Hepatoma Hep3B cell lines stably expressing a temperature-sensitive p53 species (p53-Val-135) displayed a reduced response to interleukin-6 (IL-6) when cultured at the wild-type (wt) p53 temperature (Wang, L., Rayanade, R., Garcia, D., Patel, K., Pan, H., and Sehgal, P. B. (1995) J. Biol. Chem. 270, 23159–23165). We now report that in such cultures IL-6 caused a rapid (20–30 min) and marked loss of cellular immunostaining for STAT3 and STAT5 immunostaining was transient (lasted 120 min) and tyrosine kinase-dependent, and even though the loss was blocked by the proteasome inhibitors MG132 and lactacystin it was not accompanied by changes in cellular levels of STAT3 and STAT5 proteins suggesting that IL-6 triggered a rapid masking but not degradation of these transcription factors. STAT3 and STAT5 masking was accompanied by a reduction in IL-6-induced nuclear DNA-binding activity. The data suggest that p53 may influence Jak-STAT signaling through a novel indirect mechanism involving a wt p53-dependent gene product which upon cytokine addition is activated into a “STAT-masking factor” in a proteasome-dependent step.

The cellular protein p53 has been implicated in the regulation of mammalian cell processes such as proliferation, apoptosis, and DNA repair either directly as a transcription factor which modulates expression of specific regulatory genes or indirectly as a transcription factor which up-regulates the expression of genes whose products, in turn, then regulate other cellular regulatory proteins (1–8). As an example of the latter, the inhibitory effect of p53 on the cell cycle is attributed to the ability of p53 to increase the transcriptional expression of p21, a cellular protein which inhibits cyclin kinases, thus inhibiting the function of cyclins, a class of regulatory proteins required for cell proliferation (5–8). Despite clear evidence of the influence of cytokines upon p53-induced cellular processes, for example the rescue of p53-induced apoptosis in myeloid cells by the cytokine interleukin-6 (IL-6)1 (9–14), there is little information concerning the influence, direct or indirect, of p53 upon cytokine-elicited cellular signaling through the Janus kinase-signal transducers and activators of transcription (Jak-STAT) pathway.

We previously utilized a constitutive expression vector for the temperature-sensitive p53 mutant (p53-Val-135) (2, 9) to obtain a series of stably transfected human hepatoma Hep3B cell lines that displayed a reduced response to IL-6 at the wild-type (wt) p53 temperature (32.5 °C) (14). The temperature dependence of the secretion of plasma proteins such as β-fibrinogen and α1-antichymotrypsin in response to IL-6 by these p53-Val-135-expressing Hep3B lines, but not by the pSVneo control cell lines or the p53-free parental Hep3B cells, suggested that wt p53 negatively influenced cytokine signaling (14). In as much as the transcriptional response to IL-6 of the β-fibrinogen and α1-antichymotrypsin genes is primarily through activation of STAT3 and STAT5 transcription factors through the Jak-STAT signaling pathway (15–18), our previous data suggested that p53 might, directly or indirectly, influence Jak-STAT signaling in these p53-Val-135-expressing Hep3B cell lines at 32.5 °C (the wt p53 temperature).

In the present study, we used confocal immunofluorescence microscopy to investigate the fate of transcription factors involved in Jak-STAT signaling upon addition of IL-6 to cultures of mutant or wt p53-containing Hep3B cells. It has been shown previously that the multicatalytic 26 S proteasome is involved in degradation or processing of many cellular proteins which include the transcription factors NF-κB (p50), I-κBα, c-Jun, and c-Fos (19–28). The use of proteasome inhibitors such as MG132 (19–24) and lactacystin (25–28) has greatly assisted in delineating the role of proteasomes in the turnover/processing of cellular transcription factors. For example, the cytokine-induced phosphorylation-dependent degradation of I-κBα through the ubiquitin-proteasome pathway is now well characterized (21–23). The data obtained in the present study suggest that p53 may influence Jak-STAT signaling through a novel indirect mechanism involving a wt p53-dependent gene product which upon IL-6 addition is rapidly activated into a STAT3 and STAT5 “masking” factor in a proteasome-dependent step.

**MATERIALS AND METHODS**

p53-Val-135-expressing Hepatoma Hep3B Cell Lines—A series of 11 stably transfected cell lines constitutively expressing p53-Val-135 and seven control cell lines expressing pSVneo alone were derived from the p53-free human hepatoma Hep3B line as has been described earlier (14). Line 1 and Line 5 cells which express p53-Val-135 were used in the data shown in this communication.

**Immunofluorescence Analyses for STAT Transcription Factors**—For immunofluorescence assays, cells were planted in wells of 8-chamber slides (Nunc; 2 × 10⁶ cells/chamber) in 0.3 ml of Dulbecco’s modified Eagle’s high glucose medium supplemented with 1 mm l-glutamine, 2 mm sodium pyruvate, 1% non-essential amino acids, and 10% (v/v) fetal bovine serum (all obtained from Life Technologies, Inc.). The cultures were incubated at 37 °C for 18–24 h and then either continued at 37 °C

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‡‡ To whom correspondence should be addressed: Dept. of Cell Biology & Anatomy, Basic Science Bldg., New York Medical College, Valhalla, NY 10595. Tel.: 914-993-4196; Fax: 914-993-4925.

1 The abbreviations used are: IL-6, interleukin-6; Jak, Janus kinase; mAb, monoclonal antibody; PBS, phosphate-buffered saline; STAT, signal transducer and activator of transcription; ts, temperature-sensitive; wt, wild type.
or shifted to 32.5 °C for 18–24 h. All cultures were then washed twice with phosphate-buffered saline (PBS) previously equilibrated to the respective temperatures and replenished with 0.3 ml of serum-free medium also previously equilibrated at the respective temperatures.

Four hours after the switch to serum-free medium, the cells were treated with human IL-6 (Escherichia coli-derived, a gift from Sandoz) (30 ng/ml) for 30 min. At the end of the IL-6 treatment, the cultures were washed with ice-cold PBS, drained, and fixed with cold methanol/acetic acid (2:1) for 10 min, the fixative was drained, and the slides were allowed to air dry. Immunostaining using various monoclonal and polyclonal antibodies was carried out as per the instructions provided by the respective antibody suppliers using fluorescein-tagged goat anti-mouse IgG or rhodamine-tagged goat anti-rabbit IgG (Cappel Organon Teknika). Cellular immunofluorescence was evaluated using a Bio-Rad MRC 1000 dual laser confocal microscopy system. Murine monoclonal antibodies to STAT1, STAT3, and STAT5a (marketed as anti-"STAT5") were purchased from Transduction Laboratories, murine monoclonal antibody to STAT5b and rabbit polyclonal antibody to STAT3 were purchased from Santa Cruz Biotec, and anti-p53 monoclonal antibodies PAb240 and PAb421 were purchased from Oncogene Research Products/Calbiochem.

**RESULTS AND DISCUSSION**

The confocal immunofluorescence data in Fig. 1A confirm the temperature-sensitive (ts) phenotype of the conformation of p53-Val-135 expressed in Line 5: mutant conformation at 37 °C and wt conformation at 32.5 °C. All 11 p53-Val-135-expressing Hep3B cell lines showed this ts phenotype with respect to p53 conformation. None of the seven pSVneo control lines nor the parental Hep3B cells showed immunostaining for p53. When cultures of Line 5 cells were first incubated at 37 °C for 1 day, shifted to 32.5 °C for 18–20 h, and then exposed to IL-6 for 30 min, there was a marked loss of STAT3 and STAT5 immunostaining but not that of STAT1 (Fig. 1B). Cultures kept throughout at 37 °C did not display the loss of STAT3 and STAT5 immunostaining but did not of STAT1 (Fig. 1B). Cultures kept throughout at 37 °C but not at 32.5 °C for 1 day, showed a loss of STAT3 and STAT5 immunostaining 30 min after IL-6 addition at 32.5 °C. All 11 of the p53-Val-135-expressing Hep3B cell lines showed a loss of STAT3 and STAT5 immunostaining 30 min after IL-6 addition at 32.5 °C, whereas none of the 7 pSVneo control lines nor the parental Hep3B cells displayed any loss of STAT3 or STAT5 immunostaining upon IL-6 addition at either 32.5 or 37 °C (data not shown).

The loss of STAT3 and STAT5 immunostaining as illustrated in Fig. 1B (i) was IL-6-induced and rapid that a reduction in immunostaining was observed within 10 min and there was almost complete loss by 20–30 min after IL-6 addition, (ii) was transient in that STAT3 and STAT5 immunostaining returned 120–240 min after IL-6 addition, (iii) was dependent upon cytokine concentration in that, when assayed 30 min after IL-6 addition, the loss of immunostaining was elicited by IL-6 at 0.3 ng/ml, was near-maximal at 3–10 ng/ml, and was still evident at 100 ng/ml, (iv) was selective in that a loss of immunostaining was not observed for STAT1, STAT4, STAT6, RelA (p65), C/EBPα, β, γ, and Sp1 transcription factors, (v) was cytokine-specific in that interferon-γ elicited a modest reduction in STAT3 immunostaining but no loss of STAT5 or STAT1 immunostaining, and epidermal growth factor elicited no changes in immunostaining of any STAT transcription factors, (vi) required that the p53-Val-135-expressing Hep3B cells be incubated for at least 18–20 h at the wt p53 temperature (32.5 °C) in that a 5- or 10-h incubation at 32.5 °C was insufficient to elicit this phenotype, and (vii) was not dependent upon rapid de novo protein synthesis upon IL-6 addition in that the protein synthesis inhibitor cycloheximide added 30 min prior to IL-6 did not inhibit the cytokine-induced loss of STAT3 or STAT5 immunostaining (data not shown).

The loss of IL-6-triggered STAT3 immunostaining was ob-
served using an anti-STAT3 monoclonal antibody or an anti-STAT3 polyclonal antibody each raised to different peptides from different regions of the STAT3 amino acid sequence (data not shown). Similarly, the loss of IL-6-triggered STAT5 immunostaining was observed using an anti-STAT5a monoclonal antibody (which cross-reacts with STAT5b in its native form) as well as using an anti-STAT5b monoclonal antibody (data not shown). In these Hep3B cell lines, significant nuclear immunostaining for STAT1 and STAT5, as in Fig. 1B, was observed even in the absence of IL-6 addition. IL-6 addition did not result in any clear cytoplasm to nuclear translocation of STAT1 or STAT5 immunostaining. In the case of STAT3, although cytoplasmic immunostaining predominated, some nuclear staining was always seen even in the absence of IL-6 addition. There was increased STAT3 nuclear staining upon IL-6 addition at 37°C (as in Fig. 1B).

The dramatic loss of STAT3 and STAT5 immunostaining upon IL-6 addition to Line 5 cells at 32.5°C was dependent upon tyrosine kinase activity in that it was blocked by the tyrosine kinase inhibitor genistein and by staurosporine but not by the protein kinase C inhibitor H7 (Fig. 2) (15–18). Tyrosine phosphorylation of STAT3 and STAT5 upon IL-6 addition is known to occur within 1–5 min and to reach a maximum by 20–30 min (15–18) consistent with the time course of disappearance of STAT3 and STAT5 immunostaining (Fig. 1 and data not shown).

The cytokine-triggered loss of STAT3 and STAT5 immunostaining factors suggested the involvement of proteasomes in this phenomenon. Fig. 3 shows that the proteasome inhibitors MG132 and lactacystin both blocked the IL-6-induced loss of STAT3 and STAT5 immunostaining providing evidence for a proteasome-dependent step in this mechanism.

The possibility that the loss of STAT3 and STAT5 immunostaining was the result of a degradation of the respective STAT proteins was evaluated by Western blot analyses. The total cellular amounts of STAT3 and STAT5b (Hep3B cells contain STAT5b but little STAT5a; Refs. 15–18 and 30)2 showed no change upon addition of IL-6 (Fig. 4A) or upon addition of IL-6 in combination with MG132 or lactacystin (Fig. 4B). Thus the marked loss of STAT3 and STAT5 immunostaining was not accompanied by a loss of STAT3 and STAT5 Western blottable proteins suggesting that the loss of immunostaining was an IL-6-triggered masking of STAT3 and STAT5 proteins and not the result of the degradation of these transcription factors. The observation that p53-Val-135-containing cultures had to be incubated at the wt p53 temperature (32.5°C) for at least

2 R. J. Rayanade and P. B. Sehgal, unpublished data.
18–20 h before the IL-6-induced loss of STAT3 and STAT5 immunostaining could be elicited suggested that a wt p53-induced gene product which accumulated in these cells in the 18–20-h period was activated in a proteasome- and tyrosine phosphorylation-dependent step upon IL-6 addition into a novel STAT-masking factor. The simplest possibility is that upon IL-6 addition a wt p53-induced protein is proteasomally processed into the STAT-masking factor which binds tyrosine-phosphorylated STAT3 and STAT5.

The functional consequences of STAT-factor masking and the effect of proteasomal inhibitors upon nuclear STAT-DNA-binding activity were evaluated in experiments in which nuclear extracts prepared from IL-6-treated p53-Val-135-expressing Hep3B cells incubated at either 37 °C or 32.5 °C were evaluated for their ability to bind a canonical STAT-binding DNA element. Fig. 5 shows that, consistent with the masking phenomenon illustrated in Fig. 1, IL-6 had a reduced ability to elicit STAT3 homodimer DNA-binding activity ("Complex A") in nuclear extracts prepared from cells at 32.5 °C compared to cells at 37 °C. Similar data were obtained in experiments with both Line 1 and Line 5 cell lines. As a control, there was no difference in the levels of interferon-γ-activated STAT1 DNA-binding activity detected in the nuclear extracts of Line 1 or Line 5 cells cultured at either 37 °C or 32.5 °C (data not shown), consistent with the observation that immunostaining for STAT1 was not "masked" upon interferon-γ-treatment of Line 5 (or Line 1) cells at 32.5 °C.

Fig. 5 also shows that, at both temperatures, the inclusion of MG132 increased STAT-DNA-binding corresponding to Complex C (STAT1 homodimer), Complex B (STAT1/3 heterodimer), Complex A (STAT3 homodimer), as well as a slower mobility complex (Complex A*, presumably corresponding to STAT5), an observation consistent with the effect of MG132 on STAT-specific cellular immunofluorescence (Fig. 3). The increase in STAT1 DNA-binding complexes ("Complex C") in IL-6-treated cells which had also been exposed to MG132 extends a recent report implicating proteasomes in the turnover/degradation of ubiquitinated and tyrosine-phosphorylated STAT1 protein in interferon-γ-treated cells (31).

Prior data from many laboratories have clearly established the ability of cytokines such as IL-6 to influence p53-dependent cellular events and vice versa (9–14). The underlying mechanisms have remained elusive. The cytokine-triggered proteasome- and p53-dependent masking of STAT-family transcription factors described in this report represents a novel indirect mechanism for the regulation of cytokine responses by a wt p53-induced gene product. The eventual identification and characterization of the p53-induced STAT-masking factor and the elucidation of its physiological function is likely to add a new, proteasomal, dimension to our understanding of the regulation of cytokine-activated Jak-STAT signaling.

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