THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 272, No. 22, Issue of May 30, pp. 14098–14103, 1997
Printed in U.S.A.

Prolactin Stimulates Serine/Tyrosine Phosphorylation and Formation of Heterocomplexes of Multiple Stat5 Isoforms in Nb2 Lymphocytes*

(Received for publication, November 21, 1996, and in revised form, March 26, 1997)

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Transcription factors of the Stat gene family are selectively activated by many hormones and cytokines. Stat5 originally was cloned as a prolactin-stimulated DNA-binding protein, but is also activated by non-lactogenic cytokines in many cell types. The recent identification of two distinct Stat5 genes, which encode a 94-kDa Stat5a and a 92-kDa Stat5b as well as several lower molecular weight isoforms, suggests additional complexity and combinatorial possibilities for transcriptional regulation. We now report a biochemical analysis of prolactin activation of Stat proteins in Nb2 lymphocytes, which was associated with: 1) rapid tyrosine phosphorylation of Stat5a, Stat5b, a COOH-terminally truncated 80-kDa Stat5 form, Stat1α, and Stat3; 2) rapid and selective formation of Stat5a/b heterodimers, without involvement of Stat1α or Stat3; 3) marked serine, but not threonine phosphorylation of Stat5a and Stat5b; and 4) the appearance of two qualitatively distinct Stat5 protein complexes, which discriminated between oligonucleotides corresponding to the prolactin response elements of the β-casein and interferon regulatory factor-1 gene promoters. Collectively, our analyses showed that Stat5a and Stat5b respond similarly to prolactin receptor activation, but also suggested that the two genes have evolved unique properties that may contribute to the specificity of receptors that utilize Stat5 signaling proteins.

A variety of polypeptide hormones and cytokines use cytoplasmic signal transducers and activators of transcription (Stats)[1] to regulate expression of specific genes (1, 2). The ability of individual cytokine receptors to activate overlapping, but distinct sets of homo- and heterodimerizing Stat proteins contributes to their signal specificity. For example, interferon-α activates Stat1, Stat2, and Stat3 and exerts antiviral and growth-inhibitory effects in target cells (3). Prolactin (PRL), on the other hand, activates Stat1, Stat3, and Stat5 (4–6), and stimulates β-casein synthesis in mammary epithelial cells and proliferation of Nb2 lymphocytes (7–9).

Two distinct Stat5 genes have recently been identified which encode the highly homologous 94-kDa Stat5a and 92-kDa Stat5b proteins, as well as shorter 78–80-kDa isoforms of each gene product (10–14). Interestingly, the COOH-terminally truncated forms of Stat5a and Stat5b have transdominant negative effects on transcription (15, 16). Similarly, shorter forms of Stat1 and Stat3 have been identified, and result from alternative splicing of mRNA (11, 12, 17, 18). This multiplicity of Stat isoforms adds further combinatorial possibilities to receptor-mediated transcriptional regulation. Thus, preferential activation of Stat5a over Stat5b by granulocyte colony-stimulating factor has suggested receptor selectivity in Stat5 recruitment (19). Knowledge of how different combinations of Stat5 isoforms may be used by distinct receptors therefore becomes critical to our understanding of gene regulation by a large number of hormones and cytokines.

Thus far all Stat proteins have been shown to require phosphorylation of a positionally conserved tyrosine residue corresponding to Tyr-701 of human Stat1, which in turn facilitates dimerization and binding to DNA response elements (20, 21). Furthermore, inducible serine phosphorylation of Stat1α or Stat3 is needed for full transcriptional activation (22, 23), and the mitogen-activated protein kinase (MAPK) $p42^{ERK2}$ has been implicated in the phosphorylation of Stat1α (22, 24). However, Stat5 homologues lack the putative MAPK phosphorylation site (*X*-Pro-*X*-Ser-Pro) corresponding to Ser-727 of human Stat1α (22). This finding raises the possibility that Stat5 activities are regulated different than other Stats.

The present study specifically set out to define and compare the molecular activation of different Stat5 gene products by PRL in Nb2 lymphocytes, a well characterized model of PRL-induced cell proliferation and signal transduction. We now show that multiple forms of Stat5, including $p94^{Stat5a}$, $p92^{Stat5b}$, and a COOH-terminally truncated 80-kDa Stat5 isoform, undergo marked phosphorylation on tyrosine and serine residues in response to PRL. Furthermore, PRL induced rapid and selective heterocomplex formation without involving Stat1α and Stat3, which also became tyrosine-phosphorylated. Finally, we demonstrate the formation of a Stat5a/b-containing complex that bound equally well to oligonucleotide probes corresponding to the PRL-response elements of the β-casein and interferon regulatory factor (IRF1) gene promoters. In contrast, a slower migrating complex that contained Stat5b and not

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[1] The abbreviations used are: Stat, signal transducers and activators of transcription; PRL, prolactin; oPRL, ovine prolactin; MAPK, mitogen-activated protein kinase; PAGE, polyacrylamide gel electrophoresis; mAb, monoclonal antibody; EMSA, electrophoretic mobility shift assay.

Stat5a bound exclusively to the β-casein promoter and not to the IRF-1 probe. Collectively, these observations demonstrate that the highly homologous Stat5a and Stat5b proteins respond biochemically in a similar manner to PRL receptor activation. However, the data also suggest that the two gene products, which differ most in their COOH-terminal transactivation domains, have evolved unique properties that may contribute specificity to the large number of Stat5-activating receptors.

EXPERIMENTAL PROCEDURES

Materials—Ovine PRL (NIDDK-oPRL-19, AFP-9221A) and human PRL (NIDDK-hPRL-SIAFP-B2, AFP-2969A) were supplied by the National Hormone and Pituitary Program, National Institutes of Health, Bethesda, MD. Polyclonal rabbit antisera specific to peptides corresponding to the unique COOH termini of Stat1α, Stat2, Stat3, Stat5a, and Stat5b were generated as described previously (10, 25, 26). A polyclonal antiserum that recognized both Stat5a and Stat5b was also developed as described previously (27). Each of these antisera was useful for detection of rat, mouse, and human Stat proteins by immunoprecipitation and immunoblotting. In addition, a monoclonal anti-pan-Stat5 antibody, directed to a distinct internal region shared by Stat5a and Stat5b, and monoclonal antibodies for immunoblotting of Stat1 and Stat3, were purchased from Transduction Laboratories, Inc. (catalog nos. S21520, S21120, and S21320, respectively).

Cell Culture and Treatment—The Nb2 cell line (8) was originally developed by Dr. Peter Gout (Vancouver, Canada), the Nb2-SP clone used in this work was provided by Dr. Henry Friesen (University of Manitoba, Canada). Cells were grown in RPMI 1640 medium (Mediatech, catalog no. 15-040-LM) containing 10% fetal calf serum (Intergen, catalog no. 1020-90), 2 mM L-glutamine, 5 mM HEPES, pH 7.3, and penicillin-streptomycin (50 IU/ml and 50 μg/ml, respectively), at 37 °C with 5% CO_2. Nb2 cells at a density of 1–1.5 × 10^6/ml were incubated for 20 h in lactogen-free medium consisting of RPMI 1640, which instead of 10% fetal calf serum contained 1% gelded horse serum (Sigma, catalog no. H-1895). Cells were brought to a density of 5 × 10^7 cells/ml and preincubated at 37 °C for 10 min prior to stimulation with 100 nM of ovine PRL.

Solubilization of Proteins, Immunoprecipitation, and Immunoblotting—Frozen pellets from 1 × 10^8 Nb2 cells were thawed on ice and solubilized in 1 ml of lysis buffer containing 10 mM Tris-HCl, pH 7.6, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 1 μg/ml pepstatin A, and 2 μg/ml leupeptin. Cell lysates were incubated at 4 °C for 60 min, and insoluble material was pelleted at 12,000 × *g* for 30 min at 4 °C. Depending on the experiment, clarified lysates were incubated with for 3 h at 4 °C with either monoclonal mouse anti-phosphotyrosine antibodies (clone 4G10; Upstate Biotechnology, Inc. (UBI), catalog no. 05-321; 5 μg/ml), or polyclonal rabbit antisera (2 μl/ml) to individual Stat proteins as specified. Antibodies were captured by incubation for 60 min with protein A-Sepharose beads (Pharmacia Biotech Inc., catalog no. 17-0780-01) and washed, and samples were subjected to 7.5% SDS-PAGE under reducing conditions according to Laemmli (27). The proteins were then transferred to polyvinylidene difluoride membrane (Millipore, catalog no. 1PVH 00010), using a semidry transfer unit (Multiphor Novablot, Pharmacia) at constant current of 195 mA for 90 min. After transfer, the blots were incubated for at least 1 h at room temperature in blocking buffer (0.02 M Tris-HCl, pH 7.6, 0.137 M NaCl, 1% bovine serum albumin, and 0.01% sodium azide) before immunoblotting. Blots were exposed for 90 min to primary antibodies diluted in blocking buffer at the following concentrations: anti-phosphotyrosine mAb 4G10 (UBI, catalog no. 06-321, 1 μg/ml), anti-Stat1 mAb (1:2,500 dilution), anti-Stat3 mAb (1:2,500 dilution), and anti-pan-Stat5 mAb directed to a distinct internal region common to Stat5a and Stat5b (1:250 dilution). The blots were then incubated twice for 5 min in wash buffer (50 mM Tris-HCl, pH 7.6, 200 mM NaCl, 0.25% Tween 20), followed by incubation for 30 min with horseradish peroxidase-conjugated goat antibodies to mouse or rabbit IgG (Kirkegaard and Perry Laboratories, catalog nos. 074-1806 and 074-1506, respectively) at 500 ng/ml in blocking buffer, followed by four 15-min incubations in wash buffer. The blots were then incubated for 1 min with enhanced chemiluminescence substrate (ECL) mixture according to the manufacturer's instructions (Amersham, catalog no. RPN2106), and exposed to x-ray film for 1–5 min (Eastman Kodak Co., catalog no. 165 1454).

Phosphoamino Acid Analysis—[^{32}P]Orthophosphate labeling of cell proteins were carried out as described earlier (28). Labeled Stat5a and Stat5b proteins were excised from polyvinylidene difluoride membranes and hydrolyzed in 6 N HCl at 110 °C for 90 min. Phosphoamino acid analysis was then performed as described earlier (29).

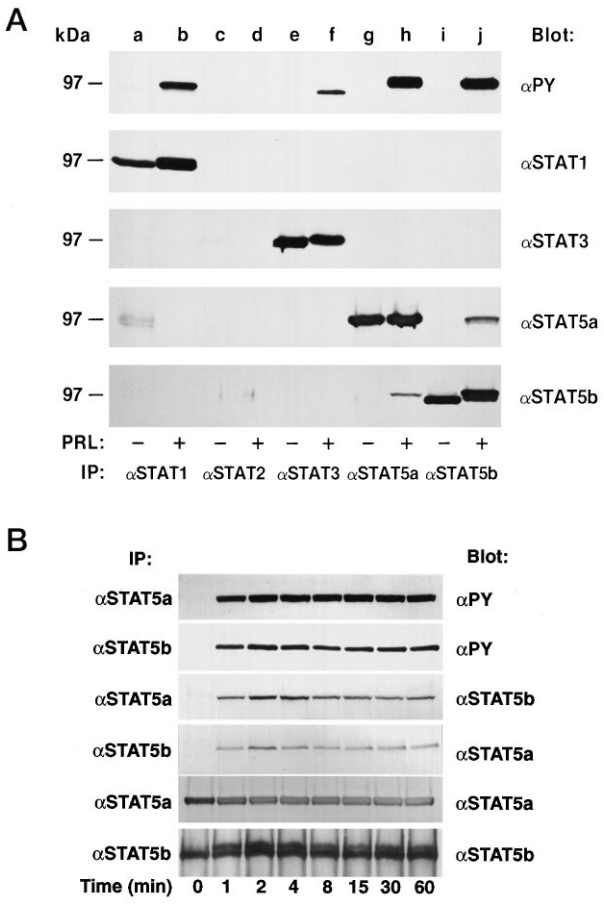

FIG. 1. **PRL induces tyrosine phosphorylation and heterodimerization of Stat5a and Stat5b.** *A*, general screening for Stat tyrosine phosphorylation and dimerization. Quiescent Nb2 cells were incubated with medium (−) or 100 nM oPRL (+) for 15 min at 37 °C, and lysates were immunoprecipitated (*IP*) with anti (α)-Stat1α (*lanes a* and *b*), α-Stat2 (*lanes c* and *d*), α-Stat3 (*lanes e* and *f*), α-Stat5a (*lanes g* and *h*), or α-Stat5b (*lanes i* and *j*). Parallel samples were blotted for either phosphotyrosine (α*PY*; *panel 1*), α-Stat1α (*panel 2*), α-Stat2 (not shown), α-Stat3 (*panel 3*), α-Stat5a (*panel 4*), or α-Stat5b (*panel 5*). *B*, kinetic analysis of tyrosine phosphorylation and heterodimerization of Stat5a and Stat5b. Quiescent Nb2 cells were incubated with medium (−) or 100 nM oPRL (+) at 37 °C for various times as indicated, and lysates were immunoprecipitated (*IP*) with either α-Stat5a (*panels 1*, *3*, and *5*), or α-Stat5b (*panels 2*, *4*, and *6*). Parallel samples were blotted for either phosphotyrosine (α*PY*; *panels 1* and *2*), α-Stat5a (heterologous antiserum; *panels 3* and *6*), α-Stat5b (homologous antiserum; *panels 4* and *5*). Note that Stat5a/b heterodimerization occurs immediately after PRL stimulation, and parallels the tyrosine phosphorylation kinetics.

Electrophoretic Mobility Shift Assay (EMSA)—Quiescent Nb2 cells were treated with or without PRL (100 nM) for 10 min, pelleted by centrifugation, and immediately solubilized in EMSA lysis buffer (20 mM HEPES, pH 7.0, 10 mM KCl, 1 mM $MgCl_2$, 20% glycerol, 0.2% Nonidet P-40, 1 mM orthovanadate, 25 mM NaF, 200 μM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 1 μg/ml pepstatin A, and 2 μg/ml leupeptin). Lysates were incubated on ice for 20 min, then clarified by centrifugation at 20,000 × *g* for 20 min at 4 °C. For the EMSA (30), 1 ng of oligonucleotides corresponding to the PRL response elements of the rat β-casein (5′-agatttctaggaattcaaatc-3′) or human IRF-1 (5′-gatccatttccccgaaatga-3′) genes that had been end-labeled using polynucleotide kinase and [γ-^{32}P]ATP, were incubated with 10 μg of protein from cellular lysates in 30 μl of binding mixture (50 mM Tris-Cl, pH 7.4, 25 mM $MgCl_2$, 5 mM dithiothreitol, 50% glycerol) at room temperature for 20 min, with preincubation of samples with 1 μl of either normal rabbit serum or antisera specific to Stat proteins as indicated. Polyacrylamide gels (5%) containing 5% glycerol and 0.25 × TBE were prerun in 0.25 × TBE buffer at 4–10 °C for 1.5 h at 270 V. After loading of samples, the

gels were run at room temperature for approximately 3 h at 250 V. Gels were dried by heating under vacuum and exposed to x-ray film (X-Omat, Kodak).

RESULTS AND DISCUSSION

PRL Induces Tyrosine Phosphorylation and Heterodimerization of Stat5a and Stat5b—Cytokine-induced tyrosine phosphorylation plays a key role in the activation of Stat transcription factors by facilitating Stat dimerization, which is needed for nuclear translocation and DNA binding (17). Thus, both homo- and heterodimerization of Stats have been reported after interferon receptor stimulation (31, 32). To assess whether the two recently identified highly homologous Stat5 genes are differentially regulated by PRL, we chose a biochemical approach and utilized the PRL-sensitive rat Nb2 lymphoma cell line (8). The first step was to analyze PRL-inducible tyrosine phosphorylation of major Stats and to compare their dimerization patterns by immunopreciptation from lysates of stimulated and unstimulated Nb2 cells with specific antisera. Immunoblotting with anti-phosphotyrosine antibodies revealed that both Stat5a and Stat5b were markedly tyrosine-phosphorylated following PRL treatment for 15 min (Fig. 1*A*, *panel 1*, *lanes g–j*). The treatment also stimulated tyrosine phosphorylation of Stat1α and Stat3, but not of Stat2 (Fig. 1*A*, *panel 1*, *lanes a–f*), consistent with previous results (5, 6). Thus, by the use of specific antibodies, these data extend the previous evidence of Stat5 activation by PRL to define specific products of the two homologous Stat5a and Stat5b genes.

There is considerable evidence to suggest that the DNA binding activity of Stat proteins is critically dependent upon selective dimerization after tyrosine phosphorylation. To test for inducible heterodimerization among PRL-activated Stat transcription factors, we performed sequential cross-blotting of parallel sets of samples to those analyzed for tyrosine phosphorylation (Fig. 1*A*, *panels 2–5*). These experiments showed that a significant fraction of Stat5a and Stat5b molecules heterodimerized and could be immunoprecipitated with antibody to the heterologous Stat5 from lysates of PRL-stimulated cells (Fig. 1*A*, *panels 4* and *5*). In contrast, PRL induced no detectable heterodimerization of Stat1α, Stat2, or Stat3 (Fig. 1*A*, *panels 2–5*). Based upon these results and the dimerization model proposed by Darnell and colleagues (22, 31), we conclude that PRL specifically induces heterodimerization of Stat5a and Stat5b, and homodimerization of Stat1α and Stat3. Furthermore, we observed that PRL induced a significant retardation of the electrophoretic mobility of Stat5b, but not of Stat5a, resulting in a Stat5b protein doublet (Fig. 1*A*, *panels 4* and *5*). PRL induced similar shifts of Stat1α and Stat3 proteins. This type of mobility shift on SDS-PAGE is often caused by protein phosphorylation (33–35), and similar observations have been reported for Stat1α and Stat3 after stimulation with interferon and insulin (36, 37). Intriguingly, Stat5a showed a significantly lesser mobility retardation than did Stat5b in PRL-stimulated Nb2 cells (Fig. 1*A*, *lanes g–j*, *bottom two panels*), possibly reflecting functional differences between Stat5a and Stat5b.

PRL Induces Tyrosine Phosphorylation and Heterodimerization of Stat5a and Stat5b with Similar Rapid Kinetics—Having demonstrated that PRL selectively induced heterodimerization of Stat5a and Stat5b, we investigated how the tyrosine phosphorylation kinetics of these two Stats corresponded with their dimerization kinetics. Nb2 cells were stimulated with PRL for varying times up to 60 min. Stat5a and Stat5b were immunoprecipitated from cell lysates with specific antibodies and blotted with anti-phosphotyrosine antibodies or specific antisera to Stat5a or Stat5b. As shown in Fig. 1*B* (*panels 1* and *2*), marked tyrosine phosphorylation of both Stat5a and Stat5b was detected within 1 min of PRL exposure, and maximal levels were

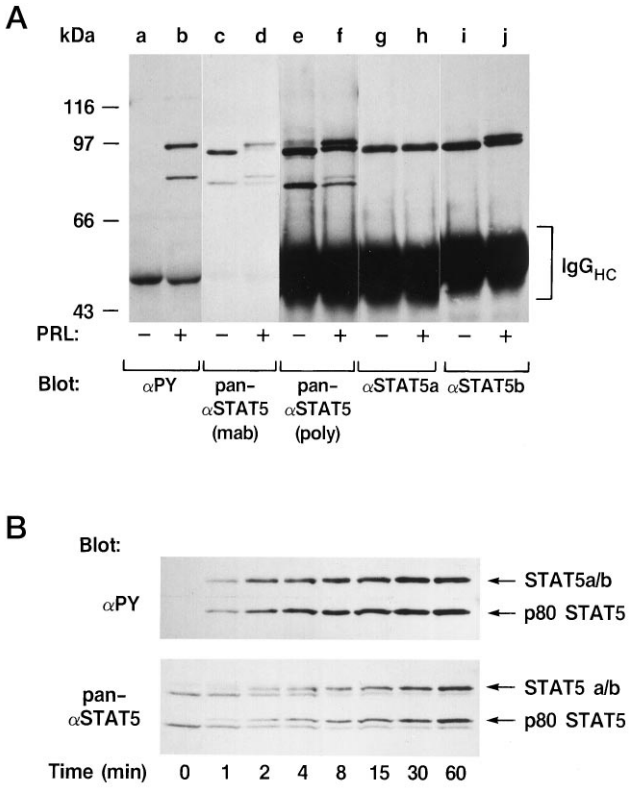

FIG. 2. **PRL induces tyrosine phosphorylation of an 80-kDa form of Stat5.** *A*, quiescent Nb2 cells were incubated with medium (−) or 100 nM oPRL (+) for 15 min at 37 °C, and lysates were immunoprecipitated with a polyclonal α-Stat5 serum that does not discriminate between Stat5a and Stat5b. Five identical sets of samples were separated by SDS-PAGE, and blotted in parallel against either phosphotyrosine (*αPY*; *lanes a* and *b*), monoclonal α-pan-Stat5 (*lanes c* and *d*), polyclonal α-pan-Stat5 (*lanes e* and *f*), α-COOH terminus of Stat5a (*lanes g* and *h*), or α-COOH terminus of Stat5b (*lanes i* and *j*). Note that an inducibly tyrosine-phosphorylated 80-kDa protein that is immunoprecipitated with α-pan-Stat5 serum is recognized by two α-pan-Stat5 antibodies, but not by antisera directed to the unique COOH termini of either Stat5a or Stat5b. *B*, kinetic analysis of tyrosine phosphorylation of p80^{Stat5}. Quiescent Nb2 cells were incubated with medium (−) or 100 nM oPRL (+) at 37 °C for various times as indicated, and lysates were immunoprecipitated with α-pan-Stat5 serum and blotted with phosphotyrosine antibodies (*upper panel*) or with monoclonal α-pan-Stat5 antibodies (*lower panel*). Note that tyrosine phosphorylation of p80^{Stat5} is parallel to that of p92–94^{Stat5} proteins, and also that p80^{Stat5} undergoes a gradual mobility shift with time.

reached within 2 min of stimulation. Interestingly, these elevated tyrosine phosphorylation levels were sustained for at least 60 min. Cross-probing the stripped immunoblots with antibodies to the heterologous Stat5 protein showed that heterodimerization of Stat5a and Stat5b paralleled their tyrosine phosphorylation kinetics (Fig. 1*B*, *panels 3* and *4*). This supports the concept of Stat dimerization via phosphotyrosyl-binding SH2-domains (22, 31, 38). Furthermore, reprobing of the same blots with antibodies to the homologous Stat5 showed that the levels of immunoprecipitated Stat5 proteins remained constant over the 60-min time course (Fig. 1*B*, *panels 5* and *6*). Collectively, experiments using reciprocal co-immunoprecipitation and immunoblotting as shown in Fig. 1 suggest that PRL induces a significant degree of heterodimerization of Stat5a and Stat5b, although further studies are needed to elucidate the stoichiometry of homo- and heterodimers.

PRL Induces Tyrosine Phosphorylation of an 80-kDa, COOH-terminally Truncated Stat5 Isoform—The results reported in Fig. 1 are based on the use of specific antisera directed to the unique COOH termini of Stat5a or Stat5b (10). Interestingly, when a polyclonal anti-Stat5 serum that recognizes shared

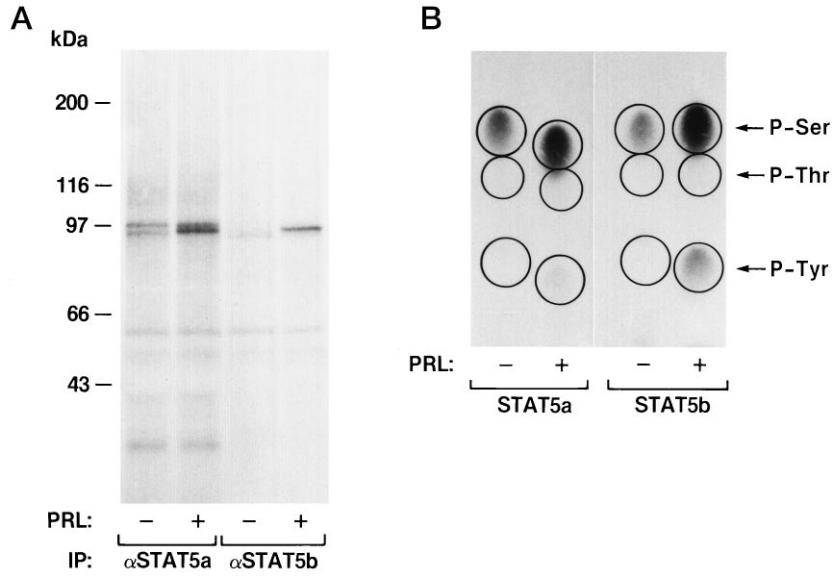

FIG. 3. **PRL induces serine phosphorylation of Stat5a and Stat5b.** *A*, autoradiography of immunoprecipitated (*IP*) Stat5a and Stat5b from [^{32}P] orthophosphate-labeled cells incubated with (+) or without (−) PRL (100 nM) for 15 min at 37 °C. *B*, phosphoamino acid analysis of Stat5a and Stat5b with (+) or without (−) PRL stimulation. *P-Ser*, phosphoserine; *P-Thr*, phosphothreonine; *P-Tyr*, phosphotyrosine. Radioactive bands corresponding to either Stat5a or Stat5b were excised and subjected to acid hydrolysis and thin layer electrophoresis, and phosphate incorporated into amino acids was visualized by autoradiography.

internal epitopes of Stat5a and Stat5b (6) was used to immunoprecipitate lysates of PRL-treated Nb2 cells, we observed a second inducibly tyrosine-phosphorylated protein with an apparent molecular mass of 80 kDa in addition to the tyrosine-phosphorylated 92–94-kDa forms of Stat5a/b (Fig. 2*A*, *lane b*). We identified this 80-kDa protein as a truncated Stat5 molecule by immunoblotting with either a monoclonal anti-pan-Stat5 antibody whose epitope is located in the central portion of both Stat5a and Stat5b (Transduction Laboratories catalog no. S21520; Fig. 2*A*, *lanes c* and *d*), or the polyclonal anti-pan-Stat5 serum (Ref. 6; Fig. 2*A*, *lanes c–f*). The 80-kDa protein was not recognized by antibodies specific to the COOH termini of either Stat5a or Stat5b (Fig. 2*A*, *lanes g–j*). Furthermore, the kinetics of tyrosine phosphorylation of the p80 Stat5-like protein paralleled that of Stat5a and Stat5b (Fig. 2*B*, *upper panel*). Based upon these observations and the reported existence of alternatively spliced 80-kDa variants of Stat5 (11–14), we conclude that PRL activates a COOH-terminally truncated form of Stat5 in Nb2 cells. Further studies are needed to determine whether p80^{Stat5} represents a short form of Stat5a or Stat5b. This is in contrast to an 80-kDa short form of Stat5a with a presumed internal deletion that was inducibly tyrosine-phosphorylated by granulocyte colony-stimulating factor in myeloid cells and could be recognized by a COOH-terminal anti-Stat5a serum (19).

The COOH-terminally truncated forms of Stat molecules, including the 80-kDa splice form Stat3β and 78–80-kDa forms of Stat5, act as dominant negative regulatory partners that lack a transactivation domain (15, 16, 39). It is therefore reasonable to anticipate that the PRL-sensitive p80^{Stat5} in Nb2 cells exerts a transdominant negative activity. Interestingly, in parallel experiments conducted with PRL-responsive human breast cancer cell lines, we have not observed PRL-induced p80^{Stat5} activation, whereas the protein response is present in 32D myeloid cells stably transfected with PRL receptors (data not shown). Collectively, these observations suggest that p80^{Stat5} is variably expressed in PRL target cells and may constitute an important modulator of the transcriptional potency of Stat5 complexes. Cellular variability in Stat isoform expression may markedly influence the signaling repertoire of individual receptors.

PRL Induces Serine but Not Threonine Phosphorylation of Stat5a and Stat5b—In addition to phosphorylation of the positionally conserved tyrosine residue corresponding to Tyr-701 of human Stat1, it has been reported that Stat1 requires phosphorylation of Ser-727 for full transcriptional activity (22, 23). Specifically, the serine-threonine kinase ERK2, one of the MAPKs, has been implicated in IFNα activation of Stat1 (24). However, unlike other known Stats, Stat5a and Stat5b lack classical *X*-Pro-*X*-Ser-Pro MAPK phosphorylation sites. To the best of our knowledge, no direct experimental evidence for inducible serine or threonine phosphorylation of either Stat5a or Stat5b has thus far been presented. On the other hand, the inducible shift in apparent molecular size of Stat5b, but not of Stat5a, on SDS-PAGE is consistent with increased protein phosphorylation (33–37), and could reflect a difference of the extent of phosphorylation between Stat5a and Stat5b. We therefore carried out phosphoamino acid analysis of Stat5a and Stat5b proteins in Nb2 cells treated with or without PRL for 10 min. Stat5a and Stat5b were individually immunoprecipitated from lysates of Nb2 cells that had been metabolically labeled with [^{32}P]orthophosphate, and examined for PRL-inducible phosphorylation by SDS-PAGE (Fig. 3*A*), followed by phosphoamino acid analysis after acid hydrolysis of the Stat5a and Stat5b proteins (Fig. 3*B*). As seen from Fig. 3*A*, PRL stimulated incorporation of phosphate into both Stat5a and Stat5b, and subsequent phosphoamino acid analysis revealed that both Stat5a and Stat5b were inducibly phosphorylated on serine and tyrosine, but not on threonine residues (Fig. 3*B*). Longer exposure times were generally required to visualize phosphotyrosine than phosphoserine, and based upon repeated analyses we propose that there is a higher number of phosphorylated serine residues than phosphorylated tyrosines in both Stat5a and Stat5b. Future studies will attempt to define the exact stoichiometry and localize the phosphorylation sites.

Thus far, we have only examined PRL-inducible serine phosphorylation in Nb2 cells. However, we have now extended these observations to include IL2 induction of serine phosphorylation of both Stat5a and Stat5b in several target cells (44). This supports the notion of serine phosphorylation of Stat5 molecules as a general activation mechanism. On the other hand, cell specific differences may exist. In a separate study of HC11 mouse mammary epithelial cells, constitutive rather than PRL-inducible serine phosphorylation of Stat5a or Stat5b was observed (40).

PRL Induces Binding of Two Distinct Stat5-containing Protein Complexes with PRL Response Elements of the β-Casein and IRF1 Gene Promoters—We next evaluated the ability of PRL-activated Stat5a and Stat5b to bind to oligonucleotide probes corresponding to the PRL response elements of the

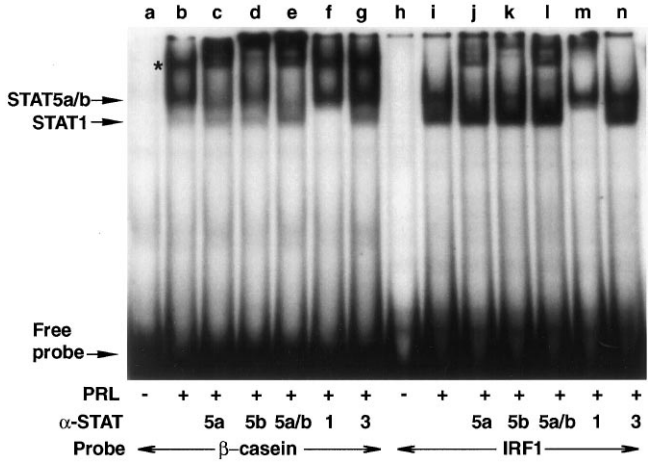

FIG. 4. **Electrophoretic mobility shift assay of PRL-inducible protein binding to PRL response elements of the β-casein and IRF1 gene promoters.** Quiescent Nb2 cells were incubated with medium (−) or 100 nM oPRL (+) for 10 min at 37 °C, and lysates corresponding to 10 μg of protein were incubated either with normal rabbit serum (*lanes a*, *b*, *h*, and *j*), α-Stat5a (*lanes c* and *j*), α-Stat5b (*lanes d* and *k*), α-Stat5a plus α-Stat5b (*lanes e* and *l*), α-Stat1α (*lanes f* and *m*), or α-Stat3 (*lanes g* and *n*) in combination with oligonucleotide probes corresponding to the PRL response elements of either β-casein (*lanes a–g*) or IRF1 (*lanes h–n*). Note qualitative differences in PRL-inducible complexes bound to the two different response elements. An *asterisk* (*lane b*) denotes a complex that preferentially binds to the β-casein promoter and is supershifted by α-Stat5b serum. A faster migrating complex that is supershifted most efficiently by a combination of Stat5a and Stat5b sera is indicated as *Stat5a/b*, and the lower complex (Stat1α) is efficiently supershifted with α-Stat1α serum. α-Stat3 serum does not have any detectable effect on any of these complexes.

β-casein and IRF1 genes. The abilities of antisera to Stat1, Stat3, and Stat5a/b to supershift individual complexes were tested. Nb2 cells were stimulated with PRL for 10 min, lysed and clarified by centrifugation, and incubated with either normal rabbit serum or with specific antisera as indicated. Intriguingly, cellular Stat complexes from PRL-stimulated cells incubated in the presence of normal rabbit serum displayed marked differences in their ability to bind to the two oligonucleotide probes (Fig. 4). PRL induced the formation of three discernible complexes that bound to the β-casein probe: a slowly migrating complex indicated with an *asterisk* (Fig. 4, *lanes a* and *b*) that did not bind to the IRF1 probe (*lane i*), an intermediate complex that bound comparably well to either probe, and a fast migrating complex that bound weakly to the β-casein probe and strongly to the IRF1 probe. Coincubation with anti-Stat5a or anti-Stat5b sera alone supershifted significant portions of the intermediate complex bound to the β-casein probe (*lanes c* and *d*), and also the corresponding IRF1 complex (*lanes j* and *k*). In both instances, the anti-Stat5a serum was particularly efficient, although the two antisera combined depleted the complex entirely (*lanes e* and *l*). Intriguingly, the slow-migrating complex unique to the β-casein probe was almost completely supershifted by anti-Stat5b serum. The effect of anti-Stat5a serum on the slow-migrating complex was obscured by the appearance of a supershifted complex containing Stat5a from the middle complex, but careful analysis of several experiments indicates that the slow-migrating Stat5b complex did not contain Stat5a. Although further work is needed to analyze the binding of PRL-inducible Stat-DNA complexes and the accompanying physiological impact on transcription rates of particular genes, these data clearly indicate qualitative differences in PRL-induced Stat5a and Stat5b complex formation within regulatory DNA sites. Finally, the fast-migrating complex was shown to contain Stat1α, because it was completely supershifted by anti-Stat1α serum (*lanes f* and *m*). In contrast, an antiserum capable of supershifting DNA-complexed Stat3 had no detectable effect on any of the PRL-inducible complexes bound to either probe, indicating that Stat3 does not constitute a significant part of these particular complexes (Fig. 4, *lanes g* and *n*). This is also consistent with the observation that PRL-activated Stat3 did not heterodimerize with Stat1α, Stat5a, or Stat5b (Fig. 1, *panel 3*), and that each of the PRL-induced protein complexes binding to the β-casein and IRF1 probes have been accounted for by supershift analysis. However, PRL-activated Stat3 probably can interact with these or other response elements under different conditions, but this remains to be established. Most importantly, the data clearly indicate marked differences in the ability of PRL-inducible Stat5 complexes to bind to β-casein and IRF1 probes, and that Stat5a and Statb appear to exist in several distinct complex combinations.

Biological Implications of Nonoverlapping Functions of Stat5a and Stat5b—The present study provides initial evidence to suggest a differential involvement of Stat5a and Stat5b molecules in PRL receptor-mediated signal transduction. Particularly intriguing is the finding of a Stat5b complex that bound preferentially to an oligonucleotide corresponding to the PRL response element of the β-casein gene, but not to the PRL response element of IRF1. If Stat5a or Stat5b were to have certain unique functions, or Stat5a/b heterodimers constitute critical mediators, one would predict that mice deficient in either transcription factor will have a phenotype. Consistent with this notion, Stat5a-deficient mice are unable to lactate (41). More specifically, in mammary glands of pregnant Stat5a-deficient mice, the expression of β-casein is markedly less affected than expression of the whey acidic protein, another mouse milk protein under PRL control (42, 43). Studies are in progress to further define the specific functions of Stat5a and Stat5b.

In conclusion, we demonstrate that PRL stimulates rapid tyrosine phosphorylation of multiple forms of Stat5, including $p94^{Stat5a}$, $p92^{Stat5b}$, and a COOH-terminally truncated 80-kDa Stat5 variant. This event is paralleled by heterodimerization of Stat5a and Stat5b, and consistent with the model of phosphotyrosyl-dependent intermolecular bridging via SH2-domains of Stat proteins. Furthermore, we demonstrated inducible phosphorylation of both Stat5a and Stat5b on serine and tyrosine, but not threonine residues, thus providing direct evidence for the existence of a PRL-activated Stat5 serine kinase. Finally, we report the formation of a Stat5a/b complex that binds equally well to the promoters of β-casein and IRF1, and a slower migrating complex that contains Stat5b and not Stat5a, which binds exclusively to the β-casein promoter. Collectively, these data suggest that although Stat5a and Stat5b respond biochemically very similarly to PRL and probably have a significant functional overlap, the two proteins may also have evolved unique properties that contribute to the specificity of signals via receptors for PRL and possibly other cytokines.

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