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Role of Signal Transducer and Activator of Transcription 5 in Nucleophosmin/Anaplastic Lymphoma Kinase-mediated Malignant Transformation of Lymphoid Cells[1]

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ABSTRACT

The *NPM/ALK* fusion gene, formed by the t(2;5) translocation in anaplastic large-cell lymphoma, encodes a M_r 75,000 hybrid protein that contains the amino-terminal portion of the nucleolar phosphoprotein nucleophosmin (NPM) joined to the entire cytoplasmic portion of the receptor tyrosine kinase anaplastic lymphoma kinase (ALK). *NPM/ALK* encodes a constitutively activated tyrosine kinase that belongs to the family of tyrosine kinases activated by chromosomal translocation. Our studies show that NPM/ALK, similar to other members of this family, activates signal transducer and activator of transcription 5 (STAT5) and that this activation is essential for lymphomagenesis. NPM/ALK-mediated activation of STAT5 was demonstrated by detection of: (*a*) constitutive tyrosine phosphorylation and enhanced DNA binding ability of STAT5 in NPM/ALK-transformed cells; and (*b*) NPM/ALK-dependent stimulation of STAT5-mediated transactivation of the β-casein promoter. Retroviral infection of NPM/ALK+ cells with a dominant-negative STAT5B mutant (STAT5-DNM) inhibited the antiapoptotic activity of NPM/ALK in growth factor and serum-free medium. In addition, STAT5-DNM inhibited proliferation and diminished the clonogenic properties of NPM/ALK-positive cells. Finally, SCID mice injected with NPM/ALK+ cells infected with a virus carrying STAT5-DNM survived significantly longer than mice inoculated with NPM/ALK+ cells infected with the empty virus. Necropsy identified a widespread ALK+ lymphoma in lymph nodes and liver of the affected animals. Together, our data indicate that NPM/ALK-induced activation of STAT5 may play an important role in NPM/ALK-mediated lymphomagenesis.

INTRODUCTION

Tyrosine kinases play an essential role in cell proliferation, apoptosis, differentiation, and malignant transformation (1). Tyrosine kinase enzymatic activity, which stimulates numerous signaling pathways, is tightly regulated by various components such as growth factors and/or stress factors that are responsible for turning on/off the kinase function. Constitutive activation of these kinases can lead to prolonged stimulation of the signaling pathways, resulting in neoplastic transformation.

Chromosomal translocations are responsible for the expression of abnormal fusion proteins that possess constitutive tyrosine kinase activity, *i.e.*, OTKs.[4] These translocations occur often in hematopoietic cells, leading to their transformation and the development of leukemias or lymphomas (2). The best characterized example of an OTK is the BCR/ABL fusion protein, a product of the t(9;22) chromosomal translocation (Philadelphia chromosome), which is responsible for the induction of chronic myelogenous leukemia and a subset of acute lymphocytic and myelogenous leukemia (3, 4). Other members of the BCR/ABL-related OTK family are: TEL/ABL (5), TEL/JAK2 (6), TEL/PDGFβR (7) and NPM/ALK (8). All of these OTKs have structural similarities, which include an NH_2-terminal domain responsible for constitutive oligomerization and activation of the associated tyrosine kinase of the COOH-terminal fusion partner. BCR/ABL, TEL/ABL, TEL/PDGFβR, and TEL/JAK2 induce acute and chronic leukemias usually of the myeloid lineage (2–7). In contrast, the NPM/ALK protein produced by the t(2;5) translocation is found in approximately two-thirds of the Ki(CD30)-positive ALCL (8–10). NPM is ubiquitously expressed and responsible for protein shuttling between the cytoplasm and the nucleus (11). ALK is a receptor tyrosine kinase physiologically expressed by neural tissues (12, 13). *NPM/ALK* encodes a M_r 75,000 fusion protein that contains the amino-terminal 117 residues of NPM joined to the entire cytoplasmic domain of ALK (8). The NPM/ALK fusion protein displays constitutive tyrosine kinase activity and is able to induce lymphomas in mice injected with bone marrow cells infected with retrovirus-containing *NPM/ALK* (14).

The mechanisms of NPM/ALK-mediated transformation are not fully understood. Although NPM/ALK can associate with and phosphorylate several adaptor proteins such as SHC, Grb2, and IRS-1 (15, 16), their role, if any, in NPM/ALK-induced transformation is uncertain. Recently, Bai *et al.* (17) demonstrated that NPM/ALK constitutively activates phospholipase C-γ to induce cell proliferation. In addition to stimulating proliferation, we (18) and others (19) have demonstrated that NPM/ALK protects the IL-3 dependent hematopoietic cell line BaF3 from apoptosis in growth factor-free conditions by activating PI3k/Akt pathway. Because it has been demonstrated previously that in addition to PI3k and Akt (20, 21), hematopoietic cells transformed by BCR/ABL required activation of the signal transducer and activator of STAT5 protein (22) to become growth factor-independent, we felt that examination of the potential role of STAT5 in NPM/ALK-mediated lymphomagenesis was warranted.

STAT5 is a member of a STAT signal transduction protein family, which, on tyrosine phosphorylation, dimerize, translocate to the nucleus, and bind to specific response elements in the promoters of target genes to induce their transcription (23). There are two STAT5 proteins: STAT5A and STAT5B, which are 95% identical in amino acid sequence and have both distinct and redundant roles in cytokine

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[4] The abbreviations used are: OTK, oncogenic tyrosine kinase; ALCL, anaplastic large-cell lymphoma; NPM, nucleophosmin; ALK, anaplastic lymphoma kinase; STAT5, signal transducer and activator of transcription 5; STAT5-DNM, dominant-negative STAT5B mutant; IL, interleukin; PI3k, phosphatidylinositol 3-kinase; FBS, fetal bovine serum; DNM, dominant-negative mutant.

responses (24–26). STAT5 proteins are activated by many growth factors (23), and their stimulation is regulated by tyrosine and, to a lesser degree, by serine phosphorylation (23). In addition, COOH-terminus truncated forms of STAT5 proteins described in different cell types (27) may act in a dominant-negative manner to regulate STAT5 activity. The findings regarding the biological consequences of STAT5 activation in hematopoietic cells are somewhat controversial; although results from STAT5A+B double knockout mice suggested that STAT5 proteins are expendable in the development and maintenance of normal hematopoiesis (24), the biological effects observed after acute inhibition of STAT5 by transient expression of the dominant-negative STAT5 mutant implicated STAT5 in the growth of hematopoietic cells (28–31). In addition, constitutive activation of STAT5 has been found in malignant lymphoma and leukemia cells (32, 33). Finally, growth of BCR/ABL- and TEL/JAK2-induced leukemias could be inhibited by the STAT5-DNM (6, 22, 34–36).

Here we show that STAT5 is constitutively activated by NPM/ALK and that it is essential for the growth factor-independence and lymphomagenesis mediated by NPM/ALK.

MATERIALS AND METHODS

Plasmids

Retroviral constructs pSRαMSVtkneo containing cDNAs encoding full-length NPM/ALK, the K210R kinase-defective NPM/ALK mutant, and full-length BCR/ABL have been described (14, 16, 22). The cDNAs for TEL/JAK2 and TEL/ABL were obtained from Dr. Gary Gilliland (Harvard Medical School, Boston, MA), and the cDNA for TEL/PDGFβR was from Dr. Martin Carroll (University of Pennsylvania, Philadelphia, PA). These cDNAs were subcloned into the pMSCV retroviral construct (37). The wild-type and the DNM of STAT5B (ΔSTAT5B, truncated after Tyr683) have been described previously (29).

Cells

The murine growth factor-dependent pro-B lymphoid cell line BaF3 was maintained in RPMI 1640 supplemented with 10% FBS and 15% WEHI-conditioned medium as a source of IL-3. BaF3 cell lines transfected with NPM/ALK (BaF3-NPM/ALK cells) or with empty virus (BaF3-neo) were obtained by electroporation of BaF3 parental cells with pSRαMSVtkneo-NPM/ALK or pSRαMSVtkneo plasmid, respectively, and selection in 800 μg/ml G418. Expression of NPM/ALK was confirmed by Western blot assay. BaF3 cells expressing TEL/JAK2 or TEL/ABL were obtained from Dr. Gary Gilliland (Harvard Medical School, Boston, MA), and cells expressing TEL/PDGFβR were from Dr. Martin Carroll (University of Pennsylvania, Philadelphia, PA) and these expressing BCR/ABL were from Dr. Richard Van Etten (Harvard Medical School, Boston, MA). BaF3 cell lines transfected with various OTKs were maintained in RPMI 1640 supplemented with 10% FBS and 15% WEHI-conditioned medium. Mouse CTL cell line CTLL-2, human chronic myelogenous leukemia cell line K562, and human acute promyelocytic leukemia cell line HL-60 were from the American Type Culture Collection (Manassas, VA). Human non-Hodgkin anaplastic large-cell T-lymphoma cell lines SUP-M2 and SU-DHL-1 were described previously (16). Human T-lymphoma cells SUP-M2 and SU-DHL-1, human K562, HL-60, and mouse CTLL-2 cell lines were all cultured in RPMI 1640 supplemented with 10–20% of FCS. In addition, mouse recombinant interleukin-2 was added at 10 units/ml for the culture of CTLL-2 cells. Pools of CTLL-2-NPM/ALK and CTLL-2-neo cell clones were established by transfecting parental cells with 10 μg of NPM/ALK cDNA construct (in pcDNA3) or empty pcDNA3 plasmid followed by selection in G418. The expression of NPM/ALK in selected CTLL-2 cells was tested by Western blot assay. For cell culture in the presence of tyrosine phosphatase inhibitor, sodium orthovanadate was added to cultures for 6 h at a final concentration of 0.1 mM. Tk$^-$ts13 hamster fibroblasts were cultured in DMEM supplemented with 10% FBS.

STAT5 Activation Assays

Cell lysates were obtained after 8-h starvation of the cells from growth factor and serum.

Tyrosine Phosphorylation. STAT5 proteins were immunoprecipitated with rabbit anti-STAT5 antibody that reacts with both A and B forms of STAT5 (C-17; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Immunoprecipitates were analyzed by SDS-PAGE and Western blot assays using anti-P-Tyr antibodies (4G10; Upstate Biotechnology, Lake Placid, NY, and PY20; Oncogene Research Products, Cambridge, MA), followed by anti-STAT5 antibody.

DNA Binding Capacity. The DNA binding activity of STAT5 was examined by electrophoretic mobility shift analysis as described previously (22, 38) using the FcγRI GAS motif as a probe.

Transactivation Activity. STAT5-mediated transactivation was assayed as described (22) using STAT5-responsive luciferase reporter construct containing a minimal β-casein promoter, the SV40 promoter, and the luciferase gene (β-casein-SV40-luc). The negative control reporter plasmid encoded only the SV40 promoter fused to the luciferase gene (*SV40-luc*). Briefly, Tk$^-$ts13 cells were transiently cotransfected by calcium phosphate with empty plasmid (control) or with plasmid-encoding indicated OTK, as well as the reporter plasmid. When OTK was cotransfected with the STAT5-DNM (or empty plasmid) the OTK:DNM (or empty) ratio = 1:4. A β-galactosidase plasmid was also transfected as a transfection efficiency control. A total of 20 μg of cDNA/10 cm plate was always used. Twenty four h after transfection, serum-free medium containing 0.1% BSA was added to the cells. Thirty six h later, luciferase was quantified by the Luciferase Assay System (Promega, Madison, WI). Transfection efficiency was normalized by measuring β-galactosidase activity. Transactivation units were calculated as a ratio of the counts from β-casein-SV40-luc to the counts from SV40-luc in particular groups.

Immunoprecipitation and Immunoblotting

Whole cell lysates were prepared from 2×10^7 cells in 1 ml of 1% Triton X-100 lysis buffer [30 mM HEPES (pH 7.4), 150 mM NaCl, 1% Triton X-100, 44 μg aprotinin/ml, 1.4 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, and 1 mM sodium orthovanadate; Ref. 16]. The lysates were first incubated with anti-STAT5B polyclonal antibody (Santa Cruz Biotechnology, Inc.) for 2 h at 4°C, then with protein A-Sepharose beads for an additional 1 h. The precipitated protein complex was separated by 10% SDS-PAGE and probed with anti-P-Tyr antibody (4G10) or anti-ALK (ALK#11) rabbit polyclonal antibody (13) followed by anti-STAT5b antibody.

Retroviral Infections

Infections were performed as described previously (22) with some modifications. To increase the concentration of the virus, BaF3-NPM/ALK cells were suspended in medium collected from the culture of Bosc23 packaging cells transfected with the appropriate constructs and cocultivated in the presence of IL-3 on a monolayer of freshly transfected Bosc23 cells. Bulk cultures obtained 72 h after infection were incubated for 24 h with 1 μg/ml of puromycin. After an additional 48 h, viable cells were isolated by centrifugation on Lympholyte M gradient medium (Cedarlane Laboratories Ltd., Hornby, Ontario, Canada) and used for experiments. Expression of the ΔSTAT5B mutant in transfected cells was confirmed by Western blot assay with use of anti-STAT5 antibody (N20; Oncogene Research Products), detecting the presence of the truncated (exogenous) and full-length (endogenous) forms of STAT5 (data not shown).

Apoptosis and Proliferation

Cells were incubated in the absence of IL-3 for 48 h. Viable cells were detected using trypan blue exclusion vital dye. The percentage of apoptotic cells was determined at the same time using the TACS1 Klenow *in situ* apoptosis detection kit (Trevigen, Inc., Gaithersburg, MD) according to the manufacturer's protocol. Cell cycle distribution was assayed by flow cytometry after staining of DNA with propidium iodide, as described (22). Clonogenic assay was performed in MethoCult H4230 semisolid medium (Stem Cell Technologies, Vancouver, Canada), as described (22), in the absence of IL-3. Colonies were counted after 7 days.

Tumorigenesis in Mice

SCID and BALB/c female mice (Taconic Farms, Inc., Germantown, NY) were injected i.v. with 10^6 or 5×10^6 BaF3-NPM/ALK cells, respectively, infected with ΔSTAT5B retrovirus or with empty retrovirus (15 SCID mice/group and 10 BALB/c mice/group). Visibly ill mice were sacrificed, and their organs were examined grossly and microscopically for the presence of malignant tumors, as described (21, 22, 38). In brief, tissue sections from femoral bone to evaluate bone marrow, lymph node, spleen, liver, lungs, kidneys, brain including meninges, testes, thymus, and any identified tumor masses were fixed in phosphate-buffered formalin and embedded in paraffin block. Two to three levels from each block were cut and stained with H&E. In addition, selected slides were stained immunohistochemically after heat-induced antigen retrieval using a standard streptavidin-biotin complex technique (Research Genetics, Huntsville, AL) and an antibody specific for ALK protein (DAKO, Carpinteria, CA) to confirm the tissue involvement by NPM/ALK lymphomatous cells.

RESULTS

NPM/ALK Activates STAT5. Tyrosine phosphorylation of STAT5 in IL-3-starved BaF3-neo cells and BaF3 cells expressing various OTKs was examined by Western blot analysis of anti-STAT5 A + B immunoprecipitates with anti-P-Tyr antibodies. In the absence of growth factor, BaF3 cells transfected with NPM/ALK or other members of the BCR/ABL-related OTK family, but not BaF3 cells transfected with empty vector, expressed tyrosine-phosphorylated STAT5 (Fig. 1*A*). To examine the ability of OTKs to stimulate the transactivation activity of STAT5, a reporter plasmid carrying luciferase cDNA under the control of the STAT5-regulated β-casein promoter was overexpressed in Tk⁻ts13 cells with the various OTKs or with empty plasmid (control), and the transactivation ability of STAT5 was measured by luciferase assay. All of the OTKs tested, including NPM/ALK, were able to induce transactivation of the β-casein promoter (Fig. 1*B*). To confirm that this effect is attributable to STAT5, in parallel experiments the DNM of STAT5 (ΔSTAT5B) was coexpressed together with the OTKs and the reporter construct. STAT5B-DNM inhibited almost completely the activation of luciferase induced by BCR/ABL, TEL/ABL, TEL/JAK2, and TEL/PDGFβR, and reduced by 2.5-fold transactivation triggered by NPM/ALK (Fig. 1*B*).

As shown in Fig. 2*A*, NPM/ALK and STAT5B were constitutively activated and physically associated in human T-cell lymphoma cell lines. These features could be fully appreciated after the lymphoma cells were precultured in the presence of the tyrosine phosphatase inhibitor, sodium orthovanadate. This finding suggests that the activated STAT5B is rapidly dephosphorylated in the NPM/ALK T-cell lymphoma cells. Similar results were obtained in NPM/ALK-transformed CTLL-2 cells. CTLL-2-neo cells require IL-2 to grow in culture, whereas introduction of NPM/ALK allowed these cells to grow in an IL-2-independent fashion (data not shown), a sign of cellular transformation. Accompanying this cytokine-independent growth was the constitutive phosphorylation and physical association of NPM/ALK and STAT5B detected in anti-STAT5B immunoprecipitates (Fig. 2*A*). As seen in human T-cell lymphoma cells, STAT5 activation was markedly enhanced by sodium orthovanadate preincubation *in vivo*. Interestingly, the NPM/ALK-STAT5B complex could be seen in anti-STAT5B but not in anti-NPM/ALK immunoprecipitates (data not shown), suggesting that the fragment of NPM/ALK used as an immunogen to obtain the antibody (amino acids 419–520; Ref. 13) may contain the STAT5B binding site.

NPM/ALK-dependent activation of STAT5 was additionally confirmed by showing that STAT5 possesses a DNA-binding activity in BaF3-NPM/ALK cells, but not in BaF3-neo cells, cultured in the absence of growth factor (Fig. 2*B*). In addition to stimulation of the DNA-binding ability of STAT5, we demonstrated that NPM/ALK-induced transactivation of the β-casein promoter is dependent on the kinase activity of NPM/ALK (Fig. 2*C*).

STAT5 Is Involved in NPM/ALK-mediated Growth Factor Independence. A DNM strategy was used to determine whether STAT5 plays a role in growth factor independence (susceptibility to apoptosis and proliferation potential) of NPM/ALK-transformed BaF3 cells. Terminal deoxynucleotidyl transferase (Tdt)-mediated nick end labeling assay revealed that BaF3-NPM/ALK cells, but not BaF3-neo cells, were protected from apoptosis induced by growth factor withdrawal (Fig. 3*A*). A time-dependent gradual increase in the percentage of apoptotic BaF3-NPM/ALK cells transfected with STAT5B-DNM was observed (Fig. 3*A*, *NPM/ALK panel*). After day 3 of culture, the percentage of apoptotic cells started to decrease (data not shown), indicating the possibility of activation of redundant signaling pathways responsible for the outgrowth of remaining cells resistant to STAT5B-DNM. Expression of STAT5-DNM facilitated apoptosis in BaF3-neo cells (Fig. 3*A*, *Control panel*).

The influence of STAT5B-DNM on the proliferation potential of BaF3-NPM/ALK cells and BaF3-neo cells was investigated by counting the number of living cells cultured in the absence or presence of IL-3, respectively (Fig. 3*B*). STAT5B-DNM inhibited growth factor-independent proliferation of BaF3-NPM/ALK cells during the first 2 days of culture (Fig. 3*B*, *NPM/ALK panel*). After that time cells started to regrow slowly, again suggesting that redundant mechanisms had been turned on. "Reactivation" of the growth ability of these cells was not attributable to the loss of expression of STAT5-DNM, as confirmed by Western blot assay (data not shown). STAT5-DNM exerted only modest effect on the proliferation of BaF3-neo cells cultured in the presence of IL-3 (Fig. 3*B*, *Control panel*). The levels of ΔSTAT5 protein were similar in BaF3-NPM/ALK cells and in

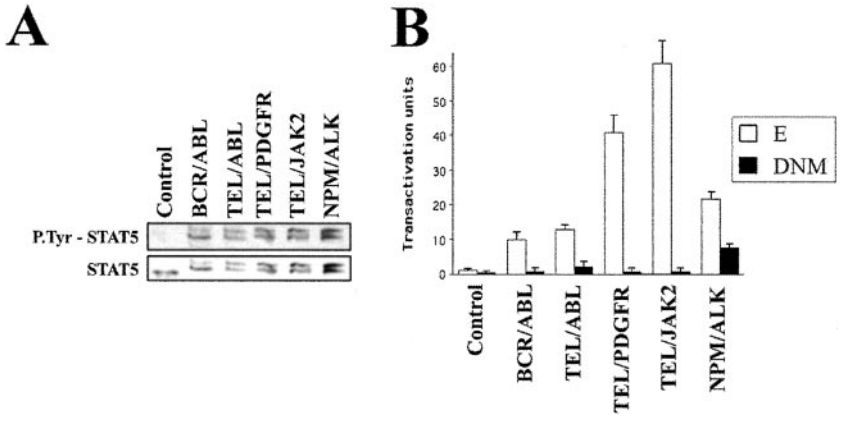

Fig. 1. NPM/ALK and other OTKs activate STAT5. BaF3-neo cells (*Control*) and BaF3 cells expressing various OTKs were starved from growth factors and serum for 8 h. Tyrosine phosphorylation of STAT5A and B was detected by Western blot assay with anti-P-Tyr antibodies of anti-STAT5 A and B immunoprecipitates (*panel A, top line*). The presence of STAT5 proteins in immunoprecipitates was confirmed by Western blotting with anti-STAT5 antibody (*panel A, bottom line*). STAT5 transactivation activity (*panel B*) was measured by luciferase assay in Tk⁻ts13 cells transiently transfected with the plasmid containing the indicated OTK or the empty plasmid (*Control*) along with the plasmid carrying STAT5-DNM or empty plasmid (*E*) and the plasmid encoding luciferase reporter gene driven by the STAT5-regulated β-casein promoter. Luciferase activity is expressed in arbitrary units. Results represent three independent experiments.

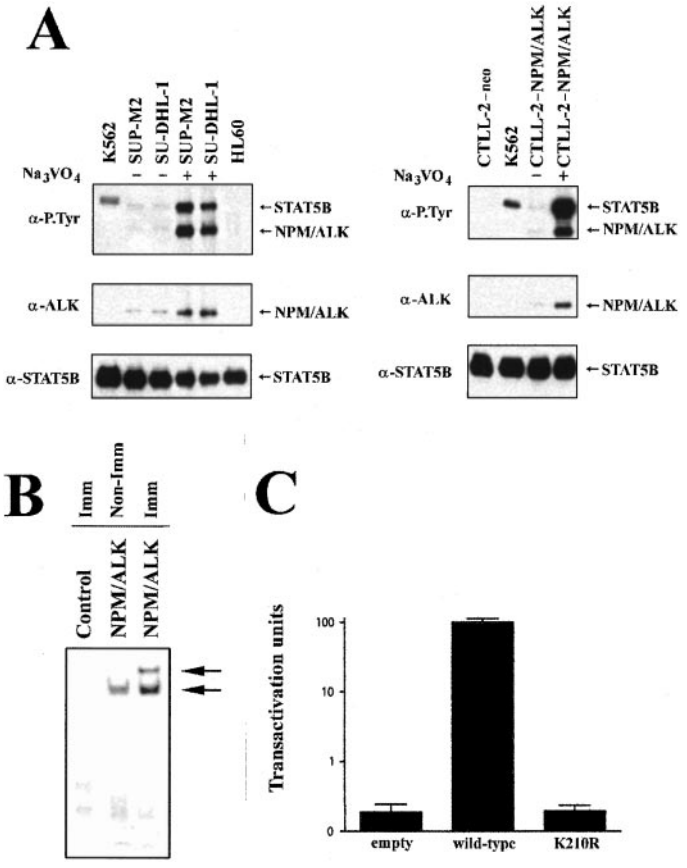

Fig. 2. Constitutive activation and association of STAT5 and NPM/ALK. *A*, phosphorylation and association of NPM/ALK and STAT5B were detected in anti-STAT5B immunoprecipitates by SDS-PAGE and Western blot assay. Phosphorylation of NPM/ALK and STAT5B in cells with (+) or without (−) the presence of sodium orthovanadate is shown. Immunoprecipitations of STAT5B protein from K562 and HL60 cells represent positive and negative controls, respectively. *B*, BaF3-neo cells (*Control*) and BaF3-NPM/ALK cells (*NPM/ALK*) were starved from growth factor and serum for 8 h before the STAT5 DNA binding activity was assayed by electrophoretic mobility shift analysis using the GAS sequence as a probe (*lower arrow*). Anti-STAT5 IgG (*Imm*) or nonimmunized IgG (*Non-Imm*) were added to the reaction mixture to detect STAT5-dependent supershift (*upper arrow*). *C*, ability of NPM/ALK kinase to induce STAT5-dependent transactivation was examined by luciferase assay in Tk$^-$ts13 cells transiently transfected with the reporter plasmid (see Fig. 1*B*, *legend*) and the empty plasmid (*empty*) or the plasmid containing the wild-type (*wild-type*) or the kinase-defective K210R mutant (*K210R*) of NPM/ALK. Luciferase activity is expressed in arbitrary units. Results represent three independent experiments.

BaF3-neo cells (data not shown). Taken together, this suggests that NPM/ALK-induced cell proliferation may be more dependent on STAT5 than that regulated by a growth factor (*e.g.*, IL-3).

To examine the effect of STAT5B-DNM in BaF3-NPM/ALK cells at the single-cell level, cells were infected with retroviruses carrying wild-type STAT5B, STAT5B-DNM, or with the empty virus, and plated in methylcellulose. The DNM of STAT5B dramatically reduced the clonogenic capability of BaF3-NPM/ALK cells in growth factor-free conditions, in comparison with cells infected with the wild-type STAT5 or with the empty virus (Fig. 3*C*). In addition, STAT5B-DNM reduced the size of colonies (data not shown).

Cell cycle analysis revealed that the presence of STAT5B-DNM caused accumulation of BaF3-NPM/ALK cells in a subdiploid peak (indicative of apoptosis) from 4 to 50%, reduced the percentage of cells in S + G_2-M phase from 48 to 26%, and increased the population of cells in G_0/G_1 phase from 52 to 74% (Fig. 3*D*). In contrast, transfection of the STAT5B wild type or the empty vector did not induce any significant changes in apoptosis and cell cycle distribution. These findings are in agreement with the results of apoptosis and proliferation assays.

STAT5 Is Required for the Tumorigenic Activity of BaF3-NPM/ALK Cells. To determine the role of STAT5 in NPM/ALK-mediated tumorigenesis, BaF3-NPM/ALK cells infected with the retrovirus carrying STAT5B-DNM or with the empty virus were inoculated into SCID mice or syngeneic BALB/c mice. All of the SCID mice injected with 10^6 BaF3-NPM/ALK + empty virus cells succumbed to neoplastic disease in 21–24 weeks (Fig. 4*A*, *left panel*) By contrast, inoculation of BaF3-NPM/ALK + STAT5B-DNM cells induced fatal disease in these mice after 35–43 weeks. BALB/c syngeneic mice inoculated with 5×10^6 BaF3-NPM/ALK cells died after 6–10 weeks (Fig. 4*A*, *right panel*) and the same number of BaF3-NPM/ALK + STAT5B-DNM cells induced fatal disease after 17–27 weeks. Visual examination of internal organs of the SCID mice revealed the presence of tumor foci in lymph nodes, liver, and extranodal sites such as meninges and orbit. Histologically, involved lymph nodes showed diffuse infiltrates of malignant lymphoid cells (Fig. 4*B, panel a*) with large nuclei, prominent nucleoli, and a moderate amount of cytoplasm (Fig. 4*B, panel d*). In the liver, the lymphoid cells formed large sheets in the periportal tracts (Fig. 4*B, panel b*); the morphology of the malignant cells was similar to that seen in the lymph nodes (Fig. 4*B, panel e*). The lymphoid tumor was clearly highly aggressive as determined by generalized lymphadenopathy and involvement of extranodal sites such as the orbital cavity (Fig. 4*B, panel c*). Immunohistochemical staining of liver (Fig. 4*B, panel f*) and other involved organs (data not shown) confirmed the expression of ALK protein in the malignant lymphoid cells.

DISCUSSION

NPM/ALK is an OTK resulting from the t(2;5) chromosomal translocation associated with ALCL (8). The transforming capacity of NPM/ALK was confirmed by the studies of Kuefer *et al.* (14), demonstrating that NPM/ALK induces lymphoma in mice injected with bone marrow cells infected with *NPM/ALK* retrovirus. However, the exact mechanisms of NPM/ALK-mediated malignant transformation are largely unknown.

Recently, the signal transduction protein PLC-γ (17) has been implicated in NPM/ALK-induced transformation. In previous studies we (39) and others (19) have demonstrated that another signaling molecules, specifically PI3k and its downstream effector Akt, are also essential for lymphomagenesis mediated by NPM/ALK. Because the PI3k/Akt pathway often collaborates with STAT5 in the transformation of hematopoietic cells (40), and both proteins are activated by the BCR/ABL OTK and are important for its leukemogenic potential (20–22), we decided to investigate whether STAT5 is a downstream effector of the NPM/ALK fusion kinase.

In this paper, we show that STAT5 is phosphorylated on tyrosine in the murine lymphocytic cell line BaF3 transformed not only by NPM/ALK and BCR/ABL but also by other members of the BCR/ABL-related OTK family including TEL/ABL, TEL/JAK2, and TEL/PDGFβR. This finding, in addition to other reports (6, 22), suggests that activation of the STAT5 pathway may be a common phenomenon occurring in hematopoietic cells transformed by OTKs. Furthermore, we demonstrate that STAT5 is phosphorylated on tyrosine and physically associated with NPM/ALK in the murine T-cell line CTLL-2 and in human ALCL cell lines bearing the t(2;5) chromosomal translocation SU-DHL-1 and SUP-M2. Association of STAT5 with NPM/ALK and its tyrosine phosphorylation led to the activation of STAT5-mediated DNA binding ability and transactivation activity.

To provide direct evidence for the role of STAT5 in NPM/ALK-mediated lymphomagenesis, a STAT5B-DNM (29) was used to perturb the function of endogenous STAT5 in NPM/ALK-positive cells. Transient expression of STAT5B-DNM induced apoptosis and ar-

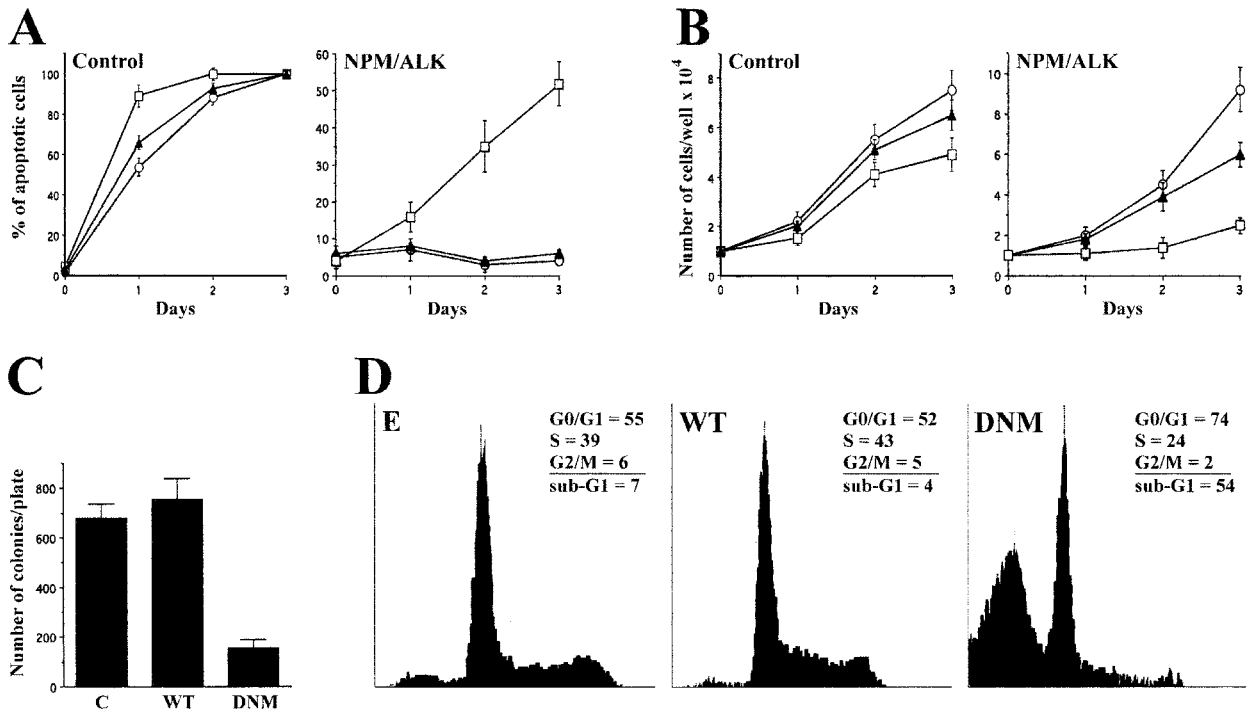

Fig. 3. STAT5-DNM induces apoptosis and inhibits proliferation of NPM/ALK-transformed cells. *A* and *B*, BaF3-neo (*Control*) and BaF3-NPM/ALK (*NPM/ALK*) cells were infected with retroviruses carrying STAT5B-DNM □, wild-type STAT5B ○, or were infected with empty retrovirus ▲. *A*, apoptotic cells were detected after incubation in IL-3-deficient medium using terminal deoxynucleotidyl transferase (Tdt)-mediated nick end labeling assay. *B*, proliferation was examined by counting alive cells incubated in the presence (*Control*) or absence (*NPM/ALK*) of IL-3. *C* and *D*, BaF3-NPM/ALK cells were infected with empty virus (*C*) or the viruses carrying STAT5B wild-type (*WT*) or STAT5B-DNM (*DNM*). *C*, cells were plated in methylcellulose in the absence of IL-3. Colonies were counted after 7 days. *D*, cell cycle distribution was assayed after 72 h of incubation without IL-3. Results represent three independent experiments.

rested cell cycle progression in a substantial number of NPM/ALK-positive cells cultured in the absence of IL-3. In addition, expression of STAT5B-DNM inhibited the tumorigenic capability of NPM/ALK-transformed cells in mice. The STAT5-dependent mechanisms involved in protection from apoptosis, growth factor-independent proliferation, and transformation are not known, but the possible role of genes transcriptionally regulated by STAT5, such as A1 (41), Bcl-xL (42), pim-1 (43), and oncostatin M (44) should be considered. A1 and Bcl-xL belong to the Bcl-2 family and protect cells from apoptosis (45, 46). In addition, the human homologue of A1 (Bfl-1) cooperates with the *E1A* oncogene in transformation (47). Activation of pim-1 correlates with mitogenesis and growth factor-independent survival, probably by phosphorylation of Cdc25A cell cycle phosphatase (48) and enhancement of expression of Bcl-2 (43). Moreover, pim-1 synergizes with c-*myc* in leukemogenesis (49). Oncostatin M is able to stimulate growth of various cells (50). Accordingly, inhibition of STAT5 activity may seriously impair the NPM/ALK-induced cell transformation by affecting function of several key downstream effectors.

Two published studies using STAT5A+B double-null mice have recently examined the necessity for STAT5-mediated signaling in transformation mediated by other OTKs such as BCR/ABL (51) or TEL/JAK2 (52). Whereas TEL/JAK2 failed to induce disease in reconstitution studies using bone marrow derived from STAT5-deficient mice suggesting an essential requirement for the STAT5 proteins in malignant transformation by this fusion tyrosine kinase (52), experiments performed in an identical manner using BCR/ABL demonstrated retention of transforming ability despite the absence of the two STAT5 proteins (51). Interestingly, BCR/ABL induced B-cell leukemias and rarely myeloid-derived leukemias when expressed in STAT5A+B double-null marrow cells, but no T-lineage disease was observed (51) despite the well-established ability of this OTK to transform T cells in mice (53, 54). Although this finding may potentially be explained by the use of host and conditioning regimens thought perhaps to be relatively unfavorable for T-cell transduction and transformation, it may reflect requirements for the STAT5A+B proteins that are specific to T cells. For example, STAT5-null peripheral T cells are markedly deficient in their proliferation, fail to undergo normal cell cycle progression, and lack expression of genes that control cell cycle progression such as cyclin D2 and D3 and Cdk6 (55). Retroviral transduction/transplantation experiments performed at St. Jude Children's Research Hospital at the same time (and using identical methods) as those noted above for TEL/JAK2 and BCR/ABL using STAT5-null bone marrow as targets for NPM/ALK-induced transformation resulted in the rapid onset (<5 weeks) of lethal hematopoietic malignancies that were characterized especially by marked hepatic infiltration with neoplastic cells. Interestingly, all of these malignancies were of the myeloid lineage as defined by expression of the markers Gr1 and/or Mac1, with neither B- nor T-cell tumors observed.[5] Thus, whereas the STAT5A+B proteins appear to be dispensable for NPM/ALK-mediated transformation of myeloid cells (an irrelevant target for the development of NPM/ALK-associated human lymphomas, which typically possess a mature, activated T-cell phenotype), it remains uncertain from these experiments whether NPM/ALK-mediated lymphoid cell transformation can in fact proceed in the absence of STAT5A+B proteins, and additional studies that specifically target NPM/ALK to STAT5A/B-null T-lineage cells will ultimately be required to unequivocally resolve this issue.

Interestingly, NPM/ALK-positive cells surviving the initial period of time despite STAT5B-DNM expression were eventually able to

[5] L. Xue and S. W. Morris, unpublished data.

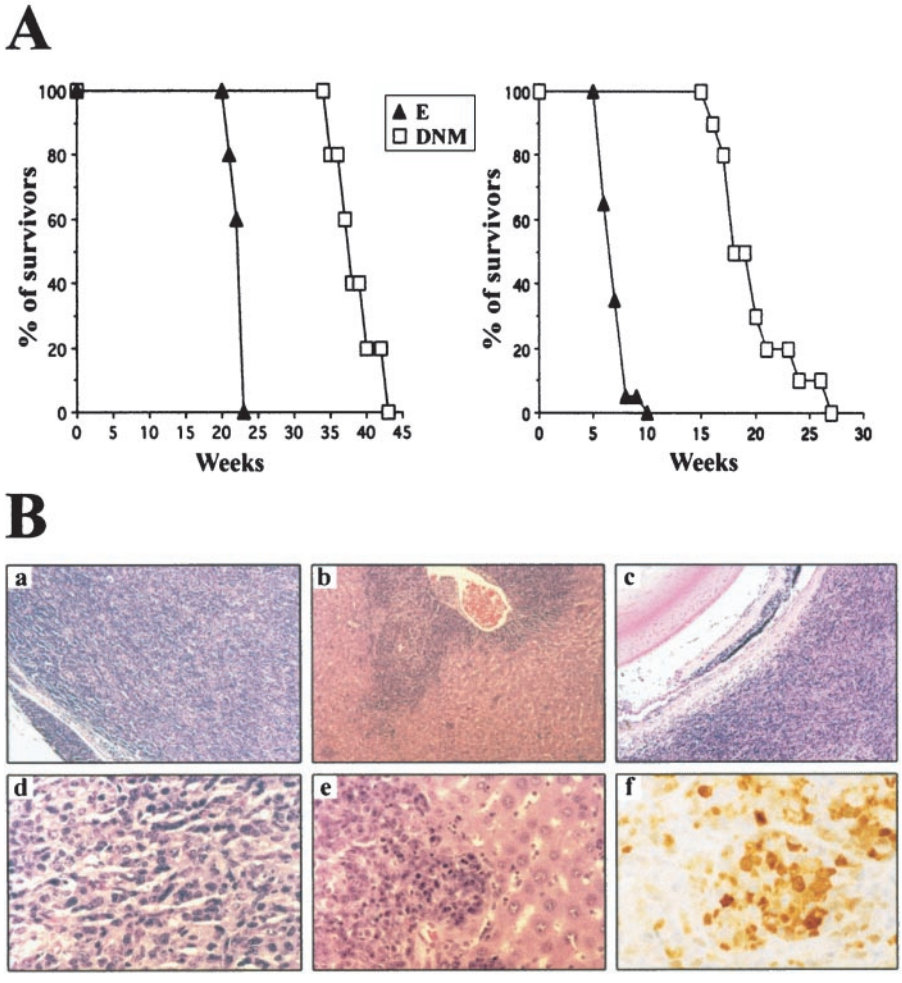

Fig. 4. STAT5 is essential for the development of NPM/ALK-mediated hematological malignancies. *A*, BaF3-NPM/ALK cells infected with STAT5B-DNM virus (*DNM*) or with empty virus (*E*) were injected i.v. into SCID mice (*left panel*) or syngeneic BALB/c mice (*right panel*). Survival time of the animals was monitored weekly. *B*, tissue sections from various organs were fixed in phosphate-buffered formalin, embedded in paraffin block, and stained with H&E. Shown are representative morphological findings of the malignant lymphoid cell infiltrates in lymph nodes (*a*, ×40; *d*, ×600), liver (*b*, ×40; *e*, ×600), and orbital cavity (*c*, ×40). Sections of liver containing the malignant lymphoid cells were stained for expression of ALK protein, which is visualized by *brown staining* (*f*, ×600).

"regain" the ability to proliferate *in vitro* and apparently also *in vivo*, but their growth rate was always lower than that of NPM/ALK-positive cells not expressing the mutant (data not shown). This phenomenon was not dependent on the loss of expression of STAT5B-DNM. Similar observations have been made in studies of the role of STAT5 in BCR/ABL-mediated leukemogenesis (22). Therefore, these observations suggest that an "acute" deficiency of the functional STAT5 caused by the expression of STAT5B-DNM could be overcome by activation of signaling pathways that are able to "bypass" STAT5. However, an alternative possibility is that the transient inhibitory effect of the STAT5-DNM could be attributable to the selection of cells with expression levels of STAT5B-DNM insufficient to block STAT5 activity. Moreover, we cannot exclude the possibility that STAT5 may be absolutely required only for the initiation of T-cell malignancy by NPM/ALK but not for its maintenance. In addition, because NPM/ALK induces not only STAT5 but also STAT3,[6] the latter one may eventually stimulate overlapping pathways and maintain NPM/ALK-lymphomagenesis in the absence of functional STAT5.

In conclusion, we show here that NPM/ALK, similar to other members of the BCR/ABL-related OTK family, activates the STAT5 pathway, which appears to be important for its transforming ability.

[6] Q. Zhang, P. N. Raghunath, M. Majewski, D. F. Carpentieri, L. Xue, N. Odum, S. W. Morris, T. Skorski, and M. A. Wasik. NPM/ALK oncoprotein mediates continuous activation of STAT3 in ALK+T/null-cell lymphoma, submitted for publication.

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