G_i-mediated activation of the Ras/MAP kinase pathway involves a 100 kDa tyrosine-phosphorylated Grb2 SH3 binding protein, but not Src nor Shc

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Mitogenic G protein-coupled receptors, such as those for lysophosphatidic acid (LPA) and thrombin, activate the Ras/MAP kinase pathway via pertussis toxin (PTX)-sensitive G_i, tyrosine kinase activity and recruitment of Grb2, which targets guanine nucleotide exchange activity to Ras. Little is known about the tyrosine phosphorylations involved, although Src activation and Shc phosphorylation are thought to be critical. We find that agonist-induced Src activation in Rat-1 cells is not mediated by G_i and shows no correlation with Ras/MAP kinase activation. Furthermore, LPA-induced tyrosine phosphorylation of Shc is PTX-insensitive and Ca²⁺-dependent in COS cells, but undetectable in Rat-1 cells. Expression of dominant-negative Src or Shc does not affect MAP kinase activation by LPA. Thus, G_i-mediated Ras/MAP kinase activation in fibroblasts and COS cells involves neither Src nor Shc. Instead, we detect a 100 kDa tyrosine-phosphorylated protein (p100) that binds to the C-terminal SH3 domain of Grb2 in a strictly G_i- and agonist-dependent manner. Tyrosine kinase inhibitors and wortmannin, a phosphatidylinositol (PI) 3-kinase inhibitor, prevent p100–Grb2 complex formation and MAP kinase activation by LPA. Our results suggest that the p100–Grb2 complex, together with an upstream non-Src tyrosine kinase and PI 3-kinase, couples G_i to Ras/MAP kinase activation, while Src and Shc act in a different pathway.
Keywords: G protein-coupled receptors/Grb2/MAP kinase/Shc/Src

Introduction

The mechanisms by which G protein-coupled receptors activate the mitogen-activated protein (MAP) kinase cascade are not well defined. Pertussis toxin (PTX)-sensitive G_i-coupled receptors, such as those for lysophosphatidic acid (LPA), thrombin and α 2-adrenergic agonists, initiate a pathway that involves stimulation of Ras-GTP accumulation through intermediate tyrosine kinase activity (Alblas *et al.*, 1993; van Corven *et al.*, 1993; Hordijk *et al.*, 1994; Moolenaar, 1995a; van Biesen *et al.*, 1995; Luttrell *et al.*, 1996). Recent studies have identified $\beta\gamma$ subunit released from G_i as the primary initiator of this pathway (Crespo *et al.*, 1994; Koch *et al.*, 1994).

Several candidate tyrosine kinases have been proposed to serve as intermediaries between G_i and Ras/MAP kinase

activation, including Src family kinases (Luttrell *et al.*, 1996), Syk (in B cells; Wan *et al.*, 1996), Pyk2 (in PC12 cells; Dikic *et al.*, 1996) and even the epidermal growth factor (EGF) receptor (Daub *et al.*, 1996). Src family kinases are rapidly and transiently activated by various G protein-coupled receptor agonists, including LPA, thrombin, bombesin and bradykinin (Jalink *et al.*, 1993; Chen *et al.*, 1994; Luttrell *et al.*, 1996; Rodriguez-Fernandez and Rozengurt, 1996). Active c-Src is thought to mediate Ras/MAP kinase activation through tyrosine phosphorylation of the adaptor protein Shc and its subsequent association with the Grb2 adaptor (Luttrell *et al.*, 1996), which directs the GDP/GTP exchanger Sos to Ras (Lowenstein *et al.*, 1992; Buday and Downward, 1993). Consistent with this, overexpression of the C-terminal Src kinase (CSK), a negative regulator of Src family kinases, inhibits LPA-induced MAP kinase activation in COS and PC12 cells (Dikic *et al.*, 1996; Luttrell *et al.*, 1996). On the other hand, antibody inhibition of Src family tyrosine kinases in quiescent fibroblasts does not interfere with the mitogenic response to either LPA or bombesin (Roche *et al.*, 1995).

An alternative mechanism has been proposed by Daub *et al.* (1996), who suggested that G protein-coupled receptors activate MAP kinase in Rat-1 cells through 'transactivation' of the EGF receptor in an EGF-independent manner. The activated receptor would then serve as a scaffold for recruitment of the Grb2–Sos complex via tyrosine phosphorylation of Shc.

In addition to protein tyrosine kinase activity, phosphatidylinositol (PI) 3-kinase activity has also been implicated in G_i-mediated mitogenic signaling upstream of Ras, a notion largely based on the use of specific PI 3-kinase inhibitors (Pace *et al.*, 1995; Hawes *et al.*, 1996).

PTX-insensitive G proteins can also mediate MAP kinase activation. This mode of activation is mediated by the phospholipase C (PLC)/Ca²⁺/protein kinase C (PKC) pathway in a Ras-independent manner (Blumer and Johnson, 1994; Hawes *et al.*, 1995). Since many receptors couple to more than one G protein subtype, their activation will initiate multiple effector systems, which hampers unraveling of the downstream biochemical pathways. In Rat-1 cells, however, LPA- and thrombin-induced activation of the MAP kinase pathway is mediated solely by G_i-regulated Ras activation, with no detectable contribution of the PLC/Ca²⁺/PKC pathway (Alblas *et al.*, 1993; van Corven *et al.*, 1993; Hordijk *et al.*, 1994).

In this study, we set out to further investigate the signaling pathway that couples G_i to Ras-dependent MAP kinase activation. Using Rat-1 fibroblasts and COS cells, we show that G_i-mediated MAP kinase activation requires neither Src nor Shc. We report the detection of a 100 kDa tyrosine-phosphorylated protein, p100, that preferentially binds to the C-terminal SH3 domain of Grb2 following

LPA or thrombin stimulation. Our findings suggest a model in which the p100–Grb2 complex, together with an upstream non-Src tyrosine kinase and a wortmannin-sensitive PI 3-kinase, links G_i to Ras/MAP kinase activation, with Src and Shc functioning in a separate pathway.

Results

Agonist-induced MAP kinase activation: effects of tyrosine kinase inhibitors and wortmannin

In Rat-1 cells, LPA- and thrombin-induced activation of Ras and the downstream MAP kinase cascade is inhibited by genistein and staurosporine at doses that have little or no effect on EGF-induced Ras/MAP kinase activation (van Corven *et al.*, 1993; Hordijk *et al.*, 1994). We assessed the specificity and dose dependence of these inhibitors in COS-7 cells transfected with epitope-tagged MAP kinase and stimulated by either LPA or EGF. As shown in Figure 1A, LPA-induced MAP kinase activation is much more sensitive to genistein than is the response to EGF: at a dose (10 μ M) that inhibits the response to LPA by >80%, EGF action is virtually unaffected. Similarly, staurosporine inhibits LPA signaling approximately five times more potently than EGF signaling (Figure 1B).

We also tested the effect of the PI 3-kinase inhibitor wortmannin (Wymann *et al.*, 1996). Wortmannin inhibits LPA-induced MAP kinase activation in COS cells with an IC_{50} of ~10 nM, whereas the response to EGF is much less affected (Figure 1C). We found that in Rat-1 cells, wortmannin inhibits LPA-induced MAP kinase activation with a similar IC_{50} value to that in COS cells (data not shown). These results support the notion that G_i -mediated Ras/MAP kinase activation involves both a protein tyrosine kinase and a wortmannin-sensitive PI 3-kinase.

Agonist-induced Src activation: uncoupling from Ras/MAP kinase activation

To assess the role of Src activation in G protein-mediated mitogenic signaling, we measured agonist-induced association of c-Src with tyrosine-phosphorylated proteins, rather than Src *in vitro* kinase activity. In chicken fibroblasts, activated Src forms a complex with two tyrosine phosphoproteins of 110 and 130 kDa (p110 and p130; Reynolds *et al.*, 1989; Kanner *et al.*, 1991). In Rat-1 cells, LPA rapidly and transiently induces the association of c-Src with two tyrosine phosphoproteins of 110 and 130 kDa (Figure 2A), indicative of Src 'activation'. The response reaches a maximum within 1 min of LPA stimulation and returns to pre-stimulation levels between 10 and 30 min.

We also tested the effect of endothelin, which strongly activates the PLC/ Ca^{2+} /PKC pathway (van der Bend *et al.*, 1992) but fails to stimulate Ras–GTP accumulation and MAP kinase activation in Rat-1 cells (van Corven *et al.*, 1993; Hordijk *et al.*, 1994). As shown in Figure 2B, endothelin is even more effective than LPA in stimulating the association of c-Src with p110 and p130, whereas the peptide does not detectably activate MAP kinase. PTX pre-treatment of the cells did not inhibit Src–p110–p130 association induced by either LPA or endothelin (not shown).

To abrogate endogenous Src signaling, we used a kinase-inactive c-Src mutant (K295M mutation; Roche *et al.*, 1995), which should act in a dominant-negative manner

Fig. 1. Differential effects of kinase inhibitors on LPA- and EGF-induced MAP kinase activation. COS-7 cells were transfected with 1 μ g pEXVmycERK2. After transfection, cells were incubated overnight in serum-free medium. Prior to stimulation with LPA or EGF, the cells were treated with the indicated concentrations of (A) genistein (30 min), (B) staurosporine (10 min) or (C) wortmannin (10 min). Transfected MAP kinase (ERK2) was subsequently precipitated and the kinase activity towards MBP assayed. The data are depicted as percentage activity of control non-treated cells and are mean values of at least three different experiments, each performed in duplicate.

by binding to proteins that interact with activated Src family members. Expression of Src(K295M) in COS cells has no detectable effect on LPA- and EGF-induced MAP kinase activation (Figure 2C), despite high expression levels of Src(K295M) (not shown); in parallel assays the inhibitory effect of dominant-negative Ras (RasN17) was readily observed (Figure 2C). Likewise, LPA-induced MAP kinase activity in Rat-1 cells stably expressing dominant-negative Src was not detectably inhibited (Figure 2D), whereas Src-mediated gap junction closure was completely prevented by this Src mutant (F.Postma, T.Hengeveld, J.Alblass, G.Zondag and W.Moolenaar, submitted for publication). Furthermore, we used fibroblasts derived from mice that lack c-Src (Bockholt and Burridge, 1995). In these Src-deficient cells, LPA-induced MAP kinase activation is readily detectable (as is the response to peptide growth factors; Figure 2E). Taken together,

Fig. 2. MAP kinase activation does not correlate with c-Src activation. (A) Serum-starved Rat-1 cells were stimulated with LPA (1 μ M). At the indicated time points, the cells were harvested and assayed by anti-Src immunoprecipitation, followed by anti-phosphotyrosine Western blotting. A rapid and transient interaction of c-Src with p110 and p130 tyrosine-phosphorylated proteins is observed in response to LPA stimulation. (B) Serum-starved Rat-1 cells were stimulated with either LPA or endothelin (100 nM) for 1 min. Subsequently, Src was immunoprecipitated and the precipitates analyzed as in (A) (upper panel). Simultaneously, 50 μ g total lysate were analyzed for MAP kinase activation by mobility shift assay (lower panel). Endothelin induces a much stronger increase in Src-p110-p130 complex formation than does LPA, but fails to activate MAP kinase. (C) COS cells were co-transfected with pEXVmycERK2 and either pCMV-lacZ (control), pMT2-N17Ras or pMT2-K295Msrc. After serum starvation (overnight), cells were stimulated with LPA and MAP kinase activity was determined as in Figure 1. The data are means of three independent transfection experiments, all performed in duplicate. Clearly, LPA-induced MAP kinase activation critically depends on Ras but not Src. (D) Stable cell lines transfected with either control vector or pMT2-src(K295M) (dn c-src) were incubated in serum-free medium and stimulated with LPA (5 min). MAP kinase activation was analyzed by mobility shift assay. Three different K295Msrc-expressing cell lines were tested and all three showed comparable MAP kinase activation in response to LPA. A representative experiment is shown. (E) MAP kinase activation in Src-deficient fibroblasts (Bockholt and Burridge, 1995), analyzed by mobility shift assay. Concentrations used: LPA, 10 μ M; EGF, 50 ng/ml; PDGF, 100 ng/ml.

these results indicate that Src does not function in G_i-mediated Ras/MAP kinase activation.

Tyrosine phosphorylation of Shc: uncoupling from G_i-mediated MAP kinase activation and dependence on Ca²⁺

Tyrosine-phosphorylated Shc has been implicated in G_i-mediated Ras signaling by virtue of its ability to bind the Grb2-Sos complex (Touhara *et al.*, 1995; Chen *et al.*, 1996; Luttrell *et al.*, 1996). As shown in Figure 3 (right panel), stimulation of COS cells with either LPA or EGF causes rapid tyrosine phosphorylation of the three Shc protein isoforms (p46, p52 and p66). However, pretreatment of the cells with PTX has no effect on LPA-induced Shc phosphorylation, whereas MAP kinase activation is completely inhibited (Figure 3, right panels).

Figure 3 also shows that wortmannin has no effect on Shc phosphorylation induced by either LPA or EGF.

In contrast to what is observed in COS cells, LPA-induced Shc phosphorylation in Rat-1 cells is not detectable under conditions where the response to EGF is pronounced (Figure 3, left panels). To further assess the role of Shc, we used a mutant version of Shc consisting of the isolated SH2 domain, designed to compete with endogenous Shc for binding to upstream tyrosine-phosphorylated proteins (Baldari *et al.*, 1995). Transfection of Shc-SH2 into COS cells attenuates EGF-induced MAP kinase activation by ~50%, without affecting the response to LPA (Figure 4).

We next examined whether Shc may function in the PTX-resistant G_q-mediated PLC/Ca²⁺/PKC pathway. Figure 5A shows that both phorbol ester and the Ca²⁺

Fig. 3. Activation of Shc and MAP kinase through different pathways. Serum-starved Rat-1 (left panel) and COS-7 (right panel) cells were either left untreated or were pre-incubated with PTX (P; 100 ng/ml, overnight) or wortmannin (W; 100 nM, 10 min) and then stimulated with LPA or EGF. Shc tyrosine phosphorylation was assayed by anti-Shc immunoprecipitation, followed by anti-phosphotyrosine Western blotting (upper panel). Simultaneously, MAP kinase activation was tested either by gel shift assay (Rat-1, lower left panel) or by analysis of transfected myc-tagged MAPK by MBP phosphorylation (COS-7, lower right panel). Clearly, LPA-induced Shc phosphorylation is cell type dependent and, unlike MAP kinase activation, insensitive to PTX and wortmannin.

ionophore ionomycin induce tyrosine phosphorylation of Shc. When intracellular stores are depleted of Ca^{2+} by treatment with either thapsigargin or ionomycin/EGTA, LPA-induced Shc phosphorylation is inhibited (Figure 5B); in contrast, EGF-induced Shc phosphorylation is not affected in Ca^{2+} -depleted cells (Figure 5C). Thus, Ca^{2+} signaling is required for LPA-induced, but not EGF-induced Shc phosphorylation. Chronic phorbol ester treatment, in an attempt to down-regulate PKC, stimulated Shc phosphorylation to the same extent as acute treatment (Figure 5B and results not shown), suggesting that PKC down-regulation was incomplete.

From these results we conclude that LPA-induced tyrosine phosphorylation of Shc is not involved in the G_i -mediated MAP kinase pathway, but is secondary to G_q -mediated Ca^{2+} mobilization and PKC activation.

Involvement of a 100 kDa tyrosine-phosphorylated Grb2 binding protein

If Shc is not involved in G_i -mediated Ras signaling, which tyrosine phosphorylation event links G_i to the recruitment of Grb2? To answer this question we set out to identify Grb2 binding proteins. Cellular proteins that bind to GST-Grb2 fusion proteins were purified on glutathione beads and analyzed by anti-phosphotyrosine immunoblotting. Using this protocol, we detected a tyrosine-phosphorylated protein of 100 kDa, termed p100, that associates with

Fig. 4. Dominant-negative Shc-SH2 attenuates EGF- but not LPA-induced MAPK activation. COS-7 cells were transiently transfected with pEXVmycERK2 and either pCMV-lacZ or pMT2-Shc-SH2 at a 1:5 ratio. After serum starvation, cells were stimulated with either LPA or EGF for 5 min and the activity of transfected MAP kinase was subsequently determined by MBP phosphorylation. The data are presented as percentage activity of control (lacZ-transfected) cells. The data are means of two independent experiments, performed in duplicate. Dominant-negative Shc inhibits EGF-induced, but not LPA-induced MAP kinase activation.

Grb2 in response to LPA stimulation (Figure 6A). Using GST alone, no specific signals were detected (not shown). If p100 functions in the G_i /Ras pathway, its association with Grb2 should be sensitive to PTX. Indeed, pre-treatment of the cells with PTX prevents the interaction between Grb2 and p100 (Figure 6A). In addition to LPA,

Fig. 5. Shc tyrosine phosphorylation requires a Ca^{2+} signal. (A) Serum-starved COS cells were incubated with either PdBu (P; 200 nM, 5 min), thapsigargin (T; 200 nM, 30 min) a combination of ionomycin (5 μM , 2 min) and EGTA (3 mM, 1 min) (I/E) or ionomycin alone (I; 50 nM). Tyrosine phosphorylation state of the Shc proteins was assayed by anti-Shc immunoprecipitation followed by anti-phosphotyrosine immunoblotting. Both PKC activation and elevation of intracellular Ca^{2+} causes tyrosine phosphorylation of Shc. Serum-starved COS cells were incubated with either PdBu (200 nM, overnight), thapsigargin or EGTA/ionomycin prior to stimulation with either LPA (B) or EGF (C) and Shc tyrosine phosphorylation was assayed as in (A).

thrombin and serum (which contains LPA; Moolenaar, 1995b) also induce p100–Grb2 complex formation (Figure 6B). In marked contrast, the peptide endothelin, which activates the G_q/PLC but not the G_i/Ras pathway in Rat-1 cells, fails to induce p100–Grb2 complex formation.

We were unable to assess whether the Grb2–100 complex is also induced by EGF, because of the many bands present in the 100–120 kDa region upon EGF stimulation (Figure 6B). Among the multiple tyrosine-phosphorylated Grb2 binding proteins in this region, we identified the p120^{cb1} proto-oncogene product (cf. Galisteo *et al.*, 1995; Meisner and Czech, 1995). However, tyrosine-phosphorylated c-Cbl was not detected in LPA-stimulated cells; we also excluded the possibility that p100 is an isoform of dynamin, a Grb2 binding GTPase involved in receptor-mediated endocytosis (Wang and Moran, 1996; O.Kranenburg and I.Verlaan, unpublished observations).

We next examined the effects of genistein, staurosporine and wortmannin, at doses that inhibit G_i -mediated MAP kinase activation (cf. Figure 1). Treatment of the cells

Fig. 6. Detection of p100, an intermediate in $\text{G}_i/\text{Ras}/\text{MAP}$ kinase signaling. (A) Purified GST and GST–Grb2 proteins were used for FISH in lysates of serum-starved Rat-1 cells that were either unstimulated, LPA-stimulated or EGF-stimulated and were either pre-treated with PTX or left untreated. The GST proteins were isolated on glutathione beads and were analyzed by anti-phosphotyrosine Western blotting. A 100 kDa tyrosine-phosphorylated protein binds to Grb2 in a PTX-sensitive manner. (B) Serum-starved Rat-1 cells were treated with the indicated agonists with or without prior pre-treatment with PTX. Induction of the p100–Grb2 interaction was then assayed as in (A). (C) Serum-starved cells were either left untreated or were pre-treated with either genistein (G; 50 mM, 30 min), staurosporine (S; 100 nM, 10 min) or wortmannin (W; 100 nM, 10 min). After LPA stimulation, induction of p100–Grb2 interaction was determined as in (A). All three agents inhibit both LPA-induced MAP kinase activation (Figure 1) and p100–Grb2 complex formation. (D) LPA-induced binding of tyrosine-phosphorylated proteins to the various domains of Grb2. Binding of cellular proteins to 2 μg of the indicated fusion proteins was assayed as in (A). p100 preferentially binds to the C-terminal SH3 domain of GST–Grb2, as does p90, whereas p110 binds to the N-terminal SH3 domain.

with any of these inhibitors prevents p100–Grb2 complex formation upon LPA stimulation (Figure 6C). In some experiments we observed two additional tyrosine-phosphorylated proteins of ~90 and 110 kDa (p90 and p110) that also bind to Grb2 in response to LPA. However, although inhibited by genistein and staurosporine, the latter interactions are insensitive to either PTX or wortmannin (Figure 6B and C). We conclude that p100–Grb2, but not p90–Grb2 or p110–Grb2, lies on the route from G_i to Ras/MAP kinase activation in fibroblasts.

p100 preferentially binds to the C-terminal SH3 domain of Grb2

Finally, we examined which Grb2 domain(s) mediates interaction with p100. Figure 6D shows the triplet of tyrosine-phosphorylated proteins that bind to full-length

Grb2 in response to LPA, with the strongest signal corresponding to p100. Using three different GST-Grb2 deletion mutants, we found that p100 preferentially binds to the C-terminal SH3 domain of Grb2 in an LPA-dependent manner (Figure 6D), although it is seen that there is also some binding affinity for the N-terminal SH3 domain of Grb2. We further note that p110 also binds to the C-terminal Grb2-SH3 domain, whereas p90 binds only to N-terminal Grb2-SH3 (Figure 6D). No detectable interaction was observed with the isolated SH2 domain of Grb2 (Figure 6D).

Discussion

The present study provides new insights into the signaling events that link G_i -coupled receptors to activation of the Ras/MAP kinase pathway. We have built on previous data showing that G_i -mediated Ras/MAP kinase activation in fibroblasts stimulated by either LPA or thrombin involves genistein- and staurosporine-sensitive tyrosine kinase activity (van Corven *et al.*, 1993; Hordijk *et al.*, 1994). Subsequent studies have shown that the G_i /Ras/MAP kinase cascade is initiated by $\beta\gamma$ subunits and involves not only protein tyrosine kinase activity but also a wortmannin-sensitive PI 3-kinase; these steps somehow lead to recruitment of the Grb2 adaptor protein, which targets the nucleotide exchanger Sos to Ras at the plasma membrane (for references see Introduction).

Using LPA and thrombin as prototypic agonists, we have established that, contrary to previous views, G_i -coupled receptors utilize neither Src nor tyrosine-phosphorylated Shc to activate the Grb2-mediated Ras/MAP kinase pathway in fibroblasts and COS cells. Instead, we find a tyrosine-phosphorylated 100 kDa protein (p100) that binds to the C-terminal SH3 domain of Grb2 and meets the criteria for linking G_i to Ras/MAP kinase activation.

Src activation

Our conclusion that G protein-mediated Src activation is not involved in MAP kinase activation is based on the following findings: (i) transient or stable expression of dominant-negative Src in COS cells or Rat-1 cells respectively does not interfere with LPA-induced MAP kinase activation; (ii) LPA-induced Src activation, as inferred from complex formation with p110 and p130, is insensitive to PTX and hence not downstream of G_i ; (iii) the G_q -coupled endothelin receptor mediates activation of c-Src, but not that of Ras nor MAP kinase (Figure 2; van Corven *et al.*, 1993; Hordijk *et al.*, 1994); (iv) LPA-induced MAP kinase activation is readily detected in Src-deficient fibroblasts (Figure 2E). Our conclusion is supported by a previous report showing that Src family kinases are not required for LPA-induced DNA synthesis in 3T3 cells (Roche *et al.*, 1995).

A recent report showed that transient overexpression of the C-terminal Src kinase (CSK), a negative regulator of Src family kinases, inhibits LPA-induced Shc phosphorylation and MAP kinase activation in COS cells (Luttrell *et al.*, 1996); dominant-negative Src was not used in that study. We have no explanation for the discrepancy with the present results. In PC12 neuronal cells, Src family kinases may act in concert with the cell type-specific Pyk2

tyrosine kinase to activate MAP kinase in response to LPA or bradykinin (Dikic *et al.*, 1996).

In contrast to the situation with G protein-coupled receptors, Src family kinases are required for PDGF and EGF receptor mitogenic signaling (Roche *et al.*, 1995; Broome and Hunter, 1996; Erpel *et al.*, 1996), where they may control the transcriptional activation of Myc in a Ras-independent manner (Barone and Courtneidge, 1995). G protein-regulated Src activation is likely to play a role in non-mitogenic events mediated by PTX-insensitive $G_{q/11}$ and/or $G_{12/13}$, such as cytoskeletal remodeling and gap junction closure (F.Postma, T.Hengeveld, J.Alblas, G.Zondag and W.Moolenaar, submitted for publication).

Daub *et al.* (1996) suggested that transactivation of the EGF receptor links G protein-coupled receptors to Ras-dependent MAP kinase activation in Rat-1 cells. Inconsistent with their model, the authors found strong EGF receptor activation by endothelin, which fails to activate Ras as well as MAP kinase in Rat-1 cells, as mentioned above. In agreement with others using PC12 cells (Dikic *et al.*, 1996), we have been unable to detect LPA-induced phosphorylation of the EGF receptor in both Rat-1 and COS cells (data not shown). Furthermore, it is noteworthy that EGF- and LPA-induced MAP kinase activation show differential sensitivity to tyrosine kinase inhibitors (Figure 1).

Shc phosphorylation

The adaptor protein Shc participates in a network of protein-protein interactions not necessarily leading to Ras activation (Bonfini *et al.*, 1996). We have shown that Shc tyrosine phosphorylation is not involved in G_i -mediated MAP kinase activation. First, although LPA-induced tyrosine phosphorylation of Shc is detectable in COS cells, PTX does not inhibit this response under conditions where MAP kinase activation is blocked. Second, expression of the dominant-negative Shc-SH2 domain (Baldari *et al.*, 1995) has no effect on LPA-induced MAP kinase activation, whereas the response to EGF is significantly attenuated. Finally, in Rat-1 cells, LPA-induced MAP kinase activation occurs in the absence of detectable tyrosine phosphorylation of Shc. In agreement with this finding, a recent study showed that a transfected G_i -coupled receptor mediates MAP kinase activation in the absence of Shc phosphorylation in mouse fibroblasts (Torres and Ye, 1996). LPA-induced Shc phosphorylation in COS cells is mimicked by Ca^{2+} ionophore and phorbol ester and, furthermore, is abrogated in Ca^{2+} -depleted cells. This suggests that LPA-induced tyrosine phosphorylation of Shc is secondary to G_q -mediated inositol lipid breakdown.

p100-Grb2 interaction

In an effort to identify tyrosine-phosphorylated signaling intermediates involved in Grb2 recruitment, we have detected several tyrosine-phosphorylated proteins that bind to Grb2 in an agonist-dependent manner. Of these, only p100 associates with Grb2 in a PTX-sensitive manner. In other words, p100 is part of a G_i -mediated signaling cascade. Furthermore, pre-treatment of cells with any of the pharmacological inhibitors of LPA-induced Ras/MAP kinase activation (genistein, staurosporine and wortmannin) precludes detection of Grb2-associated p100.

The wortmannin sensitivity is of note, as it supports

Fig. 7. Proposed model of G_i-mediated Ras/MAP kinase activation. The βγ subunits released from G_i are proposed to activate the γ-isotype of PI 3-kinase, which then may lead to recruitment of an SH2 domain-containing protein tyrosine kinase. This non-Src tyrosine kinase phosphorylates p100, presumably leading to an enhanced affinity of p100 for Grb2. With Grb2, SOS is translocated to the plasma membrane to promote GDP/GTP exchange on Ras, leading to activation of the MAP kinase cascade. In a parallel but independent pathway, activated G_qα stimulates PLC activity, leading to PKC activation and Ca²⁺ release, followed by tyrosine phosphorylation of Shc. Whether Src also participates in the G_q pathway remains to be established. For details see text.

the notion that the tyrosine phosphorylation step requires upstream PI 3-kinase activity (Hawes *et al.*, 1996; see also Pace and Bourne, 1995). Antibody microinjection experiments in fibroblasts suggest that the α isoform of PI 3-kinase is not required for LPA mitogenic signaling (Roche *et al.*, 1994). Furthermore, constitutively activated PI 3-kinase α activates Jun kinase and the p70 S6 kinase, but not MAP kinase in COS cells (Klippel *et al.*, 1996). It therefore seems more likely that the γ isoform, which directly binds to G protein subunits (Stephens *et al.*, 1994; Stoyanov *et al.*, 1995), rather than the α isoform is involved in G_i-mediated signaling. Whether the phosphatidylinositol 3-phosphate produced by PI 3-kinase γ is different from that produced by PI 3-kinase α is currently not known. Some caution is needed, however, since PI 3-kinase inhibitors such as wortmannin may affect enzymes other than PI 3-kinase that function upstream of MAP kinase activation (Scheid and Duronio, 1996). After submission of this paper, Lopez-Illasaca *et al.* (1997) reported that PI 3-kinase γ (but not α), when overexpressed in COS cells, activates MAP kinase in a Gβγ-dependent manner.

The importance of p100–Grb2 interaction in mitogenic signaling is underscored by the finding that all activators of the Ras/MAP kinase pathway in Rat-1 cells, notably LPA, thrombin and serum, induce p100–Grb2 association, whereas endothelin does not. Thus, there is an excellent correlation between G protein-mediated activation of the Ras/MAP kinase pathway and p100–Grb2 complex formation. We are currently purifying p100 in order to establish its identity. Preliminary *in vitro* kinase experiments suggest that p100 is not a tyrosine kinase (data not shown). One possibility is that p100 is a multisite adaptor or docking protein, such as c-Cbl or Gab1 (Holgado-Madruga *et al.*, 1996); but p100 is not c-Cbl, as Cbl has a slightly lower electrophoretic mobility than p100 and, furthermore, is not phosphorylated in response to LPA. We also excluded the possibility that p100 is dynamin, a Grb2 binding GTPase mediating endocytosis (Wang and Moran, 1996; O.Kranenburg and I.Verlaan, unpublished observations).

In conclusion, our data support a model as depicted in Figure 7. Upon activation of G_i-coupled receptors, βγ subunits stimulate the interaction of p100 with Grb2 via

the consecutive activation of a wortmannin-sensitive PI 3-kinase (most likely the γ isoform) and a non-Src tyrosine kinase. As a result of phosphatidylinositol 3-phosphate production in the plasma membrane, tyrosine kinase activity might be recruited (see for example Rameh *et al.*, 1995), which then phosphorylates p100. This may then lead to cooperative binding between p100 and the SH2 and SH3 domains of Grb2. Although we do not observe association of the isolated Grb2-SH2 domain with p100, it may well be that SH3–p100 interaction is required for enhanced binding through phosphotyrosine–Grb2-SH2 interaction. In a parallel but independent pathway, G_q-mediated activation of PLC causes PKC activation and Ca²⁺ mobilization, somehow leading to enhanced tyrosine kinase activity and subsequent tyrosine phosphorylation of Shc. Whether Src also participates in G_q-mediated signaling is currently not clear. Our future experiments are directed towards establishing the identity of p100 and the kinase that mediates its tyrosine phosphorylation.

Materials and methods

Cell culture and transfection

COS-7, Rat-1 cells and immortalized Src-deficient fibroblasts (Bockholt and Burrige, 1995) were routinely grown in Dulbecco's modified Eagle's medium containing 8% fetal calf serum and antibiotics. Transfection of COS-7 cells was performed by the DEAE method. After transfection cells were exposed to serum-free medium overnight. Before stimulation of either COS-7 or Rat-1 cells with LPA (10 mM; Sigma), EGF (50 ng/ml; Collaborative Biomedical Products) or endothelin (50 nM; Cambridge Research Biochemicals), the cells were pre-incubated with either PTX (100 ng/ml; List Biological Laboratories) wortmannin (Sigma), genistein (Calbiochem), staurosporine (Sigma), PdBU (Calbiochem), ionomycin (Calbiochem) or thapsigargin (Calbiochem) at the indicated concentrations.

Western blotting

Cells were washed twice in ice-cold phosphate-buffered saline (PBS) and were lysed in ice-cold lysis buffer (50 mM Tris, pH 7.4, 50 mM NaCl, 0.5% NP40, 0.1% SDS supplemented with 1 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml trypsin inhibitor, 20 mM leupeptin, 0.1 mg/ml NaF, 0.5 mM Na₃VO₄ and 1 mg/ml Na₄P₂O₇). After a 30 min incubation on ice the lysates were cleared by centrifugation (13 000 r.p.m., Eppendorf centrifuge, 15 min) and the protein concentration of the supernatant was determined with the BioRad assay kit. Equal amounts of lysate were run out on SDS–polyacrylamide gels and blotted onto nitrocellulose filters (Schleicher & Schuell). The filters were blocked in either 5% milk or in 1% bovine serum albumin. First antibodies were anti-p44/p42 MAPK (polyclonal rabbit serum 600) and anti-phosphotyrosine PY20 (ICN). Second HRP-conjugated antibodies were from DAKO and signals were visualized using the Amersham ECL kit.

Myelin basic protein (MBP) phosphorylation assay

COS-7 cells (5 cm dishes) were transfected with 1 mg pEXVmycERK2, encoding myc-tagged ERK2. ERK2 was then immunoprecipitated with the monoclonal anti-myc antibody 9E10 and the kinase activity towards MBP was measured as described. Co-transfection of pEXVmycERK2 with pMT2-N17Ras, pMT2-K295Msrc, pMT2-Shc(SH2) or pCMV-lacZ in the controls was performed at a 1:5 ratio. Expression from all constructs was routinely confirmed by Western blotting.

Src-p110–p130 complex formation

Rat-1 cells were incubated in serum-free medium overnight and were subsequently stimulated with either LPA or endothelin. The cells were rinsed twice in PBS and were lysed in a buffer containing 100 mM Tris, pH 7.4, 0.1% Triton X-100 and 10 mM EGTA supplemented with protease and phosphatase inhibitors. The lysates were then incubated with 1 μg purified monoclonal anti-src antibody (Oncogene Science Ab1, c1.327) and rocked in a cold room for 2 h. Protein A beads, pre-coated with rabbit anti-mouse antiserum, were then used to collect the immunocomplexes for 1 h. The beads were subsequently washed three

times in lysis buffer and the immunocomplexes were assayed for tyrosine-phosphorylated src-associating proteins by immunoblotting.

Shc phosphorylation assay

Rat-1 or COS7 cells were incubated in serum-free medium overnight, pre-treated with inhibitors and subsequently stimulated with LPA or EGF. The cells were then rinsed twice in ice-cold PBS and were lysed in Shc lysis buffer (50 mM Tris, pH 7.4, 1% NP40, 1 mM EDTA and 150 mM NaCl supplemented with protease and phosphatase inhibitors). The lysates were incubated on ice for 30 min and subsequently cleared by centrifugation (13 000 r.p.m.). Equal amounts of lysate were then incubated for 2 h with 1 µg rabbit polyclonal anti-Shc antibody (kindly provided by Dr H.Bos) and the immunocomplexes were collected on protein A beads (1 h). The immunocomplexes were washed three times in lysis buffer. The tyrosine phosphorylation state of the Shc proteins was then assayed by anti-phosphotyrosine immunoblotting.

GST FISH experiments

Cell lysates were prepared in a buffer containing 50 mM Tris, 250 mM NaCl, 1 mM EDTA and 0.1% Triton X-100 supplemented with protease and phosphatase inhibitors. Aliquots of 2 µg purified GST–Grb2 fusion proteins or GST alone were then added to the lysates and were incubated for 30 min on ice. Glutathione-coated beads were then used to collect the GST fusion proteins. The beads were washed three times in lysis buffer and were analyzed by anti-phosphotyrosine immunoblotting.

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References

- Alblas, J., van Corven, E.J., Hordijk, P.L., Milligan, G. and Moolenaar, W.H. (1993) Gi-mediated activation of the p21ras-mitogen-activated protein kinase pathway by alpha 2-adrenergic receptors expressed in fibroblasts. *J. Biol. Chem.*, **268**, 22235–22238.
- Baldari, C.T., Pellicci, G., Di Somma, M.M., Milia, E., Giuli, S. and Pellicci, P.G. (1995) Inhibition of CD4/p56lck signaling by a dominant negative mutant of the Shc adaptor protein. *Oncogene*, **10**, 1141–1147.
- Barone, M.V. and Courtneidge, S.A. (1995) Myc but not Fos rescue of PDGF signalling block caused by kinase-inactive Src. *Nature*, **378**, 509–512.
- Bockholt, S.M. and Burridge, K. (1995) An examination of focal adhesion formation and tyrosine phosphorylation in fibroblasts isolated from src[−], fyn[−], and yes[−] mice. *Cell Adhesion Commun.*, **3**, 91–100.
- Bonfini, L., Migliaccio, E., Pellicci, G., Lanfrancone, L. and Pellicci, P. (1996) Not all Shc's roads lead to Ras. *Trends Biochem. Sci.*, **21**, 257–261.
- Blumer, K.J. and Johnson, G.L. (1994) Diversity in function and regulation of MAP kinase pathway. *Trends Biochem. Sci.*, **19**, 236–240.
- Broome, M.A. and Hunter, T. (1996) Requirement for c-Src catalytic activity and the SH3 domain in platelet-derived growth factor BB and epidermal growth factor mitogenic signaling. *J. Biol. Chem.*, **271**, 16798–16806.
- Buday, L. and Downward, J. (1993) Epidermal growth factor regulates p21ras through the formation of a complex of receptor, Grb2 adapter protein, and Sos nucleotide exchange factor. *Cell*, **73**, 611–620.
- Chen, Y.H., Pouyssegur, J., Courtneidge, S.A. and Van Obberghen-Schilling, E. (1994) Activation of Src family kinase activity by the G protein-coupled thrombin receptor in growth-responsive fibroblasts. *J. Biol. Chem.*, **269**, 27372–27377.
- Chen, Y., Grall, D., Salcini, A.E., Pellicci, P.G., Pouyssegur, J. and Van Obberghen-Schilling, E. (1996) Shc adaptor proteins are key transducers of mitogenic signaling mediated by the G protein-coupled thrombin receptor. *EMBO J.*, **15**, 1037–1044.
- Crespo, P., Xu, N., Simonds, W.F. and Gutkind, J.S. (1994) Ras-dependent activation of MAP kinase pathway mediated by G-protein beta gamma subunits. *Nature*, **369**, 418–420.
- Daub, H., Weiss, F.U., Wallasch, C. and Ullrich, A. (1996) Role of transactivation of the EGF receptor in signalling by G-protein-coupled receptors. *Nature*, **379**, 557–560.
- Dikic, I., Tokiwa, G., Lev, S., Courtneidge, S.A. and Schlessinger, J. (1996) A role for Pyk2 and src in linking G-protein-coupled receptors with MAP kinase activation. *Nature*, **383**, 547–550.
- Erpel, T., Alonso, G., Roche, S. and Courtneidge, S.A. (1996) The Src SH3 domain is required for DNA synthesis induced by platelet-derived growth factor and epidermal growth factor. *J. Biol. Chem.*, **271**, 16807–16812.
- Galisteo, M.L., Dikic, I., Batzer, A.G., Langdon, W.Y. and Schlessinger, J. (1995) Tyrosine phosphorylation of the c-cbl proto-oncogene protein product and association with EGF receptor upon EGF stimulation. *J. Biol. Chem.*, **270**, 20242–20245.
- Hawes, B.E., van Biesen, T., Koch, W.J., Luttrell, L.M. and Lefkowitz, R.J. (1995) Distinct pathways of Gi- and Gq-mediated mitogen-activated protein kinase activation. *J. Biol. Chem.*, **270**, 17148–17153.
- Hawes, B.E., Luttrell, L.M., van Biesen, T. and Lefkowitz, R.J. (1996) Phosphatidylinositol 3-kinase is an early intermediate in the G beta gamma-mediated mitogen-activated protein kinase signaling pathway. *J. Biol. Chem.*, **271**, 12133–12136.
- Holgado-Madruga, M., Emlet, D.R., Moscatello, D.K., Godwin, A.K. and Wong, A.J. (1996) A Grb2-associated docking protein in EGF- and insulin-receptor signalling. *Nature*, **379**, 560–564.
- Hordijk, P.L., Verlaan, I., van Corven, E.J. and Moolenaar, W.H. (1994) Protein tyrosine phosphorylation induced by lysophosphatidic acid in Rat-1 fibroblasts. Evidence that phosphorylation of map kinase is mediated by the Gi–p21ras pathway. *J. Biol. Chem.*, **269**, 645–651.
- Jalink, K., Eichholtz, T., Postma, F.R., van Corven, E.J. and Moolenaar, W.H. (1993) Lysophosphatidic acid induces neuronal shape changes via a novel, receptor-mediated signaling pathway: similarity to thrombin action. *Cell Growth Differentiat.*, **4**, 247–255.
- Kanner, S.B., Reynolds, A.B., Wang, H.-C. and Parsons, J.T. (1991) The SH2 and SH3 domains of pp60src direct stable association with tyrosine phosphorylated proteins p130 and p110. *EMBO J.*, **10**, 1689–1698.
- Klippel, A., Reinhard, C., Kavanaugh, W.M., Apell, G., Escobedo, M.A. and Williams, L.T. (1996) Membrane localization of phosphatidylinositol 3-kinase is sufficient to activate multiple signal-transducing kinase pathways. *Mol. Cell Biol.*, **16**, 4117–4127.
- Koch, W.J., Hawes, B.E., Allen, L.F. and Lefkowitz, R.J. (1994) Direct evidence that Gi-coupled receptor stimulation of mitogen-activated protein kinase is mediated by G beta gamma activation of p21ras. *Proc. Natl Acad. Sci. USA*, **91**, 12706–12710.
- Lopez-Illasaca, M., Crespo, P., Pellicci, P.G., Gutkind, J.S. and Wetzker, R. (1997) Linkage of G protein-coupled receptors to the MAPK signaling pathway through PI 3-kinase γ . *Science*, **275**, 394–397.
- Lowenstein, E.J., Daly, R.J., Batzer, A.G., Li, W., Margolis, B., Lammers, R., Skolnik, E.Y., Bar-Sagi, D. and Schlessinger, J. (1992) The SH2 and SH3 domain-containing protein GRB2 links receptor tyrosine kinases to ras signaling. *Cell*, **70**, 431–442.
- Luttrell, L.M., Hawes, B.E., van Biesen, T., Luttrell, D.K., Lansing, T.J. and Lefkowitz, R.J. (1996) Role of c-Src tyrosine kinase in G-protein-coupled receptor and G $\beta\gamma$ subunit-mediated activation of mitogen-activated protein kinases. *J. Biol. Chem.*, **271**, 19443–19450.
- Meisner, H. and Czech, M.P. (1995) Coupling of the proto-oncogene product c-cbl to the epidermal growth factor receptor. *J. Biol. Chem.*, **270**, 25332–25335.
- Moolenaar, W.H. (1995a) Lysophosphatidic acid signalling. *Curr. Opin. Cell Biol.*, **7**, 203–210.
- Moolenaar, W.H. (1995b) Lysophosphatidic acid, a multifunctional phospholipid messenger. *J. Biol. Chem.*, **270**, 12949–12952.
- Pace, A.M., Faure, M. and Bourne, H.R. (1995) G(i2)-mediated activation of the MAP kinase cascade. *Mol. Biol. Cell*, **6**, 1685–1695.
- Rameh, L.E., Chen, C.S. and Cantley, L.C. (1995) Phosphatidylinositol (3,4,5)P3 interacts with SH2 domains and modulates PI 3-kinase association with tyrosine-phosphorylated proteins. *Cell*, **83**, 821–830.
- Reynolds, A.B., Kanner, S.B., Wang, H.R. and Parsons, J.T. (1989) Stable association of activated pp60src with two tyrosine-phosphorylated cellular proteins. *Mol. Cell Biol.*, **9**, 3951–3958.
- Roche, S., Koegl, M. and Courtneidge, S.A. (1994) The phosphatidylinositol 3-kinase alpha is required for DNA synthesis induced by some, but not all, growth factors. *Proc. Natl Acad. Sci. USA*, **91**, 9185–9189.
- Roche, S., Koegl, M., Barone, M.V., Roussel, M.F. and Courtneidge, S.A. (1995) DNA synthesis induced by some but not all growth factors requires Src family protein tyrosine kinases. *Mol. Cell Biol.*, **15**, 1102–1109.

- Rodriguez-Fernandez, J.L. and Rozengurt, R. (1996) Bombesin, bradykinin, vasopressin, and phorbol esters rapidly and transiently activate Src family tyrosine kinases in Swiss 3T3 cells. *J. Biol. Chem.*, **271**, 27895–27901.
- Scheid, M.P. and Duronio, V. (1996) Phosphatidylinositol 3-OH kinase activity is not required for activation of mitogen-activated protein kinase by cytokines. *J. Biol. Chem.*, **271**, 18134–18139.
- Stephens, L., Smrcka, A., Cooke, F.T., Jackson, T.R., Sternweis, P.C. and Hawkins, P.T. (1994) A novel phosphoinositide 3 kinase activity in myeloid-derived cells is activated by G protein beta gamma subunits. *Cell*, **77**, 83–93.
- Stoyanov, B. *et al.* (1995) Cloning and characterization of a G protein-activated human phosphoinositide-3 kinase. *Science*, **269**, 690–693.
- Torres, M. and Ye, R.D. (1996) Activation of the mitogen-activated protein kinase pathway by f-Met-Leu-Phe in the absence of Lyn and tyrosine phosphorylation of Shc in transfected cells. *J. Biol. Chem.*, **271**, 13244–13249.
- Touhara, K., Hawes, B.E., van Biesen, T. and Lefkowitz, R.J. (1995) G protein beta gamma subunits stimulate phosphorylation of Shc adapter protein. *Proc. Natl Acad. Sci. USA*, **92**, 9284–9287.
- van Biesen, T., Hawes, B.E., Luttrell, D.K., Krueger, K.M., Touhara, K., Porfiri, E., Sakaue, M., Luttrell, L.M. and Lefkowitz, R.J. (1995) Receptor-tyrosine-kinase- and G beta gamma-mediated MAP kinase activation by a common signalling pathway. *Nature*, **376**, 781–784.
- van Corven, E.J., Hordijk, P.L., Medema, R.H., Bos, J.L. and Moolenaar, W.H. (1993) Pertussis toxin-sensitive activation of p21ras by G protein-coupled receptor agonists in fibroblasts. *Proc. Natl Acad. Sci. USA*, **90**, 1257–1261.
- van der Bend, R.L., de Widt, J., van Corven, E.J., Moolenaar, W.H. and van Blitterswijk, W.J. (1992) The biologically active phospholipid, lysophosphatidic acid, induces phosphatidylcholine breakdown in fibroblasts via activation of phospholipase D. Comparison with the response to endothelin. *Biochem. J.*, **285**, 235–240.
- Wan, Y., Kurosaki, T. and Huang, X.-Y. (1996) Tyrosine kinases in activation of the MAP kinase cascade by G protein-coupled receptors. *Nature*, **380**, 541–544.
- Wang, Z. and Moran, M.F. (1996) Requirement for the adapter protein GRB2 in EGF receptor endocytosis. *Science*, **272**, 1935–1939.
- Wymann, M.P., Bulgarelli-Leva, G., Zvelebil, M.J., Pirola, L., Vanhaesebroeck, B.W. and Panayotou, G. (1996) Wortmannin inactivates phosphoinositide 3-kinase by covalent modification of Lys-802, a residue involved in the phosphate transfer reaction. *Mol. Cell. Biol.*, **16**, 1722–1733.

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