Conditional Inhibition of the Mitogen-activated Protein Kinase Cascade by Wortmannin

DEPENDENCE ON SIGNAL STRENGTH*

(Received for publication, April 16, 1997, and in revised form, August 27, 1997)

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Phosphoinositide (PI) 3-kinase and the mitogen-activated protein (MAP) kinase cascades are activated by many of the same ligands. Several groups have reported involvement of PI 3-kinase in the activation of Erk1 and Erk2, whereas many other groups have shown that activation of Erk1 and Erk2 is not sensitive to inhibitors of PI 3-kinase such as wortmannin. Here we show that wortmannin inhibition of the MAP kinase pathway is cell type- and ligand-specific. Wortmannin blocks platelet-derived growth factor (PDGF)-dependent activation of Raf-1 and the MAP kinase cascade in Chinese hamster ovary cells, which have few PDGF receptors, but has no significant effect on Erk activation in Swiss 3T3 cells, which have high levels of PDGF receptors. However, wortmannin blocks activation of Erk proteins if Swiss 3T3 cells are stimulated with lower, physiological levels of PDGF. These results suggest that PI 3-kinase is in an efficient pathway for activation of MAP kinase, but that MAP kinase can be stimulated by a redundant pathway when a large number of receptors are activated. We present evidence that a protein kinase C family member downstream of phospholipase Cγ is involved in the redundant pathway.

PI3 3-kinase has been implicated as being involved in the signal transduction of virtually all growth factors studied and in the transformation of cells by several oncoproteins (1, 2). Activation of PI 3-kinase with growth factor results in the appearance of the lipid products of this enzyme, PtdIns-3,4-P2 and PtdIns-3,4,5-P3, within seconds to minutes. There is also a correlation between the elevation in PtdIns-3,4-P2 and PtdIns-3,4,5-P3 levels and cell transformation. These correlations have suggested an important role for PI 3-kinase in signal transduction pathways leading to cell growth and transformation. Indeed, the catalytic subunit of PI 3-kinase (p110α) has recently been identified as a retroviral oncogene (3). Overexpression of p110α in chick embryo fibroblasts results in constitutive elevation of PtdIns-3,4-P2 and PtdIns-3,4,5-P3 and cell transformation.

The MAP kinase pathway is another key component in the transduction of signals leading to growth and transformation. This pathway consists of a linear cascade of the protein kinases Raf, MEK, and MAP kinase/Erk; like PI 3-kinase, Erk1 and Erk2 are acutely activated by growth factors and are found constitutively activated in many transformed cell lines. The Erk proteins are phosphorylated and activated by the dual specificity kinase MEK (MAP kinase/Erk kinase), which is phosphorylated and activated by the serine/threonine kinase Raf. Raf is recruited to the membrane of activated cells by direct binding to Ras-GTP. This recruitment by activated Ras is necessary but not sufficient for full activation of Raf (4); therefore, other factors that are necessary for activation of the MAP kinase pathway may feed into the pathway at Raf. A search for other sources that feed into the MAP kinase pathway has yielded several candidates including Src, members of the PKC family, and PI 3-kinase (45).

The relevance of PI 3-kinase for activation of Erk has been controversial. Expression of activated forms of p110α PI 3-kinase has been reported to stimulate the MAP kinase pathway in one case (5), but not in others (6–9). A recent study found that overexpression of the p110γ type of PI 3-kinase resulted in the activation of Erk, but that p110α did not (9). Inhibition of PI 3-kinase with wortmannin (10–16) or dominant-negative PI 3-kinase (9) has been shown to block activation of MAP kinase in some but not all cells (17–19).

Several direct targets of the lipid products of PI 3-kinase have been identified that could act as intermediates between PI 3-kinase and the MAP kinase pathway. Members of the novel PKC family (calcium-independent), PKCc, PKCd, and PKCγ (20), have been shown to be activated in vitro by two lipid products of PI 3-kinase, PtdIns-3,4-P2 and PtdIns-3,4,5-P3. Inhibition of thrombin-dependent phosphorylation of pleckstrin by wortmannin, combined with the ability of these lipids to stimulate phosphorylation of pleckstrin when added to permeabilized platelets (21, 22), suggests that they activate PKC family members in vivo. PDGF activates PKCe (23) and PKCa (24) by a mechanism that requires PI 3-kinase. Several PKC family members (25–27) have already been implicated in the activation of the MAP kinase pathway via phosphorylation of Raf. Another potential intermediate is the serine/threonine protein kinase Akt/PKB, which binds to and is activated by PtdIns-3,4-P2 in vitro (28, 29), and PDGF stimulates this enzyme via activation of PI 3-kinase (30). The serine/threonine kinase p70S6K has been shown to be downstream of PI 3-kinase (31). Thus, a potential means for PI 3-kinase to feed into the MAP kinase pathway could be via activation of one (or more) of these targets of PI 3-kinase.

We have attempted to better understand the role that PI 3-kinase plays in the activation of the MAP kinase pathway.
We show here that the effect of wortmannin on the activation of Erk varies not only between cell lines, but within a single cell line depending on both the kind and concentration of the growth factor used. We have found that Erk activity is sensitive to wortmannin when relatively few receptors are activated, but resistant if a large number of receptors are activated. We have also found that a down-regulatable PKC contributes to wortmannin-resistant MAP kinase activation.

**MATERIALS AND METHODS**

**Cell Lines—**CHO/HIR cells are a CHO clone expressing the human insulin receptor and were maintained in RPMI 1640 medium and 10% fetal calf serum. Balb/c/3T3 clone A31 fibroblasts and NIH/3T3 fibroblasts were grown in Dulbecco's modified Eagle's medium and 10% fetal calf serum. Quiescent cell cultures were established by changing the serum to calf serum. Quiescent confluent cells were pretreated with inhibition of MAP kinase activity in A31 cells (Fig. 2). In A31 cells, —70% inhibition of MAP kinase was observed. Inhibition of PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 in vivo production correlated with inhibition of MAP kinase activity in A31 cells (Fig. 2). In contrast, wortmannin had little effect on PDGF-dependent activation of Erk proteins in Swiss 3T3 cells (Fig. 1). A trivial explanation for this could be that wortmannin is more effective in vivo than in vitro. Microscopy Image of MAP Kinase Activity

**RESULTS**

**Inhibition of MAP Kinase by Wortmannin Varies by Cell Line and Stimulus—**The effect of wortmannin on PDGF-dependent activation of MAP kinase was examined in three cell lines (Fig. 1). In CHO/HIR cells, wortmannin (100 nM) almost completely blocked PDGF-dependent activation of Erk2. In A31 cells, —70% inhibition of MAP kinase was observed. Inhibition of PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3, in vivo production correlated with inhibition of MAP kinase activity in A31 cells (Fig. 2). In contrast, wortmannin had little effect on PDGF-dependent activation of Erk proteins in Swiss 3T3 cells (Fig. 1). A trivial explanation for this could be that wortmannin is more effective...
in inhibiting PI-3 kinase in CHO and A31 cells than in Swiss 3T3 cells. However, we find that 100 nM wortmannin is an effective inhibitor of PI-3 kinase activity in all three cell lines as judged by inhibition of PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 production in intact cells (A31 and CHO/HIR) or immune complex kinase assays using 4G10 anti-phosphotyrosine antibody (all cell lines) (data not shown). Consistent with this result, insulin-stimulated MAP kinase activation in Swiss 3T3 cells is almost completely inhibited by wortmannin. In contrast, in CHO/HIR cells, where the insulin receptor is overexpressed, wortmannin is less effective in blocking insulin-dependent activation of the MAP kinase pathway in CHO and A31 cells, but that in Swiss 3T3 cells, other pathways are utilized for its activation.

These surprising results might be explained by the different levels of expression of receptors for PDGF in the three cell types. CHO cells express low levels of PDGF receptors (<20,000/cell) (33), and wortmannin is an effective inhibitor of the PDGF response in these cells. Swiss 3T3 cells express high levels of PDGF receptors (400,000/cell) (34), and the PDGF response is resistant to wortmannin in these cells. A31 cells have intermediate levels of PDGF receptors (150,000/cell) (35), and MAP kinase activation is partially inhibited by wortmannin. Likewise, the relative resistance of CHO/HIR cells to wortmannin inhibition of insulin-stimulated MAP kinase activity could be explained by the high level of insulin receptor expression in these cells.

### Fig. 3. Wortmannin inhibits MAP kinase activation in Swiss 3T3 cells at low PDGF concentrations
Quiescent Swiss 3T3 cells were pretreated or not with 100 nM wortmannin (Wort) for 10 min and then stimulated with the indicated concentrations of PDGF for 5 min. The cells were lysed, and an immune complex MAP kinase assay was done as described in the legend to Fig. 1. The upper panel shows the level of MAP kinase (MAPK) activity relative to cells treated with 10 ng/ml PDGF (no wortmannin). The results are the average of three experiments. The white bars show the results for control cells, and the black bars show those for cells treated with 100 nM wortmannin. The middle panel is an autoradiogram from one experiment, showing the levels of myelin basic protein phosphorylation. The lower panel shows the percent inhibition of MAP kinase activity in wortmannin-treated cells relative to control (no wortmannin) at each PDGF concentration used, i.e. for each concentration of PDGF used.

Wortmannin inhibits MAP kinase activation in Swiss 3T3 cells at low PDGF concentrations—an alternative explanation of the results in Fig. 1 is that the cell lines differ in their composition of PDGF α- and β-receptors, and this could account for differences in wortmannin sensitivity. On the basis of activation with PDGF-AA versus PDGF-BB and immunoprecipitation of Tyr-phosphorylated PDGF receptors with isomform-specific antibodies, CHO cells contain mainly PDGF β-receptors, and Swiss 3T3 cells contain mainly PDGF α-receptors (data not shown). To test the idea that wortmannin sensitivity of MAP kinase activation depends on the number and not the type of receptors that become stimulated, we reexamined MAP kinase activation in Swiss 3T3 cells at suboptimal concentrations of PDGF. That is, we kept the number of receptors stimulated, but not the type. As shown in Fig. 3, when low concentrations of PDGF are used to stimulate the cells, MAP kinase activation is sensitive to wortmannin inhibition. These results suggest that to efficiently activate MAP kinase at low concentrations of growth factor or in cells that have few receptors, PI-3 kinase must be activated. However, PI-3 kinase becomes less important for this pathway when a large number...
of receptors become activated, presumably because a second pathway that requires more receptors provides a redundant signal.

**PI 3-Kinase Is Activated in Swiss 3T3 Cells at Low PDGF Concentrations**—To test this model, we examined whether PI 3-kinase was activated in Swiss 3T3 cells at the low PDGF concentrations at which wortmannin inhibition of MAP kinase is observed. As shown in Fig. 4, recruitment of PI 3-kinase to an anti-Tyr(P)-precipitable complex occurs at very low concentrations of PDGF (half-maximal at 2–3 ng/ml), consistent with a role for PI 3-kinase in the activation of MAP kinase at low PDGF concentrations. In contrast, anti-Tyr(P) precipitation of PLCγ1 saturates at higher concentrations of PDGF (half-maximal at 10 ng/ml). It is possible that PLCγ1 is the redundant signal, parallel to PI 3-kinase, leading to wortmannin-resistant MAP kinase activation at higher PDGF concentrations. This idea is consistent with reports that some PKC family members, which are downstream of PLCγ, can feed into the MAP kinase pathway at Raf (25–27).

**Inhibition of PKC and PI 3-Kinase Together, but Neither Alone, Leads to Inhibition of MAP Kinase in Swiss 3T3 Cells**—The possibility that a PKC family member contributes to PDGF-dependent activation of MAP kinase was investigated. Prolonged treatment of cells with the phorbol ester 12-O-tetradecanoylphorbol-13-acetate results in degradation of PKC family members. Under the conditions used here, PKCoα is down-regulated >95%, and PKCd is down-regulated 80%. PKC down-regulation alone had little effect on PDGF-dependent activation of MAP kinase in Swiss 3T3 cells (Fig. 5). However, the MAP kinase activity in the 12-O-tetradecanoylphorbol-13-acetate-treated cells was inhibited by wortmannin. These results are consistent with two redundant pathways for activation of MAP kinase in Swiss 3T3 cells at high PDGF concentrations: a PI 3-kinase-dependent pathway and a PKC-dependent pathway that is likely downstream of PLCγ.

**Wortmannin Blocks Activation of Raf Kinase**—To better define the contribution of PI 3-kinase to the activation of the MAP kinase pathway, the effect of wortmannin on Raf activation was examined in CHO cells. Raf kinase activity peaked within 2 min of PDGF addition (Fig. 6), and wortmannin almost completely inhibited the activation.

**DISCUSSION**

The MAP kinase pathway plays a central role in the transduction of signals for growth, differentiation, and other cellular responses. This modular cascade of kinases is a link between receptor signaling events and nuclear events. One of the important links in this pathway to be established was the connection between receptor signaling and the MAP kinase cascade. This link appeared to be found when studies showed that receptor activation of Ras results in Ras-GTP binding to Raf, recruiting it to the membrane (36–39). While subsequent studies have shown that recruitment of Raf to the membrane is sufficient for its activation (40), other studies have shown that once recruited to the membrane, additional steps are required for full activation of Raf (4). Thus, Ras probably contributes to the activation of Raf by recruiting it to the membrane and inducing a conformational change that facilitates activation by other factors. One of the most pressing challenges in signal transduction is to identify the other factors that contribute to the activation of Raf and the MAP kinase cascade.

Many studies have used wortmannin or dominant-negative forms of PI 3-kinase to address the involvement of this enzyme in the activation of the MAP kinase pathway. While several studies have found that wortmannin (10–15) or dominant-negative PI 3-kinase (9) effectively inhibits activation of the MAP kinase cascade, many other studies have found that inhibition of PI 3-kinase has no effect on the activation of Erk proteins (17–19). The results in Fig. 1 address this apparent contradiction. We show that wortmannin sensitivity is clearly dependent on the system under study. The agreement between the results with wortmannin and those with dominant-negative PI 3-kinase have firmly established that PI 3-kinase is required for MAP kinase activation by growth factors in certain
cells. However, as we show here, in some cells, inhibition of PI 3-kinase does not affect activation of MAP kinase.

The results that we present are consistent with a model in which PI 3-kinase is required for activation of the MAP kinase cascade under conditions in which relatively few receptors are activated, but is redundant with a parallel pathway when many receptors become activated. This model is supported by comparison of cells with different numbers of PDGF and insulin receptors (Fig. 1) and is directly addressed by varying the number of activated receptors in a single cell type (Fig. 3).

Wortmann blocks acute (2 min) PDGF-dependent activation of Raf in CHO cells, indicating that, in these cells, PI 3-kinase is required for an early step in the cascade. This result is in agreement with a previous study showing that wortmannin blocks Raf kinase activation by nerve growth factor (10), but not Ras activation by nerve growth factor (41), insulin-like growth factor-1 (10), or insulin (42).

The redundant signal for MAP kinase activation that emerges when a large number of receptors are activated apparently involves a PKC family member. Down-regulation of PKCs by reducing the magnitude of PLC phosphoinositides and also inhibit activation of conventional PKCs by reducing PLC activity. This latter effect is unlikely to explain the wortmannin inhibition of insulin-stimulated MAP kinase since insulin does not activate PLCy.

Finally, although a requirement for PI 3-kinase in MAP kinase activation is now firmly established in several systems, our results do not imply that activation of PI 3-kinase alone is sufficient to fully activate Raf or MAP kinase. In fact, we do not find significant elevation of MAP kinase activity in chick embryo fibroblasts stably transformed with viral or cellular forms of p110a PI 3-kinase,3 even though these cells have significantly elevated levels of PtdIns-3,4-P2 and PtdIns-3,4,5-P3 (3) and increased Akt/PKB protein kinase activities (3). This result is in contrast to the recent report that overexpression of p110y PI 3-kinase stimulates MAP kinase in COS-7 cells (9). There are several problems with interpreting these types of experiments. It is possible that acute activation of PI 3-kinase is sufficient to activate MAP kinase, but that continual elevation of D3 lipids results in a feedback pathway that inhibits MAP kinase. Conversely, it is possible that the lipid products of PI 3-kinase alone are not sufficient to activate MAP kinase, but that certain cells transformed with PI 3-kinase secrete factors that stimulate MAP kinase in an autocrine manner. Further work will be necessary to address the sufficiency of PI 3-kinase for MAP kinase activation.

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