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Phosphatidylinositol 3-Kinase Mediates Epidermal Growth Factor-Induced Activation of the c-Jun N-Terminal Kinase Signaling Pathway

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The signaling events which mediate activation of c-Jun N-terminal kinase (JNK) are not yet well characterized. To broaden our understanding of upstream mediators which link extracellular signals to the JNK pathway, we investigated the role of phosphatidylinositol (PI) 3-kinase in epidermal growth factor (EGF)-mediated JNK activation. In this report we demonstrate that a dominant negative form of PI 3-kinase as well as the inhibitor wortmannin blocks EGF-induced JNK activation dramatically. However, wortmannin does not have an effect on JNK activation induced by UV irradiation or osmotic shock. In addition, a membrane-targeted, constitutively active PI 3-kinase (p110β) was shown to produce in vivo products and to activate JNK, while a kinase-mutated form of this protein showed no activation. On the basis of these experiments, we propose that PI 3-kinase activity plays a role in EGF-induced JNK activation in these cells.

Phosphatidylinositol (PI) 3-kinases have been implicated in such biologically diverse processes as mitogenesis, membrane trafficking, insulin-stimulated glucose transport, and cell survival (for reviews, see references 22, 38, and 40). A scenario in which one enzyme activity could play a role in all these processes seems unlikely but may be clarified by the recent realization that there are multiple isoforms of PI 3-kinase, which belong to different families with respect to their substrate specificities and mechanisms of activation. The p110α and p110β PI 3-kinase isoforms both bind p85 regulatory subunits and are stimulated by both receptor and nonreceptor protein tyrosine kinases. The N-terminal region of p110 has been shown to bind to the region of p85 which separates two src homology (SH2) domains (inter-SH2 region) (10, 20, 23). p85 mediates the interaction of PI 3-kinase with other proteins via an SH3 domain (35) and two SH2 domains which bind proline-rich regions and tyrosine phosphoproteins, including activated growth factor receptors, respectively. Another PI 3-kinase isoform, p110γ, does not bind p85 and is stimulated by G proteins (41). All three enzymes phosphorylate the 3′ position of the inositol ring and contribute to the rapid cellular accumulation of PI 3,4-diphosphate (PI 3,4-P_2) and PI 3,4,5-triphosphate (PI 3,4,5-P_3) upon appropriate stimulation. On the other hand, resting cells have a constitutive pool of PI 3-phosphate (PI 3-P), possibly due to the activity of kinases such as PI 3-kinase p100, (46) which can phosphorylate PI to produce PI 3-P but not PI 4-P or PI 4,5-P_2 to produce PI 3,4-P_2 or PI 3,4,5-P_3.

Variations in the domain structures of PI 3-kinases suggest different modes of interaction with lipids or proteins. For example, while p110α and p110β are probably localized to the plasma membrane via p85, a p170 PI 3-kinase may be localized by an intrinsic C2 domain, previously identified in membrane-associated proteins (31, 45). Such variety in protein structure suggests variation in the way PI 3-kinases will function as signaling molecules.

Little is understood regarding the signaling events downstream of any of the known PI 3-kinases. However, proteins which are candidate targets of PI 3-kinase action are the protein kinase Akt (Rac, protein kinase B [PKB]) (11), $p70^{S6}$ kinase (6, 47), and noncanonical isoforms of PKC (32, 42). Furthermore, both Akt and the PKC isoforms appear to be activated by the 3′-phosphorylated lipid products of PI 3-kinase, supporting the contention that these phosphorylated lipids function as second messengers.

Recently, a kinase cascade in which many extracellular stimuli result in the activation of c-Jun N-terminal kinase (JNK) or stress-activated protein kinase (SAPK) has been described (9, 27). This kinase phosphorylates Jun family transcription factors to cause changes in transcription. Most of the work characterizing the JNK/SAPK signaling pathway has focused on cellular responses to stress, since JNK is most strongly activated by stimuli such as UV irradiation, ionizing radiation, and osmotic shock. However, it is also clear that inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), growth factors, and activated forms of the small G proteins Rac and Ras can activate JNK (7, 30). Furthermore, there is likely cross talk between signaling mediated by the Rho family (of which Rac is a member) and the Ras pathway (12), which are both upstream of JNK. Therefore, understanding the mechanism of growth factor-induced JNK activation will likely provide important clues regarding cell growth control and transformation.

Epidermal growth factor (EGF) has previously been shown to activate the JNK pathway (30). EGF receptor also activates PI 3-kinase, although PI 3-kinase does not bind directly, as is the case with the platelet-derived growth factor (PDGF) receptor, but rather binds through adapter proteins such as $p120^{cbl}$ and Gab1 (17, 28). Alternatively, PI 3-kinase may interact with heterodimers of other members of the EGF receptor family, such as erbB3 (4, 5). In this report, we demonstrate that a membrane-targeted form of PI 3-kinase can activate JNK and that a dominant inhibitory mutant of PI 3-kinase blocks EGF-induced JNK activation. The present experiments

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demonstrate that PI 3-kinase activated by EGF is responsible for JNK activation.

MATERIALS AND METHODS

Expression vectors. The following mammalian expression plasmids have been previously described: p85 (20), hemagglutinin (HA)-tagged p110 (18), and p85INT Tag (20). M2-JNK was obtained from M. Karin (described in reference 9). Membrane-targeted p110β was constructed by inserting a sequence encoding the Ras farnasylation signal in frame at the 3′ end of the cDNA before the stop codon. The sequences encoding the farnasylation signal and the mutant farnasylation signal are identical to those shown in reference 1. The kinase-inactive p110 variant (p110kd) was made by using an in vitro mutagenesis system (Altered Sites II; Promega). Asp919 was replaced by alanine (D919A), and Asp937 was replaced by glutamic acid (D937E).

Cell culture, transfection, and immunoblotting. For transient transfections, HeLa cells were grown to 80% confluence in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and treated with Lipofectamine reagent according to the manufacturer's instructions. EGF-stimulated cells were grown for 18 h in 0.1% fetal bovine serum prior to treatment for 5 min with EGF (100 ng/ml; Invitrogen). In experiments utilizing wortmannin, the cells were pretreated with 100 nM wortmannin (Sigma) for 10 min at 37°C prior to EGF stimulation. Following treatment, the cells were washed in ice-cold phosphate-buffered saline and 0.2 ml of lysis buffer (50 mM HEPES [pH 7.5], 10% glycerol, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 0.2 mM EGTA, 20 mM β-glycerol phosphate, 5 mM sodium orthovanadate, 20 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, aprotinin [10 mg/ml], leupeptin [10 mg/ml], and 1% Triton X-100) was added. Cells were incubated for 5 min on ice, scraped from the plates, transferred to microcentrifuge tubes, and clarified by centrifugation at 13,000 × *g* for 10 min, and the supernatants were transferred to new tubes. For immunoblot analysis, immunoprecipitates were washed three times with ice-cold lysis buffer, boiled in sodium dodecyl sulfate (SDS) sample buffer for 3 min, subjected to SDS-polyacrylamide gel electrophoresis (PAGE), and transferred to nitrocellulose filters (MSI). Proteins were visualized by enhanced chemiluminescence (Renaissance system; Dupont NEN).

Kinase assays. For solid-phase JNK assays, lysates were prepared as described above and processed as described in reference 43. For immunocomplex JNK kinase assays, 1/10 of the lysate was immediately added to the SDS gel loading buffer, boiled, and used for analysis of protein expression. The remainder of the lysate was immunoprecipitated with 1.5 μg of M2 antibody (Kodak) on ice for 1 h. Protein A-G agarose beads (20 μl; Santa Cruz Biotechnology) were added, the total volume was adjusted to 0.5 ml with lysis buffer, and the samples were incubated at 4°C for 1 h. The beads were then washed four times with lysis buffer and once with kinase buffer (20 mM HEPES [pH 7.5], 20 mM β-glycerol phosphate, 10 mM *p*-nitrophenyl phosphate, 10 mM $MgCl_2$, 10 mM dithiothreitol, 50 mM sodium vanadate) and transferred to new tubes. The samples were divided into two parts; one part was used for immunoblot analysis, and the other part was used for kinase assays. Kinase assays were performed by incubating the beads with 30 μl of kinase buffer to which 20 mM cold ATP, 5 μCi of [γ-^{32}P]ATP, and 2 μg of purified c-Jun (1-79) were added. The preparation of glutathione *S*-transferase (GST)–c-Jun and c-Jun (1-79) is described in reference 43. Samples were incubated at 30°C for 15 min, 15 μl of 3× SDS gel loading buffer was added to the reaction mixture, the mixture was heated to 100°C for 3 min, and the products were analyzed by SDS-PAGE. The gel was dried and exposed to X-ray film (Dupont) at room temperature. Each experiment was repeated at least three times, and quantification of the amount of c-Jun phosphorylation was performed with a Molecular Dynamics PhosphorImager and ImageQuant software (Molecular Dynamics, Incorporated). PI 3-kinase assays were performed as described in reference 19.

In vivo labeling of phosphoinositides. Following transfection, HeLa cells were labeled for 24 h with [^{3}H]inositol. Lipids were extracted, deacylated, and separated by high-performance liquid chromatography as described in reference 39.

RESULTS

Activation of JNK by EGF is blocked by a dominant interfering mutant of PI 3-kinase. The mechanism by which JNK is activated to cause phosphorylation of c-Jun is not well understood. Since PI 3-kinase has been shown to be activated by several small G proteins which play a role in JNK activation (36, 44, 48), we investigated whether PI 3-kinase might also mediate JNK activation. Because EGF can activate PI 3-kinase as well as the JNK signaling pathway, we used a dominant negative mutant of PI 3-kinase to examine its role in EGF-mediated JNK signaling. To abrogate endogenous PI 3-kinase activity, we used the inter-SH2 domain of p85α (p85INT), which was shown previously to contain the binding region for p110α and p110β and to inhibit PI 3-kinase in vivo (20). To test the effect of dominant negative PI 3-kinase on JNK activity, HeLa cells were cotransfected with an epitope-tagged (FLAG) variant of JNK that is recognized by the monoclonal antibody M2 (M2-JNK) along with p85INT. Approximately 48 h after transfection, the transiently expressed M2-JNK was isolated by immunoprecipitation and kinase activity with c-Jun (1-79) as a substrate was determined. Expression levels of M2-JNK and p85INT were determined by immunoblotting (Fig. 1A). In addition, in this and all other immunocomplex kinase assays described in this report, the amount of c-Jun substrate was determined to be evenly distributed in the samples, as judged by Coomassie blue staining of the gels on which the reaction products were separated. The results show that dominant negative PI 3-kinase inhibited EGF-induced JNK activation by about 60% but did not inhibit TNF-α-induced JNK activation (Fig. 1A and C). This is consistent with earlier observations (29) indicating that TNF-α induction of JNK is regulated via a different pathway than EGF induction of JNK.

Since dominant negative PI 3-kinase interfered with EGF-induced JNK activation, we expected that wortmannin would inhibit EGF-induced JNK activation as well. To test this, we incubated HeLa cells with or without wortmannin, prior to stimulation with EGF. In vitro kinase assays using a solid-phase JNK assay show that 100 nM wortmannin inhibits the ability of endogenous JNK to phosphorylate GST–c-Jun dramatically after EGF treatment (Fig. 1B). The effect is specific, as wortmannin did not inhibit JNK activation by TNF-α or anisomycin in the same experiment. Experiments utilizing the PI 3-kinase inhibitor, wortmannin, need to be interpreted with caution, since wortmannin appears to inhibit other enzymes with 50% inhibitory concentrations similar to those used to inhibit PI 3-kinase (8, 33). However, taken together, these results indicate that PI 3-kinase is an important mediator of EGF-induced JNK activation.

To see if wortmannin also inhibited stress activation of the JNK pathway, we incubated HeLa cells with or without wortmannin, prior to stimulation with sorbitol or UV. While wortmannin strongly inhibits EGF activation of JNK, it has no effect on sorbitol or UV activation of JNK (Fig. 1D). Thus, PI 3-kinase appears to play a role in the growth factor activation, but not stress activation, of the JNK pathway.

Membrane-targeted p110β causes production of PI 3,4-P_2 in vivo. To further test the hypothesis that PI 3-kinase plays a role in JNK activation, we constructed a membrane-targeted version of PI 3-kinase which we reasoned would be constitutively active due to the proximity of lipid substrates. To target p110β to the membrane, we fused the sequence which specifies farnasylation of c-HA-Ras (1, 14) to its C terminus (p110F). As controls, we used unmodified p110 and a membrane-targeted p110 containing an inactivating point mutation in the farnasylation signal (1, 13). In addition, we constructed p110F-kd, a membrane-targeted version of p110 which has two point mutations in the kinase domain, either of which abolishes enzyme activity (38) (Fig. 2). For ease of analysis, all p110β constructs were tagged with the HA epitope (19). Visualization by immunofluorescence microscopy using the anti-HA antibody verified that the majority of p110F is localized in the plasma membrane (data not shown), similar to the localization of Sos, which was associated with the plasma membrane by using the identical farnasylation signal (1). Following transient transfection of the PI 3-kinase plasmids into 293 cells, lysates were prepared and a portion of the whole-cell lysates was used for immunoblot analysis to determine the levels of p110 and p85 expression (Fig. 2A). The remainder of the lysate, immunoprecipitated with an anti-HA antibody, was used for SDS-PAGE immunoblotting and PI 3-kinase assays. Figure 2A shows that all the

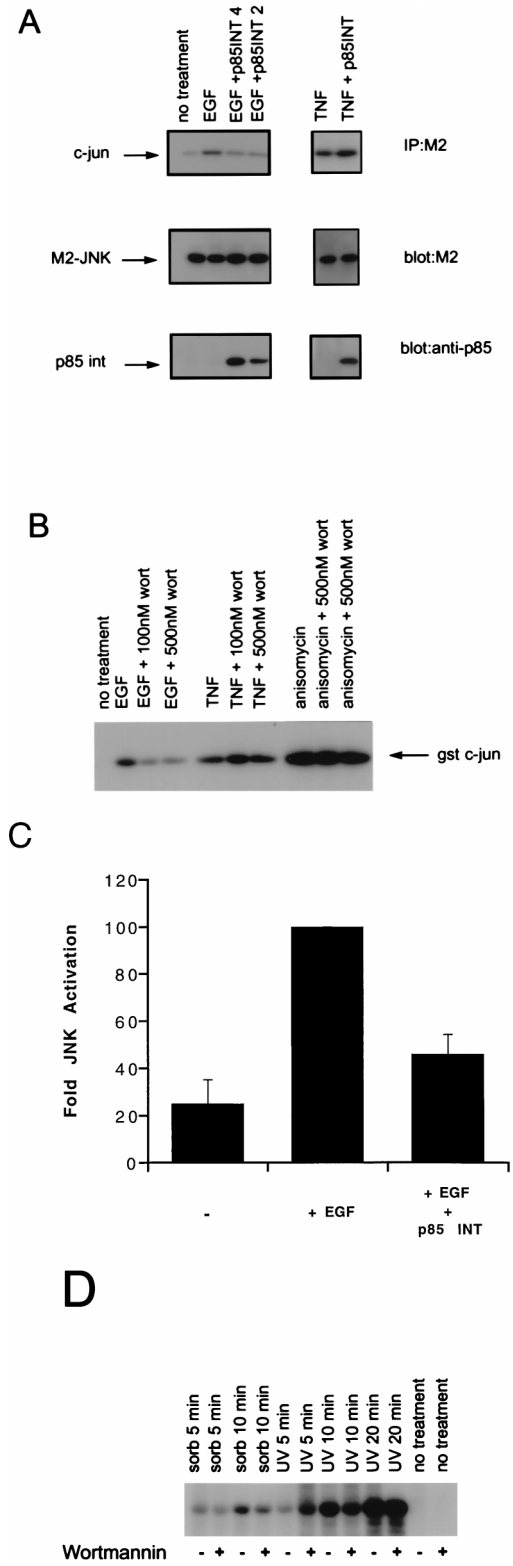

FIG. 1. Inhibition of EGF-induced JNK activation by a dominant interfering inhibitor of PI 3-kinase and by wortmannin. (A) HeLa cells were transfected with 0.15 μg of M2-JNK and either empty vector alone or 2 μg of p85INT or 4 μg of p85INT. The total amount of DNA in all transfections was held constant by the addition of empty vector. JNK activity was measured by an immunocomplex kinase assay using purified c-Jun (1-79) as a substrate (top panels). The phosphorylated proteins were separated by SDS-PAGE and visualized by autoradiography. JNK activity in this and subsequent experiments was determined by quantitation of the amount of c-Jun phosphorylation with a Molecular Dynamics PhosphorImager and ImageQuant software (Molecular Dynamics, Incorporated). The levels of M2-JNK and p85INT protein expression were determined by immunoblotting. Antibodies used for each part of the experiment are indicated to the right. (B) HeLa cells were incubated in the presence or absence of 100 nM wortmannin (wort) for 10 min followed by EGF treatment for 5 min. The cells were lysed and used to perform solid-phase JNK assays with 2 μg of GST–c-Jun (1-79) immobilized on glutathione-agarose beads as the substrate. The phosphorylated proteins were separated by SDS-PAGE and visualized by autoradiography. The experiment was repeated three times, and wortmannin inhibition of EGF activation of JNK was 61% +/− 10%. (C) The graph depicts the fold difference in JNK activation in EGF-treated cells in the presence (+) and absence (−) of a dominant interfering p85 (p85INT). The highest level of JNK activity was given a value of 100%, and the other values are shown relative to this amount. Quantitation was performed on three independent experiments as described for panel A. Error bars, standard errors of the means. (D) HeLa cells were incubated in the presence or absence of 100 nM wortmannin for 10 min, and this was followed by sorbitol (sorb) or UV treatment. Cells were allowed to recover for the indicated times before they were lysed and used to perform solid-phase JNK kinase assays as described for panel B. This experiment was repeated three times with similar results.

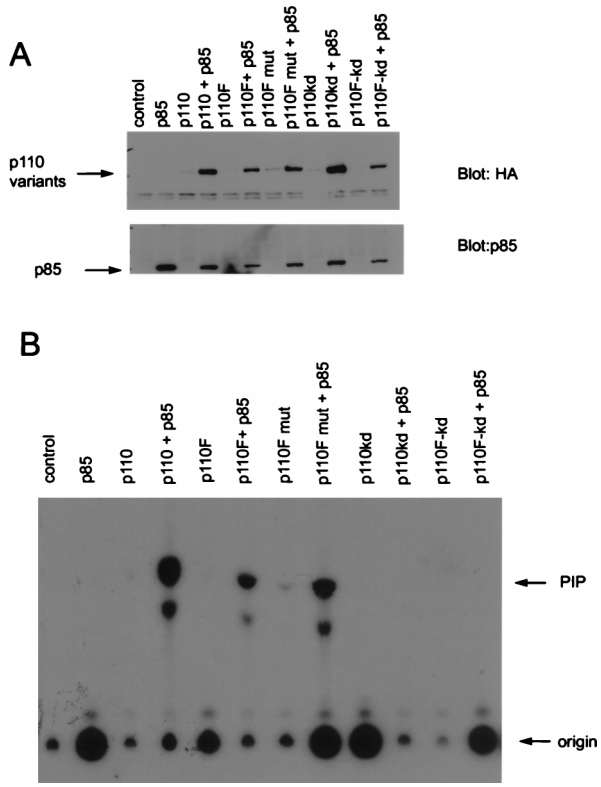

FIG. 2. Analysis of membrane-targeted and kinase-inactivated variants of PI 3-kinase in vitro. (A) 293 cells were transfected with the indicated plasmids. Lysates were made, and 10% of the sample was used for Western blot analysis to determine the levels of p110 and p85 protein expression. Protein expression was assessed by subjecting the samples to SDS–8% PAGE, transferring them to nitrocellulose, and blotting them with an antibody against HA. The blot was then stripped and reprobed with antibody against p85. The remainder of the lysates was immunoprecipitated with anti-HA antibody and used for the PI 3-kinase assay. The ratios of protein expression in the immunoprecipitates are indistinguishable from those in the whole-cell lysates (data not shown). (B) PI 3-kinase activity in anti-HA immunoprecipitates from 293 cells transfected with the indicated constructs. mut, mutant.

proteins are expressed, but in every case the level of protein expression of the p110 variant is greatly enhanced by coexpression with p85. Greater protein stability of p110 in the presence of p85 has been observed previously (18), and for this reason

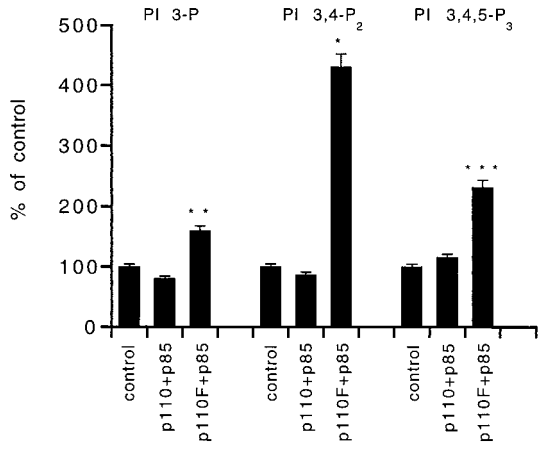

FIG. 3. In vivo labeling of HeLa cells transfected with a wild-type or membrane-targeted p110β to determine abundance of 3′-phosphorylated lipids. HeLa cells were transfected with 3 μg of p110 and 3 μg of p85, 1.5 μg of p110F and 1.5 μg of p85, or vector alone (control). These DNA ratios resulted in equal levels of protein expression as determined by immunoblotting (not shown). The total amount of DNA in each transfection was held constant by the addition of empty vector. The experiment was done three times in triplicate. The third plate of each set was used to determine protein expression. Cells on the other plates were labeled 24 h with [^{3}H]inositol. The lipids were deacylated, extracted, separated by HPLC, and quantitated by liquid scintillation counting. The graph shows the ratio of 3′ phosphoinositol products in cells transfected as indicated. The levels of phosphoinositides (in counts per minute) in the control samples are as follows: for PI, 167,235; for PI 3-P, 331; for PI 4-P, 10,345; for PI 3,4-P_2, 34; for PI 4,5-P_2, 7,358; and for PI 3,4,5-P_3, 97. Approximately 20% of the cells were transfected in these experiments. Error bars, standard errors of the means; *, $P < 0.001$; **, $P < 0.003$; ***, $P < 0.025$.

subsequent experiments designed to evaluate the role of PI 3-kinase in activation of the JNK pathway were done by coexpressing both p110 and p85 subunits. PI 3-kinase assays (Fig. 2B) demonstrate that all of the active enzyme variants (p110, p110F, and p110Fmut) can phosphorylate PI to generate PI 3-P, while the kinase-inactive variants (p110kd and p110F-kd) cannot phosphorylate PI.

To evaluate the catalytic activity of p110F, we transfected and in vivo labeled HeLa cells with [^{3}H]inositol. The labeled lipids were isolated, deacylated, and separated by high-performance liquid chromatography. Protein expression from transfected plasmids was determined to be equivalent in each experiment by inclusion of a triplicate plate, the lysate of which was used for immunoblot analysis (data not shown). Lysates from cells transfected with p110F and p85 showed a 4.3-fold increase of PI 3,4-P_2, a 2-fold increase in PI 3,4,5-P_2, and a 1.6-fold increase in PI 3-P relative to control cells transfected with vector alone (Fig. 3). Cells transfected with wild-type (nontargeted) p110 and p85 exhibited no increase in 3′-phosphorylated products. Levels of PI, PI 4-P, and PI 4,5-P_2 were not statistically different among the samples, indicating that similar levels of cell lysate were compared in the assays and confirming our expectation that overexpression of PI 3-kinase should result in changes only in 3′-phosphorylated products. Similar results were obtained when this experiment was done with 293 cells (data not shown).

The results presented above indicate that, although lysates from cells transiently overexpressing wild-type p110 and p85 show substantial phosphorylation of PI in a PI 3-kinase assay (Fig. 2), such overexpression does not result in much of an increase in in vivo-labeled products. This observation is consistent with the previous suggestion that increased levels of PI 3-kinase activity in in vitro kinase assays do not necessarily reflect the status of in vivo-phosphorylated products (16). And in fact, we observe that membrane-targeted PI 3-kinase does not show any more activity in in vitro kinase assays than does wild-type PI 3-kinase, yet it produces much greater amounts of 3′-phosphorylated lipids in vivo.

We conclude that localization of PI 3-kinase to the plasma membrane creates a constitutively active enzyme which produces greater amounts of 3′-phosphorylated lipids in vivo.

Membrane-targeted p110β activates JNK. To further determine the role that PI 3-kinase plays in JNK activation, an analysis of JNK activation in cells overexpressing constitutively active PI 3-kinase was done. HeLa cells were cotransfected with M2-JNK along with membrane-targeted or nontargeted versions of PI 3-kinase. JNK was immunoprecipitated from cell lysates with M2 antibody and divided into two equal parts. One part was used in a JNK assay, and the other part was used to ascertain the levels of M2-JNK expression in the various transfections (Fig. 4). A small portion of each lysate was also immunoblotted to show that p110, p110F (blotted with anti-HA antibody), and p85 (blotted with anti-p85 antibody) were expressed at expected levels. Consistent with our previous findings, our results show an increase in JNK activation in cells cotransfected with p110F and p85 but not in cells transfected with unmodified p110 and p85, p85 alone, or vector alone (Fig. 4A). In addition, there is no JNK activation in lysates from cells transfected with p85 and a catalytically inactive form of membrane-targeted PI 3-kinase (p110F-kd) (Fig. 4B). Thus, JNK is activated in the presence of a PI 3-kinase variant which we have shown produces greater amounts of lipid products in vivo, and this activation is dependent on the catalytic activity of p110 (Fig. 4C). We conclude that the PI 3-kinase signaling pathway alone is sufficient to activate JNK, since transient expression of a constitutively active PI 3-kinase stimulates JNK in the absence of other stimuli. Activation of JNK in these experiments requires coexpression of both p110 and p85. While this may simply be due to an increase in protein stability of p110 when coexpressed with p85 (Fig. 2), it may also indicate a regulatory function of p85.

Constitutively active PI 3-kinase does not activate ERK. A previous report (21) suggested that a constitutively active form of PI 3-kinase, called p110*, was able to act upstream of Ras to mediate mitogen-activated protein kinase (MAPK) activation. However, subsequent experiments using membrane-targeted PI 3-kinase showed no effect on MAPK activation (24). To determine if membrane-targeted p110β could activate extracellular signal-regulated kinase 1 (ERK1) (also known as MAPK), we cotransfected p110F and 5′-Sos-F along with HA-tagged ERK1 (Fig. 5). This variant of Sos has been shown to be a strong activator of the Ras/MAPK pathway (1) and therefore serves as a positive control for the experiment. Phosphorylation of HA-ERK1 was demonstrated by a shift in electrophoretic mobility in the presence of 5′-Sos-F but not in the presence of p110F. We conclude that p110β does not play a role in activation of the Ras/MAPK signaling pathway.

DISCUSSION

In an effort to examine PI 3-kinase-mediated cell signaling in the absence of activation of other pathways, we constructed a constitutively active, membrane-targeted PI 3-kinase. The experiments described in this report utilize the p110β isoform. A similar targeting approach was used to examine the signaling properties of p110α (24). Biochemical characterization has revealed no apparent differences in these two enzymes to date. However, our study suggests that p110α and p110β prefer different substrates in vivo (24). While the major phosphory-

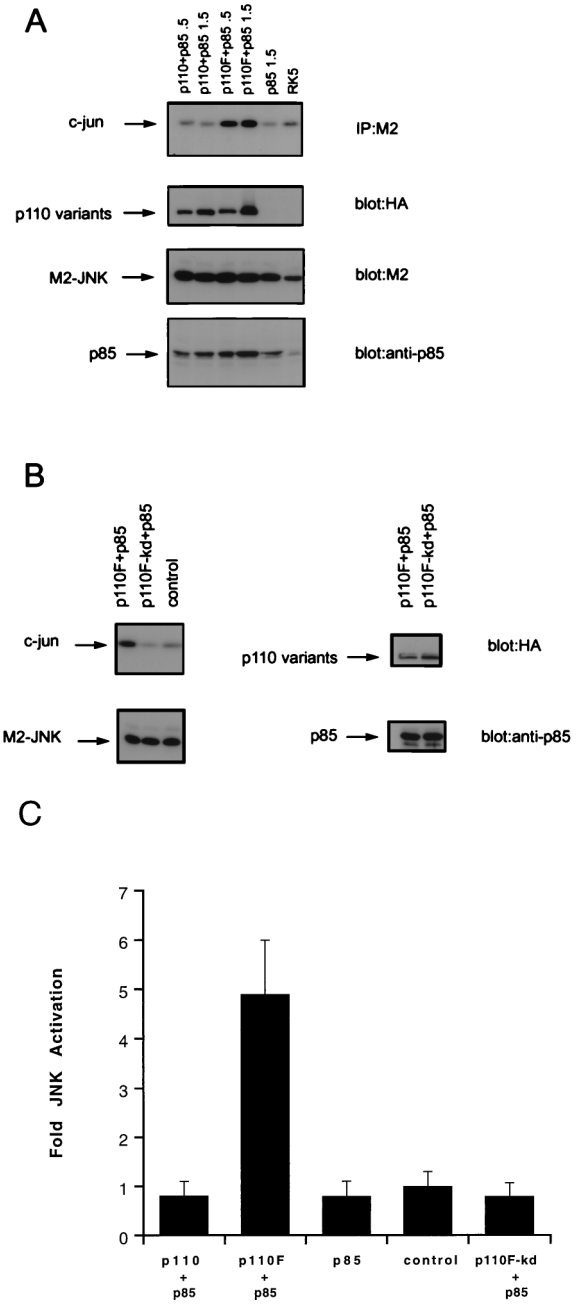

FIG. 4. Membrane-targeted PI 3-kinase activates JNK. (A) HeLa cells were transfected with 0.15 μg of M2-JNK and the indicated amounts of the various plasmids (.5 and 1.5 indicate the amount [in micrograms] of each plasmid transfected; for example, cells used to make lysates analyzed in the far left lane were transfected with 0.5 μg of p110 and 0.5 μg of p85). The total amount of DNA in each transfection was held constant by the addition of empty vector. JNK activity was measured by the immunocomplex kinase assay using purified c-Jun (1-79) as a substrate. Levels of protein expression of M2-JNK, p110 variants, and p85 are shown by immunoblots which were incubated with the antibodies indicated to the right of each panel. (B) Cells were transfected with the indicated plasmids. JNK activity was measured as described for panel A. Levels of protein expression of M2-JNK, p110 variants, and p85 are shown by immunoblots which were incubated with the antibodies indicated to the right of each panel. (C) The graph depicts the average fold difference in JNK activation in cells transfected with indicated plasmids in three independent experiments. Quantitation was performed as described in the legend to Fig. 1A. Error bars, standard errors of the means.

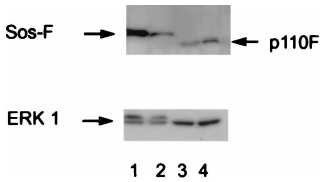

FIG. 5. Constitutively active PI 3-kinase does not activate ERK. HeLa cells were transfected with 1 μg of HA-ERK1 in addition to 0.5 μg 5′-Sos-F (lane 1) or 0.25 μg of 5′-Sos-F (lane 2) or both 2.5 μg of p110F and 2.5 μg of p85 (lanes 3 and 4). Levels of expression of the proteins were determined by immunoblotting using HA antibody since 110F, ERK1, and 5′-Sos-F were all tagged with HA.

lated lipid product in cells constitutively overexpressing membrane-targeted p110α appears to be PI 3,4,5-P_3, overproduction of membrane-targeted p110β results in greater accumulation of PI 3,4-P_2 than of PI 3,4,5-P_3. A similar pattern of lipid accumulation was observed in both HeLa and 293 cells overexpressing membrane-targeted p110β, suggesting that the result is not specific for one cell type. This difference in substrate specificity of these two enzymes may well specify a difference in signaling function.

The EGF activation of JNK is likely to be mediated by the small G proteins Rac and Ras as well as through PI 3-kinase. Previous results have shown that EGF-induced JNK activation can be completely inhibited by dominant negative Ras (RasN17) and inhibited approximately 50% by dominant negative Rac (Racn17) (30). These findings are supported by additional reports demonstrating physical or functional interaction between PI 3-kinase and Rac, Rho, and Cdc42. For example, PI 3-kinase has been shown to interact with GTP-bound Ras, and cotransfection of p110α with Ras results in accumulation of 3′-phosphorylated lipids in vivo (25, 36, 37). In addition, both GTP-Rac and GTP-Cdc42 have been shown to bind PI 3-kinase (44, 48). Functionally, PDGF stimulates an increase in the GTP-bound form of Rac, which appears to require the activity of PI 3-kinase, suggesting that PI 3-kinase is upstream of Rac. This is supported by the fact that both wortmannin and a dominant negative p85 block PDGF-induced membrane ruffling, which can be restored by the activity of a constitutively active Rac (15). Thus, it seems likely that one or more members of the small G protein family mediate PI 3-kinase activation of JNK. Although PDGF is a potent activator of PI 3-kinase, PDGF does not stimulate the JNK signaling pathway. It is not clear why PDGF is unable to stimulate the JNK signaling pathway. One possibility, among several explanations, is that PDGF induces additional intracellular events that somehow prevent the activation of the JNK signaling pathway. We attempted to determine whether PI 3-kinase is upstream of small G proteins in JNK activation. In these experiments, we coexpressed dominant negative forms of Rho, Rac, Ras, and Cdc42 with p110F and p85 to see if they would inhibit the activation of M2-JNK. We fully expect that RasN17 would interfere with PI 3-kinase activation of JNK, as it has been shown previously that RasN17 inhibits EGF activation of JNK (30). In addition, it seems likely that RacN17 would also interfere with PI 3-kinase-induced JNK activation. However, the results were inconclusive because coexpression of all the molecules necessary for the experiment (p110F, p85, M2-JNK, and a dominant interfering molecule) consistently resulted in uneven expression of one or more molecules.

Recent work suggests that PI 3-kinase may be a downstream effector of Ras (25, 36, 37). In intact cells, cotransfection of Ras along with PI 3-kinase results in enhanced production of 3′-phosphorylated lipids in vivo. This accumulation of phos-

phorylated lipids is prevented by a dominant negative Ras mutant N17 (36). The physical interaction between p110 and Ras occurs between the effector domain of Ras and the catalytic subunit of p110, with a dissociation constant of approximately 150 nM (37). For both the p110α and p110β isoforms, the site of interaction with Ras occurs between amino acids 133 and 314. A point mutation (K227E) in this region of p110α blocks the in vitro interaction between Ras and p110α as well as the ability of Ras to activate PI 3-kinase in intact cells. Thus, although it has also been suggested that PI 3-kinase activates Ras through the MAPK pathway (21), this seems unlikely. It is more likely that PI 3-kinase is one of the multiple signaling pathways activated by Ras.

In this report we find no activation of the JNK pathway unless both membrane-targeted p110 and p85 are coexpressed. It is difficult to assess the role of p85 in PI 3-kinase activation since it clearly increases the stability of p110 protein. These results are consistent with the notion that p85 is not simply an adapter which links PI 3-kinase to activated growth factor receptors but also plays a regulatory role. Indeed it was demonstrated that binding of phosphotyrosine peptides to p85 increases PI 3-kinase activity (2, 3). Furthermore, the addition of the p85 inter-SH2 domain of the carboxy terminus of p110α appears to create a more active enzyme (21). Our results are in contrast to a report (24) which demonstrated activation of the JNK pathway by overexpression of myristoylated p110α in the absence of expression of p85. One reason for this apparent discrepancy might be that myristoylation of p110α may localize it to src family members which are known to activate PI 3-kinase. This colocalization may circumvent the need for p85, which has been shown to interact with src via its SH3 domain (35).

A comparison of membrane-targeted forms of p110α and p110β suggests that both of these isoforms of PI 3-kinase can activate the JNK pathway. We extend this observation to show that PI 3-kinase appears necessary for growth factor (EGF) activation of JNK. The JNK pathway has typically been studied in the context of stress activation (UV irradiation, osmotic shock, etc.) rather than growth factor activation. Recently however, EGF receptor has been shown to play an antiapoptotic role in response to UV irradiation (26). In addition, Sek1 (JNKK/MKK4), the direct activator of JNK, has been shown to protect against apoptosis in mouse development (34). Thus, our observations are consistent with JNK's playing a role in the antiapoptotic response generated by PI 3-kinase.

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