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P210 and P190$^{BCR/ABL}$ Induce the Tyrosine Phosphorylation and DNA Binding Activity of Multiple Specific STAT Family Members*

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The products of the Philadelphia chromosome translocation, P210 and P190$^{BCR/ABL}$, are cytoplasmic protein tyrosine kinases that share the ability to transform hematopoietic cytokine-dependent cell lines to cytokine independence but differ in the spectrum of leukemia induced *in vivo*. We have analyzed the Janus kinase (JAK) and signal transducer and activator of transcription (STAT) pathways in hematopoietic cells transformed by Bcr/Abl. STAT5 and, to a lesser extent, STATs 1 and 3 were constitutively activated by tyrosine phosphorylation and induction of DNA binding activity in both P210 and P190$^{BCR/ABL}$-transformed cells, but P190 differed in that it also prominently activated STAT6. There was low level tyrosine phosphorylation of JAKs 1, 2, and 3 in Bcr/Abl-transformed cells, but no detectable complex formation with Bcr/Abl, and activation of STAT5 by P210 was not blocked by two different dominant-negative JAK mutants. These results suggest that P210 and P190$^{BCR/ABL}$ directly activate specific STAT family members and may help explain their overlapping yet distinct roles in leukemogenesis.

The Philadelphia chromosome-positive (Ph$^+$)[1] hematologic malignancies are characterized by the reciprocal translocation between the *BCR* gene on chromosome 22 and the nonreceptor tyrosine kinase c-*ABL* on chromosome 9 (1). The resulting chimeric fusion protein, Bcr/Abl, demonstrates increased tyrosine kinase activity (2), becomes mostly cytoplasmic in localization (3), and acquires the ability to transform hematopoietic cells *in vitro* and induce leukemia in mice (4). There are two distinct forms of Bcr/Abl, the consequence of different translocation breakpoints on chromosome 22. P210$^{BCR/ABL}$ is the hallmark of the myeloproliferative disorder chronic myelogenous leukemia (CML), whereas P190$^{BCR/ABL}$, with rare exceptions, is found only in acute lymphoblastic leukemia (5). Although they differ in the spectrum of leukemia induced *in vivo*, P210 and P190 share the ability to transform hematopoietic cytokine-

dependent cell lines to cytokine independence (6, 7). The downstream signaling events involved in Bcr/Abl transformation remain largely undefined. Members of the Janus kinase (JAK) family of protein tyrosine kinases and the signal transducer and activator of transcription (STAT) family of latent transcription factors are activated by tyrosine phosphorylation in many growth factor and cytokine receptor signaling pathways (reviewed in Refs. 8 and 9). Recently, constitutive tyrosine phosphorylation of JAKs 1 and 3 and induction of STAT DNA binding has been demonstrated in Abelson murine leukemia virus-transformed pre-B cell lines (10). P120^{v-abl} was also found to co-immunoprecipitate with JAK1 and JAK3. These results suggest that activation of the JAK/STAT pathway may play a role in transformation by oncogenic forms of Abl.

Here we have analyzed the JAK/STAT pathway in hematopoietic cell lines transformed by Bcr/Abl. There was high level constitutive activation of STAT5 and lower activation of STAT1 and STAT3 in both P210 and P190$^{BCR/ABL}$-transformed cells. Interestingly, P190 differed in that it also induced prominent activation of STAT6. There was low level tyrosine phosphorylation of JAKs 1, 2, and 3 in Bcr/Abl-transformed cell lines, but in contrast to v-Abl (10), we found no detectable complex formation between JAK family members and Bcr/Abl. Furthermore, dominant negative mutants of JAK2 did not block the activation of STAT5 by P210. These results suggest that P210 and P190$^{BCR/ABL}$ directly activate distinct STAT transcription factors in hematopoietic cells.

EXPERIMENTAL PROCEDURES

Cells and Cell Culture—BCR/ABL cDNAs in the vector pGD (4) were introduced into Ba/F3 and FDC-P1 cells by electroporation and cultured as described previously (4). For 38B9 cells, β-mercaptoethanol (50 μm) was also added to the medium. For serum and growth factor deprivation experiments, cells were washed twice in phosphate-buffered saline lacking calcium and magnesium chloride and resuspended in RPMI 1640 supplemented only with 1% (w/v) lipid-free bovine serum albumin (Sigma) for 7–8 h at 37 °C. Stimulated cells received 10% WEHI-3B-conditioned medium (v/v) as a source of IL-3 (12) for 10–15 min at 37 °C prior to harvest.

Immunoprecipitation and Western Blotting—Radioimmune precipitation buffer lysates were prepared as described previously (11). Immunoprecipitation was performed on lysates from 1–1.5 × 10^7 cells using either anti-STAT (Santa Cruz Biotechnology, Santa Cruz, CA), anti-JAK (Upstate Biotechnology, Lake Placid, NY), or anti-Abl (anti-Gex4 antisera (13)) antibodies at 4 °C for 6–16 h. Protein samples were normalized by A_{595} (Bio-Rad protein assay), resolved by 7% SDS-polyacrylamide gel electrophoresis, and transferred overnight to a nitrocellulose membrane. Western blotting was performed using anti-STAT, anti-JAK, anti-Abl (14), or anti-phosphotyrosine (4G10) antibodies (Upstate Biotechnology) and detected by Enhanced Chemiluminescence (Amersham Corp.).

Electromobility Shift Assay (EMSA)—Nuclear extracts from parental or Bcr/Abl-expressing cells were prepared as described previously (15). 6 μg of nuclear extract was incubated with 0.2 ng of a ^{32}P-labeled double-stranded oligonucleotide probe for 20 min at room temperature. The γ-interferon activated sequence (GAS) probe, based on the *FcγRI* gene promoter (16), was formed by annealing the oligonucleotides

[1] The abbreviations used are: Ph, Philadelphia chromosome; STAT, signal transducer and activator of transcription; JAK, Janus kinase; DNJAK, dominant negative Janus kinase; EMSA, electromobility shift assay; GAS, γ-interferon activated sequence; CML, chronic myelogenous leukemia; IL, interleukin; HA, hemagglutinin; EGF, epidermal growth factor.

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5′ CGTACGAGATGTATTTCCCAGAAAAG 3′ and 5′ CGTACTTTTCT-GGGAAATACATCTCG 3′. The N4 probe sequence was made by annealing the oligonucleotides 5′ GATCTGTAATTCGTGTGAATTATG 3′ and GATCCATAATTCACACGAATTACA 3′ (17). For supershift analysis, a 1:15 or 1:20 dilution of antibody (Santa Cruz Biotechnology) was added for an additional 45 min at 4 °C. The STAT6 competitor peptide Y^{P}578 (18) was added just prior to the addition of the probe at a final concentration of 100 μm. For the competition experiments, unlabeled double-strand oligonucleotides were added to nuclear extracts at the time of probe addition. The DNA-protein complexes were resolved on 4% Tris-acetate-ethylenediamine tetraacetic acid polyacrylamide gels and detected by autoradiography.

Dominant Negative JAK2 Mutants—The dominant negative JAK2 mutant containing the carboxyl-terminal truncation (DNJAK/Δ829) and the kinase domain double point mutant (DNJAK/DK) were expressed in the plasmid vector pBOS (19). A hemagglutinin (HA) tag was later added to the DNJAK/DK mutant by Dr. Alan D'Andrea. The DNJAK mutants and a plasmid conferring puromycin resistance were introduced into Ba/F3 cells by electroporation, as described previously (11). After puromycin selection in the presence of IL-3, individual clones were isolated by limiting dilution and screened for high DNJAK expression by anti-JAK and anti-HA Western blot. The Ba/F3 and Ba/F3-P210 clones that expressed the highest levels of DNJAK protein were then used for further analysis.

RESULTS

STATs 1, 3, and 5 Are Constitutively Tyrosine Phosphorylated in Ba/F3 Cells Transformed by Bcr/Abl—We chose to characterize the JAK/STAT pathway in a well defined cell type, the murine pro-B lymphoid line Ba/F3 (20). Ba/F3 cells are IL-3-dependent and non-leukemogenic but are transformed to IL-3 independence and leukemogenicity by expression of Bcr/Abl (7, 11). Therefore, differences in STAT activation between parental and Bcr/Abl-transformed Ba/F3 cells may be directly ascribed to the action of Bcr/Abl. Anti-STAT1 immunoprecipitates from parental and Bcr/Abl-transformed Ba/F3 cells were analyzed by Western blot using an antibody denoted pSTAT1 (developed by Dr. David Frank), which recognizes the tyrosine-phosphorylated form of STAT1 (Fig. 1A). STAT1 was not tyrosine phosphorylated in parental Ba/F3 cells, but Ba/F3-P210 and P190 cells demonstrated tyrosine phosphorylation of both the STAT1α (91 kDa) and STAT1β (84 kDa) forms of STAT1 (Fig. 1A, *upper panel*) (21). As expected, STAT1 was tyrosine phosphorylated at high levels in the control fibroblast cell line A431 stimulated with epidermal growth factor (EGF) after serum starvation (22, 23). Identical results were obtained when STAT1 immunoprecipitates were analyzed using an anti-phosphotyrosine antibody (data not shown). There was no significant difference in STAT1 protein levels between parental and Bcr/Abl-transformed cells (Fig. 1A, *lower panel*).

Similarly, STAT3 was not tyrosine-phosphorylated in parental Ba/F3 cells, but two different Ba/F3-P210 cell lines exhibited a low but detectable level of tyrosine phosphorylation of the 89-kDa form of STAT3 (Fig. 1B, *upper panel*) (24). Ba/F3-P190 cells demonstrated a similar level of tyrosine phosphorylation of STAT3 (data not shown). Control A431 fibroblasts demonstrated prominent tyrosine phosphorylation of the 92-kDa form of STAT3 upon stimulation with EGF (22). The total amount of STAT3 (92- and 89-kDa forms) was the same in parental and Bcr/Abl-transformed cells (Fig. 1B, *lower panel*).

Parental Ba/F3 cells deprived of growth factor had only minimal STAT5 tyrosine phosphorylation (Fig. 1C, *upper panel*) but exhibited a high level of tyrosine phosphorylation of STAT5 after stimulation with IL-3, as expected (25). In contrast, Ba/F3-P210 cells demonstrated constitutive high levels of STAT5 tyrosine phosphorylation in the absence of IL-3 (Fig. 1C). STAT5 was constitutively activated in Ba/F3-P190 cells as well, although at a lower level than in P210 cells. STAT5 was also tyrosine phosphorylated in the cell line 38B9, an Abelson murine leukemia virus-transformed pre-B lymphoid cell line
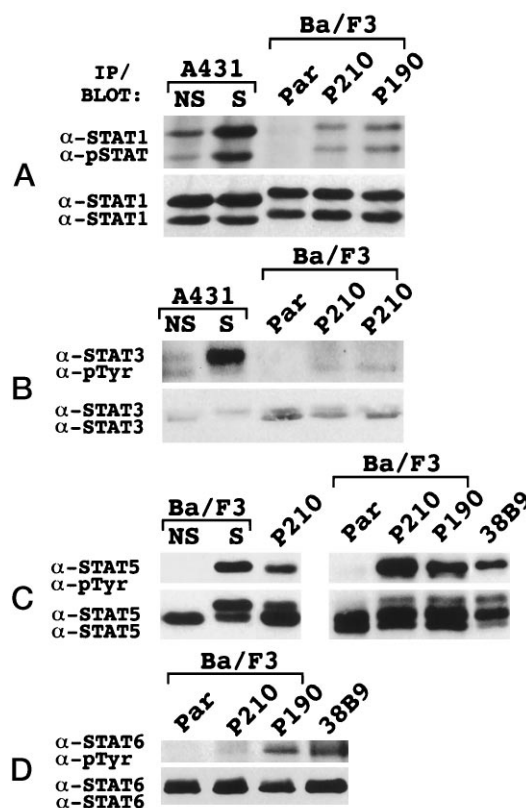

FIG. 1. **Multiple STAT family members are tyrosine phosphorylated in Bcr/Abl-transformed Ba/F3 cells.** *A*, STAT1 immunoprecipitates were analyzed by pSTAT1 antibody (*top*), which recognizes the tyrosine phosphorylated form of STAT1 (provided by Dr. David Frank), or an anti-STAT1 antibody (*bottom*), which recognizes both STAT1α (91 kDa) and STAT1β (84 kDa). A431 fibroblasts were serum starved for 48 h (*NS*) and stimulated (*S*) for 15 min with 200 ng/ml EGF (*left*). Exponentially growing parental, P210-transformed, and P190-transformed Ba/F3 cells are shown at *right*. *B*, STAT3 immunoprecipitates were analyzed by anti-phosphotyrosine antibody (*top*) or an antibody that recognizes both the 92- and 89-kDa forms of STAT3 (*bottom*). A431 fibroblasts were starved (*NS*) and then EGF stimulated (*S*) as described above (*left*). Parental or two different Ba/F3 cell lines transformed by P210 are shown at *right*. *C*, STAT5 immunoprecipitates were analyzed by anti-phosphotyrosine antibody (*top*) or an anti-STAT5 antibody which recognizes both 96- (STAT5A) and 94-kDa (STAT5B) forms of STAT5 (*bottom*). Parental Ba/F3 cells were deprived of growth factor (*NS*) and then stimulated with IL-3 (*S*) and compared with P210-transformed Ba/F3 cells (*left*). Exponentially growing parental, P210 or 190-transformed Ba/F3 cells, and the Abelson murine leukemia virus-transformed cell line 38B9 are shown at *right*. *D*, STAT6 immunoprecipitates were analyzed by anti-phosphotyrosine (*top*) or anti-STAT6 antibody (*bottom*). Parental, P210- or P190-transformed Ba/F3 cells, and 38B9 cells are compared.

expressing P120$^{v\text{-}abl}$, consistent with previous findings (10). The total level of STAT5 in parental, v-Abl-expressing, and Bcr/Abl-expressing cells was the same, and appeared as a doublet migrating at approximately 96 and 94 kDa. Often an additional band of slightly higher molecular mass was apparent in cells expressing activated STAT5 (Fig. 1C, *lower panel*), likely reflecting the effect of tyrosine and serine/threonine phosphorylation.

STAT2 was expressed at low levels in Ba/F3 cells transformed by Bcr/Abl, without evidence of appreciable tyrosine phosphorylation (data not shown). STAT4 protein was not detected in these cells (data not shown), consistent with its reported limited cellular distribution (26, 27).

Tyrosine Phosphorylation of STAT5 Is a Consistent Finding in Cells Expressing Bcr/Abl—FDC-P1/P210, a murine myeloid IL-3 or granulocyte-macrophage colony-stimulating factor de-
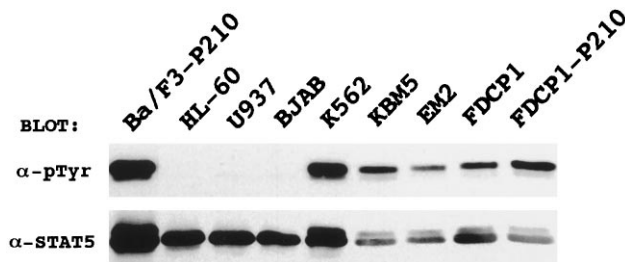
FIG. 2. **STAT5 tyrosine phosphorylation in Bcr/Abl-expressing cells.** STAT5 was immunoprecipitated from parental and P210-transformed FDC-P1 cells; the Ph-negative leukemia cell lines HL-60, U937, and BJAB; and three human CML cell lines, KBM5, K562, and EM2, and analyzed by anti-phosphotyrosine (*top*) or anti-STAT5 antibody (*bottom*). A Ba/F3 cell line transformed by P210 is shown at *left* for comparison.

FIG. 3. **STAT GAS DNA binding activity is induced by Bcr/Abl.** Nuclear extracts from parental, P210- or P190-transformed Ba/F3 cells, and 38B9 cells were analyzed by EMSA using a ^{32}P-labeled FcγRI-derived GAS probe. *A*, Ba/F3-P210 nuclear extracts were preincubated with anti-phosphotyrosine antibody (4G10) or with free phosphotyrosine, phosphoserine, or phosphothreonine added to a final concentration of 20 mm. Cold competitor GAS oligonucleotide was added in the amounts indicated. Parental Ba/F3 cells are shown at *left*. *B*, the indicated anti-STAT antibodies were added to Ba/F3-P210 nuclear extracts as described under "Experimental Procedures." Nuclear extract from IL-3-stimulated parental Ba/F3 cells produced an identical complex that was completely supershifted with anti-STAT5 antibody (data not shown). Longer exposures confirmed faint supershifted bands with pSTAT1, anti-STAT3, and anti-STAT1 and anti-STAT3 antibodies. Nuclear extract from EGF-stimulated A431 fibroblasts produced GAS-binding complexes that were readily supershifted by the anti-STAT1 and -STAT3 antibodies (data not shown). Baseline GAS DNA-binding complexes from parental and Ba/F3-P210 cells are shown at *left*. *C*, GAS DNA-binding complexes from Ba/F3-P210, Ba/F3-P190, and 38B9 cells were compared by incubation with anti-STAT antibodies or the STAT6 competitor peptide Y^P578 as described under "Experimental Procedures." The baseline GAS DNA-binding complex for each cell type is shown at *left*. The positions of STAT5 and STAT6 GAS complexes are shown by the *arrows* at *right*. Higher concentrations of Y^P578 peptide also decreased the STAT6 GAS DNA complex but were not associated with the supershift shown here.

pendent cell line (28) transformed to growth factor independence by P210 (6, 11), also exhibited constitutive tyrosine phosphorylation of STAT5 (Fig. 2), demonstrating that activation of STAT5 by Bcr/Abl is not limited to lymphoid cells. STAT5 was also tyrosine phosphorylated in the human CML cell lines K562, KBM5, and EM2 (Fig. 2). In contrast, three Ph-negative (Ph$^-$) human leukemia cell lines (HL-60 and U937, both myeloid, and BJAB, B-lymphoid), which do not express Bcr/Abl, showed no evidence of STAT5 tyrosine phosphorylation.

STAT6 Is Tyrosine Phosphorylated in Ba/F3 Cells Transformed by P190 but not by P210$^{BCR/ABL}$—While there was no significant difference in the tyrosine phosphorylation of STATs 1, 3, and 5 induced by P210 and P190, STAT6 exhibited a distinct activation pattern. Although STAT6 was not significantly tyrosine-phosphorylated in parental or P210-transformed Ba/F3 cells, P190-transformed cells demonstrated prominent activation of STAT6 (Fig. 1*D*, *upper panel*). STAT6 was also constitutively activated in the cell line 38B9, as noted by others (10). STAT6 migrated as a single band of approximately 100 kDa and was present at equal levels (Fig. 1*D*, *lower panel*).

Bcr/Abl Induces the DNA Binding Activity of Multiple STAT Family Members—Induction of STAT DNA binding activity by Bcr/Abl was assessed by EMSA using a GAS probe derived from the FcγRI promoter (16). Nuclear extracts of Ba/F3 cells expressing P210$^{BCR/ABL}$ demonstrated high levels of constitutive GAS DNA binding activity. This DNA binding activity was impaired by anti-phosphotyrosine antibody and was abolished by the addition of free phosphotyrosine but not by the addition of phosphoserine or phosphothreonine, consistent with the activation of STAT-mediated DNA binding (Fig. 3*A*). The GAS DNA binding activity was also eliminated by cold competitor GAS oligonucleotide. To identify the STATs responsible for the GAS binding activity, specific anti-STAT antibodies were used that are capable of binding to the DNA-protein complex and retarding their mobility (supershift). Incubation of Ba/F3-P210 nuclear extracts with anti-STAT5 antibody resulted in the supershift of the majority of the GAS DNA binding activity (Fig. 3*B*). Antibodies to STAT1, STAT3, or both resulted in a small but reproducible decrease in GAS DNA binding activity; on longer exposures, a faint supershift band was apparent with anti-STAT1 or anti-STAT3 antibodies (data not shown). Incubation with the combination of anti-STAT 1, 3, and 5 antibodies resulted in the abrogation of all detectable GAS DNA binding activity induced by P210$^{BCR/ABL}$. Anti-STAT2 and -STAT4 antibodies had no effect. These results demonstrate prominent activation of STAT5 DNA binding activity in P210-transformed cells, with lesser activation of STATs 1 and 3, consistent with the tyrosine phosphorylation profile.

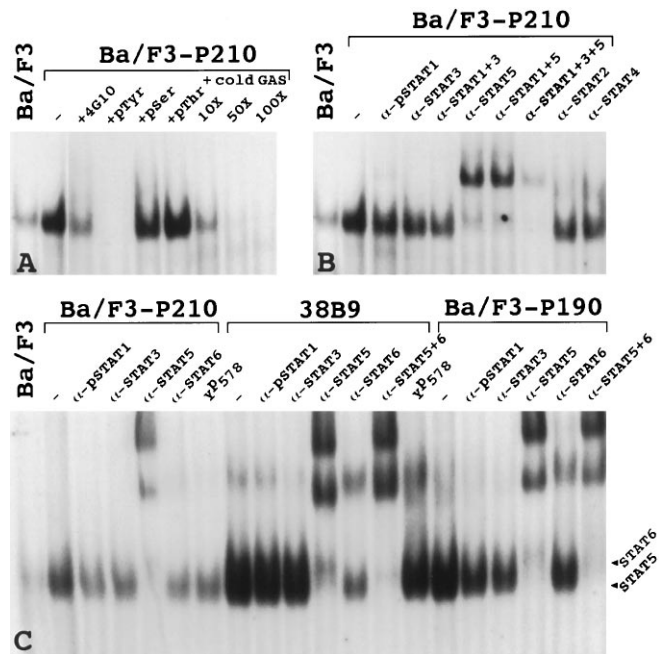Ba/F3-P190 cells demonstrated a similar spectrum of GAS

DNA binding activity except for the prominent activation of STAT6. The P190$^{BCR/ABL}$ GAS-binding complex was composed of an upper region that was supershifted with antibody to STAT6 and a lower region, the migration of which was retarded by incubation with anti-STAT5 antibody (Fig. 3*C*, *arrows*). The combination of anti-STAT5 and anti-STAT6 antibodies supershifted most of the detectable GAS DNA binding activity induced by P190$^{BCR/ABL}$. The cell line 38B9 demonstrated a similar pattern of supershift with anti-STAT5 and -STAT6 antibodies, confirming the activation of STAT5 and STAT6 DNA binding activity by P120^{v-abl} (10). The competitor peptide Y^P578, derived from the putative STAT6 binding site on the cytoplasmic portion of the IL-4 receptor (18), resulted in a supershift of the upper region of the GAS DNA-binding complex, confirming the identity of this binding activity as STAT6. In contrast, P210 GAS DNA binding activity was only slightly decreased by anti-STAT6 antibody without evidence of supershift, and incubation with the STAT6 competitor peptide Y^P578 had no appreciable effect.

To confirm differential activation of STAT6 by P190$^{BCR/ABL}$, we used the oligonucleotide probe N4, which has 4 base pairs
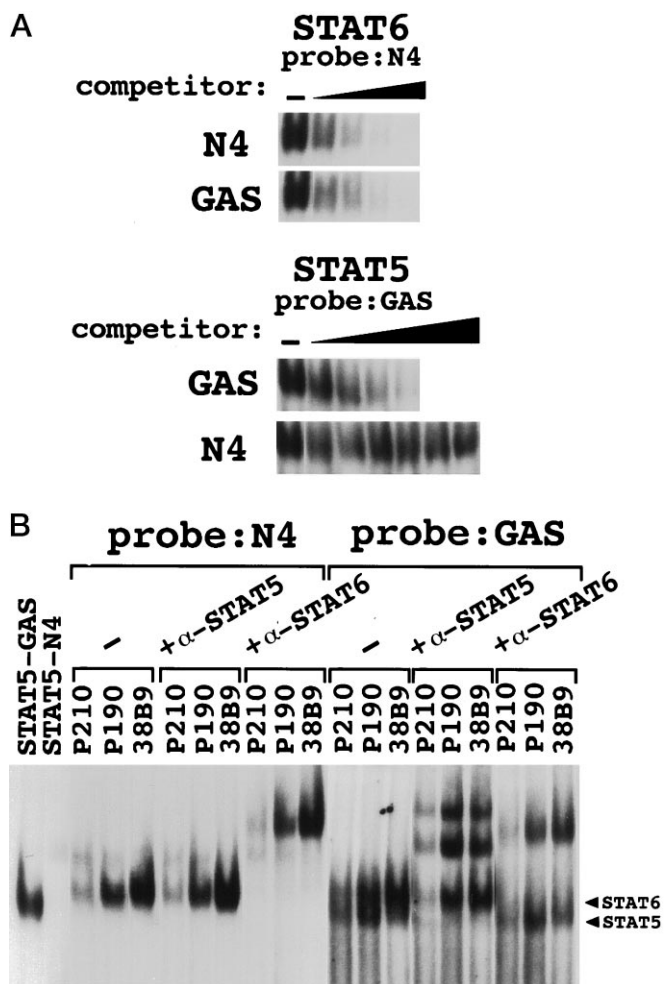
FIG. 4. **The probe N4 distinguishes STAT6 and STAT5 DNA binding activity and confirms differential activation of STAT6 by P190**[BCR/ABL]**.** *A*, nuclear extracts from either 293T cells transfected with STAT5A and JAK2 incubated with ^{32}P-labeled FcγRI-derived GAS probe (*bottom panels*) or IL-4-stimulated murine spleen cells incubated with N4 probe (*top panels*) were subjected to increasing amounts of either unlabeled GAS or N4 oligonucleotide. Samples without competitor are at *far left*. Unlabeled competitor was added starting at 3.3-fold molar excess and increasing by half-log increments. *B*, nuclear extracts from 293T cells transfected with STAT5A and JAK2 (*far left*), parental, P210- or P190-transformed Ba/F3 cells, and 38B9 cells were analyzed by EMSA using either a ^{32}P-labeled N4 (*left*) or FcγRI-derived GAS probe (*right*), with or without the indicated anti-STAT antibody. The positions of STAT5 and STAT6 GAS DNA complexes are shown by the *arrows* at *right*.

separating its region of dyad symmetry and has been shown to discriminate between STAT1 and STAT6 DNA binding activity (17). Nuclear extracts prepared from 293T cells transfected with STAT5A and JAK2 demonstrated prominent GAS DNA binding activity that was readily competed by unlabeled GAS (which has a 3-base pair separation of the dyad symmetry), but not N4 oligonucleotide, even when added at 1000-fold molar excess (Fig. 4*A*). As a source of STAT6 DNA binding activity, we used nuclear extracts from IL-4-stimulated murine spleen cells (29). Compared to unlabeled N4 oligonucleotide, GAS was approximately 3-fold less effective in the competition for N4 STAT6 DNA binding activity. Therefore, STAT6 bound with nearly equal efficiency to either N4 or GAS, but there was no detectable binding of STAT5 to N4 under these conditions.

Both Ba/F3-P190 and 38B9 nuclear extracts demonstrated prominent N4 binding activity that was not supershifted with anti-STAT5 antibody but was completely abrogated by anti-
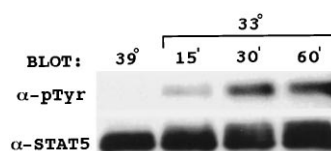

FIG. 5. **STAT5 tyrosine phosphorylation induced by a temperature-sensitive P210 mutant.** STAT5 immunoprecipitates from a temperature-sensitive P210 mutant were analyzed by anti-phosphotyrosine (*top*) or anti-STAT5 antibody (*bottom*). The nonpermissive temperature was 39 °C and the permissive temperature was 33 °C.

STAT6 antibody (Fig. 4*B*). In contrast, nuclear extracts from Ba/F3-P210 cells exhibited only faint N4-DNA binding activity. As expected, nuclear extracts from 293T cells transfected with STAT5A and JAK2 showed prominent GAS DNA binding activity but no appreciable binding to the N4 probe. Similarly, nuclear extract from parental Ba/F3 cells stimulated with IL-3 also demonstrated no significant N4 binding activity (data not shown). These results verify that P190[BCR/ABL] differs from P210 by its ability to induce high level STAT6 DNA binding activity.

Activation of STAT5 Occurs Rapidly upon Induction of Bcr/Abl Kinase Activity—The kinetics of STAT activation by Bcr/Abl was assessed using a P210 temperature-sensitive mutant (30). Ba/F3-temperature-sensitive P210 cells, grown for several days in IL-3-supplemented medium at the nonpermissive temperature (39 °C), were deprived of growth factor for 4–5 h. The cells were then shifted to the permissive temperature (33 °C) in the absence of growth factor, and the tyrosine phosphorylation status of STAT5 was examined at successive time intervals. No STAT5 tyrosine phosphorylation was detected after growth factor deprivation at the nonpermissive temperature (Fig. 5). Induction of P210 kinase activity at the permissive temperature resulted in rapid tyrosine phosphorylation of STAT5, detectable as early as 15 min and reaching a maximum by 30 min (Fig. 5). These results demonstrate that the activation of STAT5 by P210 requires Bcr/Abl tyrosine kinase activity.

JAKs 1, 2, and 3 Are Tyrosine Phosphorylated at Low Levels in Bcr/Abl-Transformed Cell Lines—To evaluate the effect of Bcr/Abl on JAK family members, anti-JAK immunoprecipitates were prepared from parental or Bcr/Abl-expressing Ba/F3 cells in the absence of serum and growth factor and after stimulation with IL-3. Parental Ba/F3 cells demonstrated no detectable JAK tyrosine phosphorylation in the absence of serum and growth factor (Fig. 6), and IL-3 stimulation resulted in prominent tyrosine phosphorylation of both JAK1 and JAK2 (31) but not of JAK3. P210 and P190[BCR/ABL]-transformed Ba/F3 cells demonstrated constitutive tyrosine phosphorylation of JAKs 1, 2, and 3 at low levels in the absence of serum and growth factor. Stimulation of these cells with IL-3 resulted in further JAK1 and 2 tyrosine phosphorylation, approaching the levels observed in growth factor-stimulated parental Ba/F3 cells. No detectable Tyk2 protein expression was found in either parental or Ba/F3-P210 cells (data not shown). JAKs 1, 2, and 3 were constitutively activated in 38B9 cells and co-immunoprecipitated with P120[v-abl] (Fig. 6), as recently demonstrated (10). In contrast, there was no evidence of co-immunoprecipitation of Bcr/Abl with any of the JAKs under these conditions (data not shown).

Constitutive Activation of STAT5 by P210[BCR/ABL] *Is Not Blocked by Dominant-Negative JAK2 Mutants*—To determine whether Bcr/Abl activated STATs indirectly through the activation of JAK family members, dominant-negative mutants of JAK2 (DNJAK) were expressed in parental and Ba/F3-P210 cells. Two different mutants were studied, one consisting of two point mutations in a conserved motif within the JAK2 kinase domain (DNJAK/DK-HA), and the other consisting of a carbox-
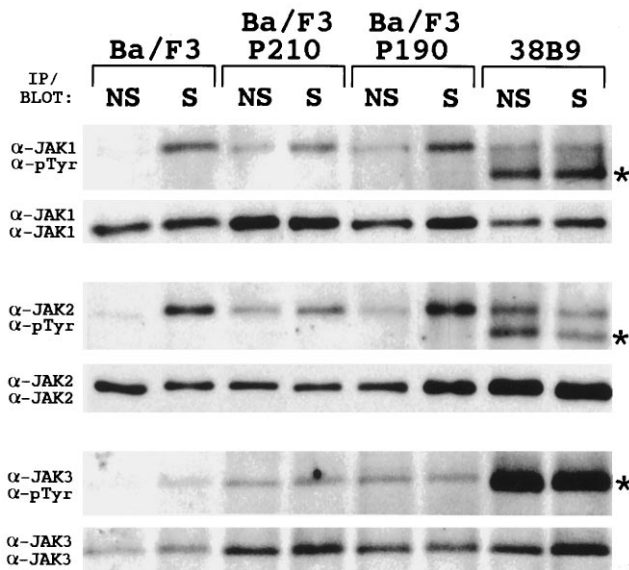
Bcr/Abl Activates Multiple STATs

FIG. 6. **Tyrosine phosphorylation of JAK family members in cells expressing activated Abl proteins.** Parental, P210- or P190-transformed Ba/F3 cells, and 38B9 cells were starved of serum and growth factor (*NS*) and stimulated (*S*) with IL-3 as described. JAK1 (*upper panel*), JAK2 (*middle panel*), or JAK3 (*lower panel*) was immunoprecipitated and analyzed with anti-phosphotyrosine antibody (*top*) or with the respective anti-JAK antibody (*bottom*). In 38B9 cells, the co-immunoprecipitation of tyrosine-phosphorylated P120[v-abl] with JAKs 1–3 was confirmed by blotting with anti-Abl antibody, and its position is indicated by the asterisk (*). Because of the similarity in molecular mass, JAK3 (125 kDa) and P120[v-abl] co-migrated as a doublet.

FIG. 7. **The effect of dominant-negative JAK2 mutants on STAT5 and JAK activation in parental and P210-transformed Ba/F3 cells.** JAK2 (*upper panels*) and STAT5 (*lower panels*) were immunoprecipitated from serum and growth factor starved (*NS*) or IL-3 stimulated (*S*) parental or P210-transformed Ba/F3 clones expressing high levels of dominant-negative JAK2 mutants, and analyzed by anti-phosphotyrosine (*top*) or anti-JAK or anti-STAT antibody (*bottom*). DNJAKΔ829 denotes the JAK2 mutant containing a carboxyl-terminal truncation deleting the entire kinase domain, including the autophosphorylation site. The DNJAK/DK-HA mutant contains two point mutations in a conserved motif within the JAK2 kinase domain. The truncation mutant DNJAK/Δ829 migrated at approximately 100 kDa (*lower arrow*) and, as expected, demonstrated no appreciable tyrosine phosphorylation (data not shown). The DNJAK/DK-HA mutant migrated just above the JAK2 protein (*upper arrow*); its identity was confirmed by blotting with an anti-HA epitope antibody (data not shown). The Ba/F3-P210 DNJAK-DK-HA mutant was expressed at very high levels and exhibited some low level tyrosine phosphorylation, likely due to the preservation of carboxyl-terminal tyrosine residues (19). The position of wild-type JAK2 is indicated at the asterisk (*). Parental and Ba/F3-P210 cells are also shown as controls for the JAK and STAT5 tyrosine phosphorylation induced by growth factor stimulation.

yl-terminal truncation deleting the entire kinase domain, including the autophosphorylation site (DNJAK/Δ829) (19). In the cell line DAER, both mutants inhibit the erythropoietin- and IL-3-dependent activation of JAK2, abolish erythropoietin-induced proliferation, and attenuate the mitogenic response to IL-3 (19). Parental and P210-transformed Ba/F3 clones expressing high levels of DNJAK were starved of serum and growth factor and then stimulated with IL-3. Both DNJAK mutants blocked the IL-3-induced tyrosine phosphorylation of JAK2 and STAT5 in parental Ba/F3 cells (Fig. 7); a similar inhibition of IL-3 induced JAK1 tyrosine phosphorylation was also observed (data not shown). In Ba/F3-P210 cells, both mutants also abrogated the IL-3-dependent increase in JAK2 and STAT5 tyrosine phosphorylation but did not affect the prominent activation of STAT5 or the low level JAK2 tyrosine phosphorylation observed in the absence of IL-3, despite somewhat higher DNJAK expression in these cells. There was no evidence for a compensatory increase in JAK1 or 3 tyrosine phosphorylation in Ba/F3-P210 cells expressing either of the DNJAK mutants (data not shown).

DISCUSSION

We have demonstrated constitutive tyrosine phosphorylation of STATs 1, 3, and 5 in Ba/F3 cells transformed to growth factor independence by both P210 and P190[BCR/ABL]. Tyrosine phosphorylation of STAT5 by Bcr/Abl was the most pronounced, with its level of constitutive activation approaching the maximum achieved with IL-3 stimulation. STAT5 activation was a consistent finding in human Ph-positive leukemia cell lines, but not in Ph-negative myeloid and lymphoid leukemia cells, suggesting that STAT activation may be important in the pathogenesis of human Ph-positive leukemias. Consistent with this, others have recently found activation of STATs 1 and 5 in a murine myeloid cell line transformed by P210[BCR/ABL] (32). Interestingly, we have found that P190 differed from P210
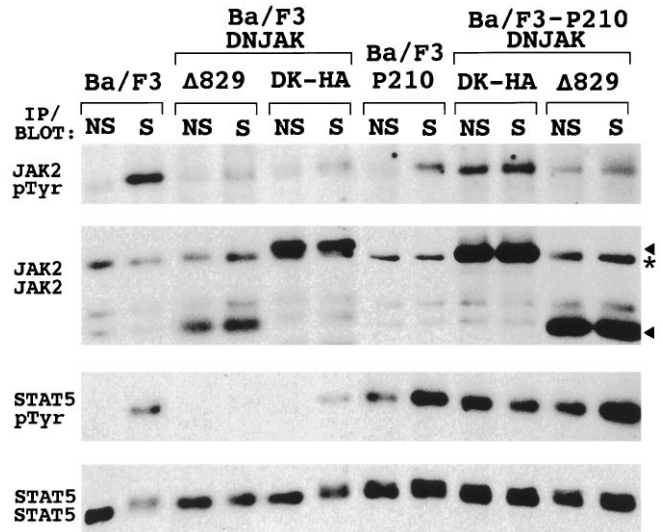
by its prominent tyrosine phosphorylation of STAT6. Bcr/Abl also induced STAT DNA binding activity, with activation of STATs 1, 3, and 5; STAT5 accounted for the majority of the GAS binding activity by antibody supershift analysis. P190-transformed Ba/F3 cells exhibited a distinct GAS binding complex composed of both STAT5 and STAT6 DNA binding activity. In addition, P190[BCR/ABL], but not P210, also induced prominent binding to an N4 probe that distinguished STAT6 from STAT5. The pattern of STAT DNA binding activity therefore correlated well with the tyrosine phosphorylation of STATs.

In addition to prominent activation of STAT5, we also found low level tyrosine phosphorylation of JAKs 1, 2, and 3 in Bcr/Abl-transformed Ba/F3 cells. However, there was no evidence of complex formation between Bcr/Abl and JAK family members under conditions in which co-immunoprecipitation of P120[v-abl] and JAKs (10) and of P210 and Grb2 (33) (data not shown) were readily detected. Therefore, the low level constitutive activation of JAKs by Bcr/Abl does not require stable complex formation. The fact that v-Abl differs from Bcr/Abl by the presence of retroviral Gag sequences and the absence of the Src homology domain 3 and Bcr suggests that the interaction of v-Abl and JAKs is mediated by Gag or is inhibited by Src homology domain 3 or Bcr.

There are several possible mechanisms by which Bcr/Abl might activate STAT family members. One possibility is that Bcr/Abl-transformed Ba/F3 cells secrete IL-3 or another cyto-

kine or growth factor, resulting in STAT activation by an autocrine mechanism. Several observations argue against this model. Although Bcr/Abl is known to induce cytokine secretion in some cell types (6, 34), concentrated conditioned medium from Ba/F3-P210 cells is not capable of supporting growth, delaying apoptosis (11), or inducing tyrosine phosphorylation of STAT5 (data not shown) in parental Ba/F3 cells. Furthermore, the rapid activation of STAT5 by the temperature-sensitive P210 mutant is inconsistent with the induction of cytokine gene expression by Bcr/Abl. Also, dominant-negative JAK2 mutants would be expected to interfere with IL-3 (βc) receptor-dependent STAT activation, and these mutants had no effect on the constitutive activation of STAT5 by Bcr/Abl.

Another possibility is that the activation of JAK family members by Bcr/Abl, albeit at low levels, is sufficient to account for the observed constitutive STAT activation. The dominant-negative JAK experiments were not able to exclude this possibility because although they did block the IL-3-dependent activation of JAK2 (and JAK1; data not shown), they did not eliminate the low level constitutive JAK tyrosine phosphorylation associated with P210 expression, suggesting that their dominant-negative effect is due to competition with endogenous JAKs for βc-binding sites. There are several reasons why the activation of STATs by Bcr/Abl is likely to be JAK-independent. First, there was discordance between the levels of constitutive JAK and STAT tyrosine phosphorylation in Ba/F3 cells transformed by Bcr/Abl. That is, the extent of STAT5 activation in Bcr/Abl-transformed Ba/F3 cells was similar to the maximum achieved by IL-3 stimulation, whereas JAK activation by Bcr/Abl was considerably lower. Also, in experiments with exponentially growing cells, parental and P210-transformed Ba/F3 exhibited similar levels of JAK2 tyrosine phosphorylation, despite much higher levels of STAT1 and 5 activation (data not shown). Finally, Ba/F3 cells transformed by P210 and P190 had identical patterns of JAK tyrosine phosphorylation, yet differed in their activation of STAT6. A less likely possibility is that another signal transduction pathway may be differentially activated by P210 and P190, resulting in tyrosine phosphorylation of STAT family members. Further experiments would be necessary to determine whether any non-JAK tyrosine kinase downstream of Bcr/Abl is required for the activation of STAT family members.

Overall, our findings are most consistent with a mechanism by which Bcr/Abl activates STAT family members directly. This model would account for the fact that P210 and P190 differed in their activation of STAT6, despite similar profiles of JAK tyrosine phosphorylation. It would also explain why dominant-negative JAK2 mutants did not block the constitutive activation of STAT5 by Bcr/Abl, yet were capable of interfering with IL-3-dependent STAT5 tyrosine phosphorylation. There was no evidence for the co-immunoprecipitation of Bcr/Abl with any STAT family member (data not shown); therefore, stable complex formation is not required for Bcr/Abl to activate STATs. Furthermore, STAT activation did not require the Src homology domain 2 or Grb2-binding functions of Bcr/Abl (data not shown).

The activation of specific STAT family members may play an important role in Bcr/Abl-mediated transformation and leukemogenesis. It is intriguing that Bcr/Abl possesses both the ability to transform hematopoietic factor-dependent cell lines to growth factor independence and to strongly activate STAT5, which has been implicated in cytokine receptor signaling pathways such as IL-3, GM-CSF, and erythropoietin (9, 25). Therefore, some of the effects of Bcr/Abl on proliferation might be explained by its ability to substitute for cytokine-induced JAK/STAT activation. However, the role of STATs in proliferative responses is controversial. Mutation of STAT5 binding sites on the erythropoietin receptor and a dominant-negative STAT5 mutant have been reported to inhibit erythropoietin- and IL-3-dependent growth, respectively (35, 36). Furthermore, in *Drosophila*, a dominant gain-of-function mutation (*tumorous-lethal*) (37, 38) in the JAK homolog *hopscotch* (39) has been shown to induce a leukemia-like illness in flies, which is suppressed by a loss-of-function mutation in the STAT homolog *marelle* (40, 41). In contrast, others have described mutations in cytokine receptors that dissociate JAK and STAT activation from proliferative signals (42). In parental Ba/F3 cells, STAT5 may contribute to mitogenesis but is not absolutely required because DNJAK expression had only a modest inhibitory effect on growth rate (data not shown). In addition, activation of Ras, which is likely to be independent of STAT activation, is required for the proliferation of factor-dependent hematopoietic cell lines (43) (data not shown). Therefore, STAT activation by Bcr/Abl may contribute to proliferation, but its effect may not be apparent until other mitogenic pathways, such as Ras, are inhibited. Alternatively, STATs could influence apoptosis or differentiation signals in Bcr/Abl-transformed cells. For example, interferon-α, an important activator of STAT1, has been used therapeutically in patients with CML and occasionally induces a cytogenetic remission. Perhaps the ratio of STAT1 and STAT5 tyrosine phosphorylation in Bcr/Abl-expressing cells is important in balancing proliferative, anti-apoptotic, and differentiation signals. Experiments with dominant-negative STAT mutants will be necessary to determine the role of STATs in Bcr/Abl transformation. STAT activation by Bcr/Abl might also contribute to the pathogenesis of human Ph$^+$ leukemia. We and others (32) collectively observed constitutive STAT activation in four Ph$^+$ cell lines but not in five human Ph$^-$ leukemia cell lines. We have also observed constitutive tyrosine phosphorylation of STAT5 in peripheral blood mononuclear cells from several patients with CML (data not shown). However, a recent report failed to find constitutive STAT activation in three Ph$^+$ acute lymphoblastic leukemia cell lines (44), suggesting that STAT activation may not be a universal finding in human Ph$^+$ leukemia. Studies of STAT activation in primary hematopoietic cells from patients with CML and Ph$^+$ acute lymphoblastic leukemia will help clarify the role of STAT activation in these diseases.

Although P210 and P190 both demonstrate high level activation of STAT5 and more modest activation of STATs 1 and 3, P190 differs by its prominent activation of STAT6. Interestingly, STAT6, activated by IL-4 signaling in lymphoid cells (18, 29, 45), was also constitutively tyrosine phosphorylated in cells transformed by P120 (10) and P160^{v-abl} (data not shown), which share with P190$^{BCR/ABL}$ the propensity for lymphoid transformation. One explanation for the difference in STAT6 activation might involve the greater intrinsic tyrosine kinase activity of P190 and v-Abl compared with P210 (11, 46). However, other factors must also influence Bcr/Abl and v-Abl STAT specificity, because the level of STAT5 tyrosine phosphorylation induced by different activated Abl proteins did not parallel their intrinsic tyrosine kinase activity. The activation of STAT6 by P190 but not by P210 represents one of the first demonstrations of a difference in substrate specificity between these chimeric fusion proteins; whether the activation of STAT6-induced genes explains the distinct leukemic phenotypes induced by P190 and P210$^{BCR/ABL}$ awaits further investigation.

Bcr/Abl Activates Multiple STATs

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