SHP2 Mediates the Protective Effect of Interleukin-6 Against Dexamethasone-Induced Apoptosis in Multiple Myeloma Cells

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Running title: Functional interaction between SHP2 and Pyk2/RAFTK

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Summary

Our previous studies have shown that activation of a related adhesion focal tyrosine kinase (RAFTK) (also known as Pyk2) is required for dexamethasone (Dex)-induced apoptosis in multiple myeloma (MM) cells, and that human interleukin-6 (IL-6), a known growth and survival factor for MM cells, blocks both RAFTK activation and apoptosis induced by Dex. However, the mechanism whereby IL-6 inhibits Dex-induced apoptosis are undefined. In this study, we demonstrate that protein tyrosine phosphatase SHP2 mediates this protective effect. We show that IL-6 triggers selective activation of SHP2, and its association with RAFTK in Dex-treated MM cells. SHP2 interacts with RAFTK through a region other than its SH2 domains. We demonstrate that RAFTK is a direct substrate of SHP2 both in vitro and in vivo, and that Y906 in the C-terminal domain of RAFTK mediates its interaction with SHP2. Moreover, overexpression of dominant negative (DN)-SHP2 blocked the protective effect of IL-6 against Dex-induced apoptosis. These findings demonstrate that SHP2 mediates the anti-apoptotic effect of IL-6 and suggest SHP2 as a novel therapeutic target in MM.
Introduction

Related Adhesion Focal Tyrosine Kinase (RAFTK), also known as Proline-Rich Tyrosine Kinase 2 (Pyk2), calcium-dependent tyrosine kinase, and cellular adhesion kinase β, is a cytoplasmic tyrosine kinase and member of the Focal Adhesion Kinase (FAK) family (1-3). RAFTK is activated in response to diverse stimuli, such as tumor necrosis factor α, ultraviolet light, hyperosmolarity, and β1 integrins (1, 4-6), and it is regulated by pathways involving calcium and protein kinase C (7). Importantly, recent studies have implicated an isoform of Pyk2, Pyk2-H, in chemokine and antigen receptor signaling (8). Overexpression of RAFTK induces activation of c-Jun N-terminal protein kinase (JNK) and p38 MAPK (4, 5). Moreover, activation of RAFTK has also been associated with induction of apoptosis (9).

Our recent study demonstrated that RAFTK mediates Dex-induced apoptosis in human multiple myeloma (MM) cells (10). Importantly, Interleukin-6 (IL-6) inhibits both Dex-induced RAFTK activation and apoptosis in these cells. However, the molecular mechanisms by which IL-6 inhibits Dex-induced apoptosis are undefined. Interleukin-6 binds to its receptor (IL-6R/gp80) which interacts with the signal transducer gp130. The cytoplasmic domain of gp130 contains phosphotyrosine motifs for recruitment of a protein tyrosine phosphatase SHP2, also referred to as SHPTP2 (11, 12). SHP2 is widely expressed and is required as a positive (i.e., signal enhancing) component of growth factor and cytokine signal transduction pathways (13-15). For example, our prior studies have demonstrated that IL-6 triggers proliferation of MM cells via
the mitogen activated protein kinase (MAPK) cascade, which includes SHP2 activation (16). Moreover, fibroblasts from SHP2 mutant mice exhibit impaired MAPK activation in response to fibroblast growth factor, epidermal growth factor, and insulin growth factor (17, 18). Although SHP2 may directly bind and dephosphorylate cytokine receptors or other cytoplasmic protein tyrosine kinases (14, 19–20), its role in regulating apoptotic signaling is at present undefined.

The present study examined the role of SHP2 in mediating the inhibitory effects of IL-6 on Dex-induced apoptosis. We demonstrate that IL-6 activates SHP2 in MM cells, and that treatment of MM cells with IL-6 and Dex induces binding of SHP2 with RAFTK, resulting in dephosphorylation of RAFTK. Importantly, we show that RAFTK is a substrate of SHP2, both in vitro and in vivo. Overexpression of dominant negative (DN)-SHP2 abrogates the protective function of IL-6 against Dex-induced apoptosis. Taken together, these findings demonstrate that SHP2 mediates anti-apoptotic signaling, and suggest SHP2 as a novel therapeutic target in MM.
Experimental Procedures

Cell culture and reagents  Human MM.1S (Dex-sensitive) cells (21) were grown in RPMI-1640 media supplemented with 10% heat inactivated fetal-bovine serum (HI-FBS), 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine. Cells were treated with 10 µM Dex (Sigma Chemical Co, St. Louis, MO) in the presence or absence of 100 ng/ml of IL-6.

Immunoprecipitation and immunoblot analysis  Cells were washed with PBS and lysed in 1 ml of lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM sodium vanadate, 1 mM PMSF, 1 mM DTT and 10 µg/ml of leupeptin and aprotinin) as previously described (10). Lysates were subjected to immunoprecipitation with anti-RAFTK (2) anti-SHP2, anti-SHP1, or anti-PTP1B (Santa Cruz Biotech, Santa Cruz, CA) for 1 h at 4°C, and then for 45 min after the addition of protein-G-sepharose. Immune complexes were analyzed by immunoblotting with anti-P-Tyr (RC20, Transduction Laboratories, Lexington, KY), anti-RAFTK or anti-SHP2. Total cell lysates were also subjected to Western blot analysis with anti-CPP32 (Transduction Labs) or anti-PKC-δ (Santa Cruz).

Transient transfections  MM.1S cells were transiently transfected using Superfect™ (Quiagen, Santa Clarita, CA) with vector containing Green-Fluorescence Protein (GFP) alone or with wild-type-SHP2 (WT-SHP2), dominant negative-SHP2 (DN-SHP2) containing cysteine to serine (Cys→Ser) mutation in the catalytic domain of SHP2, dominant negative-SHP1 (DN-SHP1) or wild-type SHP1. Following transfections, GFP-positive cells were selected by flow
cytometry and treated with 10 µM Dex in the presence or absence of IL-6 for 24 h. Cells were also transfected with Flag-RAFTK, Flag-RAFTK-Y402F, Flag-RAFTK-Y881F or Flag-RAFTK-Y906F (2) and treated with 10 µM Dex and IL-6 for 12h. Lysates were subjected to immunoprecipitation with anti-Flag and analyzed by immunoblotting with anti-RAFTK, anti-Flag, or anti-SHP2. Additionally, MM.1S cells were cotransfected with Flag-RAFTK and c-Abl or SHP2. Transfected cells were treated with Dex and IL-6, and total cell lysates were subjected to immunoprecipitation with anti-Flag. The protein precipitates were then analyzed by immunoblotting with a, anti-c-Abl, anti-SHP2, or anti-Flag. To determine whether RAFTK associate with SHP2 in vivo, MM.1S cells were cotransfected with Flag-RAFTK and HA-SHP2-PD (phosphatase-dead) or HA-SHP2-WT and then treated with Dex and IL-6. Cell lysates were subjected to immunoprecipitation with anti-Flag and analyzed by immunoblotting with anti-P-Tyr, anti-HA, or anti-Flag.

In vitro dephosphorylation assays MM.1S cells were transiently transfected with Flag-RAFTK and treated with 10 µM Dex for 12h. Total cell lysates were subjected to immunoprecipitation with anti-RAFTK, and immunoprecipitates were incubated with purified SHP2 or phosphatase-dead SHP2 (kindly provided by Dr. Haihua Gu and Benjamin G. Neel, Harvard Medical School, Boston, MA), SHP1 or PTP1B in an assay buffer containing cold ATP (22). The reaction products were analyzed by immunoblotting with anti-P-Tyr.

RAFTK immune complex kinase assays MM.1S cells were treated with 10 µM Dex and harvested at different time intervals. RAFTK immune
complex kinase assays and anti-P-Tyr immunoblotting were performed as previously described (10).

**Far Western Blotting** GST fusion proteins [GST-SHP2 and GST-SHP2 (N+C)] were expressed in Escherichia coli, purified on glutathione-Sepharose beads as previously described (22), and eluted by incubating with 20 mM glutathione in 150 mM NaCl, 50 mM Tris-Cl, pH 7.4. Protein blots on nitrocellulose filters were blocked with 5% bovine serum albumin in Tris-buffered saline/Tween, incubated with GST-SHP2 and GST-SHP2 (N+C), probed with monoclonal anti-GST (1:1000) or anti-Flag, and developed using appropriate secondary Abs and ECL.

**Flow cytometric analyses** Dual fluorescence staining with DNA-binding fluorochromes Hoechst 33342 (HO) and propidium iodide (PI) was used to quantitate the percentage of apoptotic (HO+PI-) cells using flow cytometry ('The Vantage', Becton Dickinson), as previously described (23).

**DNA fragmentation assays** Following transfections, genomic DNA was isolated from control and Dex-treated GFP-selected MM.1S cells. Briefly, 1-2 x 10^6 cells were washed two times with phosphate buffered saline (PBS) and lysed in buffer (100 mM NaCl, 10 mM Tris-HCl pH 8.0, 25 mM EDTA, 0.5% SDS, and 0.1 mg/ml proteinase K) at 37°C for 18hr. DNA was extracted with an equal volume of phenol/chloroform (1:1) and precipitated at -70°C for 2hr with 0.3 M NaAcetate and 2.5 volume of absolute ethanol. DNA pellets were resuspended in 20 ul of 10 mM Tris (pH 7.8) 1 mM EDTA buffer
and incubated for 1 hr at 37°C with 1 ug/ml RNase (Boehringer Manheim Corporation, Indianapolis, IN).

DNA-end labelling was performed as previously described (24). Briefly, 0.5-1.0 ug of DNA was treated with 5U of Klenow polymerase with 0.5 uCi of [γ32P]-dCTP in reaction mixture (Tris-HCL pH 7.5, 5 mM MgCl₂) for 10 min at room temperature and terminated by addition of 10 mM EDTA. Unincorporated nucleotides were removed by three consecutive precipitation cycles with ammonium acetate/isopropanol, and labelled DNA was resuspended in 10 mM Tris-HCL (pH 7.5) 1 mM EDTA. Labelled DNA probes were electrophoresed for 2-3 hr at 90 Volts on 1.8% agarose gels, which were dried and exposed for autoradiography.
Results and Discussion

Our recent studies have shown that RAFTK mediates Dex-induced apoptosis in MM cells: treatment of MM.1S cells with 10 \( \mu \)M of Dex induces maximal RAFTK activation as well as apoptosis in MM.1S cells (10). Since IL-6 can inhibit Dex-induced apoptosis (24-26), we first examined the effect of IL-6 on Dex-induced activation of RAFTK. MM.1S MM cells were treated with Dex (10 \( \mu \)M) in the presence or absence of IL-6 (100 ng/ml) and harvested at different time intervals. Total cell lysates were subjected to immunoprecipitation with anti-RAFTK and analyzed by immunoblotting with anti-P-Tyr. Dex induces tyrosine phosphorylation of RAFTK in MM.1S cells, which is inhibited by IL-6 (Fig 1A, upper panel). Treatment of MM.1S cells with Dex and IL-6 does not alter the levels of RAFTK protein (Fig 1A, lower panel). These results, taken together with prior studies (10, 24-27), demonstrate that IL-6 blocks both Dex-induced RAFTK activation and apoptosis in MM.1S cells.

The mechanism whereby IL-6 inhibits Dex-induced RAFTK activation and apoptosis is unknown. We and others have shown that IL-6 induces growth in MM cells via the MAPK pathway, which includes activation of protein tyrosine phosphatase SHP2 (16, 28). To determine whether SHP2 may also mediate the inhibitory effect of IL-6 on Dex-induced activation of RAFTK, MM.1S cells were treated with IL-6 for 8h, 12h, or 24h, and cell lysates were subjected to immunoprecipitation with anti-SHP2, anti-PTP1B or anti-SHP1 and analyzed by immunoblotting with anti-P-Tyr. Low to undetectable levels of SHP2 tyrosine phosphorylation were observed.
in cells cultured in media alone (Fig.1B, upper panel). In contrast, treatment of MM.1S cells with IL-6 induced SHP2 tyrosine phosphorylation as early as 8h; densitometry showed that SHP2 tyrosine phosphorylation levels peaked at 12h (9.0 ± 0.5 fold increase) and declined thereafter (Fig.1B, upper panel). There were no associated changes in SHP2 protein levels (Fig. 1B, lower panel). In contrast, IL-6 failed to induce tyrosine phosphorylation of PTP1B (Fig 1C, upper panel) or SHP1 (Fig 1D, upper panel). Moreover, IL-6 did not alter the protein levels of PTP1B or SHP1 (lower panels of Fig. 1C, and Fig. 1D, respectively).

In order to determine whether different doses of IL-6 affect SHP2 tyrosine phosphorylation, MM.1S cells were treated with various doses of IL-6. Total cell lysates were subjected to immunoprecipitation with anti-SHP2 and analyzed by immunoblotting with anti-P-Tyr. Treatment of MM.1S cells with 100 ng/ml of IL-6 is associated with maximal activation of SHP2 (data not shown). These findings are in concert with our prior studies which showed that 100 ng/ml of IL-6 induces maximal SHP2 activation (16). To determine whether IL-6 induces SHP2 enzymatic activity, SHP2 was immunoprecipitated from control and IL-6-treated MM.1S cells and phosphatase activity was measured. A 2.1 ± 0.5 fold increase in phosphatase activity was observed in IL-6 treated MM.1S cells relative to untreated cells (p <0.05, n=3).

Previous studies have demonstrated that SHP2 binds to and dephosphorylates cytokine receptors and other cytoplasmic Protein Tyrosine Kinases (PTKs) (14, 19-20). We therefore next determined whether IL-6 induces association of SHP2 and RAFTK. MM.1S cells
were treated with Dex in the presence or absence of IL-6 for different time intervals, and cell lysates were subjected to immunoprecipitation with anti-SHP2 and immunoblotting with anti-RAFTK (Fig 2A, upper panel) or immunoprecipitated with anti-RAFTK and analyzed by immunoblotting with anti-SHP2 (Fig 2B, upper panel). Treatment of cells with Dex and IL-6 induces an association between SHP2 and RAFTK, which was maximal at 12h (Fig 2A and 2B upper panels and data not shown). In contrast, treatment of cells with Dex alone fails to induce binding of SHP2 with RAFTK. Equivalent protein loading was confirmed by reprobing the filters with anti-SHP2 or anti-RAFTK (Fig 2A and 2B, lower panels).

To further demonstrate the interaction between RAFTK and SHP2, MM.1S cells were transiently transfected with Flag-RAFTK-WT. Following transfection, cells were treated with Dex and IL-6, subjected to immunoprecipitation with anti-Flag, and analyzed by immunoblotting with anti-SHP2. The results confirmed that Dex and IL-6 induces association of SHP2 with RAFTK (Fig. 3). Since tyrosine at 906 in the C-terminal domain of RAFTK forms the best consensus site essential for SHP2 binding, we next examined whether mutation of tyrosine 906 abrogates interaction between SHP2 and RAFTK triggered by Dex and IL-6. MM.1S cells were transiently transfected with constructs containg mutations at tyrosine 906, 881 or 402 residues. Following transfection, cells were treated with Dex and IL-6, and subjected to immunoprecipitation with anti-Flag. The protein precipitates were analyzed by immunoblotting with anti-SHP2. As can be seen in
Fig. 3A, overexpression of RAFTK-Y906F, but not of RAFTK-Y881F or RAFTK-Y402F, abrogates binding of SHP2 and RAFTK (Fig. 3). Reprobing the filters with either anti-RAFTK or anti-Flag demonstrated equal protein levels (Fig. 3, middle and lower panels). Taken together, these findings confirm that treatment of MM.1S cells with Dex and IL-6 induces an association between SHP2 and RAFTK. Moreover, Y906 in the C-terminus domain of RAFTK is required for its interaction with SHP2.

The interaction between RAFTK and SHP2 was further examined using GST-SHP2 fusion proteins by Far Western blot analyses. MM.1S cells were transiently transfected with Flag-RAFTK, Flag-RAFTK-Y906F, or Flag-RAFTK-Y881F mutants, and treated with Dex and IL-6. Following treatment, total cell lysates were subjected to immunoprecipitation with anti-Flag. The precipitated proteins were separated by SDS-PAGE, transferred onto nitrocellulose filters and the filters were incubated with either GST-SHP2-WT fusion protein and analyzed by immunoblotting with anti-GST or anti-Flag.

Incubation of WT-RAFTK was associated with binding to wild-type SHP2 (Fig. 4A, upper panel). Reprobing the filters with anti-Flag demonstrated equal RAFTK protein levels (Fig. 4A, lower panel).

We next determined whether association of SHP2 with RAFTK is SH2-dependent. A similar experiment, as described above, was performed and filter was incubated with GST-SHP2 (N+C) which contains both N- and C-terminal SH2 domains of SHP2. The results demonstrate that GST-SHP2 (N+C) fails to bind WT-RAFTK (Fig. 4B, upper panel). Total cell lysates from Baf3-p210 transfomed cells served as a positive control demonstrating binding of GST-SHP2-
(N+C) to p97 KD protein, as previously shown (22). Reprobing the filter with anti-Flag demonstrated equal RAFTK protein levels (Fig. 4B, lower panel). Probing similar blots with GST alone revealed no reactive bands. These data are consistent with recent findings (29), and further support that SH2 domains of SHP2 are not essential for the interaction of RAFTK with SHP2.

To determine whether other SH2-domain containing proteins associate with RAFTK, MM.1S cells were cotransfected with Flag-RAFTK, c-Abl or SHP2. Transfected cells were treated with Dex and IL-6, and total cell lysates were subjected to immunoprecipitation with anti-Flag. The protein precipitates were then analyzed by immunoblotting with anti-c-Abl, anti-SHP2 or anti-Flag. SHP2, but not c-Abl, associated with RAFTK (Fig. 4C).

To define the functional significance of SHP2-RAFTK complex formation, we next asked whether SHP2 affects Dex-induced tyrosine phosphorylation of RAFTK. RAFTK was immunopurified from Dex-treated MM.1S cells transfected with WT-RAFTK and incubated with purified wild-type SHP2 protein (SHP2-WT) or phosphatase-dead SHP2 (SHP2-PD) in the presence of ATP. RAFTK tyrosine phosphorylation was determined by immunoblotting with anti-P-Tyr. As can be seen in Fig 5A (upper panel), incubation of immunopurified RAFTK with SHP2-WT, but not SHP2-PD, inhibits tyrosine phosphorylation of RAFTK. Immunoblotting with anti-Flag confirmed equivalent RAFTK protein levels (Fig. 5A, middle panel).

Since purified SHP2 dephosphorylates RAFTK in vitro, we next asked whether dephosphorylation of RAFTK affects its kinase activity. To address this issue, we first incubated RAFTK
immunoprecipitated from Dex-treated MM.1S cells with either SHP2-WT or SHP2-PD in the presence of ATP. After washing, RAFTK activity was measured in a kinase reaction containing $\gamma^{32}$-P ATP, with GST-HEF as a substrate (10). As shown in Fig. 5A (lower panel), SHP2-WT, but not SHP2-PD, inhibited RAFTK-mediated phosphorylation of GST-HEF. Taken together, these data indicate that 1) RAFTK is a substrate of SHP2 in vitro, and 2) dephosphorylation of RAFTK by SHP2 inhibits its kinase activity.

To determine whether SHP2 related proteins such as SHP1 and PTP1B also regulate RAFTK; we performed RAFTK in vitro dephosphorylation assays using purified SHP1 or PTP1B. Cells were transiently transfected with Flag-RAFTK and treated with Dex. Anti-Flag immunoprecipitates were resuspended in buffer containing ATP and purified SHP1, PTP1B or SHP2. Both SHP1 and SHP2, but not PTP1B, dephosphorylate RAFTK in vitro (Fig. 5B). This data is consistent with our prior study suggesting that RAFTK is also an in vitro substrate of SHP1 (30). To further demonstrate SHP2-mediated dephosphorylation of RAFTK in response to Dex and IL-6, MM.1S cells were cotransfected with Flag-RAFTK and HA-SHP2-WT or HA-SHP2-PD and then treated with Dex and IL-6. Cell lysates were subjected to immunoprecipitation with anti-Flag and analyzed by immunoblotting with anti-P-Tyr, anti-HA, or anti-Flag. Expression of SHP2-PD resulted in enhanced tyrosine phosphorylation of RAFTK, whereas expression of SHP2-WT significantly abrogates RAFTK tyrosine phosphorylation (Fig. 5C). The filters were stripped and reprobed with anti-HA or anti-Flag. No alteration in the protein levels of SHP2 or RAFTK were observed (Fig. 5C, lower panel and data not
shown, respectively). These findings suggest that RAFTK is a substrate of SHP2 in vivo.

We next directly assayed the functional significance of the interaction between SHP2 and RAFTK in mediating the protective effect of IL-6 against Dex-induced apoptosis. MM.1S cells were transiently cotransfected with GFP and either vector alone or DN-SHP2. GFP positive cells were selected, treated with Dex in the presence or absence of IL-6, and assayed for apoptosis by both DNA fragmentation and flow cytometric analysis. The genomic DNA was end-labelled with $\alpha^{32}$P-dCTP and DNA fragmentation was assessed by autoradiography. Dex treatment of cells overexpressing vector alone induced DNA fragmentation, which was markedly reduced in the presence of IL-6 (Fig 6A, upper panel). In contrast, IL-6 failed to rescue Dex-induced apoptosis in cells overexpressing DN-SHP2. Similar results were obtained using PI and HO dual staining to determine the percentage for PI- and HO+ apoptotic cells (Fig. 3A, lower panel). To further confirm the role of SHP2 in conferring resistance to Dex-induced apoptosis, we transiently transfected MM.1S cells with GFP and either vector alone or WT-SHP2, treated selected GFP positive cells with Dex, and assessed for apoptosis. Overexpression of WT-SHP2 significantly inhibits Dex-induced apoptosis, confirmed both by DNA fragmentation and percentage of PI- HO+ cells (Fig 6B, upper and lower panels). To confirm the selective inhibitory effect of SHP2, MM.1S cells were also transiently transfected with dominant-negative SHP1 (DN-SHP1) and similarly treated. As can be seen in Fig 6B upper and lower panels, IL-6 blocks Dex-induced apoptosis in cells overexpressing
DN-SHP1, but not in cells overexpressing DN-SHP2. To determine whether SHP1-WT affect Dex-induced apoptosis, MM.1S cells were transiently cotransfected with vectors containing GFP and SHP2 or SHP1. GFP positive cells were selected and treated with Dex. Following treatment, cells were analyzed for apoptosis by DNA fragmentation and propidium iodide staining. Overexpression of SHP2, but not SHP1, blocks Dex-induced apoptosis (Fig. 6B). Collectively, these findings demonstrate that SHP2 specifically regulate RAFTK-mediated apoptosis.

To further confirm the role of SHP2 in mediating the protective effect of IL-6 against Dex-induced apoptosis, we determined the effect of SHP2 blockade on Dex-induced proteolytic cleavage of CPP32 and its substrate PKC-δ, additional hallmarks of apoptosis (31, 32). MM.1S cells were transiently transfected with GFP vector alone or GFP-DN-SHP2. GFP positive cells were treated with Dex and IL-6, and total lysates were analyzed by immunoblotting with anti-CPP32 or anti-PKC-δ. IL-6 significantly inhibits Dex-induced cleavage of CPP32 and PKC-δ in cells overexpressing vector alone; however, IL-6 failed to block cleavage of CPP32 and PKC-δ in cells expressing DN-SHP2 (Fig 6C).

Our prior studies have established an obligatory role for RAFTK in Dex-induced apoptosis as well as the protective effect conferred by IL-6 (10, 24, 27). By contrast, γ-irradiation (IR), UV, or Fas, is not associated with activation of RAFTK and IL-6 fails to block apoptosis of MM cells triggered by IR or UV (10). We and others have shown that IL-6 is a growth and anti-apoptotic factor for MM cells (10, 16, 26, 27). IL-6 uses JAK-STAT and RAS-
dependent mitogen-activated protein kinase (MAPK) signaling cascades to mediate biologic sequelae (33-35). Prior studies have demonstrated that IL-6-activates JAKs, STAT-3, SHP2 as well as MAPK in MM cells (16, 35). A recent study reported interaction between JAKs and SHP2 (36). The interplay between SHP2 and JAK-STAT signaling molecules in response to IL-6 stimulation of MM cells remains to be defined. Moreover, it is not yet known which tyrosine residue(s) in SHP2 are phosphorylated upon IL-6 stimulation of MM cells. The identification of IL-6-induced tyrosine phosphorylation sites in SHP2 will further elucidate the role of SHP2 in mediating its anti-apoptotic effects.

In the present study, we show that IL-6 activates SHP2, which then associates, with RAFTK in Dex-treated MM cells. SHP2 interacts with RAFTK through a region other than its SH2 domains. We demonstrate that RAFTK is a substrate of SHP2, and that Y906 in the C-terminal domain of RAFTK mediates its interaction with SHP2. Furthermore, overexpression of dominant negative (DN)-SHP2 blocked the protective effect of IL-6 against Dex-induced apoptosis. Collectively, the present model suggests that the IL-6-activated SHP2 dephosphorylate RAFTK, thereby blocking Dex-induced apoptosis (Fig 7). These studies provide the framework for novel treatment approaches targeting SHP2 in MM.
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Footnotes

1. Supported by PHS grant CA 50947 (KA) and CA 75216 (SK) awarded by the National Cancer Institute, DHHS; and by a Multiple Myeloma Research Foundation Fellowship (DC); and the Doris Duke Distinguished Clinical Research Scientist Award (KA).

2. Abbreviations: SHP2, SH2 domain containing protein tyrosine phosphatase; RAFTK, related adhesion focal tyrosine kinase; Dex, dexamethasone; MM, multiple myeloma; IL-6, interleukin-6; Pyk2, proline-rich tyrosine kinase 2; FAK, focal adhesion kinase; JNK, c-Jun N-terminal protein kinase; MAPK, mitogen activated protein kinase; DN, dominant negative; HI-FBS, heat inactivated fetal bovine serum; WT, wild type; PD, phosphatase dead; PTP, protein tyrosine phosphatase; PTK, protein tyrosine kinase; CPPD32, cysteine protease protein with a molecular mass of 32 kDa; PKC-δ, protein kinase C δ; GFP, green fluorescence protein
Figure Legends

Figure 1: (A) IL-6 inhibits Dex-induced RAFTK tyrosine phosphorylation. MM.1S Cells were treated with 10 µM Dex in the presence or absence of IL-6 (100 ng/ml) and harvested at 12h. Total cell lysates were subjected to immunoprecipitation with anti-RAFTK and analyzed by immunoblotting with anti-P-Tyr (upper panel,) or anti-RAFTK (lower panel). (B-D) IL-6 induces tyrosine phosphorylation of SHP2, but not PTP1B or SHP1. MM.1S cells were treated with 100 ng/ml of IL-6 and harvested at the indicated time periods. Total cell lysates were subjected to immunoprecipitation with anti-SHP2, anti-PTP1B or anti-SHP1 and analyzed by immunoblotting with anti-P-Tyr (B, C and D, upper panels, respectively), anti-SHP2, anti-PTP1B or anti-SHP1 (B, C, and D lower panel, respectively).

Figure 2: RAFTK associates with SHP2. MM.1S cells were treated with 10 µM Dex in the presence or absence of IL-6 (100 ng/ml) for 12h. (A) Total cell lysates were subjected to immunoprecipitation with anti-SHP2 and analyzed by immunoblotting with anti-RAFTK (upper panel) or anti-SHP2 (lower panel). (B) Total cell lysates were subjected to immunoprecipitation with anti-RAFTK and analyzed by immunoblotting with anti-SHP2 (upper panel) or anti-RAFTK (lower panel).

Figure 3: SHP2 binds at tyrosine 906 in the C-terminus domain of RAFTK. Cells were transfected with Flag-RAFTK constructs as indicated and treated with 10 µM Dex. Total cell lysates were
subjected to immunoprecipitation with anti-Flag and analyzed by immunoblotting with anti-SHP2 (upper panel), anti-RAFTK (middle panel), or anti-Flag (lower panel).

**Figure 4.** (A and B) SH2 domains of SHP2 are not essential for its interaction with RAFTK. MM.1S cells were transiently transfected with Flag-RAFTK or Flag-RAFTK-Y906F mutant, and treated with Dex and IL-6. Following treatment, total cell lysates were subjected to immunoprecipitation with anti-Flag and the precipitated proteins were separated by SDS-PAGE and transferred onto nitrocellulose filters. Filters were incubated with GST-SHP2-WT (A) or GST-SHP2 (N+C) (B) fusion proteins and analyzed by immunoblotting with anti-GST or anti-Flag. (C) RAFTK binds to SHP2, but not c-Abl. MM.1S MM cells were cotransfected with Flag-RAFTK and c-Abl or SHP2. Cells were treated with Dex and IL-6, and total cell lysates were subjected to immunoprecipitation with anti-Flag. The protein precipitates were then analyzed by immunoblotting with anti-SHP2, anti-c-Abl or anti-Flag.

**Figure 5.** (A and B) RAFTK is a substrate for SHP2 in vitro. Cells were transfected with Flag-RAFTK constructs and treated with 10 µM Dex for 12h. Total cell lysates were subjected to immunoprecipitation with anti-Flag. The immunoprecipitates were incubated with phosphatase-dead SHP2 (SHP2-PD) or phosphatase-active SHP2 (SHP2-WT), SHP1-WT or PTP1B-WT proteins in the presence of ATP for 30 min at 30°C. The proteins were separated by 7.5% SDS-PAGE, transferred to nitrocellulose filter, and analyzed
by immunoblotting with anti-P-Tyr (upper panels) or anti-Flag (lower panels). (C) **SHP2 catalytic domain is required for downregulation of RAFTK kinase activity.** Total cell lysates were subjected to immunoprecipitation with anti-RAFTK. The immunoprecipitates were incubated with purified SHP2-PD or SHP2-WT proteins for 20 min at 30°C, washed three times, and incubated in a kinase buffer containing γ-32P ATP and GST-HEF as a substrate at 30°C for 15 min. The proteins were separated by 10% SDS-PAGE, dried, and analyzed by autoradiography. (D) **RAFTK is substrate of SHP2 in vivo.** MM.1S cells were cotransfected with Flag-RAFTK and HA-SHP2-PD (phosphatase-dead) or HA-SHP2-WT and then treated with Dex and IL-6. Cell lysates were subjected to immunoprecipitation with anti-Flag and analyzed by immunoblotting with anti-P-Tyr (upper panel), or anti-HA (lower panel).

**Figure 6:** SHP2 mediates the protective effect of IL-6 against Dex-induced apoptosis (A) MM.1S cells were transiently transfected with vector alone or DN-SHP2, and cotransfected with vector containing GFP alone. The GFP-positive cells were sorted by flow cytometry and treated with 10 µM Dex in the presence or absence of IL-6 (100 ng/ml). At 24 h, cells were harvested and analyzed for apoptosis. DNA fragmentation was assayed by isolating genomic DNA from control and treated GFP selected cells. The genomic DNA was end-labelled with [α32-P]-dCTP and DNA fragmentation was assessed by autoradiography (upper panel). Flow cytometric analysis of the control and treated GFP selected cells was used to determine the percentage of PI- and HO+ apoptotic cells (lower panel). Results
are representative of three independent experiments. (B) MM.1S cells were transiently transfected with vector alone, WT-SHP2, DN-SHP2, DN-SHP1, or WT-SHP1. GFP positive cells were isolated, treated, and analyzed for apoptosis as described above. Results are representative of three independent experiments. (C) MM.1S cells were transiently transfected with vector alone or DN-SHP2. GFP positive cells were isolated, treated, and analyzed for cleavage of CPP32 and PKC δ, as a further evidence for apoptosis. Results are representative of three independent experiments. FL, full length; CF, cleaved fragment.

**Figure 7:** Cross talk between Dex-induced apoptotic signaling and IL-6-induced survival signaling We and others have shown previously that protein tyrosine phosphatase SHP2 is involved in growth signaling via the MAPK cascade (16). Our studies to date have also demonstrated that Dex-induced apoptosis in MM cells is 1) mediated by RAFTK (10); 2) associated with proteolytic cleavage and activation of CPP32 (as evidenced by cleavage of its substrate PKC δ); 3) not associated with activation of c-Jun-N-terminal kinase (JNK/SAPK) or release of mitochondrial protein Cytochrome-c (Cyto-c) (24, 27); and 4) significantly blocked in the presence of IL-6, which correlates with decreased RAFTK activity (10). The current study provides evidence that SHP2 mediates the protective effect IL-6 against Dex-induced apoptosis via downregulation of Dex-induced RAFTK activity. Thus, we propose a model in which IL-6 activated SHP2 binds and dephosphorylates Dex-induced RAFTK, thereby inhibiting Dex-induced apoptotic signaling.
Figure 1
Figure 2
Figure 4
Figure 5
Figure 6
DEXAMETHASONE

RAFTK

INTERLEUKIN-6

Activated SHP2

MAPK Cascade

Inactivated RAFTK

APOPTOSIS

NO APOPTOSIS

PROLIFERATION

+ CPP32, PKCδ and PARP cleavage
- Mitochondrial Cyto-c release
- JNK/SAPK activation
