Critical Role for Hematopoietic Cell Kinase (Hck)-mediated Phosphorylation of Gab1 and Gab2 Docking Proteins in Interleukin-6-induced Proliferation and Survival of Multiple Myeloma Cells*

Interleukin-6 (IL-6) is a known growth and survival factor in multiple myeloma via activation of extracellular signal-regulated kinase and phosphatidylinositol 3-kinase signaling cascade. In this report we show that Grb2-associated binder (Gab) family adapter proteins Gab1 and Gab2 are expressed by multiple myeloma cells; and that interleukin-6 induces their tyrosine phosphorylation and association with downstream signaling molecules. We further demonstrate that these events are Src family tyrosine kinase-dependent and specifically identify the role of hematopoietic cell kinase (Hck) as a new Gab family adapter protein kinase. Conversely, inhibition of Src family tyrosine kinases by the pyrazolopyrimidine PP2, as in kinase-inactive Hck mutants, significantly reduces IL-6-triggered activation of extracellular signal-regulated kinase and Akt, leading to significant reduction of multiple myeloma cell proliferation and survival. Taken together, these results delineate a key role for Hck-mediated phosphorylation of Gab1 and Gab2 docking proteins in IL-6-induced proliferation and survival of multiple myeloma cells and identify tyrosine kinases and downstream adapter proteins as potential new therapeutic targets in multiple myeloma.

Interleukin-6 (IL-6) is a potent growth and survival factor in multiple myeloma (MM) initiating signaling pathways via binding to the IL-6 receptor. Early IL-6 signaling events that ultimately lead to the activation of extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K) are poorly understood. The IL-6 receptor is composed of two α-chains (IL-6 receptor α, gp80, 80 kDa), which are the binding molecules, and two β-chains (gp130, 130 kDa), which are the signal transducers. IL-6 binding to its receptor triggers both the association of IL-6α with gp130 and gp130 phosphorylation. The binding and activation of Src homology-containing tyrosine phosphatase (SHP)-2 and signal transducer and activator of transcription (STAT) 3 are dependent on the phosphorylation of cytoplasmic gp130 domain residues Tyr-759 and Tyr-767, Tyr-814, Tyr-905, and Tyr-915 respectively (1, 2). Hematopoietic cell kinase (Hck) is a member of the highly conserved Src family of protein-tyrosine kinases (SFKs), which mediate mitogenesis, differentiation, survival, migration, and adhesion (3). In contrast to the ubiquitous expression pattern of Src, Yes, and Fyn, the expression of Hck is restricted to the hematopoietic system; specifically, Hck is preferentially expressed in hematopoietic cells of the myeloid and B-lymphoid lineages (4, 5). In B-lymphoid lineages Hck is a common feature of pro-B-cells and its expression decreases with B-cell differentiation (6). Importantly, Hck binds to an “acidic” domain comprising amino acids 771–811 of gp130 and is independent of STAT3 and SHP2 association via tyrosine residues 759, 767, and 814. Functionally, this region is responsible for the activation of Hck and subsequently for the phosphorylation of ERK and the dephosphorylation of Pyk2, therefore mediating cell proliferation and cell survival. Deletion of this region consequently leads to significant reduction of cell proliferation, thereby supporting a critical role of Hck in mediating IL-6-induced proliferative signals (7).

The mechanisms whereby the gp130/Hck-containing complex mediates IL-6-induced activation of both ERK and PI3-kinase are poorly understood. One explanation could be that Hck can create docking sites for signaling molecules such as growth factor receptor-bound protein (Grb) 2 and SHP2 and/or by activating non-enzymatic adapter molecules that diversify and localize signals downstream of tyrosine kinase or cytokine receptors by their ability to assemble multiprotein complexes (8). These activating adapter molecules belong to a growing family of scaffolding and docking proteins including mammalian Grb2-associated binders (Gab)-1, -2, -3, Drosophila daughter of sevenless, Caenorhabditis elegans Suppressor of clear 1, insulin receptor substrates-1, -2, and -3, Downstream of kinase (Dok), Dok-related (Dok-R), fibroblast growth factor receptor substrate 2, and linker of T cell. Typically, they contain an amino-terminal pleckstrin homology domain with the capacity to bind membrane phospholipids, a central proline-rich domain, and multiple potential binding sites for phosphotyrosine domains or SH2 domains of p85 subunit of PI3K, SHP2, phospholipase Cγ, or Crk. Gab1 and Gab2 act downstream of gp130.
in transmitting signals to ERK mitogen-activated protein kinase (9, 10).

Phosphorylated Gab1 and Gab2 bind phosphatidylinositol-3-kinase via its 85-kDa (p85) regulatory subunit (11), as well as Grb2 and SHP2. In this report we show that the scaffolding adapter Gab family proteins are both expressed by multiple myeloma cells and phosphorylated upon IL-6 stimulation. Moreover, we demonstrate for the first time that gp130-associated SFK Hck forms a complex with Gab proteins in MM cells, thereby regulating their IL-6-stimulated phosphorylation and subsequent recruitment of downstream signaling molecules. These events ultimately lead to the activation of ERK and PI3K. Our studies therefore identify Hck and downstream Gab adapter proteins as potential new therapeutic targets in MM.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human IL-6 was purchased from PEPRO-TECH, Inc. (Rocky Hill, NJ). Antibodies against pERK, ERK2, actin, SHP2, Grb2, Hck, pSTAT3 (Y705), STAT3, and p27 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal antibodies against Gab1, PI 3-kinase p85, and gp130 were purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal antibodies raised against pAKT-1 (S473), AKT-1, and pFKHR (Ser-256) were purchased from Cell Signaling Technology (Beverly, MA). Anti-HA antibody was purchased from Roche Diagnostics, and anti-FLAG antibody was purchased from Sigma. Anti-pY (4G10) monoclonal antibody was kindly provided by Dr. T. Roberts (Dana-Farber Cancer Institute, Boston, MA); the Gab2 antibody was obtained from Drs. H. Gu and B. G. Neel (Harvard Medical School, Boston, MA). Mouse IgG horseradish peroxidase-linked and rabbit IgG horseradish peroxidase-linked antibodies were used as secondary antibodies (Santa Cruz and Amersham Biosciences).

Cell Culture and IL-6 Stimulation—RPMI 8226 and U266 human MM cell lines were obtained from American Type Culture Collection (Manassas, VA). Dexam-sensitive MM.1S and Dexam-resistant MM.1R human MM cell lines were kindly provided by Dr. Steven Rosen (Northwestern University, Chicago, IL). The RPMI 8226 human MM cell line resistant to Dox (Dox40) was kindly provided by Dr. William Dalton (Moffit Cancer Center, Tampa, FL). The ARP-1 MM cell line was a gift from Joshua Epstein (University of Arkansas, Little Rock, AK). All human MM cell lines, as well as primary MM cells, were cultured in RPMI 1640 media (Cellgro, Herndon, VA) and supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Harlan, Indianapolis, IN), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 m M -glutamine (Cellgro). Human embryonic kidney (HEK) 293 cells were cultured in Dulbecco’s modified Eagle’s medium (Cellgro, Herndon, VA) and supplemented with 10% heat-inactivated FBS (Harlan, Indianapolis, IN), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 m M -glutamine (Cellgro). Prior to stimulation of cells with IL-6 (100 ng/ml) they were incubated overnight in RPMI 1640 or Dulbecco’s modified Eagle’s medium with 2% FBS and then for an additional 3 h in medium without FBS.

Isolation of Tumor Cells from the Patient—After appropriate informed consent MM patient cells (CD138+) were obtained from patient bone marrow samples by antibody-mediated negative selection using RosetteSep (StemCell Technologies, Vancouver, BC, Canada), as previously described (12, 13).

Kinase Inhibitors—The pyrazolopyrimidine PP2, a potent inhibitor of Src family tyrosine kinases (17), and PP3, a negative control for PP2 (18), were obtained from Calbiochem-Novabiochem (San Diego, CA). PP2 and PP3 were dissolved in dimethyl sulfoxide (Me2SO) and stored as 5 m M stock solutions at −20 °C.

Receptor-Polymerase Chain Reaction (RT-PCR) Analysis—Total RNA was extracted from 2 × 106 cells using Trizol® reagent (Invitrogen). cDNA was synthesized by means of the SuperScript™ One-Step RT-PCR system with Platinum Taq (Invitrogen). Primers (30 pg) were used as Gab1 (forward, 5′-CCTGTTGCTCATCACTGCTAAGC-3′; reverse, 5′-CTACACTGAGATTCGAGG-3′), and Gab2 (forward, 5′-GCCACTGTTGGAGATTCGAGC-3′; reverse, 5′-GTCAGGCTTGGAGTGTTG-3′) (19). The expected sizes of the amplified fragments were 316 (Gab1) and 512 bp (Gab2). Expression of glyceraldehyde-3-phosphate dehydrogenase was used as a control to measure integrity of the RNA samples. To ensure that RNA samples were not contaminated with DNA, purified RNA was incubated with the appropriate primers and Taq polymerase, without reverse transcriptase. PCR products were separated on a 1% agarose gel and photographed.

Expression of Hck Wild-type and Mutants in MM.1S and HEK 293 Cells—MLV-based transfer vectors were created by subcloning of wild-type and kinase-defective Hck (K269E = KE) IRES-GFP or IRES-GFP fragments into the retroviral vector pMigR1 (14). High titer retroviral pseudotype stocks were generated by triple co-transfection of 293T cells with transfer vectors together with helper vectors expressing in trans the MLV gag-pol and the vesicular stomatitis virus envelope glycoprotein (VSV-G). Transfection was performed using Trans-IT® 293 (MIRUS Corp., Madison, WI) solution according to the manufacturer’s protocol. Viral supernatants were concentrated by ultracentrifugation 100-fold prior to infections. For each infection 1 × 106 MM.1S cells or 0.1 × 106 HEK 293 cells were seeded in 10 × 10 cm plates and incubated with viral stocks in the presence of 5 μg/ml Polybrene (37 °C, 5% CO2). GFP-positive cells were isolated by fluorescence-activated cell sorting after 20 h.

Plasmids and Constructs and Transfection—Wild-type and kinase-defective (K269E = KE) forms of human Hck were subcloned into the retroviral vector pMigR1 as the Hck-IRES-GFP sequence as previously described (14). HEK 293 cells were transfected with pBART expression vector containing FLAG-tagged Gab1 cDNA (15) and pEBB expression vector containing HA-tagged Gab2 cDNA (16) using LipofectAMINE® 2000 solution (Invitrogen) according to the manufacturer’s protocol. Transient transfection of wild-type Src and kinase-inactive Src (Upstate Biotechnologies, Lake Placid, NY) was performed using Nucleofector Kit V, according to manufacturer’s instructions (Amaxa Biosystems, Cologne, Germany).

Cell Lysis, Immunoprecipitation, and Immunoblotting—After stimulation with IL-6, cells were washed three times with phosphate-buffered saline and lysed with lysis buffer (10 mM Tris, 50 mM NaCl, sodium pyrophosphate, 1% Triton X-100, 1 mM Na3VO4, and 1× protease inhibitor mixture (Roche Applied Science)) and further processed for immunoprecipitation and immunoblotting, as in previous studies (15, 20, 21). As controls for immunoprecipitation, 1 nonspecific immunoreactive antibody bands were determined by incubating protein A-Sepharose™ CL-4B beads with lysis buffer and specific antibody only; and 2 nonspecific immunoreactive protein bands were determined by incubation of whole cell lysates with preimmune rabbit serum or monoclonal antibody against an irrelevant epitope (interferon γ), followed by incubation with protein A-Sepharose™ CL-4B beads. Densitometry was performed using the NIH Image Analysis program (version 1.62) by Wayne Rasband.

DNA Synthesis and Cell Proliferation Assay—Cell growth was assayed by measuring [3H]thymidine uptake as described in prior studies (22).

RESULTS

Inhibition of IL-6-induced Tyrosine Phosphorylation of Cellular Proteins in MM.1S Cells by the Pyrazolopyrimidine PP2—IL-6 is a potent autocrine and paracrine growth and survival factor in multiple myeloma (23–25) that triggers signaling pathways via binding to the IL-6 receptor and triggering phosphorylation of the IL-6 receptor signaling chain gp130. However, early IL-6 signaling events that ultimately lead to the activation of ERK and PI3K are poorly understood. The use of pyrazolopyrimidine PP2, a potent inhibitor of the SFK p56Lck, p59Fyn, and p56/59 Hck (17, 26), demonstrated the functional relevance of Hck for gp130-mediated proliferation (7). Although PP2 was reported as a potent and specific inhibitor of Lck, Fyn, and Hck (17), it also blocks other kinases with high potency (28). After determining potential Hck targets using the PP2 model, this study therefore utilizes MM cells infected with wild-type Hck and kinase-inactive KE-Hck to limit the potential lack of PP2 specificity. We first examined MM.1S cells for changes in IL-6-induced tyrosine phosphorylation after pretreatment with PP2. As shown in Fig. 1, IL-6 stimulation of MM.1S cells (1, 5, and 30 min) induced rapid and transient tyrosine phosphorylation of proteins with apparent masses of ~60, 72, 85, 100, and 110 kDa. Importantly, tyrosine phosphorylation of these proteins triggered by IL-6 was markedly decreased after pretreatment with the PP2. In addition, Fig. 1 shows the expression of Gab1 (110 kDa), Gab2 (97/100 kDa), and Gab3. These observations are consistent with published data showing effects of PP2 on Gab family protein phosphorylation in the absence of IL-6 stimulation.
SHP2 (72 kDa), and Hck (56/59 kDa) as possible candidates for these tyrosine-phosphorylated proteins in the MM.1S model cell line.

**Gab1 and Gab2 Are Expressed in MM Cell Lines and MM Patient Cells**—To investigate a potential role of the 110-kDa protein Gab1 and the p97/100 Gab2 in IL-6-mediated MM cell signaling, we first determined the expression of Gab1 and Gab2 proteins in several MM cells by RT-PCR. As shown in Fig. 2a, all MM cell lines investigated, as well as patient MM cells, express both Gab1 and Gab2. The bands obtained for Gab1 (316 bp) and Gab2 (512 bp) were specific for cDNA, as no bands were detected in the absence of reverse transcriptase during the cDNA synthesis step (data not shown). Control PCR amplification with primers to glyceraldehyde-3-phosphate dehydrogenase confirmed that the isolated RNA could be PCR amplified and the quantity of RNA used was similar for all cells.

**IL-6 Mediates Tyrosine Phosphorylation of the Gab Adapter Proteins and Their Association with Other Signaling Proteins in MM Cells**—Gab1 and Gab2 act downstream of cytokine and growth factor receptors as well as the antigen receptors on T and B cells (10). However, whether IL-6 signaling in MM cells is mediated through Gab1 and Gab2 as downstream signaling proteins is to date unknown. We therefore next investigated whether IL-6 can induce: 1) phosphorylation of Gab1 and Gab2; and 2) the association of Gab adapter molecules with other signaling proteins in MM cells. Serum-starved cells were stimulated with IL-6 and cell lysates were immunoprecipitated with anti-Gab1 and anti-Gab2 antibody, respectively, followed by immunoblotting with antibodies directed against phosphotyrosine, Gab1, Gab2, and SHP2. To picture Gab-1 specific and Gab2-specific immunoreactive bands on the same blot, the membrane was extensively washed after Gab-1 detection (with no visible specific Gab2 band) and rebotted with anti-Gab2 antibody (Fig. 2b, second panel). Our results show that IL-6 mediates both early Gab1 and Gab2 tyrosine phosphorylation. Co-immunoprecipitation studies further demonstrate that IL-6 induces the association of Gab1 and Gab2 with tyrosine-phosphorylated SHP2 (Fig. 2b, third panel) and CRKL (data not shown) in MM cells. Gab proteins constitutively associate with Grb2, as expected (16, 27, 28) (Fig. 2b, fourth panel).

**IL-6-induced Tyrosine Phosphorylation of Gab1 and Gab2 Is Down-regulated by the Pyrazolopyrimidine PP2**—We next sought to characterize upstream IL-6-induced signaling events that regulate Gab1 and Gab2 tyrosine phosphorylation. Signal transduction of IL-6 involves activation of SFKs in MM cells (29). However, the mechanisms whereby SFKs participate in mediating the mitogenic response in MM cells are unknown. Possible candidates for proteins whose IL-6-induced tyrosine phosphorylation is markedly decreased upon pretreatment with the pyrazolopyrimidine PP2 are Gab1 and Gab2 (Fig. 1). We therefore next tested the hypothesis that SFKs are required for IL-6-induced tyrosine phosphorylation of Gab1 and Gab2 in MM cells. Immunoprecipitations were performed using anti-Gab1 (Fig. 3, a and b) and anti-Gab2 (Fig. 3, c and d) antibodies on cell lysates prepared from control and IL-6-stimulated MM cells pretreated with PP2 or left untreated; followed by immunoblotting with an anti-phosphotyrosine-specific antibody and densitometric analysis. To confirm that equal amounts of Gab1 and Gab2 were immunoprecipitated, the filter was rebotted with Gab1 and Gab2 antisera, respectively; loading of whole cell lysate served as a size control. Our results indicate that the pyrazolopyrimidine PP2 inhibits IL-6-triggered rapid tyrosine phosphorylation of both Gab1 and Gab2.

**IL-6-induced Association of Gab1 and Gab2 with SHP2 in MM Cells Is Blocked by the Pyrazolopyrimidine PP2**—In contrast to SH2 domain containing protein-tyrosine phosphatase (SHP)-1, protein-tyrosine phosphatase SHP-2 is a positive regulator of signaling from receptor tyrosine kinases and cytokine receptors (30–33). SHP2 binds Gab family proteins through the COOH-terminal consensus binding motif YXXV/I/L. Furthermore, previous studies underscore the importance of Gab-SHP2 interaction, strongly suggesting that the primary role of Gab proteins is to recruit SHP2 phosphatase to activate down-
whole cell lysates, and preimmune rabbit serum; immunoprecipitation (IP) with protein A-Sepharose, lysis buffer, and specific antibody only; cells were either pretreated with PP2 (5 μM) or Me₂SO (−) for 1 h and then stimulated with IL-6 (100 ng/ml) for the indicated intervals. a and c, cell lysates were immunoprecipitated with either anti-Gab1 (a) or anti-Gab2 (c) antibody and analyzed by immunoblotting with anti-phosphotyrosine-specific antibody (4G10) or anti-Gab1 and anti-Gab2 antibodies, respectively. b and d, densitometric analysis of blots from three separate experiments equivalent to those presented in panels a and c are shown in panels b and d as -fold change of phosphorylation (mean ± S.D.). C, immunoprecipitation (IP) with protein A-Sepharose, lysis buffer, and specific antibody only; S, immunoprecipitation with protein A-Sepharose, whole cell lysates, and preimmune rabbit serum; WCL, whole cell lysate.

As SHP2 with p85 in MM cells (35). We therefore next investigated whether: 1) IL-6 can trigger the association between Gab proteins and SHP2 in MM cells; and if so, whether 2) inhibition of SFKs by PP2 abrogates this IL-6-triggered complex formation. Anti-SHP2 immunoprecipitates from cell lysates of IL-6-stimulated MM cells, pretreated with PP2 or left untreated, were analyzed by immunoblotting with anti-Gab1, anti-Gab2, and anti-SHP2 antibodies. As shown in Fig. 4a, IL-6-induced tyrosine phosphorylation of proteins with molecular masses of 100–115 kDa, which was markedly decreased by PP2 treatment. In addition, IL-6-induced complex formation between both Gab1 and Gab2 and SHP2 was abrogated upon PP2 treatment.

Grb2 is a small adapter protein comprised of an SH2 domain flanked by two SH3 domains. Gab proteins constitutively associate with the COOH-terminal SH3 domain of Grb2, allowing the recruitment of further downstream proteins via the interaction of the SH2 domain of Grb2 (16, 36, 37). In IL-6-induced growth signaling, the phosphorylation of residue Tyr-759 of gp130 is required for binding and tyrosine phosphorylation of SHP-2, its subsequent association with Grb2, and the activation of mitogen-activated protein kinase by linking Grb2 to the Ras pathway through Sos (1). As expected, our data shows constitutive association of Gab proteins with Grb2. In contrast IL-6-induced SHP-2-Grb2 association and tyrosine phosphorylation of Gab proteins was decreased after pretreatment with PP2 (Fig. 4b). Taken together, these data indicate that IL-6-induced Gab protein tyrosine phosphorylation and Gab1/Gab2-SHP2 complex formation is dependent on SFK activity.

IL-6-induced Tyrosine Phosphorylation of Gab1 and Gab2 in MM Cells Is Hck-mediated—Previous studies have shown that 1) Hck is activated by IL-6 stimulation in murine B-9 hybridoma cells and human LP-1 MM cells (29); and that 2) Hck is bound to an acidic domain (comprising amino acids 771–811) of gp130 (7). These results suggest that Hck is involved in gp130 signaling by creating docking sites for signaling molecules such as SHP2 and Grb2, as well as by activating adapter molecules. In our previous experiments, we observed a 60-kDa band in both anti-Gab1 and anti-Gab2 immunoprecipitates (data not shown). We therefore next examined whether Hck is associated with these proteins. Immunoprecipitations using anti-Hck antibody were performed on cell lysates prepared from control and IL-6-stimulated MM cells, either pretreated with PP2 or untreated; followed by immunoblotting with the indicated antibodies. Our results show that Hck, which is bound to gp130, is also constitutively associated with both endogenous Gab1 and Gab2. Pretreatment of MM cells with PP2 abrogated the phosphorylation of 110-, 97/100-, and 56/59-kDa proteins corresponding with molecular masses of Gab1, Gab2, and Hck.
Role of Gp130-Hck-Gab Protein Complex in MM Pathogenesis

(Fig. 4c). In ongoing studies we are investigating the proline-rich SH3 domain of Hck as a potential candidate binding site for both adapter proteins.

Besides Hck, the SFK Src and Lyn are highly expressed in MM.1S cells. Moreover, a previous report indicates that, similar to Hck, Lyn also binds to gp130 (29). To verify whether these SFKs are associated with Gab adapter proteins, and if so, can be phosphorylated by IL-6 stimulation, immunoprecipitations were performed on lysates of MM cells triggered with IL-6. Importantly and in contrast to Hck, our results show that neither Src nor Lyn kinase co-immunoprecipitates Gab adapter proteins (data not shown). The present data using PP2 therefore support previous findings that show that signaling through gp130 mediates cell proliferation and activation of Hck and ERK kinases (7) and additionally indicate the involvement of Gab adapter proteins in this signaling cascade. Although PP2 was initially reported as a potent and specific inhibitor of Lck, Fyn, and Hck (17), previous studies show that PP2 also blocks other kinases with high potency (38).

To verify our data, to omit the potential lack of specificity of PP2, and to investigate the relevance of a gp130-Hck-Gab containing complex in HEK 293 cells (Fig. 5, e and f). HEK 293 cells, which express IL-6 receptor (39) but not Hck, were infected, using recombinant retrovirus, with either wild-type Hck or kinase-inactive Hck. GFP-positive cells were transiently transfected with wild-type Flag-Gab1 (wt-Flag-Gab1) or wild-type HA-Gab2 (wt-HA-Gab2) constructs, respectively. After serum starvation, cells were either left unstimulated or stimulated with IL-6 (100 ng/ml). Cell lysates were immunoprecipitated with anti-Flag (e) or anti-HA (f) antibody and immunoblotted with the antibodies indicated. C, immunoprecipitation with preimmune rabbit serum; I, immunoprecipitation with protein A-Sepharose, whole cell lysates, and irrelevant antibody; pY, phosphotyrosine.

IL-6-induced Association of Gab1 and Gab2 with SHP2 Is Hck-mediated—To further confirm that IL-6-triggered complex formation between SHP2 and Gab proteins is mediated via...
kinase-active Hck, MM cells infected with either wild-type Hck or kinase-inactive Hck were stimulated with IL-6; anti-SHP2-immunoprecipitates were then analyzed by immunoblotting with anti-Gab1, anti-Gab2, or anti-SHP2 antibodies. As shown in Fig. 6a, IL-6-induced tyrosine phosphorylation of Gab1 and Gab2, as well as their association with SHP2, were markedly decreased in MM cells with kinase-inactive Hck versus MM cells with wild-type Hck. In anti-Grb2 immunoprecipitates, IL-6-induced tyrosine phosphorylation of Gab1 and Gab2, but not their association with Grb2, was abrogated (Fig. 6b). Taken together, these data further confirm that IL-6-induced Gab protein tyrosine phosphorylation and formation of Gab/Grb2-SHP2-containing protein complexes is dependent on Hck activity.

IL-6-induced Activation of ERK and PI 3-Kinase/AKT-1 Is Dependent on Kinase-active Hck—IL-6 activates ERK, STAT3, and PI3K signaling pathways (40). We next examined whether these pathways are mediated via Hck-Gab adapter proteins. Pretreatment with PP2, but not with the diluent control Me2SO, inhibited IL-6-induced phosphorylation of ERK (Fig. 7, a and b) and AKT-1 (Fig. 7, c and d). Equal protein loading was verified by reblotting the membrane with anti-ERK and anti-AKT-1 antibodies, respectively. In contrast to ERK and AKT-1, phosphorylation of STAT3 was not decreased after pretreatment with PP2 (Fig. 7a). These effects were dependent on the dose of PP2 and were not seen in the presence of PP3, which
served as a negative control (18) (Fig. 7, b and d). As expected, phosphorylation of FKHR, a known downstream kinase in the AKT-1 signaling cascade (Fig. 7d), was also decreased. IL-6-induced ERK phosphorylation was significantly decreased in MM cells infected with kinase-inactive Hck, but not with wild-type Hck (Fig. 7e). In contrast, IL-6-induced ERK-phosphorylation was not decreased in MM cells transfected with kinase-inactive Src compared with MM cells transfected with wild-type Src. These results show that inhibition of ERK and PI3K/ AKT-1 pathways by PP2 mainly acts via Hck.

To further define the functional significance of these findings and to compare the properties of uninfected MM cells with MM cells infected with wild-type Hck, we next tested whether IL-6-mediated MM cell proliferation is influenced by treatment with PP2. As shown in Fig. 7g, IL-6-induced MM cell proliferation in both wild-type Hck infected and non-infected MM.1S cells was inhibited in a similar dose-dependent fashion by the pyrazolopyrimidine PP2. As shown in Fig. 7h, IL-6-induced G1/S phase transition (35) was abrogated in the presence of PP2 (data not shown). Taken together, these studies therefore show a key role for Hck-mediated phosphorylation of docking proteins Gab1 and Gab2 mediating IL-6-induced proliferation and survival of MM cells, identifying SFK Hck and downstream adapter proteins as potential new therapeutic targets in MM.

**DISCUSSION**

We have previously shown that IL-6 induces growth and survival in MM cells via the activation of ERK and PI3K signaling cascades, and in this study we define a role of Hck mediating these sequelae (35). Hck is a member of the highly conserved SFK that induce mitogenesis, differentiation, survival, migration, and adhesion (3, 41). SFKs contain a SH2 domain that binds phosphotyrosine residues and a SH3 domain that binds proline-rich sequences (41). In addition, Hck binds to an acidic domain (comprising of amino acids 771–811) of gp130, with resultant activation of Hck, phosphorylation of ERK, and dephosphorylation of related adhesion focal tyrosine kinase proline-rich tyrosine kinase 2 (RAFTK). Deletion of this domain leads to a significant reduction of cell proliferation, thereby supporting the critical role of Hck in mediating IL-6-induced proliferative signals (7). The present study provides further insight into molecular mechanisms by which the gp130/ Hck-containing complex mediates IL-6-induced activation of both ERK and PI3K.

We show for the first time that Gab adapter proteins Gab1 and Gab2 are expressed in MM cells. The Gab scaffolding proteins recruit multimeric cytosolic protein complexes containing SHP2, Grb2, CRKL, and p85(8) to stimulate and modulate downstream signaling molecules, i.e. ERK and PI3K. Interestingly a recent study in rat primary hepatocytes shows that Src kinase is constitutively associated with Gab2, and that epidermal growth factor-induced Gab2 phosphorylation is Src kinase-dependent (42). The present study shows that Hck is constitutively associated with both Gab1 and Gab2 in MM cells, which may implicate the SH3 domain of Hck in this interaction. Besides Hck, the Src family tyrosine kinases Src and Lyn are also highly expressed in MM.1S cells. Moreover, a previous report indicates that, similar to Hck, Lyn also binds to gp130 (29); however, in contrast to Hck, neither Src nor Lyn kinase co-immunoprecipitates Gab adapter proteins. We further demonstrate that IL-6-induced Gab protein phosphorylation in MM cells is Hck-dependent, as confirmed both by the inhibitory effects of the pyrazolopyrimidine PP2, and use of a kinase-inactive Hck mutant. Previous studies underscore the importance of Gab-SHP2 interactions and suggest that the primary role of Gab proteins is to recruit SHP2 phosphatase to activate downstream signaling molecules (8). Importantly, Gab1 (27) and Gab2 (10, 16) are among the substrates of the activated SHP2. Moreover the carboxyl terminus of SHP-2 also contains the YXXM motif, a consensus sequence for binding Grb2 (43, 44), which is constitutively associated with Gab proteins (16, 36, 37). Our results show that both Gab protein-associated IL-6-induced complex formation and phosphorylation of SHP2-Grb2 are decreased in the presence of inactive Hck. Consequently, Gab-associated activation of downstream signaling molecules ERK and AKT-1 mediating MM cell growth and survival are significantly decreased. Residual Grb2-associated tyrosine phosphorylation of SHP2 and phosphorylation of ERK and AKT-1 in the absence of kinase-active Hck may be because of the direct effects of SHP2 on the Grb-2/Sos/Ras-mitogen-activated protein pathway. In contrast to ERK and AKT-1, our results show that IL-6-induced STAT3 activation is not dependent on Hck. This observation is consistent with a previous report that shows that deletion of the acidic Hck-binding domain of p130 blocks IL-6-induced activation of ERK, but not of STAT3, signaling cascades (7).

Mice-lacking Gab1 die during embryogenesis and show defective responses to several stimuli (45, 46). In contrast, Gab2/−/− mice are viable and generally healthy; however, the response (degranulation, cytokine gene expression) to stimulation of Gab2/−/− mast cells is defective (47). In MM cells, both Gab1 and Gab2 are expressed, phosphorylated after IL-6 stimulation, and associated with similar signaling proteins. This indicates a potential redundancy in function. Distinct recruitment and function of Gab1 and Gab2 was observed in Met receptor-mediated epithelial morphogenesis (48). Our own unpublished data indicate that IL-6-triggered PI3K activation is mediated mainly via Gab2, and that IL-6-triggered ERK activation is predominantly mediated via Gab1. In ongoing studies using small interfering RNA, we will further confirm and investigate the potential distinct functional roles of Gab1 and Gab2 in MM pathogenesis. Taken together, these results not only provide further insights into IL-6-mediated cell growth and survival, but also identify Hck and downstream Gab adapter proteins as potential new therapeutic targets in MM.

**Acknowledgments**—We thank Melissa Simoncini and Guilan Li for technical assistance, Drs. Skorski and Smithgall (Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine) for providing retroviral Hck constructs, Drs. Schaeper and Birchmeier (Max Delbruck Center for Molecular Medicine Berlin-Buch, Berlin) for providing Gab1 constructs, and Drs. Gu and Neel (Cancer Biology Program, Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Institute of Medicine, Boston) for providing Gab2 antibodies.

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Critical Role for Hematopoietic Cell Kinase (Hck)-mediated Phosphorylation of Gab1 and Gab2 Docking Proteins in Interleukin 6-induced Proliferation and Survival of Multiple Myeloma Cells

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J. Biol. Chem. 2004, 279:21658-21665.
doi: 10.1074/jbc.M305783200 originally published online March 9, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M305783200

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