A Phosphatidylinositol 3-Kinase-dependent Pathway That Differentially Regulates c-Raf and A-Raf*

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Cytokines trigger the rapid assembly of multimolecular signaling complexes that direct the activation of downstream protein kinase cascades. Two protein kinases that have been linked to growth factor-regulated proliferation and survival are mitogen-activated protein/ERK kinase (MEK) and its downstream target Erk, a member of the mitogen-activated protein kinase family. Using complementary pharmacological and genetic approaches, we demonstrate that MEK and Erk activation requires a phosphatidylinositol 3-kinase (PI3-K)-generated signal in an interleukin (IL)-3-dependent myeloid progenitor cell line. Analysis of the upstream pathway leading to MEK activation revealed that inhibition of PI3-K did not block c-Raf activation, whereas MEK activation was effectively blocked under these conditions. Furthermore, agents that elevated cAMP suppressed IL-3-induced c-Raf activation but did not inhibit MEK activation. Because c-Raf activation and MEK activation were inversely affected by PI3-K- and cAMP-dependent pathways, we examined whether IL-3 activated the alternative Raf isoforms A-Raf and B-Raf. Although IL-3 did not activate B-Raf, A-Raf was activated by the cytokine. Moreover, A-Raf activation, like MEK activation, was blocked by inhibition of PI3-K but was insensitive to cAMP. Experiments with dominant negative mutants of the Raf isoforms showed that overexpression of dominant negative c-Raf did not prevent MEK activation. However, dominant negative A-Raf effectively blocked MEK activation, suggesting that activation of the MEK-Erk signaling cascade is mediated through A-Raf. Taken together, these results suggest that IL-3 receptors engage and activate both c-Raf and A-Raf in hematopoietic cells. However, these intermediates are differentially regulated by upstream signaling cascades and selectively coupled to downstream signaling pathways.

Interleukin-3 (IL-3) is a growth and survival factor for immature and developing myeloid precursors. IL-3 and the related cytokines granulocyte-macrophage colony stimulating factor and IL-5 bind to heterodimeric receptors, which are composed of unique α subunits and shared β subunits (1). Ligand-induced receptor subunit heterodimerization activates receptor-bound protein-tyrosine kinases and initiates a series of intracellular signaling cascades, including the Erk activation pathway (2, 3). Although commonly depicted as a simple linear pathway, the Ras-Erk signaling cascade receives regulatory inputs from several other receptor-triggered signaling pathways. One key site for signal integration is at the level of c-Raf. A requisite signal is provided by the interaction of GTP-bound Ras with c-Raf. Although necessary, Ras interaction with c-Raf is insufficient for maximal c-Raf activation, and other signaling pathways impinge on c-Raf (4). The precise identities of these other pathways remain unclear, but protein kinase C (PKC) isoforms (5), ceramide-activated protein kinase (6), Src (7), and JAK family protein-tyrosine kinases (8) have all been implicated in c-Raf activation.

c-Raf is the most-studied isoform; however, mammalian cells also express the related isoforms A-Raf and B-Raf (9), which phosphorylate and activate MEK. Although much is known about the stimuli that regulate and activate c-Raf and B-Raf, comparatively little is known about A-Raf. Relatively few stimuli have been shown to activate A-Raf, and these limited studies suggest that A-Raf regulation is similar to c-Raf regulation (10, 11). Like c-Raf, A-Raf associates with GTP-bound Ras, suggesting that A-Raf is a downstream Ras effector, although it currently remains unclear whether A-Raf has unique functions or is redundant with c-Raf.

In addition to the Raf protein kinases, another Ras effector is the lipid kinase phosphatidylinositol 3-kinase (PI3-K) (12). Several forms of PI3-K have been identified, including the heterodimeric PI3-K, which is composed of a regulatory 85-kDa subunit and a catalytic 110-kDa subunit (12). PI3-K activation has pleiotropic effects on cell function and downstream signaling pathways (13). The lipid kinase is required for growth factor-induced activation of the protein kinases PDK1 (14) and AKT (15, 16). Both PDK1 and AKT bind 3'-phosphorylated lipids through their pleckstrin homology domains. PDK1 is required for p70S6K activation (17) and AKT activation. AKT participates in transducing cellular survival signals by phosphorylating and inhibiting the pro-apoptotic action of BAD, a Bcl-2 family member (18, 19). However, PI3-K also has functions that are cell type-specific. For example, PI3-K is essential for receptor-driven activation of the MEK-Erk signaling cascade in some cell types (20–22) but not in others (23). In cells that require PI3-K for MEK and Erk activation, analysis of the pathway has demonstrated that in most instances PI3-K was not required for Ras activation, but the lipid kinase was required for c-Raf activation. Taken together, these studies suggest that PI3-K

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1 The abbreviations used are: IL, interleukin; PKC, protein kinase C; PI3-K, phosphatidylinositol 3-kinase; mitogen-activated protein/ERK kinase (MEK); mitogen-activated protein/ERK kinase; GST, glutathione S-transferase; PMA, phorbol myristate acetate; HA, hemagglutinin; IBMX, isobutylmethylxanthine; PCR, polymerase chain reaction; GFP, green fluorescent protein.
may deliver a cooperative activation signal to c-Raf. However, the nature of the signal and how it interacts with c-Raf are currently unknown.

Using complementary pharmacologic and genetic strategies, the present results demonstrate that PI3-K is an essential element of IL-3-induced activation of the MEK-Erk signaling cascade in primary mouse bone marrow cells and in the IL-3-dependent cell line FDC-P1. Additional analyses revealed that agents that elevate cAMP did not block MEK and Erk activation. These findings prompt us to examine the upstream signaling pathways that regulate MEK and Erk activation. Our results demonstrate that, unlike MEK activation, c-Raf activation was not blocked by PI3-K inhibition. Moreover, MEK activation was not sensitive to cAMP, whereas c-Raf activation was sensitive to the drug, suggesting that other MEK kinases activate MEK. Thus, we examined the Raf isoforms A-Raf and B-Raf. Although B-Raf was not activated by IL-3, A-Raf activity was induced by the cytokine. Moreover, A-Raf activation, like MEK activation, required PI3-K and was not blocked by cAMP. Using dominant negative mutants of both A-Raf and c-Raf, we found that only kinase-inactive A-Raf blocked MEK activation. Together, these results document that IL-3 activates both c-Raf and A-Raf and that only A-Raf activation requires a PI3-K-dependent signal. Furthermore, even though both isoforms are activated by IL-3, only A-Raf relays MEK- and Erk-activating signals, suggesting that other molecular mechanisms determine how activated Raf isoforms engage downstream signaling pathways.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Preparation**—WEHI-3-conditioned medium was used as a source of IL-3 for growth of FDC-P1 cells. WEHI-3 cells were cultured in complete RPMI medium containing additions as described previously (24). To prepare WEHI-3-conditioned medium, cells were grown to stationary phase and then incubated for 2 days at 37 °C in a 5% CO2 atmosphere. The medium was then centrifuged, filtered through a 0.2 μm filter, and stored at 4 °C. FDC-P1 cells were propagated in RPMI 1640 medium containing 10% fetal calf serum and supplemented with 10% WEHI-3-conditioned medium. To prepare primary bone marrow cells, the femurs and tibias from four BALB/c mice were flushed with 10% WEHI-3-conditioned medium. To prepare primary bone marrow cells, the femurs and tibias from four BALB/c mice were flushed with 10% WEHI-3-conditioned medium. The resulting cell suspension was incubated with FDC-P1 growth medium. The supernatant was removed and centrifuged at 600 × g for 8 min, and the cell pellet was resuspended in FDC-P1 cell growth medium containing 40 μg/ml gentamycin, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells (1 × 107/ml) were cultured in tissue- and fibronectin-coated 100-mm plastic plates for 2 days, then fresh medium was added to the cultures each day. After 3 days, the cells (1–1.5 × 106 per assay point) were deprived of growth factors as described previously (24) for 4 h.

**Cell Transfections**—FDC-P1 cells were transfected by electroporation. Cells (1 × 106 per transfection) were mixed with DNA in 400 μl of complete growth medium. Total DNA was kept constant (40 μg) for all transfections by addition of empty vector, if required. The DNA-cell suspension was transferred to a 4-mm cuvette and electroporated with a 10-ms, 350-V pulse using a BTX T820 square wave electroporator (BTX Inc., San Diego, CA).

**Reagents**—Recombinant murine IL-3 was purchased from R&D Systems (Minneapolis, MN). Rabbit polyclonal immunoglobulin G (IgG) antibodies specific for Erk2 (C-14), c-Raf (C-12), B-Raf (C-19), and A-Raf (C-20) were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-MEK monoclonal antibody was from Transduction Laboratories (Lexington, KY). Murine monoclonal antibody preparations AU1 and 12CA5 (anti-HA) were purchased from Babco (Berkeley, CA). Monoclonal antibody 9E10 (anti-Myc) hybridoma was from the American Type Culture Collection (Manassas, VA), and the antibody was purified over protein G-agarose from ascites fluids (Bio-Rad) and used in the neutralizing experiments. The Erk substrate, myelin basic protein, was from Upstate Biotechnology (Lake Placid, NY). The glutathione S-transferase (GST) fusion proteins GST-ErkII (MEK substrate) and GST-MEKIII (Raf substrate) were prepared as described previously (22). The c-Jun N-terminal kinase substrate was produced in Escherichia coli using an expression vector generously provided by J. S. Gutkind (National Institute of Dental Research, Bethesda, MD) (25). The c-Raf cDNA in pUC13 was purchased from American Type Culture Collection.

**Plasmid Construction**—All epitope-tagged proteins were expressed from the elongation factor 1α promoter in a modified pEF-BOS (26) vector (pEF-BOSARI). The parental vector contains two EcoRI restriction sites. To generate a plasmid (pEF-BOSARI), the EcoRI restriction site on the 3′ side of the polyadenylation sequences was removed. Removal of this restriction site allowed insertion of cDNA sequences with 5′ EcoRI and 3′ XhoI restriction sites. All PCR- and mutagenesis-derived portions were sequenced to ensure the fidelity of the amplification and mutagenic procedures, respectively.

Epitope-tagged Raf (Mycte-M) was constructed with PCR by appending a BamHI site, a Kozak consensus sequence, an initiating Met codon, and the Myc epitope in frame with the N terminus of c-Raf. A Nol site was inserted between the Myc epitope and the N terminus of c-Raf. A C-terminal XhoI site was incorporated after the stop codon. The digested PCR fragment was ligated into BamHI- and XhoI-digested pCDNA3 to generate pCDNA3-Myc-c-Raf. Because expression levels were higher with the pEF-BOSARI vector, Myc-c-Raf was transferred to the pEF-BOSARI vector using a 5′ EcoRI site and a 3′ XhoI site.

Epitope-tagged MEK (Myc-MEK) was constructed by PCR amplification using primers that fused a Nol site to the N terminus of the MEK1 cDNA, a generous gift of G. Johnson (National Jewish Center for Immunology and Respiratory Medicine, Denver, CO), and an XhoI site to the C terminus. The PCR product was then digested with NolI and XhoI and ligated into the vector backbone of NolI- and XhoI-digested pEF-BOSARI-Myc-c-Raf (removing c-Raf but not the Myc epitope tag) to generate pEF-BOSARI-Myc-MEK.

Epitope-tagged, wild-type, and kinase-inactive c-Raf (RafMyc-HA2) expression constructs were constructed by PCR amplification of the 5′ portion of the gene to remove all native 5′ untranslated sequences. These were replaced with an EcoRI site and a Kozak consensus sequence. The 3′ end of c-Raf was modified to remove the stop codon of c-Raf and to append tandem copies of the HA tag (recognized by the 12CA5 monoclonal antibody), a new stop codon, and an XhoI site. The epitope-tagged version was ligated into EcoRI- and XhoI-digested pEF-BOSARI to yield pEF-BOS-c-Raf-HA2. The kinase-inactive version (pEF-BOS-c-RafMyc-HA2) was constructed using the Transformer mutagenesis kit (CLONTECH, Palo Alto, CA) to change Lys-375 to Thr.

The human A-Raf cDNA was isolated by PCR amplification from a brain cDNA preparation (CLONTECH) using the Expand High Fidelity PCR system (Boehringer Mannheim). The 5′ PCR primer added an EcoRI restriction site and a Kozak consensus sequence. The 3′ primer removed the terminal stop codon and was engineered to provide an in-frame mutation with the same N terminus. The 3′ primers used in construction of the epitope-tagged c-Raf. The resulting A-Raf PCR product was subcloned into pEF-BOS-c-RafMyc-HA2, replacing the c-Raf coding sequence with the A-Raf PCR product to yield pEF-BOS-A-Raf-HA2. To prepare the kinase-inactive A-Raf expression construct, pEF-BOS-A-RafMyc-HA2 was mutagenized with the GeneEditor kit (Promega, Corp; Madison, WI) to replace K336 with Trp, an analogous mutation in the kinase-inactive human A-Raf, and mutagenic primers.

The AKT cDNA was obtained by PCR amplification from a commercial cDNA library from human bone marrow (CLONTECH). The epitope-tagged AKT expression vector (pEF-BOS-AU1-AKT) was prepared by appending an EcoRI site, a Kozak consensus site, a translation initiation AUG codon, and the AU1 epitope tag that was fused in frame to the second codon of AKT. The 3′ end of the PCR product contained an XbaI site. pEF-BOS-AU1-AKT was assembled by ligating the epitope-tagged AKT fragment into EcoRI- and XhoI-digested pEF-BOSARI.

The dominant negative p85 expression vector was prepared from a murine p56 cDNA clone, which was a generous gift of L. Williams (Chiron Corp., San Francisco, CA). A portion of the iSH2 domain of p85 was deleted with the Transformer kit (CLONTECH). The deletion removed amino acids 479–513 (27) and replaced them with a Glu and Phewh residue (an EcoRI site) to generate Δp85. The Δp85 cDNA was modified on the 3′ end to remove the native stop codon and append a tandem HA tag and XhoI site to Δp85. The epitope-tagged Δp85 cDNA was cloned into pEF-BOSARI to yield pEF-BOSARI-Δp85.

**Protein Kinase Assays**—Raf, MEK, and Erk assays were performed as described previously (22). c-Jun N-terminal kinase assays were performed as described by Hibi et al. (28), except that cells were deprived of IL-3 for 4 h in RPMI 1640 medium supplemented with 10% fetal calf serum. For all kinase assays, cells were deprived of growth factors as described previously (22) and stimulated with 10 or 20 ng/ml IL-3 or 10 ng/ml phorbol myristate acetate (PMA) for the indicated times. Kinase activities were quantitated using a Molecular Dynamics PhosphorImager.
Interleukin-3-induced Raf Activation

**Fig. 1.** Wortmannin blocks IL-3-induced mitogen-activated protein kinase activation in primary murine bone marrow cell cultures. Factor-deprived bone marrow cells were pretreated with Me2SO vehicle (untreated) or 100 nM wortmannin for 30 min. Cells were then restimulated with IL-3 or PMA for 5 min and lysed. Mitogen-activated protein kinase was immunoprecipitated, and catalytic activity was assayed. Data are the average of duplicate determinations. The experiment was repeated three times with similar results.

**RESULTS**

**Wortmannin Blocks IL-3-induced Erk Activation in IL-3-responsive Primary Bone Marrow Cells and IL-3-dependent FDC-P1 Cells**—Our previous results demonstrated that IL-3-induced Erk activation in T cells was partially sensitive to wortmannin, suggesting that both wortmannin-sensitive and wortmannin-resistant pathways participated in the Erk activation response in this cell type (22). We therefore extended our investigation to determine whether Erk activation induced by other hematopoietic growth factors was also sensitive to wortmannin. Our initial experiments tested whether the drug affected IL-3-induced Erk activation in primary bone marrow cells. Isolated murine bone marrow cells were deprived of exogenous growth factors for 4 h, pretreated with wortmannin and stimulated with IL-3. Wortmannin strongly inhibited IL-3-induced Erk activation in these cells (Fig. 1), but the drug had no effect on phorbol ester-induced Erk activation. These results demonstrated that the inhibitory effects of wortmannin on cytokine-induced Erk activation were not restricted to T cells and that the wortmannin-sensitive pathway played a major role in IL-3-induced Erk activation in normal bone marrow cells.

Due to the technical limitations imposed by studies in primary hemopoietic cells, we determined whether the inhibitory effects of wortmannin on Erk activation were recapitulated in the IL-3-dependent cell line FDC-P1 (Fig. 2). Factor-deprived FDC-P1 cells were treated with wortmannin and stimulated with IL-3. Erk (Fig. 2A) and MEK (Fig. 2B) were immunoprecipitated from detergent lysates for determination of in vitro kinase activities. As was observed with the primary bone marrow cells, wortmannin effectively suppressed both MEK and Erk activation, indicating that the IL-3 receptor couples to Erk largely through a wortmannin-sensitive signaling pathway. Because FDC-P1 cells are amenable to biochemical and genetic analyses, we used this cell line as a model system to dissect the signaling pathways that regulate IL-3-induced Erk activation.

**Wortmannin Blocks IL-3-induced G1-to-S Phase Progression**—In many experimental systems PI3-K plays critical roles in relaying proliferative signals from growth factor receptors. However, the situation is less clear for hemopoietic cytokine receptors. Because wortmannin effectively inhibits PI3-K, we asked how wortmannin would affect IL-3-induced DNA synthesis (Fig. 3). FDC-P1 cells were deprived of growth factors, which synchronizes them in the G1 phase of the cell cycle (data not shown). The G1 phase cells were pretreated with a single addition of the indicated concentrations of wortmannin and stimulated with IL-3. Because wortmannin is unstable in solution and PI3-K levels remain suppressed for only 9–12 h after drug addition (22), cells were pulsed after 12 h with \[^3H\]thymidine (as cells first enter S phase) and cultured an additional 6 h before harvesting. Therefore, PI3-K was maximally inhibited during the progression of the IL-3-stimulated cells through G1. Wortmannin attenuated IL-3-induced DNA synthesis at concentrations that inhibited PI3-K and blocked IL-3-induced Erk activation. Because these experiments were performed with a single addition of wortmannin, and PI3-K activity would be recovering during the later times of the experiment, these results likely do not reflect the full impact of PI3-K inhibition on G1 to S phase progression. This inhibition of DNA synthesis is not the result of cell killing, as FDC-P1 cells cultured in these concentrations of wortmannin do not undergo apoptosis, even though PI3-K and AKT activation are fully inhibited (data not shown).

To address the specificity of the effects of wortmannin, we coexpressed GFP with either empty vector or dominant negative p85 (Δp85) (27). Flow cytometric analysis of green-fluorescing cells revealed that transfection with GFP and empty vector produced cells that cycled normally through the cell cycle (58% G1, 33% S, and 9% G2/M). In contrast, cells cotransfected with GFP and Δp85 were primarily found in G1 (81% G1, 12% S, and 7% G2/M), suggesting that genetically blocking that pathway may be more effective than wortmannin in blocking cell cycle progression. Taken together with the drug study, these results suggest strongly that PI3-K plays a crucial role in promoting IL-3-induced cell cycle progression.

**Wortmannin and Dominant Negative p85 Block IL-3-induced Erk and AKT Activation**—Wortmannin blocked MEK and Erk activation by 50% (IC\_50) at a concentration of 3 μM (data not shown), which coincides with the IC\_50 for PI3-K inhibition in intact cells (30–32). These results suggested that a wortmannin-sensitive PI3-K is an upstream regulator of Erk activation in IL-3-stimulated hemopoietic cells. Therefore, as a complementary strategy to assess the role of PI3-K in Erk activation, we examined the extent of dominant negative Δp85 (Δp85-HA\(^2\)) on IL-3-induced MEK activation. As a positive control to confirm that Δp85-HA\(^2\) interrupts PI3-K-mediated signaling, we cotransfected the cells with an AU1-tagged AKT expression vector. Expression vectors for all three epitope-tagged proteins were cotransfected into FDC-P1 cells, with increasing amounts...
of Δp85-HA² as indicated (Fig. 4B). After factor deprivations, the transfected cell populations were divided into two equal sets. One set was stimulated IL-3 (20 min), the detergent lysates were immunoprecipitated with anti-AU1 antibody, and AKT activity was determined (Fig. 4A, bottom panel). The other set was stimulated with IL-3 (5 min), the lysates were immunoprecipitated with anti-Myc antibody, and MEK kinase assays were performed (see Fig. 6A, top panel). Increasing amounts of cotransfected Δp85-HA² plasmid DNA progressively blocked IL-3-induced activation of both AKT and MEK with virtually identical potencies (Fig. 4C). However, a small portion of MEK activation was not blocked by Δp85 or wortmannin (see Fig. 2), suggesting that a PI3-K-independent pathway plays a minor role in IL-3-induced MEK activation. The alterations in IL-3-induced MEK and AKT activation were not explained by differences in the expression of the epitope-tagged protein kinases (Fig. 4C). Because AKT and MEK showed nearly identical sensitivity to coexpressed Δp85-HA², these results argue strongly that PI3-K plays a pivotal upstream role in IL-3-induced MEK activation.

Dominant Negative p85 Selectively Disrupts PI3-K-dependent Signaling Pathways—One potential complication associated with the dominant negative approach is that overexpression of mutant proteins that contain abundant protein-protein interaction domains, as does p85, may nonspecifically disrupt signaling pathways. We tested this possibility by asking whether Δp85 blocked activation of a pathway that is independent of PI3-K. In FDC-P1 cells, IL-3-induced c-Raf activation is not blocked by wortmannin, indicating that c-Raf activation is independent of PI3-K-generated signals (Fig. 5). FDC-P1 cells were cotransfected with AU1-AKT, Myc-c-Raf, and either empty vector or Δp85 expression vector. Factor-deprived cells were then restimulated with IL-3. Myc-c-Raf and AU1-AKT were immunoprecipitated with anti-epitope monoclonal antibodies and subjected to kinase assays. IL-3 induced strong activation of c-Raf that was not blocked by coexpression of Δp85. In contrast, coexpression of Δp85 effectively blocked AKT activation. These results indicate that PI3-K is not required for IL-3-induced c-Raf activation. Moreover, they demonstrate that Δp85 does not nonspecifically disrupt all IL-3-triggered mitogenic signals, suggesting that its effects are specific for the PI3-K pathway.

Pharmacological Inhibitors Differentially Affect IL-3-induced c-Raf and MEK Activation—To further define the signaling pathway that mediates MEK and Erk activation, we determined whether the MEK activator, c-Raf, is also inhibited by wortmannin. Fig. 6A shows that IL-3 rapidly activated c-Raf. Surprisingly, however, wortmannin concentrations that resulted in near-complete inhibition of IL-3-triggered MEK and Erk activation (see Fig. 2) unexpectedly prolonged and enhanced IL-3-induced c-Raf activation. These results suggest that wortmannin-sensitive pathways may attenuate or termi-
nate the signals mediating Raf activation. One such pathway may be the MEK-Erk cascade, because previous work showed that pharmacological inhibition of MEK also enhanced platelet-derived growth factor-induced Raf activation (33).

The observation that IL-3-induced c-Raf activation was enhanced in wortmannin-treated cells, whereas MEK activation was abrogated, suggested one of two possibilities. Either c-Raf does not relay the MEK-activating signal in FDC-P1 cells, or wortmannin blocks the normal coupling between c-Raf and MEK in response to IL-3. To discriminate between these possibilities, we examined what effect blocking c-Raf activation would have on IL-3-induced Erk activation. Elevation of cAMP levels activates protein kinase A, which phosphorylates and inhibits activation of c-Raf (34). Thus, we tested the effect of cAMP on IL-3-induced c-Raf and MEK activation in FDC-P1 cells by incubating the cells with either the phosphodiesterase inhibitor IBMX (Fig. 6B) or the nonhydrolyzable cAMP analog 8-chlorophenyl-thio-cAMP (data not shown). Both drugs effectively blocked IL-3-induced c-Raf activation (see Fig. 6B for IBMX results). Surprisingly, IBMX and 8-chlorophenyl-thio-cAMP enhanced IL-3-triggered Erk activation, demonstrating that under conditions where c-Raf was not activated by IL-3, Erk activation was not impeded. Thus, these results suggest that c-Raf does not relay the IL-3-induced MEK-activating signal in this cell type.

Dominant Negative c-Raf Does Not Block IL-3-induced MEK Activation—As another method to rule out c-Raf as a mediator of MEK activation in these cells, we examined the effect of kinase-inactive c-Raf (c-RafKD-HA2) on IL-3- or PMA-induced MEK activation (Fig. 7). Consistent with the results obtained with IBMX-treated cells (see Fig. 6), IL-3-induced MEK activation was not blocked by coexpression of kinase-inactive c-Raf (Fig. 7, A and C). In fact, when low amounts of kinase-inactive c-Raf are cotransfected, IL-3-induced MEK activation was reproducibly enhanced. Anti-Myc immunoblotting suggested that this might be due to increased expression of the epitope-tagged Myc-cRaf, an effect that we observed when cells were transfected with either 5 or 10 μg of kinase-inactive c-Raf (Fig. 7B). In contrast, PMA-induced MEK activation was partially blocked by kinase-inactive c-Raf, even when Myc-MEK expression was slightly enhanced (5 or 10 μg of kinase-inactive c-Raf). A potential concern here is that kinase-inactive c-Raf does not function as an effective dominant negative (although the same construct blocks MEK activation in other systems; data not shown). However, only a partial block would be expected be-
cause the A-Raf isoform relays approximately 50% of the MEK-activating signal in PMA-stimulated cells (see Fig. 9).

IL-3 Does Not Activate B-Raf—In addition to c-Raf, FDC-P1 cells also express B-Raf. An earlier report indicated that IL-3 activated B-Raf in hemopoietic cells (35); however, in this study, autophosphorylation of B-Raf was used as a marker for enzyme activation, rather than using a specific Raf substrate, such as MEK1. Because Raf isoforms associate with and are phosphorylated by other protein kinases, autophosphorylation assays may actually reflect the presence of contaminating transphosphorylating activities. Consequently, we assayed B-Raf activation in FDC-P1 cells using a GST-MEKKD fusion protein as substrate (Fig. 8A). As previously reported, B-Raf exhibited high basal activity (7). However, this activity was not increased by IL-3, nor was it affected by wortmannin and IBMX treatment, suggesting that B-Raf does not transduce IL-3-triggered MEK-activating signals.

IL-3-induced A-Raf Activation Is Sensitive to Wortmannin—Because neither c-Raf nor B-Raf was a candidate as MEK activators in IL-3-stimulated cells, we also asked whether A-Raf, the third known member of the Raf kinase family, was involved in this response. We found that A-Raf is expressed and is activated by IL-3 (Fig. 8B). Surprisingly, IL-3-induced A-Raf activation was wortmannin-sensitive and IBMX-resistant (Fig. 8B), in contrast to the c-Raf activation provoked by the same cytokine (Fig. 8C). Also in the same experiment, we confirmed that Erk activation was sensitive to wortmannin but resistant to IBMX (Fig. 8D). This experiment was repeated three times, and in each case we observed that wortmannin blocked A-Raf activation. Moreover, coexpression of dominant negative Δp85 also blocked IL-3-induced activation of epitope-tagged A-Raf (data not shown), thus confirming that the effects of wortmannin on A-Raf are due to PI3-K inhibition. These results demonstrate that A-Raf and c-Raf are differentially regulated in IL-3-responsive cell lines and suggest that A-Raf, but not c-Raf, may relay the MEK-activating signal in IL-3-stimulated cells.

Dominant Negative A-Raf Blocks IL-3-induced MEK Activation—Although IL-3 activated c-Raf, our earlier results suggested that this protein kinase does not relay an activating signal to MEK. Based on these observations, we genetically blocked A-Raf function to determine its impact on IL-3-induced MEK activation. We cotransfected FDC-P1 cells with Myc-MEK and increasing amounts of the kinase-inactive A-Raf expression vector. Factor-deprived cells were restimulated with IL-3 or PMA for 5 min, and MEK kinase activities were determined (A) and quantitated (C). To determine expression levels of transfected genes, a portion of the lysates was immunooblotted with α-Myc to detect Myc-MEK (B, top panel) and α-HA to detect c-RafKD-HA2 (B, bottom panel). The results shown are representative of those obtained in three independent experiments.
is a major transducer of the IL-3-induced MEK-activating signal; however, other MEK activators may also participate in IL-3-induced MEK activation.

**DISCUSSION**

The present results demonstrate that IL-3-induced activation of the Erk signaling cascade is dependent on PI3-K. Although the PI3-K pathway was originally thought to be insulated from the MEK-Erk signaling cascade, multiple reports have since shown that the pathways are intertwined in a cell type- and stimulus-specific manner. Some cells and stimuli, such as IL-3, clearly require PI3-K for MEK activation, whereas in other cases, the intensity of the stimulus or the cell type employed determines whether PI3-K participates in MEK and Erk activation. Efforts have been made to determine at what level in the signaling cascade PI3-K is required, and several studies demonstrated that PI3-K is required for activation of c-Raf, a common MEK activator in growth factor signaling cascades (20, 21). However, the present results indicate that IL-3 triggers a PI3-K-dependent pathway in which an alternative Raf isoform, A-Raf, transduces an activating signal to MEK and, in turn, Erk. Furthermore, these results show that activated Raf isoforms do not necessarily couple to the expected downstream signaling pathways and suggest that Raf isoforms have unique functions in cytokine-stimulated cells.

Our findings are in disagreement with the conclusions of Schied and Duronio (36), who reported that wortmannin inhibited IL-3- and GM-CSF-induced activation of Erk but that the unrelated PI3-K inhibitor, LY294002, did not block Erk activation (36). Based on this finding, they suggested that the effects of wortmannin on cytokine-induced MEK activation are mediated by a target other than PI3-K. Although wortmannin is not absolutely specific for PI3-K, we substantiated our pharmacologic data by genetically blocking PI3-K activation with a documented dominant negative inhibitor of PI3-K (27). These results showed that both AKT and MEK activations were equally sensitive to increasing amounts of the dominant negative PI3-K construct. Taken together, these results strongly suggest that PI3-K is an upstream component in IL-3-induced MEK activation. This discrepancy between the present conclusions and those of Scheid and Duronio (36) may result from the use of different cell lines. In our studies, wortmannin had dramatic effects on Erk activation in both primary bone marrow cells and

**FIG. 8.** IL-3 activates A-Raf but not B-Raf in FDC-P1 cells. Factor-deprived FDC-P1 cells were pretreated with nothing or 100 nM wortmannin for 30 min and restimulated with IL-3 for 5 min. Detergent lysates were immunoprecipitated with B-Raf (A, top panel), A-Raf (B, top panel), c-Raf (C, top panel), or Erk antisera (D, top panel), and protein kinase activities were assayed. To demonstrate equal immunoprecipitation and sample loading, the membrane was immunoblotted with either anti-B-Raf (A, bottom panel), anti-A-Raf (B, bottom panel), anti-c-Raf (C, bottom panel), or anti-Erk (D, bottom panel). Identical results were obtained in four independent trials.

**FIG. 9.** Kinase-inactive A-Raf inhibits IL-3-induced MEK but not AKT activation. FDC-P1 cells were transfected with 5 μg of Myc-MEK expression vector and either 0, 5, 10, or 15 μg of kinase-inactive A-Raf expression vector. Factor-deprived cells were restimulated with IL-3 or PMA for 5 min, and MEK catalytic activities were assayed (A) and quantitated (C). To demonstrate Myc-MEK expression (B, top panel) and A-RafKD-HA2 expression (B, bottom panel), a portion of the cell lysate was immunoblotted with anti-Myc and anti-HA, respectively. D, AU1-AKT expression vector was cotransfected with the indicated amounts of kinase-inactive A-Raf expression vector. AKT activities were assayed and quantitated. Immunoblotting of kinase assay immunoprecipitates revealed that AU1-AKT was expressed equally in all samples (data not shown).
the myeloid progenitor FDC-P1 cell line. In the studies of Scheid and Duronio (36), wortmannin had more modest effects on GM-CSF- and IL-3-induced Erk activation in the mast cell-like MC-9 cell line. Possibly, MC-9 cells do not require a PI3-K-dependent input for MEK activation, and the inhibition that is observed with wortmannin is indeed due to another cellular target for wortmannin.

The regulation of all Raf isoforms is a complex multistep process, which begins with the accumulation of GTP-bound Ras. However, Ras provides only the initial activating signal, with other cooperating upstream signals required for full Raf activation (4). Thus, the Raf isoforms serve as a point of convergence for multiple receptor-triggered signaling pathways, one of which may be PI3-K-dependent. In IL-3-stimulated hemopoietic cells, both c-Raf and A-Raf are activated. However, in these cells, only A-Raf activation requires a PI3-K-mediated signal. Furthermore, how PI3-K relays an activating signal to PI3-K is uncertain. One possibility is that PI3-K produces a lipid product that can directly affect the Raf activation process, and previous work has demonstrated that c-Raf binds lipids (37, 38). Alternatively, a PI3-K-dependent effector may relay an activating signal to Raf. Several PI3-K effectors have recently been identified, including the nonclassical PKC isoforms PKCa/a (39) and PKCζ (40), the pleckstrin homology domain-containing kinases AKT (15, 16) and PDK1 (14), and the protein kinases p70S6K and mTOR (41). Previous work has demonstrated that rapamycin completely blocks the activation of p70S6K and mTOR, but the drug does not block MEK and Erk activation (42), demonstrating that these downstream PI3-K effectors do not relay activating signals to Raf or the MEK-Erk pathway. In the case of the PKC isoforms, dominant negative PKCa/a and PKCζ had no effect on IL-3-induced MEK activation, suggesting that these PKC isoforms do not participate in MEK activation in IL-3-stimulated cells. Whether the PI3-K effectors AKT and PDK1 function in MEK activation pathways is less well studied; however, constitutively active AKT had no effect on Erk activation in a cell line in which PI3-K was required for MEK activation, suggesting that in this setting, AKT is not sufficient for MEK activation (14).

The present results establish that, like c-Raf and B-Raf, A-Raf is differentially regulated by extracellular stimuli. Our AKT is not sufficient for MEK activation (14). If c-Raf does not mediate activation of MEK and Erk, what is the role of activated c-Raf in hemopoietic cells? Antisense oligonucleotides directed against c-Raf severely attenuate IL-3-induced FDC-P1 proliferation (44), suggesting that although c-Raf is not required for MEK and Erk activation, it has other critical functions in these cells. One possible target of IL-3-activated Raf is the apoptosis-promoting protein Bad (45). When phosphorylated, Bad is bound to 14-3-3 proteins in an inactive, cytoplasmic form. When dephosphorylated, Bad translocates to the mitochondria, where it dimerizes with Bcl-XL and promotes cell death (46). This function of c-Raf might explain why c-Raf was required for IL-3-induced FDC-P1 proliferation, even though c-Raf does not relay Erk-activating signals.

Although the Ras-Raf-MEK-Erk signaling cascade has become a central tenet of modern cell biology, it is becoming increasingly apparent that considerable plasticity exists with respect to Erk activation pathways. The present results indicate that PI3-K plays a crucial role in the activation of Erk by the hemopoietic growth and survival factor IL-3. Furthermore, the present data also demonstrate the differential regulation and coupling of c-Raf and A-Raf, suggesting that these two Raf isoforms perform nonoverlapping and unique functions in IL-3-responsive cells. These observations suggest that receptors rely on combinatorial associations among a limited set of signal transduction intermediates to activate partially redundant downstream signaling pathways. Although the complexity of the signaling pathways that converge on Erk seems daunting, the delineation of these pathways may uncover new cellular targets for the pharmacologic manipulation of hemopoietic cell functions in immune system dysfunctions and cancer.

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