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TYROSINE PHOSPHORYLATION OF JAK-TYK KINASES IN MALIGNANT PLASMA CELL LINES GROWTH-STIMULATED BY INTERLEUKINS 6 AND 11

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The pleiotropic cytokine interleukin (IL)-6 is a major growth factor for murine plasmacytomas/hybridomas and human myeloma cells. Here we report that IL-6 stimulated different patterns of tyrosine phosphorylation of JAK-TYK kinases in IL-6-responsive murine (B9E and T10D) and human (ANBL-6 and OCI-My4) plasma cell tumor lines. Interestingly, the Stat91 transcription factor essential for interferon signaling mediated by JAK-TYK kinases was significantly tyrosine phosphorylated in response to IL-6 in ANBL-6 cells but not in the other cell lines. We further show that IL-11, a cytokine that signals via the gp130 subunit of the IL-6 receptor, induced similar profiles of JAK-TYK tyrosine phosphorylation as IL-6 in B9E and T10D cells. These results suggest that functionally redundant JAK-TYK kinase cascades triggered through gp130 are involved in the growth regulation of plasma cell neoplasms. © 1994 Academic Press, Inc.

Interleukin (IL)-6 is a multifunctional cytokine (1) produced by a variety of cell types (for review see ref. 2). The observation that IL-6 exhibits growth stimulatory activity on murine plasmacytomas and hybridomas suggested that this factor might also play a central role in human multiple myeloma (3,4). Subsequent studies have confirmed that IL-6 is a primary growth factor for freshly isolated myeloma cells as well as myeloma cell lines established from patients with terminal disease (5-8). We previously reported the derivation of an experimental model of bone marrow metastasis, using IL-6-dependent murine B9 hybridoma cells, that resembles the terminal aggressive phase of multiple myeloma (9,10). Additional studies by us (11) and others (12,13)

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The abbreviations used are: anti-pTyr, anti-phosphotyrosine; CNTF, ciliary neurotrophic factor; FBS, fetal bovine serum; IL, interleukin; JAK, Janus kinase; LIF, leukemia inhibitory factor; mAb, monoclonal antibody; MAP kinase, mitogen-activated protein kinase; OSM, oncostatin M; SDS, sodium dodecyl sulfate; STAT, signal transducer and activator of transcription.

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have shown that IL-11, a bone marrow stromal cell-derived factor that was identified based on its ability to stimulate the proliferation of the IL-6-dependent murine T1165 plasmacytoma (14; reviewed in ref. 15), also stimulates the proliferation of B9 cells. An explanation for this overlap in activities has come from the recognition that the cognate receptors for IL-6 and IL-11 share a common signal transducing component, termed gp130 (16), which is utilized as well by the receptors for three other cytokines, leukemia inhibitory factor (LIF), oncostatin M (OSM) and ciliary neurotrophic factor (CNTF) (17,18). (We will refer to these factors collectively as IL-6-type cytokines.)

Recently, it has been demonstrated that the signaling pathways activated by IL-6-type cytokines involve members of the JAK-TYK family of nonreceptor protein tyrosine kinases (19-22). These kinases interact directly with gp130 and with a structurally related β component of the LIF receptor and are activated by tyrosine phosphorylation following receptor ligation by the cognate cytokine (17,18). At present there are three known members of this kinase family, JAK1, JAK2 and Tyk2: JAK1 and Tyk2 were first shown to be required for interferon-α signaling; JAK1 and JAK2 for interferon-γ signaling; and JAK2 for signaling by IL-3, erythropoietin and growth hormone (23,24). Notably, IL-6, LIF, OSM and CNTF have been found to activate all three JAK-TYK kinases but to elicit distinct patterns of JAK-TYK tyrosine phosphorylation in different cell types (19-22). By comparison, the JAK-TYK kinases activated by IL-11 have not been well characterized as yet, with a single report describing tyrosine phosphorylation of JAK1 in IL-11-treated rat hepatoma cells (20). In this paper we examined whether JAK-TYK kinases were among the proteins tyrosine phosphorylated in murine and human plasma cell tumor lines growth-promoted by IL-6 and IL-11.

MATERIALS AND METHODS

Cell Lines--The B9E cell line is an IL-11-dependent derivative of the IL-6-dependent murine B9 hybridoma (25) obtained by limiting dilution cloning (0.3 cells/well) in 96-well microtiter trays of a subpopulation of cells that would grow in medium with only IL-11 as the cytokine supplement. B9E cells were maintained in Iscove's modified Dulbecco's medium supplemented with 50 μM 2-mercaptoethanol, 5% heat inactivated fetal bovine serum (FBS; GIBCO BRL, Gaithersburg, MD) and 5% conditioned medium from B9/hIL-11 cells as a source of IL-11 (11) in a humidified atmosphere containing 5% CO_2 at 37 °C. The T10D cell line (supplied by Genetics Institute) is an IL-11-dependent derivative of the IL-6-dependent T1165 murine plasmacytoma cell line that was used to identify IL-11 (14,26). T10D cells were maintained as per B9E cells but with supplements of 10% FBS and 10% B9/hIL-11 conditioned medium. The recently established IL-6-dependent human myeloma cell line ANBL-6 and the IL-6-responsive human myeloma cell line OCI-My4 (a gift of M. Minden, Ontario Cancer Institute, Toronto, Ontario, Canada) have been described (7,8). These cells were maintained in Iscove's modified Dulbecco's medium containing 50 μM 2-mercaptoethanol, 10% FBS and 10% conditioned medium from S194/5 cells transfected with a cDNA encoding human IL-6 (9).

Reagents--Recombinant human IL-6 (specific activity, 9.1 x 10^7 units/mg) was kindly provided by D. Levitt (Sandoz Pharmaceuticals Corp., East Hanover, NJ) and recombinant human

IL-11 (specific activity, 1.5 x 10^6 units/mg) was from Genetics Institute. The anti-phosphotyrosine (anti-pTyr) monoclonal antibody (mAb), 4G10, as well as affinity purified rabbit polyclonal antisera to JAK1, JAK2 and Tyk2 were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Affinity purified rabbit polyclonal antiserum to Stat91 (anti-Stat91/84) was purchased from Transduction Laboratories (Lexington, KY). Horseradish peroxidase-conjugated secondary antibodies were from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). Enhanced chemiluminescence reagents for immunoblotting were purchased from Amersham Canada Ltd. (Oakville, Ontario) and polyvinylidene difluoride membranes (Immobilon-P) were from Millipore Corp. (Bedford, MA).

Immunoblotting and Immunoprecipitations--B9E and ANBL-6 cells (5 x 10^5 cells/ml) were starved for 16-18 h in medium without FBS and cytokines. T10D cells were starved for 12 h in medium containing 0.5% FBS. OCI-My4 cells were starved for 16 h in the presence of 10% FBS, then for 2 h in medium containing 0.25% FBS. Following these deprivation regimens, the viability of B9E and ANBL-6 cells was greater than 90% whereas the viability of T10D and OCI-My4 cells was approximately 50 and 75%, respectively, as determined by dye exclusion using 0.04% trypan blue. Cells were collected by centrifugation and resuspended at 2 x 10^7 cells/ml (B9E and T10D) or at 5 x 10^6 cells/ml (ANBL-6 and OCI-My4) and stimulated with IL-6 or IL-11 as indicated. After treatment, cells were washed once with ice cold phosphate buffered saline containing 1 mM sodium orthovanadate, incubated in lysis buffer (20 mM Tris-HCl, pH 7.5, 137 mM sodium chloride, 100 mM sodium fluoride, 10% glycerol, 1% Nonidet P-40, 1mM sodium orthovanadate, 1mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin and 10 μg/ml leupeptin) for 15 min at 0 °C, and supernatants were clarified by centrifugation at 14,000 x g for 10 min (27).

For analysis of global patterns of protein tyrosine phosphorylation (27), an equivalent volume of 2 x sodium dodecyl sulfate (SDS) sample buffer was added to the cell lysates, the samples were boiled for 3 min and the solubilized proteins were resolved on 7.5% SDS-polyacrylamide gels, and then transferred to Immobilon-P membranes using 25 mM Tris-HCl, pH 8.3, 192 mM glycine and 20% methanol. In immunoprecipitation experiments (28), the cell lysates were precleared by incubation with protein A-Sepharose CL-4B (Pharmacia Biotech Inc., Piscataway, NJ) for 1 h, incubated with antibodies for 1-2 h at 4 °C, and immune complexes were collected following an additional 1 h incubation with protein A-Sepharose. The immunoprecipitates were washed three times with lysis buffer and once with 10 mM Tris-HCl, pH 7.6, and bound proteins were eluted by boiling in SDS-sample buffer. Immunoprecipitated proteins were resolved by 7.5% SDS-polyacrylamide gel electrophoresis and transferred to Immobilon-P as described above. To detect proteins by immunoblotting, membranes were pretreated with Tris-buffered saline containing 0.1% Tween 20 and 6% bovine serum albumin for at least 1 h, incubated with primary antibody for 1 h, washed for 30 min with Tris-buffered saline-Tween 20, and developed with horseradish peroxidase-conjugated second antibody for 45 min. Blots were then washed for 30 min with Tris-buffered saline-Tween 20, incubated with enhanced chemiluminescence substrate solution and exposed to Kodak X-Omat AR x-ray film. In all cases, blots of immunoprecipitated proteins were initially probed with anti-pTyr mAb 4G10 then the membranes were stripped by incubating two times in 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 100 mM 2-mercaptoethanol at 50 °C for 20 min, washed with Tris-buffered saline-Tween 20, and subsequently reprobed with either anti-JAK1, -JAK2, -Tyk2 or -Stat91/84, as indicated. For studies with ANBL-6 and OCI-My4 cells, sequential immunoprecipitations were performed on individual lysates because of the difficulty in obtaining large numbers of cells. In these instances, lysates were cleared twice with protein A-Sepharose between successive immunoprecipitations to eliminate carry over between experiments.

RESULTS AND DISCUSSION

Because previous studies suggested that IL-6 and IL-11 stimulated tyrosine phosphorylation of different substrates in certain murine hybridomas (13,29), we first examined global patterns

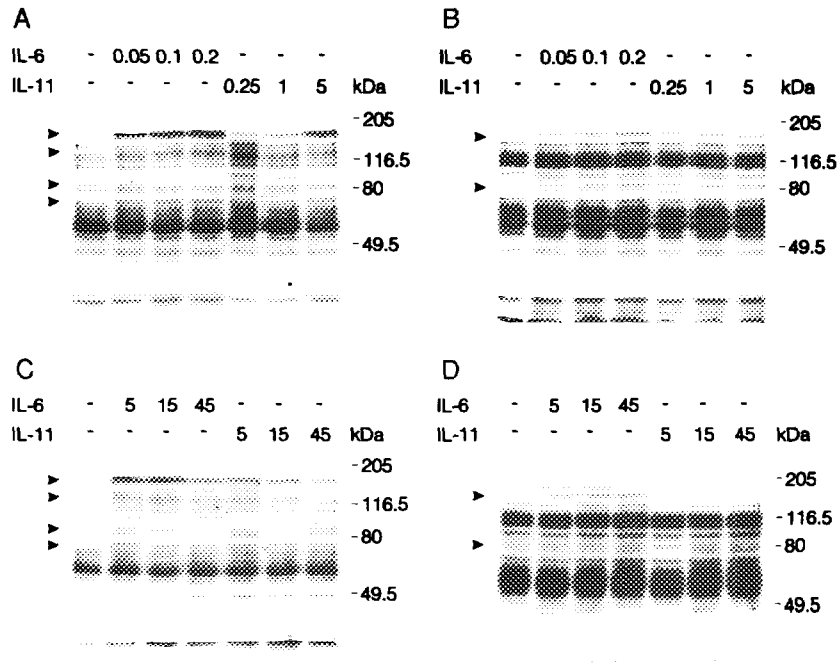

Figure 1. **IL-6 and IL-11 induce tyrosine phosphorylation of overlapping sets of proteins in murine plasma cell lines, B9E and T10D.** Samples of whole cell lysates equivalent to 1.4 x 10^5 cells were subjected to immunoblot analysis with anti-pTyr mAb 4G10 (1:1000 dilution) to detect phosphotyrosine-containing proteins. (*A*) IL-11-starved B9E cells were stimulated with or without (-) the indicated concentrations (μg/ml) of IL-6 (specific activity, 9.1 x 10^7 units/mg) or IL-11 (specific activity, 1.5 x 10^6 units/mg) for 5 min at 37 °C. (*B*) IL-11-starved T10D cells were treated as in *A*. (*C*) IL-11-starved B9E cells were stimulated with or without (-) IL-6 (0.1 μg/ml) or IL-11 (5 μg/ml) for the indicated times (min) at 37 °C. (*D*) IL-11-starved T10D cells were treated as in *C*. Arrows denote the tyrosine phosphorylated proteins discussed in the text. The sizes of molecular mass markers (Prestained SDS-PAGE standards, broad range; Bio-Rad Laboratories, Richmond, CA) are indicated.

of protein tyrosine phosphorylation induced by IL-6 and IL-11 in murine B9E hybridoma and T10D plasmacytoma cells (26; see "Materials and Methods") that require either IL-6 or IL-11 for their continued survival and proliferation in culture (30). As shown in Fig. 1*A*, IL-6 and IL-11 induced dose-dependent increases in the tyrosine phosphorylation status of a similar set of proteins with molecular masses of 160, 130, 85 and 70 kDa in B9E cells. Although T10D cells displayed a significantly different basal pattern of protein tyrosine phosphorylation, treatment with either IL-6 or IL-11 also stimulated dose-dependent increases in tyrosine phosphorylation of 160- and 85-kDa proteins and, less so, a 70-kDa protein (Fig. 1*B*). The levels of tyrosine phosphorylation of the 160- and 85-kDa proteins induced by IL-6 or IL-11 in T10D cells were lower than those observed in B9E cells treated with comparable amounts of these cytokines; overall, the biochemical responses paralleled the IL-6 and IL-11 dose dependencies of the cell lines for growth (4,13,25,26). The induction of tyrosine phosphorylation of the 160-, 130- and 70-kDa proteins by both IL-6 and IL-11 in B9E cells was rapid and transient, with maximum

levels being reached 5-15 min after stimulation then decreasing by 45 min. In contrast, tyrosine phosphorylation of the 85-kDa protein was detectable at 5 min and the level increased at 15 and 45 min (Fig. 1*C*). Similar time course analysis with T10D cells revealed that the 160- and 85-kDa proteins were tyrosine phosphorylated within 5 min in response to both factors and the levels remained the same or slightly increased at 15 and 45 min (Fig. 1*D*).

To investigate whether JAK-TYK kinases were tyrosine phosphorylated in B9E and T10D cells treated with IL-6 or IL-11, we performed immunoprecipitation experiments with antisera recognizing JAK1, JAK2 and Tyk2 followed by immunoblotting with anti-pTyr mAb. As shown in Fig. 2*A*, the phosphotyrosine content of Tyk2 and, to a lesser extent, that of JAK1 increased in a dose-dependent manner in B9E cells stimulated with IL-6, whereas there was only a marginal change in the tyrosine phosphorylation status of JAK2 at the highest dose of IL-6 employed in these experiments. To ensure that these differences were the result of tyrosine phosphorylation per se and not due to differences in quantity of protein loaded, the blots were stripped and reprobed with antisera specific for JAK1, JAK2 or Tyk2, respectively (Fig. 2*A*). In all cases approximately equal amounts of the respective kinases were detected in a particular

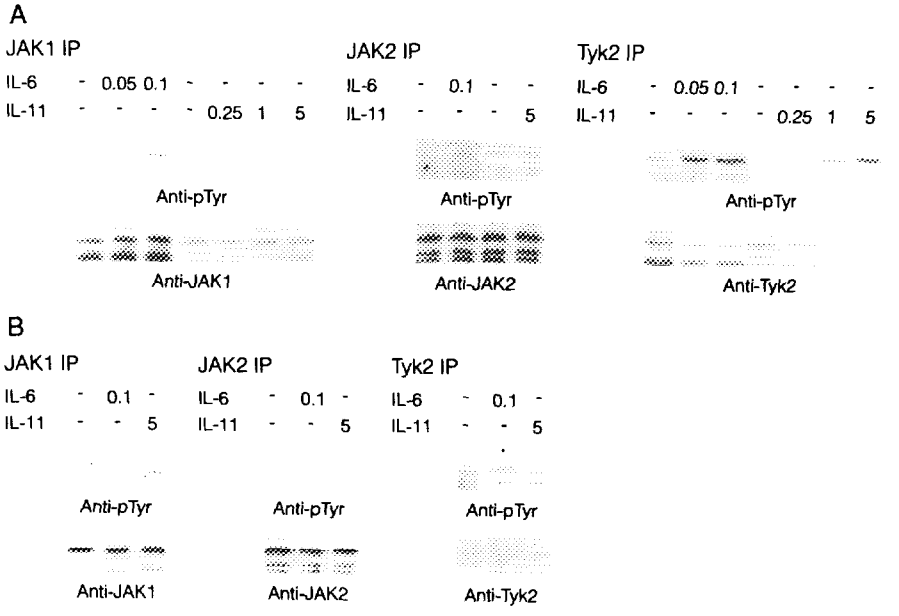

Figure 2. **Tyrosine phosphorylation of JAK-TYK kinases in response to IL-6 and IL-11 in B9E and T10D cells.** IL-11-starved B9E (*A*) and T10D (*B*) cells were stimulated with or without (-) the indicated concentrations (μg/ml) of IL-6 or IL-11 for 5 min at 37 °C. Cells were lysed and samples equivalent to 2 x 10^7 cells were immunoprecipitated (IP) with antisera to the indicated JAK-TYK kinases. Samples were initially subjected to immunoblot analysis with anti-pTyr mab 4G10 (upper panel of each pair). Membranes were then stripped and reprobed with the appropriate anti-JAK-TYK kinase antisera as indicated (lower panel of each pair) as described in "Materials and Methods".

experiment. A minor increase in the phosphotyrosine content of Tyk2 in the absence of any significant changes in JAK1 or JAK2 tyrosine phosphorylation was also detected following IL-6 treatment of T10D cells with the highest concentration of cytokine used (Fig. 2*B*). Reprobing of the blots with antisera specific for each of the JAK-TYK kinases indicated that all three kinases were present in amounts comparable to those observed in B9E cells (Fig. 2*B*). IL-11 also induced tyrosine phosphorylation of Tyk2 and JAK1 in B9E cells (Fig. 2*A*) and Tyk2 in T10D cells (Fig. 2*B*), demonstrating that the IL-11 signaling pathway shares common upstream tyrosine kinases with that of IL-6 in cells which respond to both cytokines.

We also examined two IL-6-responsive human myeloma cell lines, ANBL-6 and OCI-My4, for changes in protein tyrosine phosphorylation after stimulation with IL-6 and IL-11 (Fig. 3*A*). Treatment of ANBL-6 cells with IL-6 resulted in increased tyrosine phosphorylation of proteins with molecular masses of 160, 85, 80, 76, 70, 56, 44 and 42 kDa. A diffuse signal was also

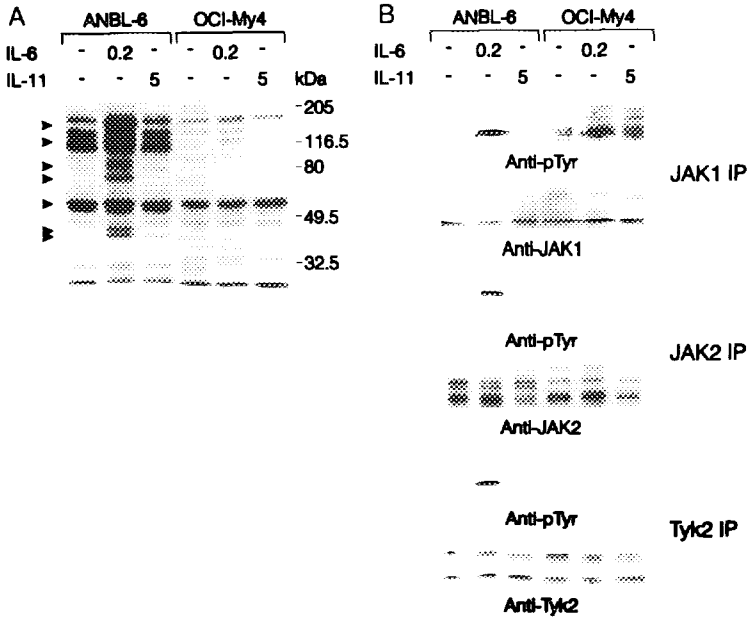

Figure 3. **JAK-TYK kinases are among the proteins tyrosine phosphorylated in response to IL-6 in human myeloma cells, ANBL-6 and OCI-My4.** IL-6-starved ANBL-6 or OCI-My4 cells were stimulated with or without (-) the indicated concentrations (μg/ml) of IL-6 or IL-11 for 10 min at 37 °C. (*A*) Proteins (20 μg) in whole cell lysates were subjected to immunoblot analysis with the anti-pTyr mab 4G10 (1:1000 dilution). Arrows denote the tyrosine phosphorylated proteins discussed in the text. The sizes of molecular mass markers are indicated. (*B*) Whole cell lysates prepared from ANBL-6 (600 μg) and OCI-My4 (850 μg) were subjected to sequential immunoprecipitations (IP) with antisera to JAK1 (4.5 μL), JAK2 (3 μL) and Tyk2 (4 μl). Immunoblot analysis with anti-pTyr mAb 4G10 (upper panel of each pair) and anti-JAK-TYK kinase antisera as indicated (lower panel of each pair) was carried out as described in Fig. 2. The anti-pTyr immunoblot of JAK1 immunoprecipitates from OCI-My4 lysates was exposed 25 times longer than the corresponding anti-pTyr immunoblot of JAK1 immunoprecipitates from ANBL-6 lysates.

detected in the 115- to 120-kDa range, suggesting the tyrosine phosphorylation of multiple comigrating proteins in this region of the blot. Elevated tyrosine phosphorylation of a subset of these proteins, including the 160-, 115- to 120- and 70-kDa species, was also observed in OCI-My4 cells stimulated with IL-6, although the magnitude of the response was substantially less than that observed for ANBL-6 cells. The lower levels of tyrosine phosphorylation detected in IL-6-treated OCI-My4 cells is in accord with biological data showing that the proliferative response of this line to IL-6 is largely restricted to the clonogenic cells which comprise less than 1% of the total cell population (7). In neither cell line did stimulation with IL-11 result in the appearance of any tyrosine phosphorylated proteins, concordant with its lack of biological activity on these cells (unpublished observations). These latter findings are congruous with the view that IL-11 is not a major growth factor for human myeloma cells (26,31,32).

While this work was in progress, Yancopoulos and colleagues (19) reported that IL-6 induced tyrosine phosphorylation of JAK1 and Tyk2 but not JAK2 in human U266 myeloma cells. By comparison, the tyrosine phosphorylation events observed following IL-6 treatment of ANBL-6 cells were accompanied by prominent tyrosine phosphorylation of all three JAK-TYK kinases (Fig. 3*B*), with the magnitude of the JAK1 response being greater than that of JAK2 and Tyk2. On the other hand, only JAK1 was tyrosine phosphorylated in IL-6-stimulated OCI-My4 cells (Fig. 3*B*), albeit to a significantly lower degree than in ANBL-6 cells. As expected from the lack of a detectable global tyrosine phosphorylation response in the immunoblotting experiments with whole cell lysates of IL-11-treated ANBL-6 and OCI-My4 cells, no apparent increase in tyrosine phosphorylation of JAK-TYK kinases was noted in either cell line after stimulation with IL-11 (Fig. 3*B*).

Interferon-induced gene expression through JAK-TYK kinases involves tyrosine phosphorylation of latent cytoplasmic transcription factors (termed STATs for signal transducers and activators of transcription) (reviewed in ref. 24). One such protein, a 91-kDa subunit (denoted Stat91) of the transcription factor interferon-stimulated gene factor-3, has been shown to be tyrosine phosphorylated in human hepatoma cells exposed to IL-6-type cytokines (20,33) and in human neuroblastoma cells stimulated with LIF and CNTF (34), suggesting that it also participates in signaling mediated by members of the IL-6 subfamily of cytokines. IL-6 treatment of ANBL-6 cells induced marked tyrosine phosphorylation of Stat91. This was recognizable as a shift in mobility in immunoblots of whole cell lysates with specific antisera recognizing Stat91 (Fig. 4*A*) and confirmed by direct probing of immunoprecipitates for phosphotyrosine with the anti-pTyr mAb (Fig. 4*B*). Tyrosine phosphorylation of Stat84, encoded by an alternatively spliced mRNA and which is usually present at lower levels than Stat91 (35), was also induced. Similar analysis revealed that the tyrosine phosphorylation status of Stat91 was unchanged in OCI-My4 cells following treatment with IL-6 (Fig. 4), while neither Stat91 nor any immunologically

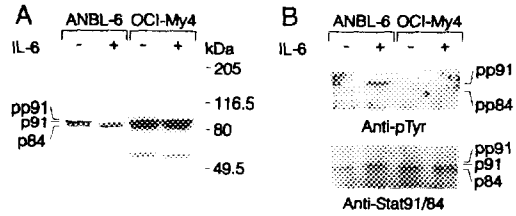

Figure 4. **IL-6 induces tyrosine phosphorylation of Stat91 in ANBL-6 cells.** IL-6-starved ANBL-6 or OCI-My4 cells were stimulated with (+) or without (-) IL-6 (0.2 μg/ml) for 10 min at 37 °C. (*A*) Proteins (20 μg) in whole cell lysates were subjected to immunoblot analysis with the anti-Stat91/84 antisera. The unmodified (p91) and modified (pp91) forms of Stat91 are indicated as is Stat84 (p84). The sizes of molecular mass markers are indicated. (*B*) Lysates prepared from ANBL-6 and OCI-My4 cells were immunoprecipitated with the anti-Stat91/84 antisera (12 μl). Samples were subjected to immunoblot analysis with anti-pTyr mab 4G10 (upper panel) and then reprobed with the anti-Stat91/84 antisera (lower panel). Slight phosphorylation of Stat84 (pp84) was also observed.

crossreacting proteins could be detected in B9E or T10D cells with the anti-Stat91/84 antisera used in this study (data not shown).

The data presented show that IL-6 and, where applicable, IL-11 consistently induced tyrosine phosphorylation of a 160-kDa protein in all murine and human plasma cell lines examined. This finding strengthens the notion that the 160-kDa protein initially reported to be tyrosine phosphorylated in response to IL-6 in another murine hybridoma (29) and subsequently observed to be tyrosine phosphorylated in a murine myeloid leukemic cell line treated with IL-6 or LIF (36) is a primary substrate of the tyrosine kinase activity induced by IL-6-type cytokines in responsive cells. Moreover, it is tempting to speculate that the 85- and 70-kDa tyrosine-phosphorylated proteins described in this report may also be important substrates of gp130-induced tyrosine kinase activity in plasma cells.

We have identified JAK-TYK kinases as potential candidates for the tyrosine kinase activity regulating the viability and/or proliferation of plasma cells whose growth is dependent on IL-6. Considered together with other data (19), the novel finding reported here is that the patterns of JAK-TYK tyrosine phosphorylation in response to IL-6 vary among plasma cell lines. Nonetheless, IL-11, the newest member of the IL-6 subfamily of cytokines, was found to induce the same patterns of JAK-TYK tyrosine phosphorylation as IL-6 in cells that are responsive to both cytokines. These results thus extend previous observations that IL-6-type cytokines induce distinct profiles of JAK-TYK kinase activation in *different cell types* while apparently exhibiting functional equivalency in stimulating tyrosine phosphorylation of the same combinations of JAK-TYK kinases in a given responsive cell (19-22). Additional variability in the JAK-TYK signaling cascades triggered through gp130 in the plasma cell lines was revealed at the level of the transcription factor Stat91, the activation of which by tyrosine phosphorylation was previously

shown to correlate with JAK-TYK tyrosine phosphorylation in cells treated with interferons (reviewed in ref. 24). These observations suggest that novel STATs identified in other cell types (20,33,34,37-39) are operative in plasma cells. It is hypothesized that the ability of different JAK-TYK/STAT signaling pathways to activate common gene sets has been exploited by this tumor for propagation of a mitogenic signal in response to IL-6-type cytokines. However, further studies are necessary to determine the relative importance of other nonreceptor tyrosine kinases in the growth control of plasma cell neoplasms using gp130 (40,41).

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