Gi Proteins Use a Novel βγ- and Ras-independent Pathway to Activate Extracellular Signal-regulated Kinase and Mobilize AP-1 Transcription Factors in Jurkat T Lymphocytes

(Received for publication, March 16, 1999, and in revised form, April 21, 1999)

Karen E. Hedin‡§, Michael P. Bell‡, Catherine J. Huntoon‡, Larry M. Karnitz‡, and David J. McKean‡

From the ‡Department of Surgery, §Department of Immunology, and †Division of Radiation Oncology, The Mayo Clinic and Foundation, Rochester, Minnesota 55905

Receptors coupled to pertussis toxin (PTX)-sensitive Gi proteins regulate T lymphocyte cytokine secretion, proliferation, and chemotaxis, yet little is known about the molecular mechanisms of Gi protein signaling in mammalian lymphocytes. Using the Jurkat T lymphocyte cell line, we found that a stably expressed GiN protein-coupled receptor (the δ-opioid receptor (DOR1)) stimulates MEK-1 and extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2) and transcriptional activity by an ERK target, Elk-1, via a mechanism requiring a PTX-sensitive Gi protein. Levels of β-adrenergic receptor kinase-1 C-terminal fragment that inhibited signaling by Gi protein βγ subunits in these cells had no effect on DOR1 stimulation of either MEK-1 or Elk-1-dependent transcription, indicating that this pathway is independent of βγ. Analysis of this βγ-independent pathway indicates a role for a herbimycin A-sensitive tyrosine kinase. Unlike βγ-mediated pathways, the βγ-independent pathway was insensitive to RasN17, inhibitors of phosphatidylinositol 3-kinase (PI 3-kinase), and constitutive PI 3-kinase activity. The βγ-independent pathway regulates downstream events, since blocking it abrogated both Elk-1-dependent transcription and mobilization of the mitogenic transcription factor, AP-1, in response to DOR1 signaling. These results characterize a novel, Ras- and PTI 3-kinase-independent pathway for ERK activation by Gi protein signaling that is distinct from ERK activation by βγ and may therefore be mediated by the αi subunit.

G protein signaling in many cell types stimulates the activity of the extracellular signal-regulated kinases (ERKs). ERK1 and ERK2 (Refs. 1–16; reviewed in Ref. 17). ERK and other mitogen-activated protein kinases phosphorylate transcription factors, thereby mediating transcriptional regulation in response to signaling by cell surface receptors (18–22). Like other G proteins, Gi proteins consist of an αi subunit and a dimeric βγ subunit and mediate activation of intracellular signaling pathways in response to the ligation of receptors with seven transmembrane-spanning domains. When stimulated by a ligand-bound receptor, the α subunit binds GTP and dissociates from βγ. This event allows both α-GTP and free βγ to directly regulate the activities of downstream effector molecules. Gi proteins contain one of the closely related αi subunits (αi1, αi2, αi3) in addition to β and γ subunits shared by other types of G proteins. Pertussis toxin (PTX) covalently modifies Gi protein αi subunits, preventing Gi protein activation by ligated receptors. Besides Giα and Giβ, only Gi proteins are inactivated by PTX.

In epithelial and fibroblast cells, certain Gi- or Gαi-coupled receptors activate ERK by liberating G protein βγ subunits that directly stimulate an isoform of phosphatidylinositol-3 kinase (PI 3-kinase) (p110-γ) (8, 12, 23). This event leads to activation of Ras and the Raf/MEK/ERK kinase cascade (1–3, 22) via a mechanism involving SHC tyrosine phosphorylation and the recruitment of GRB2-SOS (5, 6, 12, 23). Although tyrosine kinases related to PYK2 (6, 14, ZAP-70/SYK (16), and Src (6, 16–16) have been implicated in this process, a detailed mechanism explaining βγ activation by Ras is presently unavailable.

The α subunits of PTX-sensitive G proteins also have the potential to activate ERK. The gip2 oncogenes encode constitutively active α2β2 subunits with either an R179C or a Q205L point mutation that inhibits GTP hydrolysis (24). gip2 expression constitutively stimulates ERK (25) and mediates the oncogenic transformation of Rat1a fibroblasts (24, 26). Unlike βγ stimulation of ERK (1–3, 22), gip2 stimulates ERK via a mechanism that is independent of Ras (25), a result suggesting that αi and βγ activate ERK via distinct pathways. Moreover, the m1-muscarinic and platelet-activating factor receptors use the closely related PTX-sensitive αi2 subunit to activate ERK via a Ras-independent pathway in the Chinese hamster ovary epithelial cell line (10). An interesting feature of this pathway is its cell type specificity; in COS-7 fibroblasts, these same receptors use βγ and Ras to stimulate ERK (10). This resembles the cell-specific actions of gip2, which transforms Rat-1a cells but not NIH 3T3 or Swiss 3T3 fibroblasts (24). Recent evidence suggests that αi and αo activate ERK in Rat-1a and COS-7 cells via transactivation of the epidermal growth factor receptor tyrosine kinase (7, 13). Although Src family tyrosine kinases have been implicated in this process (13), additional mechanistic details of this pathway are obscure.

Lymphocytes express abundant heterotrimeric G proteins (27) that direct lymphocyte migration (28–31), proliferation (32, 33), and cytokine secretion (34, 35). Although recent work has uncovered novel mechanisms for G protein stimulation of ERK activity in fibroblast and epithelial cell types (above),
little is known about the molecular mechanisms of ERK activation by G\textsubscript{i} protein signaling in mammalian lymphocytes. We recently reported that signaling by a heterologously expressed G\textsubscript{i} protein-coupled \(\delta\)-opioid receptor (DOR1) uses a PTX-sensitive mechanism to stimulate the accumulation of AP-1 transcription factor complexes in the Jurkat human T lymphocyte cell line (35). Here, we demonstrate that DOR1 signaling activates ERK via a novel, Ras-independent, G\textsubscript{i} protein-mediated pathway. We further present evidence that this pathway is independent of \(\beta\gamma\), characterize this pathway as distinct from previously characterized \(\beta\gamma\)-mediated ERK activation pathways, and demonstrate that this pathway leads to PTX-sensitive AP-1 mobilization in these cells.

**EXPERIMENTAL PROCEDURES**

**Cells**—DOR1/Ju.1 cells were derived by fluorescence-activated cell sorting of the Ju.1 Jurkat T cell subline that was stably transfected with DOR1, as described previously (36). All cells were maintained in Medium B (RPMI 1640 supplemented with 5% fetal calf serum, 5% calf serum, 10 mM HEPES, PH 7.4, 2 mM l-glutamine, and 2 mM 2-mercaptoethanol). DOR1 expression by the DOR1/Ju.1 cell line was confirmed by assays every few months as described (35).

**Phosphorylated ERK Assays**—ERK2—Cells were preincubated 16–24 h in Medium C (RPMI 1640 supplemented with 0.5% fetal calf serum, 10 mM HEPES, PH 7.4, 2 mM l-glutamine, and 2 mM 2-mercaptoethanol) and then washed and resuspended in Medium C at a density of 10\textsuperscript{6} cells/ml. Cells were stimulated as indicated for 5 min at 37 °C, pelleted by rapid centrifugation, and lysed in SDS-PAGE sample buffer. Lysates were fractionated by SDS-PAGE (using approximately 2 \times 10\textsuperscript{6} cell equivalents/lane) and transferred to Immobilon-P membrane (Millipore, Corp., Bedford, MA). The presence of active ERK1 and ERK2 phosphorylated on threonine 202 and tyrosine 204 was detected by immunoblotting with phospho-specific p44/p42 ERK1 and ERK2 (Thr\textsuperscript{32} and Tyr\textsuperscript{392}) antisemur, while total ERK2 protein was detected using p44 ERK kinase antisemur (both antisera from New England Biolabs, Beverly, MA). Unless otherwise indicated, stimulated cells were incubated at 37 °C, unless otherwise indicated.

**Transfections**—Cells—Renilla luciferase and luciferase activity were determined following transfection of the cells as described below. 10\textsuperscript{6} cells were transfected with equal portions of each plasmid and plated in Medium B. 24 h later, MeK-1 was immunoprecipitated using an anti-Myc mAb (Babco, Berkley, CA), and an in vitro kinase assay was performed as described (43) in the presence of \(\gamma\)-\textsuperscript{32}P-ATP, using as a substrate 1 \mu\textsubscript{g}/test of kinase-inactive ERK2 GST fusion protein (GST-ERK2-KD; prepared as described (44)). Results were visualized by SDS-PAGE, Western transfer to Immobilon P membrane, and autoradiography, and/or PhosphoImager analysis using a storm model 840 (Molecular Dynamics, Inc., Sunnyvale, CA).

**Infection with Recombinant Vaccinia Viruses**—To make the recombinant Vaccinia viruses, the coding sequence of human CD56 or human Ha-RasN17 was blunt end-cloned into the Smal site of the Vaccinia virus expression vector, pSC11. The resulting vectors were introduced into the WR strain of Vaccinia via homologous recombination, and high titer viral stocks were prepared as described (45). Stocks of the WR (wild-type/parental) strain Vaccinia virus were a generous gift of Dr. Paul Leibson (Mayo Clinic, Rochester, MN). Mutant Ras expression by the RasN17 recombinant virus was confirmed by reverse transcriptase-polymerase chain reaction and DNA sequencing of the viral stock (data not shown) and by anti-Ras immunoblotting of infected cells (Fig. 5, B and C). Semipurified recombinant CD56, RasN17, or WR (control) viruses were used for infection. For each experiment, 4 \times 10\textsuperscript{6} cells were infected in 8.0 ml of RPMI 1640 for 1 h at 37 °C at a multiplicity of infection of 20:1. One volume of RPMI 1640 supplemented with 1% fetal calf serum was then added, and the cells were incubated for an additional 1 h at 37 °C. Infected cells were washed twice with RPMI 1640 supplemented with 0.5% fetal calf serum and either immediately incubated with 2 \times 10\textsuperscript{6} total cells—Renilla luciferase and luciferase activity were determined following transfection of the cells as described below. 10\textsuperscript{6} cells were transfected with equal portions of each plasmid and plated in Medium B. 24 h later, MeK-1 was immunoprecipitated using specific antisera (Santa Cruz Biotechnology) and in vitro kinase assays performed for the Myc-MeK1-1 assay above, using as substrate either 1 \mu\textsubscript{g}/test of kinase-inactive ERK2 GST fusion protein (for MeK1 assay) or 5 \mu\textsubscript{g}/test of myelin basic protein (MBP) (for ERK2 assay; Upstate Biotechnology, Inc., Lake Placid, NY).

**Akt Kinase Assay**—Cells—2 \times 10\textsuperscript{5} cells were transfected with 5 \mu\textsubscript{g} of an expression vector encoding pEF-BOS-AU1-ARK (with or without 10 \mu\textsubscript{g} of pEF-BOS-FLAG-iSH2-CAAX and plated in Medium B. 24 h later, Akt was immunoprecipitated using an anti-AU1 mAb (Babco, Berkley, CA), and an in vitro kinase assay was performed using sheep anti-Akt antisemur and horseradish peroxidase-conjugated sheep anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) as substrates for the sheep Akt kinase assay (46, 47). Akt activity was determined by phospho-Imager analysis, and/or PhosphoImager analysis using a storm model 840 (Molecular Dynamics, Inc., Sunnyvale, CA).

**RNA Blot**—Cells were pretreated and stimulated as indicated in Medium B at a density of 5 \times 10\textsuperscript{6} to 8 \times 10\textsuperscript{6} cells/ml, and total RNA was prepared using Trizol reagent according to the manufacturer’s instructions (Life Technologies, Inc.). RNA (10 \mu\textsubscript{g}/lane) was fractionated by electrophoresis in a 1% agarose-formaldehyde gel, transferred to Hybond-N membrane (Amersham Pharmacia Biotech), and hybridized with probes.
**RESULTS**

The \(G_i\) Protein-coupled Receptor, DOR1, Activates MEK-1, ERK1, and ERK2 in Jurkat T Cells via a PTX-sensitive Pathway—To address the potential for mitogenic signaling by \(G_i\) proteins in lymphocytes, we first asked whether receptor-mediated activation of endogenous \(G_i\) proteins could stimulate ERK activity in a lymphoid cell line. For this purpose, we used DOR1/Ju.1 cells, a Jurkat T cell line stably transfected with a plasmid containing the luciferase gene downstream of five meric Elk-1/Gal4 transcription factor (pFA-Elk), together with a plasmid containing the luciferase gene downstream of a minimal Gal4 DNA binding site (pFR-Luc). When activated by upstream signaling, ERK phosphorylates the Elk-1 domain of the transfected Elk/Gal4 chimera, which then stimulates luciferase expression from the Gal4-responsive promoter of pFR-Luc.

As shown in Fig. 1C, treatment of transiently transfected DOR1/Ju.1 cells with \(10^{-7}\) M deltorphin increased Elk-1-dependent luciferase activity. Elk-1 may potentially be regulated by several mechanisms; however, the deltorphin-mediated response is sensitive to the specific MEK inhibitor, PD098059. In contrast, treatment of transfected DOR1/Ju.1 cells with MEK1 but not PMA, activation of ERK was abrogated by pretreatment with PMA. DOR1 activation of ERK1 and ERK2 most likely requires upstream MEK activation, since ERK activation was sensitive to the specific MEK inhibitor, PD098059 (Fig. 1A) (47). In fact, DOR1 signaling activates MEK-1, shown by transiently transfecting a construct encoding Myc-tagged MEK-1 (Myc-MEK-1), shown by transiently transfecting a construct encoding Myc-tagged MEK-1 (Myc-MEK-1), stimulated with \(10^{-7}\) M deltorphin or 50 ng/ml PMA. C, inset, summary of multiple experiments as in D, showing the fold increase in Elk-1-dependent transcriptional activity with \(10^{-7}\) M deltorphin compared with basal levels, following pretreatment with PTX or PTX-B toxins. Each bar represents the mean \(\pm\) S.E. of three independent determinations.

Since the transcription factor, Elk-1, is activated by ERK phosphorylation (20, 21), we also tested the ability of DOR1-mediated G\(_i\) protein signaling to mediate Elk-1-dependent transcriptional activity. DOR1/Ju.1 cells were transiently transfected with a plasmid that constitutively expresses a chimeric Elk-1/Gal4 transcription factor (pFA-Elk), together with a plasmid containing the luciferase gene downstream of five Gal4 DNA binding sites (pFR-Luc). When activated by upstream signaling events, ERK phosphorylates the Elk-1 domain of the transfected Elk/Gal4 chimera, which then stimulates luciferase expression from the Gal4-responsive promoter of pFR-Luc.

As shown in Fig. 1C, treatment of transiently transfected DOR1/Ju.1 cells with \(10^{-7}\) M deltorphin increased Elk-1-dependent luciferase activity. Elk-1 may potentially be regulated by several mechanisms; however, the deltorphin-mediated response is sensitive to the specific MEK inhibitor, PD098059. In contrast, treatment of transfected DOR1/Ju.1 cells with MEK1 but not PMA, activation of ERK was abrogated by pretreatment with PMA. DOR1 activation of ERK1 and ERK2 most likely requires upstream MEK activation, since ERK activation was sensitive to the specific MEK inhibitor, PD098059 (Fig. 1A) (47). In fact, DOR1 signaling activates MEK-1, shown by transiently transfecting a construct encoding Myc-tagged MEK-1 (Myc-MEK-1), stimulated with \(10^{-7}\) M deltorphin or 50 ng/ml PMA. C, inset, summary of multiple experiments as in D, showing the fold increase in Elk-1-dependent transcriptional activity with \(10^{-7}\) M deltorphin compared with basal levels, following pretreatment with PTX or PTX-B toxins. Each bar represents the mean \(\pm\) S.E. of three independent determinations.

Since the transcription factor, Elk-1, is activated by ERK phosphorylation (20, 21), we also tested the ability of DOR1-mediated G\(_i\) protein signaling to mediate Elk-1-dependent transcriptional activity. DOR1/Ju.1 cells were transiently transfected with a plasmid that constitutively expresses a chimeric Elk-1/Gal4 transcription factor (pFA-Elk), together with a plasmid containing the luciferase gene downstream of five Gal4 DNA binding sites (pFR-Luc). When activated by upstream signaling events, ERK phosphorylates the Elk-1 domain of the transfected Elk/Gal4 chimera, which then stimulates luciferase expression from the Gal4-responsive promoter of pFR-Luc.

As shown in Fig. 1C, treatment of transiently transfected DOR1/Ju.1 cells with \(10^{-7}\) M deltorphin increased Elk-1-dependent luciferase activity. Elk-1 may potentially be regulated by several mechanisms; however, the deltorphin-mediated response is sensitive to the specific MEK inhibitor, PD098059. In contrast, treatment of transfected DOR1/Ju.1 cells with MEK1 but not PMA, activation of ERK was abrogated by pretreatment with PMA. DOR1 activation of ERK1 and ERK2 most likely requires upstream MEK activation, since ERK activation was sensitive to the specific MEK inhibitor, PD098059 (Fig. 1A) (47). In fact, DOR1 signaling activates MEK-1, shown by transiently transfecting a construct encoding Myc-tagged MEK-1 (Myc-MEK-1), stimulated with \(10^{-7}\) M deltorphin or 50 ng/ml PMA. C, inset, summary of multiple experiments as in D, showing the fold increase in Elk-1-dependent transcriptional activity with \(10^{-7}\) M deltorphin compared with basal levels, following pretreatment with PTX or PTX-B toxins. Each bar represents the mean \(\pm\) S.E. of three independent determinations.
ERK Regulation by βγ-Independent G, Protein Signaling

To directly examine the regulation of MEK activity by overexpressed βγ, we transiently transfected βγ together with a construct encoding Myc-tagged MEK-1 and then immunoprecipitated and assayed the kinase activity of the Myc-tagged MEK-1. Results shown in Fig. 3C show that overexpressing βγ in Jurkat T cells results in an approximately 2-fold activation of Myc-MEK-1 kinase activity. These results show that overexpressing G protein βγ subunits in Jurkat T cells increases both MEK-1 activity and Elk-1-dependent transcription, suggesting that βγ is capable of activating ERK in these cells.

DOR1 Stimulates MEK-1 Activity and Elk-1-dependent Transcription via a βγ-Independent Pathway—We next asked if βγ mediates MEK-1 activation by DOR1 in Jurkat T cells. βARKct binds free βγ, and βARKct expression can block βγ stimulation of ERK that is initiated by either receptor ligation or βγ overexpression (2, 3, 10). We therefore tested the effects of βARKct cotransfection on the ability of DOR1 to activate MEK-1. Fig. 3A shows that cotransfection of βARKct had no detectable effect on DOR1 signaling that increased the kinase activity of Myc-MEK-1. In multiple experiments, DOR1 activation of Myc-MEK-1 in the presence of βARKct was 105 ± 25% (n = 3) of that in the absence of βARKct (100%), which represents no significant inhibition (p = 0.86). In the same experiments, βARKct cotransfection significantly reduced βγ activation of Myc-MEK-1 to only 33 ± 6.8% (n = 3; p = 0.01; Fig. 3C) of that stimulated by βγ alone. Fig. 3B additionally shows that DOR1 activation of Myc-MEK-1 kinase activity was unaffected by βARKct throughout its time course.

We next tested the ability of cotransfected βARKct to inhibit DOR1 stimulation of the Elk-1-dependent transcriptional activity that is downstream of MEK-1 and ERK. While cotransfecting βARKct clearly inhibited the ability of overexpressed βγ subunits to stimulate Elk-1-dependent transcription, βARKct had little effect on the transcriptional activity that resulted from DOR1 ligation by deltorphin (Fig. 4A). Fig. 4B summarizes the results of multiple experiments. βARKct exerted no significant effect on the ability of deltorphin to stimulate Elk-1-dependent transcriptional activity (n = 3; p = 0.96), while in the same experiments βARKct significantly inhibited activity in response to overexpressed βγ (n = 3; p < 0.02). Together, the results in this section indicate that DOR1 uses a βγ-independent mechanism to stimulate MEK-1 kinase activity and transcriptional activation by Elk-1. Since this pathway is also sensitive to PTX (Fig. 1), these results suggest that DOR1 uses βγ rather than βγ to stimulate MEK-1 and downstream events in Jurkat T cells.

Ras Is Required for βγ Stimulation but Not DOR1 Stimulation of ERK—To characterize and compare the mechanisms used by βγ and DOR1 to stimulate ERK, we examined the effects of the dominant-negative mutant of Ras, RasN17 (48), on DOR1 and βγ signaling leading to ERK activity and downstream events in Jurkat T cells. To facilitate direct assay of the kinase activities of endogenous MEK-1 and ERK2, we employed a recombinant Vaccinia virus that expresses RasN17. Unlike transient transfection, recombinant Vaccinia virus infection of Jurkat T cells results in >95% of the cells expressing high levels of the recombinant protein. This is demonstrated by a flow cytometric assay of cell surface CD56 following infection of DOR1/Ju1 Jurkat T cells with a CD56-expressing recombinant Vaccinia virus (Fig. 5A). Using identical infection conditions, we infected Jurkat T cells with recombinant Vaccinia viruses that express either RasN17 or nothing (WR, or wild-type, strain). Following stimulation with deltorphin, the pres-
We also tested the effects of RasN17 on DOR1 signaling that leads to Elk-1-dependent transcriptional activity. An expression plasmid encoding RasN17 was transfected into DOR1/Ju.1 cells together with pFR-Luc and pFA-Elk, and the cells were stimulated and assayed for Elk-1-dependent transcriptional activity as in Fig. 1, C and D. Typical results are shown in Fig. 6A. RasN17 inhibited Elk-1-dependent transcription in response to either βγ overexpression or stimulation of the T lymphocyte antigen receptor-CD3 complex (TCR-CD3) with anti-CD3 mAb. In addition, cotransfection of this RasN17-encoding plasmid inhibited (by approximately 80%) transcription from an IL-2 promoter construct in response to TCR-CD3 and CD28 stimulation (n = 3; p = 0.02; data not shown). These results are consistent with previous reports that Ras participates in signaling by βγ (2, 3, 10) and TCR-CD3 (49, 50). In contrast to its effects on TCR-CD3 and βγ signaling, RasN17 had little or no effect on Elk-1-dependent transcriptional activity stimulated by DOR1 or PMA (Fig. 6A). Fig. 6B summarizes the results of multiple experiments. RasN17 consistently inhibited βγ, but not deltorphin, stimulation of Elk-1-dependent transcription.

To summarize, the results in this section demonstrate that DOR1 uses a RasN17-insensitive mechanism to activate MEK-1, ERK1- and ERK2-, and Elk-1-dependent transcription in these cells is sensitive to RasN17. Since DOR1, but not βγ, signaling is also insensitive to ARKct, these results suggest that DOR1 and βγ subunits stimulate ERK activation via independent signaling pathways that differ in their requirements for Ras.

Elk-1-dependent Transcription in Response to βγ, but Not DOR1, Synergizes with Constitutive PI 3-Kinase Activity—In fibroblast and epithelial cells, βγ-dependent stimulation of ERK requires activity of the p110-γ isoform of PI 3-kinase (8, 12, 23). We therefore asked whether βγ- or DOR1-mediated Elk-1-dependent transcription synergizes with constitutive PI 3-kinase activity. The binding of p85 to activated receptor tyrosine kinases normally stimulates the PI 3-kinase activity of associated p110α/β PI 3-kinases by mediating their membrane localization (51, 52). We therefore constructed an expression

ence of active, phosphorylated ERK1 and ERK2 was assayed by immunoblotting as in Fig. 1A. As shown in Fig. 5B, RasN17 had no detectable effect on the time course of ERK1 or ERK2 phosphorylation in response to deltorphin. RasN17 also had no effect on the ability of deltorphin to activate endogenous MEK-1 or ERK2 activity, as measured by immunoprecipitating these kinases and measuring their kinase activities in vitro (Fig. 5C). Fig. 5D shows that in multiple experiments, we observed no significant inhibition of DOR1-mediated activation of endogenous ERK2 or MEK-1 kinase activity in cells expressing RasN17.
plasmid encoding iSH2-CAAX, which consists of the p110αβ-binding iSH2 domain of p85 fused to the membrane targeting and isoprenylation domain of Ha-Ras. Transient expression of iSH2-CAAX enhanced the kinase activity of Akt, a downstream target of PI 3-kinase (53, 54) (Fig. 7A), indicating that like other fusion proteins that mediate membrane localization of p110αβ (51, 52, 55), iSH2-CAAX stimulates PI 3-kinase activity. Consistent with this idea, iSH2-CAAX activation of Akt was abrogated by pretreatment with the PI 3-kinase inhibitor, wortmannin. iSH2-CAAX also mimics PI 3-kinase signaling when transiently expressed in other cell types (56).

While transient expression of iSH2-CAAX increased the level of Elk-1-dependent transcriptional activity in unstimulated cells, iSH2-CAAX did not synergistically enhance Elk-1-dependent transcriptional response to either deltorphin or anti-CD3 mAb (Fig. 7B). In contrast, cotransfection of βγ and iSH2-CAAX consistently elevated luciferase activity approximately 45-fold (n = 10; Fig. 7C). This effect was synergistic, since it exceeded by 3-fold (n = 10; p = 0.02) the calculated additive effects of expressing either βγ or iSH2-CAAX alone. PD098059 inhibited Elk-1-dependent transcriptional activity stimulated by either iSH2-CAAX alone or iSH2-CAAX plus βγ, suggesting that these pathways require MEK-1. Moreover, like the activity that arises from overexpressing βγ alone (Fig. 6B), Elk-1-dependent transcriptional activity resulting from coexpressing βγ and iSH2-CAAX was sensitive to RasN17 (Fig. 7, B and C).

We also tested the effects of the PI 3-kinase inhibitor, wortmannin, on ERK activation by DOR1. As shown in Fig. 7D, wortmannin applied at doses that completely inhibit p110α or p110-γ PI 3-kinase activity (12, 57), as well as iSH2-CAAX-stimulated Akt activity (Fig. 7A), had little effect on ERK activation by deltorphin. Similarly, Elk-1-dependent transcriptional activity in response to deltorphin was unaffected by pretreatment with wortmannin (data not shown). Since the iSH2-CAAX fusion protein enhanced Elk-1-dependent transcriptional response to signaling by βγ, but not DOR1, these results provide additional support for the idea that DOR1 and βγ stimulate ERK activity via distinct pathways. In contrast, the results in this section indicate that the DOR1-mediated pathway of ERK activation is independent of PI 3-kinase activity.

**MEK-1 Activation Is Essential for DOR1 Signaling That Leads to Increased Levels of c-Fos mRNA and Transcriptionally Active AP-1 Transcription Factors**—When Elk-1 is phosphorylated by ERK, it can act together with serum response factor to mediate increased expression of c-Fos (18–21). Fig. 8A shows that deltorphin stimulated the accumulation of c-Fos mRNA but not control glyceraldehyde-3-phosphate dehydrogenase mRNA. Furthermore, this increase was abrogated by pretreatment with the MEK inhibitor, PD098059. Since PD098059 also blocks ERK activation and Elk-1-dependent transcription in

**Fig. 5.** DOR1 activation of ERK is not sensitive to RasN17. A, >95% of DOR1/Ju.1 Jurkat T cells express recombinant protein following infection with a recombinant Vaccinia virus. Cells were infected at a multiplicity of infection of 20 plaque forming units (pfu)/cell. Cell surface CD56 was assayed by incubation with anti-CD56 mAb and flow cytometry. The gray histogram shows CD56 cell surface expression by CD56-infected cells. The two open histograms are controls: uninfected cells and cells infected with wild-type Vaccinia virus and stained for cell-surface CD56 expression. B, DOR1 stimulation of active, phosphorylated ERK1 and ERK2 is not sensitive to RasN17. DOR1/Ju.1 Jurkat T cells were infected either with wild-type (WT) or RasN17-encoding recombinant Vaccinia virus as in A. 2 h later, cells were stimulated with 10⁻² M deltorphin for the indicated times. Cells were then lysed, and whole-cell lysates were analyzed by SDS-PAGE and immunoblotting for active, Thr202/Tyr204-phosphorylated ERK1 and ERK2 (bottom gel). Total ERK2 (middle gel) or RasN17 (bottom gel) in the lysates was assayed by stripping the same blot and immunoblotting with antisera specific for ERK2 or Ras. C and D, DOR1 stimulation of active, phosphorylated ERK1 and ERK2 via a RasN17-insensitive pathway. Cells were infected with either wild-type (WT) or recombinant Vaccinia viruses encoding RasN17 as in A. 2 h postinfection, cells were stimulated with 10⁻² M deltorphin for the indicated times and lysed, and endogenous MEK-1 or ERK2 was immunoprecipitated and assayed for kinase activity in the presence of γ⁻³²PATP. Substrates were kinase-deficient ERK2 fusion protein (GST-ERK2-KD; for MEK-1 assay) or MBP (for ERK2 assay). C, results of a representative experiment. Western blotting shows the amounts of MEK-1 or ERK2 immunoprecipitated in each test. The bottom gel shows the amount of RasN17 detected by immunoblotting with anti-mouse IgG of the cell lysates. D, summary of multiple experiments as in C, with the deltorphin response of RasN17-infected cells expressed as a percentage of the response of cells that were infected with wild-type Vaccinia virus in the same experiment. Each bar denotes the mean ± S.E. of three or four independent determinations.
response to deltorphin (Fig. 1), these results suggest that DOR1 requires MEK-1 and ERK activation to increase c-Fos mRNA. Consistent with this idea, DOR1 signaling leading to c-Fos mRNA induction was insensitive to wortmannin (Fig. 8A).

The c-Fos protein can participate in forming the AP-1 transcription factor complex (21, 58); therefore, we examined the regulation of AP-1 in response to DOR1 signaling. Fig. 8B shows that DOR1 signaling elevated levels of DNA-binding AP-1 complexes. Like DOR1-mediated activation of ERK and c-Fos mRNA induction, DOR1 stimulation of DNA-binding AP-1 complexes was sensitive to PD098059 but not wortmannin. In addition, we previously showed that like DOR1 activation of ERK, the DOR1-mediated increase in DNA-binding AP-1 is abrogated by PTX (35). The AP-1 complexes mobilized by DOR1 include those that are transcriptionally active, since DOR1 ligation also elevated transcription from an AP-1 reporter plasmid in a manner sensitive to PTX or PD098059 but not wortmannin (Fig. 8, C and D). These results indicate that the novel βγ, Ras-, and PI 3-kinase-independent pathway of ERK activation described above is essential for DOR1 signaling that leads to increased levels of c-Fos mRNA and transcriptionally active AP-1 transcription factors.

**A Role for a Tyrosine Kinase in ERK Activation by DOR1/oi Signaling**—Several reports indicate that tyrosine kinases can participate in G i protein-mediated stimulation of ERK (6, 7, 13–16); therefore, we examined the effects on DOR1 signaling of a tyrosine kinase inhibitor, herbimycin A. Pretreatment with herbimycin A partially inhibited the activation of ERK in response to DOR1 signaling (Fig. 9A). Herbimycin A pretreatment also partially inhibited the increase in DNA-binding AP-1 transcription factors in response to deltorphin (Fig. 9B). In contrast to its partial inhibition of DOR1 effects, herbimycin A almost completely blocked ERK activation and AP-1 mobilization in response to pervanadate, an inhibitor of tyrosine phosphatases that activates Src family tyrosine kinases in Jurkat T cells (39). Densitometric analysis shows that the DOR1-mediated increases in ERK2 phosphorylation and AP-1 DNA binding activity were inhibited 50 and 70%, respectively, by herbimycin A. These results suggest that the βγ-independent, DOR1-initiated pathway that stimulates ERK and increases AP-1 transactivation is regulated by a herbimycin A-sensitive tyrosine kinase.

**DISCUSSION**

Signaling by PTX-sensitive G proteins leads to activation of ERK1 and ERK2 (1–16; reviewed in Ref. 17), events that are important for mobilizing AP-1 and for stimulating cell cycle entry in response to growth factors (17, 20–22, 58). Most studies on the molecular mechanisms that underlie this response have focused on ERK activation by G i or G o protein βγ subunits (reviewed in Ref. 17), yet recent evidence for βγ-independent pathways (10, 13) and the oncogenic potential of mutant G i protein αi subunits (24–26) indicate that alternative mechanisms exist. Here, we present results that establish the existence in Jurkat T cells of distinct βγ-mediated and βγ-independent pathways for G i protein-mediated stimulation of MEK-1 and ERK activity. In addition, we present evidence that the βγ-independent pathway is independent of Ras and PI 3-kinase activity and demonstrate that it is required for Elk-1-dependent transcription and the mobilization of AP-1 transcription factors in response to signaling by a G i protein-coupled receptor.

We analyzed the molecular mechanisms of G i protein-mediated ERK stimulation using the DOR1/Ju.1 subline of the human T lymphocyte cell line, Jurkat. DOR1/Ju.1 cells are stably transfected with the neuronal G i protein-coupled receptor, DOR1, which we previously showed signals via a PTX-sensitive G i protein in these cells (35, 36). Here, we show that agonist stimulation of DOR1 increases ERK activity and Elk-1-dependent transcriptional activity via a PTX-sensitive pathway, establishing that this pathway requires a G i protein. We further show that these effects of DOR1 are sensitive to the MEK inhibitor, PD098059, and that DOR1 activates MEK-1, indicating that this PTX-sensitive pathway activates ERK and downstream transcriptional events via MEK-1.

Since both MEK-1 activity and Elk-1-dependent transcription were elevated by transiently transfecting G i protein βγ and γi subunits into Jurkat T cells, we asked if βγ mediates DOR1 coupling to ERK. Interestingly, βARKct inhibited Elk-1-dependent transcriptional activity in response to overexpressed βγ but had no effect on Elk-1-dependent transcription that followed ligation of DOR1. Similarly, βARKct inhibited constitutive MEK-1 activation by overexpressed βγ but had no effect on MEK-1 activation in response to DOR1 signaling. In other cell types, βARKct inhibits ERK activation in response to ligation of certain G i protein-coupled receptors, apparently because βARKct sequesters free βγ (2, 3, 10). Our results therefore indicate that although signaling by βγ subunits can stimulate MEK-1 and downstream events in Jurkat T cells, the G i protein-coupled receptor, DOR1, stimulates MEK-1, ERK-, and Elk-1-dependent transcription via a βγ-independent pathway.

We next asked if signaling intermediates that generally participate in ERK regulation also participate in the βγ-independent pathway. G i protein βγ subunits (1–3, 22), as well as receptors that signal using tyrosine kinases (22, 49), stimulate ERK via activation of Ras, a GTP/GDP-binding oncoprotein that stimulates the Raf/MEK/ERK kinase cascade. Although the DOR1- and βγ-mediated pathways were each sensitive to the MEK inhibitor, PD098059, both ERK activity and Elk-1-dependent transcription in response to DOR1 ligation were in-
sensitive to transient expression of the dominant-negative Ras, RasN17. In contrast, RasN17 inhibited Elk-1-dependent transcription in response to either βγ overexpression or TCR-CD3 ligation, consistent with previous reports that these stimuli use Ras to activate ERK (1–3, 11, 49, 50). We also explored the role of PI 3-kinase activity in the βγ-independent pathway. Interestingly, DOR1 stimulation of ERK or Elk-1-dependent transcription was insensitive to the PI 3-kinase inhibitor, wortmannin, and did not synergize with constitutively elevated PI 3-kinase activity that enhanced βγ stimulation of Elk-1-dependent transcription.

Together, these results indicate that DOR1 activates ERK or Elk-1-dependent transcription.
MEK-1, ERK1- and ERK2-, and Elk-1-dependent transcriptional activity in Jurkat T cells via a mechanism that requires neither Ras nor a PI 3-kinase. In contrast, βγ-mediated stimulation of MEK-1- and Elk-1-dependent transcription in these cells is sensitive to RasN17 and synergizes with PI 3-kinase activation. Since DOR1, but not βγ, signaling is also insensitive to βARKct, these results suggest that DOR1 and βγ subunits stimulate ERK activity via independent signaling pathways that differ in their requirements for Ras. Since DOR1 stimulation of ERK was partially sensitive to the tyrosine kinase inhibitor, herbimycin A, the βγ-independent pathway may involve a tyrosine kinase. Experiments aimed at identifying the tyrosine kinase(s) associated with DOR1 signaling are in progress. However, it seems unlikely that tyrosine kinases associated with the TCR-CD3 receptor complex are involved via transactivation in DOR1 activation of ERK, since RasN17 inhibited Elk-1-dependent transcription in response to signaling by TCR-CD3 but not DOR1.

To our knowledge, this is the first evidence for a G protein-mediated but βγ-independent pathway of ERK activation that is independent of both Ras and PI 3-kinase activity. Since DOR1 uses a PTX-sensitive mechanism to stimulate this pathway and since lymphoid cells do not express other α subunits that are PTX targets (28), it is most likely that DOR1 uses αi2 and/or αi3, rather than βγ, to activate MEK-1, ERK, and downstream transcriptional events. Consistent with this idea, previous biochemical studies have shown that DOR1 can bind and promote the activation of G proteins containing both αi2 and αi3 subunits (Ref. 59 and references therein). The DOR1-mediated pathway resembles the pathway that is stimulated by the αi2 oncogenic gig2 mutants, which activates ERK (25) and transforms Rat1a fibroblasts via RasN17-resistant pathways (24). However, unlike G protein signaling that activates ERK via the epidermal growth factor receptor in COS-7 cells (13), DOR1 activates ERK in Jurkat cells via a pathway resistant to the PI 3-kinase inhibitor drug, wortmannin. A Ras- and βγ-independent pathway was recently described in Chinese hamster ovary epithelial cells, but this pathway appears to be mediated by αo subunits and is inhibited by down-regulation of PMA-sensitive protein kinase C isoforms (10). DOR1 signaling most likely stimulates ERK via a distinct pathway, since DOR1 signaling was not sensitive to protein kinase C inhibitor drugs that inhibit PMA activation of ERK, and since lymphocytes express the G protein subunits αi2 and αi3 but are deficient in other PTX-sensitive G proteins such as αo (28).

Interestingly, DOR1 signaling showed no evidence of initiating the βγ-mediated pathway of ERK activation, although receptor activation of the heterotrimeric G protein theoretically produces equal numbers of αi and βγ signaling moieties. Other reports of putative αo-mediated ERK activation also do not address the question of why βγ-mediated signaling is not detected in these cases (10). It is possible, however, that the molecules required for βγ-mediated ERK activation, including the p110-γ PI 3-kinase and Ras, are inaccessible to βγ that is produced in the vicinity of the activated DOR1 receptor. It is also possible that free βγ is itself efficiently sequestered when it is produced in membrane subdomains associated with specific receptors. Further research will be necessary to adequately address the question of what happens to the free βγ subunits during βγ-independent signaling by DOR1 in Jurkat cells.

Although constitutively active αi2 subunits (αi2Q205L) have been shown to activate ERK in Rat1a fibroblasts (24, 25), we observed only a small elevation of Elk-1-dependent transcription increased mRNA for the AP-1 subunit, c-Fos, and direct activation PLC-β (60). In contrast, DOR1 signaling increases calcium in Jurkat T cells via a mechanism that is fully sensitive to PTX (36). Similarly, the predominantly brain-expressed αax subunit permits adenylyl cyclase regulation in a PTX-insensitive manner, while DOR1 uses a PTX-sensitive mechanism to decrease cAMP levels in Jurkat T cells (36). While we are directly addressing this question by identifying the G proteins that co-purify with DOR1 in Jurkat T cells, we consider it most likely that the βγ-independent ERK activation pathway we describe here is mediated by αi2 and/or αi3.

We also report here that βγ-mediated but not DOR1-mediated activation of ERK is synergistically enhanced by iSH2-CAAX, a fusion protein designed to constitutively activate p110α/β PI 3-kinases. Since βγ stimulates ERK via a mechanism that requires Ras (1–3, 22) and the p110-γ PI 3-kinase (8, 12, 23), we hypothesize that the D3-phosphorylated lipids produced by constitutive PI 3-kinase activity synergize with βγ signaling. RasN17 blocked ERK activity in response to either βγ alone or βγ plus iSH2-CAAX, consistent with iSH2-CAAX acting on the βγ pathway upstream of Ras. The lipid products of p110α/β PI 3-kinases may therefore participate in cross-talk that enhances βγ-mediated activation of Ras and ERK.

Finally, we show that the βγ-independent pathway for ERK activation is required for DOR1 to mobilize AP-1 transcription factors in the Jurkat T cell line, a result indicating that this novel βγ-independent pathway contributes to the regulation of a downstream event relevant to mitogenic signaling. DOR1 signaling increased mRNA for the AP-1 subunit, c-Fos, and levels of transcriptionally active AP-1, via mechanisms sensitive to a MEK inhibitor drug that also blocked DOR1 stimulation of ERK. Moreover, neither c-Fos transcription nor AP-1 mobilization in response to DOR1 signaling was sensitive to the PI 3-kinase inhibitor, wortmannin. We previously showed that DOR1 increases AP-1 in these cells via a PTX-sensitive

---

2 K. E. Hedin, unpublished observations.
pathway (35). Together with signals from TCR-CD3 and CD28, the DOR1-mobilized AP-1 complexes enhance the transcriptional activity of the NF-AT/AP-1-binding element of the interleukin-2 (IL-2) promoter and increase IL-2 synthesis and secretion (35). Others have also shown that Gβ protein-coupled receptors enhance the secretion of IL-2 (33, 34). IL-2 secretion occurs coincident with T lymphocyte immune activation and cell cycle entry and acts on T cells to promote their growth and continued immune activation. The βγ-independent pathway of ERK activation may, therefore, form part of a mechanism that permits Gβ protein-coupled receptors to regulate IL-2 and other AP-1-responsive genes in T lymphoid cell types.

Acknowledgments—We are extremely grateful to Dr. Paul Leibson and his laboratory for generosity and advice on using recombinant Vaccinia viruses.

REFERENCES

1. Alblas, J., van Corven, E. J., Hordijk, P. L., Milligan, G., and Moolenaar, W. H. (1993) J. Biol. Chem. 268, 22235–22238
2. Koch, W. J., Hawes, B. E., Allen, L. F., and Lefkowitz, R. J. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 12706–12710
3. Crespo, P., Xu, X., Simonis, W. F., and Getkind, J. S. (1994) Nature 369, 418–420
4. Faure, M., Voyo-Yasenetksaya, T. A., and Bourne, H. R. (1994) J. Biol. Chem. 269, 7851–7854
5. van Biesen, T., Hawes, B. E., Luttrell, D. K., Krueger, K. M., Touhara, K., Porfirii, E., Sakaue, M., Luttrell, L. M., and Getkind, R. J. (1995) Nature 376, 781–784
6. Dikie, I., Tokiwa, G., Lee, S., Courtneidge, S. A., and Schlessinger, J. (1996) Nature 383, 547–550
7. Dauh, H., Weiss, F. U., Wallasch, C., and Ulrich, A. (1996) Nature 379, 557–560
8. Hawes, B. E., Luttrell, L. M., van Biesen, T., and Getkind, R. J. (1996) J. Biol. Chem. 271, 12133–12136
9. Cowen, D. S., Sowers, R. S., and Manning, D. R. (1996) J. Biol. Chem. 271, 22297–22300
10. van Biesen, T., Hawes, B. E., Raymond, J. R., Luttrell, L. M., and Getkind, R. J. (1996) J. Biol. Chem. 271, 12133–12136
11. van Biesen, T., Hawes, B. E., Raymond, J. R., Luttrell, L. M., Krueger, K. M., Touhara, K., Porfirii, E., Sakaue, M., Luttrell, L. M., and Getkind, R. J. (1995) Nature 369, 418–420
12. Dikie, I., Tokiwa, G., Lee, S., Courtneidge, S. A., and Schlessinger, J. (1996) Nature 383, 547–550
13. Dauh, H., Weiss, F. U., Wallasch, C., and Ulrich, A. (1996) Nature 379, 557–560
14. Hawes, B. E., Luttrell, L. M., van Biesen, T., and Getkind, R. J. (1996) J. Biol. Chem. 271, 12133–12136
15. Cowen, D. S., Sowers, R. S., and Manning, D. R. (1996) J. Biol. Chem. 271, 22297–22300
16. van Biesen, T., Hawes, B. E., Raymond, J. R., Luttrell, L. M., Krueger, K. M., Touhara, K., Porfirii, E., Sakaue, M., Luttrell, L. M., and Getkind, R. J. (1995) Nature 369, 418–420
17. Dikie, I., Tokiwa, G., Lee, S., Courtneidge, S. A., and Schlessinger, J. (1996) Nature 383, 547–550
18. Dauh, H., Weiss, F. U., Wallasch, C., and Ulrich, A. (1996) Nature 379, 557–560
19. Hawes, B. E., Luttrell, L. M., van Biesen, T., and Getkind, R. J. (1996) J. Biol. Chem. 271, 12133–12136
20. Cowen, D. S., Sowers, R. S., and Manning, D. R. (1996) J. Biol. Chem. 271, 22297–22300
21. van Biesen, T., Hawes, B. E., Raymond, J. R., Luttrell, L. M., Krueger, K. M., Touhara, K., Porfirii, E., Sakaue, M., Luttrell, L. M., and Getkind, R. J. (1995) Nature 369, 418–420
22. Dikie, I., Tokiwa, G., Lee, S., Courtneidge, S. A., and Schlessinger, J. (1996) Nature 383, 547–550
23. Dauh, H., Weiss, F. U., Wallasch, C., and Ulrich, A. (1996) Nature 379, 557–560
24. Hawes, B. E., Luttrell, L. M., van Biesen, T., and Getkind, R. J. (1996) J. Biol. Chem. 271, 12133–12136
25. Cowen, D. S., Sowers, R. S., and Manning, D. R. (1996) J. Biol. Chem. 271, 22297–22300
26. van Biesen, T., Hawes, B. E., Raymond, J. R., Luttrell, L. M., Krueger, K. M., Touhara, K., Porfirii, E., Sakaue, M., Luttrell, L. M., and Getkind, R. J. (1995) Nature 369, 418–420
27. Dikie, I., Tokiwa, G., Lee, S., Courtneidge, S. A., and Schlessinger, J. (1996) Nature 383, 547–550
28. Dauh, H., Weiss, F. U., Wallasch, C., and Ulrich, A. (1996) Nature 379, 557–560
29. Hawes, B. E., Luttrell, L. M., van Biesen, T., and Getkind, R. J. (1996) J. Biol. Chem. 271, 12133–12136
30. Cowen, D. S., Sowers, R. S., and Manning, D. R. (1996) J. Biol. Chem. 271, 22297–22300
Gi Proteins Use a Novel βγ- and Ras-independent Pathway to Activate Extracellular Signal-regulated Kinase and Mobilize AP-1 Transcription Factors in Jurkat T Lymphocytes
Karen E. Hedin, Michael P. Bell, Catherine J. Huntoon, Larry M. Karnitz and David J. McKean

J. Biol. Chem. 1999, 274:19992-20001.
doi: 10.1074/jbc.274.28.19992

Access the most updated version of this article at http://www.jbc.org/content/274/28/19992

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 60 references, 35 of which can be accessed free at http://www.jbc.org/content/274/28/19992.full.html#ref-list-1