results are supportive of a role for Shp-2 in the regulation of PRL-induced gene expression by SIRP α . This hypothesis was further tested through the use of a potent pharmacologic inhibitor of the phosphatase Shp-2, SSG (36). To examine the effect of SSG, SIRP α , SIRP α -4YF, or control transfectant pools were plated in the presence or absence of collagen 1 before pretreatment with SSG and then stimulated with PRL. As seen in Fig. 8C, SSG treatment of SIRP α overexpression transfectants reversed its effect on PRL-induced CISH promoter luciferase activity in the absence of collagen 1. In contrast, the SIRP α overexpression transfectants plated on collagen 1 showed diminished luciferase activity equivalent to the control or SIRP α -4YF transfectants in the presence of SSG (Fig. 8D). Again, these data parallel the results obtained with DN-Shp-2, further confirming that SIRP α is a Shp-2-dependent signaling switch in the PRL/PRLr pathway in breast cancer cells.

Discussion

SIRP α 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). SIRP α 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-SIRP α signaling complex in the immune system is to inhibit macrophage phagocytosis of RBCs or platelets (19). In addition, the CD47-SIRP α 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).

SIRP α 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 SIRP α 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 SIRP α was required for recruitment of Shp-2, whereas the interaction of Jak2 and SIRP α was independent of the phosphorylation status of SIRP α (21,34). Because the GHR is the closest PRLr homologue, we hypothesized that SIRP α may affect PRLr signaling in a similar manner. In light of these data, this study sought to examine SIRP α 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 SIRP α overexpression transfectants, Jak2 is constitutively associated with SIRP α (Fig. 2B), findings that are congruent with published interaction data in COS-7 (34). In addition, the overexpression of SIRP α 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 SIRP α 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 SIRP α reveals that in the absence of collagen 1, SIRP α is a negative regulator of PRLr signaling. These results are consistent with results reported in the literature that SIRP α 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 SIRP α , the PRLr, β_1 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 β_1 integrin is required for PRLr signaling, it was hypothesized that SIRP α may function as an intermediary between the PRLr and β_1 integrin (45).

Our findings revealed that SIRP α overexpression inhibited PRL-induced CISH promoter luciferase activity in the presence of laminin and fibronectin (Table 1). In the presence of collagen 1, however, SIRP α overexpression enhanced PRL-induced CISH promoter luciferase activity, and the inhibition of β_1 integrin in the presence of collagen 1 reversed these effects (Fig. 4). These novel data suggest that collagen 1 (a β_1 integrin ligand) promotes a functional switch in the regulation of SIRP α 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 SIRP α 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.

SIRP α is phosphorylated on its four cytoplasmic tyrosine residues in response to growth factors, hormones, and integrins (46). The phosphorylation of SIRP α 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 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, SIRP α 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 SIRP α to alter Shp-2 activation in the presence or absence of collagen 1-bound integrins. Given that (a) the tyrosine residues of SIRP α are necessary for its mediation of ECM-modulated, PRL-driven signaling, (b) the tyrosine phosphorylation of SIRP α did not change in the presence or absence of collagen 1 (Fig. 5), and (c) the overexpression of DN-Shp-2 and SSG prevented SIRP α -mediated potentiation of PRLr signaling (Fig. 8A), it was hypothesized that a specific tyrosine kinase or phosphatase exists within the PRLr-SIRP α -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 SIRP α 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 SIRP α over-expression on ECM-modulated, PRL-driven gene expression (Fig. 8C and D). Collectively, our data suggest that the mechanism of action of SIRP α 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 SIRP α in PRLr-integrin-mediated signaling. This is the first report to show that SIRP α facilitates such cross-talk in ER⁺ and ER⁻ breast cancer cell lines. Importantly, data in this study revealed that SIRP α 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 SIRP α 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 SIRP α in breast cancer in its ECM-dependent regulation of PRL-induced function and suggest a potential role for SIRP α as a therapeutic target.

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FIGURE 1.

Interactions between the PRLr, SIRP α , and β_1 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. *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$.

FIGURE 2.

Interactions between SIRPα and Jak2, as well as β₁ integrin in T47D cells. A and B, T47D transfectant pools were treated with PRL (250 ng/mL) for times indicated. Whole-cell lysates were immunoprecipitated and analyzed by Western blotting. E, T47D transfectant pools were harvested and analyzed by Western blotting to show overexpression of SIRPα compared with the control transfectants. Tubulin was used as a loading control. C and D, all results reported were representative of three individual experiments. Each of the three experimental blots was quantitated. Columns, mean fold increase compared with the control transfectants; bars, SE. **, $P < 0.01$; ***, $P < 0.001$.

FIGURE 3.

PRL-induced CISH promoter luciferase activity and protein expression is enhanced by SIRPα in a collagen 1–dependent manner in T47D cells. A and B, T47D transfectant pools were plated in the presence or absence of collagen 1 and transfected with a PRL-responsive CISH luciferase promoter. The cells were treated with PRL (250 ng/mL), harvested, and analyzed for dual luciferase. Results were reported as luciferase/*Renilla* ratio (three individual experiments). Columns, mean increase or decrease in luciferase activity compared with the control transfectants; bars, SE. **, $P < 0.01$; ***, $P < 0.001$. C and D, 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 by Western blot for CISH protein expression. Tubulin was used as a loading control. E, all results reported were representative of three individual experiments. Each of the three experimental blots was quantitated. Columns, mean fold increase compared with the control transfectants; bars, SE.

FIGURE 4.

PRL-induced CISH promoter luciferase activity is enhanced by SIRPα in a collagen 1/β₁ integrin-dependent manner in T47D cells. T47D transfectant pools were plated in the presence or absence of collagen 1 in addition to collagen 1 plus an anti-β₁ integrin antibody (+ α-β₁) and transfected with a PRL-responsive CISH luciferase promoter. The cells were treated with PRL (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. The results reported were representative of three individual experiments. **, *P* < 0.01; ***, *P* < 0.001.

FIGURE 5.

SIRP α 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. **, $P < 0.01$; ***, $P < 0.001$.

FIGURE 6.

SIRP α 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 [3 H]thymidine for 4 h. Columns, mean [3 H]thymidine incorporation compared with the control transfectants; bars, SE. *, $P < 0.05$; ***, $P < 0.001$. Results are representative of three individual experiments.

FIGURE 7. SIRP α 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.

FIGURE 8.

DN-Shp-2 or SSG reversed the effect of SIRP α 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.

Table 1Effects of ECM on PRL-induced gene transcription in breast cancer cells overexpressing SIRP α

	Control	WT-SIRP α	SIRP α -4YF
No ECM	1.0	0.5 (± 2.2) [*]	0.9 (± 3.1)
Laminin	1.0	0.3 (± 2.5) [*]	1.1 (± 1.5)
Fibronectin	1.0	0.5 (± 1.5) [*]	1.0 (± 2.7)
Matrigel	1.0	0.5 (± 1.2) [*]	1.0 (± 3.7)
Collagen 1	1.0	2.5 (± 1.4) [†]	0.9 (± 5.0)

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 ($\pm$ SE) compared with the control transfectants.

^{*} $P < 0.001$.

[†] $P < 0.01$.

Table 2

Quantification of the results of the soft agar colony formation

Average number of colonies				
Collagen 1 (10%)	–	–	+	+
PRL (250 ng/mL)	–	+	–	+
WT-SIRPα	103 (±2.2)	115 (±2.7) *	125 (±3.5)	180 (±3.3) *
Control	105 (±4.3)	143 (±4.0)	125 (±5.6)	150 (±9.28)
SIRPα-4YF	102 (±5.5)	140 (±6.7)	118 (±3.2)	144 (±2.1)

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.

* $P < 0.01$.