STAT5 Activation Is Required for Interleukin-9-dependent Growth and Transformation of Lymphoid Cells

Jean-Baptiste Demoulin, Catherine Uyttenhove, Diane Lejeune, Alice Mui, Bernd Groner, and Jean-Christophe Renaud

ABSTRACT

Interleukin-9 (IL-9) is a growth factor for T cells and various hematopoietic and lymphoid tumor cells. IL-9 signaling involves activation of Janus kinase (JAK)1 and JAK3 kinases, and signal transducer and activator of transcription (STAT)1, STAT3 and STAT5. Using a dominant negative form of STAT5 (STAT5a), we demonstrated that this factor is an important mediator of IL-9-dependent Ba/F3 cell growth. Mutation of the STAT binding site of the IL-9 receptor (tyr116phe) results in an important decrease in STAT activation and inhibition of proliferation in the presence of IL-9. A small number of cells escape this inhibition, and IL-9-dependent cell lines could be derived. The selected cells required activation of STAT5 for growth, which was blocked by STAT5a expression and enhanced by overexpression of wild-type STAT5. In contrast to parental cells, Ba/F3-Phe116 cells growing in the presence of IL-9 further progress to cytokine-independent tumorigenic clones. These tumorigenic clones exhibited a strong cytokine-independent activation of JAK1 and STAT5, which most likely supports their proliferation. Transfection of a constitutively activated variant of STAT5 promoted the growth of wild-type Ba/F3 cells in the absence of cytokine. Finally, the expression of the proto-oncogene pim-1 was correlated with STAT5 activation and cell growth. Our data suggest that STAT5 is an important mediator of IL-9-driven proliferation and that dysregulation of STAT5 activation favors tumorigenesis of lymphoid cells.

INTRODUCTION

Abnormal cytokine production accompanies the onset of certain lymphomas and leukemias (1). A large number of such soluble factors, including IL-9, have been implicated in the growth and survival of hematopoietic tumors. IL-9 was first characterized as a T-cell growth factor, but it has little activity on normal T cells, which respond to IL-9 only after long-term activation (2, 3). In contrast, transformed lymphocytes, particularly freshly isolated murine lymphomas, proliferate on IL-9 stimulation (4). In line with these observations, IL-9 transgenic mice, which express large amounts of IL-9 in most organs, frequently develop T lymphomas and are highly susceptible to mutagenic treatment (5). Autocrine or paracrine IL-9 may also stimulate the growth of human acute myeloid leukemias and Hodgkin’s lymphomas, as well as some human T-cell lymphotrophic virus-1-transformed cell lines (6–10).

IL-9 binds to a receptor comprising a specific chain (IL-9R) and the γc chain, which is shared by receptors for IL-2, IL-4, IL-7, IL-9, and IL-15 (11). IL-9 effects are mediated through the activation of JAK1 and JAK3 tyrosine kinases and STAT transcription factors, namely STAT1, STAT3, and STAT5 (12–15). Activation of the JAK-STAT pathway by cytokines has been studied extensively (16). On ligand binding to hematopoietic receptors, JAK kinases are activated and phosphorylate the cytoplasmic part of the receptor, creating phosphotyrosine docking sites for STAT factors. Recruited STATs are subsequently phosphorylated by JAKs, dissociate from the receptor, and form stable dimers that migrate into the nucleus, where they bind to promoter sites and regulate the expression of genes (16). A transactivation domain has been located in the COOH terminus of most STATs. Deletion of this domain results in a dominant negative phenotype (17).

STATs are active players in malignant transformation. Fusion proteins generated by chromosomal translocation, such as BCR-ABL or TEL-JAK2, and viral oncogenes, for instance v-src, HBx, and v-Eyk, are able to activate STATs (16, 18). STAT3 is required for transformation of NIH3T3 cells by v-SRC (19, 20). A constitutively active STAT3 mutant was shown to transform fibroblasts in vitro (21). Many human leukemias and lymphomas are associated with constitutive activation of the JAK-STAT pathway (22–24). In most cases, however, the mechanism that underlies STAT activation in these tumors is unknown.

Apoptosis inhibition by STAT3 and STAT5 is well documented, and it is likely that it accounts for their role in oncogenesis (14, 25). The effect of STATs on proliferation is more complex. On the one hand, STAT1, STAT3 and STAT5 have been shown to regulate cell cycle inhibitors, such as p21wti, resulting in cell growth inhibition and differentiation (26, 27). On the other hand, STAT3 and STAT5 play a role in proliferation induced by IL-6 and IL-3, respectively (17, 28). Moreover, hematopoietic cells and T-lymphocytes from STAT5-deficient mice exhibit a decreased response to growth stimuli (29). Two recent studies have suggested that STATs simultaneously regulate genes that stimulate and inhibit cell growth, the phenotypic outcome depending on the intensity and duration of the expression of both types of genes (26, 28).

We have reported that STAT activation by IL-9 correlates with the induction of proliferation and apoptosis inhibition (12, 14). Here, we provide direct evidence that STAT5 plays an important role in IL-9-dependent growth and the malignant transformation of lymphoid cells.

MATERIALS AND METHODS

Cell Culture and Transfections. The T helper cell line TS1 and the pro-B cell line Ba/F3 were cultured as described in the presence of IL-9 or IL-3, respectively (100 units/ml; Refs. 12, 30). IL-3 was produced by transfected CHO cells (a gift from A. Burgess, Ludwig Institute, Melbourne, Australia). Recombinant human IL-9 was produced in the baculovirus system and was purified as described previously (12).

Wild-type and mutated human IL-9R cDNAs were inserted into either the pEPhos/puro plasmid (12) or pE/Myc/ctyto plasmid (Invitrogen, Carlsbad, CA), which contain a resistance gene to puromycin or to neomycin, respectively.
tTA - STAT5Δ

A

Tetracyclin

IL9

STAT5wt

STAT5Δ

B

Tetracyclin

IL9

C

Growth index

Time (days)

Tetracyclin

Δ

F

Growth index

Time (days)

Tetracyclin

Δ

Fig. 1. A dominant negative STAT5 inhibits IL-9-dependent cell growth. A, human IL-9R was transfected into Ba/F3 cells, which express a dominant negative form of STAT5, whose expression is controlled by the tetracyclin-sensitive tTA regulator (17). Induction of STAT5Δ expression 48 h after tetracyclin removal was monitored by Western blot with anti-STAT5 antibodies (Santa Cruz) directed against the NH2 terminus of the protein. B, the same cells were cultured for 48 h in the presence of IL-3 with or without tetracyclin. IL-3 was then removed for 8 h, and cells were stimulated with IL-9 (both at 500 units/ml) or with control medium for 30 min. Nuclear extracts were prepared and analyzed by EMSA with a labeled GRR probe. Anti-STAT antibodies were added to produce super-shifts. C, cells (105/3 ml) were cultured in the presence of IL-9 (300 units/ml) with (●) or without (□) tetracyclin (1 μg/ml). The number of viable cells was determined every 2 days by trypsin blue exclusion. Cells were diluted in fresh medium before confluence (107/ml). Growth indices were calculated as the ratio between the cell number at a given time and the initial cell number. D, E, F, Ba/F3-tTA-STAT5Δ-IL-9R cells overexpressing wild-type STAT5 were used as a control and treated as in A, B, and C (17). For each experiment, three independent clones of each type were analyzed, with similar results.
experiments provide direct evidences that STAT5 is an important mediator of IL-9-dependent cell growth.

Selection of Ba/F3-Phe116 Cells Growing in the Presence of IL-9. To test whether proliferation in response to IL-9 is absolutely dependent on STAT activation, we selected IL-9-dependent Ba/F3 cells transfected with a Phe116 receptor. This IL-9R is mutated in the unique STAT-binding site (tyrosine 116). The majority of Ba/F3 cells expressing the mutant receptor were unable to proliferate on IL-9 stimulation (12). After extended times of culture, we observed that some cells survived and eventually grew in the presence of IL-9 (Fig. 2A). We called these selected cells Ba/F3-Phe116/9. In the presence of high IL-9 concentrations (>100 units/ml), they proliferated at the same rate as Ba/F3-IL-9R (Fig. 2B). However, they were unable to grow in low IL-9 concentrations (1–10 units/ml, corresponding to 0.1–1 ng/ml). This selection was reproduced with cell lines and clones derived from three independent transfections.

Similar results were also obtained with cells expressing shorter IL-9R variants, truncated before tyrosine 116, such as IC115 or IC98, but not IC44 (which retains 115, 98, and 44 cytoplasmic amino acids, respectively; Fig. 2C). The 44–98 IL-9R domain is involved in the activation of JAK kinases (12, 13). As summary, it was possible to select Ba/F3 cell clones that expressed a receptor lacking the STAT-binding site, and nevertheless proliferated in the presence of IL-9.

Proliferation of Ba/F3-Phe116/9 Cells Depends on Residual STAT5 Activation. We next tested whether the selection processes affected STAT activation. Mutation of IL-9R tyrosine 116 into phenylalanine reduces STAT activation by IL-9 to a very low level (Fig. 3; Refs. 12, 15). A weak STAT5 activation was still detectable, however. After selection for IL-9-dependent growth, it was increased (Fig. 3, right). Similar results were obtained with three independent polyclonal Ba/F3-Phe116 cell populations and with three clones. The expression of IL-9R and STAT5 proteins was not affected by the selection process (not shown).

To test whether the residual STAT5 activation was involved in proliferation or if this effect depended on another signaling pathway, we used Ba/F3-STAT5ΔA transfected with Phe116. In these cells, STAT5ΔA completely blocked the activation of STAT5 and the selection of IL-9-responsive cells at high IL-9 concentrations for long observation periods (Fig. 4). As observed for the wild-type receptor, expression of a large amount of wild-type STAT5 restored—and even increased—IL-9 responsiveness (Fig. 4C). These data indicated that proliferation of Ba/F3-Phe116/9 cells requires STAT5 activation.

Cytokine-independent Tumorigenic Clones Can Be Derived from Selected Cell Lines. Ba/F3 is an immortalized pro-B-cell line that can be transformed into cytokine-independent tumorigenic cells by transfection of oncogenes such as an activated ras (34). Ba/F3 transformation does not occur spontaneously. By contrast, autonomously growing clones could be obtained with a significant frequency from Ba/F3-Phe116/9 and other IL-9-adapted cell lines expressing a truncated receptor (Table 1). Moreover, no autonomous clones were obtained from Ba/F3-IL-9R cells cultured in IL-9 for 1 month, which showed that the emergence of autonomous clones was linked to the selection process, and not to normal culture with IL-9. Autonomous clones were tumorigenic in vivo when injected into immunodeficient SCID mice (Fig. 5), whereas Ba/F3-IL-9R, Ba/F3-Phe116, and Ba/F3-Phe116/9 cell lines were not. Similar results were obtained in irradiated BALB/c mice (not shown). These experiments indicate that the selection of IL-9-responsive Ba/F3-Phe116/9 cells is correlated with the acquisition of a pretransformed phenotype.

Autonomously growing clones can be obtained from various cytokine-dependent cell lines. In some cases, IL-3 autocrine loops have been demonstrated (35). However, we failed to find evidence of an autocrine loop in Ba/F3 autonomous clones: (a) cell supernatant did not support the growth of factor-dependent cells sensitive to IL-2, IL-3, IL-4, or IL-9; (b) coculture of autonomous and parental dependent cells did not result in the proliferation of the latter; and (c) reverse transcription-PCR analysis failed to amplify any IL-3 and IL-9 mRNA from most clones (data not shown).
Constitutive Activation of STAT5 and JAK1 in Tumorigenic Clones. We next tested whether STAT5 was activated in autonomously growing Ba/F3 cells. In EMSA experiments, strong constitutive activation of STAT5 was observed in tumorigenic Ba/F3 clones (Fig. 6A). A recent report (36) suggests that constitutive activation of STAT5 in Ba/F3 cells, induced by transfecting a constitutively activated STAT5 mutant, is enough to promote proliferation in the presence of serum. We obtained similar results with a construct encoding a fusion protein composed of STAT5Δ, the transactivator domain of VP16 and the kinase domain of JAK2. This STAT5/VP16/JAK2 protein specifically mimics STAT5 activation in various cells including Ba/F3 (Fig. 6B; Refs 14, 31) and was able to support the growth of the Ba/F3 cells in the absence of cytokine (Fig. 6C). Thus, the constitutive activation of STAT5 observed in autonomous clones is
sufficient to account for the autonomous phenotype of these cells. Incidentally, neither autonomous clones nor Ba/F3-STAT5/VP16/JAK2 cells were able to grow in the absence of serum (data not shown).

In contrast to Ba/F3 cells, several cytokine-dependent cell lines, such as TS1, spontaneously give rise to autonomous tumorigenic clones (at a frequency of $10^{-2}$; Ref. 3). TS1 is an IL-9-dependent cell line derived from T-helper cells grown in the presence of keyhole limpet hemocyanin antigen and antigen-presenting cells (30). We tested the presence of activated STATs in four TS1 autonomous clones and observed a constitutive activation of STATs, including STAT5 (Fig. 6D). This is in line with our results in Ba/F3 cells. Thus, in the TS1 system, the constitutive activation of STATs also correlated with autonomous growth and tumorigenesis.

STAT activation in transformed cells has been associated with JAK tyrosine kinase activation (16, 24). In autonomous clones, we found that JAK1 was constitutively phosphorylated on tyrosines (Fig. 7). JAK2 phosphorylation was not detectable.

Pim-1 Expression Correlates with STAT Activation and Proliferation. Several STAT5-target genes, such as cyclin-D and pim-1, are related to proliferation (26, 37). The enforced expression of pim-1 has been shown to promote Ba/F3 cell growth in vitro and to favor tumor development in vivo (26, 38). Because pim-1 is up-regulated by IL-9, we analyzed its RNA levels in IL-9-adapted cells and autonomous clones by Northern blotting (14). Fig. 8 shows that the Phe116 mutation dramatically affected pim-1 induction by IL-9, as described previously (14). In IL-9-responsive Ba/F3-Phe116/9 cells, we observed significantly increased effects of IL-9 on pim-1 expression. As expected, pim-1 was constitutively expressed in tumorigenic clones derived from Ba/F3-Phe116/9 cells (Fig. 8, right). pim-1 was also present in autonomously growing Ba/F3 cells obtained by transfecting STAT5/VP16/JAK2 but not in those transformed with an activated mutant of M-ras (34). Thus, the expression of pim-1 correlated with STAT5 activation and may be a mediator of IL-9-dependent and autonomous proliferation of Ba/F3 cells.

DISCUSSION

Two activities of IL-9 may account for the tumor-promoting activity of this cytokine: apoptosis inhibition and proliferation (2). In a recent report, we showed that STAT5 activation was sufficient to block apoptosis (14). Here, we demonstrate the role of STAT5 in proliferation, using a dominant negative form of STAT5 which sig-

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**Table 1**

<table>
<thead>
<tr>
<th>Ba/F3 cell line</th>
<th>No. autonomous clones</th>
<th>No. of cells tested (million)</th>
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<tr>
<td>IL-9R/9b</td>
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<td>45</td>
</tr>
<tr>
<td>Phe116</td>
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<td>Phe116/9b</td>
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</tr>
<tr>
<td>IC115/9b</td>
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<tr>
<td>IC98</td>
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<td>60</td>
</tr>
<tr>
<td>IC98/9</td>
<td>&gt;192</td>
<td>10</td>
</tr>
</tbody>
</table>

*Cells were washed to remove ILs and transferred to multiwell plates at 50,000 cells per well. Autonomous clones were counted after 2 weeks.*

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**Fig. 5.** Autonomously growing clones were tumorigenic in vivo. SCID mice were injected with cytokine-dependent cell lines (Ba/F3-IL-9R, -Phe116, or -Phe116/9) or with autonomous clones (A1, A4). Mouse survival was followed for 3 months. This experiment was reproduced in SCID and irradiated BALB/c mice, with similar results.

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**Fig. 6.** Constitutive STAT5 activation in autonomously growing clones. Nuclear extracts were prepared from autonomous clones derived from Ba/F3 (A), Ba/F3-STAT5/VP16/JAK2 (B), or TS1 cells (D). STAT activation was analyzed by EMSA as described in Fig. 2. As controls, we used the parental factor-dependent cell lines (Ba/F3 or TS1) that were washed, starved for 8 h, and then stimulated for 30 min with IL-3 (100 units/ml), murine IL-9 (100 units/ml) or control medium. When indicated, a super-shift was induced with anti-STAT5 antibodies. C, proliferation of Ba/F3, Ba/F3-A4 and Ba/F3-STAT5/VP16/JAK2 cells was measured as in Fig. 1 in the presence IL-3 (●) or in the absence of cytokine (○). SDs for triplicate cultures are shown when they are significant.
lysates (10^5 cells/lane) by Western blot with anti-phospho-JAK2 antiserum. JAK2 phosphorylation was analyzed in crude antibody (4G10, UBI). As a control, the blot was reprobed with the anti-JAK1 antiserum (UBI), as described previously (40). JAK1 phosphorylation was tested by Western blot with an antiphosphotyrosine antibody (UBI, Biosource), or anti-JAK2 (UBI). As a positive control, we used Ba/F3-A4 cells that were stimulated with IL-3 for 5 min.

Fig. 7. JAK1 activation in tumorigenic clones. JAK1 was immunoprecipitated from the indicated cell (2.5 x 10^5; autonomous clone A1, A4, or cytokine-starved wild type Ba/F3 cells), using an anti-JAK1 antiserum (UBI), as described previously (40). JAK1 phosphorylation was tested by Western blot with an antiphosphotyrosine antibody (4G10, UBI). As a control, the blot was reprobed with the anti-JAK1 antiserum. JAK2 phosphorylation was analyzed in crude lysates (10^5 cells/lane) by Western blot with anti-phospho-JAK2 antibodies (Biosource), or anti-JAK2 (UBI). As a positive control, we used Ba/F3-A4 cells that were stimulated with IL-3 for 5 min.

Fig. 8. pim-1 expression correlates with STAT5 activation. Ba/F3-IL-9R, -Phe116, or -Phe116/9 cells were washed, cultured in the absence of cytokine for 8 h, and then stimulated with IL-3, IL-9 (500 units/ml), or control medium for 2 h. RNA was extracted and analyzed by Northern blot with a pim-1 probe. We also analyzed RNA from autonomous clones cultured in cytokine-free medium (A1, A2, A4, and A6) and RNA from Ba/F3 cells transfected with STAT5/VP16/JAK2 or with an activated form of M-ras (Q71L mutant; Ref. 34).

Fig. 9. pim-1 expression correlates with STAT5 activation. Ba/F3-IL-9R, -Phe116, or -Phe116/9 cells were washed, cultured in the absence of cytokine for 8 h, and then stimulated with IL-3, IL-9 (500 units/ml), or control medium for 2 h. RNA was extracted and analyzed by Northern blot with a pim-1 probe. We also analyzed RNA from autonomous clones cultured in cytokine-free medium (A1, A2, A4, and A6) and RNA from Ba/F3 cells transfected with STAT5/VP16/JAK2 or with an activated form of M-ras (Q71L mutant; Ref. 34).

Fig. 10. pim-1 expression correlates with STAT5 activation. Ba/F3-IL-9R, -Phe116, or -Phe116/9 cells were washed, cultured in the absence of cytokine for 8 h, and then stimulated with IL-3, IL-9 (500 units/ml), or control medium for 2 h. RNA was extracted and analyzed by Northern blot with a pim-1 probe. We also analyzed RNA from autonomous clones cultured in cytokine-free medium (A1, A2, A4, and A6) and RNA from Ba/F3 cells transfected with STAT5/VP16/JAK2 or with an activated form of M-ras (Q71L mutant; Ref. 34).
important mediators of cell growth in response to IL-9. Moreover, the mechanism of STAT5 activation may be affected during tumorigenesis, leading to a constitutive activation of this factor.

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