Prolactin Induces SHP-2 Association with Stat5, Nuclear Translocation, and Binding to the β-Casein Gene Promoter in Mammary Cells*

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The Src homology 2 (SH2) domain containing protein-tyrosine phosphatase SHP-2 contributes to prolactin receptor (PRLR) signal transduction to β-casein gene promoter activation. We report for the first time that SHP-2 physically associates with the signal transducer and activator of transcription-5α (Stat5α), an important mediator of PRLR signaling to milk protein gene activation, in the mouse mammary HC11 and the human breast cancer T47D cells when stimulated with prolactin (PRL) and human growth hormone, respectively. In addition, overexpression studies indicate that the carboxyl-terminal SH2 domain of SHP-2 is required to maintain tyrosine phosphorylation of Stat5 and its interaction with SHP-2. Furthermore, we demonstrate by nuclear co-immunoprecipitation and indirect immunofluorescence studies that PRL stimulation of mammary cells leads to the nuclear translocation of SHP-2 as a complex with Stat5α. This process was found to involve the catalytic activity of the phosphatase. Finally, using the Stat5 GAS (γ-activated sequence) element of the β-casein gene promoter in electrophoretic mobility shift assays, we demonstrate that PRL induces the SHP-2-Stat5α complex to bind to DNA. The presence of the phosphatase in the protein-bound DNA complex was verified by polyclonal antiserum to SHP-2. Our studies indicate a tight physical and functional interaction between SHP2 and Stat5 required for regulation and perpetuation of PRL-mediated signaling in mammary cells and suggest a potential role for SHP-2 in the nucleus.

PRL is a polypeptide hormone secreted by the pituitary gland and by multiple extra-pituitary sites. Numerous biological functions have been attributed to the activity of the hormone ranging from reproduction, metabolism, and immune regulation (1). Prolactin is a primary factor required for the growth and terminal differentiation of mammary epithelial cells as determined by the induction of transcription of various milk protein genes such as β-casein, β-lactoglobulin, and whey acidic protein (2).

The molecular mechanisms by which PRL induces milk protein gene transcription have been the subject of intensive research. Interaction of PRL with its receptor, the PRLR, a member of the class I cytokine receptor superfamily, induces receptor dimerization leading to activation of a member of the Janus cytoplasmic protein-tyrosine kinase family, Jak2, and members of the Src-like kinase family necessary for signal generation (3, 4). Extensive in vitro as well as gene knock out studies revealed that PRL utilizes the Jak2/Stat5 pathway to induce milk protein gene transcription (5–7).

It is known that Stat proteins are latent cytoplasmic transcription factors that are activated upon cytokine stimulation, heterodimerize and/or homodimerize, and translocate to the nucleus to bind to DNA elements in order to initiate the transcription of specific genes. The mechanisms by which Stat molecules are regulated have increasingly become more intricate with the discovery that Stat proteins interact with various cytoplasmic and nuclear proteins that may regulate Stat-mediated gene transcription. For example Stat5 has been shown to interact with steroid receptors, the glucocorticoid receptor (8), the co-activator CBP/p300 (9), N-Myc interactor (Nmi) protein (10), Erk1/2 (11), and the adaptor protein CrkL (12). It is still unclear how these interactions contribute to Stat5 activation of PRL target genes.

Another important mediator of PRL signaling to milk protein gene activation is the cytoplasmic protein-tyrosine phosphatase SHP-2. SHP-2 is a ubiquitously expressed phosphatase containing two amino-terminal SH2 domains and a carboxy-terminal phosphatase domain (13–15). A variety of studies indicate that SHP-2 interacts with an assortment of molecules that are involved in signal transduction pathways downstream of different growth factor receptors and cytokine receptors such as members of the insulin receptor substrate family of proteins (16), Grb2-associated binder proteins (17), as well as several proteins with immunoreceptor tyrosine-based inhibitory motifs such as signal regulatory protein (18), protein zero related (19), and natural killer cell inhibitory receptor (20).

Although the role of SHP-2 downstream of cytokine receptors has been investigated, the precise contribution of SHP-2 to cytokine signaling remains in large part unknown. SHP-2 is known to exert a positive effect in mediating the activation of the Jak-Stat pathway in response to different cytokines such as...
PRL (21, 22), interferon α/β (23), and growth hormone (GH) (24). However, SHP-2 can also be implicated in the negative regulation of cytokine-mediated signaling to the Jak-Stat pathway. In terms of interleukin-6 signaling, glycoprotein 130 regulates cytokine-mediated signaling to the Jak-Stat pathway. Furthermore, expression of a dominant negative variant of SHP-2 whereby the phosphatase domain is deleted led to the loss of tyrosine phosphorylation and DNA binding activity of Stat5 (22). However, the molecular events following SHP-2 recruitment to the PRLR-Jak2 complex and its regulation of Stat5 activation are not fully characterized. In our present study we demonstrate a PRL-dependent association of SHP-2 with Stat5 in mammary cells. In addition, we report for the first time PRL-mediated nuclear translocation of SHP-2, and we have shown that upon PRL stimulation SHP-2 and Stat5 exist in a complex on the Stat5-responsive element of the β-casein gene promoter. Furthermore, the catalytic activity of SHP-2 was found to regulate the formation of a SHP-2-Stat5 complex, tyrosine phosphorylation of Stat5, and translocation of SHP-2 to the nucleus. The data together indicate that SHP-2 and Stat5 as a complex participate in PRL signal transduction in mammary cells.

**EXPERIMENTAL PROCEDURES**

**Reagents**—The following reagents were used: ovine prolactin (Sigma); human growth hormone (Sigma); monoclonal antibody to phosphotyrosine 4G10 (Upstate Biotechnology Inc., Lake Placid, NY); monoclonal antibody to Stat5 (Transduction Laboratories and BD PharMingen); monoclonal antibody to phosphorylated form of Stat5ab Tyr-694/Tyr-699 (Zymed Laboratories Inc., South San Francisco, CA); polyclonal antibody specific to Stat5a (Zymed Laboratories Inc.); monoclonal antibody to SHP-2 (Transduction Laboratories and BD PharMingen); polyclonal antibody to SHP-2 (Santa Cruz Biotechnology, Santa Cruz, CA); polyclonal antibody to HA (Santa Cruz Biotechnology); goat anti-mouse horseradish peroxidase (H + L) (Sigma); goat anti-rabbit horseradish peroxidase (H + L) (Sigma); goat anti-rabbit FITC (Sigma); goat anti-mouse Cy3 (The Jackson Laboratory, Bar Harbor, ME); Lumi-light plus kit (Roche Diagnostics); protein A-Sepharose beads (Amersham Biosciences); and ECL Hyperfilm (Amersham Biosciences).

**Constructs**—Cytomegalovirus expression plasmid pB/CMV vector (Invitrogen) containing cDNAs encoding for the PRLR long form was obtained from Dr. Paul Kelly (INSERM Endocrinologie Moleculaire, Faculté de Médecine Necker, Paris, France). Expression plasmid encoding MGF/Stat5 (pX-MGF/Stat5) was obtained from Dr. Bernd Groner (Institute for Biomedical Research, Frankfurt am Main, Germany). Expression plasmids encoding for human SHP-2, SHP-2WT, catalytically inactive SHP-2, SHP-2C463A, mono-terminal SH2 domain inactivating mutant of SHP-2, SHP-2R138K, and carboxyl-terminal SH2 domain inactivating mutant of SHP-2, SHP-2R138K, were obtained from Dr. Axel Ullrich (Max-Planck Institute for Biochemistry, Martinsried, Germany). Glutathione S-transferase (GST) fusion proteins GST-N-SH2 (amino acids 6–101) and GST-C-SH2 (amino acids 112–215) of human SHP-2 were obtained from Dr. Axel Ullrich (Max-Planck Institute for Biochemistry, Martinsried, Germany).

**Cell Culture**—HC11 cells (obtained from Dr. Nancy Hynes, Friedrich Miescher Institute, Basel, Switzerland, and Dr. Bernd Groner, Institute for Biomedical Research, Frankfurt am Main, Germany) were cultured for 2 days in RPMI 1640 supplemented with 10% fetal bovine serum, 10 mg/ml epidermal growth factor, 5 μg/ml insulin, 50 units/ml penicillin, and 50 μg/ml streptomycin. At confluence HC11 cells were induced for 3 days in RPMI 1640 supplemented with 10% FBS, 5 μg/ml insulin, 1 μg hydrocortisone, 50 units/ml penicillin, and 50 μg/ml streptomycin. Prior to experimentation, HC11 cells were then starved for 2 days in RPMI 1640 supplemented with 50 units/ml penicillin and 50 μg/ml streptomycin.

293 and T47D cells were cultured in DMEM high glucose supplemented with 10% FBS, 0.1 μM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. Prior to transfection, cells were seeded at a density of 5 × 10⁵ cells per 2-cm well or 5 × 10⁶ per 6-cm dish and co-transfected with the following expression plasmids for PRLR, SHP-2, SHP-2C463A, SHP-2R132K, and SHP-2R138K using the calcium-phosphate method. After overnight post-transfection, cells were serum-starved and stimulated with PRL (1 μg/ml) for either 10 min for tyrosine phosphorylation studies or 45 min for nuclear translocation studies.

**Cell Lysis**—Cells were lysed in 300 μl of lysis buffer (10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 30 mM pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 10% (v/v) glycerol, 0.5% Triton X-100) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 mg/ml leupeptin, 5 mg/ml aprotinin) for 5 min at 4°C. The lysates were then centrifuged at 13,000 rpm for 10 min at 4°C to remove cell debris. Bradford protein assay was used to quantitate protein concentrations. Equal amounts of protein lysates were loaded and separated on SDS-PAGE followed by immunoblot analysis or for immunoprecipitations.

Nuclear extracts were prepared by initially lysing cells with a hypotonic buffer (10 mM HEPES-KOH, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 1 mM Na₃VO₄, 20 mM NaN₃, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, and 2 μg/ml leupeptin) and vortexing for 1 min. Cells were pelleted at 13,800 rpm for 1 min at 4°C, and the supernatant was discarded. The pellet was washed 3× with phosphate-buffered saline and lysed with a high salt buffer (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 1 mM NaN₃, 20 mM NaF, 5 μg/ml aprotinin, and 2 μg/ml leupeptin).

**Immunoprecipitations and Glutathione S-Transferase (GST) Pull-down Assays**—Immunoprecipitations were carried out as described previously (3). In short, cytosolic or nuclear extracts were immunoprecipitated for 2 h (overexpression studies) or overnight (endogenous studies) using polyclonal antibody to Stat5a, SHP-2, or HA and protein A-Sepharose beads. Immune complexes were separated on SDS-PAGE and prepared for immunodetection. GST fusion proteins were complexed with glutathione beads for 1 h at 4°C. Bound beads were washed twice with PBS, and two washes with PBS containing 0.3% Triton X-100. Beads were then either untreated or treated with PRL for 10 min. Complexed proteins were separated on SDS-PAGE and prepared for immunodetection with monoclonal antibody to Stat5.

**Immunoblotting**—Western blot analysis was performed using monoclonal antibodies to phosphotyrosine, P-Stat5, Stat5, or SHP-2. For repirobings, membranes were stripped and the antibodies mentioned above were used. Secondary antibodies used were goat anti-mouse IgG, which recognizes the whole IgM molecule (H + L). Proteins were detected using a chemiluminescent substrate.

**Indirect Immunofluorescence and Co-localization Experiments**—HC11 cells were seeded at a density of 5 × 10⁵ cells per well of a 24-well plate on glass coverslips pre-coated with poly-l-lysine. After a few hours, cells were co-transfected with expression plasmids coding for the long form of the PRLR, SHP-2WT, or SHP-2C463A. 24 h post-transfection, the cells were serum-starved overnight. Prior to immunostaining, cells were stimulated with PRL at 1 μg/ml for 45 min. To examine endogenous translocation of SHP-2 to the nucleus, HC11 cells were grown and stained as usual and stimulated with 1 μg/ml PRL for 45 min. Cells were fixed in acetone, blocked in 5% FBS, and incubated with polyclonal SHP-2 antibody (1:1000 overnight), and the secondary antibody used was goat anti-rabbit FITC (1:200). Coverslips were mounted on polyvinyl alcohol medium and observed under a fluorescence microscope (Nikon model Eclipse E600, Canberra-Packard, and the software application used to analyze photographs was the MetaImaging Series-MetaMorph for Microsoft Windows version 4.0). For co-localization experiments expressed in acetone-fixed in 5% FBS, and incubated overnight with polyclonal antibody to SHP-2 (1:100 dilution) and monoclonal antibody to Stat5 (1:100 dilutions). Secondary antibodies used were goat anti-rabbit FITC (1:200 dilutions) and goat anti-mouse-Cy3 (1:200 dilutions). Coverslips were mounted on PVA and observed under a confocal microscope (Zeiss LSM510, Jena, Germany. Confocal Zeiss LSM510 mounted on Axiovert. LSM510 version 2.8 software was used to analyze photographs).
Because both Stat5 and SHP-2 are critical for PRL signaling to nonspecific competitor polydeoxyinosinic-deoxycytidylic acid (25 mM monoclonal antibody to SHP-2 and reprobed with a monoclonal antibody to Stat5 (H11002 and exposed to x-ray film at 0.25 m). Stat5-response element of the nuclear extract and end-labeled double-stranded DNA containing the described above. Binding reactions were performed in a 5 mM EDTA, 5 mM dithiothreitol, 25% glycerol) containing 8–10 μg of nuclear extract and end-labeled double-stranded DNA containing the Stat5-response element of the β-casein gene promoter (5 pmol) and nonspecific competitor polydeoxynsino-deoxyctydyl acid (2 μg). For supershifts, protein extracts were incubated on ice for 30 min with polyclonal antibodies to Stat5a, SHP-2, or HA. Samples were run on a 0.25× TAE 5% non-denaturing polyacrylamide gel. The gel was dried and exposed to x-ray film at −80 °C (Fuji Film, Fisher).

RESULTS

PRL Induces SHP-2/Stat5 Interaction in Mammary Cells—Because both Stat5 and SHP-2 are critical for PRL signaling to β-casein gene activation, we were interested in determining whether these two molecules interact in mammary epithelial cells in response to PRL stimulation. In order to examine the possible formation of a PRL-mediated SHP-2-Stat5 complex, we performed co-immunoprecipitation experiments in the mammary cell line HC11, a non-tumorigenic mouse mammary epithelial cell line (27). HC11 cells were grown, induced to differentiate, and serum-starved prior to stimulation with PRL for 10 and 30 min as described under “Experimental Procedures.” Protein extracts were prepared for immunoprecipitations with a polyclonal antibody specific to the mouse isoform of Stat5α (Tyr-694) (described under “Experimental Procedures”). Protein extracts were used for immunoprecipitations with an irrelevant anti-HA tag, a polyclonal antibody to Stat5α, and a control, a polyclonal antibody to hemagglutinin (HA) epitope, as a negative control, and a polyclonal antibody to SHP-2, as a positive control. Immunoprecipitates were then separated on SDS-PAGE, and Western blot analysis was performed with an monoclonal antibody recognizing the phosphorylated form of Stat5α-Tyr-694 (western blotting). Protein lysates of HC11 or T47D cells were immunoprecipitated with a polyclonal antibody to HA (IP with polyclonal antibody to Stat5a. Protein lysates of HC11 or T47D cells were immunoprecipitated with a polyclonal antibody to SHP-2 (αSHP-2). Immune complexes were separated on SDS-PAGE, electrophoretized to a nitrocellulose membrane, and probed with a monoclonal antibody to phosphorylated Stat5α-Tyr-694. The membrane was stripped and reprobed with a monoclonal antibody to Stat5α and exposed to x-ray film at −80 °C (Fuji Film, Fisher).

To explore further whether or not this association was present in another mammary cell system, we chose to work with the T47D cell line, a human mammary carcinoma cell line, that is PRL-responsive (29). Cultures of T47D cells were stimulated with huGH, which, in addition to activating its own receptor, also has high affinity for the human PRLR (30), at various time points and protein extracts were made to immunoprecipitate with polyclonal antibody to Stat5α. As well, T47D lysates were immunoprecipitated with a polyclonal antibody to HA tag as a negative control. Immunoprecipitated proteins were separated on an 8% SDS gel. Western blot analysis was performed with a monoclonal antibody recognizing the phosphorylated form of Stat5α-Tyr-694, showing tyrosine phosphorylation of Stat5α at 10 min post-stimulation with huGH (Fig. 1B). The membrane was stripped and reprobed with monoclonal antibody to SHP-2 demonstrating a clear association with Stat5α (Fig. 1B, middle panel). Therefore, from the above data the interaction of SHP-2 with Stat5α in mammary cells was evident following lactogenic hormone stimulation.

The Carboxyl-terminal SH2 Domain of SHP-2 Is Important for the Formation of a SHP-2-Stat5 Complex and Tyrosine Phosphorylation of Stat5—The SH2 domains of SHP-2 have been shown previously to mediate various SHP-2/protein interactions. In order to understand the role of the SH2 domains of SHP-2 in the formation of a PRL-mediated SHP-2-Stat5 complex, we transiently co-transfected 293 cells with plasmids encoding for the long form of the PRLR, Stat5α, Jaks2, and either wild type SHP-2 (SHP-2WT) or forms of SHP-2 containing point mutations of the amino- or the carboxyl-terminal SH2 domains of SHP-2 where the conserved arginine residue is mutated to lysine, SHP-2R32K, and SHP-2R138K, respectively. Cells were stimulated for 10 min with PRL; protein lysates were immunoprecipitated with a polyclonal antibody to SHP-2, and the immune complexes were separated on an 8% SDS-PAGE. After electrophoretic transfer to a nitrocellulose membrane, Western analysis was performed with a monoclonal antibody to Stat5α, as shown in Fig. 2A, Stat5α was found to co-immunoprecipitate with SHP-2WT as well as SHP-2R32K. However, Stat5α was not detected in the immunoprecipitates of SHP-2R138K. As shown in Fig. 2A, middle panel, a control Western blot with a monoclonal antibody to SHP-2 showed equal immunoprecipitants of SHP-2 that were present in the different samples. As well, a Western blot of total cell lysates of the above transfections with a monoclonal antibody to Stat5α indicated similar levels of overexpression of Stat5α in the various samples (Fig. 2A, bottom panel). From the above data, it
was evident that SHP-2 formed a complex with Stat5 following PRLR activation and that the carboxyl-terminal SH2 domain but not the amino-terminal SH2 domain of SHP-2 was required for this association. To examine further the role of the SH2 domains of SHP-2 in SHP2-Stat5 complex formation, GST pull-down assays were performed using GST fusion proteins of either the amino- or the carboxyl-terminal SH2 domains of SHP-2. Protein extracts of HC11 cells unstimulated or stimulated for 10 min with PRL were incubated with glutathione bead bound-GST-N-SH2 or -GST-C-SH2 domains of SHP-2 (described under “Experimental Procedures”). Pulled down proteins were then run on SDS-PAGE and immunodetected with monoclonal antibody to Stat5. As shown in Fig. 2B, following ligand stimulation the carboxyl-terminal SH2 domain but not the amino-terminal SH2 domain of SHP-2 was able to complex with Stat5. Ligand-dependent phosphorylation of Stat5 was verified by Western blot using a monoclonal antibody recognizing the phosphorylated form of Stat5α-Tyr-694 (data not shown). Parallel SDS-PAGE of lysates of HC11 cells immunodetected with monoclonal antibody to Stat5 indicated that equal amounts of extracts were present in the different samples (Fig. 2B, bottom panel). Similar results were obtained with T47D cells (data not shown). The data confirm that PRL-mediated SHP-2/Stat5 interaction requires the carboxyl-terminal SH2 domain of SHP-2.

We then investigated whether the SHP-2/Stat5 interaction would affect the state of Stat5 tyrosine phosphorylation. Therefore, we co-overexpressed the long form of the PRLR, Jak2, Stat5, and a form of SHP-2 in 293 cells. Lysates were prepared and separated on an 8% SDS gel, and Western analysis was performed using the phospho-specific antibody to Stat5α-Tyr-694. In contrast to samples overexpressing SHP-2WT or the amino-terminal SH2 domain inactivating mutant form of SHP-2, the levels of Stat5 tyrosine phosphorylation on Tyr-694 were lost in samples overexpressing the carboxyl-terminal SH2 domain inactivating mutant form of SHP-2 (Fig. 3). Equal overexpression of SHP-2 and Stat5 in the different samples was confirmed by sequential stripping and reprobing the membrane with monoclonal antibodies to SHP-2 and to Stat5 (Fig. 3, middle and bottom panels, respectively). Therefore, the results indicate that mutation of the carboxyl-terminal SH2 domain of SHP-2 alters the tyrosine phosphorylation level of Stat5 downstream of the PRLR. Together, the data highlight a requirement for the carboxyl-terminal SH2 domain of SHP-2 in the formation of a SHP-2-Stat5 complex and the tyrosine phosphorylation of Stat5 following activation of the PRLR.

Prolactin Induces SHP-2/Stat5 Nuclear Association in Mammary Epithelial Cells—It is well established that upon PRL stimulation of HC11 cells, Stat5α is tyrosine-phosphorylated, homo- and/or heterodimerizes, and translocates to the nucleus where it binds to DNA to activate gene transcription (31). Knowing this we attempted to examine whether or not the SHP-2-Stat5α complex is present in the nucleus following PRL treatment of mammary cells. Cultures of HC11 cells were stimulated with PRL at 1 μg/ml for 30 and 60 min. Nuclear extracts were prepared, and proteins were immunoprecipitated with a
polyclonal antibody to Stat5a. In addition, unstimulated nuclear extracts of HC11 cells were immunoprecipitated with a polyclonal antibody to HA as a negative control, and unstimulated whole cell extracts were immunoprecipitated with a polyclonal antibody to SHP-2 as a positive control. Immune complexes were separated on SDS-PAGE, electroblotted to a nitrocellulose membrane, and probed with a monoclonal antibody to SHP-2. Membrane was stripped and reprobed with a monoclonal antibody to Stat5 (bottom panel). WB, Western blot.

PRL induces SHP-2 nuclear translocation in mammary epithelial cells—To confirm the presence of SHP-2 in the nucleus, we examined the subcellular localization of SHP-2 in HC11 cells following PRL stimulation. HC11 cells were serum-starved for 48 h and treated with PRL for 45 min, and the nuclear localization of SHP-2 was detected by indirect immunofluorescence after staining with a polyclonal antibody to SHP-2. As shown in Fig. 5, SHP-2 was observed to localize in the cytoplasm of unstimulated HC11 cells, and post-stimulation with PRL SHP-2 was visible in the nucleus where it was heavily stained (Fig. 5). The data support the nuclear localization of SHP-2 in mammary cells in response to PRL stimulation. Next we examined the co-localization of SHP-2 and Stat5 in HC11 cells. HC11 cells were serum-starved for 48 h, treated with PRL for 45 min, and double-immunostained with a polyclonal antibody to SHP-2 and a monoclonal antibody to Stat5. As shown in Fig. 6, in the absence of PRL stimulation SHP-2 immunostaining (green) is prominent in the cytoplasm, whereas Stat5 immunostaining (red) is distributed throughout the cell. Following stimulation with PRL both SHP-2 and Stat5 translocated to the nuclear compartment. By overlapping the green and red images, the co-localization of SHP-2 and Stat5 (yellow signal, arrowheads) is clearly visible in the nucleus of HC11 cells following ligand binding.

Nuclear translocation of SHP-2 depends on the activation of Stat5—Our next goal was to examine the role of the catalytic activity of SHP-2 in regulating its subcellular localization. We transiently co-overexpressed the long form of the PRLR and either SHP-2WT or catalytically inactive form of SHP-2, SHP-2C463A, in 293 cells. Cells were serum-starved for 24 h and treated with PRL for 45 min. By using indirect immunofluorescence, nuclear translocation of SHP-2 was examined using a polyclonal antibody to SHP-2. As shown in Fig. 7A, nuclear staining was observed in cells overexpressing SHP-2WT as SHP-2 translocated to the nucleus upon PRL stimulation. However, PRL was unable to induce nuclear translocation of the catalytically inactive mutant form of SHP-2, SHP-2C463A, and the cytoplasm remained stained for SHP-2 following PRL stimulation (Fig. 7B).

To try to understand PRL-mediated SHP-2 nuclear translocation, we examined the potential contribution of Stat5. Initially we tested whether catalytically inactive SHP-2 through point mutation in the catalytic domain, SHP-2C463A, modulated Stat5 tyrosine phosphorylation downstream of the PRLR. We co-overexpressed the long form of the PRLR, Jak2, and Stat5 along with either SHP-2WT or SHP-2C463A in 293 cells. Serum-starved cells were stimulated for 10 min with PRL at 1 μg/ml, and lysates were made and separated on an 8% SDS gel. A Western blot analysis was performed using a monoclonal antibody to phosphorylated Stat5-Tyr-694. As shown in Fig. 8, there is a pronounced decrease in the levels of tyrosine phosphorylation of Stat5 in samples overexpressing catalytically inactive mutant form of SHP-2 compared with samples overexpressing SHP-2WT. The membrane was stripped, and Western blot analyses were performed with monoclonal antibodies to SHP-2 and Stat5 showing equivalent levels of expression in the different samples (Fig. 8, middle and bottom panels, respectively). Therefore, the data indicate that catalytically active SHP-2 is required for PRL-mediated Stat5 tyrosine phosphorylation and that in the presence of inactive phosphatase Stat5 activation is lost.

We next examined whether the catalytic activity of SHP-2 may have an influence on the formation of SHP-2-Stat5 complex in response to PRL. For this reason 293 cells were either left un-transfected or co-transfected with expression plasmids encoding the long form of the PRLR, Jak2, and Stat5 along with either SHP-2WT or SHP-2C463A. Protein extracts were prepared for immunoprecipitations with a polyclonal antibody specific to Stat5a. In addition, protein extracts of 293 cells transfected with SHP-2WT were immunoprecipitated with a polyclonal antibody to SHP-2, as a positive control. Immunoprecipitated proteins were separated on SDS gel and immunodetected with a monoclonal antibody to SHP-2. As shown in Fig. 9, SHP-2 wild type but not the catalytically inactive form of SHP-2 co-immunoprecipitated with Stat5 following PRLR activation. The membrane was stripped and reprobed with a monoclonal antibody to Stat5 showing equal immunoprecipitations of Stat5 in the various samples. Furthermore, equal expression of SHP-2 in the different samples was verified by Western blotting of cell lysates of the above transfections with a monoclonal antibody to SHP-2. These data demonstrate that the catalytic domain of SHP-2 regulates the formation a SHP-2-Stat5 complex in response to PRL stimulation. Altogether, the results indicate that PRL-mediated nuclear translocation of SHP-2 correlates with Stat5 activation and the ability of...
SHP-2 to associate with Stat5.

**SHP-2-Stat5 Complex Bind to the β-Casein Gene Promoter in Response to PRL**—Based on the fact that the SHP-2-Stat5 complex is found to be present in the nucleus of mammary cells following PRL stimulation, we then examined whether this protein complex can exist on the Stat5-binding site on the β-casein gene promoter. For this reason HC11 cells were stimulated with PRL at various time points; nuclear extracts were made, and an EMSA was performed using the β-casein GAS site. As seen in Fig. 10, PRL-mediated DNA binding activity was observed from 1 to 3 h post-stimulation. Supershifts were performed with polyclonal antibody to SHP-2 (lanes 3, 5, and 7), Stat5a (lane 8), and HA (lane 9).

**FIG. 6.** SHP-2 and Stat5 colocalize in the nucleus of HC11 cells in response to PRL. HC11 cells were grown, stimulated with PRL for 45 min, and double immunostained with a polyclonal antibody to SHP-2 and a monoclonal antibody to Stat5 for confocal analysis as described under “Experimental Procedures.”

**FIG. 7.** Intact catalytic domain of SHP-2 is required for PRL-induced SHP-2 nuclear translocation. 293 cells overexpressing the long form of the PRLR and either wild type SHP-2 (A) or catalytically inactive SHP-2 and SHP-2C463A (B) were either left untreated or treated with PRL for 45 min and stained with a polyclonal antibody to SHP-2 for immunofluorescence analysis as described under “Experimental Procedures.”

**FIG. 8.** The catalytic domain of SHP-2 is required for tyrosine phosphorylation of Stat5 following activation of the PRLR. 293 cells overexpressing the long form of the PRLR, Jak2, Stat5, and either wild type SHP-2 or SHP2C463A were stimulated for 10 min with PRL prior to harvesting. Lysates were separated on a SDS-PAGE, electroblotted to a nitrocellulose membrane, and probed with a monoclonal antibody to phosphorylated Stat5a-Tyr-694. The membrane was stripped and sequentially reprobed with monoclonal antibodies to SHP-2 and Stat5 (middle and bottom panels, respectively). WB, Western blot.

**FIG. 9.** The catalytic domain of SHP-2 is required for PRL-induced SHP-2-Stat5 complex formation. 293 cells were either left untransfected (CTL) or transiently co-transfected with expression plasmids encoding for the long form of the PRLR, Jak2, Stat5, and either SHP-2WT or SHP-2C463A. Cells were starved overnight prior to stimulation. After a 10-min treatment with PRL cell lysates were prepared and immunoprecipitated (IP) with a polyclonal antibody to Stat5a. Immunoprecipitations of lysates of 293 cells overexpressing SHP-2 wild type were also performed using a polyclonal antibody to SHP-2. The immune complexes were separated on SDS-PAGE, transferred to a nitrocellulose membrane, and probed with a monoclonal antibody to SHP-2. Middle panel, the membrane was stripped and reprobed with a monoclonal antibody to Stat5. Bottom panel, cell lysates of the above transfections were separated on SDS gel and immunodetected with a monoclonal antibody to SHP-2. WB, Western blot.
readily visible when extracts were preincubated with a polyclonal antibody to Stat5α (Fig. 10, lane 8) and more importantly with a polyclonal antibody to SHP-2 (Fig. 10, lanes 3, 5, and 7) but not with a polyclonal antibody to HA. This experiment demonstrates that not only does Stat5α bind to the β-casein gene promoter in response to PRL in mammary cells, it does so in composite with SHP-2, suggesting that SHP-2 may play a role in regulating Stat5-mediated gene transcription.

DISCUSSION

Over the past decade extensive studies have indicated that the Jak/Stat pathway is one of the principal signaling pathways utilized by members of the class I cytokine receptor family to transduce their signals in target tissues. Recently it became apparent, although less studied, that other pathways and effector molecules could also play an important role in regulating this signaling cascade. The ubiquitously expressed protein-tyrosine phosphatase SHP-2 has been designated both as a positive and a negative regulator of signaling downstream of different cytokine receptors. Our present study reinforces and extends the critical role of SHP-2 in PRL signaling leading to milk protein gene activation and highlights an intricate relationship between the two molecules, SHP-2 and Stat5α, required for their activation and function.

The Jak2/Stat5α pathway has been shown to be central in mediating PRL signals to the activation of milk protein gene transcription. In this study we have demonstrated a novel physical association of SHP-2 with Stat5α in both the cytoplasm and the nucleus of mammary cells in response to PRL stimulation. Our results indicate that the interaction of SHP-2 with Stat5α depends on an intact carboxyl-terminal SH2 domain of SHP-2 and an active catalytic domain and correlates with the tyrosine phosphorylation of Stat5α on the conserved tyrosine residue (Tyr-694). We have shown previously (32) that the carboxyl-terminal SH2 domain of SHP-2 is required in order for SHP-2 to be recruited to the PRLR. Therefore, together the data indicate that Stat5α tyrosine phosphorylation downstream of the PRLR depends on the ability of the PRLR to recruit catalytically active SHP-2, further demonstrating the positive role of SHP-2 downstream of the PRLR leading to the activation of Stat5α. Also the data points to the fact that the formation of an SHP-2-Stat5α complex follows the classical SH2-phosphotyrosine interaction mechanism requiring Tyr-694 of Stat5. However, in view of the fact that tyrosine phosphorylation of Stat5 on other residues besides Tyr-694 has been documented (33), we therefore cannot implicate Tyr-694 specifically as the site of association.

However, the biochemical parameters influencing the interaction of SHP-2 with Stat5α may vary in different cell types and/or downstream of different cytokines. For example, Yu et al. (34) have demonstrated previously by GST pull-down experiments that the CS mutant of SHP-2 overexpressed in COS7 cells associated with Stat5 when preincubated with the cytosol of CTLL20 cells stimulated with interleukin-2 but were unable to show an association between SHP-2 wild type and Stat5α. Therefore, it is still to be determined whether the formation of a SHP-2-Stat5 complex and SHP-2 regulation of Stat5 activation is tissue-specific and/or ligand-specific. In this regard whereas SHP-2 has been implicated in negative regulation of signaling to Stat5b activation downstream of the growth hormone receptor (26), our results indicate that in breast cancer cells, T47D cell line, GH stimulation leads to tyrosine phosphorylation of Stat5α and to SHP-2/Stat5α interaction. This effect of huGH could be due to its ability to activate its own receptor as well as the human PRLR.

Subcellular compartmentalization and/or distribution of effector molecules occur in order for signaling proteins to target their substrates. We have shown here for the first time that SHP-2 undergoes subcellular relocalization in response to PRL stimulation in mammary cells. We found that in mammary cells as early as 45 min following PRL stimulation SHP-2 presents itself by localizing in the nucleus. Nuclear translocation of SHP-2 was found to correlate with the nuclear translocation of Stat5α in HC11 cells. This activity is not unique to SHP-2. Ram et al. (35) have demonstrated previously that GH induced nuclear localization of Stat-1, the mammalian homologue of SHP-2, and its association with GH activated tyrosine-phosphorylated Stat5b in liver cells. This study implicates SHP-1 in the dephosphorylation and recycling back of Stat5b to the cytoplasm (35). It is interesting to note that Ram et al. (35) were able to show that in liver cells nuclear translocation of SHP-1 does not depend on a simultaneous nuclear translocation of Stat5b. Nuclear translocation of SHP-1 may be due to an intrinsic activity of the phosphatase because a half-bipartite KRK nuclear localization sequence within the carboxyl-terminal distal region of SHP-1 was described (36). However, no such sequence was identified on SHP-2 (36). In fact our results indicate that SHP-2 nuclear translocation in response to PRL treatment correlates with the activation of Stat5. PRL treatment did not lead to nuclear translocation of the catalytically inactive SHP-2. This form of SHP-2 is unable to interact with Stat5. In addition, Stat5 is not tyrosine phosphorylated in this environment and therefore, is not expected to translocate to the nucleus, suggesting that Stat5 may act as a “carrier” for SHP-2 to the nucleus by providing its own nuclear translocation machinery.

We have shown that SHP-2 exists in a complex with Stat5α on the GAS-responsive element on the β-casein gene promoter (Fig. 10). We speculate that an active SHP-2 and a tyrosine-phosphorylated Stat5α localize to the nucleus and potentially function in the nucleus to regulate transcription as both SHP-2 and Stat5α as a complex binds to DNA. In the nucleus SHP-2 may potentially be involved in the transcription of Stat5α-responsive genes. Targets for SHP-2 in the nucleus have not been identified until now. It is believed that the Stat dimer becomes dephosphorylated by an unidentified protein-tyrosine phosphatase to form non-phosphorylated Stat monomers in the nucleus (37, 38). We have demonstrated that SHP-2 is required to activate Stat5α in the cytoplasm, and we find it unlikely that it would be involved in the dephosphorylation and deactivation of Stat5α in the nucleus. Nuclear localization of protein-tyrosine phosphatases may allow them to participate in regulating the state of phosphorylation of specific molecules including those involved in the transcriptional machinery of a cell such as co-activators, co-repressors, DNA/RNA polymerases, as well as chromatin adding another level of complexity in the function of protein-tyrosine phosphatases. In summary, our study indicates that PRL treatment of mammary cells induces the cytosolic and nuclear association of SHP-2 with Stat5α. This interaction is a cardinal requirement for activation and nuclear translocation of SHP-2/Stat5α in turn determining the fate of milk protein gene transcription.

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Prolactin Induces SHP-2 Association with Stat5, Nuclear Translocation, and Binding to the β-Casein Gene Promoter in Mammary Cells
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