Jak2-Stat5 Interactions Analyzed in Yeast*

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Many cytokine receptors employ Janus protein tyrosine kinases (Jaks) and signal transducers and activators of transcription (Stats) for nuclear signaling. Here, we have established yeast strains in which an autoactivated Jak2 kinase induces tyrosine phosphorylation, dimerization, nuclear translocation, and DNA binding of a concomitantly expressed Stat5 protein. Transcriptional activity of Stat5 on a stably integrated, Stat-dependent reporter gene required the C-terminal fusion of the VP16 transactivation domain. In such yeast strains, the interaction between Jak2 and Stat5 was analyzed without interference by other mammalian proteins involved in regulating Jak-Stat signaling, and mutant versions of both proteins were analyzed for their ability to productively interact. Complexes between Jak2 and Stat5 were found to be stable under stringent co-immunoprecipitation conditions. Deletion of the Jak homology regions 2–7 (JH2–JH7) of Jak2, leaving only the kinase domain (JH1) intact, reduced the ability of the kinase to phosphorylate Stat5, whereas deletion of the JH2 domain caused an increased enzymatic activity. A site-directed R618K mutation in the Stat5 SH2 domain abolished the phosphorylation by Jak2, while deletion of the C terminus led to Stat5 hyperphosphorylation. A single phosphotyrosine-SH2 domain interaction was sufficient for the dimerization of Stat5, but such dimers bound to DNA very inefficiently. Together, our data show that yeast cells are appropriate tools for studying Jak-Stat or Stat-Stat interactions. Our mutational analysis suggests that the Stat5 SH2 domain is essential for the interaction with Jak2 and that the kinase domain of Jak2 is sufficient for Jak2-Stat5 interaction. Therefore, the Jak kinase domain may be all that is needed to cause Stat phosphorylation in situations where receptor docking is dispensable.

The biological activity of cytokines generally requires changes in the pattern of gene expression. Signal transduction to the cell nucleus is therefore an essential component in a cytokine response. Many cytokine receptors contain amino acid motifs mediating constitutive binding with Jak protein tyrosine kinases (Jaks1; Refs. 1 and 2). After ligand-receptor interac-

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1 The abbreviations used are: Jak, Janus kinase; mJak, murine Jak; JH, Jak homology region; EMSA, electrophoretic mobility shift assay; GAS, γ-interferon activated site; SH2, Src homology region 2; Stat, signal transducer and activator of transcription; mStat, murine Stat; PCR, polymerase chain reaction.

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DNA.

Makino et al. for the generation of Stat5 dimer efficiently binding to the yeast cell membrane. We have shown that a functional Stat5 SH2 domain is critical for Jak2 interaction and that a functional SH2 domain is necessary to bind and phosphorylate Stat5. Rather, our results point toward a negative regulatory function of this domain.

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In mammalian cells, association with receptors usually restricts the movement of Jaks and inhibits ligand-independent kinase activity. When Jaks are overexpressed, they form a pool of cytoplasmic proteins, which are capable of ligand-independent interaction and thus autoactivation (33–35). In this study, we have exploited this feature and report the establishment of yeast cells as tools to study the interaction between activated Jaks and Stat5 as well as Stat5 dimerization. The advantage of this approach lies in the absence of an endogenous Jak-Stat signaling path in yeast, which allows us to interpret results without having to consider possible interference by receptors and/or adapter proteins. Moreover, interactions are studied in a situation of moderate overexpression of reactants, thus minimizing interactions forced by unphysiologically high concentrations.

Under these conditions, we find that the Jak2 domain is not necessary to bind and phosphorylate Stat5. Rather, our results point toward a negative regulatory function of this domain. Moreover, we show that a functional Stat5 SH2 domain is of critical importance for Jak2 interaction and that reciprocal SH2 domain-phosphotyrosine interactions are essential for the generation of Stat5 dimer efficiently binding to DNA.

**EXPERIMENTAL PROCEDURES**

**Cells and Cell Extracts—**Yeast cells (Genotype: MATa, ade2-1, trpl-1, can1-100, leu2-3, 112, his 3-11, 15, ura3, GAL, psi+ W303-1B) were grown in minimal medium containing 6.7 g/ liter yeast nitrogen base without amino acids, supplemented with necessary amino acids, 2% raffinose and/or galactose. Briefly, yeast cells were allowed to grow in 50 ml of minimal medium to an OD of 1.0 (approximately 1 x 10^7 cells/ml). In order to induce the Gal1/10 promoter, cells were grown first in minimal medium containing 2% D(+)-raffinose and shifted at an OD of 0.7 to a medium containing 2% D(+)-galactose for 4 h.

For extraction, yeast cells (1 x 10^7 cells/ml) were collected by centrifugation and resuspended in buffer C (20 mM Hapes, pH 7.9, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 25% glycerol). Approximately 1/3 volume of ice-cold glass beads were added, and cells were broken by vortexing five times for 1 min. After vortexing, the tubes were put on ice for 2 min. After vortexing the last time, the tubes were placed on ice for 30 min. Lysed cells were centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant was carefully transferred to a new Eppendorf tube.

The yeast membrane fraction was obtained by centrifugation at 100,000 g for 1 h. The supernatant was carefully transferred to a new Eppendorf tube.

**Plasmid Construction—**The cDNA encoding murine Stat5a was described previously (38). Plasmids pHS80, containing the murine Stat5b gene and pFB5f2, encoding the murine Stat5b gene, truncated at the NcoI site were kindly provided by C. Schindler (Columbia University, New York; Ref. 39). The above mentioned cDNAs were cloned into the pBluescript-SK or -KS plasmids (Stratagene). The C-terminally truncated Stat5a gene was generated by PCR mutagenesis, introducing a stop codon immediately downstream of the endogenous NsiI site. A myc tag (with three repeats of three myc epitopes) was inserted into a NheI site, which was generated by PCR mutagenesis immediately downstream of the start codon of the Stat5a gene. PCR mutagenesis allowed the generation of an EcoRI site immediately upstream of the start codon for subsequent cloning purposes. The critical tyrosine in Stat5a (Tyr694) and Stat5b (Tyr699) was mutated to phenylalanine by PCR mutagenesis. The mutation arginine 618 to lysine in Stat5a was generated by altering codon 618 from CCC to CAC by PCR mutagenesis. A VP16 fragment encoding the transactivating domain with the murine Stat5a gene (ref. 38) was grown in E. coli competent cells.

**Plasmid pK2082 containing the Gal1/10 promoter was kindly provided by K. Nasmuth (Institute of Molecular Pathology, Vienna). The Gal promoter was excised from pK2082 and cloned as a BamHI–EcoRI fragment into the BamHI–EcoRI sites of YcpLac22, -33, and -111 yeast centromeric plasmids.

mStat5a and mStat5b without the myc tag in addition to all myc-tagged cDNAs described above were cloned into EcoRI–SpeI sites of YcpLac33 and -111 yeast plasmids carrying either uracil or leucine selection markers. mJak2, kindly provided by Jim Ihle (St. Jude Children Research Hospital, Memphis, TN), was blunt end-cloned into the HindIII–SalI sites of YcpLac22 carrying a trypophane selection marker. mJak2a/JH2 was created by removal of an internal restriction fragment generated by cutting with the restriction enzymes DraI and StuI. The Stat5a/Jak2 mutant AN was generated by digestion with the restriction enzyme Asp1251 (KpnI). After removal of the restriction fragment, overhanging ends were filled with Klenow enzyme and ligated.

To generate a Stat-dependent reporter gene, a 400-base pair fragment containing three IFP-53-GAS sequences and a cdc-l minimal promoter with Smal–XbaI sites was generated by PCR and cloned into the Smal–XbaI sites of the YEp356 integrative yeast plasmid.

**Antibodies—**An antibody recognizing specifically Stat5b (5b) was generated by immunizing rabbits with a peptide comprising the C-terminal 14 amino acids of Stat5b. The Stat5a-specific antibody (5a) was described previously (41). For immunoprecipitation (IP), antibodies were used at a dilution of 1:100; for Western blots, antibodies were used at a final concentration of 1:5000. Stat5a- and Stat5b-specific antisera were used in EMSA experiments as recently described for Stat5a (41). The monoclonal anti-myc antibody (myc) was obtained from superna-
tants of the hybridoma cell line 9E10 and used in IP and EMSA at a concentration of 1:10; in Western blots, the dilution was 1:250. The antisera directed against the amino acids 144–295 of Jak2 was kindly provided by Andrew Zimiecki (University of Bern) and used in IP/ Western blotting analysis (final concentration 1:100 for IP and 1:1000 for Western blot). A goat polyclonal antibody directed against a peptide corresponding to amino acids 1110–1129 of mJak2 (Santa Cruz) was used for IP at a final dilution of 1:100 and 1:1000 for Western blotting.

The phosphotyrosine-specific antibodies PY20 and 4G10 were purchased from Transduction Laboratories and Upstate Biotechnology Inc., respectively. The final dilution for Western blotting was 1:300 (Py20) and 1:100 (4G10).

EMSA—The electrophoretic mobility shift assay was performed as described by Eilers et al. (37). In the present paper, an oligonucleotide corresponding to the GAS sequence from the rat β-casein promoter was used as a probe (42).

**Immunoprecipitation and Western Blotting**—Immunoprecipitations were carried out as described previously (41). Briefly, cells were lysed in ice-cold lysis buffer (buffer C). Nuclei were removed by centrifugation. Appropriate antibodies and protein A-Sepharose (Amersham Pharmacia Biotech) were added, and the mixture was incubated for 4 h at 4 °C. The immunoprecipitates were washed in ice-cold washing solution containing 10 mM Tris-HCl, pH 7.0, 50 mM NaCl, 30 mM NaPP, 50 mM NaF, 2 mM EDTA, and 1% Triton X-100. Proteins were separated on 7.5% gel at 420 nm, was calculated in units (1 unit catalyzes the turnover for less than 1 h at 30 °C before adding the transforming DNA. For each sample, 8 volumes of sterile water) and incubated

The membranes were washed in PBST and developed with enhanced chemiluminescence (Pierce), followed by exposure to film (Hyperfilm ECL, Amersham Pharmacia Biotech). The membranes were blocked with 5% nonfat dry milk in PBST (phosphate-buffered saline plus 0.1% Tween 20) for 1 h and subsequently incubated with relevant primary antibody for 1 h, washed in PBST, and incubated for 1 h with peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech). The membranes were washed in PBST and developed with enhanced chemiluminescence (Pierce), followed by exposure to film (Hyperfilm ECL, Amersham Pharmacia Biotech). For co-immunoprecipitations, cells were lysed in lysis buffer (buffer C). The lysates were incubated with first antibodies at 4 °C. After 2 h, protein A-Sepharose was added, and the mixture was incubated for 2 h at 4 °C. The immunoprecipitates were then processed as described above.

**DNA Transformation and Transfection**—The lithium acetate procedure was used for yeast DNA transformation. Yeast suspensions (1 × 10^7 cells/ml) were resuspended in lithium solution buffer (1 volume of 10× TE buffer (100 mM Tris-Cl, pH 7.5, 1 mM EDTA, pH 8), 1 volume of 10× lithium acetate stock solution (1 m lithium acetate, pH 7.5, adjusted with dilute acetic acid), 8 volumes of sterile water) and incubated for less than 1 h at 30 °C before adding the transforming DNA. For each transformation, 200 µg of carrier DNA and less than 5 µg of transforming DNA were mixed in a sterile microcentrifuge tube, and 200 µl of yeast suspension was added. 1.2 ml of freshly prepared polyethylene glycol solution, (8 volumes of 50% polyethylene glycol, 1 volume of 10× TE buffer, pH 7.5, 1 volume of 10× lithium acetate stock solution) was then added to each tube and shaken for 30 min at 30 °C. Heat shock for exactly 15 min at 42 °C enhanced the transformation efficiency. The cells were collected with a microcentrifuge for 5 min at room temperature, resuspended in sterile water, and incubated on the required minimal plates at 30 °C until transformants appeared.

Transfection experiments in COS-N31 cells were performed using the SuperFect transfection reagent and the manufacturer’s instructions (Qiagen). For activation of the transfectected erythropoietin, the cells were treated with human recombinant erythropoietin (7 units/ml) for 30 min.

**RESULTS**

**Expression of Activated Stat5 in Yeast Cells**—The gene encoding murine Jak2 was cloned into a yeast centromeric (single copy) plasmid under the control of a galactose-inducible promoter and introduced into yeast cells. Growth of these cells in liquid media with galactose as a carbon source or the addition of galactose to raffinose-containing medium caused expression of Jak2, and the protein was phosphorylated on tyrosine (Fig. 1a). Fig. 1a also shows that Jak2 migrates in SDS gels as two clearly distinguishable bands that react with different intensities with the anti-phosphotyrosine antibody. Jak2 is known to be phosphorylated on multiple tyrosines. Therefore, these two bands may represent phosphorylation isoforms of the kinase. Expression levels of total cellular Jak2 were somewhat higher on a protein basis compared with most mammalian cells, but Jak2 never formed a visible band when total cytoplasmic protein was analyzed by SDS-polyacrylamide gel electrophoresis. This indicated that expression of Jak2 was sufficient to cause autophosphorylation, but it did not generate a very high cytoplasmic concentration of the protein. Stat5a and Stat5b pro-

![Fig. 1](image-url)
activity in cellular extracts, despite the appearance of Stat5 EMSA activity (Fig. 2, a and b). Replacement of the Stat5 C terminus with a VP16 transactivating domain generated a Stat5-VP16 fusion protein, which was phosphorylated by Jak2 at levels comparable with those of wild-type Stat5 (Fig. 2a). As expected, antibodies specific to the authentic C terminus did not supershift the Stat5-VP16 complexes with DNA. Phosphorylation of the fusion protein resulted in transcription of the lacZ reporter gene (Fig. 2b). This result shows that Stat5 dimers in yeast are able to translocate to the cell nucleus but that the authentic Stat5 C terminus is not a transactivating domain under our experimental conditions in yeast.

Effect of Stat5 Mutations on the Interaction with Jak2—Several mutations of Stat5 were previously shown to affect Jak-mediated phosphorylation (43–45). Most obviously, mutation of Tyr<sup>694</sup> (Stat5a) or Tyr<sup>699</sup> (Stat5b) to phenylalanine abolishes phosphorylation, and in addition such mutants behave as dominant negative alleles at least in the case of some Stats. We were interested to find out whether the Stat5 YF mutant also exerted a dominant negative effect and whether this might result from an inability of the Jak kinase to release nonphosphorylatable substrates, in which case one might expect to find an increase in Jak-Stat complexes compared with the wild-type allele. A second mutation affecting the phosphorylation state in mammalian cells was generated by removing 77 and 84 amino acids from the C terminus including the putative transactivating domain. We were interested in testing whether Stat5<sup>R618K</sup> Arginine 618 within SH2 domain required for phosphorylation binding mutated to lysine.

Stat5 Activation in Yeast

![EMSA](image)

**Fig. 2.** DNA-binding activities of Stat5 proteins and transcriptional activity of Stat5 and Stat5-VP16. a, yeast cells were co-transformed with expression plasmids encoding Stat5a/Jak2 (lanes 1 and 2), Stat5aΔCVP16/Jak2 (lanes 3 and 4), Stat5b/Jak2 (lanes 5 and 6), and Stat5bΔCVP16/Jak2 (lanes 7 and 8). Nuclear extracts were used for the EMSA with a β-casein GAS probe. Specific antibodies (Ab) to Stat5a (lanes 2 and 4) and to Stat5b (lanes 6 and 8) were added for supershift analysis: These antisera recognize the C terminus of Stat5 not present in Stat5ΔCVP16. b, yeast cells containing an integrated copy of the Stat-dependent 3<sup>rd</sup>IFP-53/lacZ reporter gene were co-transformed with plasmids expressing Stat5 (lane 1), Stat5a/Jak2 (lane 2), or Stat5aΔCVP16/Jak2 (lane 3). β-Galactosidase activities were determined as described under “Experimental Procedures.”

| Designation | Description |
|-------------|-------------|
| myc-Stat5    | N-terminal tag with myc epitopes recognized by monoclonal antibody 9E10. |
| Stat5ΔC    | C-terminal deletion removing putative transactivating domain. |
| Stat5ΔCVP16 | VP16 transactivating domain fused to Stat5ΔC. |
| Stat5Y694F/699F | Tyrosine residue required for Stat5a/b activation changed to phenylalanine. |
| Stat5R618K  | Arginine 618 within SH2 domain required for phosphorylation binding mutated to lysine. |

**Table I**

**Mutated versions of Stat5 used in this study**

| Designation | Description |
|-------------|-------------|
| myc-Stat5    | N-terminal tag with myc epitopes recognized by monoclonal antibody 9E10. |
| Stat5ΔC    | C-terminal deletion removing putative transactivating domain. |
| Stat5ΔCVP16 | VP16 transactivating domain fused to Stat5ΔC. |
| Stat5Y694F/699F | Tyrosine residue required for Stat5a/b activation changed to phenylalanine. |
| Stat5R618K  | Arginine 618 within SH2 domain required for phosphorylation binding mutated to lysine. |
Stat5 Activation in Yeast

(a) Western blot analysis of Stat5a and Stat5b.  
(b) Co-immunoprecipitation of Stat5a and Stat5b with Jak2.  
(c) EMSA analysis of Stat5a and Stat5b with wild-type and mutant Jak2.

Fig. 3. Formation of complexes between Jak2 and mutant or wild-type Stat5 and DNA-binding activities of mutant and wild-type Stat5. a, yeast cells were co-transformed with expression plasmids encoding myc-Stat5a/Jak2 (lane 1), myc-Stat5aΔC/Jak2 (lane 2), myc-Stat5aY694F/Jak2 (lane 3), myc-Stat5b/Jak2 (lane 4), myc-Stat5bΔC/Jak2 (lane 5), and myc-Stat5bY699F/Jak2 (lane 6). Lysates were prepared and immunoprecipitated with an anti-myc antibody. A Western blot of the indicated immunoprecipitates was first stained with Jak2-specific antibody and subsequently reprobed with an anti-myc antibody.

The ΔC and YF mutations of either Stat5a or Stat5b were expressed at levels similar to those of wild-type Stat5. When co-immunoprecipitation analysis was performed under identical conditions as in case of wild-type Stat5, neither mutation had a significant effect on the abundance of Jak2-Stat5 complexes (Fig. 3a). Identical results were obtained when Jak2 antiserum was used for immunoprecipitation in the reciprocal experiment (data not shown). To rule out that our co-immunoprecipitation results were due to nonspecific protein interactions, we co-expressed Stat5a and Stat5b without Jak2 and precipitated the extracts with specific Stat5a (5a), Stat5b (5b), and Jak2 antisera. The precipitates were stained for the presence of Stat5a using specific antiserum (5a). Stat5a could not be co-immunoprecipitated with either Stat5b- or Jak2-specific antiserum (Fig. 3b). This indicates that co-immunoprecipitation requires specific and stable interactions between phosphorylated Stats.

EMSA analysis was performed to determine the amount of Stat5 dimers present in cellular extracts. As expected, the Y694F and Y699F mutations in Stat5a and Stat5b, respectively, completely abolished EMSA activity. When co-expressed with wild-type Stat5, this mutation did not diminish the phosphorylation of wild-type Stat5; no indication of dominant-negative activity was found (data not shown). Strongly increased amounts of Stat5 EMSA activity were detected in extracts of cells expressing the ΔC mutation of either Stat5a or Stat5b (Fig. 3c). This suggests that the hyperphosphorylation of this mutant is at least in part due to an improved ability to serve as a Jak2 substrate. As suggested from results shown in Fig. 3a, removal of the Stat5 C terminus has no bearing on the abundance of Jak2-Stat5 complexes detected by co-immunoprecipitation.

In Western blots, the amount of tyrosine-phosphorylated Stat5ΔC was found strongly enhanced compared with wild-type Stat5 (data not shown). Further controls were performed to exclude the possibility that the increased EMSA activity of the Stat5ΔC mutant was due to an altered affinity for the DNA probe. Different isoforms or Stat5 mutated proteins were bound to the radiolabeled EMSA probe and challenged with a 50-fold molar excess of cold oligonucleotide. The amount of Stat5 bound to the labeled oligonucleotide was then determined by gel retardation after various periods of incubation. In accordance with previous results with Stat1 (46), the off-rate of Stat5 was very high, with more than 50% labeled probe competed away after 5 min. However, no significant off-rate differences were found for yeast-derived Stat5a or Stat5b or for the ΔC mutations of both isoforms. Moreover, the off-rate of yeast-derived Stat5 did not differ significantly from that of Stat5 isolated from granulocyte-macrophage colony-stimulating factor-treated mouse macrophages (data not shown). This suggests that the yeast-derived dimers do not lack a specific mammalian modification(s) required for an increased affinity for the GAS sequence.

Coexpression of Jak2 with the R618K mutation of Stat5a did not produce Stat5 EMSA activity (Fig. 4a). Moreover, the Stat5aR618K mutation caused an approximately 80% reduction in the amount of co-immunoprecipitated Jak2. This was shown by expressing N-terminally myc-tagged Stat5aR618K mutant together with Jak2 protein and then using in parallel the anti-tag monoclonal antibody and Jak2 antiserum for immunoprecipitation of cellular extracts. The precipitates were stained for the presence of mutant Stat5a using myc tag anti-

co-transformed with expression plasmids as described for panel a. Cellular extracts were used for the EMSA with a β-casein GAS probe. Anti-myc antibody was added in lanes 2 and 6 for supershift analysis (+Ab).
Stat5a/Jak2. Cellular extracts were used for the EMSA with a bSion plasmids encoding mycbody (Fig. 4 not shown). Together, these results suggest that the R618K sine phosphorylation of the protein could not be detected (data not shown). Importantly, the Stat5a mutant showed no staining with an anti-phosphotyrosine monoclonal antibody (Fig. 4c). Similar results were obtained when COS-N31 cells were co-transformed with the erythropoietin receptor and Stat5aR618K mutant. The mutant Stat5a was present in cellular extracts at the same level as wild-type Stat5a, but tyrosine phosphorylation of the protein could not be detected (data not shown). Together, these results suggest that the R618K mutation interacts weakly with, and is not a substrate for, the Jak2 kinase.

**Stat5 Phosphorylation by Deletion Mutants of Jak2**—To learn about the Jak2 JH regions required for productive interaction with Stat5, deletions were introduced into the Jak2 gene-disrupting regions JH2–JH7, leaving essentially only the kinase domain intact (ΔJN), or specifically disrupting the JH2 domain (ΔJH2; Fig. 5a). Both deletion mutants were expressed at levels comparable with wild-type Jak2 and co-immunoprecipitated with Stat5 (Fig. 5b). This result suggests that the kinase domain is the only homology region that must be intact for Stat5 association and phosphorylation. Compared with wild-type Jak2, the Jak2ΔN mutant showed a reduced but significant kinase activity, measured by the appearance of Stat5 EMSA activity. By the same criteria, the activity of the Jak2ΔJH2 kinase was significantly increased, indicating that JH2 might function in the down-regulation of Jak2 kinase activity (Fig. 5c).

**Stat5 Homo- and Heterodimer Formation: The Role of SH2 Domain-Phosphotyrosine Interactions**—Previous results clearly established the potential of the two Stat5 isoforms to heterodimerize. In fact, heterodimers were suggested to have an altered potential to activate target genes compared with homodimers (47). We were interested in determining the relative rates of homodimer versus heterodimer binding to a classical Stat5 target promoter, the b-casein gene, in a situation of approximately equal expression levels of Stat5a and Stat5b (Fig. 5b) and without third party interference. EMSA supershift analysis was performed to determine the amount of Stat5 dimers present in cellular extracts (Fig. 6a). When wild-type Stat5b was co-expressed with N-terminally myc-tagged versions of Stat5a and Jak2, three different complexes were found, consisting of DNA associated with Stat5a homodimers (most abundant), Stat5a/Stat5b heterodimers, and Stat5b homodimers.

We next tested the potential of Stat5a heterodimers held by a single SH2–phosphotyrosine bond to co-immunoprecipitate and bind to DNA. This was done by co-expressing Jak2, myc-tagged Stat5aY694F mutant, and wild-type Stat5b. Cellular extracts were subjected to co-immunoprecipitation (Fig. 6b, left) and EMSA supershift analysis (Fig. 6c). The Stat5Y694F mutant, detected with anti-myc monoclonal antibody, was co-immunoprecipitated with either wild-type Stat5b or Jak2. Thus, a single phosphotyrosine-SH2 interaction is sufficient for dimerization, as suggested for Stat1 (10). Very little EMSA activity in extracts containing wild-type Stat5b and myc-tagged Stat5aY694F reacted with the myc monoclonal antibody in supershift analysis, despite a high efficiency of this antibody to supershift complexes with myc-tagged wild-type Stat5 (Fig. 5c). This result suggests that dimers between wild-type Stat5 and the Y694F mutant do form but that they have limited ability to bind to their target DNA sequence. To ensure that the complex formation was mediated by SH2-phosphotyrosine interaction, YF mutant Stat5a and wild-type Stat5b were co-expressed without Jak2. In this situation, no interaction between YF mutant and wild-type Stat5 was observed (Fig. 6b, right).

**DISCUSSION**

The experiments described here investigate protein interactions occurring in a Jak2-Stat5 signaling path as it is employed in many mammalian cytokine responses, e.g. prolactin, erythropoietin, granulocyte-macrophage colony-stimulating factor, interleukin-3, and growth hormone (48). Phosphorylation of Stat5 on tyrosine can occur in case of some cytokine receptors or experimental systems without an obvious requirement for phosphotyrosine-mediated receptor binding, as in the case of gp130, growth hormone, or prolactin (13, 15, 20, 21, 27). To investigate in how far direct interactions between Jak2 and Stat5 can contribute to Stat5 tyrosine phosphorylation, we
have established yeast strains in which interactions between the two proteins can be studied in the absence of vast overexpression or of the involvement of other proteins. A welcomed by-product of such yeast strains is the fact that activated Stat5 translocates to the nucleus and binds to DNA, without however activating transcription. This indicates that our yeast strains when optimized further may be suitable for a modified one-hybrid screening approach for Stat5 interactions involving the tyrosine-phosphorylated and dimerized protein.

Dimers of Stat5 can be formed through a single SH2 domain-phosphotyrosine interaction. This was suggested by the ability of a Y694F mutant to co-immunoprecipitate wild-type Stat5. The specificity of this interaction is suggested from control experiments demonstrating that the presence of Jak2, and thus of Stat5 tyrosine phosphorylation, is a prerequisite for co-immunoprecipitation. By contrast, dimers of phosphorylated and nonphosphorylated Stat5 bound to DNA only very inefficiently. One possible explanation is that single-bonded dimers are vastly outcompeted in abundance by the more stable dimers with reciprocal bonds. The efficient co-immunoprecipitation of Y694F with wild-type Stat5, as well as the vast excess of probe in our EMSA experiments argue against this explanation but do not entirely rule it out. We favor the assumption that the single-bonded dimers form intrinsically less stable complexes with DNA than double-bonded dimers. This possibility is suggested by the high off-rate of Stats from their cognate binding sites. One might easily imagine that the destabilization through the loss of a second bond further increases the off-rate and minimizes the ability of such dimers to form stable complexes with DNA.

We have found the phosphorylation of Stat5 to depend on an intact SH2 domain-phosphotyrosine interaction. This was suggested by the ability of a Y694F mutant to co-immunoprecipitate wild-type Stat5. The specificity of this interaction is suggested from control experiments demonstrating that the presence of Jak2, and thus of Stat5 tyrosine phosphorylation, is a prerequisite for co-immunoprecipitation. By contrast, dimers of phosphorylated and nonphosphorylated Stat5 bound to DNA only very inefficiently. One possible explanation is that single-bonded dimers are vastly outcompeted in abundance by the more stable dimers with reciprocal bonds. The efficient co-immunoprecipitation of Y694F with wild-type Stat5, as well as the vast excess of probe in our EMSA experiments argue against this explanation but do not entirely rule it out. We favor the assumption that the
due to a phosphotyrosine-independent association with Jak2 outside of the JH1 domain. Despite this possibility, there is no absolute requirement for the JH2 domain in Stat5 activation in mammalian cells. This is suggested by the results of Kohlhauer and colleagues (30), Sakai and Kraft (31), and Berchtold et al. (32), which indicate that a Jak2 kinase without JH2 domain functions in mammalian cells, particularly in activating Stat5.

Deletion of the JH2 domain resulted in an increased capability to phosphorylate Stat5. This effect is similar to what was found in case of the Hopscotch gain of function mutation (28). Interestingly, however, deletion of the entire Hopscotch JH2 domain causes a loss of enzymatic activity (28), and this is also the case for Tyk2 (29). Taken together, these results indicate that the JH2 domain serves to down-regulate kinase activity and that the effect of JH2 deletion may vary with the exact extent of the deletion, the individual Jaks, or the substrate employed to determine Jak activity. Deletion of the Stat5 C terminus also resulted in enhanced phosphorylation by Jak2. This was noted previously in mammalian cells (44). In this situation, studies on the kinetics of Stat5ΔC activation suggested a decreased rate of tyrosine dephosphorylation as a cause for the hyperphosphorylated state (44). We cannot entirely rule out the presence of tyrosine phosphatases in yeast that are similarly affected by the C-terminal truncation. However, we think it more likely that in our situation hyperactivation of Stat5ΔC results from improved interaction with Jak2. For example, the C-terminal deletion may increase the $V_{\text{max}}$ of a catalytic cycle and thus result in an increase of phosphorylated Stat5 in a given period. If our interpretation is correct, an increased phosphorylation rate may contribute to the hyperphosphorylation of Stat5ΔC also in mammalian cells and thus increase the dominant negative effect of this Stat5 allele.

The ability of Jak2 to directly bind Stat5 emphasizes the question why and when receptors do require docking of Stat5 prior to Jak2-mediated phosphorylation. Phosphorylation of Stat5 independently of receptor binding may occur in the case of many cytokine receptor-Jak2 complexes, but an amplification of the signal by receptor docking may be necessary in certain or even most situations, e.g., when receptor numbers are low or when abundant input of signals from the cell surface leads to competition of signal transducers for binding to Jak2 phosphotyrosines. Addressing these possibilities will be the direction of future work.

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