Involvement of Proteasomes in Regulating Jak-STAT Pathways upon Interleukin-2 Stimulation*

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Interleukin-2 (IL-2) activates the receptor-associated Janus family tyrosine kinases, Jak1 and Jak3, which in turn phosphorylate and activate specific STAT proteins (signal transducers and activators of transcription), such as STAT5. Activation of Jak and STAT proteins by IL-2 is transient and the mechanism for the subsequent down-regulation of their activity is largely unknown. We report here that IL-2-induced DNA-binding activity and tyrosine phosphorylation of STAT5 are stabilized by a proteasome inhibitor MG132; however, no detectable ubiquitination of the STAT proteins is observed. This sustained STAT5 activation can be blocked by protein kinase inhibitors, which is consistent with the ability of the proteasome inhibitor to stabilize IL-2-induced tyrosine phosphorylation of Jak1 and Jak3. These results suggest that proteasome-mediated protein degradation modulates protein-tyrosine phosphatase activity that negatively regulates the Jak-STAT signaling pathways.

Important insights into gene regulation by cytokines have been gained from recent studies of the Janus kinase (Jak) family of nonreceptor protein-tyrosine kinases and the signal transducers and activators of transcription (STATs) (1–3). Members of the cytokine receptor superfamily recruit the Jak family kinases to phosphorylate and activate downstream STAT proteins. STAT proteins are latent cytoplasmic transcription factors that, upon activation by tyrosine phosphorylation, translocate to the nucleus and bind to specific regulatory elements that control gene expression. Diversity in cellular responses to different cytokines is determined in part by selective association of cytokine receptors with different Jak kinases and subsequent phosphorylation of different STAT proteins (4–6).

Similar to many other mitogen-induced signaling events (7–9), cytokine-induced activation of the Jak-STAT signal transduction pathway is both rapid and transient (10–12). While the mechanisms involved in Jak and STAT activation are being delineated, the mechanisms underlying their subsequent deactivation are still largely unknown. Based on the observation that there is a good correlation between tyrosine dephosphorylation and inactivation of both Jak and STAT proteins, the roles of protein-tyrosine phosphatases in down-regulating the Jak-STAT pathways have been suggested. Indeed, there is genetic and biochemical evidence indicating that protein-tyrosine phosphatases are critical in regulating distinct Jak kinases within specific cytokine receptor complexes (13–15). The evidence for the involvement of specific protein-tyrosine phosphatases in dephosphorylating STAT proteins, however, is more indirect (16–18).

Another important mechanism in regulating signal transduction is proteolysis (19, 20). The ubiquitin-proteasome pathway, in particular, plays an important role in degrading a number of cellular proteins, including transcription factors (21–23). The target proteins are first tagged with multiple molecules of the small protein ubiquitin and then destroyed by the multisubunit proteasome complex. Recently, there have been reports indicating the potential role of proteasome-mediated proteolysis in down-regulating STAT1 activity following interferon-γ (IFN-γ) stimulation. However, one report provided evidence that STAT1 itself is ubiquitinated and degraded (24), while another report suggested that its upstream signaling pathway is the target of proteasomes (18).

In this report, we address the possible role of proteasome-mediated protein degradation in modulating STAT5 activity following stimulation with interleukin-2 (IL-2) to determine if proteasomes are involved in regulating other STAT proteins in response to diverse cytokines. Furthermore, we investigate whether the STAT proteins themselves or the upstream signaling molecules, such as the Jak kinases, are modulated through the ubiquitin-proteasome pathway.

**EXPERIMENTAL PROCEDURES**

**Cells**—Maintenance of the IL-2-dependent cell line CTLL-20 has been described earlier (25). For IL-2 stimulation experiments, exponentially growing CTLL-20 cells were washed three times in RPMI 1640 medium, resuspended in medium without IL-2, and starved for 4 h at 37 °C. IL-2-deprived CTLL-20 cells were then stimulated with 30 units/ml of recombinant human IL-2 for various times as indicated in the figure legends.

**Reagents**—Recombinant human IL-2 was a gift of Dr. J. Sepinwall (Hoffmann-La Roche Inc., Nutley, NJ). The oligonucleotides specific for STAT5 binding were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) (sc-2565). The protease inhibitor MG132 and calpain inhibitor II were from Peptide Institute, Inc. (Louisville, KY) and Boehringer Mannheim, respectively. Me₃SO, genistein, and staurosporine were from Sigma. Anti-STAT5 antibodies used for immunoprecipitation and immunoblotting were from Santa Cruz Biotechnology, Inc. (sc-835) and Transduction Laboratories (Lexington, KY) (S21520), respectively. Anti-Jak1 antibodies used for immunoprecipitation and immunoblotting were from Upstate Biotechnology, Inc. (Lake Placid, NY) (06-272) and Transduction Laboratories (J24320), respectively. Anti-Jak3 antibodies used for immunoprecipitation and immunoblotting were from Upstate Biotechnology (06-342) and Santa Cruz Biotechnology, Inc. (sc-1079), respectively. Horseradish peroxidase-conjugated anti-phosphotyrosine antibody RC20H was obtained from Transduction Laboratories (Santa Cruz, CA) (sc-2565). The proteasome inhibitor MG132 and calpain inhibitor II were from Peptide Institute, Inc. (Louisville, KY) and Boehringer Mannheim, respectively. Me₃SO, genistein, and staurosporine were from Sigma. Anti-STAT5 antibodies used for immunoprecipitation and immunoblotting were from Santa Cruz Biotechnology, Inc. (sc-835) and Transduction Laboratories (Lexington, KY) (S21520), respectively. Anti-Jak1 antibodies used for immunoprecipitation and immunoblotting were from Upstate Biotechnology, Inc. (Lake Placid, NY) (06-272) and Transduction Laboratories (J24320), respectively. Anti-Jak3 antibodies used for immunoprecipitation and immunoblotting were from Upstate Biotechnology (06-342) and Santa Cruz Biotechnology, Inc. (sc-1079), respectively. Horseradish peroxidase-conjugated anti-phosphotyrosine antibody RC20H was obtained from Transduction Laboratories.

**Electrophoretic Mobility Shift Assay (EMSA)**—CTLL-20 cells were washed once with PBS supplemented with 1 mM Na₂VO₄ (ortho) and once with PBS supplemented with 1 mM NaF (orthos) and 5 mM NaF. Nuclear extracts were prepared from washed cell pellets essentially as described earlier (26), except for the modification of the hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, and 10 mM KCl). STAT5 gel shift

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oligonucleotides were labeled with T4 polynucleotide kinase in the presence of [γ-32P]ATP. EMSA was performed as described previously (26).

**Immunoprecipitation and Immunoblotting**—CITLL-20 cells were washed once with PBS supplemented with 1 mM Na3VO4 (ortho) and 10 mM NaF. Preparation of cell lysates, immunoprecipitation, and immunoblotting were done essentially as described elsewhere (24, 27). Dilutions of different antibodies for immunoprecipitation and immunoblotting and subsequent detection by the enhanced chemiluminescence (ECL) system were performed as recommended by the manufacturers.

**RESULTS**

**Proteasome Inhibitor MG132 Stabilizes IL-2-induced STAT5 Activation**—To determine if proteasome-mediated proteolysis is involved in modulating STAT activity in response to cytokines, such as IL-2, we examined the effects of a proteasome inhibitor (MG132) on STAT5 activation induced by IL-2 (28, 29). An IL-2-dependent cell line CITLL-20 was deprived of IL-2, pretreated with the carrier Me2SO, MG132, or a cyssteine protease inhibitor (calpain inhibitor II) for 1 h and then stimulated with IL-2 for 15 min to 2 h. We analyzed nuclear extracts by EMSA using a labeled probe specific for STAT5 DNA binding activity (Fig. 1). In Me2SO-treated cells, IL-2 induced rapid and transient STAT5 DNA binding activity (lanes 1–6). This DNA binding activity involves both STAT5a and STAT5b, as determined by supershift assays using specific antibodies (data not shown). This IL-2-induced STAT5 DNA binding activity was sustained in the presence of MG132 (lanes 7–12), but not calpain inhibitor II (lanes 13–15). This result demonstrates that proteasome-mediated protein degradation, but not proteinolysis in general, is involved in modulating IL-2-induced STAT5 activity.

**Tyrosine-phosphorylated, Active STAT5 Proteins Are Not Directly Degraded through the Ubiquitin-Proteasome Pathway**—In many cases, phosphorylation of target proteins is required for their ubiquitination and subsequent degradation by proteasomes (24, 27, 30, 31). To determine whether active STAT5 is directly degraded by proteasomes, we prepared cell lysates from IL-2-stimulated cells in the absence or presence of MG132 under conditions that should preserve ubiquitinated proteins (24, 27). Anti-STAT5 immunoprecipitates were prepared from cell lysates and analyzed by protein immunoblotting with antibody to phosphotyrosine or anti-STAT5 mAb (Fig. 2). The anti-STAT5 antibodies used for immunoprecipitation and immunoblotting recognize both STAT5a and STAT5b. As a result, STAT5 proteins appear as broad bands on immunoblots, and the bands become even broader when some of the STAT5 proteins are phosphorylated and migrate slower in SDS-polyacrylamide gels (32).

Consistent with its DNA binding activity, IL-2-induced tyrosine phosphorylation of STAT5 is prolonged by MG132 (Fig. 2, lanes 9–14 compared with lanes 3–8). Proteins conjugated with multiple molecules of ubiquitin appear as a ladder of higher molecular mass proteins in SDS-polyacrylamide gels (24, 27). We could not detect any ubiquitinated STAT5 in the presence of MG132 either in phosphotyrosine or STAT5 immunoblots. This result suggests that inactivation of STAT5 following IL-2-induced activation is mostly due to dephosphorylation, but not removal of active STAT5 through a ubiquitin-proteasome pathway.

To further confirm that tyrosine-phosphorylated STAT5 is not the direct target of proteasomes, we examined the effects of protein kinase inhibitors on IL-2-induced DNA-binding activity (Fig. 3A) and tyrosine phosphorylation (Fig. 3B) of STAT5. Two protein kinase inhibitors were tested: genistein, a protein-tyrosine kinase inhibitor, and staurosporine, a broad spectrum protein kinase inhibitor. Both inhibitors have been shown to block cytokine-induced STAT activation (18, 24, 33). We first stimulated CITLL-20 cells, which were pretreated with either Me2SO or MG132, with IL-2 to accumulate a pool of active STAT5 (lanes 2 and 9). Genistein, staurosporine, or carrier Me2SO were then added to block further upstream signaling that would activate STAT5.

As expected, in the absence of MG132, treatment with both protein kinase inhibitors results in rapid dephosphorylation of STAT5 with a rapid decrease in STAT5 DNA binding activity (lanes 5–8). If active STAT5 proteins are directly destroyed by proteasomes, MG132 should still be able to stabilize these pre-activated proteins, even without continuous generation of active STAT5. Consistent with our previous conclusion that proteasomes do not directly degrade tyrosine-phosphorylated,
Proteasomes Modulate Jak-STAT Signaling Induced by IL-2

FIG. 3. Protein kinase inhibitors abolish sustained IL-2-induced STAT5 activation in the presence of MG132. Equal numbers of IL-2-deprived CTLL-20 cells were pretreated with 0.1% MeSO (DMSO) (lanes 1–8) or 50 μM MG132 (lanes 9–15) for 1 h and then left unstimulated (lane 1) or stimulated with IL-2 for 15 min (lanes 2 and 9). At the same time, 0.1% MeSO (lanes 3, 4, 10, and 11), 100 μg/ml of genistein (lanes 5, 6, 12, and 13), or 1 μM staurosporine (lanes 7, 8, 14, and 15) was added into IL-2-stimulated cell suspensions and incubated for another 45 min (lanes 3, 5, 7, 10, 12, and 14) or 105 min (lanes 4, 6, 8, 11, 13 and 15) at 37 °C. Panel A, nuclear extracts were prepared and subjected to EMSA using a STAT5-specific probe as described in the legend to Fig. 1. Panel B, anti-STAT5 immunoprecipitates were analyzed by immunoblotting with anti-phosphotyrosine antibody RC20H (top panel) or anti-STAT5 mAb (bottom panel) as described in the legend to Fig. 2.

active STAT5, both protein kinase inhibitors almost completely abolished STAT5 phosphorylation and activation in the presence of MG132 (lanes 12–15). Moreover, this result indicates that protein kinases are important in maintaining IL-2-induced prolonged STAT5 activation by MG132.

IL-2-induced Tyrosine Phosphorylation of Jak1 and Jak3 Is Prolonged by MG132—Jak1 and Jak3 are the two Janus family nonreceptor tyrosine kinases that become tyrosine-phosphorylated and activated by IL-2 stimulation, which in turn activate the STAT proteins (34, 35). To determine if sustained Jak activation contributes to prolonged STAT5 activation by IL-2 in the presence of MG132, anti-Jak1 and anti-Jak3 immunoprecipitates were prepared from cell lysates and analyzed by immunoblotting with antibody to phosphotyrosine. As shown in Fig. 4, IL-2-induced tyrosine phosphorylation of both Jak1 (panel A) and Jak3 (panel B) was prolonged in the presence of MG132 (compare lanes 3 and 6) and correlated well with sustained STAT5 activity. Without IL-2 stimulation, however, MG132 itself does not enhance tyrosine phosphorylation of either Jak1 or Jak3 above background (lane 7).

Furthermore, under conditions that preserve protein ubiquitination (24, 27), we were unable to detect any ubiquitinated Jak1 or Jak3 in the presence of MG132 (lanes 5 and 6). This observation suggests that inactivation of Jak kinases following IL-2-induced activation is mostly due to tyrosine dephosphorylation instead of removal of active Jak proteins through the ubiquitin-proteasome pathway. Consistent with this explanation, there was no detectable loss of either Jak proteins after IL-2 stimulation in the absence of MG132 (lanes 2 and 3, lower panels). These findings suggest that MG132 may sustain IL-2-induced STAT5 activation by maintaining tyrosine phosphorylation of Jak1 and Jak3.

FIG. 4. MG132 maintains IL-2-induced tyrosine phosphorylation of Jak1 and Jak3. Equal numbers of IL-2-deprived CTLL-20 cells were pretreated with 0.1% MeSO (DMSO) (lanes 1–3) or 50 μM MG132 (lanes 4–7) for 1 h and then either left unstimulated (lanes 1 and 4) or stimulated with IL-2 for 15 min (lanes 2 and 5) or 2 h (lanes 3 and 6). As a control, the same number of MG132-pretreated cells were left unstimulated for another 2 h (lane 7). Proteins from cell lysates were immunoprecipitated with either anti-Jak1 antibody (αJak1, panel A) or anti-Jak3 antibody (αJak3, panel B) and resolved on a 7% SDS-polyacrylamide gel. The blots were first probed with the anti-phosphotyrosine mAb 4G10 (αP-Tyr, panels A and B, top), then stripped and reprobed with anti-Jak antibodies (panels A and B, bottom). The arrows indicate the Jak proteins.

DISCUSSION

Recent studies have shown that proteasome-mediated proteolysis down-regulates STAT1 activity subsequent to IFN-γ-induced activation either by degrading active STAT1 (24) or by blocking upstream signaling (18). Our results that the proteasome inhibitor MG132 stabilizes IL-2-induced STAT5 activation further support the notion that the ubiquitin-proteasome pathway does play an important role in regulating STAT activity in response to multiple cytokines. Even though active STAT1 may be removed through this pathway in some cases (24), we provide further evidence indicating that this mechanism is not generalizable to all STAT proteins in response to different cytokines. First, Haspel et al. (18) demonstrated that STAT1 is quantitatively preserved through the activation-inactivation cycle following IFN-γ stimulation. Second, as demonstrated by our findings, proteasomes modulate IL-2-initiated signaling pathways upstream of STAT5, instead of directly destroying active STAT5 proteins.

Haspel et al. (18) also reported that proteasome inhibitors prolonged signaling from the IFN-γ receptors after ligand stimulation by showing sustained tyrosine phosphorylation of the receptors in the presence of MG132. They proposed that proteasome inhibitors may prevent the internalization and subsequent proteolysis of the ligand or receptor or both. The alternative, but not mutually exclusive explanation, is that the proteasome inhibitor MG132 may stabilize active Jak kinases, which are the kinases phosphorylating both the cytokine receptors and the downstream STAT proteins. Consistent with this possibility, we clearly demonstrate here that IL-2-induced tyrosine phosphorylation of Jak1 and Jak3 is maintained in the presence of MG132.

It has become increasingly evident that protein-tyrosine phosphatases play important roles in modulating Jak-STAT signaling pathways in response to different cytokines (13–15). Even though we cannot completely rule out the possibility that small amounts of tyrosine-phosphorylated, active Jak kinases are subjected to proteasome-mediated protein degradation, our results are more consistent with the involvement of protein-
tyrosine phosphatases in modulating Jak activity. Therefore, we propose that the ubiquitin-proteasome pathway down-regulates a phosphatase inhibitor(s), which normally inhibits protein-tyrosine phosphatase activity directed toward the Jak kinases. The proteasome inhibitor MG132 stabilizes the phosphatase inhibitor(s), prevents dephosphorylation of the Jak kinases, and results in prolonged signaling by activating downstream STAT proteins.

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