Biphasic activation of p21^{ras} by endothelin-1 sequentially activates the ERK cascade and phosphatidylinositol 3-kinase

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Endothelin-1 (ET-1) induces cell proliferation and differentiation through multiple G-protein-linked signaling systems, including p21^{ras} activation. Whereas p21^{ras} activation and desensitization by receptor tyrosine kinases have been extensively investigated, the kinetics of p21^{ras} activation induced by engagement of G-protein-coupled receptors remains to be fully elucidated. In the present study we show that ET-1 induces a biphasic activation of p21^{ras} in rat glomerular mesangial cells. The first peak of activation of p21^{ras}, at 2–5 min, is mediated by immediate association of phosphorylated Shc with the guanosine exchange factor Sos1 via the adaptor protein Grb2. This initial activation of p21^{ras} results in activation of the extracellular signal-regulated kinase (ERK) cascade. We demonstrate that ET-1 signaling elicits a negative feedback mechanism, modulating p21^{ras} activity through ERK-dependent Sos1 phosphorylation, findings which were confirmed using an adenovirus MEK construct. Subsequent to p21^{ras} and ERK deactivation, Sos1 reverts to the non-phosphorylated condition, enabling it to bind again to the Grb2/Shc complex, which is stabilized by persistent Shc phosphorylation. However, the resulting secondary activation of p21^{ras} at 30 min does not lead to ERK activation, correlating with intensive, ET-1-induced expression of MAP kinase phosphatase-1, but does result in increased p21^{ras}-associated phosphatidylinositol 3-kinase activity. Our data provide evidence that ET-1-induced biphasic p21^{ras} activation causes sequential stimulation of divergent downstream signaling pathways.

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Introduction

Ras proteins are members of the superfamily of small GTPases claimed to play a key role in signaling pathways leading to cell proliferation, differentiation and transformation (Sato *et al.*, 1992; Boguski and McCormick, 1993). p21^{ras} activity is regulated by guanosine nucleotides, the active form being bound to GTP and the inactive form being bound to GDP. Activation of p21^{ras} and the exchange

of GTP for GDP, thereby increasing the GTP/GDP ratio, is promoted by guanosine nucleotide exchange factors (GEF) (Boguski and McCormick, 1993). The altered conformation which GTP-bound p21^{ras} adopts enables it to associate with other signaling proteins such as Raf-1 (Barnard *et al.*, 1995) and phosphatidylinositol 3-kinase (PI-3-K) (Rodriguez-Viciano *et al.*, 1994). The interaction of p21^{ras} with Raf-1 leads to phosphorylation and activation of downstream protein kinase MAP kinase/ERK kinase (MEK) and extracellular signal-regulated kinase (ERK) (Payne *et al.*, 1991; Alessi *et al.*, 1994), whereas PI-3-K activation leads to phosphorylation of phosphatidylinositides whose role, although not yet completely understood, seems to be important in regulation of cellular growth, differentiation, adhesion and migration (Carpenter and Cantley, 1996).

Most growth factors and insulin are shown to activate p21^{ras} by recruitment of the GEF Son of Sevenless (Sos) to a complex with adaptor protein Grb2 (Sato *et al.*, 1992; Buday and Downward, 1993). Grb2 is subsequently able to associate with Shc through its Src homology 2 (SH2) domain, recognizing phosphoTyr317 on Shc, while binding proline-rich domains on Sos through its SH3 domains (Egan *et al.*, 1993). Recently it has been shown that cell stimulation with growth factors and insulin results in serine/threonine phosphorylation of Sos1 and its dissociation from Grb2, leading to p21^{ras} deactivation (Cherniack *et al.*, 1995). The C-terminal domain of Sos1 contains multiple ERK consensus phosphorylation sites. Moreover, the ability of ERK to phosphorylate Sos1 *in vitro* and *in vivo* has been shown (Porfiri and McCormick, 1996). These data suggest Sos1 phosphorylation as a possible negative feedback mechanism in p21^{ras}/Raf/MEK/ERK signaling. An additional mechanism of regulation of ERK activity is achieved by dual specificity phosphatases which exhibit catalytic activity toward phosphotyrosine and phosphothreonine (Hunter, 1995). MAP kinase phosphatase-1 (MKP-1), also referred to as 3CH134 (Charles *et al.*, 1992), the mouse homolog of human dual specificity phosphatase CL100 (Keyse and Emslie, 1992), shows high affinity for ERK and is responsible for its inactivation *in vivo* (Sun *et al.*, 1993). Expression of MKP-1 in COS cells (Sun *et al.*, 1993) or rat embryonic cells (Sun *et al.*, 1994) prevents activation of ERK not only by different extracellular stimuli but also by oncogenic v-Ras and constitutively active Raf.

Recent findings suggest that p21^{ras} activation plays a role in mitogenic signal transduction by G-protein-coupled receptors (Alblas *et al.*, 1993; Chen *et al.*, 1996; Dikic *et al.*, 1996; Sadoshima and Izumo, 1996). Endothelin-1 (ET-1) is a member of a family of 21 amino acid peptides possessing potent vasoconstrictor properties which are also known to modulate gene expression as well as cell growth and differentiation (Simonson and Dunn, 1993;

Schramek and Dunn, 1997). ET-1 binds to a specific heterotrimeric G-protein-coupled receptor evoking several intracellular pathways that, in rat glomerular mesangial cells (GMC), include protein tyrosine phosphorylation, activation of different phospholipases, mobilization of Ca^{2+} and activation of protein kinase C (Schramek and Dunn, 1997). In GMC ET-1 is able to activate transiently both isoforms of ERK, p42^{ERK} and p44^{ERK} , with a peak of activation at 2–5 min (Wang *et al.*, 1992), and to induce nuclear signaling through p21^{ras} (Herman and Simonson, 1995), since expression of a dominant-negative form of Ras inhibits ET-1-stimulated gene induction (Herman and Simonson, 1995). Further, ET-1 has been shown to induce Shc phosphorylation in astrocytes and the association of tyrosine-phosphorylated Shc with the SH2 domain of a Grb2 fusion protein (Cazaubon *et al.*, 1994).

To date, no detailed studies have appeared on the molecular mechanism involved in p21^{ras} activation and deactivation following ET-1 stimulation. In the present study we demonstrate that ET-1 is able to activate p21^{ras} in a biphasic manner with the first peak at 2–5 min and a second, longer lasting activation beginning at 30 min. ET-1 induces persistent tyrosine phosphorylation of the adaptor protein Shc, enabling it to associate with the adaptor protein Grb2 and thus the guanine exchange factor Sos1. The first recruitment of Sos1 to a complex with Shc and Grb2 results in activation of the ERK cascade. Activation of the MEK/ERK pathway correlates with Sos1 phosphorylation, causing its dissociation from the Shc/Grb2 complex. Subsequent to p21^{ras} and ERK deactivation Sos1 returns to the dephosphorylated condition, enabling it to again associate in complex with Grb2 and Shc, in turn leading to the second activation of p21^{ras} . However, consequent to the second activation of p21^{ras} at 30 min a secondary ERK activation does not occur, possibly due to ET-1-induced expression of MKP-1. We also demonstrate that the second activation of p21^{ras} results in increased p21^{ras} -associated PI-3-K activity, providing evidence that biphasic activation of p21^{ras} induced by ET-1 in GMC activates multiple signaling pathways.

Results

Endothelin-1 induces biphasic activation of p21^{ras}

The activity of p21^{ras} is regulated through its binding of guanine nucleotides, the GTP-bound active form of the protein being able to interact with effector proteins like Raf-1 and PI-3-K (Rodriguez-Viciana *et al.*, 1994; Barnard *et al.*, 1995). The kinetics of ET-1 stimulated p21^{ras} activation have, as yet, not been studied. In the present study p21^{ras} activation was assessed by two different methods: (i) measuring the relative amount of nucleotides bound to p21^{ras} ; (ii) evaluating the quantity of Raf-1 protein able to associate with p21^{ras} . The relative amount of [^{32}P]GTP and [^{32}P]GDP bound to p21^{ras} was evaluated over a 90 min period following ET-1 (100 nM) stimulation of GMC. Cell lysates were subjected to immunoprecipitation with an anti- p21^{ras} monoclonal antibody (Y13-259) and guanine nucleotides bound to immunoprecipitated p21^{ras} were separated by thin layer chromatography (TLC). As shown in Figure 1A, the initial increase in p21^{ras} -bound GTP, which peaks at 2–5 min, is followed by a recovery to baseline values at 15 min and thereafter by a

Fig. 1. Biphasic activation of p21^{ras} by ET-1. (A) Quiescent GMC labeled with [^{32}P]orthophosphate were stimulated with ET-1 (100 nM) for the times indicated. p21^{ras} was isolated by immunoprecipitation and p21^{ras} -bound guanine nucleotides were eluted and separated by TLC. Results were quantitated by phosphorimager analysis and expressed as $\text{GTP}/(\text{GTP} + \text{GDP}) \times 100$. Data are the mean of five separate experiments ($n = 3$ for the extended 90 min time course). Bars represent standard deviations. The inset represents Western blot analysis of GMC lysates immunoprecipitated with anti- p21^{ras} and immunoblotted with the same antibody to confirm that equal amounts of p21^{ras} protein are immunoprecipitated throughout the time course of ET-1 stimulation. (B) GTP-bound active p21^{ras} was isolated from lysates of quiescent and ET-1-stimulated GMC by affinity precipitation with a GST-RBD fusion protein followed by immunoblot analysis with anti- p21^{ras} antibody. The fusion protein (~42 kDa) was detected by Coomassie brilliant blue staining of the polyacrylamide gel. Shown is a representative result; the experiment was repeated three times.

second and more stable reassociation of GTP with p21^{ras} , which is maximal at 30 min and then decreases again to the basal level at 90 min post-stimulation. When guanine nucleotides were eluted from immunoprecipitates performed without addition of the specific antibody or following substitution of normal rabbit serum for rabbit anti-rat antibody (refer to Materials and methods for the procedure) only background levels of ^{32}P activity were found (not shown), indicating that the GTP and GDP detected on TLC were originally complexed with p21^{ras} . Further, fetal bovine serum (17%) and platelet derived growth factor $\beta\beta$ (50 ng/ml) were included as positive controls for p21^{ras} activation in several experiments (data not shown). Binding of p21^{ras} with the Ras binding domain (RBD) of Raf-1 was evaluated over a 2 h time course. Cell lysates of GMC stimulated with 100 nM ET-1 were subjected to affinity precipitation with a glutathione *S*-transferase-RBD fusion protein (see Materials and methods). The eluted proteins were then subjected to SDS-PAGE and immunoblotted with anti- p21^{ras} antibody. As is shown in Figure 1B, the GST-RBD of Raf-1 associates in a biphasic manner with p21^{ras} , with a first peak at 2 and 5 min and

Fig. 2. Monophasic activation of ERK by ET-1. Quiescent GMC were stimulated with ET-1 (100 nM) for the times indicated. **(A)** Immunoblot detection of the two isoforms of ERK. Activation is identifiable by the appearance of bands with delayed mobility related to the phosphorylated form of the protein. **(B)** ERK activity assayed by the ability of immunoprecipitated p42^{ERK} to phosphorylate MBP as substrate.

a second at 30 min following ET-1 stimulation. Thereafter, the baseline level is gradually attained and recovery is complete at 2 h. These data provide evidence that ET-1 activates p21^{ras} in a biphasic mode characterized by a first sudden and transient peak followed by a second more stable activation.

Endothelin-1 induces a time-dependent activation of ERK

p21^{ras} interacts with effector proteins through a stretch of amino acids, known as the effector region, that assume an active conformation when the protein is bound to GTP (Barnard *et al.*, 1995). To date, the p21^{ras} effectors known to mediate predominantly growth stimulatory signals are the serine/threonine Raf kinases (Marshall, 1994). p21^{ras} is reported to interact, through the effector region, with the N-terminal domain of Raf, regulating its kinase activity (Vojtek *et al.*, 1993; Marshall, 1994). Raf exhibits high substrate specificity toward MEK, phosphorylating its two regulatory serine residues and achieving MEK activation (Alessi *et al.*, 1994). MEK1 and MEK2 are dual specificity kinases capable of phosphorylating regulatory serine/threonine and tyrosine residues on ERK (also referred to as p44 and p42 MAP kinases) (Payne *et al.*, 1991). Thus activated, ERK is itself able to phosphorylate serines and threonines of a number of cytoplasmic and nuclear proteins, such as transcription factors, thereby transducing proliferative and differentiation signals to the nucleus (Seger and Krebs, 1995). In this study we investigated the kinetics of ERK activation following ET-1 stimulation. ERK activation was assessed both by delayed mobility of the phosphorylated form on SDS-PAGE and the ability of immunoprecipitated p42^{ERK} to phosphorylate myelin basic protein (MBP). As shown in Figure 2, data obtained by both these methods are consistent with an ET-1-induced peak of ERK activation occurring at 5 min. Quantification of ³²P incorporation into MBP revealed a 1.7-fold increase in ET-1-stimulated cells in comparison with their unstimulated counterparts. These experiments confirm the ability of ET-1 to induce ERK activation under our experimental conditions. The monophasic activation of ERK, over a 60 min period, suggests the presence of mechanisms

Fig. 3. Shc phosphorylation is persistent during the time period of ET-1-stimulation of rat glomerular mesangial cells. Lysates from quiescent cells stimulated with ET-1 (100 nM) for the times indicated were immunoprecipitated with anti-Shc polyclonal antibody (Transduction Laboratories). Immunoprecipitates were analyzed by SDS-PAGE and immunoblotted with anti-phosphotyrosine (anti-PY) antibody **(A)** and anti-Shc antibody **(B)** as indicated. Shown is a representative result; the experiment was repeated three times.

downstream of p21^{ras} involved in regulation of ERK activity.

Endothelin-1 stimulates tyrosine phosphorylation of Shc and its association with Grb2 and Sos1

It has been shown that Shc adaptor proteins are involved in signal transduction of mitogenic stimuli arising through G-protein-coupled receptors via p21^{ras} (Cook *et al.*, 1993; Chen *et al.*, 1996; Sadoshima and Izumo, 1996). It has also been shown that ET-1 is able to induce Shc tyrosine phosphorylation in cultured astrocytes (Cazaubon *et al.*, 1994). In the present study we evaluated whether ET-1 could bring about Shc phosphorylation in GMC. Shc immunoprecipitates were immunoblotted with an anti-phosphotyrosine monoclonal antibody. The results of these experiments demonstrate (Figure 3A) that ET-1 is indeed able to phosphorylate Shc, principally the p46 and p52 kDa isoforms, within 1 min and that this phosphorylation is persistent for at least 60 min, with a peak at 15 min. The anti-Shc antibody was able to immunoprecipitate all three isoforms of Shc, as determined by immunoprecipitation and immunoblotting with the same antibody (Figure 3B).

Shc phosphorylation creates a binding site for the SH2 domain of the SH2/SH3 domain-containing adaptor protein Grb2 (Salcini *et al.*, 1994). SH3 domains of Grb2 have been shown to interact with the proline-rich motifs located in the C-terminus of the guanine exchange factor Sos1 (Buday and Downward, 1993; Egan *et al.*, 1993; Li *et al.*, 1993), leading to recruitment of Sos1 to the membrane and its ability to activate p21^{ras}. To examine whether, and with what kinetics, Grb2 associates with Shc in GMC stimulated with ET-1 we performed immunoprecipitations with anti-Shc antibody at different time points post ET-1 stimulation, followed by immunoblotting with an anti-Grb2 antibody. As shown in Figure 4A, there is no evident association between Shc and Grb2 in unstimulated cells and a time-dependent increase in Grb2/Shc association is apparent following addition of ET-1. To confirm further

Fig. 4. ET-1 induces association of Shc with Grb2. Lysates from quiescent cells stimulated with ET-1 (100 nM) for the times indicated were immunoprecipitated with anti-Shc (A) or anti-Grb2 antibodies (B and C). The resultant immunoprecipitates were resolved by SDS-PAGE and then immunoblotted with anti-Grb2 (A), anti-Shc (B) and anti-PY antibodies (C). (B) The anti-Shc antibody specifically recognizes the three Shc isoforms in the lysate. The band between the p52 and p66 isoforms is characterized as non-specific, since it is still recognized when immunoprecipitation is carried out in the absence of antibody (No abs). (C) GMC lysate was subjected to SDS-PAGE analysis and immunoblotted with anti-Shc antibody (far right lane) in order to define the positions of the Shc isoforms in the corresponding anti-phosphotyrosine immunoblot (left lanes).

association of Shc with Grb2, Grb2 immunoprecipitates were subjected to SDS-PAGE and blotted with anti-Shc antibodies. As shown in Figure 4B, Grb2 binds rapidly to Shc following ET-1 stimulation of GMC and the association lasts throughout the time course evaluated. Western blot analysis shows that the p52^{Shc} isoform, the most prevalent in GMC, plays a major role in association of Shc with Grb2. The identity of the band as p52^{Shc} was confirmed by comparison with Western blots of cell lysates and anti-Shc immunoprecipitates probed with anti-Shc antibody (not shown). Moreover, to further support the ability of Grb2 to associate with the phosphorylated form of p52^{Shc}, Grb2 immunoprecipitates were subjected to SDS-PAGE and immunoblotted with an anti-phosphotyrosine antibody. Figure 4C shows that after ET-1 stimulation

Fig. 5. A biphasic increase in Grb2/ Sos1 association induced by ET-1. Serum-starved cells were stimulated with ET-1 for the indicated times. Cell lysates were immunoprecipitated with anti-Sos1 antibody (A) or anti-Grb2 antibody (B). Immunoprecipitates were analyzed by SDS-PAGE and then immunoblotted with anti-Sos1 antibody and anti-Grb2 antibody. Shown is a representative result; the experiment was repeated three times.

Grb2 associates with the phosphorylated p52^{Shc} isoform. The onset of association is rapid and lasts throughout the 60 min time course. These data indicate that Grb2 forms a complex with Shc after ET-1 stimulation in GMC and that this association is persistent for at least 60 min.

Although Grb2 associates with the phosphorylated form of Shc, in ET-1-stimulated GMC the amount of Grb2 detected in Shc immunoprecipitates does not completely correlate with the amount of phosphorylated Shc. This apparent discrepancy may be explained in a number of ways. Several agonists have been shown to induce Shc phosphorylation not only on Tyr317 but also on Tyr239 and Tyr240 (Gotoh *et al.*, 1996, 1997; van der Geer *et al.*, 1996). Phosphorylation on positions Tyr239 and Tyr240 has been reported not to contribute to p21^{ras}/ERK activation and to have little involvement in Grb2 association (Gotoh *et al.*, 1997). Therefore, it may be assumed that Shc phosphorylation does not exclusively lead to Grb2/ Shc association. Moreover, the SH2 domain and phosphotyrosine binding (PTB) domain of Shc have been shown to associate with a vast array of tyrosine-phosphorylated proteins, some not yet characterized (Kavanaugh *et al.*, 1996). In GMC ET-1 induces rapid and transient tyrosine phosphorylation of a large number of proteins (Force *et al.*, 1991) that may compete with Grb2 for Shc interaction in the early phase but not at later time points of ET-1 stimulation.

In order to examine association of Grb2 with Sos1, Sos1 immunoprecipitates were immunoblotted with anti-Grb2 antibody. Figure 5A shows that Sos1 is able to associate with Grb2 in quiescent cells and this association strongly and rapidly increases following ET-1 stimulation, in a biphasic manner. The first peak of association, observed at 1 and 2 min, is followed by a second and

Fig. 6. Grb2 mediates the association of Sos1 and Shc. Lysates from quiescent cells stimulated with ET-1 (100 nM) for the times indicated were immunoprecipitated with anti-Sos1 antibody (A) or anti-Shc antibody (B). The resultant immunoprecipitates were resolved by SDS-PAGE and then immunoblotted with anti-Sos1 antibody and anti-Shc antibody. Shown is a representative result; the experiment was repeated three times.

longer lasting association at 30 and 60 min. The identity of the band as Grb2 was confirmed by comparison of cell lysates probed with anti-Grb2 antibody (not shown). To further confirm the bimodal association of Grb2 with Sos1, Grb2 immunoprecipitates were blotted with anti-Sos1 antibody, showing a rapid Grb2/Sos1 association at 1 and 2 min, followed by a new association predominant at 30 min (Figure 5B). Quantitative immunoprecipitation experiments indicate that only a small fraction of cellular Grb2 is in complex with Sos1 (Waters *et al.*, 1996). The relative excess of cytosolic Grb2 could account for the small amount of Sos1 immunoprecipitated with Grb2 under our experimental conditions. These results indicate that ET-1 increases the Grb2/Sos1 complex in a biphasic manner that correlates temporally with p21^{ras} activation.

We next examined whether Sos1 is complexed with Shc, as a result of association of Grb2 with both Sos1 and Shc, after ET-1 stimulation and whether association of Sos1/Shc follows the kinetics of association of Shc with Grb2. Sos1 was immunoprecipitated from quiescent and ET-1-stimulated GMC and immunoblotted with anti-Shc antibody. During ET-1 stimulation a biphasic association of Sos1 with Shc occurs; the first transient association is evident within the first minute and the second phase of association starts at 30 min and lasts at least until 60 min (Figure 6A). Association of Sos1 is mainly with the p52^{Shc} isoform. In our experiments p46^{Shc} and p52^{Shc} were the predominant isoforms phosphorylated on tyrosine, while

only the p52^{Shc} isoform associated with Grb2 during ET-1 stimulation. Differential recruitment of the three isoforms of Shc in the signaling leading to p21^{ras} activation is consistent with data obtained by other authors using different cell lines (Langlois *et al.*, 1995; Chen *et al.*, 1996; Sadoshima and Izumo, 1996). It is interesting to note that in our experiments Sos1 is able to associate with the p66^{Shc} isoform, although this protein appears to be poorly phosphorylated and not associated with Grb2 in ET-1-stimulated GMC. The p66^{Shc}/Sos1 association could, however, be mediated by other SH2/SH3-containing proteins involved in signaling by ET-1 through p21^{ras}. Shc immunoprecipitates blotted with anti-Sos1 antibody (Figure 6B) confirm the biphasic association of Sos1 with Shc with the same time course displayed by Sos1 immunoprecipitation. These data demonstrate the ability of ET-1 to induce a biphasic Shc/Sos1 association. When comparing the time course of Shc/Grb2/Sos1 association with that of p21^{ras} activation it is evident that there is a close correlation between these events, suggesting that Sos1 dislocation and association with Shc and Grb2 are responsible for both phases of p21^{ras} activation.

The kinetics of association of Sos1 with Grb2 and p21^{ras} desensitization are determined by Sos1 phosphorylation

It has been shown that exposure of cells to growth factors and cytokines results in a reduction in the electrophoretic mobility of Sos1 on SDS-PAGE as a result of protein phosphorylation (Cherniack *et al.*, 1994, 1995; Waters *et al.*, 1995). Phosphorylation of Sos1 occurs on serine/threonine residues located in the C-terminal portion of the protein. Phosphorylation of Sos1 acts as a feedback mechanism, leading to dissociation of Sos1 from the complex with Grb2 and Shc (Cherniack *et al.*, 1994, 1995; Waters *et al.*, 1995). Although ERK has been shown to be able to phosphorylate Sos1 (Porfiri and McCormick, 1996), it was recently reported that an as yet undefined MEK-dependent, ERK-independent kinase might phosphorylate Sos1 in CHO cells stimulated by insulin (Holt *et al.*, 1996). Here we show that during ET-1 stimulation of GMC Sos1 is phosphorylated in a time-dependent manner. Phosphorylation of Sos1, resulting in delayed mobility on SDS-PAGE, is evident at 5 min and reaches a peak at 15 min (Figure 7A). Comparing the time course of Sos1 phosphorylation with that of its association with Grb2/Shc, shown above, strongly suggests that phosphorylation is responsible for the dissociation of Sos1 from the complex with Grb2 and the desensitization of p21^{ras} we observe in our experiments.

To determine whether the MEK/ERK cascade plays a role in Sos1 phosphorylation we used the specific MEK inhibitor PD 98059 in order to block phosphorylation and hence activation of ERK. As shown in Figure 7B, PD 98059 almost completely abolished the Sos1 phosphorylation induced by ET-1. These experiments indicate that the MEK/ERK cascade plays a major role in Sos1 phosphorylation in GMC. Moreover, in order to better elucidate the mechanism of Sos1 phosphorylation, we used NIH 3T3 cells stably transfected with the wild-type or a constitutively active form of MEK (Mansour *et al.*, 1994). In serum-starved constitutively active MEK-expressing cells we detected a delayed mobility of ERK, indicating

Fig. 7. MEK-dependent mechanism of Sos1 phosphorylation in ET-1-stimulated rat glomerular mesangial cells. Quiescent GMC were left in serum-free medium or exposed to the MEK-inhibitor PD 98059 (100 μ M) for 1 h prior to being stimulated with ET-1 (100 nM) for the indicated times. Lysates were resolved by SDS-PAGE and then immunoblotted with anti-Sos1 antibody. (A) Kinetics of ET-1-induced phosphorylation of Sos1 seen as delayed mobility of the phosphorylated form on SDS-PAGE. (B) ET-1-induced phosphorylation of Sos1 is compared in GMC pretreated or not with PD 98059. (C) Phosphorylation of Sos1, as delayed motility, in serum-starved NIH 3T3 cells transfected with wild-type (WT) or constitutively active MEK (DN3-S222D). These experiments were repeated three times.

ERK phosphorylation and activation. In contrast, no shift was detected in serum-starved cells expressing wild-type MEK (data not shown). Lysates from these cell lines were analyzed by SDS-PAGE and blotted with anti-Sos1 antibody. As shown in Figure 7C, we found a delayed mobility of Sos1 in constitutively active MEK-expressing cells, while with wild-type MEK no phosphorylation of Sos1 was observed. This experiment confirms the role of the MEK/ERK cascade in Sos1 phosphorylation. Moreover, it confirms that the reduced phosphorylation of Sos1 observed in GMC preincubated with PD 98059 is wholly due to MEK inhibition and not to non-specific mechanisms. In order to define the role of Sos1 phosphorylation in the mechanism of p21^{ras} desensitization, we used a recombinant adenovirus to express a constitutively active form of MEK (AdMEK_{CA}) in GMC. A transgenic adenovirus encoding bacterial β -galactosidase activity (AdLacZ) was used to define the multiplicity of infection (MOI) transducing expression in practically 100% of the cells (Figure 8A). Lysates from serum-starved cells transduced with AdMEK_{CA} and with non-transgenic viruses (Ad-dl327) as a control were resolved by SDS-PAGE and immunoblotted with anti-ERK, anti-MEK and anti-Sos1 antibodies. As a result of strong expression of the catalytically active form of MEK in AdMEK_{CA}-transduced cells, ERK presented a delay in gel mobility, indicating phosphorylation and activation (Figure 8B). Also, cells expressing the active form of MEK show a pronounced phosphorylation of Sos1, assessed as a delay in gel mobility (Figure 8B). Further, to confirm the role of phosphorylated Sos1 in p21^{ras} desensitization and the requisite recovery of the non-phosphorylated condition of Sos1 in the second activation of p21^{ras}, we stimulated AdMEK_{CA}-transduced and control cells for 30 and 60 min with ET-1 and determined p21^{ras} activation by affinity binding to the RBD of Raf-1. As shown in Figure 8B and C, in Ad-dl327-infected cells after ET-1 stimulation for 30 and 60 min, as well as in uninfected cells, Sos1 is almost completely dephosphorylated and, consequently, the second peak of p21^{ras} activation

can be detected. In contrast, p21^{ras} activation is completely abolished and persistent phosphorylation of Sos1 is induced in AdMEK_{CA}-infected cells. These experiments confirm the role of the MEK/ERK cascade in Sos1 phosphorylation and the requirement for Sos1 phosphorylation in p21^{ras} desensitization. Further, we obtained evidence that in GMC recovery of the non-phosphorylated status of Sos1 is required for onset of the second phase of p21^{ras} activation.

Endothelin-1 induces expression of the MAP kinase-specific phosphatase MKP-1

ERK requires phosphorylation on both threonine and tyrosine regulatory residues for activation. Protein phosphorylation is a dynamic process that can be reversed by phosphatases that catalyze dephosphorylation. Recently a new dual specificity phosphatase has been cloned that is able to dephosphorylate tyrosine- and threonine-phosphorylated ERK 15- to 200-fold more rapidly than the other tyrosine-phosphorylated substrate examined (Charles *et al.*, 1993; Sun *et al.*, 1993) and for this reason this phosphatase has been named MKP-1. In order to assess if MKP-1 plays a role in preventing second phase activation of ERK, we evaluated MKP-1 expression during the time course of ET-1 stimulation. MKP-1 was immunoprecipitated from quiescent and ET-1-stimulated GMC and immunoblotted with the same polyclonal antibody. As shown in Figure 9, ET-1 induces expression of MKP-1 30 min post-stimulation, with expression persisting for at least 90 min. These results confirm our previous finding that ET-1 induces the MKP-1 mRNA transcript with a peak at 30 min in GMC (our unpublished data). These experiments suggest that MKP-1 may play an important role in dephosphorylation of ERK and deactivation of the ERK pathway during ET-1 stimulation in GMC. The increased and persistent expression of MKP-1 after 30 min exposure to ET-1 could be one of the factors explaining why late phase activation of p21^{ras} does not result in a secondary activation of ERK.

Endothelin-1 increases p21^{ras}-associated PI-3-K activity

PI-3-K, which is responsible for phosphorylation of the 3'-OH of the inositol ring of phosphatidylinositides, is present in the cell as a heterodimer composed of a 110 kDa subunit, accounting for catalytic activity, and a 85 kDa subunit, with a regulatory role. Although the biological function of PI-3-K has not yet been completely elucidated, it has been implicated in transduction of signaling leading to cell proliferation, differentiation, adhesion and migration by several growth factors (Carpenter and Cantley, 1996). Several regulatory mechanisms involving the p85 subunit of PI-3-K have been proposed to be involved in controlling the activity of this lipid kinase in response to extracellular signals. In H-ras-transformed rat liver epithelial cells PI-3-K activity has been detected in p21^{ras} immunoprecipitates (Sjolander *et al.*, 1991). Recently p21^{ras} has been demonstrated to directly interact with the catalytic subunit of PI-3-K. The interaction between p21^{ras} and PI-3-K is regulated in a GTP-dependent manner, occurring through the effector region of p21^{ras} (Rodriguez-Viciano *et al.*, 1994). In order to verify if ET-1, secondary to its ability to activate p21^{ras}, can regulate PI-3-K activity

Fig. 8. p21^{ras} desensitization is determined by Sos1 phosphorylation. (A) GMC were infected with adenoviral constructs at a MOI of 60 for 24 h. As assessed by X-gal staining, AdLacZ infects ~100% of cells. (B) Lysates from AdMEK_{CA}- or Ad-dl327-infected cells, stimulated or not for the indicated times with ET-1, were resolved by SDS-PAGE and immunoblotted with anti-Sos1, anti-MEK and anti-ERK antibodies. An ERK shift in AdMEK_{CA}-infected cells is evidence of ERK activation. (C) Lysates, equalized for protein, from AdMEK_{CA}- and Ad-dl327-infected cells were subjected to affinity precipitation with GST-RBD fusion protein. p21^{ras} proteins were then detected by immunoblotting with anti-p21^{ras} antibody. The fusion protein (~42 kDa) was detected in polyacrylamide gels by Coomassie brilliant blue staining. This experiment was repeated twice with identical results.

we evaluated PI-3-K activity in p21^{ras} immunoprecipitates following ET-1 stimulation. In quiescent and ET-1-stimulated GMC p21^{ras} was immunoprecipitated with monoclonal antibody Y13-259 and subsequently incubated with phosphatidylinositol. Lipids were extracted from the reaction product and resolved by TLC. Following ET-1 stimulation p21^{ras}-associated PI-3-K activity shows a major increase at 30 min (Figure 10A and B). When immunoprecipitates were performed without addition of specific antibody no activity was found (Figure 10, lane 9), indicating that the PI-3-K activity measured is specifically associated with p21^{ras}. The kinetics of activation of PI-3-K do not directly follow the kinetics of p21^{ras} activation following ET-1 stimulation. Even if an increase in PI-3-K activity is present at 5 min, it appears that PI-3-K is predominantly recruited by p21^{ras} during its second phase

Fig. 9. ET-1 induces expression of the dual specificity phosphatase MKP-1. Lysates from quiescent cells stimulated with ET-1 (100 nM) for the times indicated were analyzed by immunoprecipitation and successive immunoblotting with anti-MKP-1 antibody. Shown is a representative result; the experiment was repeated twice.

Fig. 10. ET-1 induces PI-3-K activity in anti-p21^{ras} immunoprecipitates. Lysates from quiescent cells stimulated with ET-1 (100 nM) for the times indicated were immunoprecipitated with either anti-p21^{ras} antibody (lanes 1–6), anti-p85 antibody (lane 7 and 8) or with neither (lane 9). PI-3-K activity was assayed by its ability to phosphorylate phosphatidylinositol. In lane 7 phosphatidylinositol 4-phosphate was used as substrate (refer to Material and methods). Ori, origin. (A) A quantitative presentation (bars represent standard deviation). (B) A representative result ($n = 4$).

of activation (Figure 10A). These experiments demonstrate the ability of ET-1 to induce a p21^{ras}-associated PI-3-K activity in GMC with a peak at 30 min. The kinetics of PI-3-K activity suggest that mechanisms other than the GTP binding state of p21^{ras} contribute to regulation of p21^{ras}-associated PI-3-K activity.

Discussion

Our data represent the first demonstration of biphasic p21^{ras} activation; further, we provide evidence that this biphasic model of activation results in sequential activation of two downstream pathways, ERK and PI-3-K.

The proto-oncogene product p21^{ras} has been shown to act as a pivotal point in transmission of signals from tyrosine kinase receptors to downstream kinase cascades, ultimately leading to cell proliferation, differentiation and gene expression (Satoh *et al.*, 1992; McCormick, 1994). More recently it has been shown that engagement of G-protein-coupled receptors also leads to activation of p21^{ras} (Alblas *et al.*, 1993; Herman and Simonson, 1995; Sadoshima and Izumo, 1996). Indeed, in yeast which lacked tyrosine kinase receptors, activation of a GTPase analog of p21^{ras} was promoted by binding of a mating pheromone to a G-protein-coupled receptor. These data indicate that transduction of signals from G-protein-coupled receptors via p21^{ras} phylogenetically predates even the link between this GTPase and tyrosine kinase receptors (Herskowitz, 1995).

ET-1-induced biphasic activation of p21^{ras} represents a

new model of p21^{ras} activation that is facilitated by persistent tyrosine phosphorylation of Shc over a 60 min period. Prolonged phosphorylation of Shc induced by several agonists has been described (Chen *et al.*, 1996; Holt *et al.*, 1996) and the crucial role of Shc phosphorylation and its association with Grb2 in ERK pathway activation, secondary to G-protein-coupled receptor engagement, has been extensively documented (Chen *et al.*, 1996). However, the role of prolonged phosphorylation of Shc has not been addressed. In the present study we show that persistent phosphorylation of Shc, stabilizing it in complex with Grb2, results in a permanent docking site for sequential recruitment of Sos1 and hence biphasic activation of p21^{ras}. Rapid and transient initial activation of p21^{ras} is followed by an equally rapid and transient deactivation, as a direct consequence of Sos1 phosphorylation and its release from the Shc/Grb2/Sos1 complex (Figure 11). We have also demonstrated ET-1-induced MEK/ERK-dependent phosphorylation of Sos1, leading to its release from the Grb2/Shc complex and the return of p21^{ras} to the inactive GDP-bound form. Dephosphorylation and deactivation of ERK is followed by recovery of the non-phosphorylated form of Sos1, with its ability to again complex with Shc/Grb2, leading to secondary activation of p21^{ras} (Figure 11). In fact, transgenic adenovirus-mediated persistent activation of ERK, responsible for prolonged phosphorylation of Sos1, blocks agonist-induced p21^{ras} activation. Serine/threonine phosphorylation of Sos1 has been shown to play a role in feedback mechanisms leading to p21^{ras} desensitization in insulin- and epidermal growth factor (EGF)-stimulated cells. Rapid activation of p21^{ras} with a peak at 1.5–2 min is followed by recovery of the resting condition within 10 min (Klarlund *et al.*, 1995, 1996; Waters *et al.*, 1996). Return of p21^{ras} to the inactive form correlates with Sos1 phosphorylation and dissociation from the Grb2/Shc complex (Langlois *et al.*, 1995; Klarlund *et al.*, 1996). Recruitment of Sos1 to the membrane seems to be the main requisite for p21^{ras} activation, since expression in cells of Sos mutants containing membrane anchoring myristoylation or farnesylation signals results in persistent stimulation of p21^{ras} signaling and consequent oncogenic transformation (Aronheim *et al.*, 1994). Therefore, Sos1 phosphorylation appears to desensitize the p21^{ras} pathway, resulting in dissociation of Sos1 from Grb2 and its release from the membrane. However, C-terminal deletion generates a considerably more active form of Sos (Aronheim *et al.*, 1994; Wang *et al.*, 1995) and *in vitro* phosphorylation of Sos1 does not appear to affect its binding to Grb2, although the ability of Sos1 to associate with Shc and with the epidermal growth factor receptor is strongly reduced (Porfiri and McCormick, 1996). These data suggest that Grb2 interacting with the C-terminal region of Sos1 plays a more complex role than merely recruiting it to the membrane. In accordance with these findings, in the present study we also demonstrated an ET-1-dependent increase in Grb2/Sos1 association. Initial results obtained in mammalian cells indicated that Grb2 exists constitutively in complex with Sos1 and that the degree of association does not change upon ligand stimulation (Buday and Downward, 1993; Egan *et al.*, 1993; Li *et al.*, 1993). Recently, however, the ability of ligands to increase association of Grb2 with Sos1 has been reported in several

Fig. 11. Schematic representation of the proposed signaling through p21^{ras} induced by ET-1. ET-1 induces a first phase of p21^{ras} GTP binding which drives ERK pathway activation. ERK phosphorylation of Sos1 leads to p21^{ras} desensitization. Dephosphorylation and deactivation of ERK, corresponding to increased expression of MKP-1, correlates with the renewed ability of Sos1 to complex with Shc/Grb2, leading to the secondary activation of p21^{ras} and hence of PI-3-K. The PI-3-K products could act by regulating ribosomal function through the involvement of Akt and p70^{S6} kinases.

studies using a wide range of agonists (Cherniack *et al.*, 1995; Waters *et al.*, 1995, 1996; Hu and Bowtell, 1996).

Other mechanisms of desensitization are known to operate at the receptor level in G-protein-mediated signaling determining agonist-induced down-regulation of receptor function and/or expression. Desensitization at the receptor level, which occurs within a few minutes, is mediated by the sequential action of the G-protein-coupled receptor kinases, which phosphorylate agonist-occupied receptors, facilitating binding of a second family of proteins, the arrestins, which block receptor signaling and facilitate receptor internalization (Lefkowitz, 1993). Receptor desensitization has been shown to take place following ET A receptor engagement and has been hypothesized to modulate ET responsiveness (Cyr *et al.*, 1993). Although ET receptor desensitization could down-regulate ET-induced ion transport, protein kinase A and C and phospholipase activation, we show here that ET-1-stimulated p21^{ras} signaling undergoes an independent mechanism of desensitization.

To date, no other studies on the kinetics of p21^{ras} activation subsequent to G-protein-coupled receptor engagement have been reported, although persistent phosphorylation of Shc in response to thrombin has been documented (Chen *et al.*, 1996) and transient activation of ERK consequent on G-protein-coupled receptor activation is a common finding. It is possible, therefore, that the biphasic p21^{ras} activation that we observe in cells stimulated with ET-1 may represent a general event in cellular responses to G-protein-coupled receptor agonists. It is noteworthy that a single activation of p21^{ras} is apparent in the available kinetic data on tyrosine kinase receptor-mediated signaling. This could be due to the fact that following tyrosine kinase receptor engagement the status of the signaling molecules does not favor the

biphasic kinetics of p21^{ras} activation or, alternatively, that biphasic activation of p21^{ras} in response to growth factors escaped the attention of investigators because the time course considered was not long enough. While phosphorylation of the receptor is a transient phenomenon, Shc phosphorylation has been shown to be persistent under stimulation with EGF and to reach a maximum at 60 min during insulin stimulation (Waters *et al.*, 1996). Thus, prolonged phosphorylation of Shc persisting after Sos1 dephosphorylation could, in theory, lead to its association with Grb2/Sos1, driving a second activation of p21^{ras}.

Activation of ERK follows promptly on the first p21^{ras} stimulation but not after the second phase of p21^{ras} activity. This lack of late phase ERK activation suggests that other factors involved in regulation of its activity come into play. MKP-1, which selectively dephosphorylates ERK, has been shown to be regulated mainly at the transcriptional level by different MAP kinase pathways (Bokemeyer *et al.*, 1997). We demonstrate that, following 30 min ET-1 stimulation, there is strong and persistent expression of MKP-1. The onset of expression of MKP-1 correlates with deactivation of ERK following the second phase activation of p21^{ras} activity, suggesting that MKP-1 may play a part in blunting ERK activity (Figure 11). However, further studies will be needed to verify if other mechanisms are involved in this process.

In the present study we show increments in PI-3-K activity in p21^{ras} immunoprecipitates with a peak at 30 min, corresponding to the second peak of p21^{ras} activation. Our data support the involvement of p21^{ras} in regulating PI-3-K activity in ET-1-stimulated cells. The biological significance of this activation remains to be defined and, considering that the functions of the phosphorylated inositide products of PI-3-K activity are largely unknown, the issue is clearly a difficult one. Some lines of evidence

indicate that PI-3-K regulates the translation process via other effector molecules, such as Akt kinase and/or p70^{S6} kinase, known to regulate ribosomal function (Burgering and Coffer, 1995). In accordance with these data and our findings one could formulate a working hypothesis in which the first activation of p21^{ras} drives the ERK cascade, inducing gene transcription, whereas the second activation of p21^{ras}, through the effector PI-3-K, could regulate gene translation (Figure 11).

While in this study we have determined the proteins involved in transduction of the signal from phosphorylation of Shc to p21^{ras} activation, some steps in ET-1-induced activation of p21^{ras} remain to be elucidated. For example, the signaling pathways that lead to activation of intracellular tyrosine kinase by ET-1 are still under investigation, as are which kinase(s) phosphorylates Shc on tyrosines. It has been suggested that the $\beta\gamma$ subunit of Gi and Gq proteins (G $\beta\gamma$) mediates activation of p21^{ras} by the G-protein-coupled receptor through recruitment of the Src family kinases (Crespo *et al.*, 1994; van Biesen *et al.*, 1995). Moreover, evidence that a dominant interfering Src kinase mutant blocks ET-1-stimulated *c-fos* transcription in GMC supports the involvement of Src in ET-1 signaling (Simonson *et al.*, 1996), although the involvement of other tyrosine kinases cannot be ruled out. Further, as shown in stimulated rat fibroblasts overexpressing the human EGF receptor, ET-1 appears to be able to induce EGF receptor phosphorylation, which in turn could be responsible for adaptor protein recruitment (Doub *et al.*, 1996). We were not able to detect ET-1-induced tyrosine phosphorylation of the EGF receptor in primary cultures of GMC (data not shown), possibly due to low-level expression of the EGF receptor itself.

In conclusion, as schematically represented in Figure 11, the ET receptor, like tyrosine kinase receptors, is capable of recruiting the signaling proteins Shc, Grb2 and Sos1, leading to an initial first activation of p21^{ras}, which in turn results in activation of the downstream ERK pathway. As negative feedback mechanisms, Sos1 is phosphorylated and MKP-1 is expressed, playing determining roles in p21^{ras} and ERK deactivation respectively. Inactivation of ERK correlates with dephosphorylation of Sos1 and its return to the Shc/Grb2 complex. The consequence is a second delayed activation of p21^{ras}, resulting in PI-3-K activation. Therefore, ET-1 in GMC results in sequential activation of the ERK and PI-3-K pathways.

Materials and methods

Materials

Tissue culture media and reagents were from Life Technologies Inc. (Grand Island, NY). Purified human ET-1 was from Calbiochem-Novabiochem Corp. (La Jolla, CA). ECL reagent was supplied by Amersham (Little Chalfont, UK). PD 98059 was kindly provided by Dr A.Saltiel (Parke-Davis, Ann Arbor, MI). Free carrier ³²P, [γ -³²P]ATP and [³H]GDP were from Dupont-NEN Research Products (Boston, MA). Polyethyleneimine (PEI)-cellulose and silica TLC sheets were from J.T.Baker Inc. (Phillipsburg, NJ) and Whatman (Clifton, NJ) respectively. The BCA protein assay kit was from Pierce (Rockford, IL). All other reagents were from Sigma Chemical Co. (St Louis, MO).

Cell culture

Primary GMC from male Sprague-Dawley rats were isolated and characterized as previously reported (Simonson and Dunn, 1990). Cells were cultured in RPMI 1640 medium supplemented with 17% fetal

bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml each insulin and transferrin and 5 ng/ml selenite at 37°C in a 5% CO₂ incubator. NIH 3T3 cells expressing wild-type and catalytically active (DN3-S222N) MEK were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a 5% CO₂ incubator. As previously described, the catalytically active mutant of MEK was obtained by substitution of Asp for the regulatory Ser222 and by deletion of a predicted α -helix encompassing residues 32–51 (Mansour *et al.*, 1994). All experiments were performed with cells in 100 mm Petri dishes made quiescent for 24 h in serum-free medium before stimulation with 100 nM ET-1. Rat hybridoma Y13-259 cells and human embryonic kidney (HEK) 293 cells were purchased from the American Type Culture Collection (Rockville, MD) and cultured according to the supplier's protocols.

Antibodies

Rat monoclonal anti-Ras antibodies which react with both H- and K-Ras were obtained by culturing rat hybridoma Y13-259 cells. Polyclonal anti-Sos1 and polyclonal anti-Grb2 antibodies, both raised against C-terminal peptides of the respective proteins, and rabbit normal IgG, used as non-specific antibody, were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-Shc antibodies, generated with a GST-Shc fusion protein, and rabbit polyclonal anti-p85 antibodies directed against a GST fusion protein containing amino acids 265–523 of human p85 were kindly provided by Dr J.Schlessinger (New York University Medical Center, New York, NY) (Hu *et al.*, 1992; Pelicci *et al.*, 1992). Monoclonal anti-phosphotyrosine antibodies and a polyclonal anti-Shc antibody (used where indicated) raised against a C-terminal peptide of Shc protein were from Transduction Laboratories (Lexington, KY). Polyclonal anti-p42^{ERK}/p44^{ERK} antibodies were raised by immunizing rabbits with synthetic peptides (Wang *et al.*, 1992). Anti-MKP-1 antibody was produced by immunizing rabbits with a synthetic peptide derived from the C-terminus of the protein (Bokemeyer *et al.*, 1997).

Determination of GTP/GDP ratio

Determination of the GTP/GDP ratio from nucleotides immunoprecipitated with p21^{ras} was essentially as described elsewhere (Downward *et al.*, 1990). Briefly, cells were rendered quiescent for 24 h in serum-deprived medium and subsequently labeled for 4 h with 400 µCi [³²P]orthophosphate/dish in phosphate-free/serum-free medium. Cells were stimulated with ET-1 (100 nM) for the times indicated. Following ET-1 stimulation cells were placed on ice and rapidly washed with ice-cold Tris-buffered saline and lysed in 50 mM HEPES buffer, pH 7.4, 1% Triton X-100, 100 mM NaCl, 5 mM MgCl₂, 1 mg/ml bovine serum albumin (BSA), 10 µg/ml leupeptin, 10 µg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride. Nuclei were removed by centrifugation at 15 000 g for 3 min and 0.5 M NaCl, 0.5% deoxycolate and 0.05% SDS added to the lysates. Immunoprecipitation with Y13-259 rat anti-p21^{ras} monoclonal antibody (300 µl hybridoma supernatant) was carried out for 1 h. Rabbit anti-rat or normal rabbit serum, as a control, coupled to protein A-Sepharose, was added to the lysate and incubation carried out for an additional 1 h at 4°C with rotation. Immunoprecipitates were collected and washed eight times with 1 ml 50 mM HEPES buffer, pH 7.4, 0.1% Triton X-100, 500 mM NaCl, 5 mM MgCl₂ and 0.005% SDS. Nucleotides were eluted in 2 mM EDTA, 2 mM dithiothreitol, 0.2% SDS, 0.5 mM GDP and 0.5 mM GTP at 65°C for 20 min and separated on PEI-cellulose resin plates developed in 1 M KH₂PO₄, pH 3.4. The positions of GTP and GDP were monitored by chromatography of standards. The results are expressed as percentage GTP, which is the percentage of the amount of GTP relative to total GTP plus GDP determined by scintillation counting and phosphorimager analysis.

Activated Ras affinity precipitation assay

The expression vector encoding the fusion protein GST-RBD was obtained by ligation of the portion of the *raf-1* gene encoding the first 149 amino acids of the protein into the *Sma*I site of the pGEX 2T vector (Pharmacia Biotech, Piscataway, NJ) (Taylor and Shalloway, 1997). GST-RBD expression was induced in transformed bacteria with 1 mM isopropyl β -D-thiogalactoside (IPTG) for 3–4 h, after which time bacteria were harvested and lysed by sonication. The GST-RBD fusion protein was then purified on glutathione-Sepharose beads. Affinity precipitation of activated p21^{ras} was performed as described elsewhere (Taylor and Shalloway, 1997). Briefly, lysates, equalized for protein, were incubated on a rocker plate at 4°C for 30 min with 50–60 µg GST-RBD bound to glutathione-Sepharose beads. The beads were then extensively washed with 20 mM HEPES, pH 7.5, 120 mM NaCl, 10% glycerol, 0.5% Triton X-100, 2 mM EDTA, 10 µg/ml leupeptin and 10 µg/ml aprotinin. The

eluted proteins were resolved on an 11% polyacrylamide gel. Comassie brilliant blue was used to stain the fusion protein in the gel (molecular weight ~42 kDa). The polyacrylamide gel was processed as described in the Western blot analysis section and probed with anti-p21^{ras} antibody.

Immunoprecipitation and Western blot analysis

Stimulation of cells was terminated by washing twice with ice-cold phosphate-buffered saline (PBS). Cells were then lysed with 50 mM HEPES buffer, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 1 mM EGTA, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride and 0.2 mM sodium orthovanadate for 20 min at 4°C. Cells were scraped from dishes and centrifuged at 15 000 g for 20 min at 4°C. Supernatants were either loaded for SDS-PAGE or subjected to further analysis.

Immunoprecipitation of samples standardized for proteins was performed for 1 h at 4°C with constant rotation. After this time protein A-Sepharose was added and incubation was carried out for an additional 1 h. Immunoprecipitates were washed five times with 1 ml ice-cold 50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% Triton X-100, 0.5 mM MgCl₂ and 0.5 mM CaCl₂ and resuspended in 50 µl 2× sample buffer. The sample were boiled for 5 min, subjected to SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA).

For Western blotting the membrane was first blocked with 20 mM Tris-HCl, pH 7.8, 150 mM NaCl and 2% BSA for 1 h at 42°C for all antibodies or with 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1% BSA and 0.1% Tween 20 for anti-phosphotyrosine antibody and then probed in the same solution with primary antibody for 2 h at room temperature. After three washes membranes were incubated for 45 min at room temperature with horseradish peroxidase-conjugated protein A in 20 mM Tris-HCl, pH 7.8, 150 mM NaCl and 5% non-fat dry milk. The signal from immunoreactive bands was detected by ECL.

ERK activity assay

Cells were stimulated and harvested as above. Lysates, containing 400 µg protein, were immunoprecipitated with 2 µl anti-p42^{ERK} antibody. Immunoprecipitation was carried out at 4°C for 1 h before adding protein A-Sepharose and incubating for an additional 1 h. Immunoprecipitates were washed twice in lysis buffer and twice in 20 mM HEPES, pH 7.4, 10 mM MgCl₂ and 0.2 mM sodium orthovanadate and incubated in 0.25 mg/ml MBP, 50 µM ATP and 5 µCi [γ -³²P]ATP for 15 min at 30°C. The reaction was terminated by addition of 4× sample buffer and the proteins subjected to electrophoresis in a 12.5% SDS-polyacrylamide gel and visualized by autoradiography. The bands corresponding to phosphorylated MBP were cut out and radioactivity measured using liquid scintillation counting.

Recombinant adenoviral vectors

The recombinant adenovirus vector AdMEK_{CA}, expressing the catalytically active form of MEK, was constructed from replication-deficient adenovirus type 5 (Ad5) with deletions in the *E1* and *E3* genes, Ad-dl327 and a plasmid containing Ad5 sequences from bp 22 to 5790 with a deletion of the *E1* region from bp 342 to 3523, a polycloning site under control of the CMV promoter, the mutated human *MEK* gene and the SV40 polyadenylation signal. As previously described, the catalytically active mutant of MEK was achieved by substitution with Glu and Asp respectively of the regulatory residues Ser218 and Ser222 and by deletion of a predicted α -helix encompassing residues 32–51 (Mansour *et al.*, 1994). Aliquots of 10 µg linearized plasmid were co-transfected with 10 µg of the large fragment of *Clal*-digested Ad-dl327 DNA into HEK 293 cells, to allow homologous recombination to occur, followed by replication and encapsidation of recombinant adenoviral DNA into infection virions and formation of plaques as a result of lysis of the infected cells. Individual plaques were isolated, amplified in HEK 293 cells and viral DNA isolated (Hirt, 1967) and analyzed for identification of recombinant viruses by *HindIII* DNA restriction analysis and DNA sequencing. Recombinant viruses were propagated in 50 T175 flasks of HEK 293 cells infected at a MOI of five. Cells were recovered 36–48 h after infection and viruses released by five cycles of freeze-thawing. All viral preparations were purified by CsCl density gradient centrifugation (Graham and Van Der Eb, 1973), dialyzed and stored at –70°C in 10 mM Tris-HCl, pH 7.4, 1 mM MgCl₂ and 10% glycerol until used. Titers of the viral stocks were determined by plaque assay using HEK 293 cells (Graham and Van Der Eb, 1973).

Adenoviral infection

GMC were infected with varying titers of AdLacZ in 1 ml RPMI 1640 medium containing 2% fetal bovine serum. After 1 h infection culture

medium was added to the plate. Twenty-four hours after infection cells were washed twice with PBS and fixed in 1% glutaraldehyde, 1 mM MgCl₂ in PBS for 15 min. Cells were then washed three times with PBS and stained with 5 mM K₄Fe(CN)₆·3H₂O, 5 mM K₄Fe(CN)₆, 2 mM MgCl₂, 0.2% X-gal in PBS for 1–2 h at 37°C.

GMC were infected with AdMEK_{CA} at a MOI of 60. After 1 h infection serum-free medium was added to the plate and the cells maintained for 24 h before agonist stimulation.

Phosphatidylinositol 3-kinase activity assay

The determination of PI-3-K activity was performed essentially as described elsewhere (Fukui and Hanafusa, 1989). Briefly, cells were made quiescent and then stimulated with ET-1 (100 nM) as described above. Stimulation of cells was terminated by washing twice with ice-cold PBS. Cells were lysed with 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 1 mM sodium orthovanadate and 1 mM phenylmethylsulfonyl fluoride for 20 min at 4°C. Lysates, containing an equal amount of protein, were immunoprecipitated with Y13-259 rat anti-p21^{ras} (800 µl hybridoma supernatant) previously coupled with rabbit anti-rat antibody and protein A-Sepharose. The Sepharose beads containing the immunoprecipitates were washed twice with lysis buffer, once with PBS, once with 0.5 M LiCl, 100 mM Tris-HCl, pH 7.5, 1 mM sodium orthovanadate, once with distilled water and once with 20 mM Tris-HCl, pH 7.5, 0.5 mM EGTA, 100 mM NaCl, and resuspended in 50 µl final wash solution to which was added 0.5 µl 20 mg/ml phosphatidylinositol (Avanti Polar Lipids Inc., Alabaster, AL). Anti-p85 immunoprecipitates were finally resuspended either in phosphatidylinositol or in phosphatidylinositol 4-monophosphate in order to identify phosphatidylinositol monophosphate and biphosphate respectively on TLC. Samples were incubated at 25°C for 10 min, at which time [γ -³²P]ATP (10 µCi/sample) and MgCl₂ (20 mM final concentration) were added and incubation allowed to continue at 25°C for an additional 15 min. The reaction was stopped by addition of 150 µl chloroform/methanol/11.6 N HCl (100:200:2) and subsequently 100 µl chloroform was added to separate the organic phase. Reaction products were separated by TLC and resolved in chloroform/methanol/ammonium hydroxide/water (129:114:15:21). Spots were visualized by autoradiography of TLC plates and quantified by phosphorimager analysis.

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