

Blockade of Mitogen-activated Protein Kinase Cascade Signaling in Interleukin 6-independent Multiple Myeloma Cells¹

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ABSTRACT

Interleukin 6 (IL-6) is a growth factor for multiple myeloma (MM) cells, yet not all MM cell lines or patient cells require IL-6 for their growth. It is well known that IL-6 activates the signal transducers and activators of transcription (stat) 1-stat3 heterodimer, stat3 homodimer, and Ras-dependent mitogen-activated protein kinase (MAPK) cascades in multiple cell systems. We have shown previously that the MAPK pathway is an important pathway for IL-6-mediated MM cell growth. In this study, we delineate the pattern of upstream MAPK cascade activation in IL-6-responsive B9 cells and in IL-6-nonresponsive U266, OCI-My5, and RPMI8226 MM cells to define sites of blockade of this pathway associated with loss of responsiveness to IL-6. In B9 cells, IL-6 triggered the following in sequence: gp130 phosphorylation, gp130-to-protein tyrosine phosphatase 1D (PTP1D) binding, PTP1D phosphorylation, PTP1D complex formation with Grb2-Son of sevenless 1 (Sos1), and Sos1 phosphorylation. gp130 phosphorylation, gp130-to-PTP1D binding, PTP1D phosphorylation, and PTP1D-to-Grb2 binding are also induced by IL-6 in all IL-6-independent MM cell lines studied. However, Grb2 is not associated with Sos1, and neither Grb2-to-Sos1 binding nor Sos1 phosphorylation is triggered by IL-6 in OCI-My5 MM cells. On the other hand, Grb2 and Sos1 are associated constitutively in U266 and RPMI8226 MM cells, but phosphorylation of Sos1 is not induced by IL-6. These data suggest that lack of Sos1 activation is associated with loss of IL-6 responsiveness in MM cell lines that grow independently of IL-6.

INTRODUCTION

IL-6³ is an autocrine and paracrine growth factor for MM cells (1, 2). An autocrine growth mechanism was postulated initially because MM cells produce, secrete, and specifically proliferate to IL-6 *in vitro* (1). On the other hand, IL-6-mediated paracrine MM cell growth is supported by the observations that BMSCs are the major source of IL-6 in MM (2), and that adhesion of MM cells to BMSCs up-regulates IL-6 secretion by BMSCs (3, 4). However, several reports have demonstrated a proliferative response to IL-6 in only 40–60% patients with advanced MM (1, 2, 5). The observation that IL-6Rs are present even on MM cells that do not proliferate in response to IL-6 suggests that there may be blockade of IL-6 signaling in MM cells growing independently of IL-6.

IL-6 binds specifically to a cell surface receptor consisting of two subunits, the ligand-binding gp80 IL-6R and the signal transducing gp130 components (6). Binding of IL-6 to IL-6R induces homodimerization of gp130 and activation of the JAK family of tyrosine kinases (JAK1, JAK2, and/or Tyk2), which phosphorylate gp130. Following activation of these tyrosine kinases, three downstream pathways have been identified, as follows (7, 8). (a) Phosphorylated gp130 binds to stat3, and homodimers of phosphorylated stat3 migrate rapidly to the nucleus and bind to DNA. (b) IL-6 induces stat1 phosphorylation, and heterodimers of tyrosine-phosphorylated stat1 and stat3 bind to DNA (9, 10). (c) IL-6 activates the Ras-dependent MAPK cascade, with sequential activation of gp130, PTP1D, or Src homology and collagen, Grb2-Sos1 complex, Ras, Raf, mitogen-activated protein/extracellular signal-regulated kinase, and MAPK (11–17).

We have shown activation of stat1 and stat3 in both IL-6-dependent B9 cells and IL-6-independent (U266, OCI-My5, and RPMI8226) MM cell lines,⁴ suggesting that stat1 and stat3 may not be mediating IL-6-dependent growth. In contrast, MAPK activation in response to IL-6 was evident only in IL-6-dependent proliferating B9 cells and was not present in MM cells that do not proliferate in response to IL-6. Furthermore, MAPK antisense (but not sense) oligodeoxynucleotide inhibited proliferation of B9 cells triggered by IL-6. These data suggest that the MAPK cascade mediates IL-6-dependent MM cell proliferation.

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³ The abbreviations used are: IL-6, interleukin 6; IL-6R, IL-6 receptor; MM, multiple myeloma; BMSC, bone marrow stromal cell; JAK, Janus-activated kinase; stat1 and stat3, signal transducers and activators of transcription 1 and 3, respectively; MAPK, mitogen-activated protein kinase; PTP1D, protein tyrosine phosphatase 1D; Sos1, Son of sevenless 1; FBS, fetal bovine serum; L-glu, L-glutamine; pen, penicillin; strep, streptomycin; Ab, antibody; [³H]dThd, tritiated thymidine; SI, stimulation index; GST, glutathione S-transferase.

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Although IL-6 triggers activation of gp130 and JAK family kinases, MAPK phosphorylation was not induced by IL-6 in IL-6-nonresponsive U266, OCI-My5, and RPMI8226 cells,⁴ suggesting that IL-6 signaling is blocked in the Ras-dependent MAPK cascade. However, the site of blockade of IL-6 signaling is not defined.

In the present study, we demonstrate activation of the upstream MAPK cascade in IL-6-responsive B9 cells and in IL-6-nonresponsive U266, OCI-My5, and RPMI8226 MM cells. Gp130 phosphorylation, gp130-to-PTP1D binding, PTP1D phosphorylation, and PTP1D-to-Grb2 binding are induced by IL-6 in both IL-6-responsive cells and IL-6-nonresponsive MM cells. In B9 cells, IL-6 induced PTP1D and Grb2-Sos1 complex formation, as well as Sos1 phosphorylation. However, Grb2 and Sos1 are dissociated, and Sos1 is not phosphorylated in OCI-My5 cells cultured with IL-6. Moreover, although Grb2 and Sos1 are associated constitutively in U266 and RPMI8226 MM cells, Sos1 is not phosphorylated in these cell lines. These data suggest that blockade of Sos1 activation is associated with lack of responsiveness to IL-6 and growth of MM cells in an IL-6-independent fashion.

MATERIALS AND METHODS

MM-derived and B9 Cell Lines. RPMI8226 (18) and U266 (19) human MM-derived cell lines, which grow independently of exogenous IL-6, were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in RPMI 1640 (Mediatech, Washington, DC) containing 10% FBS (PAA Laboratories, Inc., Newport Beach, CA), L-glu, 100 units/ml pen, and 100 units/ml strep. The OCI-My5 human MM cell line (Ref. 20; provided kindly by Dr. H. A. Messner, Ontario Cancer Institute, Toronto, Canada) grows independently of IL-6 and was maintained in Iscove's modified Dulbecco's medium containing 10% FBS, L-glu, 100 units/ml pen, and 100 units/ml strep. The B9 murine IL-6-dependent hybridoma/plasmacytoma cell line (Ref. 21; a kind gift of Dr. Lucien Aarden, Netherlands Red Cross Blood Transfusion Service, Amsterdam, the Netherlands) was maintained in RPMI 1640 supplemented with 10% FBS, 50 μ M 2-mercaptoethanol (Sigma Chemical Co.), L-glu, 100 units/ml pen, 100 units/ml strep, and 1 ng/ml recombinant IL-6 (Genetics Institute, Cambridge, MA).

Reagents. The anti-gp130 Ab and GST-Grb2 fusion protein were obtained from Upstate Biotechnology (Lake Placid, NY). The anti-Sos1 and anti-Grb2 Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-PTP1D Ab and antiphosphotyrosine Ab (RC20) were obtained from Transduction Laboratories (Lexington, KY).

Assays of DNA Synthesis. DNA synthesis of MM cells stimulated by IL-6 was measured by [³H]dThd incorporation. Briefly, 1×10^5 cells in 200 μ l of RPMI 1640 without FBS were cultured for 72 h in 96-well culture plates in the presence or absence of 50 ng/ml IL-6. Cells were labeled with 1 μ Ci/well of [³H]dThd (Dupont, Wilmington, DE) during the last 12 h of culture, harvested onto glass filters with the aid of the Harvester 96 Mach II (Tomtec, Inc., Orange, CT) and counted on a 1205 Betaplate β counter (Wallac, Finland). Proliferation was defined by the SI, calculated as [³H]dThd uptake of sample in media + IL-6/[³H]dThd uptake of control sample in media alone.

Table 1 Proliferation of myeloma cell lines in response to IL-6^a

Cell line	IL-6		SI
	(-)	50 ng/ml	
B9	7406 \pm 3193	92444 \pm 4532	12.5
U266	24580 \pm 925	33570 \pm 2495	1.4
OCI-My5	29121 \pm 457	34441 \pm 1241	1.2
RPMI8226	10890 \pm 1861	10996 \pm 2729	1.0

^a Cells (1×10^5) were cultured for 72 h in the absence (-) or presence of 50 ng/ml IL-6. Cells were labeled with 1 μ Ci/well of [³H]dThd during the last 12 h of culture, harvested onto glass filters, and then counted on a β counter. SI was calculated as [³H]dThd uptake of sample in media + IL-6/[³H]dThd uptake of control sample in media alone.

Immunoprecipitation and Immunoblotting. For immunoprecipitation experiments, 1×10^7 cells were cultured for 2 h in the absence of serum and growth factors. Cells were next stimulated with 100 ng/ml IL-6 for 15 min at 37°C. Cells were then washed twice with ice-cold Tris-buffered saline containing 1 mM sodium orthovanadate and resuspended for 30 min at 4°C in 1 ml of lysis buffer [0.5% NP40, 10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 5 mM EDTA, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 5 μ g of aprotinin per ml, and 1 mM NaF]. Cell lysates were centrifuged (10,000 \times g at 4°C) and then precleared for 2 h with normal rabbit serum and protein A-Sepharose CL-4B (Pharmacia, Sweden). The precleared supernatant was then immunoprecipitated overnight at 4°C with specific Abs and pelleted by protein A-Sepharose or protein G-Sepharose. The immunoprecipitates were washed with lysis buffer three times and then boiled for 5 min, using a modified Laemmli method, in loading buffer [0.0625 M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.001% bromophenol blue]. The supernatants were analyzed by SDS-PAGE and subsequent immunoblotting with specific Abs using an enhanced chemiluminescence detection system (ECL; Amersham Corp., Arlington Heights, IL).

RESULTS

Effects of IL-6 on Proliferation of IL-6-responsive and IL-6-nonresponsive MM Cell Lines. The effect of IL-6 on DNA synthesis of B9, U266, OCI-My5, and RPMI8226 cells was analyzed by [³H]dThd incorporation. As shown in Table 1, significantly increased proliferation of B9 cells (SI, 12.5) was noted in response to IL-6 (50 ng/ml). In contrast, no significant increase in DNA synthesis (SI, 1.0–1.4) was observed in U266, OCI-My5, and RPMI8226 cells.

Activation of the Upstream Ras-dependent MAPK Cascade in IL-6-responsive B9 Cells. Our earlier studies demonstrated that IL-6 triggered activation of gp130 and JAK kinases without MAPK activation in IL-6-nonresponsive MM cells.⁴ Prior to delineating the sites of blockade of the MAPK cascade in IL-6-nonresponsive MM cells, we first characterized upstream MAPK signaling in IL-6-responsive B9 cells. As shown in Fig. 1A, phosphorylation of gp130 was triggered at 1 min by IL-6. Peak phosphorylation is at 5 min, with phosphorylation decreasing thereafter to undetectable levels by 60 min. Binding of gp130 to PTP1D was correlated temporally with

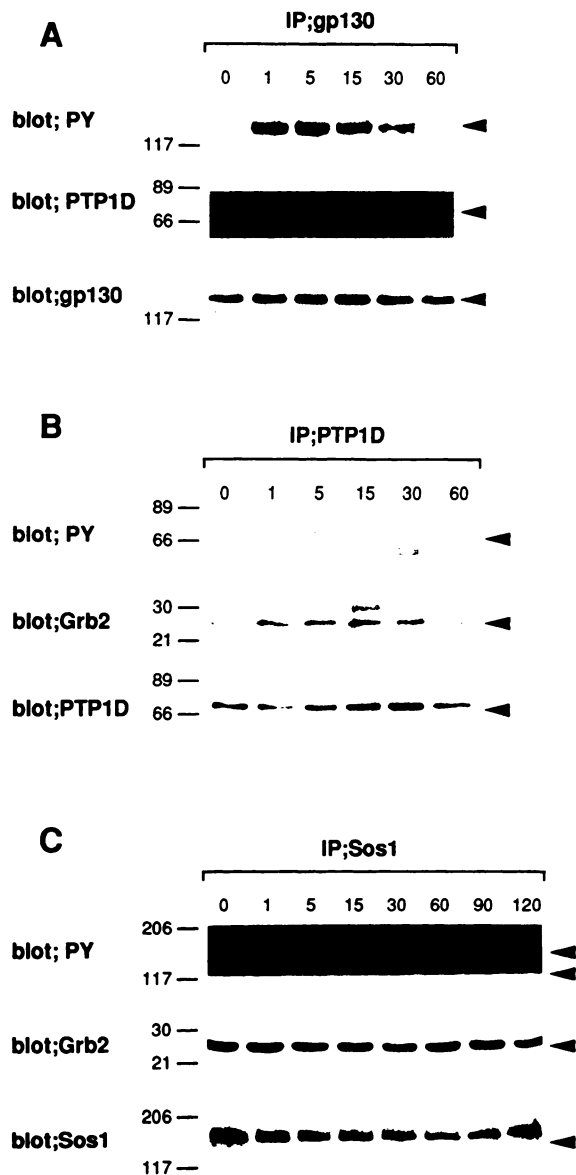


Fig. 1 Activation of the upstream Ras-dependent MAPK cascade in IL-6-responsive B9 cells. B9 cells were cultured with or without 100 ng/ml IL-6 for 1, 5, 15, 30, and 60 min. **A**, cell lysates were immunoprecipitated with anti-gp130 Ab and immunoblotted with antiphosphotyrosine Ab (*top*), anti-PTP1D Ab (*middle*), and anti-gp130 Ab (*bottom*). **B**, cell lysates were immunoprecipitated with anti-PTP1D Ab and immunoblotted with antiphosphotyrosine Ab (*top*), anti-Grb2 Ab (*middle*), and anti-PTP1D Ab (*bottom*). **C**, cell lysates were immunoprecipitated with anti-Sos1 Ab and immunoblotted by antiphosphotyrosine Ab (*top*), anti-Grb2 Ab (*middle*), and anti-Sos1 Ab (*bottom*). PY, antiphosphotyrosine Ab; IP, immunoprecipitation.

gp130 phosphorylation; however, peak gp130-to-PTP1D binding is at 15 min. Blotting with gp130 confirmed equal protein loading.

PTP1D was also phosphorylated in B9 cells in response to IL-6, with peak phosphorylation at 15 min (Fig. 1*B*). Increased PTP1D-Grb2 complex formation was correlated temporally

with PTP1D phosphorylation. Equal protein loading was confirmed by blotting with PTP1D.

As shown in Fig. 1*C*, Grb2 is associated constitutively with Sos1. Phosphotyrosine immunoblotting confirmed peak Sos1 phosphorylation triggered by IL-6 at 60–90 min; SDS-PAGE revealed a shift in Sos-1 at 90–120 min. Immunoblotting confirmed equal protein loading. Furthermore, a M_r 120,000 protein was phosphorylated and coimmunoprecipitated with Sos1 in IL-6-triggered B9 cells.

Effect of IL-6 on Activation of gp130 and PTP1D in IL-6-nonresponsive U266, OCI-My5, and RPMI8226 MM Cell Lines. To determine whether gp130 and PTP1D are also activated by IL-6 stimulation in IL-6-nonresponsive U266, OCI-My5, and RPMI8226 MM cell lines, cells were cultured in the presence or absence of IL-6 for 15 min. Cell lysates were then immunoprecipitated with Ab to gp130. Tyrosine phosphorylation of gp130 and its association with PTP1D were detected by immunoblotting using antiphosphotyrosine Ab and anti-PTP1D Ab, respectively. As shown in Fig. 2*A*, IL-6 induced phosphorylation of gp130 in B9, U266, OCI-My5, and RPMI8226 cells. IL-6 also induced gp130-to-PTP1D binding in all cell lines. Blotting with anti-gp130 confirmed equal protein loading.

Next, we analyzed the phosphorylation of PTP1D triggered by IL-6. Expression of PTP1D is greater in U266 and RPMI8226 cells than in B9 and OCI-My5 cells (Fig. 2*B*). PTP1D phosphorylation is induced by IL-6 in all cell lines. As shown in Fig. 2*C*, all MM cell lines expressed similar amounts of Grb2 protein constitutively. Furthermore, IL-6 induced PTP1D-Grb2 complex formation binding in all cell lines. Reprobing the blots with anti-PTP1D Ab revealed equivalent amounts of protein loading for both IL-6-treated and nontreated cells.

Effect of IL-6 on Activation of Sos1 in IL-6-nonresponsive U266, OCI-My5, and RPMI8226 MM Cells. Because activation of gp130 and PTP1D is normal in all IL-6-nonresponsive MM cells, we analyzed the next step of the Ras-dependent MAPK cascade: Sos1 activation and Sos1 binding to Grb2. As shown in Fig. 3*A*, Grb2 is associated constitutively with Sos1 in B9, U266, and RPMI8226 cells; however, Grb2 is dissociated from Sos1 in OCI-My5 cells. In contrast to the activation of Sos1 induced in B9 cells by IL-6, Sos1 activation was not triggered by IL-6 in U266, OCI-My5, and RPMI8226 cells.

Although OCI-My5 cells express both Grb2 (Fig. 2*C*) and Sos1 (Fig. 3*A*), there is no detectable Grb2-Sos1 association (Fig. 3*A*), suggesting decreased binding by either Grb2 or Sos1. To determine whether Grb2 or Sos1 protein abnormalities accounted for this lack of Grb2-to-Sos1 binding, we next analyzed binding of recombinant GST-Grb2 fusion protein to Sos1 immunoprecipitated from OCI-My5 cells. If Grb2 protein is abnormal and Sos1 protein is normal in OCI-My5 cells, then recombinant GST-Grb2 protein should bind Sos1. As shown in Fig. 3*B*, recombinant GST-Grb2 protein can bind to Sos1 protein in OCI-My5 cells, suggesting that Sos1 protein within these cells can bind normally to Grb2.

DISCUSSION

It is well known that IL-6 activates gp130 and JAK family kinases with downstream signaling via the stat3 homodimer, the

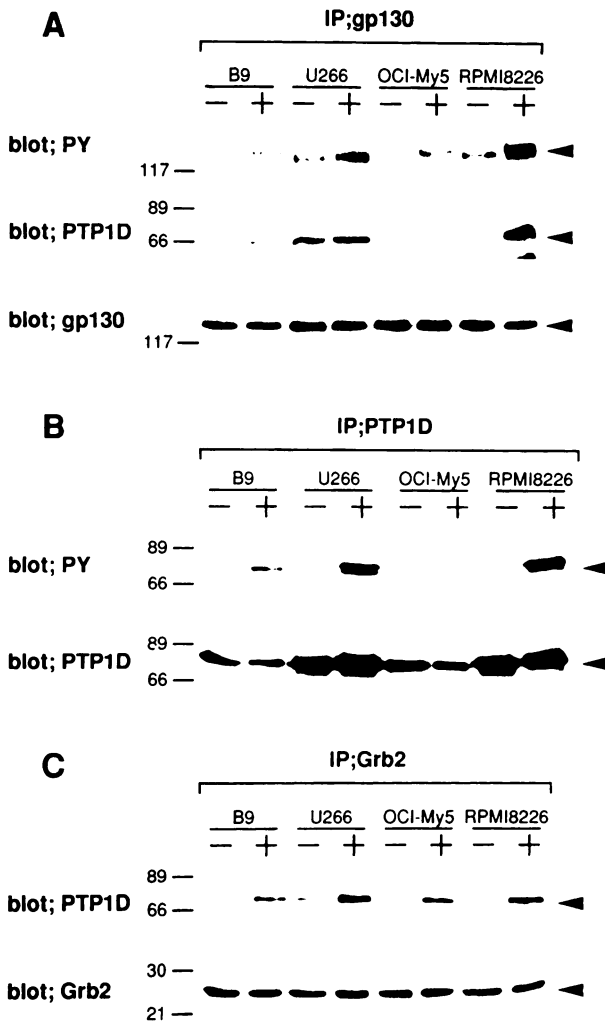


Fig. 2 Effect of IL-6 on activation of gp130 and PTP1D in IL-6-responsive B9 and IL-6-nonresponsive U266, OCI-My5, and RPMI8226 MM cell lines. B9, U266, OCI-My5, and RPMI8226 cells were cultured either in media alone (control) or with 100 ng/ml IL-6 for 15 min. **A**, cell lysates were immunoprecipitated with anti-gp130 Ab and immunoblotted with antiphosphotyrosine Ab (*top*), anti-PTP1D Ab (*middle*), and anti-gp130 Ab (*bottom*). **B**, cell lysates were immunoprecipitated with anti-PTP1D Ab and immunoblotted with antiphosphotyrosine Ab (*top*) and anti-PTP1D Ab (*bottom*). **C**, cell lysates were immunoprecipitated with anti-Grb2 Ab and immunoblotted with anti-PTP1D Ab (*top*) and anti-Grb2 Ab (*bottom*). PY, antiphosphotyrosine Ab; IP, immunoprecipitation.

stat1-stat3 heterodimer, and the Ras-dependent MAPK pathways. To date, however, few reports have examined the functional role of each of these signaling pathways. Studies using dominant negative stat3 mutants to block stat3 expression have demonstrated the role of stat3 in mediating IL-6-induced differentiation and growth inhibition of murine M1 leukemia cells (22, 23). On the other hand, we have demonstrated that the Ras-dependent MAPK cascade mediates IL-6-triggered MM cell growth, because this cascade is activated in tumor cells proliferating to IL-6, whereas inhibition of MAPK expression abrogates IL-6-mediated growth.⁴ Additional evidence of the

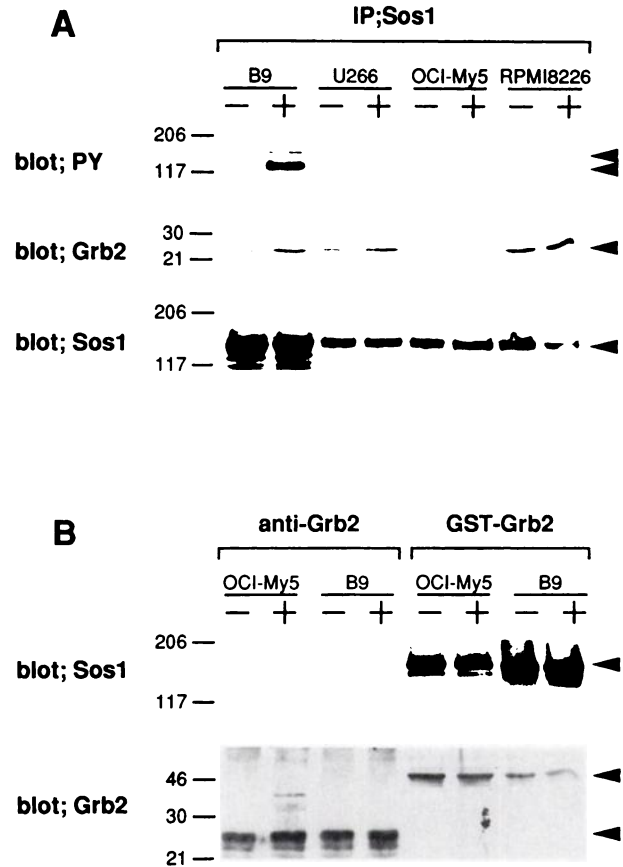


Fig. 3 Effect of IL-6 on activation of Sos1 in IL-6-responsive B9 and IL-6-nonresponsive U266, OCI-My5, and RPMI8226 MM cell lines. B9, U266, OCI-My5, and RPMI8226 cells were cultured either in media alone (control) or with 100 ng/ml IL-6. **A**, cell lysates were immunoprecipitated with anti-Sos1 Ab and immunoblotted with antiphosphotyrosine Ab (*top*), anti-Grb2 Ab (*middle*), and anti-Sos1 Ab (*bottom*). **B**, cell lysates were immunoprecipitated with anti-Grb2 Ab or GST-Grb2 and immunoblotted with anti-Sos1 Ab (*top*) or anti-Grb2 Ab (*bottom*). PY, antiphosphotyrosine Ab; IP, immunoprecipitation.

importance of the MAPK cascade in IL-6-mediated MM cell growth is provided by the demonstration that the ANBL6 IL-6-dependent MM cell line can be relieved of its IL-6 dependence by transfection with activated mutant *N-ras* (16). In the present study, we identified sites of blockade in the upstream Ras-dependent MAPK cascade in MM cells in which gp130 and JAK family kinase activation is triggered by IL-6, without cell proliferation.

We first characterized early events in the Ras-dependent MAPK cascade in IL-6-responsive B9 cells to serve as a basis for comparison with IL-6-nonresponsive MM cells. IL-6 sequentially induced gp130 phosphorylation, gp130-to-PTP1D binding, PTP1D phosphorylation, PTP1D binding to Grb2-Sos1 complex, and Sos1 activation in B9 cells. In our study, tyrosine phosphorylation of Sos1 was triggered by IL-6 in B9 cells. This is in contrast to previous studies using *Drosophila* Sos, which demonstrated that phosphorylation of Sos was limited to serine and threonine residues (24). However, tyrosine phosphorylation of Sos was detected in epidermal growth factor-treated A431 human epidermoid carcinoma cells (25), consistent with our

results in B9 cells. In our study, Sos1 activation was evident both by antiphosphotyrosine Ab staining and by gel shift. Moreover, Sos1 associated with a phosphorylated M_r 120,000 protein in IL-6 triggered B9 cells. Preliminary studies have shown that this protein is not Cas or Cbl (data not shown), and its characterization is currently under active investigation.

We next investigated IL-6-related MAPK signaling in MM cells that do not proliferate in response to IL-6. The impetus for these studies is the demonstration in multiple studies that although IL-6 is a growth and survival factor for MM cells, some tumor cells that express functional IL-6R do not proliferate to IL-6 as expected. Because many IL-6-nonresponsive MM cells express IL-6R and gp130, abnormalities in IL-6 signaling may account for their lack of responsiveness to IL-6. This study is the first report of abnormalities in IL-6 signaling cascades in MM cells that do not proliferate in response to IL-6. We specifically demonstrated activation of upstream MAPK signaling, but importantly, the lack of Sos1 activation in U266, OCI-My5, and RPMI8226 cells. The observation that these three IL-6-nonresponsive MM cell lines have in common blockade of the MAPK cascade at Sos1, with no downstream activation of MAPK cascade or proliferation triggered by IL-6 as in B9 cells, confirms the importance of blockade at this site in IL-6-independent MM cell growth.

The mechanisms underlying blockade of Sos1 activation appear to vary in these cell lines. Grb2 binds Sos1 without activating Sos1 in U266 and RPMI8226 cells, suggesting an intrinsic abnormality in Sos1. In contrast, Grb2 is not bound constitutively to Sos1, and IL-6 does not trigger Sos1 activation in OCI-My5 cells. Our data demonstrate that the recombinant GST-Grb2 fusion protein binds Sos1 in OCI-My5 cells, suggesting that Sos1 protein is normal and that Grb2 protein is abnormal in these cells. The mechanism of signaling blockade remains undefined. One possibility is that Sos1 or Grb2 is mutated in U266 and RPMI8226 or OCI-My5 cells, respectively; if this is this case, growth of these IL-6-nonresponsive MM cells may not be mediated via intrinsic activation of the MAPK cascade. Alternatively, the downstream MAPK cascade may be activated constitutively in these IL-6-nonresponsive MM cells, and there may be a negative feedback mechanism inhibiting Sos1 activation. It has recently been shown that insulin can trigger dissociation of the Sos1-Grb2 complex and decreased Ras activation, demonstrating such a negative feedback loop within the MAPK cascade (26). We have recently confirmed constitutive activation of MAPK in RPMI8226 cells (27) and OCI-My5 cells.⁵ Moreover, Ras mutation has been demonstrated in RPMI8226 cells, but not in U266 cells (28), which may constitutively activate the MAPK cascade downstream from Sos1. In the setting of constitutive activation of MAPK cascade, the lack of Sos1 phosphorylation in these MM cells may be related to a negative feedback loop rather than a primary block in signaling upstream of Sos1.

At present, MM remains incurable with conventional therapy, and there is urgent need for new treatment approaches. Because IL-6 both promotes growth and inhibits apoptosis of MM cells, novel therapeutic approaches may be based on interruption of IL-6 signaling in MM cells (29, 30). However, IL-6 responsiveness of MM cells, both between patients and within individual patients, is heterogeneous. Therefore, further characterization of the growth-signaling cascades in MM cells will attempt to identify the molecular basis underlying the evolution from IL-6-dependent to IL-6-independent tumor cell growth that occurs with progressive disease and may suggest innovative treatment strategies that target MM cells unresponsive to IL-6.

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