Src-family tyrosine kinases, phosphoinositide 3-kinase and Gab1 regulate extracellular signal-regulated kinase 1 activation induced by the type A endothelin-1 G-protein-coupled receptor

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The multisubstrate docking protein, growth-factor-receptor-bound protein 2-associated binder 1 (Gab1), which is phosphorylated on tyrosine residues following activation of receptor tyrosine kinases and cytokine receptors, regulates cell proliferation, survival and epithelial morphogenesis. Gab1 is also tyrosine phosphorylated following activation of G-protein-coupled receptors (GPCRs) where its function is poorly understood. To elucidate the role of Gab1 in GPCR signalling, we investigated the mechanism by which the type A endothelin-1 (ET-1) GPCR induced tyrosine phosphorylation of Gab1. Tyrosine phosphorylation of Gab1 induced by endothelin-1 was inhibited by PP1, a pharmacological inhibitor of Src-family tyrosine kinases. ET-1-induced Gab1 tyrosine phosphorylation was also inhibited by LY294002, which inhibits phosphoinositide 3-kinase (PI 3-kinase) enzymes. Inhibition of Src-family tyrosine kinases or PI 3-kinase also inhibited ET-1-induced activation of

the mitogen activated protein kinase family member, extracellular signal-regulated kinase (ERK) 1. Thus we determined whether Gab1 regulated ET-1-induced ERK1 activation. Overexpression of wild-type Gab1 potentiated ET-1-induced activation of ERK1. Structure–function analyses of Gab1 indicated that mutant forms of Gab1 that do not bind the Src homology (SH) 2 domains of the p85 adapter subunit of PI 3-kinase or the SH2-domain-containing protein tyrosine phosphatase 2 (SHP-2) were impaired in their ability to potentiate ET-1-induced ERK1 activation. Taken together, our data indicate that PI 3-kinase and Src-family tyrosine kinases regulate ET-1-induced Gab1 tyrosine phosphorylation, which, in turn, induces ERK1 activation via PI 3-kinase- and SHP-2-dependent pathways.

Key words: cross-talk, growth regulation, signal transduction.

INTRODUCTION

Endothelin-1 (ET-1) was originally isolated as a potent vascular-smooth-muscle contractile agonist [1]. Two ET-1 receptors (ET_A and ET_B), which belong to the family of G-protein-coupled receptors (GPCRs) and bind ET-1 with equal affinity, have also been cloned [2,3]. ET-1 is a multifunctional peptide. ET-1 and both ET_A and ET_B are required for normal embryonic development [4]. ET-1 also regulates gene expression, cell contraction, proliferation and survival [5–10]. Aberrant ET-1-induced cell proliferation and/or survival are implicated in the pathophysiology of many diseases, including atherosclerosis, asthma and human tumorigenesis [11–13]. Nevertheless, the mechanism(s) by which ET-1 regulates cell growth are poorly understood, although ET-1-induced activation of the mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinase (ERK) 1 and ERK2, regulates cell proliferation and survival in some cell types [8–10]. ERK1 and ERK2 are key signal transducers activated by a wide range of extracellular stimuli [14–17]. In the case of GPCRs, following ligand binding, the receptor-associated G α subunit exchanges GDP for GTP and dissociates from the $\beta\gamma$ subunits. Both GTP-bound activated G α subunits and free $\beta\gamma$ subunits regulate GPCR-induced MAPK activation [18].

One mechanism by which GPCRs, including the ET_A, induce MAPK activation involves GPCR-induced activation of receptor

tyrosine kinases (RTK), such as the epidermal growth factor (EGF) receptor (EGFR) [19–23]. GPCRs also induce the activation of Src-family tyrosine kinases, which may subsequently tyrosine phosphorylate and ‘transactivate’ RTKs [22,23]. Once activated, Src and/or the RTK induce tyrosine phosphorylation of the Shc adapter protein. Both tyrosine-phosphorylated Shc and the activated RTK are then able to bind growth-factor-receptor-bound protein 2 (Grb2) and its associated Ras guanine nucleotide exchange factor, Son of sevenless (Sos) [24]. RTK- or Shc-associated Grb2/Sos induces exchange of GDP for GTP on Ras, thus initiating the well-described Ras/MAPK pathway containing Raf and MAPK/ERK kinase (MEK) as intermediates [24].

GPCR-induced tyrosine phosphorylation of Shc and activation of MAPK are also dependent upon phosphoinositide 3-kinase (PI 3-kinase) activity [25,26]. Pharmacological inhibition of PI 3-kinase with wortmannin or LY294002 inhibits GPCR- or $\beta\gamma$ subunit-induced Shc tyrosine phosphorylation and MAPK activation, suggesting that PI 3-kinase may be required for activation of the kinase that tyrosine phosphorylates Shc [26]. However, under conditions where inhibition of PI 3-kinase with wortmannin blocks GPCR-induced MAPK activation, wortmannin does not inhibit GPCR-induced EGFR tyrosine phosphorylation [20]. This suggests that the requirement for PI 3-kinase in GPCR-induced MAPK activation may be downstream

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; GPCR, G-protein-coupled receptor; Grb2, growth-factor-receptor-bound protein 2; Gab1, Grb2-associated binder 1; PI 3-kinase, phosphoinositide 3-kinase; ET-1, endothelin-1; ETR, endothelin-1 receptor; LPA, lysophosphatidic acid; GST, glutathione S-transferase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; PH, pleckstrin homology; PKC, protein kinase C; RTK, receptor tyrosine kinase; EGF, epidermal growth factor; EGFR, EGF receptor; HA, haemagglutinin; HRP, horseradish peroxidase; Sos, Son of sevenless; SH, Src homology; SHP-2, SH2-domain-containing protein tyrosine phosphatase 2; ECL[®] (Amersham Pharmacia Biotech), enhanced chemiluminescence; FBS, fetal bovine serum.

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of the EGFR and upstream of the kinase that tyrosine phosphorylates Shc. Other PI 3-kinase-dependent signalling pathways also regulate GPCR-induced activation of MAPK. Activation of MAPK by the G_q -coupled bradykinin receptor is dependent upon PI 3-kinase β and protein kinase C (PKC) ϵ [27]. Activation of MAPK by the G_i -coupled lysophosphatidic acid (LPA) receptor is regulated by PI 3-kinase γ -induced activation of PKC ζ [28]. Overall, these data indicate that PI 3-kinase can modulate GPCR-induced MAPK activation via several distinct signalling pathways.

Multisubstrate docking proteins, such as Gab1 and insulin receptor substrate ('IRS') 1/2, are important regulators of PI 3-kinase activity induced by RTKs [29–32]. Although there is limited sequence homology between members of this family, each protein has an N-terminal pleckstrin homology (PH) domain, proline-rich sequences that bind Src homology (SH) 3-domain-containing proteins and multiple tyrosine residues that, when phosphorylated, bind SH2-domain containing proteins, including the p85 subunit of Class IA PI 3-kinase. Gab1 is tyrosine phosphorylated in cells following activation of RTKs, the B cell antigen receptor and cytokine receptor family members [29,31,33–37]. Notably, Gab1 is also tyrosine phosphorylated following activation of LPA and thrombin GPCRs [20,21,38]. The tyrosine kinase(s) that phosphorylate(s) Gab1 have not been defined. However, inhibition of Src-family tyrosine kinases or the EGFR inhibits LPA-induced Gab1 tyrosine phosphorylation [20]. These kinases also regulate GPCR-induced MAPK activation raising the possibility that Gab1 may be a general regulator of GPCR-induced MAPK activation. Support for this is provided by data indicating that Gab1 enhances MAPK activity following stimulation of interleukin 6 receptor family members and the EGF and nerve growth factor ('NGF') RTKs [36,39,40].

Gab1 was recently shown to enhance the activation of MAPK following stimulation of the Edg2 GPCR by LPA [38]. However, the mechanism by which Gab1 and its associated signalling molecules regulate MAPK activation is poorly understood. Our data presented in this study indicate that ET-1 stimulation of NIH3T3 fibroblasts, which express the ETR_A GPCR [41], induced the tyrosine phosphorylation of Gab1 as well as the activation of MAPK. ET-1-dependent proliferation of NIH3T3 fibroblasts is dependent upon both PI 3-kinase and MAPK [42]. Because tyrosine-phosphorylated Gab1 regulates both cell proliferation and activation of PI 3-kinase and MAPK following stimulation of other receptor types, we set up a model system in HEK-293T cells to better understand the relationship between PI 3-kinase activity, Gab1 tyrosine phosphorylation and MAPK activation following stimulation of the ETR_A GPCR by ET-1. In the present study we report that ET-1-induced Gab1 tyrosine phosphorylation and activation of the MAPK, ERK1, were dependent upon Src-family tyrosine kinases as well as PI 3-kinase. Moreover, overexpression of Gab1 potentiated ET-1-induced activation of ERK1 via PI 3-kinase- and SH2-domain-containing protein tyrosine phosphatase 2 (SHP-2)-dependent signalling pathways.

MATERIALS AND METHODS

Materials

LY294002, wortmannin, PD98059 and rapamycin were purchased from Biomol (Plymouth Meeting, PA, U.S.A.). PP1 (a pharmacological inhibitor of Src-family tyrosine kinases) was purchased from Calbiochem (Foster City, CA, U.S.A.). Polyclonal antibodies that recognize Gab1 and the p85 subunit of PI 3-kinase were kindly provided by Dr. Morag Park (Departments of Biochemistry, Oncology and Medicine, McGill University,

Montreal, QC, Canada) and Dr. X. Johné Liu (Loeb Research Institute, University of Ottawa, ON, Canada) respectively. Polyclonal antibodies that recognize the total and phosphorylated forms of MAPK were purchased from New England Biolabs (Beverly, MA, U.S.A.) and monoclonal anti-haemagglutinin (HA) antibodies (HA11) were purchased from BabCO (Richmond, CA, U.S.A.). For anti-phosphotyrosine immunoblotting, RC20H, a recombinant horseradish peroxidase (HRP)-conjugated anti-phosphotyrosine Fab fragment, was used (Transduction Laboratories, Lexington, KY, U.S.A.). Protein A-Sepharose, Protein G-Sepharose, HRP-conjugated Protein A and enhanced chemiluminescence (ECL[®]) reagents were from Amersham Pharmacia Biotech (Baie d'Urfé, QC, Canada).

Cell culture and plasmid DNAs

NIH3T3 fibroblasts and HEK-293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10 % (v/v) fetal bovine serum (FBS; Gibco BRL, Burlington, ON, Canada) and antibiotics. HEK-293T cells (1×10^6) were plated on to 100-mm-diameter cell-culture dishes 24 h prior to transfection. Cells were then transfected using a standard calcium phosphate procedure with expression plasmids encoding ETR_A (in pcDNA3.1), glutathione S-transferase (GST)–ERK1 (in pEBG) and HA-tagged murine Gab1 (in pcDNA3.1). These plasmids were kindly provided by Dr Tomoh Masaki (Department of Pharmacology, Kyoto University, Kyoto, Japan), Dr Bruce Mayer (Department of Genetics and Development Biology, University of Connecticut Health Center, Farmington, CT, U.S.A.) and Dr Albert Wong (Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, PA, U.S.A.) respectively. After overnight incubation, cells were washed twice with PBS and the medium was replaced with DMEM/10 % (v/v) FBS. After a further 24 h incubation, the cells were washed twice with PBS and then starved overnight in serum-free DMEM before being stimulated with ET-1 (0.01–100 nM) or LPA (10 μ M) for 1–30 min and lysed. For NIH3T3 fibroblasts, cells grown in 100-mm dishes were allowed to become confluent, washed twice with PBS and starved overnight in serum-free DMEM. They were then stimulated with ET-1 (100 nM) for 1–30 min and lysed. Inhibitors or appropriate vehicles were added to the cells prior to ET-1 or LPA treatment, as indicated in the Figure legends.

Cell lysates, immunoprecipitation and Western blotting

Following stimulation, cells were washed twice in ice-cold PBS and harvested in lysis buffer [50 mM Hepes (pH 8.0), 1 % Triton X-100, 150 mM NaCl, 10 % (v/v) glycerol, 2 mM EGTA, 1.5 mM $MgCl_2$, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 1 mM PMSF and 1 mM sodium orthovanadate]. Following a 10-min incubation on ice, the lysates were clarified by centrifugation at 14000 *g* for 10 min, and protein concentrations were determined by the method of Bradford. To detect activation of MAPK in NIH3T3 fibroblasts, equal amounts of whole-cell lysates were solubilized in boiling SDS-gel loading buffer [100 mM Tris/HCl (pH 6.8), 100 mM dithiothreitol, 4 % (w/v) SDS, 20 % (v/v) glycerol and 0.2 % (w/v) Bromophenol Blue]. To monitor tyrosine phosphorylation of Gab1, Gab1 was immunoprecipitated by incubating lysates with a polyclonal anti-Gab1 antibody for 1 h at 4 °C with mixing. Protein A-Sepharose was added [20 μ l of a 50 % (w/v) solution] and mixed for an additional 1 h at 4 °C. Immunoprecipitates were washed three times in lysis buffer and solubilized in SDS-gel loading buffer.

To detect activation of ERK1 and tyrosine phosphorylation of Gab-1 or HA–Gab1 in HEK-293T cells, equal amounts of protein were incubated with either glutathione-Sepharose, anti-

Gab1 or anti-HA antibodies for 1 h at 4 °C with mixing. In the case of anti-Gab1 and anti-HA immunoprecipitations, Protein A-Sepharose or Protein G-Sepharose respectively, were added [20 μ l of a 50 % (w/v) solution] and mixed for an additional 1 h at 4 °C. Glutathione-Sepharose-associated proteins or immunoprecipitated proteins were washed three times in lysis buffer and solubilized in boiling SDS-gel loading buffer.

For Western blotting, the samples obtained above were subjected to SDS/PAGE and transferred on to nitrocellulose (Bio-Rad, Mississauga, ON, Canada). Membranes were blocked with 1 % BSA in Tris-buffered saline [10 mM Tris/HCl (pH 7.4) and 150 mM NaCl] containing 2.5 mM EDTA and 0.1 % (v/v) Tween-20 for 1 h at 20 °C. To detect activated MAPK (NIH3T3 fibroblasts) or GST-ERK1 (HEK-293T cells), blots were incubated with anti-phosphoMAPK antibodies overnight at 4 °C followed by HRP-conjugated Protein A for 1 h. For anti-phosphotyrosine immunoblotting, the membranes were blocked for 1 h as above, followed by the addition of a recombinant HRP-conjugated anti-phosphotyrosine Fab fragment. Immuno-reactive bands were detected by ECL[®] and X-ray film. Where indicated, protein phosphorylation or protein levels were visualized and quantified on a FluorChem 8000 Imaging System (Alpha Innotech, San Leandro, CA, U.S.A.) using AlphaEase software (Alpha Innotech).

RESULTS

Activation of the ETR_A GPCR induces MAPK activation and Gab1 tyrosine phosphorylation

We (S. Bisotto and E. Fixman, unpublished work) and others [19,43] have demonstrated previously that proteins in the molecular-mass range of 110–120 kDa are phosphorylated on tyrosine residues in ET-1-stimulated cells. To determine whether Gab1, which is 110 kDa, represented one of these tyrosine-phosphorylated proteins, the tyrosine phosphorylation status of Gab1 was assessed in NIH3T3 fibroblasts, which express the ETR_A GPCR, following stimulation with ET-1 for 1–30 min. An increase in Gab1 tyrosine phosphorylation was detected at 1 min of stimulation with ET-1 (an average 2.5-fold increase) (Figure 1A, top panel). Gab1 tyrosine phosphorylation remained elevated at 10 and 20 min (an average 2.3- and 1.7-fold increase respectively) and returned to baseline by 30 min (an average of 1.3-fold). Gab1 was also tyrosine phosphorylated following ET-1 stimulation of primary rat airway smooth-muscle cells, which express the ETR_A GPCR (results not shown). Immunoblot analysis indicated that the tyrosine-phosphorylated protein that co-immunoprecipitated with Gab1 (indicated by * in Figure 1A, upper panel) co-migrated with the p85 subunit of PI 3-kinase, whose association with Gab1 also increased following ET-1 stimulation of NIH3T3 cells (Figure 1A, lower panel). ET-1-induced activation of MAPK in NIH3T3 cells was assessed using phosphoMAPK-specific antibodies that recognize only the dually phosphorylated and activated forms of MAPK. ET-1-induced MAPK activation was detected at 1 min (4.4 ± 0.9 -fold), maximal at 5 min (17.5 ± 6.8 -fold) and returned to basal levels by 30 min (1.5 ± 0.4 -fold) (Figure 1B).

Following stimulation of other receptor families, tyrosine-phosphorylated Gab1 regulates activation of PI 3-kinase and MAPK [30,31,39,40]. Moreover, following activation of GPCRs, PI 3-kinase regulates MAPK activation [25,26]. Thus to address the role of PI 3-kinase and Gab1 in ET-1-induced activation of MAPK, we established a transient transfection assay in HEK-293T cells. Because HEK-293T cells do not express ETRs, cells were transfected with an expression plasmid encoding the

Figure 1 Activation of the ETR_A GPCR induces MAPK activation and Gab1 tyrosine phosphorylation in NIH3T3 fibroblasts

Confluent NIH3T3 fibroblasts were serum-starved overnight prior to being stimulated with 100 nM ET-1 for 1–30 min. Following stimulation, cells were washed and lysed. (A) Endogenous Gab1 was immunoprecipitated (IP) with a polyclonal anti-Gab1 antibody followed by the addition of Protein A-Sepharose. Protein complexes were washed with lysis buffer and analysed by SDS/PAGE and immunoblotting (Blot). Tyrosine-phosphorylated Gab1 was detected with a recombinant anti-phosphotyrosine antibody (pTYR). A tyrosine-phosphorylated protein that co-immunoprecipitated with Gab1 is shown (*). The positions of the molecular-mass markers (in kDa) are indicated on the left. The blot was stripped and reprobed with the polyclonal anti-Gab1 antibody and then polyclonal anti-p85 antibody (p85; lower panel). (B) Whole-cell lysates (20 μ g) were solubilized in sample buffer and analysed by SDS/PAGE and immunoblotting. Activated MAPK was detected with an anti-phosphoMAPK antibody. The blot was stripped and reprobed with a polyclonal anti-MAPK antibody to detect total MAPK protein. Proteins were visualized by ECL[®] on an Alpha Innotech FluorChem 8000 Imaging system. Results are representative of two (A) or three (B) experiments. The fold induction is given for the representative experiment shown and the means $\pm$ S.E.M. for (B) are presented in the text.

ETR_A GPCR. To selectively assess activation of MAPK in ETR_A transfected cells, an expression vector encoding GST-ERK1 was also transfected. Similar to ETR_A-induced MAPK activation in NIH3T3 fibroblasts, ET-1-induced activation of ERK1 in a concentration- and time-dependent manner in HEK-293T cells (Figures 2A and 2B). Maximal activation of ERK1 was induced with 10^{-7} M ET-1 (17.6 ± 4.1 -fold; Figure 2A, lane 6) and following 5 min of ET-1 stimulation (11.9 ± 1.9 -fold; Figure 2B, lane 3).

The ETR_A GPCR is able to associate with several G α subunits, including G_{i1}, G_q and G_s [44,45]. Thus to determine which G α subunit(s) coupled ET-1 stimulation of ETR_A with activation of ERK1 in HEK-293T cells, cells were pre-treated with pertussis toxin to ADP-ribosylate and inhibit G_{i/o} subunits. Alternatively,

Figure 2 ERK1 activation induced by ET-1 is mediated by multiple G α subunits

HEK-293T cells were transfected with expression plasmids (1 μ g each) encoding ETR_A and GST-ERK1. After incubation for 2 days, the cells were serum-starved overnight before being stimulated with 10^{-11} – 10^{-7} M ET-1 for 5 min (A), or with 100 nM ET-1 for 1–30 min (B). Cells were either pretreated for 18 h with vehicle (lane 3) or 100 ng/ml pertussis toxin (lane 4) (C), or pretreated for 30 min with DMSO (lane 3) or 10 μ M Ro-31-8220 to inhibit PKC (lane 4) (D), and then stimulated with ET-1 as indicated. After ET-1 treatment, cells were washed twice in ice-cold PBS and lysed. GST-ERK1 was purified by incubating 1 mg of lysate with glutathione-Sepharose for 30 min at 4 °C. Associating proteins were washed with lysis buffer, solubilized in sample buffer and analysed by SDS/PAGE and immunoblotting. Phospho-GST-ERK1 was detected with an antibody that recognizes dually phosphorylated, activated ERK1. Proteins were visualized by ECL[®] on an Alpha Innotech FluorChem 8000 Imaging system (A–C) or on X-ray film (D). Results are representative of two (D), three (A, C) or five (B) experiments. The fold induction (A, B) or the percentage of maximum activation (C, D) are given for the representative experiment shown. Means $\pm$ S.E.M. for (A–C) are presented in the text.

cells were pretreated with Ro-31-8220 to inhibit PKC to determine if ETR_A-induced activation of ERK1 was regulated by G α_q . Pertussis toxin (100 ng/ml) attenuated ET-1-induced ERK1 activation by an average of 70.6 ± 1.3 % (Figure 2C). Similarly, inhibition of PKC with Ro-31-8220 (10 μ M) attenuated ET-1-induced ERK1 activation by an average of 63.7 % in two experiments (Figure 2D). These data indicate that ERK1 activation induced following ET-1 stimulation of the ETR_A is regulated by both pertussis-toxin-sensitive (G $\alpha_{i/o}$) and -insensitive (most likely G α_q - and PKC-dependent) signalling pathways in these cells.

To determine if ET-1 induced tyrosine phosphorylation of Gab1 in HEK-293T cells, cells were transiently transfected with an expression vector encoding ETR_A and stimulated with ET-1 for 0–30 min. Similar to ET-1 stimulation of NIH3T3 fibroblasts (Figure 1A), 5 min after the addition of ET-1, tyrosine phosphorylation of Gab1 increased (an average of 2.2-fold in two experiments; Figure 3A). Gab1 tyrosine phosphorylation remained elevated at 10–20 min post ET-1 stimulation (an average of 2.8- and 1.9-fold respectively in two experiments). When cells were co-transfected with expression vectors encoding ETR_A and HA-tagged murine Gab1, HA-Gab1 was also tyrosine phosphorylated (Figure 3B). An increase in tyrosine phosphorylation of the HA-Gab1 was first detectable at 5 min stimulation (2.6 ± 0.1 -fold), was maximal at 10–20 min (4.2 ± 0.4 -fold), and remained elevated at 30 min (2.4 ± 0.8 -fold).

ET-1-induced ERK1 activation, but not Gab1 tyrosine phosphorylation, is dependent upon MEK

To better define the signalling pathways that regulated ET-1-induced ERK1 activation and Gab1 tyrosine phosphorylation, HEK-293T cells were pretreated with the MEK inhibitor, PD98059, prior to ET-1 stimulation. As predicted, ET-1-induced

ERK1 activation was dependent upon activation of MEK (Figure 4A). Pretreatment of cells with PD98059 inhibited ET-1-induced ERK1 activation (Figure 4A, top panel; compare lanes 5 and 6 with lanes 7 and 8). However, PD98059 did not inhibit ET-1-induced Gab1 tyrosine phosphorylation, indicating that MEK activity was not required for ET-1-induced Gab1 tyrosine phosphorylation (Figure 4B, top panel; compare lanes 5 and 6 with lanes 7 and 8). ET-1-induced ERK1 activation and Gab1 tyrosine phosphorylation were also not inhibited by rapamycin (results not shown) indicating their independence from target of rapamycin ('TOR') kinases.

PI 3-kinase activity regulates GPCR-induced ERK1 activation and Gab1 tyrosine phosphorylation

The N-terminus of Gab1 contains a PH domain, which binds the PI 3-kinase product, PtdIns(3,4,5)P₃, *in vitro* [40,46,47]. Moreover, the Gab1 PH domain localizes Gab1 to the plasma membrane in a PI 3-kinase-dependent manner and is required for efficient tyrosine phosphorylation of Gab1 induced by EGF [31,40]. Following activation of RTKs, Gab1 may also be recruited to the plasma membrane in a PH domain-independent manner mediated, in part, by the Grb2 adapter protein [31,35,40,48].

To determine if PI 3-kinase activity was required for ET-1-induced Gab1 tyrosine phosphorylation and/or ERK1 activation, cells were incubated 30 min prior to ET-1 stimulation with LY294002 to inhibit PI 3-kinase activity. ET-1-induced tyrosine phosphorylation of Gab1 was nearly abolished in the presence of LY294002 (Figure 5A, top panel; compare lanes 7 and 8 with lanes 9 and 10). Pretreatment of cells with LY294002 also inhibited activation of ERK1 induced by ET-1 (Figure 5B, top panel; compare lanes 7 and 8 with lanes 9 and 10). Similar results were obtained when cells were preincubated with the

Figure 3 ET-1 induced tyrosine phosphorylation of HA-Gab1

HEK-293T cells were transfected with an expression plasmid (1 μ g) encoding ETR_A without (A) or with (B) 1 μ g of HA-tagged, murine Gab1. After incubation for 2 days, the cells were serum-starved overnight before being stimulated with 100 nM ET-1 for 1–30 min. Cells were then washed twice in ice-cold PBS and lysed. Lysates (1 mg) were incubated with polyclonal anti-Gab1 antibody and Protein A–Sepharose to immunoprecipitate (IP) endogenous Gab1 (A) or with anti-HA antibody and Protein G–Sepharose to immunoprecipitate HA-Gab1 (B). In each case, protein complexes were washed three times with lysis buffer, solubilized in sample buffer and analysed by SDS/PAGE and immunoblotting (Blot). Tyrosine-phosphorylated Gab1 or HA-Gab1 were detected with a recombinant anti-phosphotyrosine antibody (pTYR). The blots were stripped and reprobed with an anti-Gab1 antibody to demonstrate that equal protein levels were present. Proteins were visualized by ECL[®] on X-ray film (B, top panel) or on an Alpha Innotech FluorChem 8000 Imaging system. Results are representative of two (A) or three (B) experiments and the fold stimulation is given for the representative experiment shown. Means $\pm$ S.E.M. for (B) are presented in the text.

structurally distinct PI 3-kinase inhibitor, wortmannin (results not shown). These data indicate that PI 3-kinase activity was required for ET-1-induced tyrosine phosphorylation of Gab1 and activation of ERK1.

Src-family tyrosine kinases regulate GPCR-induced ERK1 activation and Gab1 tyrosine phosphorylation

The Src tyrosine kinase regulates MAPK activation downstream of several GPCRs as well as Gab1 tyrosine phosphorylation induced by LPA GPCR [20–22,49,50]. To address the role of Src-family tyrosine kinases in ET-1-induced ERK1 activation and Gab1 tyrosine phosphorylation, cells were pretreated with PP1 prior to stimulation with ET-1. As a control, cells were also stimulated with LPA. PP1 inhibited ET-1-induced Gab1 tyrosine phosphorylation (Figure 6A, top panel, lanes 7–9) and ERK1 activation (Figure 6B, top panel, lanes 7–9). As demonstrated previously, pretreatment of cells with PP1 also inhibited LPA-induced Gab1 tyrosine phosphorylation (Figure 6A, top panel, lanes 4–6) and ERK1 activation (Figure 6B, top panel, lanes 4–6) [20]. These data demonstrate that inhibition of Src-family tyrosine kinases blocks both ET-1-induced Gab1 tyrosine phosphorylation and ERK1 activation.

Figure 4 ET-1-induced ERK1 activation is dependent upon MEK

HEK-293T cells were transfected with expression plasmids (1 μ g each) encoding GST-ERK1, HA-tagged, murine Gab1 and ETR_A. After incubation for 2 days, the cells were serum-starved overnight before being pretreated for 30 min with vehicle (DMSO, lanes 1, 2, 5 and 6) or PD98059 (10 μ M, lanes 3 and 7; 50 μ M, lanes 4 and 8) to inhibit MEK activity. Cells were then stimulated with 100 nM ET-1 (lanes 5–8), washed twice in ice-cold PBS and lysed. (A) GST-ERK1 was isolated by incubating 1 mg of lysate with glutathione–Sepharose for 30 min at 4 °C. (B) HA-Gab1 was immunoprecipitated (IP) by incubating lysates (1 mg) with anti-HA antibody followed by Protein G–Sepharose at 4 °C. In each case, proteins were washed three times with lysis buffer, solubilized in sample buffer and analysed by SDS/PAGE and immunoblotting (Blot). Activated ERK1 (phospho-GST-ERK1) was detected by immunoblotting with an anti-phosphoMAPK antibody. Total ERK1 (GST-ERK1) was detected using an anti-MAPK antibody. Tyrosine-phosphorylated Gab1 was detected with recombinant anti-phosphotyrosine antibody (pTYR). The blots were stripped and reprobed with a polyclonal anti-Gab1 antibody to detect total Gab1 (HA-Gab1) protein. Proteins were visualized with ECL[®] on an Alpha Innotech FluorChem 8000 Imaging System. Results are representative of three experiments.

Gab1 regulates ET-1-induced ERK1 activation

Inhibition of PI 3-kinase or Src inhibited both ET-1-induced Gab1 tyrosine phosphorylation and ERK1 activation. Moreover, Gab1 has been shown to potentiate MAPK activation induced by other receptor families [36,38–40]. To determine if Gab1 regulated ET-1-induced ERK1 activation, cells were transfected with either a Gab1 expression plasmid or its parent vector (pcDNA3.1), and the ability of ET-1 to induce ERK1 activation assessed. As shown in Figure 2, ET-1 induced maximal ERK1 activation at 5 min stimulation (Figure 7A, top panel, and Figure 7B). In the presence of co-transfected wild-type Gab1, ERK1 activation induced by ET-1 was potentiated at all time points examined (Figure 7A, bottom panel, and Figure 7B).

To define the Gab1-dependent signals involved in ERK1 activation, structure–function analyses of Gab1 were performed. A mutant Gab1 protein lacking the N-terminal PH domain retained the ability to potentiate ET-1-induced ERK1 activation (results not shown). Gab1 also contains nine tyrosine residues whose downstream sequences are predicted to bind SH2 domain-

Figure 5 PI 3-kinase regulates GPCR-induced ERK1 activation and Gab1 tyrosine phosphorylation

HEK-293T cells were transfected with expression plasmids (1 μ g each) encoding GST-ERK1 and HA-tagged, murine Gab1 and ETR_A. After incubation for 2 days, the cells were serum-starved overnight before being pretreated for 30 min with DMSO (lanes 3, 4, 7 and 8) or LY294002 (10 μ M, lanes 5 and 9; 50 μ M, lanes 6 and 10) to inhibit PI 3-kinase activity. Cells were then stimulated with 100 nM ET-1 (lanes 2, 7–10), washed twice in ice-cold PBS and lysed. **(A)** HA-Gab1 was immunoprecipitated (IP) by incubating lysates (1 mg) with an anti-HA antibody followed by Protein G-Sepharose at 4 °C. **(B)** GST-ERK1 was purified by incubating 1 mg of lysate with glutathione-Sepharose for 30 min at 4 °C. In each case, proteins were washed three times with lysis buffer, solubilized in sample buffer and analysed by SDS/PAGE and immunoblotting (Blot). **(A)** Tyrosine-phosphorylated Gab1 was detected with a recombinant anti-phosphotyrosine antibody (pTYR). The blot was stripped and reprobed with a polyclonal anti-Gab1 antibody and total Gab1 (HA-Gab1) protein was visualized with ECL[®] on an Alpha Innotech FluorChem 8000 Imaging System. **(B)** Activated ERK1 (phospho-GST-ERK1) was detected with anti-phosphoMAPK antibody. Blots were stripped and reprobed with anti-MAPK antibody to determine total ERK1 (GST-ERK1) protein levels. Proteins were visualized by ECL[®] and X-ray film. Results are representative of three experiments.

containing proteins, including the p85 adapter subunit of PI 3-kinase, SHP-2, Crk, phospholipase C ('PLC') γ , and Grb2 [29]. Mutation of Tyr⁶²⁸ of murine Gab1 abolishes the association between Gab1 and SHP-2 [51]. Similarly, a murine Gab1 mutant in which Tyr⁴⁴⁸, Tyr⁴⁷³ and Tyr⁵⁹⁰ are each mutated to phenylalanine is no longer able to bind p85 and PI 3-kinase [30]. Thus tyrosine-to-phenylalanine mutant Gab1 proteins that are unable to associate with PI 3-kinase (Δ p85/Gab1) or SHP-2 (Δ SHP-2/Gab1) were assessed for their ability to potentiate ET-1-induced ERK1 activation. Although expressed at equivalent levels compared with the wild-type Gab1 protein (results not shown), both of the Gab1 mutant proteins were impaired in their ability to potentiate ET-1-induced ERK1 activation (Figures 8A and 8B). Following a 10-min ET-1 stimulation, wild-type Gab1 enhanced ET-1-induced ERK1 activation approx. 2.5-fold. However, both the Δ p85/Gab1 and the Δ SHP-2/Gab1 mutants were impaired in their ability to enhance ET-1-induced ERK1 activation. Thus these data indicate that PI 3-kinase and SHP-2 are downstream regulators of Gab1-potentiated ERK1 activation induced by ET-1.

Figure 6 Inhibition of Src-family tyrosine kinases inhibits GPCR-induced ERK1 activation and Gab1 tyrosine phosphorylation

HEK-293T cells were transfected with expression plasmids (1 μ g each) encoding GST-ERK1 and HA-tagged murine Gab1. In addition, pcDNA3.1 (1 μ g; lanes 1–6) and the ETR_A expression plasmid (1 μ g; lanes 7–9) were also transfected. After incubation for 2 days, cells were serum-starved overnight before being pretreated for 30 min with DMSO (lanes 1, 4 and 7) or PP1 (5 μ M, lanes 2 and 5 and 8; 10 μ M lanes 3, 6 and 9) to inhibit Src. Cells were then stimulated with 10 μ M LPA (lanes 4–6) or 100 nM ET-1 (lanes 7–9), washed twice in ice-cold PBS and lysed. **(A)** HA-Gab1 was immunoprecipitated (IP) by incubating lysates (1 mg) with an anti-HA antibody followed by Protein G-Sepharose at 4 °C. **(B)** GST-ERK1 was purified by incubating 1 mg of lysate with glutathione-Sepharose for 30 min at 4 °C. In each case, proteins were washed three times with lysis buffer, solubilized in sample buffer and analysed by SDS/PAGE and immunoblotting. **(A)** Tyrosine-phosphorylated Gab1 was detected with a recombinant anti-phosphotyrosine antibody (pTYR). The blot was stripped and reprobed with polyclonal anti-Gab1 antibody to detect total Gab1 (HA-Gab1) protein. **(B)** Activated ERK1 (phospho-GST-ERK1) was detected with an anti-phosphoMAPK antibody. Blots were stripped and reprobed with an anti-MAPK antibody to detect the levels of ERK1 (GST-ERK1) protein. Proteins were visualized by ECL[®] on an Alpha Innotech FluorChem 8000 Imaging System. Results are representative of three experiments.

DISCUSSION

One emerging common theme in the activation of MAPK by GPCRs is the requirement for PI 3-kinase. It has been proposed that PI 3-kinase regulates GPCR- or $\beta\gamma$ -subunit-induced activation of MAPK by stimulating the activation of Src-family tyrosine kinases [22,26]. However, the mechanism remains poorly defined. More recently, following GPCR activation, PI 3-kinase has been shown to induce the activation of members of the PKC family, which subsequently induce MAPK activation [27,28]. Our data reported in this study now suggest that another function of PI 3-kinase in GPCR-induced MAPK activation is to induce tyrosine phosphorylation of the multisubstrate docking protein, Gab1, which subsequently regulates signalling pathways leading to MAPK activation.

One possible role of PI 3-kinase in ET-1-induced tyrosine phosphorylation of Gab1 and activation of MAPK is to recruit Gab1, via its PH domain, to the plasma membrane where it would subsequently be tyrosine phosphorylated. Once at the plasma membrane, tyrosine-phosphorylated Gab1 and its associated signalling molecules would induce activation of MAPK. However, in our experiments the PH domain of Gab1 was dispensable for Gab1-mediated potentiation of ET-1-induced ERK1 activation in HEK-293T cells (results not shown). This suggests that PI 3-kinase-dependent membrane translocation of Gab1 may not be required for Gab1 to potentiate ET-1-induced

Figure 7 Gab1 potentiates ET-1-induced ERK1 activation

(A) HEK-293T cells were transfected with a combination of expression plasmids encoding GST-ERK1 (1 μ g), ETR_A (1 μ g) and either 2 μ g of pcDNA3.1 (A, upper panel) or 2 μ g of HA-tagged, murine Gab1 (A, lower panel). After 2 days the cells were serum-starved overnight before being stimulated with 100 nM ET-1. Following ET-1 stimulation, cells were washed twice in ice-cold PBS and lysed. GST-ERK1 was purified by incubating 1 mg of lysate with glutathione-Sepharose for 30 min at 4 °C. Protein complexes were washed three times with lysis buffer, solubilized in sample buffer and analysed by SDS/PAGE and immunoblotting. Activated ERK1 (phospho-GST-ERK1) was visualized with anti-phosphoMAPK antibody and ECL[®] on an Alpha Innotech FluorChem 8000 Imaging System. (B) ET-1-induced activation of ERK1 in the presence of co-transfected pcDNA3.1 (stippled bars) or HA-Gab1 (grey bars) is presented with results expressed as means $\pm$ S.E.M. for three experiments.

ERK1 activation, or that other PH domain-independent mechanisms regulate membrane translocation of Gab1 following activation of the ETR_A. This appears to be the case for RTK-induced Gab1 tyrosine phosphorylation. Following activation of the Met RTK, Gab1 can be tyrosine phosphorylated and recruited to the plasma membrane in a PH domain-independent manner by associating with the Met RTK directly, and indirectly via the Grb2 adapter protein [31,35,47,48]. It is unclear if the association of Gab1 with Grb2 is required for ET-1-induced Gab1 tyrosine phosphorylation or Gab1-dependent potentiation of ET-1-induced ERK1 activation.

Another possible role of PI 3-kinase in GPCR-induced Gab1 tyrosine phosphorylation and ERK1 activation is to regulate activation of the Gab1 tyrosine kinase [52]. This is consistent with data indicating that GPCR-induced Shc tyrosine phosphorylation is also dependent upon PI 3-kinase [26]. Shc does not contain a PH domain and thus its subcellular localization is not predicted to be directly regulated by PI 3-kinase. LPA- and ET-1-induced tyrosine phosphorylation of Gab1 is dependent upon Src-family tyrosine kinases and EGFR [19,20]. However, it is unclear if EGFR and Src act in the same or different pathways. Inhibition of PI 3-kinase does not inhibit GPCR-induced ac-

Figure 8 Gab1 association with p85 and SHP-2 is required for potentiation of ET-1-induced ERK1 activation

(A) HEK-293T cells were transfected with a combination of the expression plasmids encoding GST-ERK1 (1 μ g), ETR_A (1 μ g) and either 2 μ g of pcDNA 3.1 or HA-tagged wild-type Gab1 (+ WTGab1), 2 μ g of Δ p85/Gab1 or 2 μ g of Δ SHP-2/Gab1. After 2 days the cells were serum-starved overnight before being stimulated with 100 nM ET-1 for 0–30 min. Cells were then washed twice in ice-cold PBS and lysed. GST-ERK1 was purified by incubating 1 mg of lysate with glutathione-Sepharose for 30 min at 4 °C. Proteins were washed three times with lysis buffer, solubilized in sample buffer and analysed by SDS/PAGE and immunoblotting. Activated ERK1 (phospho-GST-ERK) was visualized with anti-phosphoMAPK antibody and ECL[®] on an Alpha Innotech FluorChem 8000 Imaging System. (B) ERK1 activation in the presence of co-transfected pcDNA3.1, wild-type HA-Gab1 (wtGab1), Δ p85/Gab1 or Δ SHP-2/Gab1 is presented with results expressed as means $\pm$ S.E.M. for at least three experiments.

tivation of the EGFR [20], and our data in the present study indicate that tyrosine phosphorylation of Gab1 is dependent upon PI 3-kinase activity. This would suggest that EGFR is not the tyrosine kinase that phosphorylates Gab1 and that PI 3-kinase is in a pathway downstream of the EGFR and upstream of Src. Interestingly, efficient Gab1 tyrosine phosphorylation induced by the EGFR itself is also dependent upon PI 3-kinase [40], suggesting that both the EGFR and ET-1 GPCR may use similar mechanisms (i.e. Src-family tyrosine kinases) to induce tyrosine phosphorylation of Gab1. The possibility that other GPCR-induced tyrosine kinases phosphorylate Gab1 has not yet been addressed. Overall, these data suggest that GPCR-induced Gab1 tyrosine phosphorylation is regulated by the EGFR as well as Src, although it is unclear if either of these kinases phosphorylates Gab1 directly.

Our data in the present study indicate that there is a correlation between tyrosine phosphorylation of Gab1 and ET-1-induced ERK1 activity. Furthermore, overexpression of Gab1 in HEK-293T cells potentiated the activation of ERK1 induced by ET-1. Gab1 associates with several molecules that induce activation of Ras following stimulation of other receptor families, including p85, Shc, Grb2 and SHP-2 [29,31,34,36]. Experiments are in

progress to determine if the ability of Gab1 to potentiate ET-1-induced ERK1 activation is due to potentiation of Ras activation. Recent data [27,28] indicates that PI 3-kinase α and PI 3-kinase β regulate GPCR-induced MAPK activation via Ras-independent activation of the PKC isoforms, ϵ and ζ . Thus our data in the present study raise the possibility that Gab1 may be a potential adapter protein coupling the activation of PI 3-kinase with PKC and subsequent MAPK activation.

Disrupting the association between Gab1 and either PI 3-kinase or SHP-2 attenuated the ability of Gab1 to potentiate ET-1-induced MAPK activation in HEK-293T cells. In agreement with our data presented in this study, Cunnick et al. [38] recently showed that association between Gab1 and SHP-2 was required for Gab1-dependent potentiation of LPA-induced MAPK activation in HEK-293 cells. However, while our data indicated that PI 3-kinase was also a downstream effector of Gab1 in ET-1-induced MAPK activation, their data indicate that the ability of Gab1 to potentiate LPA-induced MAPK activation is not dependent upon the association between Gab1 and PI 3-kinase. Although the reason for this discrepancy is unclear, one possible explanation for the difference is that ETR α activates MAPK via multiple signalling pathways, one or more of which depend upon PI 3-kinase. On the other hand, PI 3-kinase may not regulate activation of MAPK following LPA-induced activation of the Edg-2 GPCR in HEK-293 cells, and thus disrupting the association of Gab1 with PI 3-kinase would be predicted to have no effect. Gab1 is a crucial regulator of PI 3-kinase activity induced by RTKs [30,31,39,40]. Thus our data in the present study suggest that Gab1 may also be a critical regulator of PI 3-kinase activity induced by ET-1. Consistent with the data in Figure 8, blocking the association between Gab1 and PI 3-kinase may alter PI 3-kinase activity and thus modulate MAPK activation induced by ET-1 stimulation of ETR α . Identification of the G-proteins that are activated by ETR α , and which of these regulate Gab1 tyrosine phosphorylation and PI 3-kinase activation, may be key to understanding the mechanism by which each of these signalling pathways regulates MAPK activation induced by ETR α , Edg-2 and other GPCRs.

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