Phosphoinositide 3-Kinase γ Is a Mediator of Gβγ-dependent Jun Kinase Activation* 

(Received for publication, November 24, 1997) 

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Jun kinases (JNK) are involved in the stress response of mammalian cells. Stimulation of JNK can be induced by stress factors and by agonists of tyrosine kinase and G protein-coupled receptors. G protein-dependent receptors stimulate JNK via Gβγ subunits of heterotrimeric G proteins, but the subsequent signaling reaction has been undefined. Here we demonstrate JNK activation in COS-7 cells by Gβγ-stimulated phosphoinositide 3-kinase γ (PI3Kγ). Signal transduction from PI3Kγ to JNK can be suppressed by dominant negative mutants of Ras, Rac, and the protein kinase PAK. These results identify PI3Kγ as a mediator of Gβγ-dependent regulation of JNK activity. 

Interaction of cells with a wide variety of agonists results in a stimulation of intracellular mitogen-activated protein kinase (MAPK) cascades. MAPKs control the expression of genes that are important for the regulation of many cell functions including proliferation and differentiation. In mammalian cells three parallel MAPK pathways have been characterized so far (1). The canonical MAPK cascade composed of Raf, MEK, and ERK is regulated by the Ras GTPase in response to agonists of tyrosine kinase and G protein-coupled receptors. ERK species catalyze the phosphorylation of transcription factors including Elk-1, thus controlling the expression of several genes (2). 

A second MAPK cascade that is stimulated by osmotic stress regulates the activity of the protein kinase p38. Signal transduction to p38 and the function of this pathway are still unclear. Like p38 the elements of the Jun kinase (JNK) cascade as a third MAPK pathway are involved in the stress response of mammalian cells. JNK can be stimulated by stress factors like interleukin 1 and tumor necrosis factor but also by agonists of tyrosine kinase and G protein-coupled receptors (3, 4). Available evidences point to an involvement of the small GTPase Rac and several protein kinases in the regulation of JNK activity (5). In some cellular systems the STE 20 homologue PAK was found to act as a JNK kinase kinase kinase (6). PAK is able to activate JNK via sequential stimulation of the protein kinases MEKK and SEK. 

The mechanism of signal transduction from G protein-coupled receptors to the JNK cascade is only partially understood. Using COS-7 cells as a transient expression system a recent report showed the involvement of Gβγ subunits of heterotrimeric G proteins in the stimulation of JNK by agonists of the G protein-coupled muscarinergic receptor m2 (7). Additionally the small GTPases Ras and Rac have been demonstrated to mediate signal transduction from G protein-coupled receptor to JNK, but the topology of the signaling path from Gβγ to Rac and Ras remains unknown (7–9). 

One candidate for the link of G protein-coupled receptor and JNK cascade is phosphoinositide 3-kinase γ (PI3Kγ). This sub-species of the PI3K family is stimulated in vitro by Gβγ and recently has been shown to be involved in signal transduction from G protein-coupled receptor to the ERK path of MAPK cascades (10). We now present evidences that JNK stimulation by an agonist of the m2 muscarinergic receptor and by Gβγ also implies a PI3K. Overexpression of PI3Kγ in COS-7 cells induces a significant increase of JNK activity. Stimulation of JNK by Gβγ can be suppressed by the PI3K inhibitor wortmannin and a lipid kinase negative mutant of PI3K. Dominant negative mutants of Ras, Rac, and PAK significantly reduce the stimulatory effect of PI3Kγ on JNK. Thus PI3Kγ seems to act as an intermediate connecting G protein-coupled receptors to the JNK cascade. 

EXPERIMENTAL PROCEDURES 

Cell Culture and Transfection—COS-7 cells (ATCC) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Cells were split the day before transfection. Subconfluent cells were transfected with pcDNAIII-HA-JNK or pcDNAII-HA-MAPK and additional DNAs, following the DEAE-dextran technique, adjusting the total amount of DNA to 5 μg per plate with vector DNA when necessary. Assays were performed 48 h after transfection (9). For JNK assays the cells were serum-starved for 2 h, whereas for MAPK the cells were starved overnight in serum-free medium. 

DNA Constructs—Expression plasmids for an epitope-tagged JNK and MAPK, pcDNA3 HA-JNK and pcDNA3 MAPK, respectively, as well as expression plasmids for the dominant negative mutants of the small GTP-binding proteins Ras, RhoA, Rac1, and Cdc42 have been described (9, 11). PI3Kγ and dominant negative PI3Kγ were used as described previously (10). pcDNA3-p101 was kindly provided by Dr. Len Stephens and prepared as published recently (12). An expression plasmid used for the N-terminal 150-amino acid noncatalytic domain of Pak1, which contains the Rac/Cdc42 binding region, was cloned into pcDNA3 vector coding the NH2-terminal myristoylation membrane localization signal from c-Src (13, 14). The final construct was designated pcDNA3-myr-PAR1N. Efficacy of dominant negative mutants of PI3Kγ, Ras, RhoA, Rac1, Cdc42, and PAK to suppress muscarinergic response in COS-7 cells has been established recently (7, 10, 14). 

Kinase Assays—JNK assays in cells transfected with an epitope-tagged JNK construct (HA-JNK) was determined as described previously (9) using purified, bacterially expressed GST-ATF2(96) fusion protein as a substrate. Briefly, serum-starved cells left untreated or stimulated with various agents were lysed, and after centrifugation, clarified supernatants were immunoprecipitated with an anti-hemagglutinin monoclonal antibody 12CA5 (Babeo, Richmond, CA) for 1–2 h at 4 °C, and immunocomplexes were recovered with the aid of GammaBind (Pharmacia, Uppsala). Pellets were then washed three times with phosphate-buffered saline solution, supplemented with 1% Nonidet
In the stimulation of JNK by G protein-coupled receptor.

FIG. 1. Inhibition of JNK activation in COS-7 cells by wortmannin and LY 294002. A, effect of wortmannin on the stimulation of JNK induced by the m2 muscarinergic receptor and 1 mM carbachol (m2), Gβγ, or anisomycin. Two days after transfection with expression plasmids of HA-JNK, and as indicated m2 receptor or Gβγ cells were treated for 15 min with carbachol or anisomycin (10 μg/ml) in the presence or absence of 100 nM wortmannin. After lysis of the cells JNK kinase activity was assayed as described under “Experimental Procedures.” B, dose-response effect of the PI3K inhibitor LY294002 on JNK stimulation induced by Gβγ and anisomycin.

Results and Discussion

To investigate signaling from G protein-coupled receptor to JNK the muscarinergic m2 receptor has been transiently expressed in COS-7 cells. Confirming previous results (7) we observed an increase of JNK activity after treatment with the receptor agonist carbachol (Fig. 1A). Overexpression of Gβγ mimics the effect of carbachol on JNK. As shown in Fig. 1A both m2- and Gβγ-mediated stimulation could be effectively suppressed by wortmannin, a specific inhibitor of PI3K. In contrast JNK activation by the stress-inducing agent anisomycin was unaffected by wortmannin, demonstrating the specificity of this approach. Another PI3K inhibitor LY 294002 decreased JNK stimulation induced by Gβγ in a dose-dependent manner (Fig. 1B). Together these data point to an involvement of PI3K in the stimulation of JNK by G protein-coupled receptor.

Trying to outline the PI3K species that induces Gβγ-dependent JNK activation we expressed PI3Kγ in COS-7 cells. As shown in Fig. 2A, PI3Kγ moderately stimulated JNK activity. This effect could be significantly enhanced by coexpression of p101, a PI3Kγ-binding protein recently discovered (12). p101 alone was unable to induce JNK stimulation. For a comparison Fig. 2A expresses the individual effects of p101, PI3Kγ, and PI3Kγ + p101 on MAP kinase (ERK) activity. Interestingly p101 exhibited significantly lower potency to affect PI3Kγ-dependent MAPK activity than JNK induced by PI3Kγ. To examine possible autocrine reactions of the transfectants we analyzed the effects of conditioned media from COS-7 cells expressing PI3Kγ and/or p101 on naive cells. Supernatants of the transfected cells did not induce any measurable stimulation of JNK activity in untreated cells, thus providing evidence for direct signaling from PI3Kγ and p101 to JNK (data not shown). Fig. 2B demonstrates the dependence of JNK activation on the expression levels of p101 and PI3Kγ. Thus optimal stimulation of JNK seems to depend on PI3Kγ and p101.

The activatory effect of PI3Kγ on JNK could be completely inhibited by wortmannin suggesting an important role of the kinase activities of the PI3K (Fig. 3A). PI3Kγ recently has been shown to exhibit a significant ability to catalyze autophospho-
We next explored a possible involvement of protein kinase PAK in downstream signaling from PI3K to JNK. The coexpression of a dominant negative mutant of this protein kinase also induced a partial suppression of JNK stimulation by Gβγ, PI3K, and a constitutively active mutant of Rac (Fig. 4B).

Fig. 4. Involvement of Ras, Rac, and PAK in JNK activation by PI3Kγ. A, effects of dominant negative mutants of Ras, Rac1, Cdc42, and RhoA on JNK activation induced by PI3Kγ and p101. COS-7 cells were transfected with control vector or expression plasmids of PI3Kγ and p101. COS-7 cells were transfected with control vector or expression plasmids of PI3Kγ and p101 and with dominant negative mutants of Ras, Rac1, Cdc42, and RhoA, and JNK was assayed after 2 days. B, effect of dominant negative PAK on JNK activation by PI3Kγ. Stimulation of JNK was induced by expression of Gβγ, PI3Kγ + p101, RacQL or incubation with anisomycin (10 μg/ml, 15 min), and the effect of dominant negative PAK(N) expression on JNK activity was assayed. Levels of JNK expression were equal in every assay (data not shown).

Thus signaling from G protein-coupled receptor to JNK appears to require Gβγ, PI3Kγ, Rac, Ras, and PAK.

Nevertheless the sequence of events connecting PI3Kγ and JNK remains unclear. A PI3Kγ effect on Ras has been proposed in our previous report on ERK activation by PI3Kγ (10). In this paper we provide evidences that Ras could be activated by PI3Kγ via a Src-type tyrosine kinase, the adapter proteins Shc and Grb2, and the guanine nucleotide exchange factor Sos. Stimulated in this way Ras could act as a dissociation point for signals from PI3Kγ to ERK and JNK. Such a bifurcating function of Ras as a regulator of the Raf-ERK path and signal transduction to Rac, PAK, and JNK has been proposed (6).

Despite the attractiveness of this model some experimental data point to a more complex relation of the signaling proteins involved. Thus the differential effects of p101 on the PI3Kγ-dependent stimulation of ERK and JNK cannot be explained by simple sequential signal transduction from PI3Kγ via the lipid kinase product PIP3 to Ras and subsequent stimulation of JNK and ERK paths. One possible explanation for the distinct effects of PI3Kγ and its ligands on the MAPK cascades could be the involvement of signaling activities of the enzyme in addition to PIP3 production. Wortmannin-sensitive protein kinase activity of PI3Kγ has been reported (15). If Gβγ and p101 produce divergent effects on the lipid kinase and protein kinase activities of PI3Kγ and if both activities are important for signal transduction different downstream events could be expected. The individual signaling functions of PI3Kγ lipid kinase and protein kinase activities are currently under investigation. The effects of PI3Kγ on different MAPK cascades clearly represent another example for the complex interrelations of these intracellular signaling proteins.

Acknowledgments—We are very grateful to Drs. Len Stephens and Phill Hawkins for providing us with the pcDNA3-p101 construct.

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