

Interleukin-6 activates phosphoinositol-3' kinase in multiple myeloma tumor cells by signaling through RAS-dependent and, separately, through p85-dependent pathways

Jung-hsin Hsu¹, Yijiang Shi¹, Patrick Frost¹, Huajun Yan¹, Bao Hoang¹, Sanjai Sharma¹, Joseph Gera¹ and Alan Lichtenstein^{*1}

¹Department of Medicine, West LA VA-UCLA Medical Center and Jonsson Comprehensive Cancer Center, UCLA, Los Angeles, CA 90073, USA

The IL-6-induced activation of the phosphatidylinositol-3' kinase (PI3-K)/AKT cascade in multiple myeloma (MM) cells is critical for tumor cell proliferation and viability. Since the IL-6 receptor does not contain binding sites for the p85 regulatory portion of PI3-K, intermediate molecules must play a role. Coimmunoprecipitation studies in MM cell lines demonstrated the IL-6-induced formation of two independent PI3-K-containing complexes: one containing p21 RAS but not STAT-3 and a second containing STAT-3 but not RAS. Both complexes demonstrated IL-6-induced lipid kinase activity. IL-6 also generated kinase activity in a mutant p110 molecule that could not bind p85. Use of dominant-negative (DN) constructs confirmed the presence of two independent pathways of activation: a DN RAS prevented the IL-6-induced generation of lipid kinase activity in the mutant p110 molecule but had no effect on activity generated in the STAT-3-containing complex. In contrast, a DN p85 prevented the generation of kinase activity in the STAT-3-containing complex but had no effect on activity generated in the p110 molecule. Both DN constructs significantly prevented the IL-6-induced activation of AKT. MM cells expressing activating RAS mutations demonstrated enhanced IL-6-independent growth and constitutive PI3-K activity. These data indicate two potential independent pathways of PI3-K/AKT activation in MM cells: one mediated via signaling through RAS which is independent of p85 and a second mediated via p85 and due to a STAT-3-containing complex.

Oncogene (2004) 23, 3368–3375. doi:10.1038/sj.onc.1207459
Published online 15 March 2004

Keywords: multiple myeloma; interleukin-6; AKT; phosphatidylinositol-3'-kinase

Introduction

Interleukin-6 is an important tumor growth factor in patients with multiple myeloma (MM) (Kawano *et al.*, 1988; Anderson *et al.*, 1989). One of several signal cascades activated by IL-6 in MM tumor cells is the phosphatidylinositol 3-kinase (PI3-K)/AKT pathway (Tu *et al.*, 2000; Hideshima *et al.*, 2001). The ability of IL-6 to activate this growth-promoting pathway may somewhat explain its ability to stimulate expansion of myeloma clones. Indeed, recent studies document the importance of IL-6-dependent activation of PI3-K/AKT as it promotes proliferation (Tu *et al.*, 2000) protects survival (Hideshima *et al.*, 2001) and stimulates migration (Podar *et al.*, 2002) of myeloma cells.

IL-6 initiates intracellular responses via signaling through glycoprotein 130 (gp 130), the signal transduction portion of the IL-6 receptor. In addition to IL-6, leukemia inhibitory factor (LIF), also signaling through gp 130, can activate the PI3-K/AKT pathway (Oh *et al.*, 1998). However, the molecular mechanism by which IL-6 and gp 130 activates PI3-K has not previously been elucidated. The PI3-K enzyme is a heterodimer, made up of p85, the regulatory subunit, and p110, the catalytic subunit (reviewed in Carpenter and Cantley, 1990). Although some cytokines can activate PI3-K by interaction of their receptors with the SH2 domain of the p85 regulatory subunit, the gp 130 signal transducing portion of the IL-6 receptor does not contain consensus binding sites for this SH2 domain (Boulton *et al.*, 1994). This indicates that an intermediate signal protein or adaptor mediates PI3-K activation in IL-6-treated cells.

There are several possible candidates for intermediates that may be mediating IL-6-induced activation of PI3-K in myeloma cells. In MM.1S myeloma cells, IL-6 triggered the association of PI3-K with the SHP2 adaptor (Hideshima *et al.*, 2001) and SHP2 is reported to mediate PI3-K activation in other models (Hakak *et al.*, 2000). However, lipid kinase activity specifically present in SHP2:PI3-K complexes was not tested in these myeloma cells. IL-6 can also activate JAKs (Ogata *et al.*, 1997), STAT-3 (Catlett-Falcone *et al.*, 1999), RAS (Ogata *et al.*, 1997) and vav (Lee *et al.*, 1997) in myeloma cells and these proteins have also been

*Correspondence: A Lichtenstein, Hematology-Oncology, W111H, VA West LA Hospital, 11301 Wilshire Blvd., Los Angeles, CA 90073, USA. E-mail: alan.lichtenstein@med.va.gov

Received 3 October 2003; revised 15 December 2003; accepted 15 December 2003; Published online 15 March 2004

identified as intermediates for activation of PI3-K in previous studies (Pfeffer *et al.*, 1997; Shigematsu *et al.*, 1997; Oh *et al.*, 1998). To address this question, we evaluated several of these potential candidates in two myeloma cell lines. Our results support the existence of at least two independent pathways by which IL-6 can activate PI3-K and, subsequently, AKT, in myeloma cells. One pathway was mediated via RAS activation with direct stimulation of p110 kinase activity which was independent of p85, and a second that was mediated via p85 and a STAT-3-containing complex. Additional studies in oncogenic, mutated RAS-containing MM cells confirmed the existence of the RAS-mediated pathway of PI3-K activation.

Results

IL-6 induces formation of separate PI3-kinase:STAT-3 and PI3-kinase:RAS complexes in MM cells

To investigate the IL-6-dependent activation of PI3-K in MM cells, we first used the AF-10 MM cell line. This line responds to IL-6 with enhanced proliferation and survival. Most importantly, it also demonstrates IL-6-induced PI3-K activation as previously published (Tu *et al.*, 2000). Moreover, AF-10 cells exhibit activation of classical IL-6 signal pathways (Kishimoto *et al.*, 1995) which include several proteins that could also mediate PI3-K activation. We, thus, tested interactions between these proteins and PI3-K by coimmunoprecipitation studies in IL-6-stimulated MM cells. We initially investigated PI3-K binding to JAK-1, STAT3, SHP2 and vav because these proteins become tyrosine phosphorylated by IL-6 in MM cells, mediate activation of PI3-K in other cell types, and could, thus, act as adaptors, binding to the SH2 domain of p85. PI3-K was immunoprecipitated with an anti-p85 antibody and the precipitate was immunoblotted for these proteins. As shown in Figure 1a, SHP2 and STAT-3 become associated with PI3-K in an IL-6-dependent fashion, with increased binding evident by 5 min of exposure. In contrast, constitutive binding of JAK 1 to PI3-K was evident, which did not further increase upon exposure to IL-6. These latter binding results with JAK1 are consistent with that of Oh *et al.* (1998) who studied PI3-K activation by signaling through gp 130 with LIF in cardiac myocytes. Comparable amounts of p85 were present in each immunoprecipitate (lower panel). In contrast, by immunoblot assay of anti-p85 immunoprecipitates, we could not detect the presence of vav in PI3-K complexes (data not shown).

An additional mechanism by which cytokines might stimulate PI3-K activation is through activated RAS. Activated GTP-bound RAS can directly bind to and activate the p110 kinase domain of PI3-K (Rodriguez-Viciana *et al.*, 1994) and, since IL-6 significantly activates RAS in myeloma cells (Ogata *et al.*, 1997), we also investigated the ability of RAS to associate with PI3-K. As shown in Figure 1a, p21 RAS was also associated with PI3-K in an IL-6-dependent fashion,

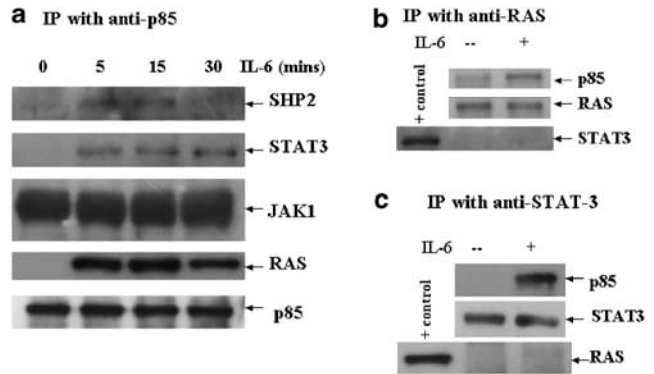


Figure 1 IL-6 induces two separate PI3-K-containing complexes in AF-10 myeloma cells. (a) AF-10 MM cells were treated with IL-6 (100 U/ml) for 0, 5, 15 or 30 min, after which PI3-K was immunoprecipitated with anti-p85 antibody and immunoblot performed for SHP2, STAT3, JAK1, p21RAS, and p85. (b), AF-10 cells were treated with or without IL-6 for 15 min, RAS was immunoprecipitated and immunoblot assay performed for p85, RAS and STAT3. The '+ control' for STAT3 is lysate from ANBL-6 MM cells. (c) STAT3 was immunoprecipitated from the same cells and immunoblot assay performed for p85, STAT3 and RAS. The '+ control' for RAS is KNRK cell lysate purchased from Santa Cruz Biochemicals

with increased binding rapidly detected by 5 min of treatment.

To confirm this IL-6-dependent binding, we first immunoprecipitated RAS in IL-6-treated MM cells and immunoblotted the precipitate for p85. As shown in Figure 1b, the IL-6-dependent interaction between RAS and p85 was again demonstrated. However, immunoblotting the precipitate for STAT-3 demonstrated that the RAS-p85 complex did not contain any STAT-3 in the presence or absence of IL-6. In likewise fashion, when we immunoprecipitated STAT-3 from IL-6-treated MM cells (Figure 1c), an IL-6-dependent interaction between STAT-3 and p85 was again demonstrated but there was no p21 RAS associated in this complex (bottom panel, Figure 1c). These coimmunoprecipitation studies, which were all repeated twice more with identical results, demonstrate the possibility that two separate complexes formed upon IL-6 stimulation: One containing ras and PI3-K but not STAT-3 and a second containing STAT-3 and PI3-K but not RAS. However, we could not rule out that one protein complex consisting of STAT-3, p85, p110 and RAS forms when RAS and STAT-3 are activated by IL-6 but the full complex does not survive the entire coimmunoprecipitation procedure.

To ensure that the above results were not peculiar to this particular MM cell line, we also performed a limited number of coimmunoprecipitation studies in the ANBL-6 MM line. ANBL-6 cells also respond to IL-6 with enhanced proliferation (Billadeau *et al.*, 1995) and demonstrate IL-6-induced PI3-K activation (see below, Figure 7). As shown in Figure 2a, STAT-3 also associates with p85 PI3-K upon IL-6 treatment of ANBL-6 cells but RAS is not present in this complex (Figure 2a). In addition, PI3-K also associates with RAS

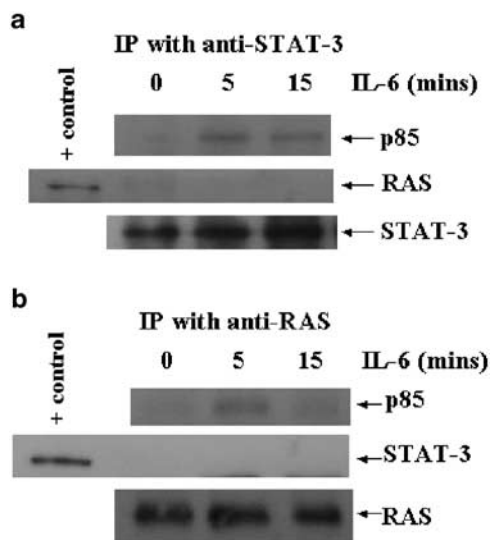


Figure 2 IL-6 induces two separate PI3-K-containing complexes in ANBL-6 MM cells. ANBL-6 MM cells were treated with IL-6 (100 U/ml) for 0, 5 or 15 min, after which STAT3 (in (a)) or RAS (in (b)) was immunoprecipitated. Immunoblot assays performed for p85, RAS and STAT3. '+ controls' for RAS and STAT-3 were as in Figure 1

upon IL-6 stimulation and STAT-3 is not present in this latter complex (Figure 2b). Thus, results in IL-6-treated ANBL-6 cells are similar to those in AF-10 cells, indicating the generation of at least two separate PI3-K-containing complexes upon IL-6 stimulation.

IL-6-induced generation of lipid kinase activity in MM cells

To determine if the above-described proteins that interacted with PI3-K contribute to IL-6-induced lipid kinase activity, we first immunoprecipitated them from AF-10 cells and tested the precipitates for the ability to phosphorylate phosphoinositol. Figure 3a is a representative of three separate experiments that had identical results. Although containing constitutive lipid kinase activity, SHP-2 immunoprecipitates did not demonstrate any IL-6-induced increase in activity (top panels of Figure 3a). In contrast, a strong IL-6-dependent increase in kinase activity was evident in STAT-3, and RAS immunoprecipitates. A smaller transient IL-6-dependent increase in activity was also seen in anti-JAK precipitates (bottom panels of Figure 3a). Immunoblot assay demonstrated comparable amounts of SHP2, STAT-3, RAS and JAK1 were present in these immunoprecipitates at the varying time points of IL-6 exposure (panels below each lipid kinase assay). Again, a limited number of experiments in the ANBL-6 MM cell line model (Figure 3b) revealed similar results in that immunoprecipitated RAS or STAT-3 also demonstrated IL-6-dependent lipid kinase activity.

In a separate set of experiments, we transiently transfected AF-10 MM cells with epitope-tagged STAT-3 or p110 PI3-K constructs, treated the cells with

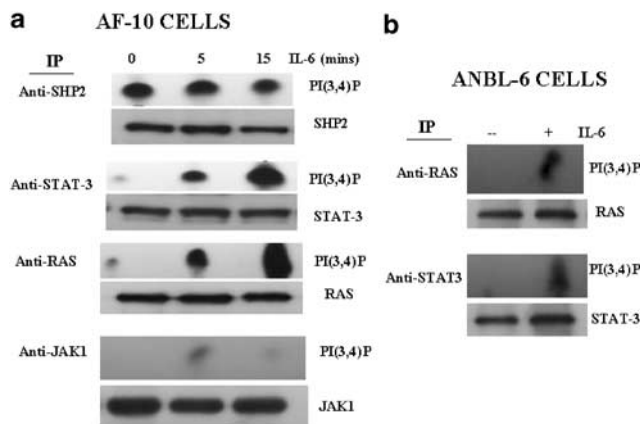


Figure 3 IL-6 induces lipid kinase activity in anti-RAS and anti-STAT-3 immunoprecipitates. (a) AF-10 cells were treated with IL-6 for 0, 5 or 15 min and, (b) ANBL-6 MM cells were treated with or without IL-6 for 15 min. At designated time points, SHP2, STAT-3, RAS or JAK-1 was immunoprecipitated and the immunoprecipitates were tested for their ability to phosphorylate phosphatidyl-4-monophosphate (PI(4)) in a lipid kinase assay as described in Materials and methods. For each immunoprecipitate, the reaction product, PI (3, 4)P, is shown in the top panel and immunoblot for the precipitated protein is shown in the bottom panel

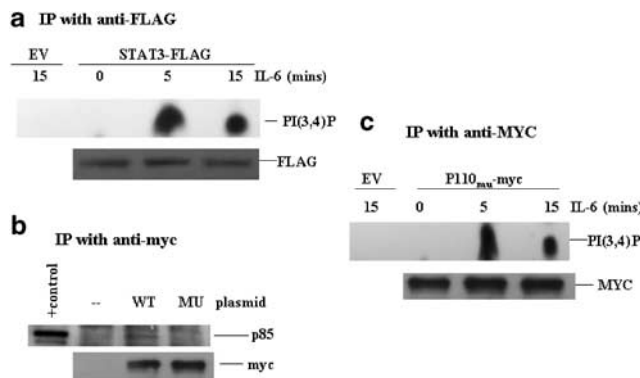


Figure 4 Ability of epitope-tagged constructs to demonstrate IL-6-induced lipid kinase activity. (a) AF-10 cells were transiently transfected with FLAG-tagged STAT-3 or empty vector (EV), treated with IL-6 for 0, 5 or 15 min and STAT-3 immunoprecipitated with anti-FLAG antibody. Immunoprecipitates were tested for lipid kinase activity (upper panel) and presence of FLAG-STAT-3 (lower panel). (b) AF-10 cells were transiently transfected with myc-tagged WT p110 (WT) or a mutant p110 (MU) which cannot bind p85. After 24 h, the expressed proteins were immunoprecipitated with anti-myc antibody and immunoblotted for p85 or myc. The '+ control' for p85 is Jurkat cell lysate purchased from Santa Cruz Biochemicals. (c) AF-10 cells were transiently transfected with the myc-tagged mutant p110 (p110_{mu}) or empty vector (EV), treated with IL-6 for 0, 5 or 15 min and p110_{mu} was immunoprecipitated with anti-myc antibody. Immunoprecipitates were tested for lipid kinase activity (top panel) and presence of myc-p110_{mu} (lower panel)

IL-6, immunoprecipitated the proteins and performed the lipid kinase assay. As expected, immunoprecipitated STAT-3 demonstrated a clear IL-6-dependent generation of lipid kinase activity (Figure 4a). The p110 PI3-K construct we used is a deletion mutant that lacks the

minimal p85 binding site ($\Delta 3-125$) so that it cannot bind to p85 (Rodriguez-Viciana *et al.*, 1996). To first confirm the inability of this expressed protein to bind p85, we transfected this construct or the p110 wild-type (WT) gene into AF-10 cells, immunoprecipitated the respective proteins and immunoblotted for p85. The mutant p110 was unable to bind any p85 while significant amounts were bound to WT p110 (Figure 4b). Also shown in Figure 4 is that this mutant p110, which cannot bind to p85, developed IL-6-dependent lipid kinase activity in AF-10 cells (Figure 4c). These latter results demonstrate that at least part of the lipid kinase activity generated in IL-6-treated MM cells is due to a direct activation of the p110 kinase domain, which is independent of p85. This is consistent with the known ability of activated GTP-bound RAS to directly activate p110 (Rodriguez-Viciana *et al.*, 1994).

Effects of dominant-negative RAS or p85 constructs

The above results suggested that, during IL-6 exposure, two separate signal protein complexes form with PI3-K-activating potential: one containing STAT-3 and a second containing RAS. To confirm that the lipid kinase activity of these two complexes were completely independent of each other, we used transient transfections with dominant-interfering constructs. In the first set of experiments, AF-10 cells were cotransfected by electroporation with a dominant-negative (DN) RAS construct (or empty vector control) along with either the epitope-tagged mutant p110 construct that cannot bind to p85, or epitope-tagged STAT-3. After IL-6 treatment, p110 or STAT-3 were immunoprecipitated and a lipid kinase assay was performed. As shown in Figure 5a, IL-6 activated lipid kinase activity in both p110_{mutant} and STAT-3 immunoprecipitates. Cotransfection with 20 μg of the DN RAS construct significantly inhibited activity in p110 immunoprecipitates while 40 μg completely abrogated activity. In contrast, the DN RAS had no effect on lipid kinase activity in the STAT-3 immuno-

precipitate (lower panel of Figure 5a). With a similar design, we next cotransfected a DN p85 PI3-kinase construct (or empty vector) with the tagged STAT-3 or p110 constructs and again tested their ability to inhibit IL-6-induced lipid kinase activity. This DN p85 cannot bind p110 and, thus, will competitively inhibit endogenous PI3-K activation through proteins that interact via binding to the SH2 domain. As shown in Figure 5b, the DN p85 successfully prevented activity in the STAT-3 immunoprecipitate but had no effect on activity in the p110 immunoprecipitate. In fact, the DN p85 increased IL-6-induced lipid kinase activity in anti-p110 immunoprecipitates. In a prior study (Sharma *et al.*, 1998), a DN p85 caused a significant increase in GTP loading of RAS possibly due to inhibitory effects on GTPase-activating protein (GAP). By inhibiting the GAP inactivator of RAS, the DN p85 may heighten RAS activation, thus increasing the activation of lipid kinase activity in p110_{mutant} immunoprecipitates.

The p110_{mutant} protein is incapable of binding p85 and the DN p85 is incapable of binding to p110. Thus, the absence of inhibition of IL-6-dependent lipid kinase activity in the p110_{mutant} immunoprecipitate by the DN p85 cannot be taken as evidence that p85 has no role in IL-6-induced ras-mediated kinase activity but only as further support for the existence of two independent pathways. Clearly, however, the ability of IL-6 to activate kinase activity in a p110 mutant that cannot bind p85 (Figure 4) and the ability of the DN RAS to inhibit such activity (Figure 5a), demonstrates an IL-6-dependent ras-mediated mechanism of lipid kinase activation which is independent of p85 function. In summary, these results collectively support the notion of two independent pathways of PI3-kinase activity in IL-6-treated AF-10 myeloma cells. One is mediated by RAS and is independent of the p85 regulatory portion of the kinase and a second is mediated through p85 and a STAT-3-containing complex.

Both PI3-K-stimulating pathways contribute to activation of AKT

A major downstream target of PI3-K and its phospholipid second messengers is AKT. This is an important target of PI3-K in IL-6-treated MM cells as well since dominant-interfering AKT alleles prevent IL-6-induced stimulation of MM cell expansion (Hsu *et al.*, 2001; Hsu *et al.*, 2002). We, thus, tested if the two independent mechanisms by which IL-6 activates PI3-K, also contribute to downstream activation of AKT. AF-10 MM cells were cotransfected with an epitope-tagged AKT gene along with either the DN RAS or p85 constructs (or their corresponding empty vectors). After IL-6 stimulation, AKT was immunoprecipitated and its activity tested by its ability to phosphorylate the GSK-3 substrate. As shown in Figure 6, IL-6 successfully stimulated AKT activity in untransfected AF-10 cells (left panels) as well as in MM cells cotransfected with tagged AKT and empty vector constructs. However, both the DN RAS and DN p85 constructs prevented IL-6-induced activation of AKT kinase activity. Thus, both

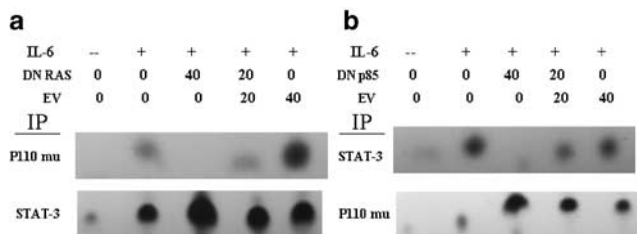


Figure 5 Effect of DN constructs on PI3-K activity. (a) AF-10 MM cells were transfected with tagged p110_{mu} or STAT-3 and addition of either 20 or 40 μg of DN RAS construct or empty vector (EV). Cells were treated with or without IL-6 (100 U/ml, 15 min), after which, p110_{mu} or STAT-3 was immunoprecipitated and a lipid kinase assay performed. (b) Cells were similarly transfected with tagged p110_{mu} or STAT-3 and addition of either 20 or 40 μg of DN p85 construct or empty vector (EV). After similar treatment with or without IL-6, immunoprecipitates were tested for lipid kinase activity. In both experiments, when 20 μg of DN DNA was used, it was added to 20 μg of EV DNA so that 40 μg of DNA was always cotransfected along with the tagged constructs

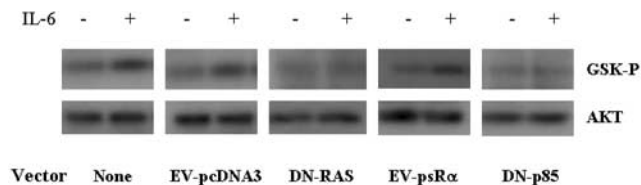


Figure 6 Effect of DN constructs on AKT activity. AF-10 cells were transfected with myc-tagged AKT either alone (VECTOR = NONE) or with addition of DN RAS (DN-RAS), DN p85 (DN-p85) or their respective empty vectors (EV). Cells were treated with or without IL-6 for 15 min, after which AKT was immunoprecipitated and tested for its ability to phosphorylate the GSK-3 substrate, shown as immunoblot with an antibody specific for GSK when it is phosphorylated (GSK-P). The immunoprecipitate was also immunoblotted for AKT

RAS-dependent and p85-dependent pathways significantly contribute to AKT activation induced by IL-6 in MM cells.

Activated RAS activates PI3-kinase in myeloma cells

The above results indicated that RAS activity participates in IL-6-induced PI3-kinase/AKT activation in myeloma cells. To provide further support for the ability of RAS to activate PI3-kinase in the myeloma model, we tested whether activating RAS mutations result in heightened PI3-kinase activity. This is also a clinically relevant question as activating mutations of N-RAS or K-RAS genes occur frequently in myeloma patients (up to 50% of cases) and are associated with particularly aggressive tumors (Liu *et al.*, 1996). To address this question, we again utilized the ANBL-6 myeloma cell line. As previously described (Rowley *et al.*, 2000) ANBL-6 cells were stably transfected with mutant N-RAS or K-RAS genes (termed N-RAS or K-RAS cells) or empty vector (termed WT cells) and tested for PI3-kinase activity. While the three cell lines were continuously cultured in 100 U/ml of IL-6, they maintained identical cell growth. The cell lines were then depleted of IL-6 for 48 h, PI3-kinase was immunoprecipitated with an anti-p85 antibody and the lipid kinase assay was performed. As shown in Figure 7, lipid kinase activity was absent in IL-6-depleted WT ANBL-6 cells but was constitutively present in mutant N-RAS and K-RAS-expressing myeloma cells. At this time point after 48 h of IL-6 depletion, viabilities of all three cell lines were comparable (>85%). To further ensure that IL-6-depleted WT cells were healthy with intact PI3-kinase-activating cascades, they were re-exposed to recombinant IL-6. This restimulation effectively stimulated PI3-kinase activity (lanes 2–4 in Figure 7). This experiment was repeated once with identical results. Thus, activating mutations of RAS in myeloma cells result in constitutive PI3-kinase activity. A previous publication (Hu *et al.*, 2003) demonstrated that N-RAS or K-RAS mutation in MM cells was also associated with constitutive AKT activation.

As shown in previous publications (Billadeau *et al.*, 1995; Hu *et al.*, 2003), ectopic expression of oncogenic ras resulted in IL-6-independent growth. At 6 days after



Figure 7 Mutant activated RAS constitutively activates PI3-K. ANBL-6 MM cells, either stably transfected with mutant K-ras or N-ras genes or empty vector (WT cells). WT, mutant N-ras-containing (N-RAS) or mutant K-RAS-containing (K-RAS) MM cells were depleted of IL-6 for 48 h and PI3-K was immunoprecipitated with anti-p85 antibody and lipid kinase assay performed. Separate groups of IL-6-depleted WT cells were also re-stimulated with IL-6 for 5, 10 or 15 min prior to immunoprecipitation of PI3-K

seeding 10^5 cells and depleting IL-6, the recovery of WT RAS-expressing cells (empty vector-transfected) had greatly decreased (to $4 \pm 0.3 \times 10^4$ cells, mean \pm s.d., $n = 3$), while recovery of mutant N-ras ($2.5 \pm 0.7 \times 10^5$ cells) and mutant K-ras ($2.9 \pm 0.5 \times 10^5$ cells)-clones demonstrated continual growth. Thus, the development of constitutive, IL-6-independent, PI3-K activation in myeloma cells harboring oncogenic RAS mutations parallels the development of IL-6-independent growth.

Discussion

Our previous study (Tu *et al.*, 2000) and the work of others (Oh *et al.*, 1998; Chen *et al.*, 1999) established the ability of IL-6, and other cytokines signaling through gp 130, to activate PI3-kinase in several different cell types. The current study was prompted by the fact that gp 130 does not contain a binding site for the p85 regulatory portion of PI3-kinase, indicating the requirement of intermediate adaptor molecules. Our results indicate at least two independent pathways in MM cells by which IL-6 can activate the PI3-kinase enzyme. Two independent complexes formed in both AF-10 and ANBL-6 myeloma cell models when exposed to IL-6, one containing PI3-K complexed to STAT-3 and another where PI3-K interacted with p21RAS. The YXXM p85 binding site is present on STAT-3 at residues 656–659 (Pfeffer *et al.*, 1997) and this presumably served as a docking site when phosphorylated by IL-6. In contrast, p21 RAS likely interacted with PI3-kinase via binding to the p110 kinase domain at a site spanning residues 130–315 in the amino-terminal region as previously described (Rodriguez-Viciano *et al.*, 1994). IL-6-induced lipid kinase activity was contained in both these complexes attesting to their functional significance.

The mechanistic independence of these two pathways was confirmed by use of DN constructs. A DN RAS construct prevented the IL-6-induced generation of lipid kinase activity in a p110 molecule that could not bind to p85. This demonstrates that a RAS-mediated pathway can activate lipid kinase activity in IL-6-treated myeloma cells which is independent of any p85 function. In contrast, the DN RAS had no effect on IL-6-induced lipid kinase activity in anti-STAT-3 immunoprecipi-

tates. On the other hand, a DN p85 construct that should disrupt complex formation between endogenous PI3-K and all its SH2 domain binding targets, prevented IL-6-induced lipid kinase activity in anti-STAT-3 immunoprecipitates but had no effect on activity generated in anti-RAS precipitates. This is consistent with a prior study (Pfeffer *et al.*, 1997) in IFN-treated cells where STAT-3 served as an adaptor for p85 PI3-kinase and mediated its activation. The lack of effect of the DN p85 on RAS-associated lipid kinase activity further supports the notion that RAS activates lipid kinase activity in myeloma cells via a direct activation of p110 which is independent of p85.

In contrast to our results on RAS and STAT-3 as intermediates for activation of PI3-K, the significance of IL-6-induced formation of PI3-K complexes with JAK-1 or SHP2 is unclear. JAK-1 constitutively bound PI3-kinase but binding did not increase in the presence of IL-6 although lipid kinase activity in anti-JAK-1 immunoprecipitates increased minimally and transiently (Figure 3a). These results are similar to those of Oh *et al.* (1998). Although the YXXM p85 binding motif is present on JAK-1, it would not be phosphorylated in the absence of IL-6. Thus, the ability of p85 to bind constitutively to JAK-1 in the absence of IL-6 is difficult to explain. Furthermore, it is clear that this constitutive binding itself does not induce constitutive activity (Figure 3). The slight increase in lipid kinase activity seen in anti-JAK-1 immunoprecipitates upon IL-6 exposure may be due to further recruitment of another PI3-kinase-activating molecule into JAK-1:PI3-kinase complexes upon stimulation. Additional questions arise concerning the relevance of the ability of SHP-2 to coimmunoprecipitate with PI3-kinase. Coimmunoprecipitation studies confirm an IL-6-dependent interaction between SHP-2 and PI3-kinase which is consistent with a prior study in MM.1S myeloma cells (Hideshima *et al.*, 2001). However, we could not demonstrate any IL-6-induced increase in lipid kinase activity contained within anti-SHP-2 immunoprecipitates.

There are several other potential mediators of PI3-kinase activation possibly stimulated by IL-6. IL-6 can induce tyrosine phosphorylation of vav in U266 myeloma cells (Lee *et al.*, 1997) and vav has been reported to interact with PI3-kinase (Shigematsu *et al.*, 1997). However, in several experiments, we could not demonstrate any vav:PI3-kinase binding in our myeloma cells in the presence of IL-6 (results not shown). Other potential PI3-kinase activators that are worth examining include src-like kinases and c-cbl. In B-lymphocytes, surface IgM activated c-cbl via interaction of src family kinases and Grb2/Shc adaptors (Panchamoorthy *et al.*, 1996). Activated c-cbl could bind to p85 PI3-kinase and stimulate its kinase activity (Ueno *et al.*, 1998). In a second model of PI3-kinase/AKT kinase activation, v-src interacted with SHP-2 ultimately resulting in c-cbl mediating PI3-kinase activation (Hakak *et al.*, 2000). IL-6 is known to activate Grb2/Shc and SHP-2 in myeloma cells (Ogata *et al.*, 1997) and recent work suggests it could activate src family kinases in these cells as well (Podar *et al.*, 2003). Thus, this is a

third potential pathway of PI3-kinase activation in IL-6-stimulated myeloma cells.

The presence of at least two independent pathways utilized by IL-6 to activate PI3-kinase provides for a potential additive or synergistic stimulation. This notion was further supported by the results shown in Figure 6 where interruption of each pathway with the appropriate dominant interfering construct significantly inhibited the activity of the downstream substrate of PI3-kinase, AKT. This confirms that each pathway provides a significant contribution to ultimate lipid kinase activity in the intact MM cell. Several previous reports document a synergistic interaction between RAS and phosphotyrosine-stimulated PI3-K activity, but it has not always been clear that the interaction at the level of PI3-K is due to completely separate upstream activating pathways. For example, although a DN RAS inhibits PDGF-stimulated AKT activation, activation was primarily mediated by upstream PI3-K activity stimulated by direct binding to the PDGF receptor (Franke *et al.*, 1995). In addition, an activated RAS allele was not sufficient by itself to activate AKT (Franke *et al.*, 1995). These results suggest the RAS contribution occurs by enhancing the PI3-K activation mediated by PDGF-R rather than via a separate RAS-dependent pathway. In similar manner, activating H-RAS increases insulin-stimulated PI3-K activity in anti-phosphotyrosine and anti-insulin receptor precipitates (Sjolander *et al.*, 1991) also suggesting an enhancement of the interaction between p85 and the insulin receptor or the IRS-1 adaptor. In contrast, our results are more consistent with a prior study (Rodriguez-Viciano *et al.*, 1994) in NGF- or EGF-stimulated PC12 cells, where a RAS-dependent pathway was completely independent from a phosphotyrosine-dependent pathway and the two pathways interacted synergistically to optimally activate phosphorylation of phosphoinositols. Similar to the current study, an activated RAS allele was sufficient to activate lipid kinase activity and a DN RAS prevented RAS-mediated activity but had no effect on lipid kinase activity in anti-phosphotyrosine precipitates. The presence of two completely independent mechanisms by which PI3-K/AKT can be activated in myeloma cells has therapeutic implications. Thus, interventions targeting only one or the other of the upstream activators of the pathway may be unsuccessful in completely shutting down PI3-K/AKT activity.

Activating mutations of N-RAS or K-RAS in myeloma cells occur frequently and are associated with aggressive tumors (Liu *et al.*, 1996). In the ANBL-6 model, when MM cells are engineered to express oncogenic K-RAS or N-RAS, they demonstrate enhanced continued cell growth which is IL-6-independent. Thus, oncogenic RAS expression may be one mechanism by which MM clones undergo IL-6-independent expansion in addition to other mechanisms such as stimulation by the IGF-1 growth factor. Our results on the ability of these consistently activated mutated RAS proteins to stimulate PI3-K activity constitutively may partially explain this growth advantage. This enhanced growth is reduced by treatment with

the PI3-K inhibitors wortmannin or LY294002 (Hu *et al.*, 2003), further supporting a tumor-stimulatory role for constitutive lipid kinase activity in mutant ras-expressing MM cells. Thus, PI3-K and its downstream targets PDK1 and AKT may be particularly appropriate targets for future therapy of patients with mutant RAS-expressing tumors.

Materials and methods

Cell lines, reagents, plasmids

The AF-10 MM cell line was maintained *in vitro* as previously described (Tu *et al.*, 2000). The ANBL-6 cell line was maintained in 100 U/ml of recombinant IL-6 as previously described (Billadeau *et al.*, 1995). The ANBL-6 parental line was stably transfected by retroviral transfection with virus expressing N-RAS or K-RAS genes mutated at codon 12 as described (Rowley *et al.*, 2000). ANBL-6 cells were also identically transfected with retrovirus lacking the transgene to serve as a control. The parental, control-transfected and oncogenic RAS-transfected lines were kind gifts of Dr Brian Van Ness, University of Minnesota. These transfected cell lines were also routinely maintained in 100 U/ml IL-6. A FLAG-tagged STAT-3 cloned into pRcCMV was a gift of Curt Horvath (Mt Sinai School of Medicine, NY, USA). The N17 DN H-RAS, cloned in pcDNA3 was a gift from Dr Hong-Gang Wang of the University of South Florida. The DN p85 that cannot bind to p110, cloned in pSR α , was a generous gift from Dr Erich Gulbins of the University of Tuebingen. Myc-tagged WT and mutant p110 constructs cloned in pSG5 were kind gifts of Dr Julian Downward (Imperial Cancer Research Fund, UK). The mutant p110 (Δ 3–125) lacks the minimal p85 binding site (Rodriguez-Viciano *et al.*, 1996). Recombinant IL-6 was purchased from R&D Systems (Minneapolis, MN, USA). Immunoprecipitating antibodies were purchased from Cell Signaling Labs (Boston, MA, USA) or Santa Cruz Labs (Santa Cruz, CA, USA). Radioisotopes were from Amersham Corp (Arlington Heights, IL, USA). All other reagents were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Coimmunoprecipitation assays

Briefly, cells were lysed in 0.65 ml of NP-40 lysis buffer (10 mM HEPES, pH 7.5, 142.5 mM KCl, 5 mM MgCl₂, 1 mM EGTA, 0.2% NP-40) containing 1 mM PMSF, 5 μ g/ml leupeptin and 5 μ g/ml aprotinin. After preclearing with normal rabbit antiserum (50 μ l/ml) and 50 μ l Protein A-Sepharose at 4°C for 1 h, immunoprecipitations were performed by incubating 0.2 ml of lysate with 20 μ l of Protein A-Sepharose preadsorbed with 10 μ l of antibody or normal rabbit antiserum as a negative control at 4°C for 3 h. After washing in NP-40 lysis buffer, beads were boiled in 60 μ l of Laemmli buffer and 20 μ l of the eluted proteins were subjected to SDS/PAGE immunoblot analysis.

References

Anderson K, Jones R, Morimoto C, Leavitt P and Barut B. (1989). *Blood*, **73**, 1915–1922.
Billadeau D, Jelinek D, Shah N, LeBien T and Van Ness B. (1995). *Cancer Res.*, **55**, 3640–3646.
Boulton T, Stahl N and Yancopoulos GD. (1994). *J. Biol. Chem.*, **269**, 11648–11657.

Lipid kinase assay

The assay was performed as previously described (Tu *et al.*, 2000). Briefly, immunoprecipitates were washed 3 \times with lysis buffer, 3 \times with 0.5 M NaCl and 25 mM Tris-HCl (pH 7.5) and 2 \times with 25 mM Tris-HCl (pH 7.5). The kinase reaction was run in a reaction mixture containing 10 mM Tris (pH 7.5), 100 mM NaCl, 20 mM MgCl₂, 0.2 mM EGTA, 20 μ g of phosphatidyl-4-monophosphate as substrate, 10 μ M ATP, 10 μ Ci of (γ -³²P)ATP and phosphatase inhibitors. After up to 30 min, the reaction was terminated and lipids extracted in chloroform:methanol:HCl (100:200:2). The organic phase was collected, dried and re-dissolved in chloroform:methanol (1:1) and spotted on TLC plates. The plates were developed with chloroform:methanol:H₂O:NH₄OH (43:38:7:5), dried and exposed to film. The location of PI (3, 4)P was determined by comparison with standards in iodine-stained TLC plates.

Transient transfections

Transient expression of genes in myeloma cells was accomplished by electroporation. Briefly, cells were resuspended to a concentration of 10⁷/ml and DNA to 20 μ g/100 μ l. A volume of 5 μ l of cell suspension was added to 100 μ l of DNA in cuvettes and the mixture kept at room temperature for 5 min. After electroporation was performed using 250 V and 25 ms pulse duration, cuvettes were kept at room temperature for 15 min and then added to culture plates for overnight incubation. After 24 h, cells were treated with or without IL-6 and immunoprecipitations performed. Viabilities of MM cells after electroporation routinely approximated 60–75% with transduction efficiencies of 15–25%, as monitored by electroporation with a plasmid expressing the enhanced green fluorescent protein.

AKT kinase assay-3

The myc-tagged WT AKT gene was cotransfected with DN or empty vectors control constructs into AF-10 MM cells by electroporation. After 24 h, cells were treated with or without IL-6 for 15 min and AKT was then immunoprecipitated. The AKT *in vitro* kinase assay utilized a nonradioactive kit (New England Biolabs). Immunoprecipitated AKT was incubated with GSK-3 fusion protein in the presence of ATP and kinase buffer. AKT-dependent GSK-3 phosphorylation was then measured by immunoblotting using a phospho-GSK-3 antibody that recognizes GSK-3 only when it is phosphorylated.

Acknowledgements

We thank Dr Julian Downward, Imperial Cancer Research Fund, Dr Erich Gulbins, University of Tuebingen, Germany, Dr Hong-Gang Wang, University of South Florida and Dr Curt Horvath, Mt Sinai School of Medicine, for their generous gifts of plasmids. The parental ANBL-6 MM cell line and stable transfectants expressing mutated N-RAS or K-RAS genes were generous gifts from Dr Brian Van Ness, Minneapolis, MN, USA.

Carpenter CL and Cantley LC. (1990). *Biochemistry*, **29**, 11147–11156.
Catlett-Falcone R, Landowski T, Oshiro MM, Turkson J, Levitzki A, Savino R, Ciliberto G, Moscinski L, Fernandez-Luna JL, Nunez G, Dalton W and Jove R. (1999). *Immunity*, **10**, 105–115.

- Chen R-H, Chang M-C, Su Y-H, Tsai Y-T and Kuo M-L. (1999). *J. Biol. Chem.*, **274**, 23013–23019.
- Franke T, Yang S-I, Chan TO, Datta K, Kazlauskas A, Morrison DK, Kaplan DR and Tschlis PN. (1995). *Cell*, **81**, 727–738.
- Hakak Y, Hsu YS and Martin GS. (2000). *Oncogene*, **19**, 3164–3171.
- Hideshima T, Nakamura N, Chauhan D and Anderson K. (2001). *Oncogene*, **20**, 5991–6000.
- Hsu Jh, Shi Y, Hu L, Fisher M, Franke T and Lichtenstein A. (2002). *Oncogene*, **21**, 1391–1400.
- Hsu J-h, Shi Y, Krajewski S, Renner S, Fisher M, Reed JC, Franke T and Lichtenstein A. (2001). *Blood*, **98**, 2853–2855.
- Hu L, Shi Y, Hsu J-h, Gera J, Van Ness B and Lichtenstein A. (2003). *Blood*, **101**, 3126–3135.
- Kawano M, Hirano T, Matsuda T, Taga T, Horii Y, Iwato K, Asaoku H, Tang B, Tanabe O, Tanaka H, Kuramoto A and Kishimoto T. (1988). *Nature*, **332**, 83–87.
- Kishimoto T, Akira S, Narazaki M and Taga T. (1995). *Blood*, **86**, 1243–1254.
- Lee I-S, Liu Y, Narazaki M, Hibi M, Kishimoto T and Taga T. (1997). *FEBS Lett.*, **401**, 133–143.
- Liu P, Leong T, Quam L, Billadeau D, Kay N, Greipp P, Kyle R, Oken M and Van Ness B. (1996). *Blood*, **88**, 2699–2706.
- Ogata A, Chauhan D, Urashima M, Teoh G, Hatziyanni M, Vidrales VMB, Schlossman R and Anderson K. (1997). *J. Immunol.*, **159**, 2212–2220.
- Oh H, Fujio Y, Kunisada K, Hirota H, Matsui H, Kishimoto T and Yamauchi-Takahara K. (1998). *J. Biol. Chem.*, **273**, 9703–9710.
- Panchamoorthy G, Fukazawa T, Miyake S, Soltoff S, Reedquist K, Druker B, Shoelson S, Cantley L and Band H. (1996). *J. Biol. Chem.*, **271**, 3187–3194.
- Pfeffer LM, Mullersman JE, Pfeffer SR, Murti A and Yang CH. (1997). *Science*, **276**, 1418–1425.
- Podar K, Mostoslavsky G, Tai YT, Sattler M, Catley LP, Hideshima T, Chauhan D, Mulligan RC and Anderson KC. (2003). *Hematol. J.*, **4**, S140.
- Podar K, Tai Y-T, Lin BK, Narsimhan RP, Sattler M, Kijima T, Salgia R, Gupta D, Chauhan D and Anderson KC. (2002). *J. Biol. Chem.*, **277**, 7875–7881.
- Rodriguez-Viciana P, Warne PH, Vanhaesebroeck B, Gout I, Fry MJ, Waterfield MD and Downward J. (1994). *Nature*, **370**, 527–532.
- Rodriguez-Viciana P, Warne PH, Vanhaesebroeck B, Waterfield MD and Downward J. (1996). *EMBO J.*, **15**, 2442–2451.
- Rowley M, Liu P and Van Ness B. (2000). *Blood*, **96**, 3175–3180.
- Sharma P, Egawa M, Huang Y, Martin JL, Huvar I, Boss GR and Olefsky J. (1998). *J. Biol. Chem.*, **273**, 18528–18530.
- Shigematsu H, Iwasaki H, Otsuka T, Ohno Y, Arima Y and Niho Y. (1997). *J. Biol. Chem.*, **272**, 14334–14340.
- Sjolander A, Yamamoto K, Huber BE and Lapetina EG. (1991). *Proc. Natl. Acad. Sci. USA*, **88**, 7908–7912.
- Tu Y, Gardner A and Lichtenstein A. (2000). *Cancer Res.*, **60**, 6763–6770.
- Ueno H, Sasaki K, Honda H, Nakamoto T, Yamagata T, Miyagawa K, Mitani K, Yazaki Y and Hirai H. (1998). *Blood*, **91**, 46–53.