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Constitutive activation of a slowly migrating isoform of Stat3 in mycosis fungoides: Tyrphostin AG490 inhibits Stat3 activation and growth of mycosis fungoides tumor cell lines

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ABSTRACT **Mycosis fungoides (MF) is a low-grade cutaneous T cell lymphoma of unknown etiology. In this report, the Jak/Stat (Janus kinase/signal transducer and activator of transcription) signaling pathway was investigated in tumor cell lines established from skin biopsy specimens from a patient with MF. Jaks link cytokine receptors to Stats, and abnormal Jak/Stat signaling has been observed in some hemopoietic cancers. In MF tumor cells, a slowly migrating isoform of Stat3, Stat3^{sm}, was found to be constitutively activated, i.e., (*i*) Stat3^{sm} was constitutively phosphorylated on tyrosine residues, and tyrosine phosphorylation was not enhanced by growth factor stimulation; (*ii*) band shift assays and immunoprecipitations of DNA/Stat complexes showed constitutive DNA-binding properties of Stat3^{sm}; and (*iii*) Stat3^{sm} was constitutively associated with Jak3. The abnormal activation of Stat3^{sm} was highly specific. Thus, neither the fast migrating isoform of Stat3 (Stat3^{fm}) nor other Stats (Stat1, Stat2, and Stat4 through Stat6) were constitutively activated. The Jak kinase inhibitor, tyrphostin AG490, blocked the constitutive activation of Stat3^{sm} and inhibited spontaneous as well as interleukin 2-induced growth of MF tumor cells. In conclusion, we have provided evidence for an abnormal Jak/Stat signaling and growth regulation in tumor cells obtained from affected skin of an MF patient.**

Cutaneous T cell lymphomas include a wide spectrum of lymphomas of which mycosis fungoides (MF) and Sézary syndrome are the most common representatives (reviewed in ref. 1). The lymphocytic infiltrate in MF consists mainly of tumor cells of T cell origin intermixed with nonmalignant (inflammatory) T cells. The tumor cells are genetically unstable, and they often show an aberrant karyotype and/or phenoype (e.g., loss of surface markers) (2). In contrast to resting T cells, MF cells are not activated through the T cell receptor but through antigen-independent pathways, e.g., cytokine receptors such as the interleukin 2 receptor (IL-2R) (3). This antigen-independent activation may contribute to the constitutive activation found in plaques from affected skin in MF patients. Although viral transformation by human adult T cell leukemia virus type 1 (HTLV-1) has been reported in some cases of cutaneous T cell lymphoma (4), it does not seem to be a general characteristic in MF, and the primary etiological agent(s) of MF still remains to be found (5–7).

The Stats (signal transducers and activators of transcription) are members of a newly identified family of transcription factors activated in response to most growth factors and cytokines (reviewed in ref. 8). After receptor ligation, receptor-associated Jaks (Janus kinases) are activated by tyrosine (auto)phosphorylation. Subsequently, the activated Jaks tyrosine-phosphorylate the latent cytoplasmic Stats, which then homo- or heterodimerize and translocate to the nucleus, where they function as transcription factors. At present, four Jak (Jak1 through Jak3 and Tyk2) and six Stats (Stat1 through Stat6) have been identified. In addition, two isoforms of Stat5 and three isoforms of Stat3 can be expressed in hemopoietic cells (ref. 9 and unpublished observations). Because the Jak/Stat pathway is involved in regulation of cellular responses to cytokines and growth factors, it has been hypothesized that dysregulation and/or chronic activation of Jak/Stat proteins could play a key role in malignant transformation of cells. Recently, it was reported that *in vitro* transformation of cells with HTLV-1 or v-Src correlated with constitutive activation of the Jak/Stat signaling pathway (10, 11). In HTLV-1-transformed T cells, the change from IL-2-dependent to IL-2-independent growth correlated with an increased level of Stat activation. In leukemic cell lines expressing the fusion product Bcr/Abl, Stat proteins were found to be constitutively activated (12, 13), and recently, two studies reported on dysregulation of Jak/Stat signaling in malignant and/or nonmalignant cells obtained from peripheral blood cells from patients with acute lymphoblastic leukemia, acute myeloid leukemia, Sézary syndrome, and/or anaplastic large T cell lymphoma (14, 15).

In the present study, Jak/Stat signaling was examined in tumor cell lines established directly from skin biopsies taken from a patient with MF (3). We show that MF tumor cell lines express a constitutively active, slowly migrating isoform of the Stat3 protein, Stat3^{sm}. In addition, we show that Stat3^{sm} is constitutively associated with Jak3 and that Stat3^{sm} activation and mitogenesis are inhibited by tyrphostin AG490, a newly identified inhibitor of Jaks.

MATERIALS AND METHODS

Antibodies and Other Reagents. Recombinant IL-2 was a generous gift from Craig W. Raynolds and John Detrich (Biological Response Modifiers Program, National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, MD). Tyrphostin AG490 and A1 were from LC Laboratories Europe (Lãufelfingen, Switzerland). Antibodies against Jak1, Jak3, and P-tyr (4G10) were from Upstate Biotechnology (Lake Placid, NY). Antibodies against Stat1,

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Abbreviations: MF, mycosis fungoides; Stat, signal transducer and activator of transcription; Jak, Janus kinase; IL-2, interleukin 2; IL-2R, IL-2 receptor; HTLV-1, human adult T cell leukemia virus type 1.

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Stat2, Stat3 (S21320), Stat4, Stat5, and Stat6 were from Transduction Laboratories (Lexington, KY). Antibodies against Tyk2, Jak2, $Stat3^{fm}$ (K-15), and $Stat3^{sm}$ (C-20) were from Santa Cruz Biotechnology. The enhanced chemiluminesense (ECL) kit was from Amersham.

Cells. A tumor cell line was established from a plaque biopsy specimen of a patient with MF as described in detail elsewhere (3). A combinatorial approach that included phenotyping, genotyping, HLA-typing, and karyotyping verified the tumor cell origin of the cell line (2, 3). Because the tumor cell line during growth is genetically unstable, we compared an early culture of the tumor cells (My-la 2039) with a long-term culture (My-la 2059). The My-la 2039 cell line was studied between cell population doublings 10 and 100, whereas the My-la 2059 cell line has more than 1,000 population doublings. The karyotypic changes of the tumor cell line during this continuous culture period has previously been described (2, 3). From another biopsy taken 3 months later, the My-la 1929 cell line was established which karyotypically closely resembled the My-la 2039 cell line. An early culture of My-la 1929 was used in this study. All the cell lines were sero-negative for HTLV-1 and HTLV-negative as determined by reverse transcriptase–PCR (3). MF cell lines, which constitutively expressed IL-2Rs, were cultured in RPMI 1640 (GIBCO) supplemented with 100 units/ml IL-2, 10% fetal calf serum, 2 mM L-glutamine, 100 μg/ml penicillin, and 100 μg/ml streptomycin. MF cells were "starved" for IL-2 for 4–18 hr before initiation of the experiments described below.

Proliferation Assays. Assays were performed in culture medium (RPMI 1640 medium, GIBCO) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 μg/ml penicillin, and 100 μg/ml streptomycin (Novo, Copenhagen) in 96-well round-bottom tissue culture plates (Nunc) with a final volume of 200 μl (16). T cells were cultured at 5×10^4 cells per well for 16 hr with or without lymphokines or reagents as indicated. Twelve hours before harvest, [^{3}H]thymidine (1 μCi per well; 1 Ci = 37 GBq) was added. The cells were harvested onto glass fiber filters, [^{3}H]thymidine incorporation was measured in a scintillation counter, and the results were expressed as median counts per minute from triplicate cultures (16).

Protein Extraction, Immunoprecipitation, and Western Blotting. When chemical enzyme inhibitors were used, the cells were incubated for 16 hr in culture medium with IL-2 and 1% fetal calf serum and with or without the inhibitor. Before analysis, cells were starved for 4 hr in IL-2-free medium with or without inhibitor. After starvation, cells were either lysed directly or stimulated for 15 min with IL-2 (500 units/ml). For preparation of cytoplasmic lysates, 20×10^6 cells per experiment were lysed on ice in 900 μl of lysis buffer [1% Nonidet P-40/20 mM Tris, pH 8.0/137 mM NaCl/5 mM $MgCl_2$/10% glycerol and the following inhibitors: 5 mM EDTA, 1 mM Na_3VO_4, 10 μg/ml aprotinin, 4 μM iodoacetamide, and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. For preparation of nuclear lysates, cells were lysed for 15 min on ice in 0.05% Nonidet P-40/20 mM Tris, pH 8.0/137 mM NaCl/5 mM $MgCl_2$/10% glycerol and the following inhibitors: 1 mM Na_3VO_4, 10 μg/ml aprotinin, 4 μM iodoacetamide, and 1 mM PMSF. After three rounds of washings and pelleting at 500 $\times$ *g* for 5 min, pellets were resuspended in high salt buffer (300 mM NaCl/50 mM KCl/2% glycerol/40 mM Tris-base/2 mM EGTA/5 mM $MgCl_2$/1 mM PMSF/0.1 mM Na_3VO_4/10 mM NaF/0.1 mM NH_4-Molybdate/10 μM pepstatin/10 mM β-glycerophosphate/5 mM DTT/15 mM *p*-nitrophenylphosphate/10 μM leupeptin) for 30 min on ice. After the samples were spun for 15 min at 10,000 $\times$ *g*, the supernatants were used as nuclear lysates. Before immunoprecipitation from nuclear lysates, the samples were diluted with the lysis buffer used for preparing cytoplasmic lysates.

Immunoprecipitation and immunoblotting were conducted as described (17). In brief, lysates were precleared 2 hr with protein A beads before incubation overnight with the relevant antibody. Complexes were recovered by incubation with protein A beads for 3 h followed by repeated washing of the precipitates in lysis buffer. Samples were boiled in reducing SDS sample buffer, separated on a 10% polyacrylamide gel, and transferred electrophoretically to a nitrocellulose membrane. The membrane was blocked in 3% skim milk and 1% BSA in PBS and incubated with primary antibody in blocking buffer followed by washing in PBS and incubation with peroxidase-conjugated secondary antibody. Blots were evaluated by using ECL, stripped, and reprobed according to the manufacturer's manual (Amersham).

Electrophoretic Mobility-Shift Assays. Crude nuclear extracts were prepared by lysing cells for 30 min in hypotonic buffer (20 mM Tris/1 mM EGTA/2 mM $MgCl_2$/500 μM PMSF/50 μM Na_3VO_4/5 mM NaF/50 μM NH_4Molybdate/5 μM pepstatin/5 μM leupeptin/5 mM β-glycerophosphate/2 mM DTT, pH 7.0). After a quick spin, the pellets were resuspended and incubated for 30 min in hypertonic buffer (hypotonic buffer with 300 mM NaCl, 50 mM KCl, and 1.5% Ficoll). Lysates were then sonicated and spun at 10,000 $\times$ *g* for 10 min. The supernatant was used in band-shift assays. Lysates were incubated with ^{32}P-labeled double-stranded oligonucleotides corresponding to the SIE m67 sequence 5′-tgcaGTCGACATTTCCCGTAAATCGTCGA-3′ (18) or a probe corresponding to a GAS-motif in the IL-2Rβ gene promoter 5′-tgcaAGCTTGAATTCATGGAAATGGGATC-3′ (19) (lowercase letters indicate the 5′ overhang used for labeling with the Klenow fragment). In competition assays, unlabeled probe was added in a 50-fold excess. DNA-binding complexes were resolved on a 6% nondenaturing polyacrylamide gel, dried, and exposed to autoradiography.

Oligonucleotide Affinity Purification of Stats. Cell lysates were prepared as described above for immunoprecipitations. Lysates were precleared with streptavidin-coated beads and incubated with biotinylated double-stranded hSIE (high-affinity sis-inducible element) or IL-2Rβ oligonucleotide at 10 μg of oligonucleotide per ml. Stat/oligonucleotide complexes were recovered using streptavidin-conjugated beads. Purified oligonucleotide-binding proteins were boiled in reducing SDS sample buffer and analyzed by gel electrophoresis and Western blotting as described above.

RESULTS

Stat3 Is Constitutively Tyrosine-Phosphorylated in Cytoplasmic and Nuclear Extracts of MF Cells. Three continuous tumor cell lines, which all had the malignant characteristics of MF, were obtained from two independent plaque biopsies from a patient with classical MF (3). These cell lines constitutively express IL-2R and show a low spontaneous growth that is strongly enhanced in response to cytokines, especially IL-2 (3). To examine whether the growth regulation in MF tumor cells was associated with disturbances in Jak/Stat activation, MF cells were starved in medium without IL-2 before preparation of cytoplasmic or nuclear extracts. Tyrosine phosphorylated proteins from cytoplasmic extracts were immunoprecipitated with anti-phosphotyrosine mAb and analyzed by Western blotting by use of antibodies against all the known Stats, i.e., Stat1 through Stat6. As shown in Fig. 1*A* (*Upper*), Stat3 was heavily tyrosine-phosphorylated in the cytoplasm of starved MF cells (My-la 2059). In contrast, none of the other Stats appeared to be tyrosine-phosphorylated. A strong overexposure of the film did not reveal tyrosine phosphorylation of any Stats other than Stat3 (data not shown). Stat3 was also constitutively tyrosine-phosphorylated in two other MF cells, My-la 1929 and My-la 2039 (Fig. 1*A Lower*). The level of Stat3 expression, relative to other Stats, was normal in MF cells compared with that in nonmalignant T cells (data not shown). IL-2 had little or no effect on Stat3 phosphorylation in MF

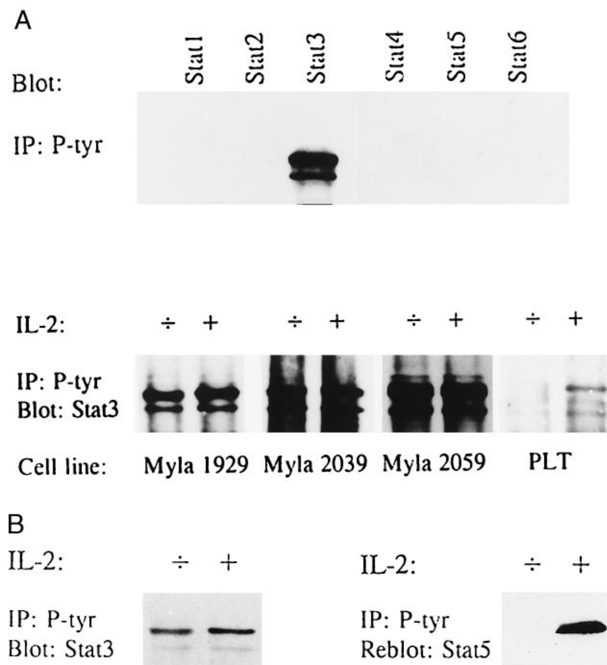

FIG. 1. (*A*) Stat3^{sm} (*Upper*) is constitutively tyrosine-phosphorylated in MF cells. Cytoplasmic extracts from IL-2-starved cells were analyzed by immunoprecipitation with anti-phosphotyrosine mAb (4G10) and subsequent blotting with use of mAbs against Stat1 through Stat6. Tyrosine phosphorylation of Stat3^{sm} cannot be further induced in My-la cells (*Lower*). Three MF cell lines and a nonmalignant T cell line (PLT) were IL-2-starved before stimulation with IL-2 (500 units/ml) for 10 min. The cytoplasmic lysates were analyzed by immunoprecipitation with an anti-phosphotyrosine mAb and by subsequent blotting with a Stat3 mAb. (*B*) After IL-2 starvation, MF cells were stimulated with IL-2 (500 units/ml) for 30 min. Nuclear extracts were analyzed by immunoprecipitation with an anti-phosphotyrosine mAb (4G10) and by subsequent blotting with a Stat3 mAb (*Left*). The blot was stripped and reprobed with an antibody against Stat5 (*Right*).

cells. In contrast, IL-2 clearly induced Stat3 phosphorylation in nonmalignant T cell lines (Fig. 1*A Lower*). It was a consistent observation that the level of Stat3 phosphorylation was higher in My-la 2059 than it was in My-la 1929 and My-la 2039. Interestingly, the spontaneous growth of My-la 2059 was also higher than that of My-la 1929 and My-la 2039 (data not shown).

Because tyrosine phosphorylation of Stats is normally followed by dimerization and nuclear translocation, we examined whether Stat3 was also constitutively tyrosine-phosphorylated in the nucleus of MF cells. As shown in Fig. 1*B*, tyrosine-phosphorylated Stat3 was indeed found to be constitutively present in the nucleus. Stripping of the membrane and reprobing with antibody against Stat5 showed that Stat5 was tyrosine-phosphorylated and translocated to the nucleus only in response to IL-2. Thus, the constitutive nuclear translocation of phosphorylated Stat3 was highly specific (Fig. 1*B*). Detection of tyrosine-phosphorylated Stat3 in the nuclear extracts was not due to contamination with cytoplasmic proteins because blotting of total nuclear extracts (before immunoprecipitation was performed) showed that Stat5 was only present after IL-2 stimulation (data not shown). Thus, Stat3 was found to be constitutively tyrosine-phosphorylated in both the cytoplasm and the nucleus of MF cells.

Constitutive DNA-Binding Activity of Stat-Related Proteins. Next, we investigated whether the constitutive tyrosine phosphorylation of Stat3 correlated with a constitutive binding to DNA. To investigate this, nuclear extracts were prepared and analyzed in electrophoretic mobility-shift assays by using two different oligonucleotide probes: one representing the Stat-binding sequence in the promoter of the IL-2Rβ gene and the other representing the high-affinity sis-inducible element (hSIE m67) derived from the c-*fos* promoter region. As shown in Fig. 2*A* (lanes 1 and 5), nuclear lysates prepared from unstimulated My-la cells contained Stat-related proteins with a very strong binding to both DNA probes. In contrast, nuclear lysates from "rested," nonmalignant T cell lines did not constitutively bind the IL-2Rβ probe, whereas some binding of the hSIE probe was observed (Fig. 2*A*, lanes 3 and 7). IL-2 induced an enhanced binding of the hSIE probe to nuclear lysates from nonmalignant T cells, whereas IL-2 had little effect on the binding of both the hSIE and IL-2Rβ probes in My-la cells (Fig. 2*A*). The binding of the IL-2Rβ probe to nuclear lysates from MF cells seemed to be specific because the binding was almost completely blocked when an excess of the

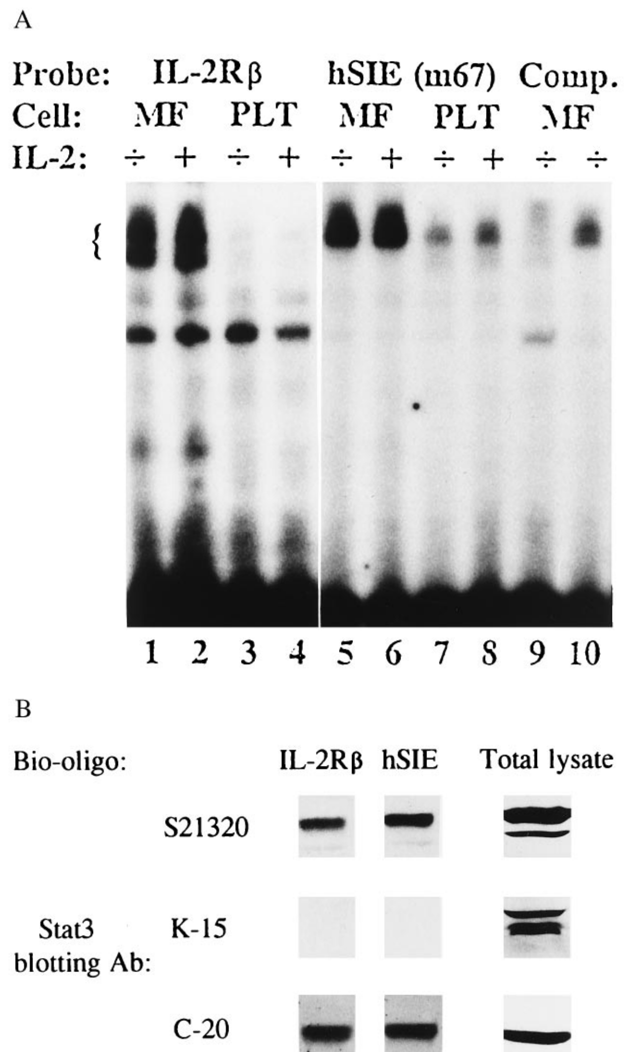

FIG. 2. (*A*) Constitutive tyrosine phosphorylation of Stat3^{sm} correlates with constitutive binding to DNA. MF cells or nonmalignant T cells (PLT) were starved in medium without IL-2 before band-shift analysis of nuclear extracts. DNA binding was determined by using ^{32}P-labeled IL-2Rβ and hSIE m67 probes. The position of Stat-related proteins is indicated by a bracket. Binding was competed out by using a 50-fold excess of unlabeled probe (not shown). (*B*) A specific form of Stat3 binds to DNA. Cytoplasmic extracts from IL-2-starved MF cells were analyzed by affinity purification of Stat proteins by using biotinylated probes (hSIE and IL-2Rβ). Stat/DNA complexes were analyzed by Western blotting with use of Stat3 antibodies directed against different parts of Stat3 (S21320, amino acids 1–178; K-15, amino acids 626–640; C-20, amino acids 750–769). The same panel of Stat3 antibodies was used for blotting of total cell lysates.

unlabeled probe was added (Fig. 2*A*, lane 9 versus lane 1). Likewise, an excess of the unlabeled hSIE probe strongly inhibited binding of the labeled hSIE probe (Fig. 2*A*, lane 10 versus lane 5).

A Specific Isoform of Stat3 Is Present in the DNA-Binding Complexes. To further characterize the DNA-binding Stats, Stat/DNA complexes were affinity-purified by using biotinylated DNA probes. The purified complexes were analyzed in Western blotting experiments by using Stat3 antibodies recognizing different parts of the Stat3 protein (S21320, amino acids 1–178; K-15, amino acids 626–640; C-20, amino acids 750–769). The C-20 antibody recognizes a slowly migrating isoform of Stat3, $Stat3^{sm}$, which is also recognized by S21320 (unpublished observations). The K-15 and S21320 antibodies recognize several Stat3 isoforms, including a fast migrating isoform that is not recognized by C-20 (20). In total cell lysates from MF cells, these antibodies recognize at least three isoforms of Stat3 (Fig. 2*B*). In contrast, only the S21320 and C-20 antibodies recognize Stat/DNA complexes precipitated with biotinylated IL-2Rβ and hSIE probes, indicating that the precipitates contained only the slowly migrating isoform of Stat3, $Stat3^{sm}$, and not the fast migrating isoforms (Fig. 2*B*). This conclusion was supported by our observation that a fast migrating isoform of Stat3, $Stat3^{fm}$, which is recognized by K-15 antibody, is not constitutively tyrosine-phosphorylated in MF cell lines (data not shown). In other cell types, the K-15 antibody has been shown to react with tyrosine-phosphorylated $Stat3^{sm}$ (20). Yet K-15 did not bind DNA-bound $Stat3^{sm}$ or tyrosine-phosphorylated $Stat3^{sm}$ in MF cells (Fig. 2*B* and data not shown). Because the S21320 and C-20 antibodies readily reacted with tyrosine-phosphorylated $Stat3^{sm}$ (cf. above), it is possible that in MF cells, $Stat3^{sm}$ is phosphorylated in a way that prevents binding of K-15 but not of C-20 and S21320 antibodies. An alternative explanation, not excluding the above, might be that mutations in the Stat3 gene or posttranslational events other than phosphorylation selectively inhibit binding of the K-15 antibody to tyrosine-phosphorylated $Stat3^{sm}$.

IL-2 Activates Stat5 and Enhances Growth of MF Cells. In nonmalignant T cell lines, growth factors such as IL-2 and IL-15 activate both Stat3 and Stat5 (17, 21). Because MF cells express the high-affinity IL-2R and because IL-2 strongly enhances the low spontaneous growth of MF cells, it was important to elucidate how cytokine-induced Stat5 activation was regulated in MF cells. As mentioned above, IL-2 induces tyrosine phosphorylation of Stat5. To compare the DNA-binding capacity of Stat3 and Stat5, starved MF cells were incubated in medium with or without IL-2. Activation of Stats was then analyzed by affinity purification with use of the biotinylated hSIE and IL-2Rβ probes and subsequently by Western blotting with antibodies against Stats. As shown in Fig. 3, IL-2 did not induce a significant increase of $Stat3^{sm}$ binding to DNA, a result that was in keeping with our observation that IL-2 did not enhance $Stat3^{sm}$ phosphorylation (cf. above). Stripping of the membrane and reprobing with antibody against Stat5 showed that IL-2 induced strong DNA binding of Stat5 to the IL-2Rβ probe (Fig. 3). Stat5 does not bind to the hSIE motif (9), and as expected, IL-2 did not induce Stat5 binding to the hSIE probe. Thus, although $Stat3^{sm}$ activation was constitutive and could not be further increased, IL-2 induced a normal activation of Stat5, indicating that the dysregulation of Stat3 proteins in MF cells is highly specific.

Tyrphostin AG490 Inhibits the Constitutive Stat3 Activation and Growth of MF Cells. Jaks are believed to be responsible for tyrosine phosphorylation of Stats in response to growth factors (8). Therefore, we examined whether the newly described Jak inhibitor, tyrphostin AG490 (22), could block the constitutive activation of $Stat3^{sm}$. The inactive tyrphostin A1 was used as a negative control. As shown in Fig. 4*A*, 16 hr of incubation with AG490, at a concentration of 100 μM,

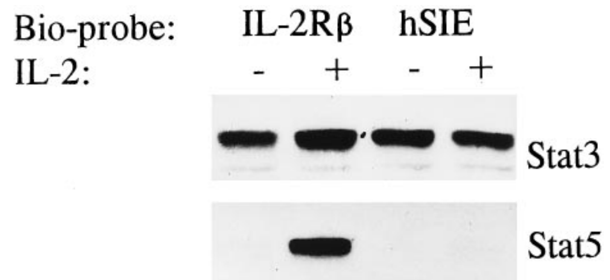

FIG. 3. Stat5 activation is inducible, whereas $Stat3^{sm}$ cannot be further induced. Cells were starved for 4 hr in IL-2-free medium before stimulation with medium or IL-2 (500 units/ml) for 15 min. DNA-binding Stats were affinity-purified by using biotinylated hSIE and IL-2Rβ probes and were subjected to Western blotting with the $Stat3^{sm}$ mAb (upper bands). The membrane was stripped and reprobed with the Stat5 mAb (lower band).

clearly inhibited the constitutive phosphorylation of $Stat3^{sm}$ (lanes 1–4), whereas incubation with the inactive control, tyrphostin A1, did not (lanes 5–7). Additional experiments confirmed that the constitutive phosphorylation of Stat3 was strongly inhibited by 60–80 μM AG490 (data not shown). Next, we examined whether proliferation of MF cells was also inhibited by AG490. Cells were cultured with or without IL-2 and with different concentrations of AG490. After 48 hr of culture, incorporation of [^{3}H]thymidine was determined. As shown in Fig. 4*B*, AG490, at concentrations at and above 50 μM, inhibited the spontaneous growth of MF cells (IC_{50} = 75

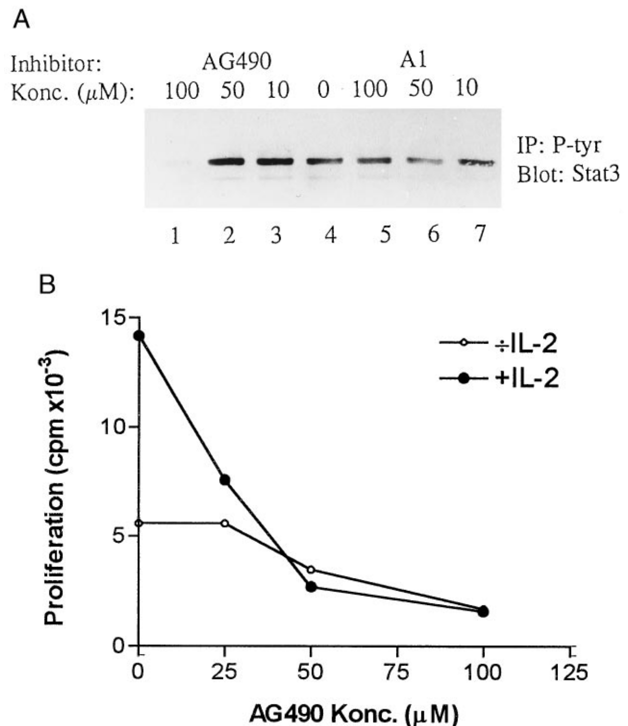

FIG. 4. Tyrphostin AG490 inhibits constitutive $Stat3^{sm}$ activation and growth of MF cells. (*A*) Cells were incubated for 16 hr in culture medium without inhibitor (lane 4), medium containing the indicated amounts of the Jak inhibitor AG490 (lanes 1–3), or the inactive control tyrphostin A1 (lanes 5–7). The cells were then starved for 4 hr in IL-2-free medium containing the relevant inhibitor before cytoplasmic lysates were prepared. Tyrosine-phosphorylated proteins were immunoprecipitated with an anti-phosphotyrosine mAb (4G10), resolved on a polyacrylamide gel, and immunoblotted with the $Stat3^{sm}$ mAb. (*B*) Cells were cultured for 16 hr with or without the indicated concentrations of Jak inhibitor AG490, and then [^{3}H]thymidine uptake was measured.

μM). In contrast, the IL-2-mediated up-regulation of MF cell growth was inhibited by lower concentrations of AG490 (IC_{50} = 20 μM) (Fig. 4*B*). IL-2-driven proliferation of nonmalignant T cells was also inhibited with an IC_{50} of 20 μM (data not shown), suggesting that the spontaneous and the IL-2-mediated growth of MF cells are mediated by pathways with different sensitivity to AG490.

Stat3^{sm} Is Constitutively Associated with Jak3 and Tyk2. The studies with the inhibitor AG490 indicated that Jaks might be involved in the constitutive activation of Stat3^{sm}. We therefore examined whether Stat3^{sm} was constitutively associated with one or more of the four currently known Jaks, Jak1, Jak2, Jak3, and Tyk2. Accordingly, Jaks were precipitated from cytoplasmic lysates, and the precipitates were analyzed by Western blotting with Stat3^{sm} antibody. As shown in Fig. 5, Jak3 specifically coprecipitated Stat3^{sm} (lane 5), whereas Stat3^{sm} was detected only at background levels in Jak1 and Jak2 precipitates (lanes 2–4). Tyk2 showed weak (yet above background level) association with Stat3^{sm} (lane 1). To examine whether Jaks were constitutively activated in MF cells, Jaks and Tyk2 were immunoprecipitated from total cell lysates from starved MF cells and nonmalignant T cell lines and blotted with anti-phosphotyrosine antibody. Surprisingly, none of the Jaks or Tyk2 were constitutively tyrosine-phosphorylated in MF cells, whereas Jak3 became tyrosine-phosphorylated after IL-2 treatment (data not shown), suggesting that Jak3 (and possibly Tyk2) may function as "docking" proteins rather than activators of Stat3^{sm} in MF cells. In light of the recent finding that Jaks localize to the nucleus as well as to the cytoplasmic fraction (23), we have performed preliminary experiments to examine whether nuclear Jaks could be involved in the constitutive activation of Stat3^{sm}. In parallel to the association of Stat3^{sm} with Jak3 in the cytoplasm, we found that trace amounts of Jak3 could be coprecipitated with Stat3 from nuclear extracts (data not shown). However, we were still not able to detect any tyrosine phosphorylation of Jak3 in the nucleus. Thus, the constitutive Stat3^{sm} activation cannot definitely be ascribed to the Jaks. In a further attempt to identify the kinase responsible for the constitutive activation of Stat3^{sm}, we focused on tyrosine kinases that had previously been reported to activate Stat3 in virus-transformed cells and cancers.

The Bcr/Abl fusion protein, which is associated with chronic myelogenous leukemia and acute lymphocytic leukemia, can activate Stat3 in leukemic cells, but the MF cells did not express the leukemic Bcr/Abl fusion protein and c-Abl was not constitutively activated in MF cells (data not shown). A series of other tyrosine kinases, including ZAP-70 and fyn, were expressed but not constitutively active in MF cells (data not shown). Coprecipitation studies did not show association of any major tyrosine-phosphorylated proteins with Stat3^{sm}. It was recently reported that Stat3 becomes constitutively activated in HTLV-1- or v-Src-transformed cell lines (10, 11). Our MF cells are HTLV-1-negative and express very low levels of the Src-family kinase p56^{lck}, as judged from Western blot analysis with lck-specific mAbs, suggesting that the expression of lck is down-regulated or that lck is modified (or mutated) in a way that affects mAb recognition in MF cells (data not shown). Thus, it is possible that a mutated kinase (Jak/p56^{lck}) or an as yet unidentified AG490-sensitive kinase is responsible for the constitutive activation of Stat3 in these cells.

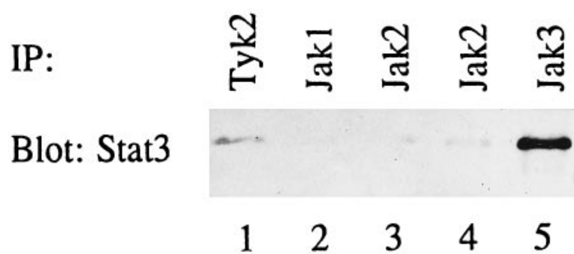

FIG. 5. Stat3^{sm} is associated with Jak3 and Tyk2. Cells were starved for 4 hr in IL-2-free medium before they were lysed. The different members of the Jak family were immunoprecipitated by using the antibodies indicated on the figure. Association with Stat3^{sm} was determined by immunoblotting with the Stat3^{sm} mAb.

DISCUSSION

In the present study, we have investigated Jak/Stat signaling in MF cell lines established from skin biopsy specimens taken from a patient suffering from MF, i.e., a low-grade malignant form of cutaneous T cell lymphoma. We found that Stat3 was constitutively tyrosine-phosphorylated in the cytoplasm of these cells. Further analysis showed that the constitutively tyrosine-phosphorylated Stat3 protein also had the ability to translocate to the nucleus and bind to DNA. The constitutive activation of Stat3 was highly selective because tyrosine phosphorylation of Stats other than Stat3 could not be detected in starved cells. The use of different antibodies against Stat3 revealed a very high degree of selectivity in the abnormal activation of Stat3. In total cellular lysates, three isoforms of Stat3 could be recognized by the well characterized antibodies, C-20, K-15, and S21320. Yet, only the slowly migrating isoform of Stat3, Stat3^{sm}, was constitutively activated, as determined from both tyrosine-phosphorylation profiles and DNA binding to the IL-2Rβ and the hSIE probes. In contrast, a fast migrating isoform, Stat3^{fm}, which was recently identified at the mRNA level and which is recognized by the K-15 but not the C-20 antibody (20), did not appear to be constitutively activated. Thus, the fast migrating Stat3 did not bind to well defined Stat3-binding motifs in the IL-2Rβ and the c-*fos* genes. Moreover, the fast migrating isoform of Stat3 was not constitutively phosphorylated on tyrosine residues, supporting our conclusion that the MF cells had a selective dysregulation of Stat3^{sm}.

The exact mechanism underlying the constitutive activation of Stat3^{sm} in MF cells is not known at present. Stat3 was found to be constantly associated with Jak3. However, Jak3 was not found to be constitutively activated, as judged by tyrosine phosphorylation. Because tyrosine phosphorylation of Jak kinases is believed to be prerequisite for kinase activation, it is possible that Jaks are not involved in the activation of Stat3^{sm} in MF cells. Another possibility might be that Jak3 in MF cells is immunologically different from Jaks in other cells, i.e., that genetic or other changes in MF cells modify the configuration of Jak3 in a way that interferes with antibody binding. Our finding that the Jak inhibitor AG490 inhibited the constitutive phosphorylation of Stat3^{sm} also suggests that Jaks or Jak-like kinases are involved in the constitutive activation of Stat3^{sm}. IL-2-enhanced growth of MF cells was inhibited at a lower concentration of AG490 than was the spontaneous growth, suggesting that spontaneous and cytokine-driven proliferation are mediated by at least partly distinct pathways with different sensitivity to AG490.

In a recent report, Zhang *et al.* (15) studied Jak/Stat activation in more advanced states of cutaneous T cell lymphoma, Sézary syndrome, and anaplastic large T cell lymphoma. In anaplastic large T cell lymphoma, Jak3 and Stat3/Stat5 were constitutively activated, whereas phosphorylation of Jak3 and Stat5 in a Sézary syndrome cell line was induced by cytokines and did not appear to be constitutive because starvation of the cells strongly decreased the level of phosphorylation. Therefore, Zhang *et al.* (15) hypothesized that the induced versus constitutive activation of the Jak/Stat pathway could be associated with disease progression. In the present study, we also observed that Stat5 was not activated in "starved" MF cells, supporting the hypothesis that Stat5 activation per se might not play a pathogenetic role in low-

grade cutaneous T cell lymphoma. In contrast, we observed that Stat3^{sm} was constitutively activated in the three MF cell lines.

In conclusion, we have shown that tumor cell lines obtained from MF skin plaque biopsy specimens have a highly selective dysregulation of the Stat signaling pathway. Although Stat3^{sm} is constitutively associated with Jak3, there is no constitutive phosphorylation of Jak3, suggesting that Jak3 might be an important "docking" protein rather than the initial activator. Because the novel Jak inhibitor, tyrphostin AG490, suppresses both spontaneous growth and the constitutive activation of Stat3^{sm}, it may be speculated that malignant transformation of MF cells involves either a novel AG490-sensitive kinase or an oncogenic form of known Jaks.

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