Differential Effects of Prolactin and src/abl Kinases on the Nuclear Translocation of STAT5B and STAT5A*

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In this study, DNA binding and tyrosine phosphorylation of STAT5A and STAT5B were compared with their subcellular localization determined using indirect immunofluorescence microscopy. Following prolactin activation, both STAT5A and STAT5B were rapidly translocated into the nucleus and displayed a detergent-resistant, punctate nuclear staining pattern. Similar to prolactin induction, src activation resulted in tyrosine phosphorylation and DNA binding of both STAT5A and STAT5B. However, nuclear translocation of only STAT5B but not STAT5A was observed. This selective nuclear translocation appears to be mediated via the carboxyl-terminal sequences in STAT5B. Furthermore, overexpression of a dominant negative kinase-inactive mutant of JAK2 prevented prolactin-induced tyrosine phosphorylation and nuclear translocation of STAT5A and STAT5B but did not block src kinase activation and nuclear translocation of STAT5B. In co-transfection assays, prolactin-mediated activation but not src kinase-mediated activation of STAT5B resulted in the induction of a β-casein promoter-driven reporter construct. These results suggest that STAT5 activation by src may occur by a mechanism distinct from that employed in cytokine activation of the JAK/STAT pathway, resulting in the selective nuclear translocation of STAT5B.

Cytokines influence a variety of cellular functions including proliferation, growth arrest, and differentiation. The neuroendocrine hormone prolactin (Prl)1 plays a central role in the development and differentiation of the mammary gland. Binding of Prl to its cell surface receptor, a member of the cytokine receptor superfamily, regulates the transcription of several milk protein genes, including the whey acidic protein (1), β-lactoglobulin (2), and β-casein (3, 4) genes. The Prl receptor (PrlR) transmits signals in part via activation of the JAK/signal transducers and activators of transcription (STAT) pathway. Interaction of Prl with PrlR induces receptor dimerization, activation of the JAK2 protein-tyrosine kinase (3, 5–7), and tyrosine phosphorylation of transcription factors that belong to the STAT family.

Among the seven mammalian STAT proteins that have been discovered (8), STAT1, STAT3, and STAT5 are capable of activation by the PrlR (9). STAT5, however, plays a key role in Prl-induced milk protein gene expression and mammary gland differentiation (10, 11). Tyrosine 700 of rat STAT5 is the site of phosphorylation by JAK2 and the primary regulator of STAT5 DNA binding (3). After tyrosine phosphorylation, STAT5 dimerizes and translocates into the nucleus, where it binds to specific DNA elements and activates transcription of target genes, such as the milk protein genes.

Two different STAT5 genes encoding STAT5A and STAT5B have been identified that share 93% identity at the amino acid level with the primary differences occurring at their carboxyl termini (12–14). STAT5A was discovered as a mediator of Prl response in mammary epithelial cells and was originally designated as mammary gland-specific factor, or MGF (4, 15). STAT5B was cloned from hematopoietic cells, mammary gland, and liver tissue (12, 14, 16, 17). STAT5A and STAT5B are ubiquitously expressed in most cell lines and tissues at comparable levels with a few exceptions (14).

In addition to Prl, both STAT5A and STAT5B are activated by many other cytokines, including growth hormone, erythropoietin, granulocyte-macrophage colony-stimulating factor (17, 18), IL-2 (19, 20), IL-3 (16, 17), IL-5 (17), IL-7, IL-15 (20), and thrombopoietin (21). STAT5 can also be activated by certain growth factors, like epidermal growth factor, through their respective receptor tyrosine kinases (22) as well as by certain nonreceptor tyrosine kinases like src and ber-abl (23, 24). STAT5A and STAT5B can, therefore, participate in many different signaling pathways leading to cell growth and differentiation. The targeted knockout of the individual genes in mice has suggested that they play essential but often redundant roles in the physiological responses associated with Prl (25). Despite their homology, there is some evidence suggesting that STAT5A and STAT5B may be differentially activated (26) and even exhibit distinct DNA binding specificities (27). However, functional differences between STAT5A and STAT5B and their precise roles in normal mammary gland development and cancer are just beginning to be elucidated.

In this study, we have compared the kinetics of DNA binding and tyrosine phosphorylation of STAT5A and STAT5B following either Prl treatment or src/abl kinase activation with their subcellular localization determined by indirect immunofluorescence microscopy. src kinase activation resulted in tyrosine phosphorylation and DNA binding of both STAT5A and STAT5B, but unlike prolactin induction, nuclear translocation of only STAT5B but not STAT5A was observed. This selective nuclear translocation appears to be mediated via the carboxyl-terminal sequences in STAT5B and was not prevented but instead stimulated by a dominant-negative kinase-inactive mutant of JAK2. These observations establish another mechanism for STAT5 activation in response to the src/abl kinase.
family that results in the selective nuclear translocation of STAT5B and possibly activation of unique gene targets.

**EXPERIMENTAL PROCEDURES**

**Expression and Reporter Constructs—**Rat STAT5A cDNA was cloned and characterized in our laboratory (13). Rat STAT5B was cloned by Guoyang Luo and Dr. Li-yan Yu-Lee at Baylor College of Medicine (28). Both STAT5A and STAT5B were subcloned into the pRCCMV expression vector (Invitrogen, Carlsbad, CA), src kinase-active (srcK⁺) and dominant negative src (srcK⁻) constructs (29) were kindly provided by Dr. Sara Courtneidge at Sugen Corp. The JAK2 mutant expression vector (JAKS29) has been described previously and was kindly provided by Dr. Nelson Horseman (30). A chimeric STAT5A:B expression construct was made by ligation of the 5’ segment of STAT5A (HindIII/XhoI 1.7-kilobase fragment) to the 3’ portion of STAT5B (XhoI/SpeI 0.9-kilobase fragment) in the HindIII/XhoI sites of the pCDNA3 vector. The –2300/+490 β-casein gene promoter-CAT construct and the expression vector for the long form of PrIR have been described previously (31). All DNA plasmids were purified using a QIAGEN maxiprep kit (Qiagen, Valencia, CA).

**Cell Culture and Transfections—**The majority of the experiments were performed using COS-1 cells maintained in Dulbecco’s modified Eagle’s medium (JRH, Lenexa, KS), which was supplemented with 10% FCS (charcoal-stripped horse serum, insulin (5 μg/ml), gentamicin (50 μg/ml), sodium deoxycholate, 0.1% SDS, 1% Nonidet P-40, 150 mM NaCl, 100 mM NaCl, 300 mM sucrose), containing protease and RNase inhibitors: 1 mM phenylmethylsulfonyl fluoride, aprotinin (2 μg/ml), leupeptin (2 μg/ml), benzamidine (2 μg/ml), and vanadyl ribonucleoside complex (2 mM; 5 Prime). Cells were rinsed twice with ice-cold PEM buffer (80 mM PIPES, 1 mM EGTA, 1 mM MgCl₂, pH 6.9) and hydrocortisone (1 μg/ml) and then (for the Prl experiments only) stimulated with ovine Prl (1 μg/ml, 70,000–150,000 Da; Sigma). Preparation of cell extracts—Cells were rinsed twice with ice-cold phosphate-buffered saline (Life Technologies) and then fixed with 4% paraformaldehyde in PEM buffer (80 mM PIPES, 1 mM EGTA, 1 mM MgCl₂, pH 6.9) for 30 min. After washing coverslips three times with PEM buffer, they were incubated for 5 min in NaBH₄ (1 mg/ml) solution in PEM buffer twice. Then they were washed with PEM twice and incubated with 0.5% Triton X-100 in CSK buffer (10 mM PIPES, pH 6.8, 1 mM EGTA, 1 mM MgCl₂, 100 mM NaCl, 300 mM sucrose), containing protease and RNase inhibitors: 1 mM phenylmethanesulfonyl fluoride, aprotinin (2 μg/ml), antipain (2 μg/ml), leupeptin (2 μg/ml), benzamidine (2 μg/ml), and vanadyl ribonucleoside complex (2 mM; 5 Prime → 3 Prime) (34). After fixing, coverslips were washed three times with PEM buffer and once with PBS-T (100 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Tween 20) and subjected to immunostaining.

For immunostaining, coverslips were blocked in 5% milk in PBS-T overnight at 4 °C and incubated with primary antibodies for 1 h at room temperature. After washing five times with PBS-T, cells were incubated with secondary antibodies conjugated with Texas Red (Molecular Probes, Inc., Eugene, OR) or fluorescein isothiocyanate (Santa Cruz Biotechnology) for 30 min in the dark at room temperature and then washed with PBS-T five times and stained by DAPI by using VECTASHIELD mounting media (VECTOR, Burlingame, CA). Images were obtained using a Zeiss Axioskop fluorescent microscope.

The primary antibodies used for immunostaining were as follows: anti-Stat5a (COOH-terminal), diluted 1:1000; anti-Stat5b (COOH-terminal), diluted 1:2000; anti-Stat5p (SA:5MV-700), diluted 1:500; and anti-phosphotyrosine (PY-20), diluted 1:1000.

**Indirect Immunofluorescence—**Cells were cultured on glass coverslips, coated with poly-L-lysine (1 mg/ml, 70,000–150,000 Da; Sigma). After trypsinization, the cells were rinsed twice with ice-cold phosphate-buffered saline (Life Technologies) and then fixed with 4% paraformaldehyde in PEM buffer (80 mM PIPES, 1 mM EGTA, 1 mM MgCl₂, pH 6.9) for 30 min. After washing coverslips three times with PEM buffer, they were incubated for 5 min in NaBH₄ (1 mg/ml) solution in PEM buffer twice. Then they were washed with PEM twice and incubated with 0.5% Triton X-100 (Sigma) in the same buffer for 20 min. Alternatively, for extraction of soluble proteins that were not tightly associated with cellular structures such as the cytoskeleton and nuclear matrix (33), cells were fixed for 30 min in 0.5% Triton X-100 in CSK buffer (10 mM PIPES, pH 6.8, 1 mM EGTA, 1 mM MgCl₂, 100 mM NaCl, 300 mM sucrose), containing protease and RNase inhibitors: 1 mM phenylmethanesulfonyl fluoride, aprotinin (2 μg/ml), antipain (2 μg/ml), leupeptin (2 μg/ml), benzamidine (2 μg/ml), and vanadyl ribonucleoside complex (2 mM; 5 Prime → 3 Prime) (34). After fixing, coverslips were washed three times with PEM buffer and once with PBS-T (100 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Tween 20) and subjected to immunostaining.

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The primary antibodies used for immunostaining were as follows: anti-Stat5a (COOH-terminal), diluted 1:400; anti-Stat5b (COOH-terminal), diluted 1:500; anti-abl (c-abl) (Santa Cruz Biotechnology), diluted 1:400; and anti-src (v-src) (Calbiochem), diluted 1:250. For anti-Stat5a and anti-Stat5b antibodies, goat anti-rabbit IgG conjugated with Texas Red (1:1000) was used as the secondary antibody. Goat anti-mouse IgG conjugated with fluorescein isothiocyanate (1:500) was used as the secondary antibody for anti-abl and anti-src antibodies.

**Analysis of STAT5 Nuclear Localization—**Two sets of coverslips were set up in the dish. One of them was pretreated with 0.5% Triton X-100 in PBS to remove the soluble proteins from the cytoplasm and nuclei prior fixing the cells, while another set was fixed without pretreatment with Triton-CSK buffer. Both sets of coverslips were subjected to immunostaining. Nuclear staining was detected only in cells pretreated with Triton-CSK buffer, while the untreated cells exhibited both cytoplasmic and nuclear staining. The percentage of nuclear localization was measured by dividing the total number of cells stained for STAT5 following Triton-CSK pretreatment by the total number of cells exhibiting STAT5 staining without pretreatment relative to the average number of transfected cells. For each time point, 4–6 fields of view were analyzed.
Tyr700 is the primary epitope on STAT5 for PY-20 and indicate that STAT5A (3)). The results illustrated in Fig. 2 suggest that the nonphosphorylated forms by 7.5% SDS-polyacrylamide gel electrophoresis was observed to parallel the changes in tyrosine phosphorylation.

To establish the time course of DNA binding activity for STAT5A and STAT5B, EMSAs were performed using whole cell extracts and a double-stranded oligonucleotide corresponding to a 34-base pair region of the β-casein proximal promoter containing the STAT5 binding site (Fig. 2, bottom panel, lanes 1–5 and lanes 8–12, respectively). Similar to the tyrosine phosphorylation results, STAT5A and STAT5B DNA binding activity reached a maximum after 30 min of PRL induction and subsequently decreased at longer times of PRL treatment. As expected, the phosphorylation status of STAT5A and STAT5B in response to PRL induction generally correlated with their DNA binding activity.

Tyrosine phosphorylation and DNA binding activity of STAT5A and STAT5B were also increased by a constitutively active src (srcK+, Fig. 2, lanes 7 and 14) but were not observed following transfection of a src kinase, dominant negative (srcK−, Fig. 2, lanes 6 and 7) construct. Direct Western blot analysis revealed phosphorylation of both STAT5A and STAT5B (Fig. 2, top two panels, lanes 7 and 14) on Tyr700 as a result of co-transfection of srcK+. Similar results were obtained from immunoprecipitation experiments for STAT5A and STAT5B (Fig. 2, third panel, lanes 7 and 14). The DNA binding activity of STAT5A and STAT5B in response to src kinase activation was also demonstrated by EMSA (Fig. 2, bottom panel, lanes 7 and 14). No significant differences in the phosphorylation status or DNA binding activity in response to src were detected between STAT5A and STAT5B using these techniques.

Cytoplasmic-Nuclear Transport of STAT5A and STAT5B in Response to PRL Induction—Using indirect immunofluorescence, it was possible to examine if the decrease in STAT5A and STAT5B tyrosine phosphorylation and DNA binding activity (Fig. 2) with time after PRL treatment was correlated with the changes in subcellular localization of these transcription factors. In cells transfected with PRLR and STAT5A or STAT5B without PRL treatment, both STAT5A and STAT5B appear to be predominantly localized in the cytoplasm in the absence of prolactin activation (data not shown). Similar observations using GFP-STAT5B constructs and confocal microscopy have been reported recently in human fibrosarcoma cells (35). However, the nuclear staining observed in the absence of PRLR in our studies was completely removed by extraction with 0.5% Triton in CSK buffer, suggesting a loose association of nonactivated STAT5A and STAT5B in the nucleus. After 30 min of PRL stimulation, both STAT5A and STAT5B translocate into the nucleus and generate detergent-resistant complexes (Fig. 3, A and B, respectively). Using deconvolution confocal microscopy, some staining for both STAT5A and STAT5B could be detected in the nucleus in the absence of prolactin activation (data not shown). Similar observations using GFP-STAT5B constructs and confocal microscopy have been reported recently in human fibrosarcoma cells (35). However, the nuclear staining observed in the absence of PRLR in our studies was completely removed by extraction with 0.5% Triton in CSK buffer, suggesting a loose association of nonactivated STAT5A and STAT5B in the nucleus. After 30 min of PRL stimulation, both STAT5A and STAT5B translocate into the nucleus and generate detergent-resistant complexes (Fig. 3, A and B, respectively). Using deconvolution confocal microscopy, some staining for both STAT5A and STAT5B could be detected in the nucleus in the absence of prolactin activation (data not shown).

Differential Localization of STAT5A and STAT5B in Re-
Src-mediated STAT5B Nuclear Translocation

Tyrosine phosphorylation and DNA binding of STAT5A and STAT5B appear similar in response to Prl and src kinase activation (Fig. 2). Thus, it was expected that STAT5A and STAT5B would also exhibit similar nuclear translocation in response to SrcK+ activation. However, surprisingly when transiently transfected COS cells co-
expressing srcK+ and STAT5A or STAT5B were examined by indirect immunofluorescent microscopy; it was discovered that only STAT5B was localized in the nucleus following src activation. STAT5A remained in the cytoplasm (Fig. 3, compare E (for STAT5A) and F (for STAT5B)). This was confirmed in experiments using double immunofluorescence staining in which STAT5A or STAT5B was localized using specific carboxyl-terminal antibodies with corresponding secondary antibodies conjugated with Texas Red and srcK+ was localized with an anti-v-src antibody recognized with a corresponding secondary antibody conjugated with fluorescein isothiocyanate. In cells co-transfected with cDNA encoding STAT5A and srcK+, predominantly yellow cytoplasmic staining formed by the combination of the green and red fluorescence was observed (Fig. 4A). In contrast, in cells co-expressing STAT5B and srcK+, green cytoplasmic staining for src and punctated red nuclear staining for STAT5B was observed (Fig. 4B). The punctate nuclear pattern observed following src activation was similar to that seen for STAT5B after stimulation with Prl. Furthermore, a similar pattern of selective nuclear translocation for STAT5B activated by c-abl, another member of the same nonreceptor tyrosine kinase family, was also detected (Fig. 4C).

Identification of the Sequences Required for the Selective Nuclear Translocation of STAT5B in Response to src Activation—Because the sequence differences between the two isoforms of STAT5 are most pronounced in the carboxyl terminus, we constructed a chimeric STAT5A:B mutant, that consisted of 545 amino acids from the NH2-terminal end of STAT5A and 241 amino acids from the COOH-terminal end of STAT5B. Based upon the structural analysis of the STAT proteins, the STAT5A/B chimeric construct was made in the linker domain between the DNA binding and Src homology 2 domains of STAT5A and STAT5B (36) and presumably does not affect the ability of STAT5 to dimerize or form tetramers on the appropriate DNA response elements (37). This chimera was co-transfected in HeLa cells with the PrlR or srcK+. In parallel, STAT5A and STAT5B were expressed with the PrlR or srcK+ as positive and negative controls. The control experiments showed similar patterns for STAT5A (data not shown) and STAT5B localization in HeLa cells (Fig. 5, A–C) as observed previously in COS cells, confirming that the localization patterns did not result from marked overexpression of these proteins in COS cells. Remarkably, the chimeric STAT5A:B mutant translocated to the nucleus in response to both Prl induction and srcK+ activation (Fig. 5, E and F, respectively). This result suggests that STAT5B contains a unique sequence in its carboxyl terminus that could be responsible for nuclear translocation in response to src activation.

Association of STAT5 with src Kinase and the Role of JAK2 in Nuclear Translocation—Although src has been shown to associate with and phosphorylate STAT3 both in vivo and in vitro (38) it was unclear whether src could associate with and phosphorylate either STAT5A or STAT5B. Accordingly, immunoprecipitation with an anti-src antibody followed by Western blotting with an antibody that will recognize either STAT5A or STAT5B was performed using extracts prepared from transiently transfected COS cells. These experiments demonstrated that src kinase is capable of in vivo association with both STAT5A and STAT5B (Fig. 6). However, this association appeared not to be dependent upon the enzymatic activity of src kinase, since it was observed in both srcK+ and srcK− cells. Furthermore, in vitro kinase assays failed to reveal an increase in STAT5A or STAT5B tyrosine phosphorylation (data not shown) in the co-immunoprecipitated complex, suggesting that this was not a direct kinase-substrate interaction.

Having determined that the activation of both STAT5A and STAT5B by src kinase is not a direct enzyme-substrate reaction, we wished to investigate whether srcK+ might activate either STAT5 indirectly through the activation of JAK2. For this purpose, a dominant negative mutant of JAK2 consisting of a carboxyl-terminal truncation deleting the entire kinase domain (JAK829) (30) was utilized. STAT5A or STAT5B and PrlR or srcK+ were co-transfected in COS cells with or without JAK829. Whole cell extracts were prepared for immunoprecipitation, Western blotting, and immunofluorescence analyses (Fig. 7). As expected (39), overexpression of the kinase-inactive, dominant negative mutant of JAK2 blocked Prl-inducible tyrosine phosphorylation of STAT5A and STAT5B (Fig. 7, A and B, lanes 1–3). However, the JAK2 dominant negative mutant did inhibit block src-inducible tyrosine phosphorylation of STAT5A (Fig. 7A, lanes 4 and 5) or STAT5B (Fig. 7B, lanes 4 and 5). Immunofluorescence analyses were consistent with these results. In cells co-transfected with STAT5A or STAT5B, PrlR, and JAK829 and treated with Prl for 30 min, exclusive cytoplasmic staining for both STAT5s was observed, similar to cells that did not receive Prl treatment (data not shown). In cells co-expressing STAT5B, srcK+, and JAK829, punctated nuclear staining similar to that seen in cells without JAK829 was observed (data not shown). The analysis of numerous fields was performed in order to calculate the percentage of cells displaying nuclear localization (Fig. 8). These results indicate that JAK829 significantly reduced the nuclear translocation of STAT5B induced by Prl, but not by src kinase. In contrast, in cells co-expressing STAT5B and srcK+, an increased level of tyrosine phosphorylation and nuclear localization of STAT5B was detected in the presence of JAK829 compared with cells not expressing the dominant-negative JAK2 mutant. These results suggest that src activation of STAT5B may occur by a mechanism independent of the conventional ligand-dependent activation of the JAK/STAT pathway (Fig. 10 and “Discussion”).

STAT5B Does Not Activate Transcription from the β-Casein
Gene Promoter after src Kinase-mediated Nuclear Translocation—Since both Prl and srcK1 activation result in STAT5B tyrosine phosphorylation, nuclear translocation, and DNA binding, it was reasonable to examine if these pathways are transcriptionally equivalent. β-Casein is a well characterized STAT5 target gene. Prl induces β-casein gene expression through STAT5A and STAT5B both in mammary epithelial cells (15, 40) and in COS cells (3) and CHO cells (4). STAT5A and STAT5B bind to the region between −105 and −75 in the β-casein gene promoter (40, 41), which contains the recognition site for STAT5, a highly conserved sequence 5′-CTTCTTG-GAATT-3′. A β-casein gene promoter fragment (−2300/+490) linked to the CAT gene as a reporter was transfected into COS cells with the PrlR or srcK1 expression vectors. Cellular extracts were prepared, and CAT activity was determined. In cells transfected with the promoter-reporter gene construct, STAT5B, and PrlR and treated with Prl overnight, an expected 4.5-fold increase (3) in CAT protein was detected as compared with cells treated with only insulin and glucocorticoids (Fig. 9). However, STAT5B co-expressed with srcK1 was unable to cause transactivation of the β-casein gene promoter (Fig. 9). Western blot and indirect immunofluorescent analyses of STAT5B were performed in parallel to these transactivation experiments to demonstrate src activation of STAT5B tyrosine phosphorylation and nuclear translocation (data not shown). These results suggest that src activation is not transcriptionally equivalent to Prl activation.

**DISCUSSION**

**Differential Effects of src/abl Kinases on the Nuclear Localization of STAT5A and STAT5B**—The general paradigm for JAK/STAT signaling is that ligand binding to cytokine receptors leads to JAK kinase activation and STAT tyrosine phosphorylation, followed by dimerization and obligatory nuclear translocation. However, as reported in this study, signals from
the src/abl family of protein kinases also led to STAT5 activation but did not result in the equivalent nuclear translocation of STAT5A and STAT5B. This appears to represent a novel property of cytokine-independent pathways for STAT activation (Fig. 10). Furthermore, recent observations suggest that there are distinct biochemical differences between the closely related STAT5A and STAT5B proteins (37) that could potentially result in the differential activation of STAT5 gene targets (27). Thus, the preferential nuclear translocation of STAT5B as well as other STAT proteins provides another level of gene regulation that may have profound biological consequences. These studies also illustrate the importance of analyzing the subcellular distribution of STAT proteins following activation in addition to merely assessing their tyrosine phosphorylation and in vitro DNA binding activity by EMSA. For example, discrepancies between cell fractionation and immunofluorescence results have been reported when analyzing the nuclear translocation of STAT chimeras (42).

Little is known about the mechanisms regulating the nuclear import and export of STAT proteins. STAT1 nuclear import following activation by interferon-γ is mediated by a Ran GTPase-dependent process involving a nuclear pore-targeting complex with NPI-1 (43, 44). However, no conventional nuclear localization signals have been identified in the STAT proteins. In fact, it has been hypothesized that a ligand-receptor complex might function as a chaperone to facilitate STAT nuclear import (45, 46). Such a mechanism appears unlikely to account for the selective nuclear import of STAT5B by the src/abl kinases.

While little is known about the mechanisms of nuclear import, even less is known about the mechanisms regulating nuclear export of STATs. Limited studies have suggested that nuclear export appears to be dependent upon a nuclear tyrosine phosphatase (47). Pulse-chase studies have indicated that STAT1 cycles into the nucleus as tyrosine-phosphorylated molecules and quantitatively returns to the cytoplasm as nonphosphorylated molecules (47).

Although STAT5A and STAT5B display 93% identity at the amino acid level, the chimeric construct in which the amino-terminal region of STAT5A was fused to the carboxyl terminus of STAT5B still exhibited src kinase-dependent nuclear translocation, and, as expected, responded to Prl activation (Fig. 5). Recent analysis of STAT chimeras has suggested that STAT amino termini provide a signal that is important for nuclear translocation and subsequently deactivation (42). However, the analysis of STAT5A/5B chimeras in our study suggests that differences at the amino termini cannot account for the selective effects of src on the nuclear import of STAT5B as compared with STAT5A. There are two regions in the carboxy-terminal regions of STAT5A and STAT5B that display significant differences (Fig. 1). The principal difference between these two STATs is at the carboxyl terminus in a region thought to be a transcriptional transactivation domain (48). In addition, there is a six-amino acid difference in STAT5B immediately amino-terminal to tyrosine 700. We thought that the sequence, PCE-PAT, might be important for the selective effects on STAT5B nuclear localization and generated the respective in frame deletion in STAT5B. Preliminary results indicate that this deletion did not inhibit src-dependent nuclear translocation (data not shown). More detailed analysis of carboxy-deleted naturally occurring splice variants of STAT5B (14) and additional chimeric constructs may be informative for the identification of the precise determinants that discriminate between the selec-

**FIG. 7.** Phosphorylation of STAT5A and STAT5B by src kinase is not inhibited by a dominant-negative mutant of JAK2. COS cells were co-transfected with STAT5A (A) or STAT5B (B) and PrlR (lanes 1–3), constitutively active src kinase (lanes 4 and 5), and the dominant negative JAK2 mutant (lanes 3 and 5) and induced by Prl for 30 min as indicated (lanes 2 and 3). STAT5 was immunoprecipitated with an NH₂-terminal anti-Stat5 antibody. The immunoprecipitated proteins were subjected to Western blot analysis using the PY-20 antiphosphotyrosine antibody. The blots were then stripped and probed with the anti-Stat5 monoclonal antibody.

**FIG. 8.** Nuclear translocation of STAT5B by src kinase is not prevented by a dominant negative JAK2 mutant. Quantitative analysis of nuclear localization of STAT5B performed by immunofluorescence staining of co-transfected COS cells as described under "Experimental Procedures."
tive nuclear localization of STAT5A and STAT5B.

**Biological Consequences of STAT5B Activation**—The src family of protein-tyrosine kinases are involved in signal transduction pathways that result in growth and differentiation (49, 50) and when dysregulated can result in a variety of pathological conditions including cancer (51–53). A number of primary tumors and tumor-derived cell lines including breast and colon cancer, melanoma, and sarcoma have been shown to possess elevated src tyrosine kinase activity (54–57). src and its family members are required for mitogenesis initiated by several growth factor receptors, including epidermal growth factor receptor (58–60), platelet-derived growth factor receptor (50, 61–63), basic fibroblast growth factor receptor (64, 65), and colony-stimulating factor-1 receptor (49, 60). A number of different substrates have been identified for src kinase including contractin (66–68), p125FAK (69), and p130Cas (70), but how src contributes to the mitogenic response is still not well understood.

It has been demonstrated that in addition to their signaling functions in normal cells, STATs can also participate in oncogenesis (71). A number of reports correlate STAT activation with the activity of nonreceptor proto-oncogenic tyrosine kinases such as v-src (38, 72, 73), v-abl (74), lyn (75), lsk (76), ber/abl (77), and fes (78). Thus, constitutive, non-cytokine activation of STATs may play an important role in the etiology of primary lymphoid and myeloid leukemias as well as in breast cancer (24, 72, 79). In support of this hypothesis, recent functional studies have demonstrated that STAT3 activation is required for transformation of mammalian fibroblasts (80). A reciprocal pattern of STAT5 and STAT3 activation has been observed during mammary gland development (10), suggesting that these STAT proteins may play different or even opposite roles in the regulation of cell proliferation, differentiation, and apoptosis (10). Conceivably, src activation may disrupt these processes.

Although it has been reported that src-transformed cells exhibit constitutive activation of JAK1 and possibly JAK2 (81), there is some evidence that v-src and ber/abl may, in addition, be directly associated with STATs (23, 24). This is consistent with the observation that dominant negative mutant of JAK2 did not prevent STAT5B activation by src (Fig. 7). In fact, in the presence of the dominant negative JAK2 mutant, increased STAT5B nuclear translocation was observed (Fig. 8). Thus, it is conceivable that JAK2 and src might compete for activation of STAT5B, and expression of the dominant negative JAK2, therefore, resulted in a small increase in src activation of STAT5B. Interestingly, a dominant negative JAK2 has also been reported not to prevent IL-3-mediated activation of STAT3, which is thought to be mediated in part by c-src (82).

Our results provide evidence that the interaction of the src kinase with STAT5 is most likely not a kinase-substrate interaction and that activation of STAT5B occurs through an undetermined indirect mechanism. The interaction of src and STAT5 observed in the co-immunoprecipitation/Western blotting experiments is most likely due to direct or indirect interaction with its Src homology 2 domains. It is possible that STAT5B is a target of tyrosine kinases activated by src and/or that src may serve as an adaptor protein facilitating STAT5B association as part of a multiprotein complex. For example, src kinase has been shown to bind to and phosphorylate the adapter protein p130Cas, an important modulator of signal transduction.

In addition to the JAK/STAT pathway, PrlR signaling may be mediated via the MAP kinase pathway and by activation of members of the src kinase family (83–85). However, despite these observations, the activation of STAT5B by src failed to activate a β-casein-CAT reporter construct that contains a consensus mammary gland-specific factor-binding site for STAT5 (Fig. 9). Prl activation of STAT5B has been reported to be sufficient for the activation of β-casein promoter-driven reporter constructs in COS cells (12). The β-casein gene pro-

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**Fig. 9.** Src-activated STAT5B does not activate a β-casein promoter-driven reporter construct. COS cells were co-transfected with a β-casein CAT construct, STAT5B and with constitutively active src kinase or with PrlR without or with induction by Prl for 24 h. CAT concentrations were determined by an enzyme-linked immunoassay assay.

**Fig. 10.** STAT5 activation mechanisms. A. cytokine-dependent pathway. Binding of Prl to its membrane receptor activates the tyrosine kinase JAK2. This kinase catalyzes the tyrosine phosphorylation of STAT5A and STAT5B. After tyrosine phosphorylation, STAT5 isoforms form homo- or heterodimers, which translocate into the nucleus, where they bind to specific DNA response elements and activate transcription of target genes presumably facilitating the proliferation and terminal differentiation of mammary epithelial cells. B. cytokine-independent pathway. STAT5B is activated by src kinase via an indirect mechanism. After tyrosine phosphorylation, STAT5B homodimers translocate into the nucleus, where they may facilitate the selective regulation of genes involved in proliferation and/or apoptosis.
Src-mediated STAT5B Nuclear Translocation

moter, however, contains binding sites for a number of other transcription factors that comprise a composite response element responsible for both lactogenic hormone and developmental regulation (86). Thus, it is conceivable that src kinase influences either directly or indirectly the activity of these other factors, leading to the inhibition of casein gene expression independently of activated STAT5B. A similar inhibition of lactogenic hormone signaling by other proto-oncogenes has been reported (87).

In conclusion, these studies have suggested that ligand-dependent and -independent signaling pathways may differentially regulate STAT5A and STAT5B nuclear translocation. One potential consequence may be the differential activation of gene targets involved in differentiation, proliferation, or apoptosis.

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